Universitat Internacional de Catalunya

# Adipose Stem Cells from Buccal Fat Pad and Abdominal Adipose Tissue for Bone tissue Engineering

Elisabet Farré Guasch

**Dipòsit Legal: B-25086-2011**
**ISBN: 978-90-5335-394-3**

**http://hdl.handle.net/10803/31987**

# AUTORITZACIÓ DELS DIRECTORS DE TESI PER A LA SEVA PRESENTACIÓ

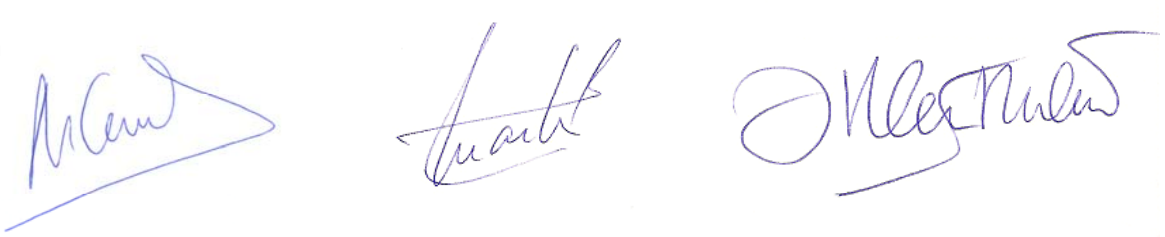
La Dra. Núria Casals Farré, el Dr. Carles Martí Pagès i la Prof. Dra. Jenneke Klein-Nulend, com a co-directors de la tesi doctoral:

"Adipose Stem Cells from Buccal Fat Pad and Abdominal Adipose Tissue for Bone tissue Engineering" realitzada a la Facultat d'Odontologia pel doctorant Sra. Elisabet Farré Guasch, autoritzen la presentació de l'esmentada tesi doctoral opció a Menció Europea, atès que reuneix les condicions necessàries per a la seva defensa.

Sant Cugat, 09 de Decembre del 2010

ELS CO-DIRECTORS DE LA TESI

Dra. Núria Casals Farré  Dr. Carles Martí Pagès  Prof. Dra. Jenneke Klein-Nulend



**The research presented in this thesis was carried out at:**

Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Universitat Internacional de Catalunya (UIC), Barcelona, Spain.

Department of Basic Sciences, Faculty of Medicine and Health Sciences, Universitat Internacional de Catalunya (UIC), Barcelona, Spain.

Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA)- University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Amsterdam, The Netherlands.

**In kind cooperation with:**

The Flow Cytometry Research Core from Institut de Biotecnologia i Biomedicina UAB, Barcelona, Spain.

Department of Orthopaedics, VU University Medical Center, Research Institute MOVE, Amsterdam, The Netherlands.

Publication of this thesis was partially financially supported by:

STEGA MOVE/Skeletal Tissue Engineering Group Amsterdam (STEGA), Amsterdam, The Netherlands.

Institut Straumann AG, Basel, Switzerland.

Roche Diagnostics Nederland BV, Almere, The Netherlands.


ISBN/EAN: 978-90-5335-394-3



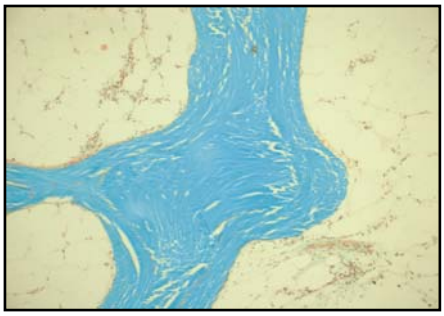
Printed by Ridderprint.

Layout and cover design by E. Farré Guasch & N. Vermeulen.

Universitat Internacional de Catalunya

Oral & Maxillofacial Department, Faculty of Dentistry

Basic Sciences Department, Faculty of Medicine and Health Sciences



# Adipose Stem Cells from Buccal Fat Pad and Abdominal Adipose Tissue for Bone Tissue Engineering

PhD dissertation European Mention

**Elisabet Farré Guasch**

born in Barcelona

Director: Dra. Núria Casals Farré

Co-Director: Dr. Carles Martí Pagès

Co-Director: Prof. Dra. Jenneke Klein-Nulend

Barcelona, 2011

Contemplant el parpelleig de les estrelles,
Trepanant la imponent volta de la nit
Com s´enfila més encara l´esperit
Fins a Vós, Senyor de tantes meravelles

I et condolen els que acluquen les parpelles
Al misteri fecundant de l´infinit
Encallats al seu món físic… Tan petit
Que no hi ha cap l´afany d´haver les coses belles
Josep Farré i Gual

**To my family, with all my love**

“Those who have a 'why' to live, can bear any 'how'”. Adaptation of Viktor Frankl

“Life would be less fun without fat”

# AGRAÏMENTS

I can not end this chapter without mentioning all the people who has contributed in some way or another to the configuration of this book. What starts has to end, and the ending is not less important than the start, although the ending remains more in the memories, a good end is always better than a good start.

I remember my start, in 2005, doubting if to begin this path, although I had always clear where to start, in my home, at the University where I learnt and I became a professional, in the International University of Catalonia, Barcelona, where brought me my best memories and my best experiences decisive to consolidate my life. Although I was in that moment far away, if it can be nowadays considered far with the changes of distances thanks to the easiness of flying, I had clear that I had to begin with it here, and left everything there, the work, the patients, the friends, and a project of life, and came here to start this project. A start is always more difficult than a continuation, but I can tell that this start has taught me a lot, and I had the satisfaction to be able to decide where to lead the path. I was very lucky to meet Dr Giner, an excellent professional and person, and he opened the door to begin with it. Esme, you are not here now, but I hope you can read this, thank you for all your support and what you did while I was here. Afterwards I contact another excellent and professional person, Dr Martí, although in that moment it was a bit difficult to start contact with him personally, because his spirit of service and professionality lead him to work at that moment in the southeast of Africa, I finally met him in person and he accepted to guide my work. His curiosity and research interest lead him to work in that moment with stem cells, and that let me be able to start the idea of project I had, which was very new here in that moment, and that was to use stem cells for regeneration of maxillars. Afterwards, and when I was still in The Netherlands, I contacted Dr Clotet, a bright mind and intriging professor, who I was lucky to have met in my first years of study at the University and one of the people who encouraged my science interest, what I guess has always been there, but like what does the wind to a fire, it can grow or extinguish. Unfortunately, he was working with bacterias, and that was incompatible with my project, specially wished to avoid as much as possible working with cells. That brought me to meet Dra Casals, another brilliant professor and researcher, and a very social person, who has helped me a lot to improve and develop some other skills. Although in that moment I was a new person here in the lab, joining to the few team we were

in the beginnings, and although I was a dentist, not a biology or biochemistry person, she decided to accept me in her team and guide me, and not less important, sustain also my research, morally and financially, and because all this, I am and I will always be thankful to her for being so brave. Afterwards I joined to the lab team, very tiny in that moment, as I said, and I met Ester, Patri, Anna and Diana. Ester, you are a great friend and collegue, and thank you a lot to motivate so much and be so hard with me, especially in the beginning, to make me learn a lot. You are really a practical person, and with a great interest to teach people. Thank you. Patri, I have also learnt a lot from you, and with you I had a lot of enjoyment with the performing of the first experiments, and you are always willing to give a hand to the next. That is really appreciated in the lab. Anna, you are not here now, but I also had a lot of fun with you, at the lunch in the bar, and I still remember your fibroblasts, which do look like stem cells, as you said. Adriana, you are far away now, in Colombia, but I will also remember you as a kind person, who always gave nice advice and you were the first to present the thesis in the department, and your funny comments about some Spanish words that were strange for you to use. Luckily I was not the only research dentist in the area, Deborah and Maria José were also there with Dr Clotet, and I also shared with them nice moments and a lot of coffees. The next year I met my master colleagues, Maher, Elena, Cris and Anna and we share many coffee breaks and productive lessons of Scientific communications. Tania, Alex, Sandra, Natalia, Aranxa, Jordi, Dani, thank you too to be here and for the nice moments and laughs we have shared, and lunchs. And Sara H and Sara the new phD, it would not be the same without you. And thanks to Dra Núria Durany and Dr Agustí Fontarnau, for all their presence, company, conversations and support they gave to me. Dear Núria Durany, it has been so sad that you had to leave us, we will never forget you and your fighting spirit, showing us what true courage is. Thank you for the opportunity to be Junior Faculty, I have learned a lot from it. And Montserrat, thanks a lot to make statistics more friendly, and the pacience to spend some intempestive hours and the time spent to make it more clear. It is very trustworthy your dedication.

I would like also to thank Dr Bowlin, from Virginia Commonwealth, for let me enter his laboratory and see the amazing technique of electrospinning, and how a complex thing can be explained in a simple way, that is the most amazing thing in science, and what great minds can do. You are a great person and very modest, I am very proud to have met you. Jenny, Trish, Michael, Parth and colleagues from the Tissue Engineering lab, thanks to all you guys

for all the tours you organize the days I was there, and thank you a lot for all the instructions you gave me those days I was there with you. You are very nice and I hope to have the chance to meet you again.

If there is a why you can stand any how, and that is a sentence has been printed these years of hard working and unstoppable reading and writing. First you have to do what is necessary, then you can do what is possible and finally you will do what is impossible. First I had the career, afterwards I completed the master, and now, finally, I made possible what seemed impossible. I would like to thank all the Department of Oral Cell Biology from ACTA-VU, Amsterdam. Specially to Vincent, for the welcome and the great koffee talks, and his valuable input in the first day I was here, for Jenneke, for giving me the opportunity to participate in this exciting work, and for bringing out the value of fundamental research to oral research, and Marco. Marco, thanks a lot to support me from the beginning and all the good advises. You have not always been in the department, because you are in the Orthopaedics department, but you have always been there when I have needed you. Jolanda, dank je wel voor alles. Voor alle steun, en hulp met de taal. Jol, het was zo leuk met jou een speld in een hooiberg te vinden. Cor, dank je voor de leuke tijd en gezelligheid in de lab, en leren om lieveheersbeestjes te tellen! Rishi, you are really a nice person!. Astrid, je hebt me zoveel geleerd, dank je, je bent echt een slim en aardig mens. Janice, Petra, Marjolein, wat kan ik zeggen? Ik had zoveel fun met jullie, we waren een hecht team. Ik zal de leukste feesten van Amsterdam nooit vergeten! En de Cosmos ook niet! Dank je. En Mark, dank je ook voor alle steun, en gezelligheid in de ACTA bespreking. En Nina, je bent een grappig mens! Ik heb echt met je gelachen. En Behrouz, dank je voor alle tips en aardigheid. En Anneke, voor alle correcties om mijn Nederlands te verbeteren. I will miss you. I enjoyed really to be there and to have met you all. Thank you to welcome me.

Thank you too to the Sant Joan de Deu Oral Maxillofacial Unit, the Clinic plastic surgery Unit, Dr Fontdevila, Dr Eloy, and Dr Hernández Alfaro, for kindly providing the samples, and Dr Balaguer and Dr Cortada for their support during the studies. Thank you also to the Flow Cytometry Core and especially to Manuela for all the technical advice, she is a great professional and she provided useful advise during the assays. And thanks to the UIC library service, for providing articles, books, and search information. Lluís i Eugenia, moltes gràcies per tot. And Isabel, thanks for showing me during the first years of clinical practice the

surgical techniques and bringing me the opportunity to join you in the operating room, there began this story…

And to close this chapter, I wish to thank my father. As cardiologist he helped people to heal hearts during a whole life, and he taught me a lot. He gave me the spirit and the strength to begin this work, and I always carry with me his wise words and tips. And my mother, moltes gràcies. Sense el teu suport no hauria pogut tenir la força d'arribar fins aquí. She is the strongest person I know and I also learnt a lot from her. Gemma, Joan, Francesc, Climment, Thank you. I am really lucky to have you as my brothers and family. And Carol and Marcel, thank you, you helped me a lot to carry out this path and without you it would not have been the same. Maite, Ester, Anna, you are far but you are always close, and I hope we can soon meet us all. Elisa and Marina. Thank you a lot for the support and patience with this always busy time. I am very lucky to have you as my friends. And thank you all that took part in this story…

# TABLE OF CONTENTS

**RESUM** ..... **15**

**CHAPTER 1 INTRODUCTION** ..... **61**

**CHAPTER 2 OBJECTIVES** ..... **73**

**CHAPTER 3 BACKGROUND** ..... **77**

**3.1. Bone embryology** ..... **80**

**3.2. Bone structure and composition** ..... **84**

**3.3. Bone dynamics** ..... **95**
**3.3.1. Bone modeling** ..... 95
**3.3.2. Bone remodeling** ..... 96

**3.4. Bone loss** ..... **98**

**3.5. Bone reconstruction** ..... **98**
**3.5.1. Two-phase theory of osteogenesis** ..... 99
**3.5.2. Immune response** ..... 100
**3.5.3. Types of grafts** ..... 100
3.5.3.1. Autogenous grafts ..... 100
**3.5.3.1.1. Block grafts** ..... 101
**3.5.3.1.2. Particulate marrow and cancellous bone grafts** ..... 101
**3.5.3.1.3. Autogeneous bone with blood supply** ..... 101
3.5.3.1.3. a) Bone graft pedicled to a muscular or muscular and skin pedicle ..... 101
3.5.3.1.3. b) Use of microsurgical techniques. ..... 101
3.5.3.2. Allogenic grafts ..... 102
3.5.3.3. Xenogenic grafts ..... 103
3.5.3.4. Alloplast grafts ..... 103
**3.5.4. Principles osteogenesis** ..... 103
3.5.4.1. Osteogenesis ..... 104
3.5.4.2. Osteoconduction ..... 104
3.5.4.3. Osteoinduction ..... 104

**3.6. Bone tissue engineering** ..... **104**
**3.6.1. Stem cells** ..... 104
3.6.1.1. Properties of Stem Cells ..... 104
3.6.1.2. Plasticity of Stem Cells ..... 105
3.6.1.3. Sources of stem cells ..... 106
**3.6.1.3.1. Embryonic stem cells (ES)** ..... 106
**3.6.1.3.2. Adult stem cells** ..... 107
**3.6.1.3.2. a) Mesenchymal stem cells (MSC)** ..... 108
**3.6.1.3.2. b) Endothelial progenitor cells (EPC) /Hemangioblasts** ..... 110
**3.6.1.3.2. c) Hematopoietic stem cells (HSC)** ..... 111
**3.6.1.3.2. d) Multipotent progenitor cells (MAPC)** ..... 112
**3.6.1.3.2. e) Induced pluripotent stem cells (iPS)** ..... 112
**3.6.1.3.2. f) Cancer stem cells** ..... 113
**3.6.2. Stem cell niche** ..... 113
**3.6.3. Scaffolds** ..... 116
3.6.3.1. Definition and types ..... 116
3.6.3.2. Properties ..... 116
**3.6.4. Growth factors, cytokines and hormones in bone** ..... 119

3.6.4.1. Bone Morphogenetic Proteins (BMPs) ..... 119
3.6.4.2. Tumor necrosis factor (TNF) ..... 120
3.6.4.3. Colony stimulating factor (CSF) ..... 121
3.6.4.4. Granulocyte colony-stimulating factor (G-CSF) ..... 121
3.6.4.5. Basic fibroblast growth factor (bFGF) ..... 121
3.6.4.6. Interleukins (IL) ..... 121
3.6.4.7. Insulin-like growth factor (IGF) ..... 121
3.6.4.8. Glucocorticoids ..... 122
3.6.4.9. Hepatocyte growth factor (HGF) and macrophage colony-stimulating factor ..... 122
3.6.4.10. Leptin ..... 122
3.6.4.11. Sex steroids ..... 123
3.6.4.12. Epidermal growth factor (EGF) ..... 123
3.6.4.13. Platelet-derived growth factor (PDGF) ..... 123
3.6.4.14. Vascular endothelial growth factor (VEGF) ..... 124
3.6.4.15. Prostaglandins (PG) ..... 124

**3.7. Adipose Tissue ..... 125**
**3.7.1. Adipose tissue development and morphology** ..... 125
**3.7.2. Physiology of the adipose tissue** ..... 126
**3.7.3. Biology of adipose tissue** ..... 130
3.7.3.1. Adipose stem cells (ASC), history and definition ..... 130
3.7.3.2. Localization of ASC ..... 131
3.7.3.3. Isolation of ASC ..... 132
3.7.3.4. Characterization of ASC phenotype ..... 133
3.7.3.5. Mesenchymal stem cell sources: bone marrow versus adipose tissue ..... 133
3.7.3.6. Stromal vascular fraction (SVF) and their potential ..... 134
3.7.3.7. Clinical trials with ASC ..... 136
**3.7.4. Endocrine and metabolic functions of adipose tissue** ..... 137

**3.8. Buccal fat pad (BFP) ..... 138**
**3.8.1. Anatomy** ..... 139
**3.8.2. Physiology** ..... 140
**3.8.3. Uses** ..... 141

**CHAPTER 4 HYPOTHESIS ..... 143**

**CHAPTER 5 MATERIALS & METHODS ..... 147**

**5.1. STUDY 1: BUCCAL FAT PAD, AN ORAL ACCESS-SOURCE OF HUMAN ADIPOSE STEM CELLS WITH POTENTIAL FOR OSTEOCHONDRAL TISSUE ENGINEERING ..... 149**
**5.1.1. ASC isolation and characterization** ..... 149
5.1.1.1. Tissue preparation and cell isolation/expansion ..... 149
5.1.1.2. Flow cytometry ..... 152
**5.1.2. ASC differentiation** ..... 153
5.1.2.1. ASC differentiation medium and seeding ..... 153
**5.1.2.1.1. Osteogenic differentiation** ..... 153
**5.1.2.1.2. Adipocyte differentiation** ..... 154
**5.1.2.1.3. Chondrogenic differentiation** ..... 154
5.1.2.2. ASC differentiation assays ..... 154
**5.1.2.2.1. Immunohistochemistry** ..... 154
**5.1.2.2.2. Histology** ..... 155
**5.1.2.2.3. Alkaline phosphatase (ALP) activity assay** ..... 155
**5.1.2.2.4. Quantitative real-time PCR** ..... 156
**5.1.3. Statistical analysis** ..... 157

**5.2. STUDY 2: OSTEOGENIC DIFFERENTIATION OF BMP-2 INDUCED HUMAN ADIPOSE STEM CELLS SEEDED ON BIPHASIC CALCIUM PHOSPHATE AND β-TRICALCIUM PHOSPHATE SCAFFOLDS ..... 158**

**5.2.1. ASC isolation and culture on different scaffolds** ..... 158
5.2.1.1. ASC isolation/expansion ..... 158
5.2.1.2. ASC culture, and seeding on scaffolds ..... 159
**5.2.2. ASC Attachment and proliferation on different scaffolds** ..... 160
5.2.2.1. Colony-forming unit fibroblasts (CFU-F) assay and CFU-F depletion assays (depl CFU-F) ..... 160
5.2.2.2. CyQUANT DNA proliferation assay ..... 161
**5.2.3. Osteogenic differentiation of ASC on different scaffolds** ..... 161
5.2.3.1. ALP activity ..... 161
5.2.3.2. Quantitative real-time PCR ..... 162
**5.2.4. Statistical analysis** ..... 162

**CHAPTER 6 RESULTS ..... 163**

**6.1. STUDY 1: BUCCAL FAT PAD, AN ORAL ACCESS-SOURCE OF HUMAN ADIPOSE STEM CELLS WITH POTENTIAL FOR OSTEOCHONDRAL TISSUE ENGINEERING ..... 165**
**6.1.1. ASC isolation** ..... 165
6.1.1.1. Enzymatic digestion protocol is effective to isolate ASC from buccal fat pad ..... 165
6.1.1.2. Reduction of 30 minutes collagenase digestion does not affect ASC cell proliferation ..... 166
6.1.1.3. Reduction of collagenase concentration by 50% seems to affect ASC proliferation ..... 167
**6.1.2. ASC characterization** ..... 168
6.1.2.1. Buccal fat pad contains stem cells with ASC characteristics ..... 168
6.1.2.2. Morphology of cultured ASC from two different sources ..... 168
6.1.2.3. Comparison between cell surface marker profile ASC from buccal fat pad and ASC from subcutaneous abdominal adipose tissue (SC) ..... 169
6.1.2.4. Comparison of ASC yield from buccal fat pad and subcutaneous abdominal adipose tissue ..... 172
6.1.2.5. SVF from BFP is a rich source of ASC. ..... 173
**6.1.3. Multilineage differentiation potential of ASC derived from buccal fat pad in vitro** ..... 175
6.1.3.1. Osteogenic differentiation ..... 175
6.1.3.2. Adipogenic differentiation ..... 179
6.1.3.3. Chondrogenic differentiation ..... 180

**6.2. STUDY 2: OSTEOGENIC DIFFERENTIATION OF BMP-2 INDUCED HUMAN ADIPOSE STEM CELLS SEEDED ON BIPHASIC CALCIUM PHOSPHATE AND β-TRICALCIUM PHOSPHATE SCAFFOLDS ..... 183**
**6.2.1. Effect of BMP-2 on attachment and proliferation of ASC on different scaffolds** ..... 183
6.2.1.1. BMP-2 increases CFU-F and does not affect cell attachment on different scaffolds ..... 183
6.2.1.2. BMP-2 affects ASC proliferation dependent on the carrier ..... 186
**6.2.2. Effect of BMP-2 on osteogenic differentiation of ASC on different scaffolds** ..... 187
6.2.2.1. BMP-2 induces ALP activity dependent on the carrier ..... 187
6.2.2.2. BMP-2 induces osteogenic gene expression dependant on the carrier ..... 188

**CHAPTER 7 DISCUSSION ..... 193**

**CHAPTER 8 CONCLUSIONS ..... 219**

**CHAPTER 9 FUTURE DIRECTIONS ..... 223**

**CHAPTER 10 ABBREVIATIONS ..... 229**

**CHAPTER 11 REFERENCES ..... 235**

**CHAPTER 12 APPENDIX ..... 287**

# Resum

# INTRODUCCIÓ

Actualment l'esperança de vida s'ha incrementat gradualment, dels 45 anys a començaments del segle 20 fins als aproximadament 80 anys que es dóna actualment en els països desenvolupats. Aquest augment en l'esperança de vida va acompanyat d'un envelliment dels òrgans que afecta la qualitat de vida de la població. Les bases histològiques d'aquest envelliment podrien estar relacionades amb la pèrdua de la capacitat proliferativa i de diferenciació de les cèl·lules mare que es troben en els òrgans, així com una reducció en el seu nombre, el que comporta malalties i processos degeneratius en els òrgans afectats.

L'enginyeria tisul·lar, també anomenada medicina regenerativa o medicina reparativa, és un camp emergent que combina els principis de bioenginyeria, transplantament cel·lular i enginyeria de biomaterials a fi de reparar, substituir, mantenir o millorar la funció dels òrgans *in vivo*, a més de desenvolupar substituts de teixits *in vitro* per a implantar-los *in vivo* com a substituts biològics, fet que comporta un enorme potencial en el tractament de malalties cròniques i processos degeneratius. Aquest camp, consolidat al segle XX, ha estat desde la dècada dels 90 acompanyat en paral·lel per una industria emergent, i s'ha format la Societat d'Enginyeria tisul·lar que ha donat lloc a la Societat Internacional de Medicina Regenerativa i Enginyeria tisul·lar, coneguda com a TERMIS. Aquesta societat ha donat lloc a la formació d'un registre internacional d'assaigs de medicina regenerativa en pacients, a més de productes innovadors, i pretén promoure la col·laboració global per fer front al gran repte de millorar la salut del pacient.[1] El gran paradigma és trobar la font adecuada de cèl·lules mare que siguin capaces d'integrar-se en els teixits de l'hoste, i que a més posseeixin les propietats del teixit a regenerar. El repte de la medicina regenerativa és el de desenvolupar *in vivo* un nou teixit u òrgan sense la presència de teixit fibrós ni cicatricial. Això conduiria al complet reemplaçament de les estructures danyades, resultant en un teixit reconstruit completament functional i acicatricial.

La medicina regenerativa engloba tres principals blocs: els biomaterials, els factors de creixement (estímuls inductius), i les cèl·lules mare. Els biomaterials actuen com un andamiatge que permet i promou el creixement i la diferenciació cel·lular, proveint suport estructural, contenció biològica i estímuls químics a les cèl·lules, i afavorint per tant la osteoconducció i osteoinducció cel·lular. Els estímuls inductius són necesaris per millorar la

proliferació i diferenciació d'aquestes cèl·lules, i engloben les hormones, els factors de creixement i les citoquines, a més a més de les senyals mecàniques.[2] Finalment, i no menys important, és trobar una font de fàcil accés amb alta disponibilitat de cèl·lules mare per aplicacions clíniques immediates i cirurgia de una sola fase, o per a intervencions múltiples que requereixen un alt nombre de cèl·lules. Dos grans problemes a resoldre són l'alt nombre de cèl·lules necessari i la mort cel·lular associada a la seva implantació en els teixits. Les cèl·lules madures expandides *in vitro* perden la seva eficàcia i la seva implantació està associada amb disrupció vascular, el que resulta en un ambient hipòxic que desencadena la mort cel·lular. Per ser efectives, les cèl·lules haurien de ser fàcilment obtenibles, expandibles, i sobreviure a la implantació amb l'aport d'una adecuada xarxa vascular. A més a més, haurien de ser immunocompetents i no provocar resposta inflamatòria a l'organisme, a més de funcionar correctament i no posseir malignitat. Finalment no haurien de generar-se debats ètics ni morals derivats del seu ús.[3]

Tradicionalment, les cèl·lules mare embrionàries (ES) han conduït la recerca en regeneració tisul·lar, però l'ús de cèl·lules mare adultes venç els obstacles principals que deriven de l'utilització d'aquestes cèl·lules, com són la formació teratogènica, la seva immunogeneitat, i els debats ètics i morals que deriven del seu ús.[4,5]

Les cèl·lules mare adultes han estat identificades en una àmplia gamma de teixits com teixit sanguini, cervell, estòmac, intestí, cor, retina, sang del cordó umbilical, villi coriònica, placenta, polpa dental, lligament periodontal, greix, pell, cabell, fetge, teixit ossi i cartilaginós i muscle esquelètic. En tots aquests teixits les cèl·lules mare adultes juguen un paper essencial en l'homeòstasi tisul·lar, formant part en els processos de regeneració dels teixits i reemplaçant les cèl·lules senescents, i en la restauració dels teixits perduts després d'una malaltia o accident. Encara que inicialment es creia que aquestes cèl·lules estaven compromeses a diferenciar-se al llinatge cel·lular d'on provenien, hi ha creixent evidència de la seva plasticitat per transdiferenciar-se i creuar llinatges germinals i diferenciar-se en un gran espectre de tipus cel·lulars diferents de la línia germinal del teixit d'on provenen. Aquestes cèl·lules també han mostrat potencial per retornar a un estat previ indiferenciat (desdiferenciació).[6] Aquesta inesperada plasticitat de les cèl·lules mare les converteix en candidates interessants per aplicacions en enginyeria tisul·lar.

Un dels factors crítics en enginyeria tissul·lar òssia, a més a més de trobar un biomaterial que permeti el creixement cel·lular i la seva diferenciació, i l'ús d'estímuls inductius que promoguin la proliferació i diferenciació d'aquestes cèl·lules, és el trobar una font adequada de cèl·lules mare. En la dècada dels 60 Friedenstein i col·legues van demostrar que l'estroma mesenquimal de la mèdul·la òssia conté una població de cèl·lules que proliferen quan són cultivades en plàstic de cultiu i es diferencien a llinatges celul·lars derivats del mesoderm, com condrocits i osteoblasts.[7] Posteriorment aquestes cèl·lules amb morfologia fibroblàstica es van anomenar cèl·lules mare mesenquimals (MSC)[8] i van mostrar habilitat per diferenciar-se a diferents llinatges cel·lulars *in vitro*[9,10] i *in vivo*,[11,12,13] el que les converteix en prometedores candidates per reparar defectes de teixits del mesoderm. Aquestes cèl·lules tenen la capacitat de crèixer adherides a plàstic de cultiu, expressen marcadors de superfície específics i posseeixen potencial de diferenciació multipotent, en concordància amb el que postula el Comitè de Cèl·lules Mesenquimals i de Teixits de la Societat Internacional de Teràpia Cel·lular (ISCT).[14] Les cèl·lules mare de la mèdul·la òssia (BM MSC) han estat la font més util·litzada de MSC per a teràpies clíniques.[15-18] L'ús de MSC no presenta rebuig del sistema immunitari, risc de formació de teratomes, ni les discussions ètiques i morals derivades de l'ús de cèl·lules mare embrionàries.

L'habilitat de diferenciar-se a cèl·lules d'altres llinatges del mesoderm com osteoblasts, condrocits, mioblasts i adipocits, en combinació amb alguns dels trets immunològics que posseeixen, com són la baixa expressió de l'antígen leucocitari humà (HLA) de tipus II, les fa candidates ideals per enginyeria tissul·lar i en tractaments alogènics, ja que no desencadenen respostes immunitàries en l'organisme.[19] No obstant presenten algunes desavantatges. El nombre estimat de BM MSC és extremadament baix, d'aproximadament 1 per cada $10^5$-$10^6$ cèl·lules nucleades de mèdul·la òssia, i la seva obtenció està associada amb dolor local i morbiditat en el lloc d'obtenció.[10,20,21] El baix rendiment cel·lular fa que sigui necessària una expansió *ex vivo* per obtenir unes dosis cel·lulars terapèutiques, el que consumeix temps, diners, i comporta risc de contaminació i pèrdua cel·lular.[22] A més d'això, el nombre de MSC també disminueix amb l'edat del pacient, d'una MSC per cada $10^4$ cèl·lules totals en neonats a 1 per cada $10^6$ cèl·lules totals en individus ancians.[23] Tot això porta cap a la recerca d'una font alternativa de cèl·lules mare per a aplicacions en teràpia basada en cèl·lules mare.

El teixit adipós, descobert més recentment, representa una font interessant de MSC, ja que està disponible en grans quantitats, la seva obtenció no requereix l'ús d'anestèsia general i resulta en un mínim discomfort pel pacient.[24] A més a més, el rendiment cel·lular del teixit és molt més elevat que en la mèdul·la òssia, el que fa que es pugui evitar l'expansió cel·lular *in vitro*.[25] La nomenclatura de les MSC de teixit adipós varia enormement, però es va arribar a un consens en la segona reunió anual de la Societat Internacional de Tecnologia Aplicada al Greix (IFATS) en el 2004, d'utilitzar l`acrònim d'ASC enlloc de cèl·lules mare estromals derivades de teixit adipós i cèl·lules mare derivades de teixit adipós, entre d'altres.[26]

El teixit adipós, com la mèdul·la òssia, deriva del mesoderm i conté una població heterogènia composta per adipocits, cèl·lules endotelials, pericits, fibroblasts, cèl·lules progenitores endotelials, cèl·lules progenitores vasculars pluripotents i ASC, a més d'un gran nombre de progenitors hematopoiètics.[27,28] Les ASC són capaces de diferenciar-se cap a múltiples tipus cel·lulars derivats del mesoderm, com condrocits, osteoblasts, adipocits, miocits, cardiomiocits i cèl·lules endotelials,[27,29] i hi ha evidència creixent de que presenten l'habilitat de donar lloc a cèl·lules d'altres llinatges, com l'ectoderm[30,31] i l'endoderm.[32,33] A més a més, el teixit adipós secreta una varietat de factors de creixement angiogènics i antiapoptòtics[34,35] i posseeix un potencial de vascularització més alt que les cèl·lules mare de mèdul·la òssia,[36] el que converteix el greix en una font prometedora per cirugia plàstica i reconstructiva. Per tot això el teixit gras seria una font interessant per utilitzar en teràpia cel·lular.

Per a enginyeria tisul·lar òssia, l'ús d'autoinjerts o teixit ossi obtingut del mateix pacient és considerat el més idoni, doncs és l'únic tipus d'injert ossi capaç d'abastir amb cèl·lules òssies essencials per participar en l'osteogènesi. Els autoinjerts es poden obtenir de diferents llocs i de diverses maneres. Al provindre del mateix individu, l'injert és reconegut com a propi i el sistema immunitari no inicia una resposta immunològica.[37] No obstant, l'ús d'autoinjerts presenta algunes desavantatges, com són la insuficient obtenció d'os, el dany en la zona donadora, el risc d'infecció, i la morbiditat al pacient. Altres enfocaments per reparar els defectes ossis, com són l'ús d'aloinjerts, os obtingut d'altres individus, xenoinjerts, os obtingut d'altres espècies, o l'ús de substituts biosintètics, estan limitats per la immunogenicitat i l'absència de cèl·lules en el material, i la manca de les propietats osteogèniques i osteoinductives que caracteritzen als autoinjerts.[38] Els substituts biosintètics com són el fosfat β-tricàlcic (β-TCP), la hidroxiapatita (HA), o les ceràmiques de fosfat càlcic

compostes de HA i β-TCP (fosfat bicàlcic o BCP), han sigut aplicades amb èxit en injerts ossis, degut a la seva biocompatibilitat i composició química, que s'assembla a la matriu òssia.[39-42] Per a tractar els defectes ossis, una prometedora alternativa és l'enginyeria tisul·lar, que combina els principis de bioenginyeria, trasplantament cel·lular i enginyeria de materials per a reparar i regenerar el teixit perdut.[38] En el camp de l'enginyeria tisul·lar òssia, les cèl·lules mare de teixit adipós o ASC han estat utilitzades amb èxit per reparar defectes esquelètics de tamany crític.[43,44,45] ASC sembrades en cubs de HA/β-TCP i implantades subcutàniament en conills, han demostrat formació d'osteoid a les 6 setmanes per immunohistoquímica i histologia.[46] Aquests resultats mostren l'ús satisfactori de les ASC en enginyeria tisul·lar. En medicina regenerativa, a més a més de les cèl·lules mare i els biomaterials adequats, és important l'ús de factors de creixement o d'estímuls inductius. Un dels principals factors de creixement són les proteïnes morfogenètiques òssies o BMPs, de les quals BMP-2 i BMP-7/OP-1 han estat aprovades per l'administració de l'alimentació i medicaments (FDA) per a l'ús mèdic. Les BMPs van ser primer descobertes en la matriu òssia desmineralitzada, i promouen la formació òssia endocondral.[47] Les BMPs promouen la diferenciació de les cèl·lules osteoprogenitores i indueixen l'osteogènesi en les cèl·lules mare mesenquimals.[48] La proteïna morfogenètica òssia-2 (BMP-2), que regula el desenvolupament, creixement, remodelament, i reparació òssia, pot promoure la proliferació i diferenciació de les cèl·lules mare i osteoblasts, i la curació intraòssia de defectes periodontals, a més a més de la curació de defectes òssis de tamany crític.[49-52] El mètode d'aplicació de les BMPs és de màxima importància en enginyeria tisul·lar òssia.[52] L'ús de BMP-2 amb biomaterials osteoinductius, a més a més de la transfecció adenoviral amb BMPs *ex vivo,* estan essent avaluats.[50,52-55] No obstant, hi ha preocupació en relació amb la seguretat en l'utilització d'adenovirus, degut a la imprevisible dinàmica en la producció de proteïnes en l'organisme i al comportament imprevisible de les cèl·lules transfectades.[56,57] A més a més, les altes dosis utilitzades en estudis clínics presenten efectes adversos, com sobrecreixement ossi i inflamació.[57,58] Prèviament s'ha demostrat que una incubació *ex vivo* amb BMP-2 a una concentració un mil·lió de vegades més baixa, de només 10 ng, durant només 15min és suficient per promoure la diferenciació osteogènica de les ASC.[2,59] Aquesta curta incubació podria encaixar fàcilment en un procediment quirúrgic d'una fase, en el qual les cèl·lules mare del propi pacient són aïllades, estimulades amb factors de creixement, i immediatament utilitzades per a regeneració òssia i del cartílag en el mateix pacient.

La bola adiposa de Bichat o "buccal fat pad" (BFP) és una massa de greix encapsulat que es troba a la cavitat oral distinta del greix subcutani.[60] Aquest teixit ha estat utilitzat amb èxit durant anys com a injert pediculat per a reconstruccions orals i tancament de comunicacions orosinusals,[61,62] una causa freqüent de complicació després d'extracció dentària i col·locació d'implants. Degut a la versatilitat d'aquest teixit, i als resultats exitosos obtinguts amb el seu ús en cirugia oral maxil·lofacial reconstructiva, vam sospitar que el teixit adipós obtingut de BFP podria ser una font excel·lent de MSC per a ús en enginyeria tisul·lar. L'obtenció de BFP és un procediment no complicat, que requereix una mínima incissió utilitzant anestèsia local, el que causa una mínima morbiditat al pacient i fa que sigui un teixit fàcilment accesible per a odontòlegs i cirugians orals. Per tant, seria interessant esbrinar la presència de cèl·lules mare en aquest teixit, doncs podria representar una font de fàcil accés per a odontòlegs i cirugians orals d'aplicació en enginyeria tisul·lar.

L'enfocament de la tesi es divideix en dues parts. La primera part anirà encaminada a determinar la presència de cel·lules mare mesenquimals en una font de fàcil accés per a cirugians orals, com és la bola adiposa de Bichat. Primerament es durà a terme l'aïllament i la caracterització de les cèl·lules mare obtingudes de boles adiposes de Bichat per citometria de fluxe, per a determinar si posseeixen característiques de MSC. Posteriorment es compararà el nombre i l'expressió de marcadors cel·lulars de superfície amb ASC obtingudes de teixit adipós subcutani abdominal (SC), per observar si hi ha diferències en les ASC obtingudes d'ambdós teixits. Finalment s'evaluarà la seva possible aplicació clínica per a enginyeria tisul·lar de defectes ossis i cartil·laginosos, estudiant el seu potencial de diferenciació *in vitro* a osteoblasts, condrocits i a adipocits. La segona part de la tesi pretén estudiar l'efecte del factor de creixement BMP-2 en ASC sembrades en diferents biomaterials de fosfat β-tricàlcic i fosfat β-tricàlcic amb hidroxiapatita, per determinar si una incubació de només 15min amb 10 ng/ml és suficient per estimular la proliferació i diferenciació osteogènica de les ASC, sense afectar la seva adhesió, en els diversos biomaterials, per a aplicacions clíniques immediates en pacients.

# OBJECTIUS

**Estudi 1: Bola adiposa de Bichat, una font de fàcil accés oral de cèl·lules mare de teixit adipós (ASC) humà amb potencial per enginyeria tisul·lar osteocondral.**

L'objectiu general és determinar si les boles adiposes de Bichat poden ser una font adecuada i de fàcil accés d'ASC, per a la seva util·lització en regeneració dels teixits mesodèrmics.

Els objectius específics són:

1) Aïllar les cèl·lules mare de bola adiposa de Bichat i cultivar-les *in vitro,* desenvolupant un protocol eficient i ràpid d'aïllament de cèl·lules mare de teixit adipós.

2) Determinar i comparar l'expressió de marcadors cel·lulars de superfície d'ASC obtingudes de teixit adipós subcutani abdominal i ASC obtingudes de bola adiposa de Bichat, a fi de determinar les similituds i diferències d'aquests dipòsits de greix.

3) Comparar el nombre d'ASC de bola adiposa de Bichat amb el nombre d'ASC de teixit adipós subcutani abdominal a la setmana de cultiu *in vitro*.

4) Determinar el percentatge d'ASC fresques obtingudes de bola adiposa de Bichat per a possibles aplicacions clíniques immediates.

5) Diferenciar les ASC de bola adiposa de Bichat a adipocits, osteoblasts i condrocits, per estudiar el seu potencial de diferenciació multipotent.

**Estudi 2: Diferenciació osteogènica d'ASC estimulades amb proteïna òssia morfogenètica-2 (BMP-2) i sembrades en biomaterials de fosfat bicàlcic i fosfat β-tricàlcic.**

L'objectiu general és analitzar l'efecte de BMP-2 sobre l'adhesió, proliferació i diferenciació osteogènica de les ASC sembrades en diferents biomaterials de fosfat càlcic, per a aplicacions en enginyeria tisul·lar òssia.

Els objectius específics són:

1) Analitzar l'efecte de BMP-2 en l'adhesió d'ASC sembrades en fosfat bicàlcic i en fosfat β-tricàlcic.

2) Analitzar l'efecte de BMP-2 en la proliferació d'ASC sembrades en fosfat bicàlcic i en fosfat β-tricàlcic.

3) Analitzar l'efecte de BMP-2 en la diferenciació osteogènica d'ASC sembrades en fosfat bicàlcic i en fosfat β-tricàlcic.

**ESTUDI 1: BOLA ADIPOSA DE BICHAT, UNA FONT DE FÀCIL ACCÉS ORAL DE CÈL·LULES MARE DE TEIXIT ADIPÓS HUMÀ AMB POTENCIAL PER ENGINYERIA TISUL·LAR OSTEOCONDRAL.**

## Materials i mètodes

### Aïllament de cèl·lules mare de teixit adipós

Durant l'estudi realitzat s'han obtingut mostres de teixit adipós subcuntani abdominal i teixit adipós de boles de Bichat de diversos pacients sans sotmesos a cirugia abdominal i ortognàtica a l'Hospital Clínic i Hospital Sant Joan de Deu de Barcelona, després d'obtenir el Consentiment informat aprobat pel Comitè Ètic de la Universitat Internacional de Catalunya (veure annexes). S'ha util·litzat BFP de 10 pacients (17-40 anys) i teixit adipós SC de 4 pacients (19-57 anys). S'han processat les mostres de teixit adipós d'acord amb un protocol d'extracció de cèl·lules mare de teixit adipós abdominal posat a punt amb mostres de rata i presentat prèviament a la tesina (Elisabet Farré Guasch, Universitat Internacional de Catalunya, Juliol 2008). Per a l'obtenció de cèl·lules mare de teixit adipós s'ha util·litzat un protocol modificat d'un procediment prèviament util·litzat.[27] Teixit adipós cru de BFP (16 ± 4.1 ml) i SC (22 ± 12.2 ml) (promig ± SD) és rentat diverses vegades amb tampó fosfat salí (PBS) i disgregat en petits fragments, per digerir-se posteriorment amb 0.075% col·lagenasa A durant 60 minuts a 37ºC de temperatura amb agitació intermitent. Després de la digestió enzimàtica, el teixit és centrifugat durant 10 minuts a 400xg per separar els adipocits de la fracció vascular estromal (SVF), que conté les ASC. El pellet cel·lular obtingut és resuspès en sol·lució de llisi de cèl·lules sanguínies compost per 8.2 g/l $NH_4Cl$, 0.84 g/l $NaHCO_3$, 0.37 g/l disodium EDTA a pH 7.4 i a continuació incubat durant 10min a temperatura ambient. Després de centrifugar la SVF és resuspesa en medi Dulbbeco's Modified Eagle's medium (DMEM) baix en glucosa (lg), que conté un 10% de sèrum boví fetal (FBS) i 100 unitats/ml d'antibiòtics i antimicòtics. Les cèl·lules són filtrades per una malla de 100 μm i s'evalua la viabilitat cel·lular amb la prova de viabilitat per tinció amb blau de tripà. Posteriorment són sembrades 5 x $10^3$ cèl·lules/$cm^2$ en discs de plàstic de 100 mm i són cultivades en un incubador humidificat a 37ºC i 5% $CO_2$. Les mostres de teixit adipós de BFP són processades de 4 maneres diferents, per a desenvolupar un protocol eficient d'aïllament d'ASC per a futures aplicacions clíniques immediates. #1: el teixit adipós disgregat és digerit durant 60min amb col·lagenasa i centrifugat a 400xg 10min, per separar la SVF que conté les ASC dels adipocits, i finalment és filtrat; #2: el teixit adipós disgregat és passat 5 vegades a través d'una

cànula de plàstic conectada a dues xeringues per a disgregar el teixit mecànicament, i a continuació és centrifugat i filtrat tal com s'ha descrit prèviament; #3: després de disgregar-se el teixit adipós és centrifugat i filtrat, omitint la digestió enzimàtica i la disgregació mecànica; #4: l'últim protocol consisteix en omitir la digestió enzimàtica i la disgregació mecànica, a més d'omitir la posterior centrifugació; el teixit disgregat és filtrat per una malla de 100 μm i posteriorment filtrat amb una malla de 40 μm. La concentració de col·lagenasa i el temps d'incubació també són estudiats, anal·litzant si una reducció a la meitat de la concentració i una reducció en el temps d'incubació poden afectar la proliferació cel·lular. Després de 3-4 dies de cultiu s'observen colònies al microscopi, i es considera a aquesta població cel·lular inicial com a passatge 0 (P0). Després d'una setmana de cultiu les cèl·lules adherents de BFP (n=5) i de SC (n=4) són contades al microscopi òptic, i el nombre de cèl·lules per gram de teixit adipós és calculat per comparar el nombre d'ASC obtingut en ambdós teixits. Les cèl·lules en passatge 2 són recollides i sembrades en medis de diferenciación a adipocits, osteoblasts i condrocits, tal com ha estat descrit en estudis anteriors.[27] Les cèl·lules són diferenciades a adipocits i osteoblasts durant 3 setmanes, i a condrocits durant 4 setmanes. Com a control s'han util·litzat adipocits, una línia cel·lular d'osteoblasts (SAOS-2) i condrocits obtinguts de nucli pulpós.

**Citometria de fluxe**

S'han anal·litzat per citometria de fluxe teixit adipós fresc de BFP (n=5) i ASC obtingudes de BFP (n=5) i SC (n=4) cultivades a diferents temps de creixement (P0= setmana de cultiu fins P5= mes de cultiu), per l'expressió de marcadors de superfície CD14, CD19, CD34, CD45, CD29, CD73, SSEA, CD90, CD146, HLA-DR i CD105, per comparar l'expressió de marcadors de superfície i percentatge d'ASC obtingudes en ambdós teixits. Aliquotes de cèl·lules incubades amb immunoglobulines (IgGs) s'han util·litzat com a control negatiu per l'assaig. El teixit adipós fresc de BFP s'ha anal·litzat per citometria de fluxe per a determinar el percentatge d'ASC que es troba en aquest teixit.

**Diferenciació a adipocits, osteoblasts i condrocits**

Després de caracteritzar la població de cèl·lules mare de BFP i determinar el percentatge obtingut en teixit fresc per a utilització en regeneració tisul·lar i el contatge cel·lular, i després de comparar l'expressió de marcadors al llarg del cultiu amb teixit adipós subcutani abdominal, s'ha procedit a diferenciar les cèl·lules amb medis amb vitamines i factors de

creixement, per a induir-les a diferenciar a cèl·lules del llinatge osteoblàstic, condrogènic i adipogènic. S'han realitzat diverses probes com tincions característiques d'adipocits (Oil Red), d'osteoblasts (Alizarin Red) i de condroblasts (Blau de Toluidin), a més de probes immunohistoquímiques per a marcadors característics d'osteoblasts (osteocalcina) i condroblasts (col·lagen II) i assaig enzimàtic per determinar l'expressió de fosfatasa alcalina (ALP), enzim secretat per osteoblasts al començar a mineralitzar la matriu extracel·lular. Finalment s'han realitzat estudis de real-time PCR quantitativa, per a determinar l'expressió de gens característics d'adipocits (receptor gamma dels peroxisomes proliferador-activat o PPARγ), d'osteoblasts (CBFA1 i osteonectina, ON o SPARC) i condroblasts (SOX-9).

**Anàl·lisi estadístic**

Els valors promig es van expressar com a promig ± SD, o bé com a promig ± SEM, indicat en la llegenda de les figures.

Les diferències en l'expressió de marcadors de superfície per citometria de fluxe entre les dues fonts d'ASC, l'expressió d'ALP i l'expressió dels gens PPARγ, CBFA1, SPARC i SOX-9 en ASC de BFP cultivades en medi osteogènic, condrogènic i adipogènic, van ser analitzades estadísticament amb una prova estadística no paramètrica, la prova de Mann-Whitney, i les diferències es van considerar significatives si $p<0.05$. Es van analitzar les dades amb el programa estadístic SPSS 17.0.

# Resultats i discussió

## 1. Aïllament de cèl·lules mare de teixit adipós

### 1.1. El protocol que inclou una digestió enzimàtica és efectiu per aïllar les cèl·lules mare de teixit adipós de bola adiposa de Bichat

Comparant els diferents mètodes d'aïllament d'ASC, només util·litzant el mètode #1 es van observar cèl·lules adherides amb morfologia allargada semblant a fibroblastes, característic d'ASC, a les 48h de cultiu (Fig. IA). En canvi no es va observar la presència d'aquestes

cèl·lules amb la resta de mètodes d'aïllament (Fig. IB-ID), indicant que la digestió enzimàtica amb col·lagenasa és fonamental per l'aïllament d'ASC.

48h

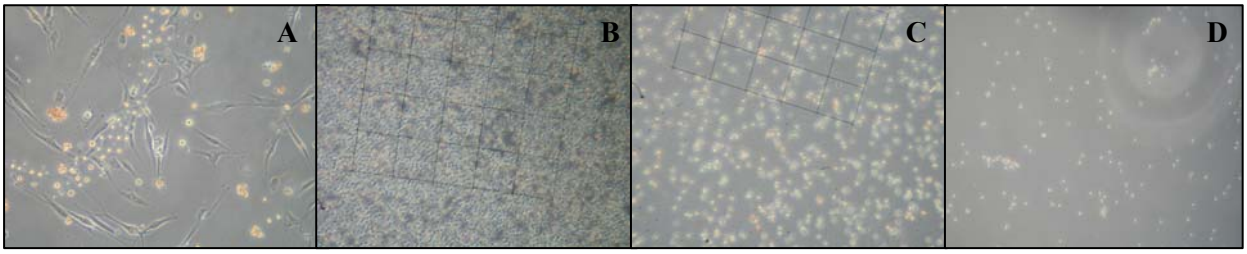



**Fig. I: Aïllament de cèl·lules mare de teixit adipós (ASC) després de 48 h de cultiu:** Es va observar la presència de cèl·lules allargades amb morfologia fibroblàstica adherides a plàstic de cultiu després de l´aïllament utilitzant el protocol #1 **(A)**. No es va observar la presència d´aquestes cèl·lules amb aquesta morfologia característica d´ASC utilitzant els protocols #2 **(B)**, #3 **(C)** i #4 **(D)**. Mirar Materials i Mètodes per descripció més detallada dels protocols. Magnificació 100x.

Després de 4 dies de cultiu encara es van observar més cèl·lules adherents amb morfologia d'ASC utilitzant el protocol #1 (Fig. IIA), indicant l'eficàcia del protocol que inclou la digestió enzimàtica amb col·lagenasa. En canvi no es van observar cèl·lules amb morfologia d'ASC en la resta de protocols que no havien inclòs la digestió enzimàtica amb aquest enzim (Fig. IIB-IID).

4d

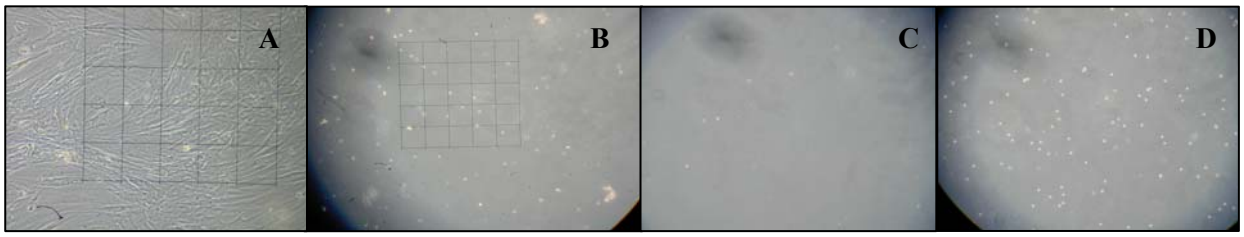



**Fig. II: Aïllament d'ASC després de 4 dies de cultiu. A)** Es va observar la presència de cèl·lules amb morfologia d'ASC després de l'aïllament utilitzant col·lagenasa, posterior centrifugació i filtració cel·lular a través d'una malla. **B,C** i **D)** No es va observar la presència d'aquestes cèl·lules en la resta de protocols que no havien inclòs la digestió enzimàtica en el procediment d'aïllament. Magnificació 100x.

### 1.2. Una reducció del 50% en el temps d'incubació amb col·lagenasa no afecta la proliferació de cèl·lules mare de teixit adipós en cultiu

Després de 24h de cultiu es va observar la presència de cèl·lules amb morfologia allargada característica d'ASC en plàstic de cultiu (Fig. IIIA). Es va observar un nombre similar d'ASC després de reduir la digestió amb col·lagenasa al 50% (Fig. IIIB). Cèl·lules no adherents arrodonides (fletxes) amb morfologia de cèl·lules hematopoiètiques també eren presents en

ambdós cultius, desapareixent amb els successius passatges cel·lulars. Als 8 dies de cultiu es va observar un nombre similar d'ASC en ambdós cultius cel·lulars (Fig. IV).

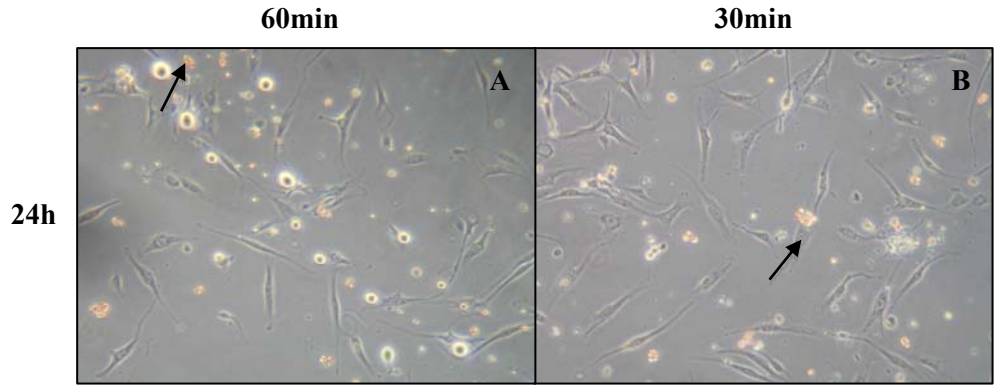



**Fig. III: ASC a les 24h de cultiu després d'utilitzar el protocol de digestió amb col·lagenasa durant 60min i 30min. A)** Després de la digestió amb col·lagenasa durant 60min, es va observar la presència d'algunes cèl·lules adherents amb característiques d'ASC, a més a més de cèl·lules no adherents arrodonides amb morfologia de cèl·lules hematopoiètiques (fletxes). **B)** Després de la reducció en l'incubació amb col·lagenasa a 30min, es va observar també la presència d'aquestes cèl·lules amb característiques de cèl·lules mare de teixit adipós. Magnificació 100x.

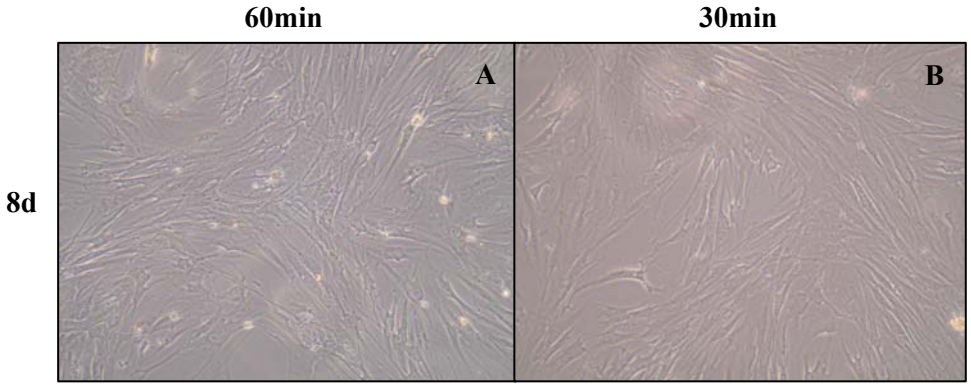



**Fig. IV: ASC als 8 dies de cultiu després d'utilitzar el protocol de digestió amb col·lagenasa durant 60min i 30min. A,B)** Es va observar un nombre similar d'ASC utilitzant ambdos procediments, indicant que es podria disminuir el periode d'incubació sense afectar les ASC. Magnificació 100x.

### 1.3. Una reducció en la concentració de col·lagenasa a la meitat afecta la proliferació de cèl·lules mare de teixit adipós en cultiu

Després de reduir la concentració de col·lagenasa a la meitat es va observar un nombre més baix de cèl·lules adherents després de 24h de cultiu (Fig. V) i als 8d de cultiu (Fig. VI).

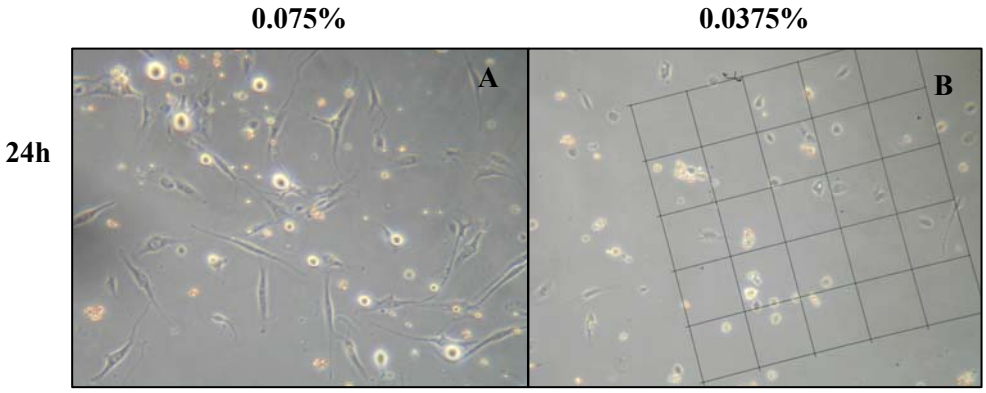



**Fig. V: ASC obtingudes amb protocol de digestió amb col·lagenasa 0.075% i 0.0375 després de 24h de cultiu. A,B)** Un nombre inferior de cèl·lules mare de teixit adipós era present en cultiu després de reduir la concentració de col·lagenasa a la meitat. Magnificació 100x.

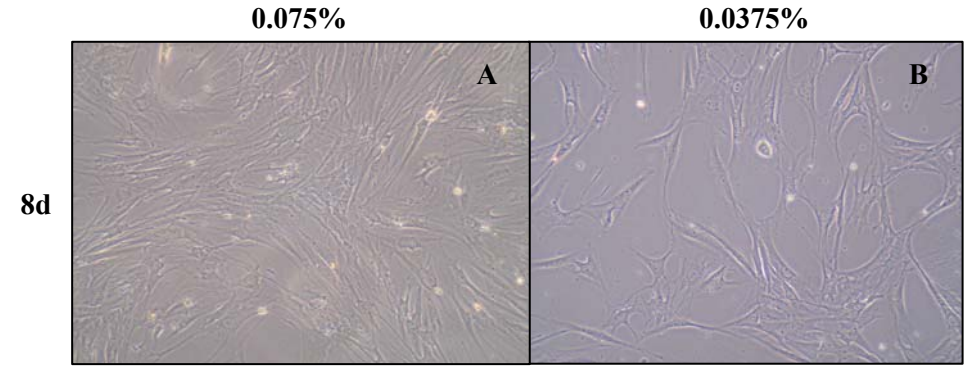



**Fig. VI: ASC obtingudes amb protocol de digestió amb col·lagenasa 0.075% i 0.0375 després de 8 dies de cultiu. A,B)** Un nombre inferior de cèl·lules mare de teixit adipós era present en cultiu després de reduir la concentració de col·lagenasa a la meitat. Magnificació 100x.

Una reducció del temps i la concentració de col·lagenasa va afectar el nombre d'ASC i la seva morfologia, i va semblar induir les ASC a la diferenciació adipogènica (Fig. VII).

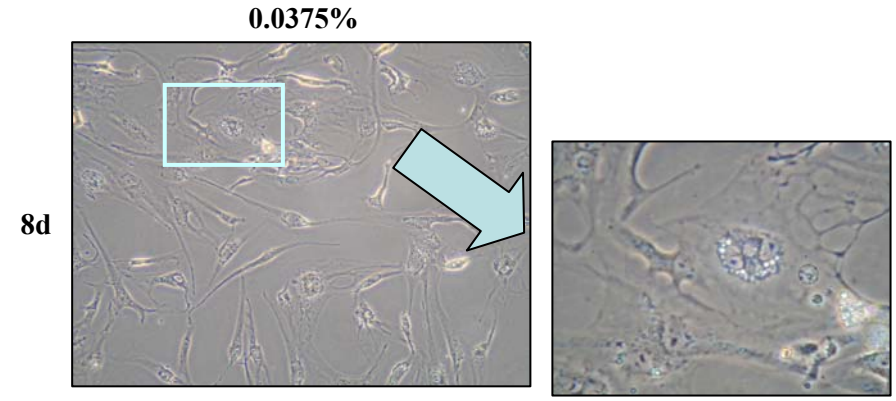



**Fig. VII: ASC obtingudes amb protocol de digestió amb col·lagenasa al 0.0375 mostren diferenciació adipogènica de les ASC.** Es va observar la presència d'adipocits entre les ASC després de reduir la concentració a la meitat, indicant la pèrdua de potencial d'ASC. Magnificació 100x.

## 2. Caracterització de cèl·lules mare de teixit adipós

### 2.1. La bola adiposa de Bichat conté cèl·lules progenitores amb característiques de cèl·lules mare de teixit adipós

Es va extirpar BFP de pacients sans sotmesos a cirugia ortognàtica (n=10), a fi d'estudiar si BFP conté cèl·lules amb característiques d'ASC. La SVF es va aïllar utilitzant el protocol amb digestió enzimàtica amb col·lagenasa 0.075% durant 60min. Es van analitzar ASC fresques i ASC cultivades per a determinar l'expressió de marcadors de superfície, i determinar si ASC de BFP comparteixen característiques amb ASC derivades d'una altra font de greix com és el greix subcutani abdominal. Després d'una setmana de cultiu de les ASC obtingudes de SC (n=4) i de BFP (n=5), es va realitzar el contatge cel·lular, i es va calcular el nombre d'ASC per gram de teixit adipós.

### 2.2. Morfologia de cèl·lules mare de teixit adipós cultivades de dues fonts de greix diferents

L'estudi pretén analitzar les cèl·lules progenitores derivades d'una font de greix oral després de varis pasatges cel·lulars, i comparar-les amb una altra font de teixit adipós, com és el greix subcutani abdominal. Primer vam determinar si les ASC de BFP són similars a les ASC de teixit adipós subcutani. Després de la digestió amb col·lagenasa i la posterior separació dels adipocits per centrifugació, vam procedir a cultivar ambdós tipus cel·lulars.

Es va observar la presència d'algunes cèl·lules adherents a les 48h després de la sembra amb visualització al microscopi (Fig. VIII, imatge superior). A més a més d'aquesta població cel·lular, unes escasses cèl·lules no adherents eren presents en cultiu (fletxes). Les cèl·lules d'ambdós teixits van romandre en una fase quiescent (adormida) durant 2-4 dies, i posteriorment van començar a multiplicar-se ràpidament, arribant a confluència com una capa de cèl·lules allargades i aplanades. Després de 7 dies (Fig. VIII, imatge inferior) les ASC d'ambdós teixits van mostrar una morfologia similar, amb morfologia fibroblàstica, característica d'ASC. En aquest moment les cèl·lules van arribar a un 90% de confluència i es va realitzar el passatge cel·lular.

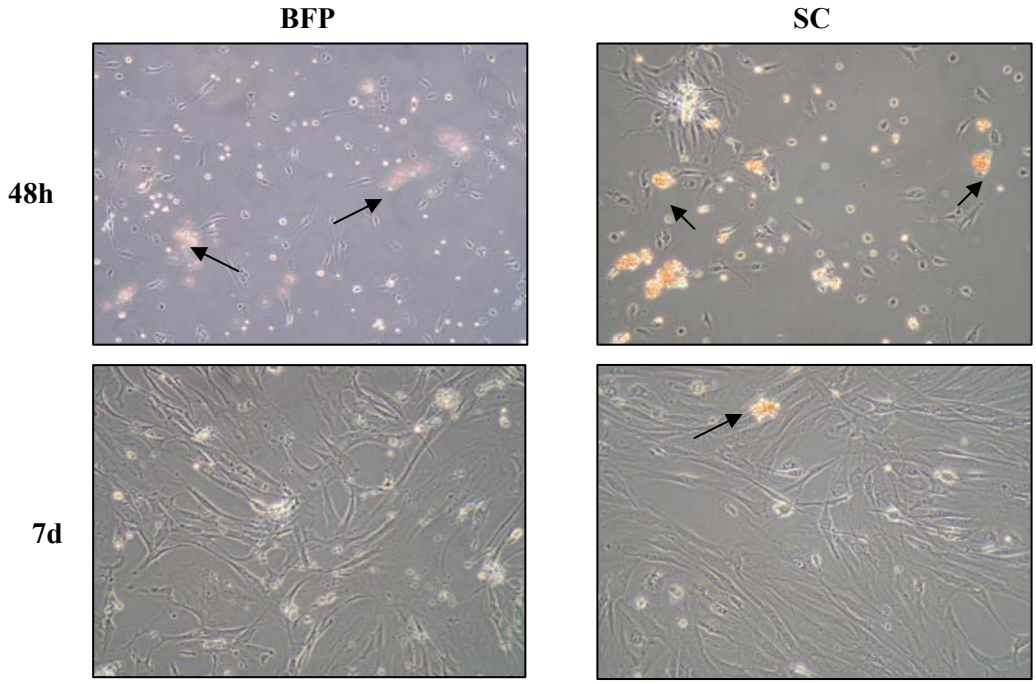



**Fig. VIII: Comparació de la morfologia d'ASC derivades de bola adiposa de Bichat (BFP) i teixit adipós subcutani abdominal (SC).** La morfologia de les cèl·lules aïllades de BFP o SC al microscopi a les 48h (imatges superiors) i als 7 dies (imatges inferiors). La morfologia de les ASC d'ambdós teixits era similar. A les 48h, havia una abundant presència de cèl·lules hematopoiètiques (fletxes) i cèl·lules amb morfologia fibroblàstica. La població als 7 dies de cultiu era homogènia i les cèl·lules hematopoiètiques escases o absents. Magnificació 100x.

## 2.3. Comparació de l'expressió de marcadors cel·lulars de superfície entre cèl·lules mare de teixit adipós de bola adiposa de Bichat i de teixit subcutani abdominal

Comparant l'expressió de marcadors característics de cèl·lules mare de teixit adipós en ASC en cultiu fins al mes de cultiu, s'observa que ASC de BFP sembrades en cultiu expressen CD73, CD90, CD29 i CD105, i no expressen CD34, CD45, CD14, HLA-DR, CD19 (Taula I), d'acord amb el Criteri Mínim de la Societat Internacional de Teràpia Cel·lular,[14] ni expressen CD146 o SSEA, marcadors característics de cèl·lules progenitores endotelials o vasculars.[63]

| Marcador | P0 | P2 | P5 | Nombre pacients |
|---|---|---|---|---|
| CD105 | 89.1± 13.6 | 99.5 ± 0.8 | 99.1± 0.2 | n=3 |
| CD90 | 75.2 ± 18.3 | 80.8 ± 12 | 83.4 ± 18.3 | n=4 |
| CD73 | 84.7 ± 16.4 | 97.2 ± 1.3 | 99.1 ± 0.4 | n=4 |
| CD29 | 96.4 ± 2.4 | 98.6 ± 0.9 | 99.3* | n=3 |
| CD34 | 32.1 ± 20.1 | 12.7 ± 1.4 | 7.5 ± 4.5 | n=3 |
| CD45 | 1.3 ± 1.3 | 0.4 ± 0.5 | 0.5 ± 0.6 | n=4 |
| CD14 | 1.6 ± 2.2 | 6.6 ± 7.4 | 0.5 ± 0.3 | n=4 |
| HLA-DR | 2 ± 2.3 | 1.1 ± 1.4 | 0.9 ± 1 | n=3 |
| CD19 | 0.9 ± 0.9 | 1.4 ± 2 | 1.9 ± 2.2 | n=3 |
| CD146 | 1.1 ± 1.4 | 0.3 ± 0.1 | 0.1* | n=3 |
| SSEA | 1.8 ± 1.7 | 0.4 ± 0.1 | 0.1* | n=3 |

**Taula I: Percentatge de l'expressió de marcadors de cèl·lules mare i de cèl·lules endotelials i hematopoiètiques d'ASC de bola adiposa de Bichat en diferents passatges de cultiu.** Les cèl·lules es van analitzar als 7d de cultiu (P0), en passatge 2 (P2) i en passatge 5 (P5) de cultiu. Per a l'expressió de CD29, CD146 i SSEA en P5 es van analitzar cèl·lules d'un pacient (*). Els resultats es mostren en promig ± SD del percentatge de cèl·lules expressant un marcador.

Es va observar la presència de poblacions contaminants en passatges inicials de les ASC derivades de BFP (Fig. IXA) i SC (Fig. IXB), que va desaparèixer en posteriors passatges cel·lulars. Es va observar també la presència d'una població CD146+CD29+ en passatges inicials d'ASC de BFP, absent en ASC obtingudes de teixit adipós subcutani abdominal. CD146 és un marcador característic de cèl·lules progenitores endotelials, que poden trobar-se presents degut a la profusa microvascularització d'aquest teixit adipós.

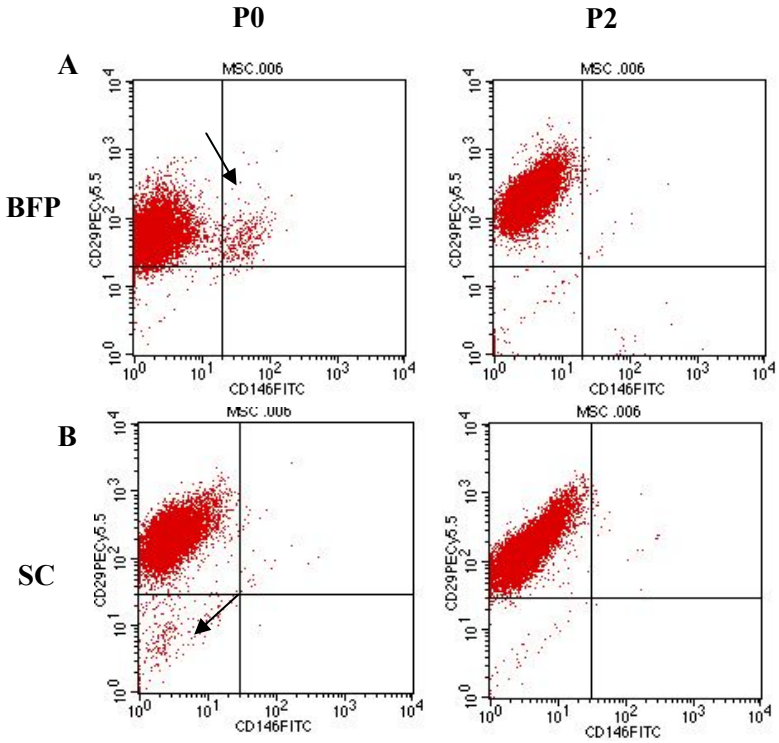



**Fig. IX: Presència d'una població contaminant en teixit adipós.** Imatges en núvol de punts de citometria de fluxe en passatge 0 (P0) i passatge 2 (P2), mostrant ASC derivades de bola adiposa de Bichat (BFP) **(A)** i teixit adipós subcutani abdominal (SC) **(B)**. La presència de poblacions contaminats (fletxa) era present en passatge 0 (P0), que representa els 7 dies de cultiu en plàstic, però va desaparèixer en passatge 2 (P2) revelant la purificació del cultiu amb el successiu passatge cel·lular.

ASC de BFP (en blau) i ASC de SC (en vermell) van mostrar una expressió de marcadors de cèl·lules mare similar en P0 i P2 de cultiu (Fig. XA). L'alta expressió de CD73, CD90, CD105 i CD29 és característic d'ASC, i es va observar a la setmana de cultivar les ASC d'ambdues fonts de teixit adipós (P0). Després de dues setmanes de cultiu de les ASC (P2), el percentatge de cèl·lules que expressaven marcadors característics d'ASC es va incrementar en ambdues fonts d'ASC, mostrant una expressió similar de marcadors de cèl·lules mare. No obstant, es va observar una expressió different en el marcador angiogènic CD34 (Fig. XB), marcador característic de cèl·lules hematopoiètiques i endotelials, a la setmana de cultiu (P0). ASC de BFP mostraven una expressió més alta d'aquest marcador (32.1 ± 20.1) en comparació amb ASC de SC, que expressaven CD34 en un percentatge més baix (24.7 ± 9.5). En P2 es va observar una diferència significativa en l'expressió de CD34 (test de Mann-Whitney, $p<0.05$) entre ambdues fonts. L'expressió de CD34 era 5 vegades més alta en ASC derivades de BFP que en ASC derivades de SC (12.4 ± 1.7 vs 2.5 ± 0.8). Encara que

l'expressió de CD34 en ASC de BFP va disminuir amb el temps en cultiu, va romandre encara un 7.5% al mes de cultiu. L'alta expressió de CD34 podria tenir efectes positius en aplicacions de regeneració òssia, on l'angiogènesis i la formació de vasos sanguinis és vital.[64,65,66]

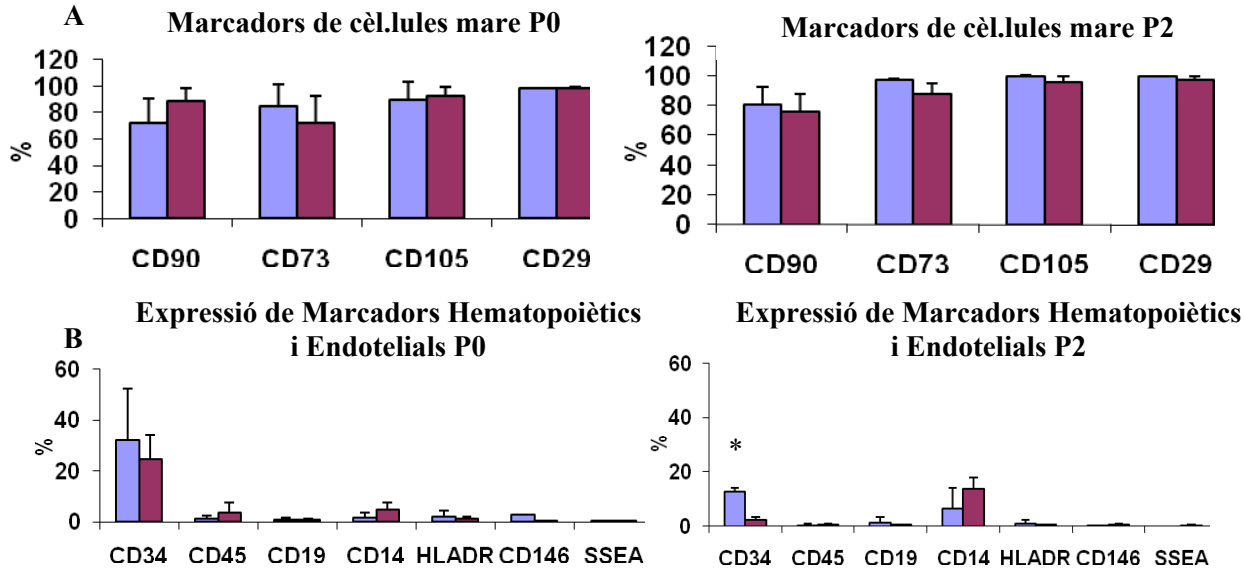



**Fig. X: Comparació del fenotip d'ASC derivades de bola adiposa de Bichat (BFP) i teixit adipós subcutani abdominal (SC).** El perfil d'expressió de marcadors de superfície d'ASC de BFP en blau (n=4) i ASC de teixit adipós subcutani abdominal en vermell (n=4) va ser analitzat per citometria de fluxe en P0 i P2. **A)** Expressió de marcadors de cèl·lules mare. **B)** Expressió de marcadors de cèl·lules hematopoiètiques i endotelials. L'expressió de marcadors de cèl·lules mare, cèl·lules hematopoiètiques i endotelials és similar en ambdues fonts de teixit adipós, excepte en l'expressió de CD34, molt més alta en ASC de BFP que en ASC de SC. * Diferència significativa en l'expressió del marcador de superfície entre BFP i SC ($p<0.05$).

## 2.4. Comparació del rendiment de cèl·lules mare de teixit adipós de dues fonts de greix diferents: bola adiposa de Bichat i teixit subcutani abdominal

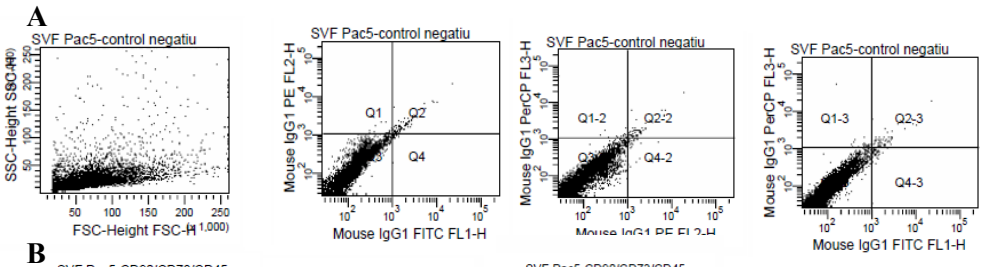
El nombre de cèlules mare obtingudes de pacients sotmesos a cirugia oral o abdominal per gram de teixit adipós és expressat en la Taula II. L'edat mitja és de 19.6 anys en pacients sotmesos a cirugia oral (n=5, 18-22 anys) i de 43.3 anys en els pacients sotmesos a cirugia abdominal (n=4, 19-57 anys). El promig d'ASC de BFP a la setmana de cultiu era de 513 ± 227 x $10^3$ cells per gram de teixit adipós, mentres que el nombre d'ASC de SC era de 253 ± 56 x $10^3$ cells per gram de teixit adipós, nombre similar al nombre d'ASC observat en altres estudis.[26] Aquest resultat indica que el nombre d'ASC de BFP dobla el nombre d'ASC obtingudes de teixit adipós subcutani abdominal.

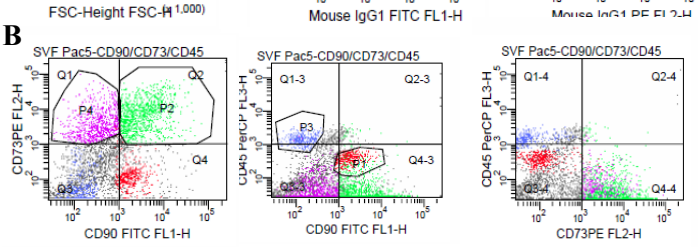
| Pacient | Font | Edat (anys) | Volum del greix (ml) | Pes del greix (g) | Nombre cel·lular | Dies de cultiu | Nombre cel·lular/ g | X± SEM |
|---|---|---|---|---|---|---|---|---|
| Home | BFP | 18 | 14 | 12 | 3.800.000 | 7 | 316.667 | |
| Home | BFP | 20 | 23 | 18.2 | 6.615.000 | 7 | 363.462 | |
| Dona | BFP | 22 | 16 | 13.6 | 11.679.999 | 7 | 858.823 | 513 ± |
| Dona | BFP | 20 | 15 | 14.5 | 9.075.000 | 7 | 625.862 | 227 |
| Dona | BFP | 18 | 12.5 | 11.9 | 4.780.000 | 7 | 401.681 | |
| Dona | SC | 19 | 14 | 12.2 | 2.339.000 | 7 | 191.721 | |
| Dona | SC | 47 | 36 | 29 | 9.045.000 | 7 | 311.897 | 253 ± |
| Dona | SC | 50 | 16 | 13.2 | 2.940.000 | 7 | 222.727 | 256 |
| Dona | SC | 57 | 5.6 | 5.5 | 1.579.999 | 7 | 287.273 | |

**Taula II: Nombre cel·lular en P0 obtingut de dues fonts diferents de teixit adipós: bola adiposa de Bichat (BFP) i teixit adipós subcutani abdominal (SC).** El nombre de cèl·lules és expressat per gram de teixit adipós a la setmana de cultiu.

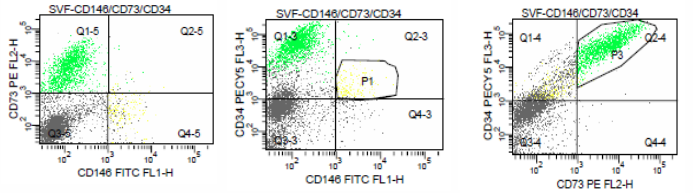
### 2.5. La fracció estromal de bola adiposa de Bichat és una font rica en ASC

El teixit adipós és pot dividir en dues fraccions, una que conté els adipocits madurs, i una altra fracció anomenada fracció estromal vascular que conté una població heterogènia de cèl·lules. La SVF de BFP no ha estat fins ara caracteritzada. Per determinar el percentatge d'ASC en BFP es va analitzar la SVF de 5 pacients (19-29 anys) i es va determinar la coexpressió de diversos marcadors per citometria de fluxe per quantificar el percentatge d'ASC fresques. La SVF de BFP conté una mescla de cèl·lules, tal com es mostra amb imatges en núvols de punts representatives de diferents pacients i amb percentatges de coexpressió de marcadors (Fig. XI).

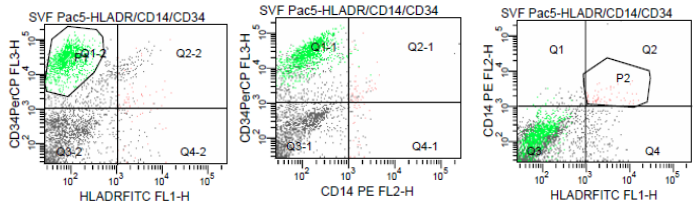


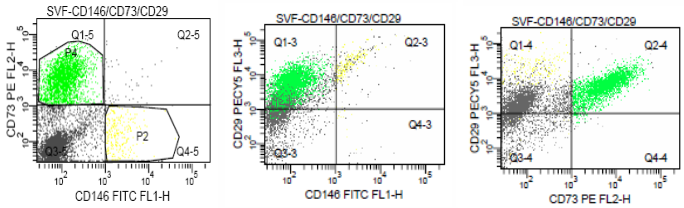

**Regió P2(CD90+CD73+CD45-) = 23.7%**



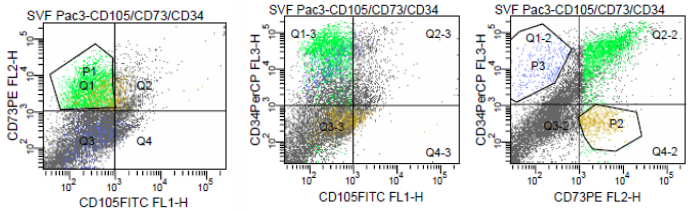

**Regió P3(CD146-CD73+CD34+) = 26%**




**Regió P1(CD34+CD14-HLA-DR-) =28.6%**




**Regió P4(CD146-CD73+CD29+) = 32.6%**




**Regió P1(CD105-CD73+CD34+) = 18.3%**

**Fig. XI: Citometria de fluxe d´ASC fresques obtingudes de bola adiposa de Bichat, analitzades amb software FACS DIVA. A)** Població general i controls d´isotip de diferents fluorocroms.Les mostres es van analitzar amb citometria de fluxe utilitzant forward scatter (FSC) per a detectar el tamany i side scatter (SSC) per detectar la granularitat. **B)** Poblacions cel·lulars mixtes van ser observades en la fracció estromal vascular (SVF), analitzant la co-expressió de 3 marcadors característics de 3 pacients representatius. S´han marcat en verd les cèl·lules que expressen marcadors característics d´ASC fresques. Cèl·lules endotelials, hematopoiètiques, fibroblastes, i cèl·lules progenitores endotelials i vasculars es van marcar respectivament en lila, blau, vermell i groc.

La SVF conté un alt percentatge de cèl·lules (21-38%) que expressen els marcadors CD90, CD73, CD29 i CD34, i que no expressen els marcadors hematopoiètics CD45, CD19, CD14, HLA-DR ni CD146 (Taula III). A més a més, aquesta població cel·lular expressa CD34, però no expressa el marcador CD105. Aquest perfil fenotípic és característic d'ASC fresques, mentres que el cultiu d'ASC en plàstic incrementa l'expressió de CD105 mentres que redueix l'expressió de CD34.[67,68] L'expressió de CD34 en ASC contrasta amb BM MSC, que no expressen aquest marcador.[69] Les cèl·lules que expressen CD34 estan relacionades amb l'angiogènesis, i intervenen en processos de neovascularització que faciliten la curació de teixits isquèmics.[70]

Aquests resultats demostren que la SVF de BFP és una font rica d'ASC (~30%), similar amb altres fonts de teixit adipós (~22%),[71] per tant BFP seria una font d'ASC idònia per a aplicacions clíniques.

| Marcadors | % ASC en SVF (Promig ± SD) | Nombre pacients |
|---|---|---|
| CD90+/CD73+/CD45- | 27.2 ± 13.9 | 4 |
| CD90+/CD73+/CD34+ | 21.5 ± 13.9 | 5 |
| CD105-/CD73+/CD34+ | 21.3 ± 14.3 | 5 |
| CD105-/CD19-/CD34+ | 38.1 ± 18.3 | 2 |
| HLA-DR-/CD14-/CD34+ | 26.5 ± 2.9 | 2 |
| CD146-/CD73+/CD34+ | 21.4 ± 6.4 | 2 |
| CD146-/CD73+/CD29+ | 23.9 ± 12.2 | 2 |

**Taula III. Percentatge promig d'ASC en la fracció estromal vascular (SVF) de bola adiposa de Bichat, analitzat per citometria de fluxe.** Les mostres (n=5) es van incubar amb 9 marcadors i es van analitzar per la co-expressió de 3 marcadors cel·lulars de superfície per citometria de fluxe.

## 3. Potencial de diferenciació multipotent in vitro de cèl·lules mare de teixit adipós de bola adiposa de Bichat

### 3.1. Diferenciació osteogènica

Per valorar el potencial de diferenciació osteogènica de les ASC de BFP, l'activitat d'ALP, marcador primerenc de diferenciació osteoblàstica, es va determinar després de 0, 7, i 21 dies

de cultiu en medi osteogènic. Cèl·lules SAOS-2 es van utilitzar com a control positiu. Després de la setmana de cultiu, les ASC van passar d'una morfologia fibroblàstica a una morfologia més poligonal, a més de mostrar increment en l'activitat d'ALP fins al dia 21 (Fig. XII). Les ASC cultivades en medi osteogènic van mostrar un increment de 2.5 vegades l'activitat ALP al dia 7, i un increment significatiu en l'activitat d'ALP de 16.5 vegades al dia 21 comparat amb el control (test de Mann-Whitney, $p<0.05$).

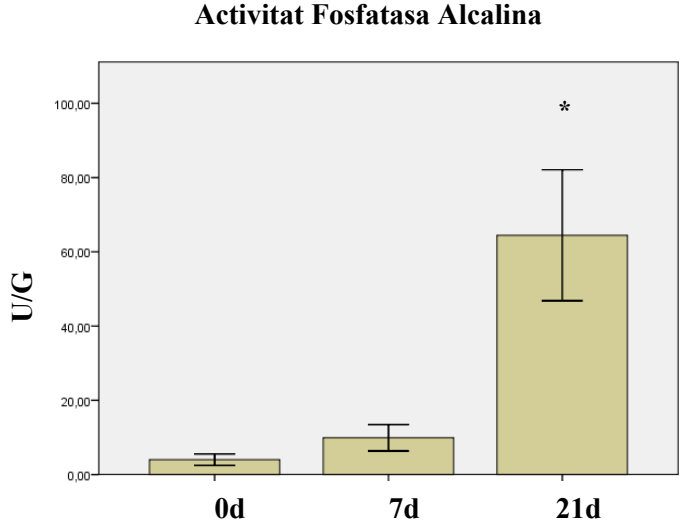



**Fig. XII: Activitat fosfatasa alcalina (ALP) en ASC de bola adiposa de Bichat**. Representació gràfica d'activitat fosfatasa alcalina en unitats per gram (U/G) en ASC després de 0, 7, i 21 dies d'inducció osteogènica. Els valors són representats en promig ± SEM. L'activitat ALP de cèl·lules SAOS-2 cells (control positiu) era de 672.7 U/G ± 125.5 (resultats no mostrats) i l'activitat ALP en ASC era de 3.9 U/G ± 1.2 als 0 dies, 9.9 U/G ± 3.5 als 7 dies i 64.4 U/G ± 17.6 als 21 dies de diferenciació osteogènica. * Diferència significativa d'activitat fosfatasa alcalina entre els 0 dies i els 21 dies ($p<0.05$).

Les ASC cultivades en medi osteogènic van mostrar àrees d'alta densitat granular després de 2 setmanes. Aquestes àrees es van tenyir intensament amb Alizarin Red, indicant la calcificació de la matriu extracel·lular (Fig. XIIIB). Les ASC cultivades en medi control no es van tenyir amb Alizarin Red (Fig. XIIIA). Visualment tampoc es va observar tinció en ASC no diferenciades (Fig. XIIIC), però sí que es va observar tinció en canvi en ASC en medi osteogènic (Fig. XIIID), tanmateix com també es va observar en SAOS-2 (Fig. XIIIE).

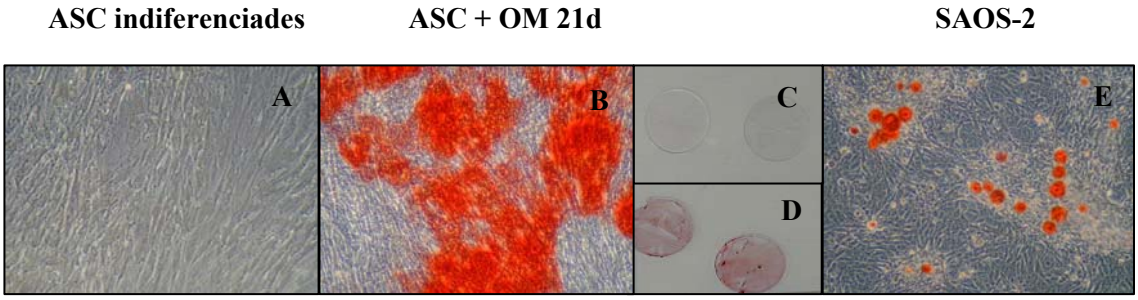



**Fig. XIII: Calcificació de la matriu extracel·lular en ASC de bola adiposa de Bichat mostrat per tinció Alizarin Red d'ASC de bola adiposa de Bichat**. ASC tenyides amb Alizarin Red en medi osteogènic (OM) **(B)** i en medi control **(A)** amb magnificació 100x. Les cèl·lules es van sembrar a $5x10^4$ cèl·lules/pou. La presència de nòduls vermells era apreciable visualment en ASC diferenciades **(D)** però era absent en ASC indiferenciades **(C)**. SAOS-2 van servir com a control positiu **(E)**.

L'expressió d'osteocalcina, marcador característic d'osteoblasts madurs i osteocits, va determinar-se per immunohistoquímica. ASC cultivades en medi osteogènic (Fig. XIVB), però no en canvi ASC indiferenciades (Fig. XIVA), van mostrar expressió d'aquest marcador. SAOS-2, utilitzades com a control positiu, van mostrar expressió d'aquest marcador (Fig. XIVC).

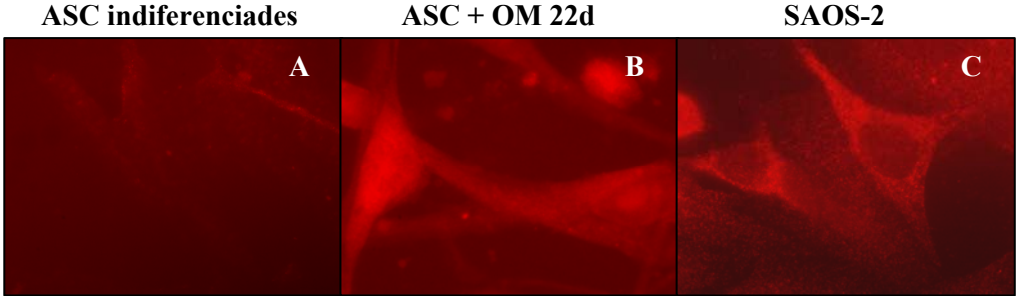



**Fig. XIV: Expressió d'osteocalcina en ASC de bola adiposa de Bichat per immunohistoquímica.** Les cèl·lules es van sembrar a $2x10^4$ cèl·lules/pou. ASC cultivades en medi osteogènic (OM) però no en medi control **(A)** durant 22 dies van mostrar expressió d'osteocalcina **(B)**. SAOS-2 van ser utilitzades com a control positiu **(C)**. Magnificació 400x.

Durant el procés de diferenciació, l'expressió dels gens osteogènics CBFA1 i SPARC es va incrementar 8 vegades i 2 vegades respectivament al dia 14 de cultiu (Fig. XV).

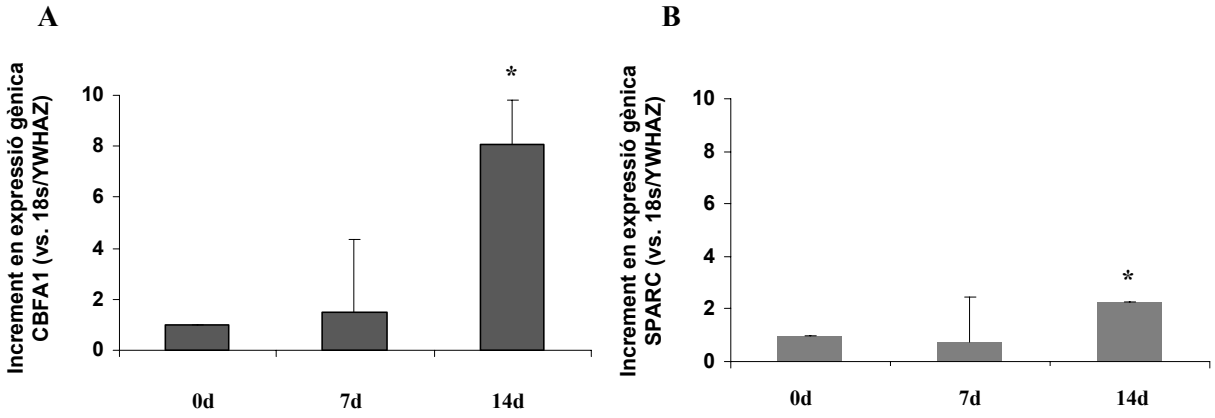



**Fig. XV: Expressió de gens osteogènics en ASC de bola adiposa de Bichat (BFP) quantificat per real-time PCR.** ASC de BFP es van cultivar en medi control i en medi osteogènic. **A)** Expressió de CBFA1 als 0, 7 i 14 dies de diferenciació en medi osteogènic. **B)** Expressió de SPARC als 0, 7 i 14 dies de diferenciació en medi osteogènic. Els nivells d'expressió gènica es van normalitzar amb 18S i YWHAZ utlilitzant el factor de normalització i es van expressar relatius amb ASC indiferenciades. Els valors són promig ± SEM, n=3. * Increment significatiu d'expressió gènica entre els 0 dies i els 14 dies ($p<0.05$).

Per altra banda es va observar un descens en l'expressió del marcador d'adipocits PPARγ als 21 en medi osteogènic (Fig. XVI). SAOS-2 es van utilitzar com a control positiu. Aquestes dades demostren que les ASC obtingudes de BFP són capaces de diferenciar-se al llinatge osteogènic *in vitro*.

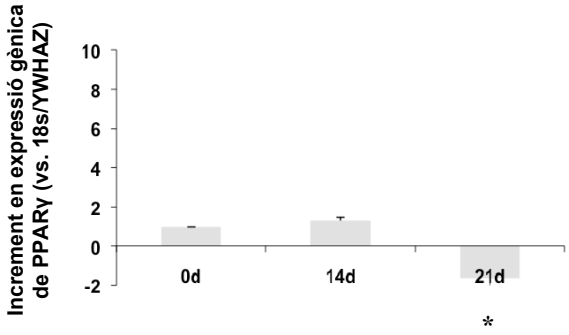



**Fig. XVI: Expressió gènica de PPARγ en ASC derivades de bola adiposa de Bichat (BFP) quantificat per real-time PCR.** ASC de BFP van ser cultivades en medi control i en medi osteogènic durant 3 setmanes. Els nivells d'expressió gènica es van normalitzar amb 18S i YWHAZ utlilitzant el factor de normalització i es van expressar relatius amb ASC indiferenciades. Els valors són promig ± SEM, n=3. * Increment significatiu d'expressió gènica entre els 0 dies i els 21 dies ($p<0.05$).

### 3.2. Diferenciació adipogènica

Després de la inducció amb medi adipogènic, es va observar la presència de vacuoles lipídiques intracel·lulars, que van augmentar en tamany i en nombre durant cultiu (Fig. XVII).

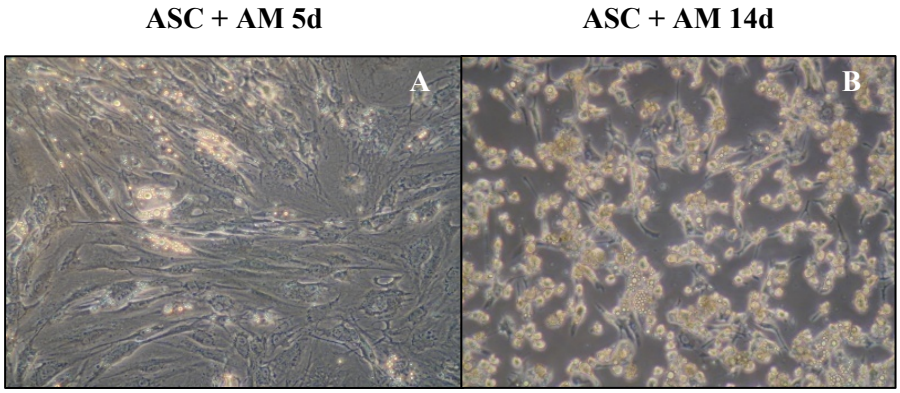



**Fig. XVII: Diferenciació adipogènica en ASC de bola adiposa de Bichat.** Les ASC es van cultivar en medi adipogènic (AM) i van ser analitzades al microscopi als 5 dies **(A)** i als 14 dies **(B)**. Es van observar vacuoles lipídiques intracel·lulars. El tamany de les vacuoles va incrementar-se amb temps en cultiu. Magnificació 100x.

La tinció d'Oil Red va revelar la presència de múltiples vacuoles lipídiques intracel·lulars en ASC cultivades en medi adipogènic (Fig. XVIIIB), però no en ASC indiferenciades (Fig.XVIIIA).

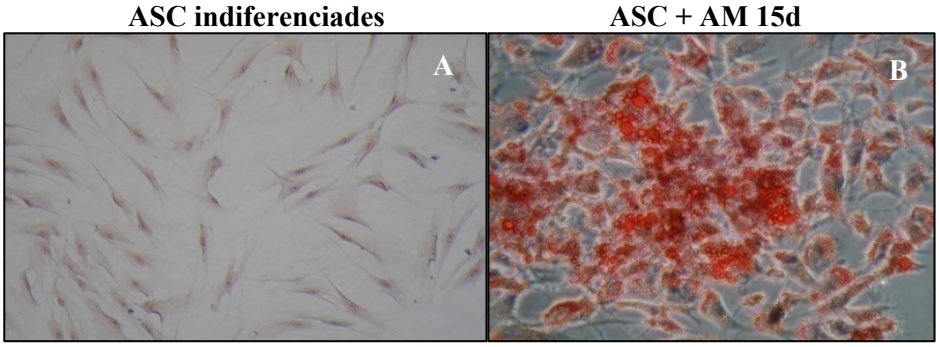



**Fig. XVIII: Tinció d'Oil Red per detectar vacuoles lipídiques característiques d'adipocits.** Al dia 15 les cèl·lules es van tenyir amb Oil Red i es van analitzar utilitzant un microscopi. Les vacuoles lipídiques es van tenyir intensament en vermell en ASC cultivades en medi adipogènic (AM) **(B)**, però no es van observar en canvi en ASC indiferenciades **(A)**.

Els nivells d'expressió del marcador específic d'adipocits PPARγ i el gen de normalització GAPDH es van mesurar per real-time PCR quantitativa utilitzant el mètode comparatiu $C_t$. L'expressió de PPARγ es va incrementar durant cultiu, abarcant un augment de ~4 vegades comparat amb ASC indiferenciades (Fig. XIX). Mostres de teixit adipós subcutani i teixit adipós de BFP es van utilitzar com a control positiu.

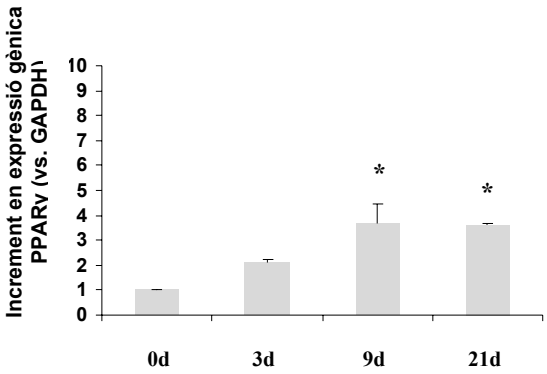



**Fig. XIX: L'expressió de PPARγ en ASC de bola adiposa de Bichat (BFP) quantificat per real-Time PCR.** L'expressió de PPARγ es va mesurar per Real-Time PCR quantitativa en ASC indiferenciades i en ASC cultivades en medi adipogènic durant 3, 9, i 21 dies. Els nivells d'expressió es van representar com a increment en comparació amb ASC no diferenciades, després de normalitzar amb el gen GAPDH. Mostres de teixit adipós subcutani i teixit adipós de BFP es van utilitzar com a controls positius, i van mostrar un increment en l'expressió de PPARγ comparat amb ASC indiferenciades de 28 ± 4.4 i de 66 ± 7.8 vegades respectivament. * Increment significatiu d'expressió gènica entre els 0 dies i els 9 dies i entre els 0 dies i els 21 dies ($p<0.05$).

### 3.3. Diferenciació condrogènica

Al diferenciar les cèl·lules a condrocits van canviar la seva morfologia a una forma més esferoidal (Fig. XXA). Les ASC cultivades en condicions control no van mostrar tinció amb Blau de Toluidin, específic de proteoglicans presents en la matriu cartilaginosa (Fig. XXB). ASC en medi condrogènic van mostrar tinció amb Blau de Toluidin (Fig. XXC), indicant la presència d'aquests proteoglicans. El nombre de nòduls es va incrementar amb temps en cultiu. Els nòduls no es van observar en ASC indiferenciades (Fig. XXD), però van ser clarament visibles en ASC cultivades en medi condrogènic (Fig. XXE).

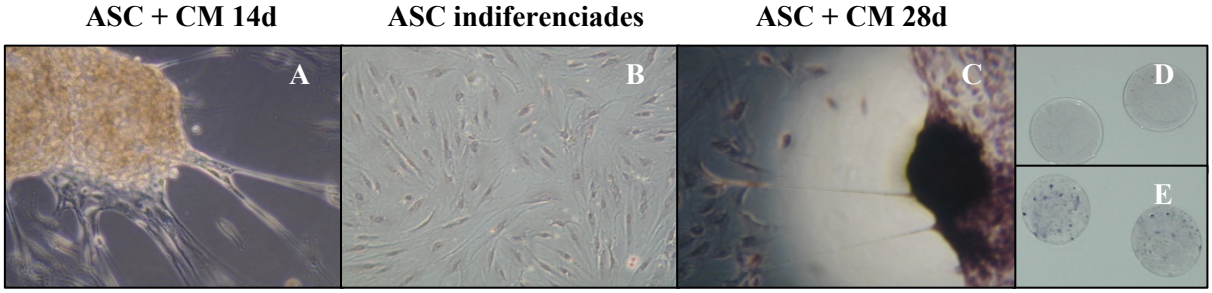



**Fig. XX: Tinció de Toluidin Blau per detectar una matriu de proteoglicans característica de condrocits.** Es va observar la presència de micromasses i el seu nombre es va incrementar amb temps en cultiu. **A)** ASC en medi de diferenciació condrogènic (CM) als 14 dies. Als 28 dies es van tenyir les cèl·lules amb Blau de Toluidin per detectar la presència d'una matriu de proteoglicans característica de condrocits. Es va observar la presència de nòduls tenyits en ASC induïdes a diferenciar **(C)**, però no es van observar en ASCs cultivades en medi control **(B)**. Magnificació 100x. Es van observar visualment nòduls tenyits amb Blau de Toluidin en ASC en medi condrogènic **(E)**. Aquests nòduls en canvi no eren presents en ASC en medi control **(D)**.

Anàlisi immunohistoquímic no va mostrar expressió de col·lagen II en ASC indiferenciades (Fig. XXIA), però sí va mostrar expressió en ASC diferenciades en medi condrogènic durant 32 dies (Fig. XXIB).

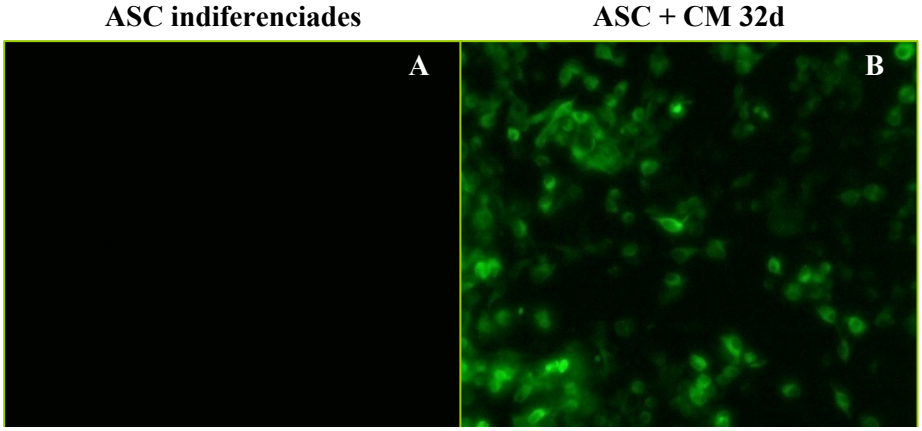



**Fig. XXI: Immunohistoquímica per col·lagen II en ASC de bola adiposa de Bichat**. Es va mostrar tinció en ASCs cultivades en medi condrogènic (CM) durant 32 dies **(B)**, no present en canvi en ASC cultivades en medi control **(A)**. Magnificació 100x.

L'expressió de SOX9, factor de transcripció clau en la diferenciació condrogènica, sembla incrementar-se 3.4 vegades en medi condrogènic en comparació amb ASC indiferenciades al dia 14 (Fig. XXII).

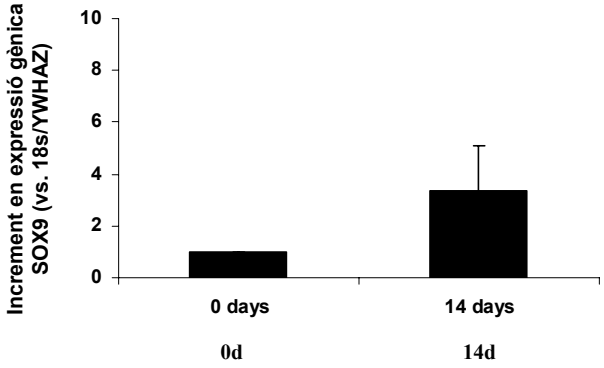



**Fig XXII: Expressió de SOX9 en ASC de bola adiposa de Bichat.** L'expressió de SOX9 es va mesurar per real-Time PCR quantitativa en ASC en medi control i ASC en medi condrogènic durant 14 dies. Els nivells d'expressió es van representar com a increment respecte als nivells presents en cèl·lules indiferenciades, normalitzats els valors amb els gens 18S i YWHAZ. Les barres representen promig ± SEM. Cèl·lules de nucli pulpós es van utilitzar com a controls positius.

L'expressió del gen d'adipocits PPARγ va disminuir durant la diferenciació condrogènica (Fig. XXIII), indicant la diferenciació al llinatge condrogènic de les cèl·lules.

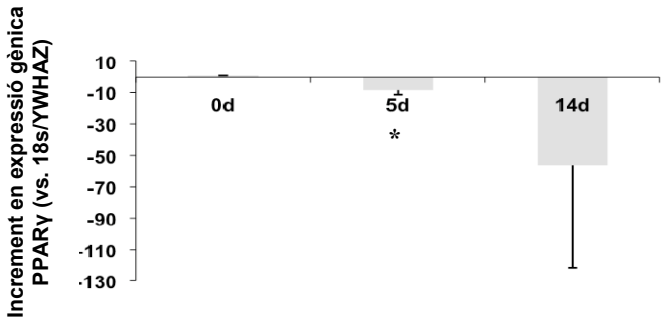



**Fig. XXIII: Expressió de PPARγ en ASC de bola adiposa de Bichat.** L´expressió de PPARγ es va mesurar per real-time PCR quantitativa en ASC en medi control i ASC en medi condrogènic durant 21 dies. Els nivells d'expressió es van representar com a increment respecte als nivells presents en cèl·lules indiferenciades, normalitzats els valors amb els gens 18S i YWHAZ. Les barres representen promig ± SEM. * Increment significatiu d'expressió gènica entre els 0 dies i els 5 dies ($p<0.05$).

**ESTUDI 2: DIFERENCIACIÓ OSTEOGÈNICA DE CÈL·LULES MARE DE TEIXIT ADIPÓS HUMÀ ESTIMULADES AMB PROTEÏNA ÒSSIA MORFOGENÈTICA-2 I SEMBRADES EN BIOMATERIALS DE FOSFAT BICÀLCIC I FOSFAT β-TRICÀLCIC.**

## Materials i mètodes

### Aïllament d'ASC

Va obtenir-se teixit adipós subcutani abdominal de pacients sans sotmesos a cirugia abdominal electiva en l'hospital Tergooiziekenhuizen Hilversum (n=9, 23-32 anys), després d'obtenir el Consentiment informat aprobat pel Comitè Ètic de la Vrije Universiteit Medisch Centrum (VUMC) d'Amsterdam, Països Baixos. Es realitza l'aïllament de les ASC de la SVF modificant un procediment prèviament descrit.[72] El material és disgregat en petits fragments i és posteriorment digerit amb 0.1% col·lagenasa A en PBS amb 1% de proteïna albúmica sèrica (BSA) durant 45 minuts a 37ºC de temperatura amb agitació intermitent. Després de la digestió enzimàtica el teixit és filtrat amb una malla de 100 μm i rentat amb PBS amb 1% de BSA. Posteriorment el teixit és sotmès a un gradient de separació Ficoll per separar els eritrocits de la SVF, i centrifugat varies vegades durant 10 minuts a 600xg per separar els adipocits de la SVF que conté les ASC. El pellet cel·lular obtingut és resuspès en medi d'expansió, DMEM amb 10% de FBS i 1 mg/ml d'antibiòtics i 2.5 μg/ml d'antimicòtics. La viabilitat cel·lular es va analitzar utilitzant el mètode d'exclusió de tripà blau. Es van contar les cèl·lules viables utilitzant un microscopi i es van cultivar immediatament en els diferents biomaterials, o van ser resuspeses en medi crioprotector i posteriorment congelades seguint el protocol de congelació, i finalment emmagatzemades en nitrogen líquid fins a la seva utilització posterior.

### Cultiu d'ASC i sembra en biomaterials

ASC obtingudes de la SVF sense cultivar (ASC fresques) i ASC fresques congelades i descongelades es van tractar durant 15 minuts amb o sense 10 ng/ml de BMP-2 (Peprotech EC Ltd, London, UK) a 37°C, i es van sembrar a una concentració de $1x10^5$ cèl·lules per 25 mg de biomaterial, i posteriorment es van cultivar en "transwells" en plaques de 12 pous amb medi d'expansió, amb 25 mg de biomaterial per cada pou. Quatre biomaterials diferents van ser utilitzats en l'estudi (Taula IV): 1) Straumann® BoneCeramic (Straumann, Basel, Switzerland), un BCP porós amb 60% d'HA i 40% de ß-TCP (BCP 60/40), 2) Straumann®

BoneCeramic, un BCP porós amb 20% d'HA i 80% de fosfat ß-TCP (BCP 20/80), 3) Ceros® TCP (Mathys, Bettlach, Switzerland), un ß-TCP porós amb tamany de partícules de 0.5-0.7 mm (ß-TCP<0.7mm), i 4) Ceros® TCP, un ß-TCP porós amb tamany de partícules de 0.7-1.4 mm (ß-TCP>0.7mm).

| Biomaterial | Composició | Tamany de partícula (μm) | Porositat (%) | Tamany de porus (μm) | Quantitat/unitat |
|---|---|---|---|---|---|
| Straumann® BoneCeramic BCP 60/40 | 60% HA/40% ß-TCP | 500-1000 | 90 | 500-1000 | 0.5g |
| Straumann® BoneCeramic BCP 20/80 | 20% HA/80% ß-TCP | 500-1000 | 90 | 500-1000 | 0.5g |
| Ceros® TCP β-TCP | 100% ß-TCP | 500-700 | 60 | 100-500 | 0.5g |
| Ceros® TCP β-TCP | 100% ß-TCP | 700-1400 | 60 | 100-500 | 0.5g |

**Taula IV:** Composició dels diferents biomaterials utilitzats en l'estudi, amb el tamany de partícules, porositat, amplitud de porus i quantitat per unitat. HA, hidroxiapatita; BCP, fosfat bicàlcic; ß-TCP, fosfat ß-tricàlcic.

**Adhesió i proliferació d'ASC cultivades en diferents biomaterials**

Suspensions cel·lulars d'ASC tractades o no durant 15 minuts amb BMP-2 es van sembrar en els diferents biomaterials durant 30 minuts. A continuació els biomaterials es van rentar amb PBS, i la concentració de DNA es va determinar com a mesura del nombre cel·lular per evaluar el nombre de cèl·lules adherides. Els biomaterials amb les cèl·lules adherides es van cultivar en medi d'expansió fins a 21 dies en un incubador a un 5% $CO_2$ i a 37°C en condicions d'humitat. Després de 4, 14 i 21 dies, la concentració de DNA es va determinar com a mesura del nombre cel·lular per evaluar la proliferació cel·lular.

**Assaig d'unitats formadores de colònies fibroblàstiques (CFU-F) i assaig de reducció de CFU-F (Depl CFU-F)**

Per valorar si la freqüència d'ASC en la SVF del teixit adipós es veu afectada amb el tractament amb BMP-2, es va realitzar l'assaig de CFU-F com ha estat prèviament descrit.[73] Un total de $1x10^3$ i $1x10^4$ cèl·lules es van sembrar en plaques de 6 pous. Després de 14 dies de cultiu les CFU-F es van fixar amb 4% paraformaldehid durant 10min i es van tenyir amb Blau de Toluidin al 0.2 % en tampó bòrax (pH 12) durant 1min. El nombre de colònies es va

quantificar utilitzant un microscòpic òptic a a 100x de magnificació. El percentatge de CFU-F es va calcular pel nombre total d'ASC sembrades.

L'adhesió de les ASC als diferents biomaterials es va determinar utilitzant l'asaig de reducció de CFU-F, tal com ha estat prèviament descrit.[74] Les cèl·lules no adherides, obtingudes dels rentats dels biomaterials amb PBS, es van recollir, i es va analitzar el percentatge de CFU-F a una densitat 10 vegades més elevada que la densitat normal de sembra, ja que ha estat establert que la sembra a una densitat normal resulta en absència de colònies.

**Assaig de proliferació CyQUANT**

Immediatament després de la sembra i l'adhesió cel·lular, i després de 4, 14 i 21 dies, la quantitat de DNA es va quantificar utilitzant el kit d'assaig de proliferació cel·lular CyQUANT. Les cèl·lules es van rentar amb PBS i es van guardar a −80°C abans de ser analitzades seguint les instruccions del fabricant. Es va utilizar una curva estàndard de referencia en l'assaig. Per quantificar la quantitat de DNA, es van afegir a la mostra 200 µl de CyQUANT GR tampó de llisi, i es va incubar durant 4 minuts a temperatura ambient i protegit de la llum. Seguidament es va mesurar la fluorescència utilitzant un filtre de fluoresceina amb filtres de 480 nm d'excitació i 520 nm d'emissió.

**Diferenciació osteogènica d'ASC cultivades en diferents biomaterials**

Per analitzar la diferenciació osteogènica d'ASC tractades o sense tractar amb BMP-2, es van sembrar $1x10^5$ cèl·lules per 25 mg de biomaterial. Les cèl·lules cultivades en monocapa van servir de control per a la prova d'activitat ALP i d'anàlisi d'expressió gènica utilitzant real-time PCR. Per a la diferenciació osteogènica, les ASC (tractades o no tractades amb BMP-2), van ser cultivades fins a 21 dies en medi DMEM suplementat amb 10% FBS, antibiòtics, antimicòtics, 10 mM de β-glicerolfosfat i 50 µg/ml d'àcid ascòrbic. Si les cèl·lules es van sembrar amb biomaterials l'adició de β-glicerolfosfat es va ometre del medi de cultiu. El medi es va canviar dues vegades per setmana. L'activitat d'ALP es va determinar als 4, 14, i 21 dies i l'expressió gènica de mRNA es va analitzar amb real-time PCR als 14 dies, per analitzar l'expressió dels següents gens: CBFA1, Col1, ALP, ON, OPN, OCN i PPARγ. Osteoblasts humans primaris van ser utilitzats com a control. L'expressió gènica es va comparar entre ASC sembrades en els diferents biomaterials.

**Anàl·lisi estadístic**

Els valors promig d'ASC sembrades en els biomaterials i en plàstic de cultiu es van expressar com a promig ± S.E.M (n=6-9).

Es va utilitzar una prova t de Student per comparar les dades dels grups d'unitats formadores de colònies fibroblàstiques en plàstic de cultiu i en l'assaig de proliferació i d'activitat fosfatasa alcalina d'ASC sembrades en biomaterials. La prova d'ANOVA va ser utilitzat per analitzar les diferències en l'assaig de reducció de CFU-F i l'assaig d'adhesió als 30min CyQUANT. Un valor de probabilitat $<0.05$ es va considerar estadísticament significatiu. Un valor de probabilitat $<0.001$ es va considerar altament significatiu. Els anàlisis es van realitzar utilitzant el programa estadístic SPSS 17.0 (SPSS Inc.) i GraphPad Prism 5.01 (GraphPad Software Inc.).

# Resultats i discussió

## 4.1. Efecte de BMP-2 en l'adhesió i proliferació d'ASC cultivades en diferents biomaterials

Per determinar si una curta estimulació de només 15min amb BMP-2 afecta la freqüència de progenitors mesenquimals en el teixit adipós, es va realitzar l'assaig d'unitats formadores de colònies fibroblàstiques, després de sembrar ASC tractades amb o sense BMP-2 en plàstic de cultiu (n=9) i els resultats es van expressar en percentatge de CFU-F. L'adhesió cel·lular als diferents biomaterials es va determinar mesurant la concentració de DNA (n=6), a fi d'evaluar el nombre de cèl·lules adherides després de sembrar ASC tractades o no tractades amb BMP-2 en els diferents biomaterials. També es van realitzar assaigs de reducció de CFU-F amb els rentats recollits dels biomaterials després de 30 minuts de sembrar les ASC tractades o no tractades amb BMP-2 (n=6), per determinar el nombre d'ASC no adherides en els diferents biomaterials. La proliferació de les ASC tractades i sense tractar cultivades en BCP i en β-TCP es va quantificar i comparar mitjançant l'assaig de DNA, al dia 4, 14 i 21 de cultiu (n=6).

### 4.1.1. BMP-2 incrementa el nombre de CFU-F sense afectar l'adhesió d'ASC cultivades en diferents biomaterials

Assaigs CFU-F van ser realitzats en ASC fresques tractades amb o sense BMP-2 després de sembrar-les en plàstic de cultiu durant 2 setmanes. Es van observar colònies als 14 dies de cultiu que es van tenyir positivament amb la tinció Blau de Toluidin, característic de CFU-F. La freqüència de CFU-F era de 0.08% ± 0.007 (promig ± SEM; Taula V), i després d'una curta incubació amb BMP-2 de només 15min es va incrementar dues vegades (0.15% ± 0.004). Vam observar que una curta incubació amb BMP-2 incrementa significativament la freqüència de CFU-F en ASC sembrades en plàstic de cultiu després de 2 setmanes de cultiu (Fig. XXIV, $p<0.001$).

| CFU | Pacients (n) | % CFU-F (promig ± SEM) | |
|---|---|---|---|
| | | -BMP-2 | +BMP-2 |
| CFU-F | 9 | 0.08 ± 0.007 | 0.15 ± 0.004 |
| Depl CFU-F BCP 60/40 | 6 | 0.01 ± 0.001 | 0.02 ± 0.002 |
| Depl CFU-F BCP 20/80 | 6 | 0.01 ± 0.002 | 0.02 ± 0.001 |
| Depl CFU-F ß-TCP<0.7mm | 6 | 0.01 ± 0.002 | 0.02 ± 0.001 |
| Depl CFU-F ß-TCP>0.7mm | 6 | 0.02 ± 0.001 | 0.02 ± 0.001 |

**Taula V: Assaigs d'unitats formadores de colònies fibroblàstiques (CFU-F) i assaigs de reducció de CFU-F (Depl CFU-F).** A les 2 setmanes de cultiu, es van tenyir les CFU-F amb Blau de Toluidin, per determinar si la freqüència d'ASC en plàstic de cultiu i en diferents biomaterials es veu afectada pel tractament amb proteïna morfogenètica humana (BMP-2). BCP 60/40, 60% hidroxiapatita i 40% fosfat ß-tricàlcic; BCP 20/80, 20% hidroxiapatita i 80% fosfat ß-tricàlcic; ß-TCP<0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.5-0.7 mm; ß-TCP>0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.7-1.4 mm. BCP, fosfat bicàlcic; β-TCP, fosfat tricàlcic.

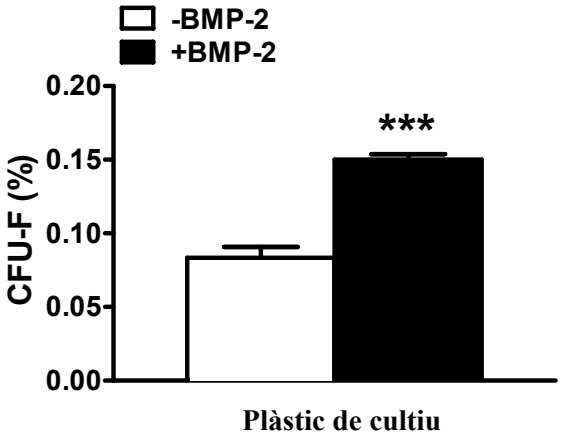



**Fig. XXIV: Efecte de la proteïna morfogenètica humana (BMP-2) en la freqüència d'ASC en teixit adipós**. El tractament amb BMP-2 d'ASC fresques incrementa significativament el percentatge de colònies que es van tenyir amb Blau de Toluidin, característic d'ASC. ***p<0.001. CFU-F, d'unitats formadores de colònies fibroblàstiques.

ASC fresques, a més a més d'ASC congelades fresques provinents de varis pacients, estimulades amb BMP-2 o sense estimular, es van sembrar en BCP i en β-TCP. Després de 30min de sembrar el DNA es va quantificar i es va expressar en nanograms (ng). No vam detectar diferències entre biomaterials després del tractament amb BMP-2 (Fig. XXV, barres negres). Sembla ser present un nombre més alt de cèl·lules en BCP que en ß-TCP.

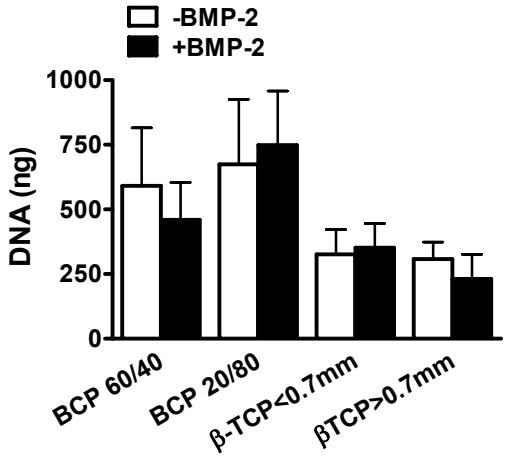



**Fig. XXV: Efecte de la proteïna morfogenètica humana (BMP-2) en l'adhesió d'ASC en diferents biomaterials.** El gràfic de barres mostra la quantitat de DNA després de 30min de sembrar ASC no estimulades amb BMP-2 (barres blanques), i ASC estimulades amb BMP-2 (barres negres). No es van observar diferències significatives entre ambdós grups. BCP 60/40, fosfat bicàlcic 60% hidroxiapatita i 40% fosfat ß-tricàlcic; BCP 20/80, fosfat bicàlcic 20% hidroxiapatita i 80% fosfat ß-tricàlcic; ß-TCP<0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.5-0.7 mm; ß-TCP>0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.7-1.4 mm. BCP, fosfat bicàlcic; ß-TCP, fosfat tricàlcic.

Aquests resultats validen els mètodes d'utilitzar la SVF després de congelar, i mostra que el mètode de congelació no afecta la proliferació ni la diferenciació osteogènica de cèl·lules mare de teixit adipós de la fracció estromal vascular, tal com ha estat prèviament descrit.[74] En relació amb l'efecte de BMP-2 en l'adhesió d'ASC fresques als biomaterials de fosfat càlcic, hem observat que els resultats són similars amb els obtinguts utilitzant ASC fresques congelades (dades no mostrades).

La recol·lació dels rentats després de sembrar i d'incubar les cèl·lules amb els biomaterials durant 30min es va sembrar en plàstic de cultiu, per mesurar les CFU-F amb l'assaig Depl CFU-F (Fig. XXVI). L'adhesió cel·lular no es va veure afectada pel tractament amb BMP-2. Es van observar més CFU-F en ß-TCP que en BCP abans i després del tractament, indicant la presència de més CFU-F adherides en BCP ($p<0.05$).

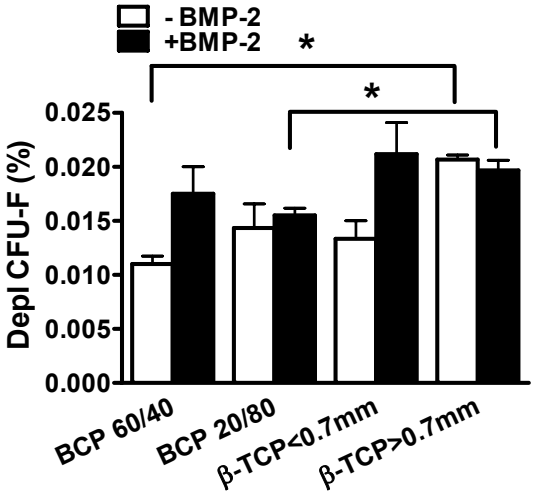



**Fig. XXVI: Efecte de la proteïna morfogenètica humana (BMP-2) en l´adhesió d´ASC en diferents biomaterials, determinat per l'assaig de reducció d'unitats formadores de colònies fibroblàstiques (Depl CFU-F); després d´estimular amb BMP-2 (barres negres) i sense estimular amb BMP-2 (barres blanques.** L´adhesió als biomaterials no es va veure afectada per BMP-2. Es van observar més cèl.lules adherides en BCP que en β-TCP abans i després del tractament. *$p<0.05$. BCP 60/40, fosfat bicàlcic 60% hidroxiapatita i 40% fosfat ß-tricàlcic; BCP 20/80, fosfat bicàlcic 20% hidroxiapatita i 80% fosfat ß-tricàlcic; ß-TCP<0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.5-0.7 mm; ß-TCP>0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.7-1.4 mm. BCP, fosfat bicàlcic; ß-TCP, fosfat tricàlcic.

### 4.1.2. BMP-2 afecta la proliferació depenent del biomaterial

Les ASC cultivades en BCP i en β-TCP es van quantificar i comparar amb l'assaig de DNA als 4, 14, i 21 dies de cultiu, i el resultat es va expressar com a increment de les ASC tractades amb BMP-2 respecte les no tractades amb BMP-2. El contingut de DNA de les cèl·lules mare

sembrades en BCP 60/40 va ser significativament més alt després del tractament amb BMP-2 als 21 dies, amb un increment de 2.4 ± 0.5 vegades respecte les ASC no tractades amb BMP-2 (Fig. XXVII, $p<0.05$). El contingut de DNA de les cèl·lules mare sembrades en BCP 20/80, ß-TCP<0.7mm i ß-TCP>0.7mm va incrementar-se 1.5 ± 0.6, 1.1 ± 0.09 i 1.9 ± 0.8 vegades respectivament després del tractament amb BMP-2. Per tant el tractament amb BMP-2 afecta la proliferació d'ASC en els biomaterials depenent del tipus de biomaterial.

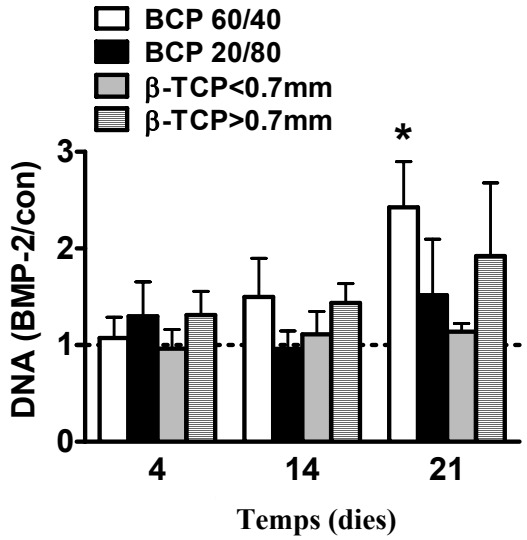



**Fig. XXVII: La proteïna morfogenètica humana (BMP-2) augmenta la proliferació d´ASC depenent del biomaterial als 21 dies de cultiu.** BMP-2 va estimular significativament el contingut de DNA en cèl·lules mare sembrades en BCP 60/40 al dia 21. *p<0.05. El contingut de DNA es va expressar com a BMP-2-tractat-respecte-no-tractat control. 1 significa no efecte de BMP-2. Con, control. BCP 60/40, 60% hidroxiapatita i 40% fosfat ß-tricàlcic; BCP 20/80, 20% hidroxiapatita i 80% fosfat ß-tricàlcic; ß-TCP<0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.5-0.7 mm; ß-TCP>0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.7-1.4 mm; BCP, fosfat bicàlcic; ß-TCP, fosfat tricàlcic.

### 4.2. Efecte de BMP-2 en la diferenciació osteogènica d'ASC en els diferents biomaterials

Per investigar si una curta incubació amb BMP-2 en ASC humanes cultivades en diferents biomaterials de fosfat càlcic és suficient per promoure la diferenciació osteogènica de les ASC *in vitro*, es va mesurar l'activitat ALP (n=6) i l'expressió de gens associats amb l'osteogènesi als 14 dies de cultiu (n=2).

### 4.2.1. BMP-2 indueix l'activitat ALP depenent del biomaterial

La diferenciació primerenca de les MSC en osteoblasts immadurs es caracteritza per l'activitat de l'enzim ALP, que és expressat en MSC tan aviat com des dels 4 dies d'inducció, i els màxims nivells d'expressió s'observen cap als 14 dies d'inducció osteogènica.[75]

L'activitat ALP de les ASC cultivades en quatre biomaterials diferents de fosfat càlcic es va mesurar utilitzant el mètode ELISA, i els resultats es van expressar com a increment en les cèl·lules tractades amb BMP-2 respecte les cèl·lules no tractades (n=6). L'activitat ALP d'ASC cultivades en BCP 60/40 es va veure incrementada durant tot el cultiu després del tractament amb BMP-2, esdevenint significativament més alta al dia 21 de cultiu (Fig. XXVIII, barres blanques, $p<0.05$), amb un increment de 1.9 ± 0.5 vegades respecte ASC no tractades amb BMP-2. Les ASC sembrades en BCP 20/80 van mostrar un increment d'activitat ALP de 1.6 ± 0.9 vegades al dia 21 de cultiu respecte les ASC no tractades amb BMP-2. Les ASC sembrades en ß-TCP<0.7mm i en ß-TCP>0.7mm van mostrar un increment en l'activitat ALP de 2.6 ± 1.5 vegades i de 1.2 ± 0.4 vegades respectivament als 21 dies, després del tractament amb BMP-2.

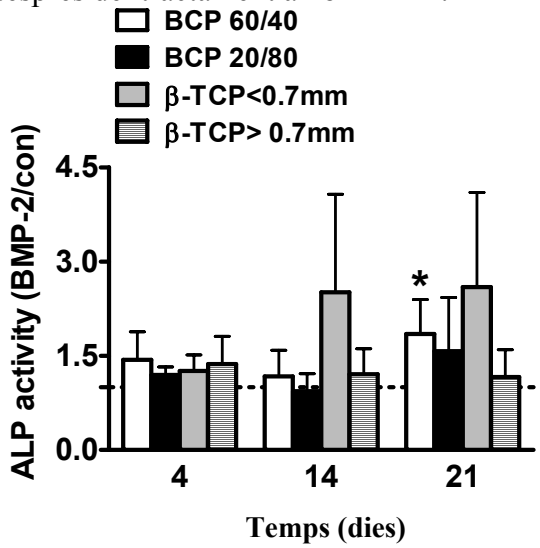



**Fig. XXVIII: La proteïna morfogenètica humana (BMP-2) augmenta l´activitat fosfatasa alcalina (ALP) d´ASC depenent del biomaterial als 21 dies de cultiu.** BMP-2 va estimular significativament l´activitat ALP en cèl·lules mare sembrades en BCP 60/40 al dia 21. *p<0.05. L´activitat ALP es va expressar com BMP-2-tractades-respecte-no-tractades control, i es va normalizar per la proteïna. 1 significa no efecte de BMP-2. Con, control. BCP 60/40, 60% hidroxiapatita i 40% fosfat ß-tricàlcic; BCP 20/80, 20% hidroxiapatita i 80% fosfat ß-tricàlcic; ß-TCP<0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.5-0.7 mm; ß-TCP>0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.7-1.4 mm; BCP, fosfat bicàlcic; ß-TCP, fosfat tricàlcic.

### 4.2.2. BMP-2 indueix la diferenciació osteogènica depenent del biomaterial

Per a confirmar la diferenciació osteogènica de les ASC tractades amb BMP-2 sembrades en diferents biomaterials de fosfat càlcic, es van mesurar amb real-time PCR l'expressió de gens associats amb l'osteogènesi, tals com CBFA1, Col1, ALP, ON, OPN, i OCN als 14 dies de cultiu (Fig. XXIX).

ASC no tractades amb BMP-2 cultivades en plàstic de cultiu semblen expressar CBFA1, Col1, i ON (Fig. XXIXA,B,D, barres blanques). Després del tractament amb BMP-2, l'expressió dels gens osteogènics CBFA1, Col1, i ON sembla incrementar-se (Fig. XXIXA,B,D, barres negres). També l'activitat ALP sembla incrementar-se després del tractament amb BMP-2, amb un increment de 3 vegades en ASC cultivades en plàstic de cultiu (Fig. XXIXC, barres negres).

Les ASC no tractades amb BMP-2 cultivades en biomaterials de BCP 60/40 semblen expressar CBFA1, Col1, ALP i ON (Fig. XXIXA-D, barres blanques). Després del tractament amb BMP-2, l'expressió dels gens osteogènics CBFA1, Col1, i ON sembla incrementar-se (Fig. XXIXA,B,D, barres negres). ASC tractades amb BMP-2 sembrades en BCP 60/40 també semblen incrementar l'expressió de OPN i OCN (Fig. XXIXE,F, barres negres). En canvi, ASC tractades amb BMP-2 cultivades en biomaterials de ß-TCP no semblen mostrar un increment en l'expressió de gens osteogènics, excepte OCN, detectada en ASC tractades amb BMP-2 sembrades en ß-TCP>0.7mm (Fig. XXIXF, barres negres).

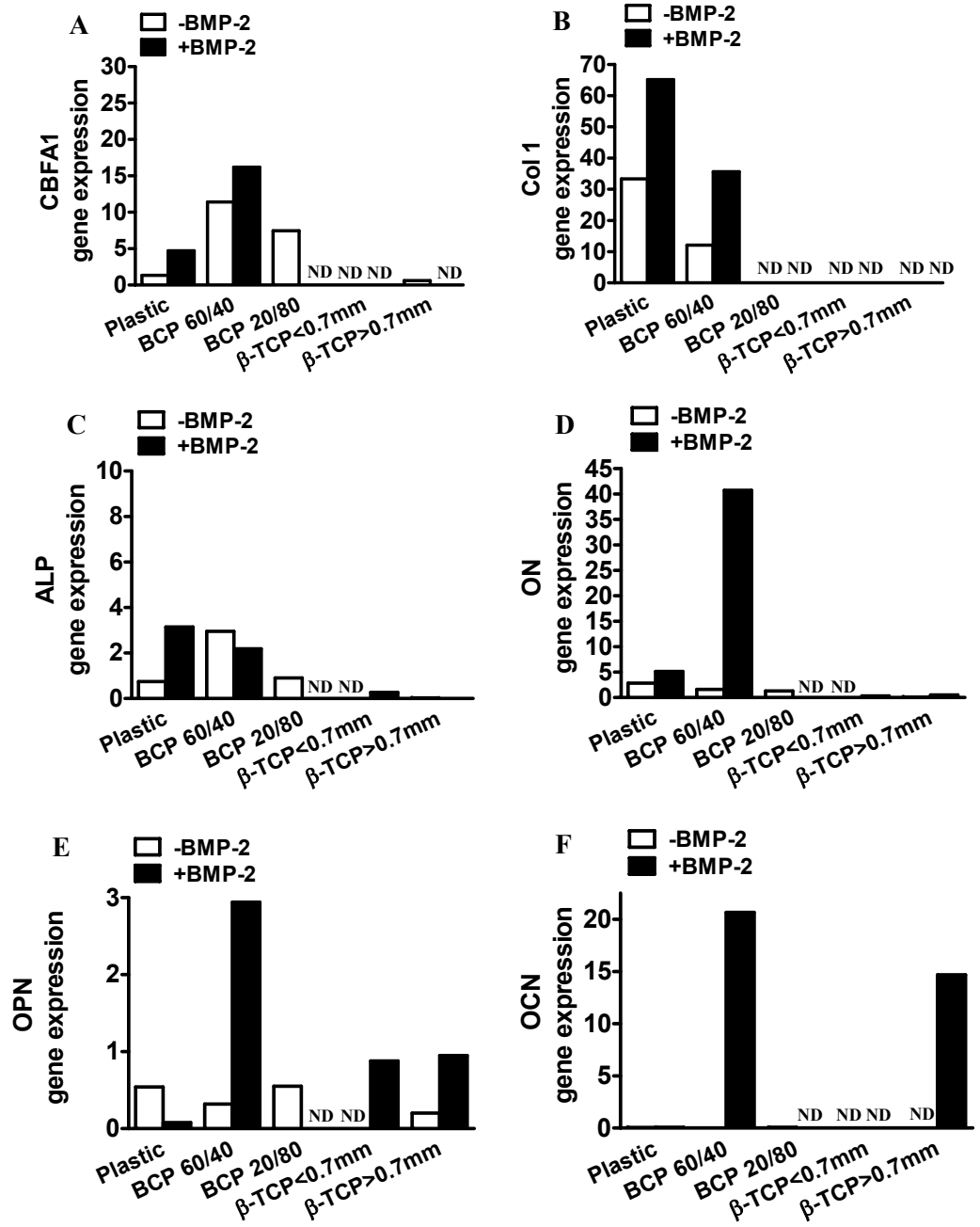



**Fig. XXIX: La proteïna morfogenètica humana (BMP-2) sembla incrementar la diferenciació osteogènica d´ASC sembrades en BCP 60/40 als 14 dies de cultiu.** ASC recentment aïllades, tractades i sense tractar amb BMP-2, es van sembrar en plàstic de cultiu i en diferents biomaterials de fosfat càlcic. **A)** Expressió gènica de CBFA1. **B)** Expressió gènica de Col1. **C)** Expressió gènica d´ALP. **D)** Expressió gènica d´ON. **E)** Expressió gènica d´OPN. **F)** Expressió gènica d´OC. Els valors d´expressió es van representar com a increment respecte ASC no diferenciades, després de normalitzar amb els gens constitutius UB i YWHAZ. UB, ubiquitina; CBFA1, cbfa1/runx2; Col1, col·lagen I; ALP, fosfatasa alcalina; ON, osteonectina; OPN, osteopontina; OCN, osteocalcina; BCP 60/40, fosfat bicàlcic amb 60% hidroxiapatita i 40% fosfat ß-tricàlcic; BCP 20/80, fosfat bicàlcic amb 20% hidroxiapatita i 80% fosfat ß-tricàlcic; ß-TCP<0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.5-0.7 mm; ß-TCP>0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.7-1.4 mm; ND, no detectat.

L'expressió del gen adipogènic PPARγ es va mesurar als 14 dies de cultiu. Aquest factor de transcripció està críticament relacionat en l'inducció de l'adipogènesi i en l'inhibició de l'osteogènesi.[76] L'expressió del gen PPARγ sembla disminuir en ASC cultivades en plàstic de cultiu i en els diferents biomaterials de fosfat càlcic, indicant el compromís al llinatge osteogènic de les cèl·lules (Fig. XXX).

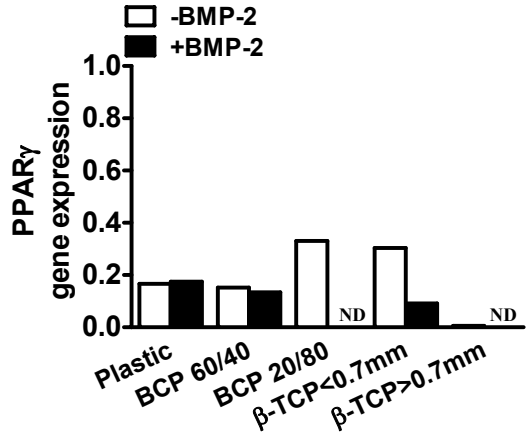



**Fig. XXX: L´expressió del gen adipogènic PPARγ va disminuir als 14 dies de cultiu, indicant la diferenciació osteogènica.** ASC recent aïllades, tractades o sense tractar amb proteïna morfogenètica humana (BMP-2), són sembrades en plàstic de cultiu i en diferents biomaterials. Els valors d´expressió es van representar com a increment respecte ASC no diferenciades, després de normalitzar amb els gens constitutius UB i YWHAZ. UB, ubiquitina; BCP 60/40, fosfat bicàlcic amb 60% hidroxiapatita i 40% fosfat ß-tricàlcic; BCP 20/80, fosfat bicàlcic amb 20% hidroxiapatita i 80% fosfat ß-tricàlcic; ß-TCP<0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.5-0.7 mm; ß-TCP>0.7mm, fosfat ß-tricàlcic amb tamany de partícules de 0.7-1.4 mm; ND, no detectat.

Com a resum, ASC sembrades en un BCP 60/40 després de ser estimulades amb BMP-2 mostren una activitat ALP més alta als 21d de cultiu. A més a més el tractament amb BMP-2 sembla estimular l'expressió gènica de CBFA1, Col1, ON, OPN, i OCN en ASC sembrades en aquest biomaterial (Fig. XXIXA-F, barres negres).

# Conclusions

**Estudi 1: Bola adiposa de Bichat, una font d'accés oral de cèl·lules mare de teixit adipós (ASC) humà amb potencial per enginyeria tisul·lar osteocondral.**

1) Hem desenvolupat un protocol per l'aïllament eficient i ràpid d'ASC de bola adiposa de Bichat que inclou com a mínim 30min de digestió amb col·lagenasa a una concentració del 0.075%. La reducció a la meitat en la seva concentració o l'el·liminació de col·lagenasa afecta l'aïllament de cèl·lules mare de teixit adipós.

2) ASC obtingudes de bola adiposa de Bichat expressen marcadors característics de MSC com CD73, CD90 i CD105, i no expressen marcadors característics hematopoiètics com el CD45, CD14, CD19 i HLA-DR, d'acord al mínim criteri d'expressió de marcadors definit pel Comitè de Cèl·lules Mesenquimals i de Teixits de la Societat Internacional de Teràpia Cel·lular en el 2006.

3) El nombre d'ASC obtingut per gram de teixit adipós de bola adiposa de Bichat a la setmana de cultiu és aproximadament de 500 x $10^3$ cèl·lules per gram de teixit adipós, el doble del nombre obtingut de teixit adipós subcutani abdominal.

4) Aproximadament un 30% de cèl·lules de la fracció estromal vascular mostren característiques de d'ASC per citometria de fluxe. Després d'anal·litzar la fracció estromal vascular per aquesta tècnica s'observa la presència d'una població heterogènia de cèl·lules.

5) ASC de bola adiposa de Bichat mostren una expressió més alta del marcador angiogènic CD34, el que podria fer-les interessants candidates per aplicacions d'enginyeria tisul·lar òssia.

6) ASC de bola adiposa de Bichat són capaces de diferenciar-se *in vitro* al llinatge osteogènic, condrogènic i adipogènic després d'aplicar l'estímul inductiu adecuat.

**Estudi 2: Diferenciació osteogènica d'ASC estimulades amb proteïna òssia morfogenètica-2 (BMP-2) i sembrades en biomaterials de fosfat bicàlcic i fosfat β-tricàlcic.**

1) BMP-2 augmenta la proliferació de les ASC.

2) BMP-2 no afecta l'adhesió de les ASC sembrades en fosfat bicàlcic i fosfat β-tricàlcic.

3) BMP-2 augmenta la proliferació de les ASC sembrades en fosfat bicàlcic compost de 60% d'hidroxiapatita i 40% de fosfat β-tricàlcic, però no en canvi en fosfat β-tricàlcic.

4) BMP-2 augmenta la diferenciació osteogènica de les ASC sembrades en fosfat bicàlcic compost de 60% d'hidroxiapatita i 40% de fosfat β-tricàlcic, però no en canvi en fosfat β-tricàlcic.

# Chapter 1 INTRODUCTION

Nowadays life expectancy has gradually increased from 45 years at the beginning of the 20th century to about 80 years in advanced industrialized countries. This lengthening of the average life expectancy forces an increasing proportion of the population to live with organs that are merely old. The histologic basis of this aging process would seem to be the loss of stemness, i.e., a progressive reduction in the number and quality of stem cells, what leads to diseases and decrease in quality of life.

Tissue engineering, also referred to as regenerative medicine or reparative medicine, has emerged over the last few decades as a growing technology, which combines the principles of bioengineering, cell transplantation and biomaterial engineering, and has evolved in what is nowadays called regenerative medicine. This involves the regeneration of tissue *in vivo* to repair, replace, maintain, or enhance organ function, as well as to engineer and grow functional tissue substitutes *in vitro* for implantation *in vivo* as biological substitutes, involving the repair and regeneration of tissues and organs and offering an enormous potential to treat disabling and chronic disorders. This field, which has consolidated in the 20th century, was since the 90s accelerated in parallel with an emerging industry, and the Tissue Engineering Society was formed and evolved into the Tissue Engineering and Regenerative Medicine International Society (TERMIS). This Society is involved in the formation of an international registry of regenerative medicine patient trials and products, and promotes worldwide collaboration in this field facing with immense challenges to improve patient health.[1] The paradigm is to find the appropriate source of stem cells able to integrate in the host tissues, and which possess the properties to the tissue to regenerate. The challenge for regenerative medicine is to grow *in vivo* a new tissue or organ without the presence of fibrous tissue and scarring. This would lead to complete replacement of the injured structures, resulting in a fully functional, scarless, reconstructed tissue.

However, tissue engineering has also been envisioned as a subset of regenerative medicine instead of being a synonym for it.[77] The glowing of this multidisciplinary field has been shown by the rapid increase in publications on this topic during the last years (Fig. 1), and is thought to become the “gold standard” for surgical procedures in the coming future.

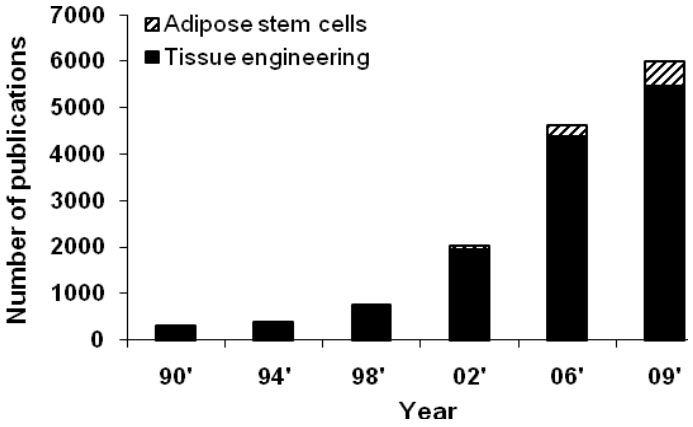



**Fig. 1: Number of publications found in PubMed on tissue engineering and adipose stem cells (ASC).** The number of publications from 1990 to 2009 referring to tissue engineering (black bars) and ASC (hatched bars) are shown.

Regenerative medicine comprises three main building blocks: biomaterials or scaffolds, biologics (inductive stimuli), and stem cells. Biomaterials act as a scaffold allowing and promoting the cell growth and differentiation, by providing structural support, biological containment and chemical stimuli to the cells, and therefore favouring osteoconduction and osteoinduction. Biologics are needed to improve proliferation and differentiation of these cells, and comprise hormones, growth factors and cytokines, as well as mechanical signals.[2] Last but not least important, is to find an easy access source with availability of stem cells in a high number for one-step and multiple-step interventions. Two major problems to overcome are associated with "scale-up" and cell death associated with implantation. Large numbers of cells are needed for tissue regeneration, and mature cells expanded *in vitro* lose efficacy. Cell implantation is associated with vascular disruption, and this results in an hypoxic environment which leads to cell death. To be effective cells should be easily obtained, effectively expanded and survive the initial implantation with an adequate vascular network. Besides this, they should be immunocompetent and function correctly, and not become malignant. No moral and ethical concerns should be generated with their use.[3]

Traditionally, embryonic stem cells (ES) lead the research in tissue regeneration, but use of adult stem cells do overcome the major drawbacks of the use of these embryonic cells, such as teratogenic formation, immunogenicity, and ethical and political concerns.[4,5] Adult stem cells have been identified in a broad range of tissues, such as blood, brain, estomach, intestine, heart, retina, umbilical cord blood, corionic villi, placenta, dental pulp, periodontal

ligament, fat, skin, hair, liver, bone, cartilage and skeletal muscle, and altought initially these cells were thought to be commited to differentiate into the cell lineage of the tissue where they reside, there is increasing evidence of their plasticity to transdifferentiate and cross germ lines, and differentiate into a whole spectrum of cell types other than the germ line lineage of the tissue they derive from. These cells also have showed the potential to return to a previous undifferentiated state, phenomen called dedifferentiation.[6]

Use of autograft, or patient's own bown, is the gold standard of bone tissue engineering, because is the only type of bone graft able to supply living bone cells essential to osteogenesis. Autograft can be taken from different sites and in several forms. Since it is from the same individual, the tissue is recognized as "self" and the immune system is not triggered with an immunological response.[37] However, autograft has some disadvantages, such as insufficient bone source, donor-site injury, risk of infection, and morbidity to the patient. Other approaches to repair bone defects, such as the use of allograft, xenograft or biosynthetic substitute, are limited by the immunogenicity and inertness of the material, and the lack of the osteogenic properties of bone autografts.[38] Biosynthetic substitutes, such as β-tricalcium phosphate (β-TCP), hydroxyapatite (HA), and mixtures of HA/β-TCP (biphasic calcium phosphate), have been successfully used as a bone graft bsubstitute, because of their good biocompatibility and chemical composition which resemble the composition of a natural bone matrix.[39-42]

Bone tissue engineering is emerging as a valid alternative to treat bone defects caused by trauma, tumors, or congenital deficiency (Fig. 2), allowing regeneration of lost tissues to recover functionality.[78] It combines the principles of bioengineering, cell transplantation, and biomaterial engineering to repair and regenerate lost or damaged tissue,[38] avoiding the major problems associated with autograft transplantation, such as insufficient bone source, donor-site injury and morbidity, and surgical risks.[79] A common challenge for reconstructive surgeons is the treatment of large bone defects resulting from traumatic injury, tumor resection, degenerative diseases, and congenital deformities. Over 1,000,000 surgical procedures were performed in 2005, as shown by Nationwide Inpatient Statistics, involving partial excision of bone, bone grafting, spinal fusion, and inpatient fracture repair. The total charges for the operations exceeded $40 billion in this year, and had a large psychosocial impact due to the orthopedic trauma and surgery.[80,81] The cost of surgical treatment and subsequent impact on patient quality of life illustrate the need to look for alternative therapies.

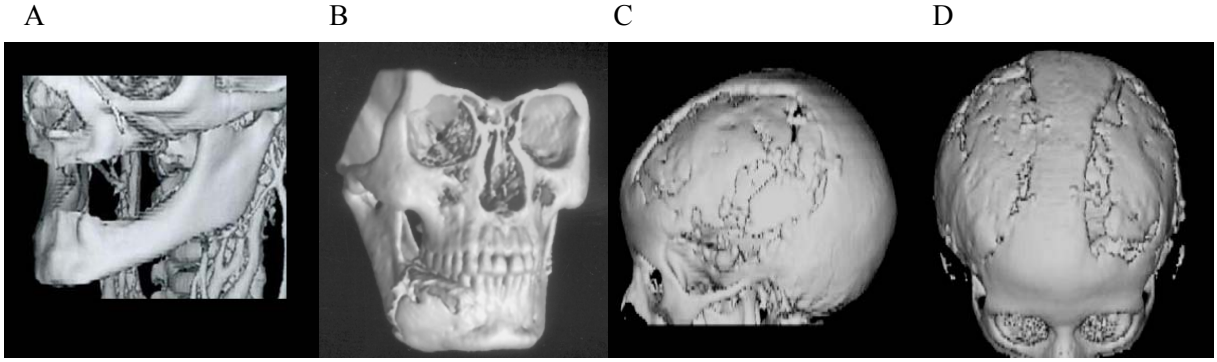



**Fig. 2 depicts different causes of bone defects. (A)** Underdeveloped jaws in ectodermal dysplasia, a congenital disease characterized by poorly developed alveolar processes as if they were old and atrophic and missing teeth. **(B)** Ameloblastoma of the right mandible. **(C,D)** Craneofacial traumatism after a fall. 3D computed tomography (CT) images. (Larheim TA et al. Maxillofacial imaging. Ed. Springer. 2008). (Alam A et al. MJAFI. 2005). (Lendeckel S et al. J Craniomaxillofac Surg. 2004).

The combination of an optimal source of osteoprogenitor cells with an appropriate biomaterial focuses the bone tissue engineering research and has gained notoriety in the last 15 years.[82] Cell-based therapy such as tissue regeneration may be useful to treat tissue defects. Two cell types are generally considered as candidates for such strategy, 1) lineage-commited progenitor cells, and 2) stem cells. The former one has important limitations, such as low number of cells that can be harvested and decrease in proliferation potential with increase in donor age.[83,84]

Fig. 3 shows healing of a critical-size defect in a sheep model after 16 weeks. A combination of a coral scaffold with *in vitro*-expanded marrow stromal cells increased osteogenesis more than that obtained with the scaffold alone or the scaffold plus fresh bone marrow (BM). Defects filled with coral cointained newly formed bone through the medullary area, but the cortical continuity was not restored. Cavities filled with coral and fresh BM were invaded by dense fibrous tissue, indicating scarring. Instead, coral loaded with Mesenchymal Stem Cells (MSC) resulted in new bone formation without fibrous tissue, with a well-differentiated marrow cavity and cortical structure, and produced more bone than with coral alone (54% vs 13%, $p<0.001$). This scaffold had almost completely disappeared after 4 months in all groups.[38]

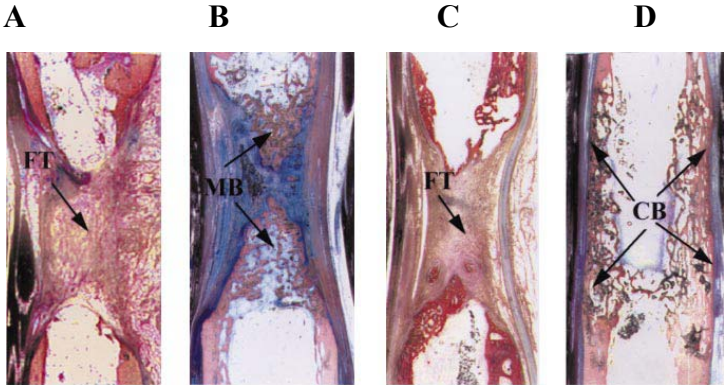



**Fig. 3: Histological sections of critical-size defects (25 mm long) in sheep metatarsus after 16 weeks of healing. (A)** Empty defects. **(B)** Defects filled with coral exoskeleton scaffold. **(C)** Defects filled with coral and 2ml ± 0.5 ml fresh bone marrow aspirate. **(D)** Defects filled with coral scaffold and MSC obtained from 14 ml ± 3 ml autologous bone marrow. Note in **(A)** and **(C)** fibrous tissue (FT) invading the defect. In defects filled with only scaffold osteogenesis occurred through the medullary channel (MB). Defects filled with coral and MSC show pheripheral cortical-like bone formation (CB), with cortical continuity in the edges. (Petite H et al. Nat Biotechnol. 2000).

The scaffold for bone tissue engineering should facilitate the vascular invasion and bone development. Ideally, the scaffold should be resorbed at a rate commensurate with new bone formation. Coral scaffold is reabsorbed rapidly, i.e. within weeks,[85] which makes hydroxyapatite and tricalcium-phosphate-hydroxyapatite or biphasic calcium phosphate (BCP), and biodegradable polymers such as polylactic acid (PLAc) and polyglycolic acid (PGA), interesting alternatives (Fig. 4). These scaffolds allow cell proliferation as well as osteogenic differentiation of the progenitor cells.[86] Besides bone formation, angiogenesis is very important to allow an adequate supply of oxygen and nutrients to the cells. A massive cell death would occur if there is lack of vascularization in the center of the implant, which would impede bone regeneration. In pioneering studies it has been shown that pore sizes less than 15–50 μm result in fibrovascular ingrowth, pore sizes of 50–150 μm favour osteoid formation, and pore sizes larger than 150 μm favour mineralized bone ingrowth.[87]

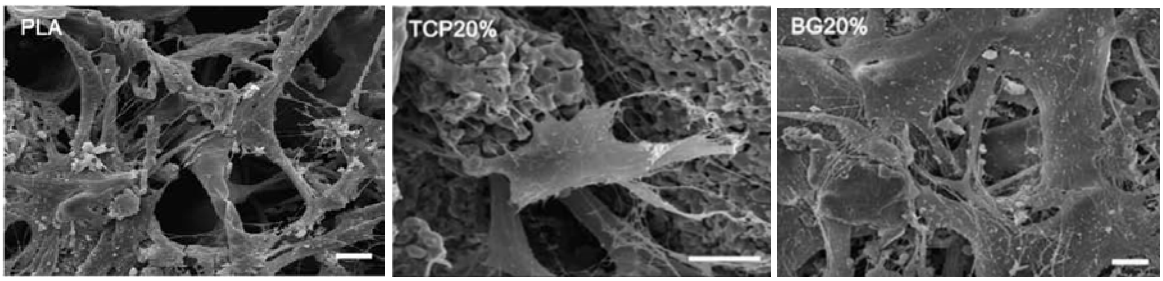



**Fig. 4: Some types of scaffolds used in bone tissue engineering.** Pictures show SEM micrographs of adipose stem cells (ASC) on polylactic acid (PLAc), PLAc/ß-TCP (ß-TCP 20%), and PLAc/20 bioactive glass (BG20%) (scale bars: 10 mm). (Haimi S et al. Tissue Engineering. 2009).

When engineering tissues *in vitro,* one of the principal constraints on the size of the tissues engineered is the short distance over which oxygen can diffuse before being consumed (a few hundred micrometers at most). Therefore, once implanted in the patient, cells consume immediately the available oxygen within a few hours, while it takes several days for the growth of new blood vessels (angiogenesis) that will deliver oxygen and nutrients to the implanted cells. Therefore, it is important the presence of growth factors, such as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), that induce and speed up angiogenesis in the regenerating tissue.[88] One main group of growth factors for bone tissue engineering are the bone morphogenetic proteins or BMPs, of which BMP-2 and BMP-7/OP-1 are approved by the food and drug administration (FDA) as medical device. BMPs were first discovered in demineralized bone matrix and are responsible for promoting endochondral bone formation.[47] BMPs promote the differentiation of osteoprogenitor cells, and also induce osteogenesis in mesenchymal stem cells.[48] Bone morphogenetic protein-2 (BMP-2), which regulates the development, growth, remodeling, and repair of bones, can promote osteoblast and stem cell proliferation and differentiation and heal intrabony periodontal defects, as well as critical size defects.[49-52] Bone morphogenetic proteins play a critical role in bone healing, by stimulating the differentiation of MSC to an osteogenic lineage. Multiple release of growth factors such as VEGF and BMP-2, may be able to mimic the conditions in bone fracture repair, by enhancing osteoblast differentiation and activation and the recruitment of osteoprogenitor cells. The release of angiogenic factors such as VEGF and osteogenic like BMP-2 factors from a biodegradable scaffold seeded with human BM MSC, has demonstrated a significant effect on bone regeneration in a murine critical size defect (Fig. 5).[89]

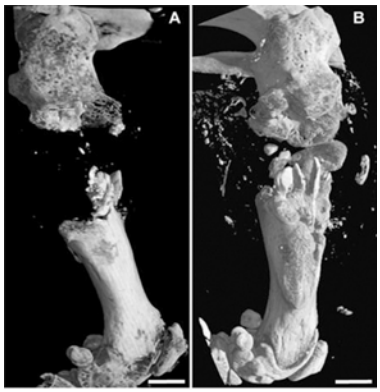



**Fig. 5: Micro-CT images of murine segmental 5 mm femur defects after 4 weeks operation.** A): Control segmental femur defect, B): Encapsulated VEGF and BMP-2 in a composite alginate / Poly (DL) lactic acid scaffold with seeded human bone marrow stromal cells implanted within the femur defect (Kanczler JM et al. Eur Cell Mater. 2008).

The delivery method and concentration of BMPs is of utmost importance for tissue engineering.[52] The use of recombinant BMP-2 on osteoinductive carriers, as well as *ex vivo* adenoviral transfection with BMPs are under evaluation.[50,52-55] However, there are safety concerns related with adenoviral vectors, i.e. unpredictable kinetics of protein production in the organism, or the unpredictability of the behavior of transfected cells.[56] Moreover the high doses used in clinical studies present some adverse effects, such as bone overgrowth and swelling.[57,58] It was previously shown that *ex vivo* incubation with BMP-2 at a million-fold lower concentration during only 15min is enough to stimulate the osteogenic differentiation of ASC, and could easily fit within a one-step surgical procedure, where autologous stem cells are isolated, triggered with growth factors, and immediately used for bone and/or cartilage regeneration in the patient.[2,59]

One of the key issues in bone tissue engineering, besides to find an adequate scaffold that allows cell growth and differentiation, and inductive stimuli that promote proliferation and differentiation of these cells, is to find an adequate source of stem cells. In the late 60s, Friedenstein and colleages demonstrated that the mesenchymal stroma from human bone marrow contains a population of cells that proliferate when cultured on plastic, and differentiate to cell lineages derived from the mesoderm, such as chondrocytes and osteoblasts.[7] Later these precursor spindle-shape cells are referred to as mesenchymal stem cells,[8] and have been shown to differentiate to several lineages *in vitro*[9,10] and *in vivo*,[11,12,13] making these cells promising candidates for mesodermal defect repair. These cells grow when

adhered to plastic, express specific surface antigens, and possess multipotent differentiation potential, according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT).[14] Bone marrow mesenchymal stem cells (BM MSC) have been until recently most commonly used source of MSC for clinical therapies. Use of BM MSC avoids the immunorejection, risk of teratoma formation, and the ethical concerns derived from use of embryonic stem cells. However, the clinical use of MSC provides several problems, such as pain associated with the harvest procedure, complexity of the technique, and low cell number upon purification, especially in elderly donors.[20,21] The low cell yield obtained upon harvest, approximately one MSC per $10^5$ adherent stromal cells,[10] makes an *ex vivo* expansion step necessary to obtain therapeutic cell doses, which is time consuming, expensive, and contains the risk of cell contamination and cell loss.[11] Besides this, MSC cell number decreases with aging, from 1 MSC per each $10^4$ nucleated cells in newborns, to 1 MSC each $10^6$ nucleated cells in elderly people.[23] All together leads to the search of alternative sources of stem cells for clinical applications in tissue engineering.

Adipose tissue represents a promising source of MSC available in large quantities, which does not require the use of general anesthesia and results in minimal patient discomfort. Furthermore, adipose tissue yields higher number of MSC than BM, which could avoid cell expansion.[25] The nomenclature of MSC from adipose tissue varies widely,[26] but the consensus reached at the Second Annual International Fat Applied Technology Society (IFATS) Meeting (2004, Pittsburgh PA) was to use the term adipose stem cells (ASC) for adipose-derived stromal cells[90] and human adipose-derived stromal cells.[91] Adipose tissue, as BM, is derived from embryonic mesoderm and contains a heterogeneous population, composed of adipocytes, mast cells, endothelial cells (EC), pericytes, fibroblasts, endothelial progenitor cells (EPC), pluripotent vascular progenitor cells, multipotent cells, and a high number of hematopoietic progenitors.[27,28] ASC are able to differentiate to multiple mesenchymal tissue cell types, such as osteoblasts, chondrocytes, adipocytes, myocytes, cardiomyocytes, and endothelial cells,[27,29] and there is growing evidence suggesting that they can also give rise to cells from other lineages, such as ectoderm[30,31] and endoderm.[32,33] In addition, adipose tissue secretes a variety of angiogenic and anti-apoptotic growth factors, that makes fat a promising source for plastic and reconstructive surgery.[34] In the bone tissue engineering field, ASC have been used successfully to repair critical size skeletal defects.[43,44,45] ASC seeded onto HA/β-

TCP cubes and implanted subcutaneously in rabbits showed osteoid formation at 6 weeks.[46] Therefore adipose tissue is an interesting source for cell-based therapy.

The oral cavity contains a mass of specialized fatty tissue, which is distinct from subcutaneous fat, named buccal fat pad (BFP) or Bichat's fat pad. This encapsulated adipose tissue was first identified more than 300 years ago by the german anatomist and surgeon Lorenz Heister, and later their histologic adipose origin and anatomic position was described by the French anatomist Xavier Bichat, who gave the name to the adipose mass.[92] This encapsulated mass of adipose tissue that fills the masticatory spaces is a remanent tissue, which serves for mastication and sucking in childhood. Buccal fat pad has an aesthetic function contributing to the 'fullness' of the cheeks. The easy accessibility and rich vascularization makes this fat mass an attractive graft widely used for the repair of bone and periodontal defects.[61,93,94] Due to the versatility of this tissue and successful results obtained with the use of BFP for reconstructive maxillofacial oral surgery, we suspected that the adipose tissue obtained from BFP could be an excellent source of MSC for tissue engineering, that is easily available for dentists and oral surgeons.

In the firsty study we analyzed if there was presence of multipotent stem cells in this tissue, performing a full characterization of the population of cells by flow cytometry to observe if they had characteristics of MSC, and studied their differentiation potential. BFP or Bichat's fat pad is a mass of specialized fatty tissue distinct from subcutaneous fat,[60] so we decided to compare both types of adipose tissue to observe if there were differences regarding stem cell populations between both sources. In this pioneering study we characterized the stem cell population found in buccal fat pad and studied *in vitro* the potential to give rise to cells derived from the mesoderm, such as bone and cartilage, for osteochondral tissue engineering. The harvesting of BFP is a non-complicated procedure, which requires a minimal incision with local anesthesia, and causes minimal donor site morbidity. Therefore it would represent an easy access source of stem cells for oral surgeons and dentists. In the second study we analyzed the effect of the growth factor BMP-2 on ASC seeded on different β-TCP and biphasic calcium phosphate scaffolds, to determine if a short incubation of only 15min with 10ng/ml is enough to promote the proliferation and osteogenic differentiation of the ASC, and therefore could be used in immediate clinical applications.

# Chapter 2 OBJECTIVES

## Study 1: Buccal fat pad, an oral access-source of human adipose stem cells (ASC) with potential for osteochondral tissue engineering

- General objective:

To analyze if buccal fat pad may be an appropriate easy access source of ASC for tissue engineering of mesodermal tissues.

- Specific objectives:

1) To isolate the adipose stem cells from adipose tissue from buccal fat pad and culture them *in vitro*, developing an efficient and rapid protocol of ASC isolation and culture.
2) To determine and compare surface marker expresion of ASC from buccal fat pad, and that of ASC from subcutaneous abdominal adipose tissue to determine the similarities and differences among these adipose depots.
3) To compare the ASC cell yield in adipose tissue from buccal fat pad and subcutaneous abdominal adipose tissue.
4) To determine the percentage of fresh ASC from buccal fat pad for possible immediate clinical applications.
5) To differentiate ASC from buccal fat pad to osteoblasts, chondrocytes, and adipocytes, to study their multipotent differentiation potential.

## Study 2: Osteogenic differentiation of bone morphogenetic protein-2 (BMP-2) induced human ASC seeded on biphasic calcium phosphate and β-tricalcium phosphate scaffolds

- General objective:

To analyze the effect of a short incubation of 15min only with the growth factor BMP-2 on attachment, proliferation, and differentiation of ASC seeded on different calcium phosphate scaffolds for bone tissue engineering.

- Specific objectives:

1) To analyze the effect of BMP-2 on attachment of ASC seeded on biphasic calcium phosphate and β-tricalcium phosphate scaffolds.
2) To analyze the effect of BMP-2 on proliferation of ASC seeded on biphasic calcium phosphate and β-tricalcium phosphate scaffolds.
3) To analyze the effect of BMP-2 on osteogenic differentiation of ASC seeded on biphasic calcium phosphate and β-tricalcium phosphate scaffolds.

# Chapter 3 BACKGROUND

For bone defect treatments, the gold standard is the use of the own body's bone, because it contains living cells that allow osteogenesis, osteoconduction, or cell migration of these cells, and induces the differentiation to osteoprogenitor cells in perivascular MSC, a phenomenon known as osteoinduction. This process, together with neovascularization and osteoclastogenesis from circulating monocytes, occur during bone healing, and allow the resorption and remodeling of tissue to incorporate the bone graft.[95] However, the surgery to harvest bone involves donor site morbidity and a limited amount of bone is obtained. This leads to a search for alternative therapies that overcome this disadvantage.

Tissue engineering appears as an interesting alternative to bone grafting, which combines the principles of bioengineering and biology using stem cells, biomaterials or scaffolds, and growth factors, to generate biological substitutes that restore, maintain, or improve tissue function or a whole organ.[78]

In biology of development, pluripotency to give rise to cells of the three germ layers and plasticity are traditionally restricted to embryonic stem cells, while adult stem cells are considered restricted to differentiate to the lineage where they reside. In larger vertebrates, the majority of adult tissues and organs contain stem cells capable of self-renewal, proliferation and differentiation to a mature and functional progeny. These stem cells are more abundant in tissues with a high renewal rate such as blood, epithelium, and vasculature, and less abundant in organs and tissues with scarce proliferative rate such as myocardial muscle or nervous central system, and proliferate and differentiate in response to growth factors.[96] In hypoxic stress, fibroblasts secrete basic fibroblast growth factor (bFGF), that acts as a chemoatractant for MSC, which mobilize to the damaged tissue.[97,98]

The properties of adult stem cells have a special impact in regenerative medicine. The possibility to use stem cells from an easy access source has a special redundance in regenerative medicine, and opens new possibilities to repair of damaged tissues such as myocardial infarct or cyrrotic liver, or tissues severely damaged by genetic diseases such as muscular dystrophy or dysplasia ossificans, and it would have a profound therapeutic impact on untreatable diseases.[99] Other potencial uses are cartilage and bone regeneration[100] or treatment of graft versus host disease.[101]

This chapter initiates with the biology of bone tissue to understand the basic processes ocuring in tissue regeneration. It contains the following sections. First, bone tissue is analyzed to understand the basic mechanisms related to bone regeneration. Second, the three key factors related to tissue engineering: stem cells, growth factors, and biomaterials or scaffolds, are exhaustively analyzed, describing the different sources of stem cells for tissue engineering and their characteristics, the different scaffolds to be used in bone tissue engineering, and the growth factors related to cell migration, proliferation, and differentiation in bone tissue healing. These are the main issues of the second study, which pretends to analyze the osteogenic differentiation of BMP-2 induced human ASC seeded on different calcium phosphate scaffolds. Then adipose tissue is described in detail, because we aim to use adipose tissue as a source of adult stem cells. Finally, buccal fat pad is described and analyzed to study the potential of this tissue as a valid source of adult stem cells for tissue engineering, which is the main objective of the first study.

### 3.1. Bone embryology

The skeletal system is derived from the mesoderm. The formation of the mesoderm begins with the migration of a layer of cells between the primitive endoderm and ectoderm. This layer spreads along the anteroposterior and dorsoventral axes of the developing embryo and forms the axial, intermediate, lateral plate, and paraxial mesoderm. The paraxial mesoderm gives rise to the axial skeleton and muscles of the trunk, after its segmentation into somites. The lateral plate mesoderm generates the skeleton and muscles of the limbs.[102]

Interestingly, the facial bones, jaws and associated connective tissues have been shown to derive from the neural crest. The neural crest is a vertebrate cell population that arises from the euroectoderm. After neural tube closure, neural crest cells undergo an epithelial-to-mesenchyme transition and migrate to diverse regions in the developing embryo, where they differentiate into various cell types. Neural cells derivatives include pigment cells, neurons and glial cells of the peripheral nervous system, as well as some endocrine cells. In the head and neck, the neural cells also yield mesenchymal precursors which differentiate into connective tissue cells, vascular smooth muscle cells, tendons, dermis, odontoblasts, cartilages and bones.[103,104] Therefore the skeletal system develops from paraxial mesoderm, lateral plate mesoderm and neural crest cells.

The process of osteoblast differentiation during embryonic development occurs through two distinct pathways. For the entire future skeleton, except the clavicles, the mandibles and certain bones of the skull, a cartilage template surrounded by a bone collar prefigures each future bone. Upon vascular invasion of the cartilage template, the apoptotic chondrocytes are replaced by osteoblasts brought in from the bone collar. This process is called endochondral ossification ("endo"=within, and "chondro"=cartilage). In contrast, in the intramembranous ossification that takes place in the condensations prefiguring the clavicles, the mandibles, and certain bones of the skull, the mesenchymal progenitor cells differentiate directly into osteoblasts without mediating of any cartilaginous template.[105]

- Endochondral Ossification

The sequence of events by which a cartilage model is replaced by bone begins in centers of ossification set up in embryonic skeletons. Blood vessels invade the cartilaginous model, and carry into it the progenitors of bone-forming cells. Eventually three such centers are established: one at each end, and one in the center, of a long bone such as the femur or humerus. Throughout embryonic life the process of replacing the cartilage model with bone takes place. At the time of birth most of the original model has been removed and replaced by bone. The principal function of hyaline cartilage is to provide a "model skeleton" which will eventually be replaced by a new one composed of bone. The old cartilaginous model is actually taken apart piece by piece while a new one is made, replacing the old one.

The vertebral column, the pelvis, and the appendicular skeleton or limbs, develop by endochondral ossification. In this ossification a cartilage template is first formed from aggregated MSC and thereafter is replaced by bone tissue. This process is divided in 5 stages:

1. The MSC differentiate through the chondrogenic pathway through expression of two transcription factors, Pax1 and Scleraxis. These are thought to activate cartilage-specific genes.
2. The chondrogenic differentiated MSC condense into compact nodules and differentiate into chondrocytes. In humans, SOX9 (sex reversal Y-related high-mobility group box protein) gene is expressed in the precartilaginous condensations. Mutations of this gene result in deformities of most bones in the body.

3. During this phase, chondrocytes rapidly proliferate to form the cartilage model that will eventually be replaced by bone.
4. Chondrocytes stop dividing and become hypertrophic. Hypertrophic chondrocytes show increased production of collagen (Col) type X and fibronectin, altering the remaining cartilage matrix so it can be mineralized by calcium carbonate.

Following this, blood vessels begin to invade the cartilage template. The hypertrophic chondrocytes undergo apoptosis, and spaces are invaded by ingrowing blood vessels. As chondrocytes die, osteoprogenitor cells differentiate into osteoblasts and begin to lay down bone matrix on the partially-degraded mineralized cartilage remains. The ossification begins in the center of the cartilage and is known as primary center of ossification (diaphysis of the long bone). In mammals it spreads along the vertical axis in both directions from the center. As bone grows in length, secondary ossification centers appear at the ends of each bone. The secondary ossification centers become the ephiphysis of the bone. The invasion by blood vessels is a critical step in the novo bone formation. Disruption of this process cause abnormalities in growth and misshapen bones. One illustration is achondroplasia, caused by a mutation in fibroblast growth factor resulting in a malfunction of the growth plate causing a short stature.

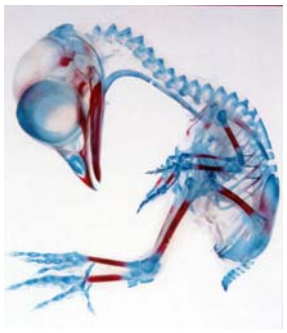


**Fig. 6: Endochondral ossification in forming skull skeleton of an embryonic chicken.** Alizarin Red stain and Alcian Blue are used to stain osseous and cartilage structure respectively. (Caceci T. 2009).

- Intramembranous Ossification

In intramembranous ossification embryonic mesenchyme (rather than embryonic cartilage) is replaced by bone. This mode of ossification is typical of flat bones such as some of those in the skull (e.g., the frontal, parietal, occipital, and temporal) and a few others. The initial stage begins when embryonic mesenchyme condenses and mesenchymal cells produce a thin matrix material among the existing collagen fibers. Cells differentiate into osteoblasts and become

active in the secretion of additional bone matrix. In intramembranous ossification, bone formation takes place simultaneously at numerous sites, one characteristic that distinguishes it from endochondral ossification, in which ossification occurs from the center of a long bone towards its ends.[106]

In intramembrous ossification, bone growth occurs by apposition of osteoblasts, derived from the osteoprogenitor cells present within the mesenchyme, lining the surfaces of growing bones. Then ossification centers develop and enhance mineralization. When the growing slows down, the bone takes a lamellar shape and Haversian remodeling occurs in adults. Within the skull, a chondroid bone forms, being an intermediate between bone and cartilage, containing both types collagen I and II. It acts as a scaffold upon which lamellar bone is deposited, associated with suture closure. A factor associated is core binding factor-alpha1 (CBFA1), responsible of osteoblast differentiation and osteocalcin (OCN) binding, resulting in maturation of bone. A mutation of this gene causes delayed ossification of cranial structures, a disorder known as dysplasia cleidocraneal.[107]

Cartilage remains in some areas such as the bridge of the nose, parts of the ribs, and the joints. Except for flat bones, which form on fibrous membranes, most develop using hyaline cartilage structures as their models. Bone formation or ossification involves two major phases. First, the hyaline cartilage is covered with bone matrix by bone-forming cells (osteoblasts). Then, the enclosed hyaline cartilage is digested away, opening up a medullary cavity within the newly formed bone. At birth, or shortly thereafter, most hyaline cartilage has been converted to bone, except for two regions, the articular cartilage that covers the bone ends, and the epiphyseal plates. The articular cartilage persists for life to reduce the friction at joint surfaces. The epiphyseal plates provide for longitudinal growth of the long bones during childhood. New cartilage is formed continuously on the external face of the articular cartilage and on the epiphyseal plate surface that is farther away from the medullary cavity, and the old cartilage adjacent to the internal face of the articular cartilage and the medullary cavity is broken down and replaced by bony matrix. Apart from lengthening, bones grow in diameter by appositional growth. Osteoblasts, located in the periosteum and bone tissue to the external face of the diaphysis, deposit bone, while osteoclasts in the endosteum remove bone from the inner face of the diaphysis wall, expanding the circumference of long bone. Growth hormones and sex hormones during puberty control the mechanism until end of adolescence.[108]

### 3.2. Bone structure and composition

Bone is a highly specialized support tissue that provides structural support for the body, protection of vital organs, an environment for marrow (both blood forming and fat storage), and acts as a mineral reservoir for calcium homeostasis in the body.

Bone is one of the most dynamic tissues in the body. Bones are continually being modified, reshaped, remodeled, and overhauled. Bone is a reservoir for calcium and phosphorus, and there is a constant flux of minerals in and out of it. Bone responds to injury by rapid healing. It can be made to grow in different ways by clever tricks of orthodontia and surgery to be remodeled, and it is greatly affected by nutritional and metabolic changes.

- Macroscopic structure of bone

The adult human skeleton is composed of 206 bones and they are divided basically in two types of osseous tissue, woven bone and cortical or lamellar bone. Woven bone, also known as trabecular, cancellous or spongy bone, is composed of small needlelike pieces and lots of open space. This bone makes up for the 20% of the human skeleton and provides structural support and flexibility, and is found in most areas of bone that are not subjected to a large mechanical stress.[109] This is a highly variable tissue, relatively weak, disorganized and poorly mineralized. However, it plays a critical role in wound healing by two mechanisms: 1) rapidly filling the osseous defects, and 2) providing initial continuity for fractures and osteotomy segments. The spaces between the trabecula are filled with red marrow- loose connective tissue containing a large number of blood vessels, some white blood cells and fat cells, and a large number of cells called erythroblasts responsible for producing red blood cells.

Compact bone, also known as cortical or lamellar bone, on the contrary, is very dense and homogeneous and is harder and stiffer than cancellous bone. When new lamellar bone is formed, a portion of the mineral component of osteoblasts (hydroxylapatite or hydroxyapatite) is deposited during primary mineralization and provides rigidity to the bones. After this primary mineralization, a slow progressive increase in mineral deposition occurs and this secondary mineralization can last months.[110] The compact bone accounts for about the 80% of the human skeleton, and provides a dense cortical layer around all bones, thereby giving support to the internal trabecular network and at the same time enclosing the bone marrow.[111]

Compact bone provides the long bones with the strength that is needed to support the body during the cycle dynamic loading that takes place during locomotion and other physical activities.[112] Trabecular bone is composed of a network of fine, interlacing partitions, called trabeculae, with a diameter ranging from 10-400 μm enclosing cavities that contain either haematopoietic or fatty marrow. Trabecular bone is remodelled much more faster (5-10 times as fast) than cortical bone.[113] Mature spongy bone also has concentric lamellae with lacunae and canaliculi housing osteocytes and their filipodia.[108] The porosity or nonbone tissue, provided by the spaces and channels whithin bone, also differs between compact and woven bone. At skeletal maturity, the porosity of compact and woven bone is approximately 2% and 50%, respectively, and the density (amount of bone tissue per unit volume) in compact bone is approximately double than that of woven bone.[114]

Bones are found in different sizes and shapes. Long bones form all the bones of the limbs (except the wrist and ankle) and are mostly compact bone. Short bones are cube-shaped and contain mostly spongy bone. The wrist bone and the ankle bone are examples of short bones. Flat bones are composed of two thin layers of compact bone sandwiching a layer of spongy bone. Most bones of skull, ribs and sternum are flat bones. Sesamoid bones develop in a muscle tendon such as the kneecap, and around joints. Finally, the bones that do not fit in the mentioned categories are considered irregular bones. Vertebral bones and hip bones are classified in this group. Long bones are composed of a diaphysis (compact bone) which is covered by a fibrous connective tissue membrane called periosteum. Sharpey's fibers, fibers of connective tissue, attach the periosteum to the bone. The epihysis or bone ends consist of a thin layer of compact bone surrounding a spongy area. The epiphyseal line is a remanent of the epiphyseal plate found in young, growing bone. This flat plate of hyaline cartilage allows the bone to grow in length until end of puberty, when it is completely replaced by bone, leaving the remanent epiphyseal line.[115] The cavity of the diaphysis (or shaft) is a storage of adipose tissue, and it is called yellow marrow, or medullary cavity. In contrast, the cavity of the epiphysis contains red marrow and is a blood cell forming area in infants. In adults red marrow is restricted to the cavities of spongy bone of flat bones and epiphysis in some long bones. The committed differentiated (trabecular) bone cells are able to express the adipose phenotype.[116] These circumstances demonstrate the reciprocal relationship between osteoblast and adipocyte differentiation. The transdifferentiation of osteoblasts to adipocytes is an important mechanism in the pathogenesis of osteoporosis.

- Microscopic structure of bone

Bone is a complex structure. The passageways that riddle the bones provide nutrients to the bone cells and a route for waste removal. In bone there are tiny cavities known as lacunae, where the osteocytes lay. In compact bone the lacunae are arranged in concentric circles named lamellae, found around central (Haversian) canals. Haversian canal and matrix rings form a complex called osteon or Haversian system, crossed by blood vessels and nerves (Fig. 7). The central canals are connected to all lacunae by tiny canals known as canaliculi, allowing the nutrient supply through the hard matrix.

**Fig. 7: Image of compact bone showing haversian systems or osteons, as concentric rings, and interstitial systems.** The central osteonal canals are visible here as round dark holes. They are the route for the blood vessels and nerves serving the osteons, known as the Haversian canal. Osteocyte lacunae are visible as elongated black spots in the bone matrix. Canaliculi radiate from the lacunae into the surrounding bone matrix. **(**Marieb EN. 2002).

Perforating Volkmann's canals connect the central canals between them, and to the periosteum and medullary cavity. The periosteum closely wraps all bone, except the bone of articulating surfaces in joints that are covered by synovial membranes (Fig. 8). Like all other internal bone cavities, these canals are linked with endosteum. Hardness of bone is due to the calcium salts and organic parts (composed mostly of collagen) that provide flexibility and tensile strength.[115]

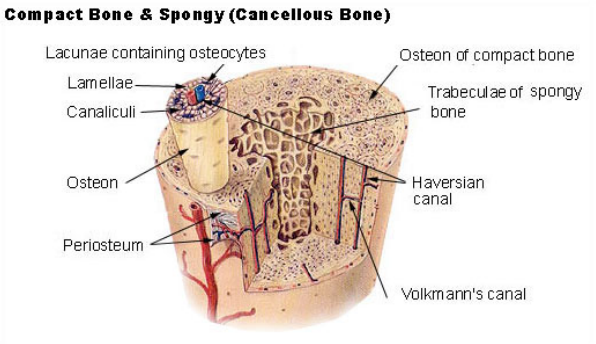



**Fig. 8: Histology of a section of mature bone**. This image shows compact and spongy bone and tissue organization. (training.seer.cancer.gov. Bethesda: SEER Training Modules, Anatomy and physiology: skeletal system. U. S. National Institutes of Health, National Cancer Institute. [updated: 19 August 2010]. http://training.seer. cancer.gov/anatomy/ skeletal/tissue.html).

Both cortical and trabecular bone contain specialized cells (Table 1), organic matrix and mineral phase.

| BONE MARROW STROMA | BONE TISSUE |
|---|---|
| Hematopoietic stem cells | Osteoblasts |
| Mesenchymal stem cells | Pre-osteoblasts |
| Adipocytes | Osteocytes |
| Macrophages | Osteoclasts |
| Mastocytes | Pre-osteoclasts |
| Endothelial cells | Bone lining cells |

**Table 1: Cells found in cortical and trabecular bone.**

1. Bone Cells

Four different cell types are found in developing bone. These are osteoblasts, osteocytes, osteoclasts, and bone lining cells, and are crucial for processes of bone formation and bone remodelling.

- Osteoblasts

Osteoblasts originate from mesenchymal stem cells present in bone marrow and periostium. The commitment of MSC to become osteoprogenitors is regulated by the tissue specific transcription factor CBFA1 and by molecules of the transforming growth factor-ß (TGF-ß) superfamily: the bone morphogenetic proteins. The bone morphogenetic proteins as well as

growth factors like fibroblast growth factor, influence the proliferation and differentiation of osteoprogenitors into osteoblasts. Factors such as parathyroid hormone-related peptide, insulin-like growth factors (IGFs) and vitamin D induce the bone-forming activity of osteoblasts. This activation includes a series of phenotypical changes resulting in production of bone matrix proteins by the bone forming osteoblasts (Fig. 9). Toward the end of the matrix secreting period, a further step is involved in osteoblast maturation. Approximately 15% of the mature osteoblasts become encapsulated in the new bone matrix and differentiate into osteocytes. In contrast, some cells remain on the bone surface, becoming flat lining cells.[117]

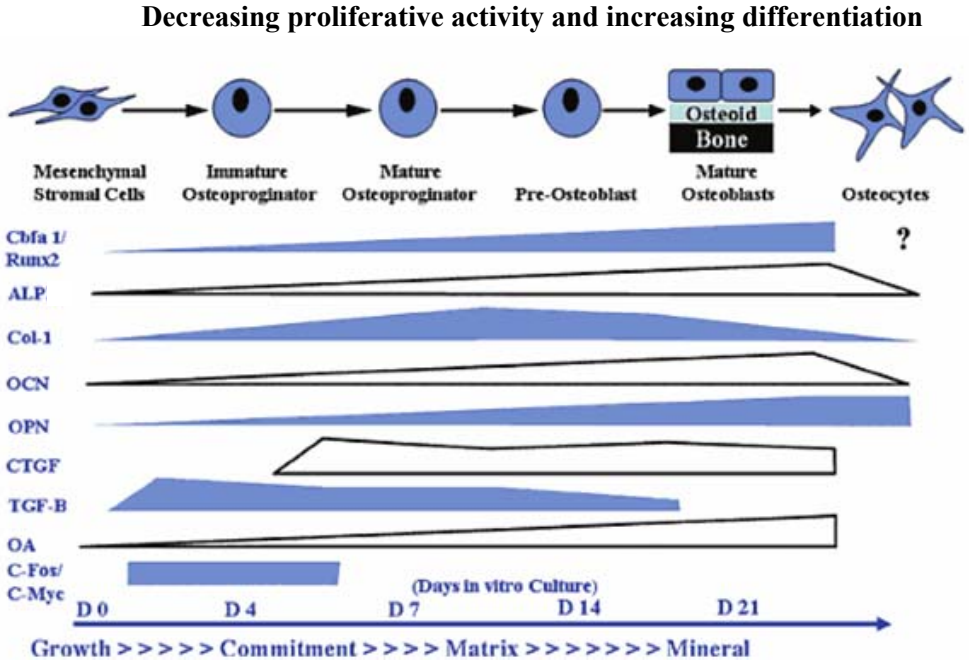



**Fig. 9: Temporal pattern of marker expression during osteoblast differentiation in culture.** As osteoblasts proliferate and differentiate, they express various proteins including growth factors, transcription factors and extracellular matrix proteins, each of which has a different temporal pattern of expression (blue line). Cbfa1/Runx2 (CBFA1), c-fos and c-myc are transcription factors. ALP, alkaline phosphatase; Col-I, collagen type I; OCN, osteocalcin; OPN, osteopontin; CTGF, connective tissue growth factor; OA, osteoactivin; TGF-β, transforming growth factor-β. (Khurana JS. Bone pathology. 2009).

Osteoblasts never appear or function individually but are always found in clusters of cuboidal cells along the bone surface (~100–400 cells per bone-forming site). Among the cytokines secreted by the osteoblast are the main regulators of osteoclast differentiation, i.e. macrophage colony-forming units, receptor activator of nuclear kappa B ligand (RANKL) and osteoprotegerin (OPG). Macrophage colony-forming units are essential in inducing the

commitment of monocytes to the osteoclast lineage whereas RANKL promotes the differentiation and activity of osteoclasts.[118]

- Osteocytes

The osteocyte is the most abundant cell type in immature bone. Osteocytes are former osteoblasts that become embedded in the bone matrix during bone formation. They are situated at regularly spaced distances throughout the matrix and they are connected via slender cytoplasmatic processes within the canaliculi. The processes form a metabolically network connected via gap junctions. This unique 3D organization of osteocytes throughout the bone suggests an important function of these cells in bone metabolism and bone maintenance. They are situated to detect and respond to mechanical loading. Pericellular fluid in the canalicular system is the coupling medium through which mechanical forces are translated into biochemical, mechanochemical, and electrochemical effects at the cellular level. Osteocytes regulate osteoclasts, osteoblasts, and bone lining cell functions by sending signals to the bone surface and secreting a molecule, sclerostin, which inhibits bone formation. This molecule is inhibited by mechanical loading.[119,120]

- Osteoclasts

Osteoclasts are multinucleated cells and can range from 10 μm up to 100 μm in diameter. As it prepares to resorb bone it attaches to the bone surface through specific integrins and then forms a specialized attachment membrane area, the sealing zone. In the center of the sealing zone the osteoclast forms another unique membrane domain, the ruffled border. The actual degradation of bone takes place in the extracellular compartment adjacent to the ruffled border, an area that is sealed off the surrounding extracellular environment. A low pH in the resorption lacuna dissolves the mineral of the bone matrix, exposing the organic matrix for proteases secreted also in the ruffled border area. The low pH of the resorption lacunae is mediated by carbonic anhydrase II and by the V-type ATPase proton pump. Enzymes like cysteine proteinases and, under certain conditions, matrix metalloproteinases (MMP) are responsible for the degradation of demineralised bone matrix in the resorption lacunae. Osteoclasts are derived from hematopoietic cells of the monocyte-macrophage lineage. Osteoblasts express two essential molecules to promote osteoclastogenesis: macrophage colony stimulating factor and RANKL. Macrophage colony stimulating factor binds to its receptor c-FMS expressed by osteoclast precursors and promotes the proliferation of these

cells. RANKL binds to activator of nuclear kappa B (RANK), which is also expressed by the precursors, and stimulates them to become osteoclasts. This process is down-regulated by a secreted member of the TNF receptor superfamily, osteoprotegerin, which binds to RANKL and blocks the interaction with RANK, thereby inhibiting the differentiation of osteoclast precursors into mature osteoclasts. Osteoprotegerin plays a pivotal role in the regulation of bone metabolism not only by inhibiting osteoclast differentiation and activation, but also by increasing osteoclast apoptosis.[118]

- Bone lining cells

Bone-lining cells may play a role in different steps involved in bone resorption and formation such as 1) recruitment of osteoclast precursors, 2) induction of formation of osteoclast at sites were resorption will take place, 3) cleaning of the bottom of Howship's lacuna following osteoclastic resorption, and finally 4) deposition of an initial layer of new bone matrix.[121]

2. Organic matrix

The organic matrix or osteoid material makes up a third of the bone mass. It is composed fundamentaly by proteins, especially collagen, about a 90% (Table 2). The matrix plays an important role within the entire bone system, a fact that becomes evident when a collagen disease such as imperfect osteogenesis appears. However, the extracellular mineralized matrix should now be considered as something more than simply a reservoir of calcium and phosphorous, since it constitutes a reserve of proteins that participate in the regulation of cellular differentiation and in the integrity and function of bone tissue.

| Markers | Immature Cell | Mature Osteoblast |
|---|---|---|
| | General | |
| ALP | ± | ± |
| Stro-1 | + | + |
| | Matrix proteins | |
| Col-III | - | - |
| Col-I | + | ++ |
| OPN | ++ | + |
| ON | + | + |
| OCN | - | + |
| BSP | - | + |
| Thrombospondin | nd | + |
| Biglycan | nd | ± |
| Decorin | nd | ± |

**Table 2: Phenotypic osteoblast markers in human osteoblasts** (Bone mechanics handbook. Cowin SC. 2001). Col, collagen; ALP, alkaline phosphatase, OPN, osteopontin; ON, osteonectin; OCN, osteocalcin; BSP, bone sialoprotein; nd, no detected.

The main composition of the extracellular matrix is collagen, above all type I (>95%) and type V (<5%). The presence of small amounts of collagen type III has also been found, related to Sharpey fibers, and type XII, formed under mechanical stress. In the collagen molecule the Arg-Gly-Asp (RGD) sequence is found and is recognised by the surface integrins of the bone cells. Characteristically collagen contains the hydroxylysine and hydroxyproline amino acids, the latter being a specific marker of all the collagen phenotypes and its urinary secretion values are directly related to the bone resorption rate. The collagen fibers are established by means of hydrogen bridges between amino acids and through the formation of pyridinoline bridges between lysines and hydroxylysines. However, collagen has no great affinity for calcium, for this reason other proteins are involved in mineral deposition.[122]

The bone matrix also contains proteoglycans, proteins with γ-carboxyglutamic acid, glycoproteins, proteins originating from plasma, and growth factors (Table 3).

- Proteoglycans: large molecules making up 10% of the non-collagen proteins. In the osteoid matrix there are four types of proteoglycans: hyaluronan and chondroitin-sulphate, large molecules that take part in the initial stages of bone

morphogenesis, and biglycan and decorin, smaller molecules which appear in the next phases of bone formation and are involved in matrix calcification.

- Proteins with γ-carboxyglutamic acid: i.e. osteocalcin and the matrix protein with γ-carboxyglutamic acid, an amino acid that binds calcium and requires vitamin K for its synthesis. OCN is a small matrix protein synthesized by osteoblasts and platelets. OCN is vitamin D and K-dependent, and it represents 15% of the non-collagen matrix proteins. Plasmatic levels of OCN have been considered as one of the biochemical markers of osteogenesis, being related with the number and activity of the osteoblasts.
- Glycoproteins: i.e. osteonectin (ON or SPARC), alkaline phosphatase (ALP), and proteins with the RGD tripeptide. Osteonectin is a glycoprotein with a strong affinity for collagen type I, calcium and hydroxyapatite. Osteonectin represents 25% of the non-collagen proteins and is thought to play a role in the regulation of cellular adhesion between the matrix and the cells. In bone osteonectin is necessary for normal mineralization. Alkaline phosphatase (ALP) is an enzyme that liberates inorganic phosphate from phosphoric esters, and is necessary for mineralization. Various isoenzymes exist; the bone one is considered a good marker of osteoblastic activity. There are fundamentally five proteins with the RGD tripeptide, also called small integrin-binding ligand, n-linked glycoprotein, osteopontin (OPN), bone sialoprotein (BSP), fibronectin, thrombospondin and vitronectin. These glycoproteins, recognized by the osteoblast and osteoclast integrins, are fundamental to bone regeneration and remodeling processes. They also act as bone cell surface receptors, allowing the adhesion of the cells to the extracellular matrix, and activating signals.
- Proteins originating from plasma: a greater proportion of these are found in the organic bone matrix. They are albumin and a2-SH-glycoprotein, and these are probably related with the deposition of calcium in the osteoid matrix.
- Growth factors: these are polypeptides synthesized within the bone itself or derived from other locations, i.e. liver, platelets, etc. and which take part in the autocrine or paracrine differentiation, growth, and proliferation of the cells. Bone morphogenetic protein -2, -3, -4, -6 and -7, fibroblast growth factor -1 and -2, insulin growth factor-1 and -2, platelet derived growth factor (PDGF), transforming growth factor-β and vascular endothelial growth factor.[122]

| Source of factor | Cellular Response |
|---|---|
| | BMP (BMP-2,-3,-4, 6 and -7) |
| Bone extracellular matrix | Activation of cortical osteoblast |
| Osteoblasts and osteoprogenitors | Initiates differentiation of osteoprogenitor cells into osteoblasts |
| | FGF (FGF-1 and -2) |
| Inflammatory cells (macrophages) | Mitogenic effects on MSC and osteoblasts |
| Mesenchymal cells | Angiogenic actions |
| Chondrocytes | Mesoderm induction |
| Osteoblasts | TGF-ß production |
| Bone extracellular matrix | |
| | IGF (IGF-1 and -2) |
| Liver | Stimulating stem cell differentiation |
| Osteoblasts | Increase bone turnover |
| Bone extracellular matrix | Mitogenic to osteoblast |
| | Stimulates type I collagen production |
| | PDGF (PDGF-AA,-AB,-BB) |
| Degranulating platelets | Macrophage chemotaxis |
| Monocytes and macrophages | Mesenchymal cell chemotaxis |
| Hypertrophic chondrocytes (PDGF-alpha) | Mesenchymal cell proliferation |
| Osteoblasts (PDGF-ß) | Angiogenic action |
| Bone extracellular matrix | Stimulates osteoblast proliferation |
| | TGF (TGF-ß-1 and -2) |
| Degranulating platelets | Pleiotropic factor |
| Bone extracellular matrix | Osteoprogenitor cell proliferation |
| Inflammatory cells | Stimulates undifferentiated mesenchymal cell proliferation |
| Chondrocytes | Stimulates extracellular matrix production |
| Osteoblasts | Mitogenic effects on osteoblast |
| | Stimulates alkaline phosphatase production |
| | Stimulates collagen synthesis |
| | VEGF |
| Osteoblasts | Controls endothelial migration |
| Osteoclasts | Controls osteoclasts/chondroclasts during endochondral ossification |
| Chondrocytes | Regulates proliferation, differentiation and/or survival of osteoblasts, osteoclasts and chondrocytes. |

**Table 3: Major growth factors in bone formation and fracture repair.** (Modified of Bone mechanics handbook. Cowin SC. 2001). BMP, bone morphogenetic protein; FGF, fibroblast growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

3. Mineral phase

The mineral phase is formed by calcium, phosphate and carbonate (in proportions of 10:6:1) in the form of small hyroxyapatite crystals $Ca_{10}(PO_4)_6(OH)_2$ and, in smaller quantities, magnesium, sodium, potassium, manganese and fluoride. The proteins with adhesive capacity favor mineralization, while the proteoglycans, magnesium, adenosine-5'-triphosphate and pyrophosphates function as inhibitors. Hydroxyapatite gives the bone its hardness and is chiefly a form of calcium phosphate (Ca/P). The trigger for the crystallization of this material is not known, but it may involve the interaction of collagen fibers with the glycosaminoglycans of the matrix material. Matured bone contains about 65% mineralized matter, the rest being collagen and matrix. The mineral gives bone its toughness and rigidity, the highly ordered fibrous component provides tensile strength and flexibility. Hydroxyapatite provides the compressive strength to bone to resist pushing forces, while collagen the tensile strength to resist pulling forces as well. Therefore this brittle structure has also a significant degree of elasticity due to these collagen fibers.[123]

The sequence of events charactherizing the differentiation process, leading the stem cell to differentiate to osteoblasts, is not well understood. Osteoblast differentiation is believed to be under the control of the transcription factor CBFA1. This transcription factor performs a dominant and nonredundant role in osteoblast differentiation and controls bone formation.[124] Osteoblasts are derived from mesenchymal stem cells in the mesenchyme or in the marrow stroma. Once committed to the osteoblast lineage, osteoblast precursor cells proliferate and then differentiate into preosteoblasts and then into mature osteoblasts. The cessation of cell growth leads to the matrix maturation stage, which induces expression of alkaline phosphatase and specialized bone proteins that render the osteoid competent for mineral deposition. Osteocalcin and bone sialoprotein are secreted in later stages of osteoblast differentiation and lead to matrix mineralization by calcium deposition.

- Bone-wound healing

Injury to vascularized bone leads to implantation of a provisional matrix, containing mainly fibrin, produced by the activation of platelets, the coagulative and thrombosis systems, as well as fibronectin and thrombospondin bound to fibrin, and platelet granule components. This

granule components derived from platelet aggregation include thrombospondin and cytoquines (TGF-alpha, TGF-β, PDGF, platelet factor 4 and platelet-derived endothelial cell growth factor). This matrix is stabilized by factor XIIIa crosslinking. Wound inflammatory product released by the complement system activates the platelets, inflammatory, and endothelial cells, and recruits inflammatory cells as well as fibroblasts to initiate the repair processes. Platelets, monocytes and lymphocytes secrete chemotactic factors like platelet derived growth factor and transforming growth factor-β. The PDGF and TGF-β growth factors released recruit fibroblasts to the wound. Fibrin plays a major role in development of vascularization, and its function is enhanced when platelet derived growth factor is incorporated in the fibrin matrix, which is critical for bone repair. The invasive growth of bone into an endosseous healing site or during remodeling is the result of osteogenic cell migration, and recruitment, preceding bone formation. The migration of osteoprogenitor cells (osteoconduction) occurs through a 3D transient, soft, biological matrix that acts as a biodegradable sustained release system of chemoatractants and cytoquines to control the wound healing process, and precedes the novo bone formation by these cells.[125]

## 3.3. Bone dynamics

### 3.3.1. Bone modeling

Bone modeling adapts structure to loading, by changing bone size and shape to maintain the bone strength, and involves independent actions of osteoblasts and osteoclasts. During childhood and young adulthood growth and modeling occur continuously, to achieve its final size and shape at the age of 25-30 years. At this age “peak bone mass” is attained and a few years later bone starts to lose mass in an age-related process (Fig. 10). This loss of bone is caused by the remodeling process, during which osteoblasts and osteoclasts work as orchestrated units, matched in time and space. This process renews bone throughout life, but during each remodeling process a small amount of bone is normally lost. The vertebral body is the loadbearing part of the vertebra. When peak bone mass has been attained, the vertebral body consists of a central trabecular network demarcated by a bony shell approximately 400–500 $\mu$m thick. In young individuals the loadbearing capacity of a lumbar vertebral body is 1000 kg or even more.[113]

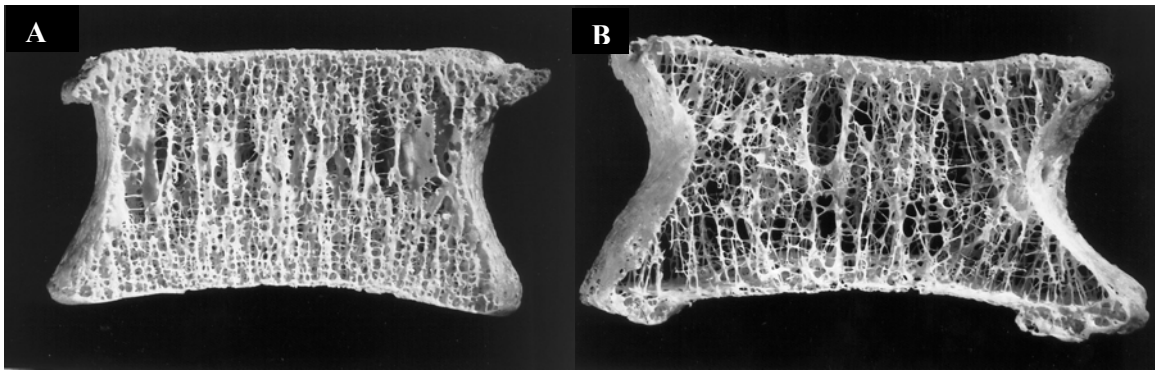



**Fig. 10: Vertebral body showing the trabecular network in a young individue (A) and elderly individue (B).** In the elderly individue trabecular bone density has decreased, showing many perforations. (Mosekilde L. Technol Health Care. 1998).

### 3.3.2. Bone remodeling

Remodeling is initiated by damage that produces osteocyte apoptosis, via the osteocyte-canalicular system to endosteal lining cells, and involves sequential action of osteoblasts and osteoclasts. Bones experiment a high variety of mechanical loadings daily, and this leads them to adapt their mass and structure to the conditions of loading to adapt to them. Osteocytes are the cells which orchestrate this biomechanical regulation, and the process involves a relation between the bone deposition, carried out by osteoblasts, and bone resorption, carried out by osteoclasts. The activity of osteoclasts and osteoblasts is regulated by the osteocytes, which comprise between 90 and 95% of the whole cell bone population in adults.[126] They lie in the hard matrix of bone, known as lacunae, and from them 50-60 processes elongate from the cell body and communicate between the surrounding osteocytes through an intercellular network of dendritic processes.[127] These processes also connect them with bone marrow, bone-lining cells, osteoblasts, and osteoclasts and are altered in bone disease such as osteoporosis or osteoarthritis, and age.[128]

Bones are also being remodeled continuously, in response to changes of calcium in blood, gravity effect and muscles on the skeleton. Bone remodeling occurs throughout life and is required for calcium homeostasis and maintenanace of the skeleton. When blood calcium levels drop below homeostatic levels, the parathyroid glands stimulate the release of parathyroid hormone into the blood, which activates osteoclasts, and calcium ions are released to blood via breaking down of the bone matrix. If levels of calcium are too high, calcium is deposited in bone matrix as hard calcium salts.[108]

The bone remodeling process occurs in small packets of cells called basic multicellular units, consisting of about 10 osteoclasts and several hundred of osteoblasts, which turn bone over in multiple surfaces and susequently form new bone to replace resorbed bone. The result of this activity is a packet of bone called the bone structural unit, and there can be many bone structural units made at different times within any bone. A bone structural unit in cortical bone is called an osteon.[129] At any time ~ 20% of the cancellous bone surface is undergoing remodelling. Autocrine or paracrine factors generated in the bone microenvironment are probably controlling activation of cellular events responsible for remodeling, because each basic multicellular units is geographically and chronologically separated from other packets of remodelling.[130]

Osteoblastic bone formation follows osteoclastic bone resorption in bone remodeling, in a process called coupled bone formation. The tumor necrosis factor (TNF) family member receptor activator of RANKL, expressed by osteoblasts and their immature precursors and its receptor RANK, expressed in osteoclast precursors and osteoclasts, are key regulators of bone remodeling, and are essential for the development and activation of bone-resorbing osteoclasts. RANKL and its receptor osteoprotegerin, produced by osteoblasts and bone marrow stromal cells, modulate the bone homeostasis. Osteoprotegerin binds to RANKL, limiting the interaction between RANKL and RANK, and inhibits the osteoclast differentiation and lymphocyte development. Osteoprotegerin has a protective effect from bone loss in mammals, and alterations of the RANKL/OPG ratio are critical in the pathogenesis of bone diseases, leading to increased bone resorption.[131]

Bone remodeling is essential for: 1) adaptation to mechanical loading, 2) maintenance of bone strength, and 3) manteinance of calcium homeostasis.[121]

Control factors involved in bone modeling are endocrine, mechanical, paracrine, and autocrine. Endocrine factors involved are bone metabolic hormones such as parathyroid hormone, vit D, calcitonin, growth hormone, somatotropin, insulin growth factor –I and -II and sex steroids, i.e., testosterone and estrogen. Mechanical factors taking part in bone modeling are disuse atrophy, bone maintenance, physiological hypertrophy, and pathological overload. A <1000 peak load in microstrain provokes more remodeling, whereas a >2000 peak load in microstrain causes less remodeling in bone.[110]

Bone remodeling can be divided into the following phases: quiescent, activation, resorption, formation, and mineralization.[133]

1. Quiescent phase: the bone remains resting. The factors that initiate the remodeling process remain unknown.
2. Activation phase: the first phenomena that occurs in this phase is the activation of the bone surface prior to resorption, through the retraction of the bone lining cells on the endosteal surface and the digestion of the endosteal membrane by collagenase action. Once exposed, the mineralized surface attracts the circulating osteoclasts coming from the nearby vessels.
3. Resorption phase: the osteoclasts then begin to dissolve the mineral matrix and decompose the osteoid matrix. This process is completed by the macrophages, and permits the release of the growth factors contained within the matrix, fundamentally transforming growth factor-β, platelet derived growth factor, and insulin-like growth factor I and II (IGF-I and II).
4. Formation phase: simultaneously in the resorbed areas the preosteoblast grouping phenomena is produced, attracted by the growth factors liberated from the matrix which act as chemotactics, and stimulate, in addition, their proliferation. The preosteoblasts synthesize a cementing substance upon which the new tissue is attached, and express bone morphogenic proteins which are responsible for osteoblast differentiation. A few days later, the already differentiated osteoblasts synthesize the osteoid material which fills the perforated areas.
5. Mineralization phase: mineralization begins thirty days after deposition of the osteoid, ending at 90 days in the trabecular bone and at 130 days in the cortical bone.
The quiescent or 'at rest' phase then begins again.

### 3.4. Bone loss

Patologic conditions, trauma, infections and congenital deformities are among the causes of bone defects, especially in the jaw. Severe maxillary atrophy has a multifactorial aetiology, including long evolution edentulism, hyperpneumatization of the maxillary sinus, post-traumatic deficit, bone loss after surgery, and periodontal problems or infection.[134]

### 3.5. Bone reconstruction

The goal of reconstructive surgical intervention is the restoration of normal structure, with improvement in the function and appearance. The tissue most commonly used to substitute

lost osseous structure is bone, with different degrees of success. The bone transplanted to the oseus defect is known as a graft. It is critical to take into account the bone physiology and individual immunologic concepts in this healing process, besides the surgical principles and banking procedures. Healing of bone is unique between connective tissues, as it arises from tissue regeneration instead of simple tissue repair with scar formation, as occurs in other connective tissues. Graft healing is termed incorporation. The quality and vascularity of the recipient bed, into which the graft is placed, plays a key role in the successful incorporation. The bed provides the cellular elements that are transformed into osteoblasts mediated by inductive factors contained within the bone graft. The bed also provides the blood vessels that bring the nutrients, which are needed to ensure the survival of the transplanted osteoblasts in the cancellous moiety of the bone graft. When bone is transplanted from one area to another, activation of several processes occur during the incorporation of the graft.[135]

### 3.5.1. Two-phase theory of osteogenesis

This theory begon on the XX century, when Barth and Axhausen studied histological specimens to better understand the process of graft incorporation. They concluded that vascularity of the host bed was critical in providing blood vessels that could invade the inert graft and replace it with living host bone. When the graft is removed from the body to the transplantation place the blood supply is ceased, what causes a considerable death of cells, but the diffusion of nutrients from the surrounding tissues involves bone regeneration from the surviving bone cells. This first phase is responsible for the formation of most of the new bone and can last up to 4 weeks. The amount of bone formed depends on the number of cells that survive, therefore it is crucial to provide the largest number of osteoprogenitor cells per given volume of tissue, to ensure an adequate bone formation. In the 2$^{nd}$ week, an intense angiogenesis begins, and fibroblast proliferation occurs from the graft bed and then osteogenesis takes place. Thereafter osteoprogenitor cells differentiate to osteoblasts, and begin to form new bone.[136] Evidence shows that some proteins found in the bone are very important in this process such as the bone morphogenetic proteins.[47] In this second phase resorption occurs, and replacement and remodeling lead to the incorporation of the graft into the host bed.

### 3.5.2. Immune response

One of the complications of transplanting tissue is the immunological response that leads to a graft rejection. When a tissue is transplanted from different individuals or from different species, the immune system becomes then an obstacle to the integration of the graft in the host tissues. If the graft is recognized as a foreign member, the host will trigger an intense response to try to destroy the graft. This response is primarily a cell-mediated respons by T-lymphocytes. This response may not occur immediately, and the incorporation of the bone graft appears to be successful until the rejection appears, soon or lately, in the bed graft.

There are two approaches to improve the success of grafting procedures. First is immunosuppression with various medications, but it is not recommended in oral and maxillofacial surgical procedures, for the potential complications from the immunosuppression. Another more helpful approach is the alteration of antigenicity of the graft to avoid stimulation of the immune response, with methods such as boiling deproteinizing, merthiolating, freezing, freeze-drying, irradiating, and dry-heating.[37]

### 3.5.3. Types of grafts

There are several types of grafts, categorized according to their nature (origin) and thus the potential to induce an immunologic response.

#### 3.5.3.1. Autogenous grafts

It is defined as tissue transplanted from one site to another within the same individual. Because the grafts are from the same individual the tissue is recognized as “self”, and the immune system is not triggered with an immunological response. Fresh autogenous bone is the most ideal bone graft material. Is the only type of bone graft able to supply living bone cells essential to phase I osteogenesis, and therefore has been considered the Gold Standard graft. It can be taken from different sites and in several forms. The only disadvantage of the autograft is that it has to be harvested from a secondary site in your body, which usually means more morbidity and a more complicated surgery overall.[37]

#### 3.5.3.1.1. Block grafts

Block grafts are solid pieces of cortical and underlying cancellous bone usually taken from iliac crest, but also taken from ribs.

#### 3.5.3.1.2. Particulate marrow and cancellous bone grafts

Particulate marrow and cancellous bone grafts are obtained by harvesting the medullary bone and the associated endosteum and hematopoietic marrow. Those grafts contain the greatest concentration of osteogenic cells. Because their particulated nature, they allow more nutrients to access the particulate surface and nourish the cells, assuring the proliferation of these cells. Particulate bone grafts can be obtained from ilium in large quantities. The diploic space of the cranial vault has also been used when small amounts of bone chips are needed, for example for alveolar cleft grafts.[37]

#### 3.5.3.1.3. Autogeneous bone with blood supply

Autogenous bone may also be transplantated with blood supply. Two methods use this procedure:

3.5.3.1.3. a) Bone graft pedicled to a muscular or muscular and skin pedicle.

In this technique the pedicle is not stripped from the soft tissue and some blood supply is preserved, thus a great number of surviving osteogenic cells can be found in the bone graft. An example of this type is a segment of the clavicle, pedicled to the sternocleidomastoid muscle and transferred to the mandible.[37]

3.5.3.1.3. b) Use of microsurgical techniques.

In this technique a block of ilium, tibia, rib, etc. can be used for grafting material. The block is removed with the overlaying soft tissue after dissecting free an artery and a vein, and the block and overlaying soft tissue are reconnected in the site with another artery and vein, through a microvascular anastomoses.

Both of these types are known as composite grafts, because they contain soft tissue and osseus elements. The bone graft pedicled to a muscular or muscular and skin pedicle is known as a pedicled composite graft, and the second type of bone graft including a block is known as a free graft, because is totally removed from the body and immediately replaced, and the blood supply restored. Composite grafts have shortcomings applied to the jaw such as some stripping, because the soft tissues attached during the procurement and placement. In occasions an inadequate bulk of bone is provided, for example for continuity defects in the jaw. Also the morbidity to the donor site is a problem. Removing the soft tissue improves the outcome of the graft. The advantages of autogenous grafts are the presence of osteogenic cells and the absence of an immune response. A disadvantage is the morbidity because another site of operation is needed to obtain the graft.[37]

#### 3.5.3.2. Allogenic grafts

These grafts are taken from another individual of the same species, usually from cadaver bone. Because the genetic differences found, it is necessary to treat the graft to reduce the antigenicity and to avoid cross contamination. The most used allogenic bone is the freeze-dried, but others include irradiation, acid washing, and other chemical treatments. All these processes destroy the osteogenic cells in the graft and reduce the immunogenicity, and therefore the phase I of osteogenesis does not occur. These grafts act merely passive, as a matrix for phase II induction. The advantage is that is not required another site of operation in the host, and similar bone and similar shape can be obtained, for example an allogenic mandible for reconstruction of a mandibulectomy defect. The disadvantage of this type of graft is that viable cells are not provided and thus phase I osteogenesis cannot take place.[37,137]

The ideal graft would have characteristics of a block graft, with osteogenic potential of particulate marrow and cancellous-bone grafts. A large block graft does not provide a high concentration of osteoprogenitor cells such as particulate marrow and cancellous–bone graft do, and a large block graft implies removing a large portion of bone in the patient, with the consequent morbidity to the site. To reconstruct defects in the mandibles a technique can be used that combines allogenic and autogenous bone grafts. The cortical of an allogenic block of an ilium or mandible is obtained, and it is used as a matrix for phase II bone formation. The cortical is filled with autogenous particulate marrow and cancellous bone, and this provides

the osteoprogenitor cells necessary for the phase I osteogenesis. The allogenic part is biodegradable and is replaced entirely by host bone. The advantages are several, i.e. there is no immunological response with this technique, the combination of grafts allows to obtain a large quantity of bone and a sufficient number of osteoprogenitor cells are provided. The disadvantage is the second site of operation in the host, which is required to obtain the autogenous particulate marrow and cancellous bone graft.[37]

#### 3.5.3.3. Xenogenic grafts

Those grafts are taken from one species and grafted to another. The antigenicity is greater than in allogenic grafts, causing more problems of rejection. Therefore the graft must be treated carefully before implanted to avoid rejection. The advantages are that do not require another site of operation in the host, and a large quantity of bone can be obtained. The disadvantages are that xenografts do not provide viable cells for phase I osteogenesis and must be rigorously treated to reduce the antigenicity. Some examples from bovine xenograft commercially available are Bio-Oss® (Geistlich AG, Wolhusen, Switzerland), Endobone® (Merck Biomaterialen, Darmstadt, Germany), Laddec® (Ost Development, Clermont-Ferrand, France), Bon-Apetite® (Bio Interfaces Inc., San Diego, USA).[37]

#### 3.5.3.4. Alloplast grafts

The Alloplast usually includes any synthetically derived graft material not derived from animal or human origin. In oral implantology this usually includes hydroxyapatite and β-tricalcium phosphate.[37]

### 3.5.4. Principles osteogenesis

Three basic principles should be considered to enhance bone healing and bone formation: osteogenesis, osteoconduction, and osteoinduction, and they should be taken into account when regenerating bone defects (Table 4).[137]

| Osteoinduction | Osteogenesis | Osteoconduction |
|---|---|---|
| Bone precursor cells | Growth factors | Porous coatings |
| TGF-β super family | Ca/P ceramics | Ca/P ceramics |
| Bone autografts | Bone autografts | Bone autografts/allografts |

**Table 4: Basic principles for enhancing of bone healing and bone formation and their mechanisms.** TGF-β, transforming growth factor-β; Ca/P, calcium phosphate. (Modification of Lind M et al. Eur Spine J. 2001).

#### 3.5.4.1. Osteogenesis

Osteogenesis is stimulated by natural biochemical processes that initiate and mantain bone formation during a healing response. The growth factors stored in the bone matrix participate in the activation and maintenance of cellular processes during bone formation and bone healing.[137]

#### 3.5.4.2. Osteoconduction

Osteoconduction is the enhanced bone formation provided by a favorable structural environment, that allows the osteoprogenitor cells to migrate.[137]

#### 3.5.4.3. Osteoinduction

In this process osteogenic precursor cells produce new bone. This can be achieved by two approaches: cell-mediated therapy and growth factor-mediated therapy. In the first, bone precursor cells are harvested and placed into the bone defect, proliferating and differentiating to mature osteoblasts and producing new bone. In the second, a subset of bone morphogenic proteins are locally applied to induce bone formation. This family of proteins stimulates MSC to differentiate towards the condrogenic and osteogenic lineages.[137,138,139]

### 3.6. Bone tissue engineering

#### 3.6.1. Stem cells

#### 3.6.1.1. Properties of Stem Cells

Stem cells have the potential to develop into many different cell types in the body during early life and growth. In many tissues they act as a internal repair system, dividing

continuously without limit, to replenish other cells during the lifetime of the organism. After stem cell division, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function such as a muscle cell, a red blood cell, or a brain cell. Stem cells differ from other cell types by two important characteristics. First, they are unspecialized cells, able to renew themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs such as the gut and bone marrow, stem cells regularly divide to repair and regenerate worn out or damaged tissues. On the contrary, in other organs such as pancreas and heart, stem cells only divide under special conditions.[140]

#### 3.6.1.2. Plasticity of Stem Cells

Stem cells can be classified into three broad categories, based on their ability to differentiate. These basic measures of the stem cell potency or plasticity are: totipotent, pluripotent, and multipotent, from less restricted to more restricted. Totipotent stem cells are found in the undifferentiated inner cell mass of the blastocyst and can give rise to any of the over 200 different cell types present in the body, including the germ cells. Pluripotent stem cells are cells that can give rise to cells derived from the three embryonic layers, mesoderm, ectoderm, and endoderm, but not the placental tissues. Multipotent stem cells are able to differentiate to only one lineage. Although their ability to differentiate is more limited than pluripotent stem cells, they already have been successfully applied in cell-based therapies.

What determines stem cell potency is dependent on genetics of the cell, and whether it contains the appropriate active or activated genes and programming ability to differentiate into a determined cell type or different cell types. However, the environment in which the stem cell is located plays also an important role on determining the cues that the cell receives. Changes in local growth factors, cytokines, hormones, chemokines, etc., as well as cell-cell and cell-matrix contacts are important in switching on and off the genes and gene pathways, and even in reprogramming these pathways, what results in changes in these cells.[141,142] This classification of stem cell potency is not rigid, becoming more evident that the distinction between pluripotent and multipotent is becoming increasingly more undefined, with some cells seeming to have greater plasticity than what previously thought.

The process of differentiation, where a cell acquires a set of characteristics enabling it to perform a determined function (e.g. insulin production by pancreatic beta cells or dopamine production by neural cells, etc.), usually involves an intermediate progenitor cell, also called a transit amplifying cell. They have properties of stem cells, like being able to divide a number of times, but usually with each division the cell becomes progressively more differentiated. The main function of progenitor cells is to increase cell numbers, and to contribute to the formation of tissue and organs in response to morphogenic stimuli. Progenitor cells may also exhibit a degree of plasticity, from being unipotent and restricted to one specific cell type, to being bi/tripotent and giving rise to several differentiated cell types. In skeletal tissue, cartilage (chondrocytes), fat cells (adipocytes) and bone forming cells (osteoblasts) cells are believed to arise from a common tri-potential progenitor, which arises itself from a stem cell located within the bone marrow microenvironment.[143]

#### 3.6.1.3. Sources of stem cells

##### 3.6.1.3.1. Embryonic stem cells (ES)

Scientists discovered ways to derive pluripotential cells from early mouse embryos nearly 30 years ago, in 1981. These cells, called embryonic stem cells, were harvested from the inner cell mass of murine blastocyst seven to ten days after fertilization.[144] The detailed study of the biology of mouse stem cells led to the discovery, in 1998, of a method to derive stem cells from human embryos and grow the cells in the laboratory. These cells derived from pre- or peri-implantation human embryo, are capable of prolonged undifferentiated proliferation, and demonstrate potential to form derivatives of all three embryonic germ layers even after prolonged culture.[145] However, culture of these cells have several important drawbacks:

1. ES are difficult to control, and it may take many attempts before researchers are able to derive the desired cell line from them.

2. The use of ES involves the destruction of 5 to 7-day-old embryos, which has raised moral and ethical issues amongst the public.

3. There is also a risk of immunogenic reaction, as stem cells from a random embryo donor are more likely to face rejection after transplantation. The bovine and murine products currently required to grow undifferentiated human ES, lead to incorporate an immunogenic

molecule of animal origin to these cells, causing immune rejection to humans.[146] Besides this, use of reagents of animal origin may cause hypersensitivity reactions in patients and they may introduce pathogens. Furthermore, there is an increased scrutiny for contamination with the causative agent of bovine spongiform encephalopathy, since the outbreak of the epidemic in the United Kingdom, and there is still no reliable test to detect the bovine spongiform encephalopathy infectious agent.[147]

4. Clear teratomas form after ES transplantation in animals, containing cell types derived from the three germ layers: keratinized epithelium from ectoderm, ciliated epithelium, goblet cells from endoderm, and cartilage from mesoderm.[148]

Recently, a sobering drawback appeared of use of fetal stem cells, showing that fetal stem cells injected into patients can cause disabling and deadly tumors.[149] Because all this reasons no clinical trials are performed using these cells.[150]

**3.6.1.3.2. Adult stem cells**

While embryonic stem cells form tissues, adult stem cells are essential for tissue maintenance and repair throughout life. Adult stem cells are found in many tissues from the body. These cells divide and differentiate to replenish the supply of cells that die or to repair the damaged tissue. Tissues like blood, skin, liver, bone, and gut are replenished and repaired almost constantly, in a process that is carried out throughout life and involves tissue aging and senescence. Many adult tissues contain stem cells that can be isolated such as bone, skin, hair follicle, retina, blood and blood vessels, intestine and gut, liver, lung, muscle, brain, pancreas, umbilical cord blood, placenta, and adipose tissue.

In the body, stem cells differentiate to a particular cell type, usually associated with the tissue in which they are located. Therefore, adult stem cells are traditionally considered multipotent stem cells. Although there is increasing evidence of a broader plasticity than thought in these cells, suggesting that adult stem cells might in fact possess pluripotency, supported by several facts:

a) Stem cells from bone marrow have been differentiated into neural cells and likewise stem cells from brain have been differentiated into blood cells.[151,152]

b) Studies on bone marrow transplantation into irradiated mice have demonstrated that small numbers of marrow-derived stem cells, probably marrow hematopoietic stem cells (HSC), can migrate to various tissues and organs around the body and have the capacity to produce non-marrow cells after engraftment.[153]
c) Bone marrow stem cells have been used in clinical trials to help promote repair of heart tissue damaged after heart attack.[154,155]
d) The expression of genes and proteins associated with pluripotency (i.e. those expressed by embryonic stem cells) have been detected in some adult stem cells.[156,157]

Adult stem cells can be divided regarding their tissue of origin in intestinal/gut, lung, epithelial, muscle, endothelial/hemangioblasts, liver, pancreatic, eye or retinal, cardiac, epidermal, neural, hematopoietic and mesenchymal stem cells. Besides these there are cells with characteristics of stem cells, called multipotent progenitor cells (MAPC), induced pluripotent stem cells (iPS) and cancer stem cells. We will review the stem cells that are found in the stromal vascular fraction (SVF) of the adipose tissue and also the MAPC, iPS and cancer stem cells.

**3.6.1.3.2. a) Mesenchymal stem cells (MSC)**

The presence of non-hematopoietic stem cells in the bone marrow was first suggested by the German pathologist Cohneim more than 130 years ago, who proposed that bone marrow can be the source of fibroblasts contributing to wound healing in numerous peripheral tissues.[158] In the early 60s, the pioneering work of Friedenstein and colleagues demonstrated that whole bone marrow transplanted under the kidney capsule of mice could generate heterotopic bone and these cells could support hematopoiesis as well.[7,159] Later in the 70s Friedenstein and co-workers showed that a population of fibroblastoid cells was found in bone marrow from pigs, and this population had clonogenic potential *in vitro*. These isolated spindle-like cells were adherent to the plastic, and were capable of forming colonies termed colony-forming unit fibroblasts (CFU-F). These cells could also make bone and reconstitute a hematopoietic microenvironment in subcutaneous transplants. Moreover, Friedenstein demonstrated that they could regenerate heterotopic bone tissue in serial transplants, thus providing evidence supporting their self-renewal potential.[160] Over the years, numerous laboratories have confirmed and expanded these findings, by showing that cells isolated according to Friedenstein's protocol were also present in the human bone marrow, and by demonstrating

that these cells could be sub-passaged and differentiated into a variety of cells of the mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes, and myoblasts *in vitro*[10,161,162,163] and *in vivo,*[11,12,13] making these cells promising candidates for mesodermal defect repair. Friedenstein had thus isolated from the bone marrow what later on would have been renamed by Caplan and colleagues "mesenchymal stem cells" or MSC.[23]

Phenotypically, MSC express a number of markers, none of which, unfortunately, are specific to MSC. Human MSC do not express the hematopoietic markers CD45, CD34, CD14, nor CD11. They also do not express the costimulatory molecules CD80, CD86 or CD40, the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule [PECAM]-1), CD18 (leukocyte function-associated antigen-1 [LFA-1]), nor CD56 (neuronal cell adhesion molecule-1), but they do express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, and Stro-1, as well as the adhesion molecules CD106 (vascular cell adhesion molecule [VCAM]-1), CD166 (activated leukocyte cell adhesion molecule [ALCAM]), intercellular adhesion molecule (ICAM)-1, and CD29.[19]

The use of a minimal criteria to define MSC by scientists would help to standardize the protocols used in obtaining the cells, and to characterize each cell type, therefore ensuring a more efficient progression in both preclinical and clinical trials. This criteria was postulated by the Mesenchymal and Tissue Stem Cell Committee of the ISCT in 2006,[14] and included three statements:

1. Adherence to plastic in standard culture conditions.
2. Phenotype positive (≥95%+): CD105, CD73, CD90.
   Phenotype negative (≤2%+): CD45, CD34, CD14 or CD11b, CD79 α or CD19, and HLA-DR.
3. *In vitro* differentiation to: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of *in vitro* cell culture).

However, the clinical use of MSC presents several problems such as pain related to the procedure, morbidity, and low cell number upon harvest, especially in the old donors.[20] The cell yield is low, approximately one MSC per $10^5$ adherent stromal cells.[22] Low stem cell

numbers need an *ex vivo* expansion step to obtain therapeutic cell doses which is time consuming, expensive, and risks cell contamination and cell loss.

MSC or MSC-like cells are not a unique feature of the bone marrow, as they are also found in tissues such as fat, periostium, bone marrow, umbilical cord blood, amniotic fluid, placenta, skeletal muscle, heart, dermis, dental pulp, liver and spleen, though the complete equivalency of such populations has not been formally demonstrated using robust scientific methods.[164,165]

Unlike most other human adult stem cells, MSC can be obtained in quantities appropriate for clinical applications, making them good candidates for use in tissue repair. Techniques for isolation and amplification of MSC in culture have been established, and the cells can be maintained and propagated in culture for long periods of time, without losing their capacity to form all the above cell types. Besides this, they show to have an anti-inflammatory and immunomodulatory effect, that makes them interesting for treatment of allogenic conflicts.[166]

Over the years, it has also become progressively clear that MSC could be the basis for an extremely powerful natural system of tissue repair.[167] MSC have been demonstrated, upon exogenous administration, to serve as effective therapeutic agents in a variety of experimental models of tissue injuries.[168,169,170] Curiously, in the vast majority of these studies the therapeutic efficacy did not correlate with the efficiency of engraftment, which was in general low. This finding suggests that the ability to repair was very likely secondary not to transdifferentiation of MSC into the appropriate cell phenotype or to cell fusion, but rather to the secretion by MSC of soluble factors that altered the tissue microenvironment.[167] In other terms, MSC may thus provide what Caplan and colleagues define as "trophic activity".[171] The properties of these cells make them interesting candidates for clinical applications, existing more than 120 clinical trials being carried out at the moment using these cells.[150]

**3.6.1.3.2. b) Endothelial progenitor cells (EPC) /Hemangioblasts**

Two vascular endothelial populations have been defined on the basis of CD31 and CD34 expression. Nonhematopoietic cells coexpressing CD31 and CD34 (endothelial progenitor), the more prevalent of the two, are located within capillaries, whereas CD31+/CD34- cells (endothelial mature) are localized into the lumen of small vessels. The phenotype of adipose derived endothelial progenitors is consistent with that reported for both bone marrow and

circulating endothelial progenitor cells (CD45-/CD34+/VEGFR2+), but the frequency in adipose stromal vascular fraction is found to be three to five orders of magnitude higher, making them excellent candidates for regenerative therapy.[172] The long-standing tenet that vasculogenesis occurs exclusively during embriogenesis has been challenged by Asahara and colleagues, that showed that EPC in adults contribut to formation of new blood vessels in the absence of pre-existing ones via *de novo* production of endothelial cells.[173]

EPC also express CD133, characteristic marker of hematopoietic stem cells, and are thought to be derived from a common precursor of hematopoietic and endothelial cells, called the hemangioblast.[174]

**3.6.1.3.2. c) Hematopoietic stem cells (HSC)**

Hematopoietic stem cells are adult stem cells found mainly in the bone marrow, but are also circulating in the blood and occupy other tissues like the spleen. HSC provide the blood cells required for daily blood turnover and for fighting infections. The proliferation and differentiation of HSC is tightly controlled in their niche by physical contact with stromal cells and osteoblasts, together with various growth factors such as stem cell factor and other factors such as angiopoietin, $Ca^{+2}$ ions, CXCL12, osteopontin, and thrombopoietin. Perivascular cells may also play an important role in HSC maintenance.[175,176] The existence of these cells was inferred from studies showing that a proportion of transplanted marrow cells could engraft in spleen and form macroscopic colonies of proliferating cells that differentiated into erythrocytes, granulocytes and megacaryocytes and rescue animals from the lethal effects of radiation. These macroscopic colonies were clonal in nature and derived from single cells, being refered later as colony forming-unit spleen. This provided evidence from existence of a common myeloid progenitor cell in bone marrow.[177] Thereafter, cytological evidence demonstrated that a single donor marrow cell could yield myeloid and lymphoid cell lineages *in vivo*, and the concept of a hierarchical model for HSC differentiation was spurred.[178] HSC express CD34+ and CD45+,[179,180] and when cultured they must grow on a support matrix such as methylcellulose, because they do not grow adhered to cell culture plates like MSC do. Besides this, growth factors like the stem cell factor must be added to culture, to help stimulate and maintain HSC proliferation.[181]

Hematopoietic stem cells have been studied by scientists for many years, and they were the first stem cells to be used successfully in clinical therapies. HSC have been used for decades to treat blood cancers (i.e. leukemia) and other blood disorders. More recently, their use in treatment of breast cancer and coronary artery diseases has also been explored. The potential for HSC to produce cell types other than blood cells has become the subject of intense scientific controversy, and it is still not clear whether they could be used on a clinical scale, to restore tissues and organs other than blood and the immune system.[182]

**3.6.1.3.2. d) Multipotent progenitor cells (MAPC)**

Multipotent progenitor cells were isolated as a subset of bone marrow MSC and exhibit a fibroblastic morphology similar to MSC. They express cell surface markers that are distinct of MSC, and express markers associated with embryonic stem cells and pluripotency, and after expansion *in vitro* they can contribute to all the three germ layers upon injection into blastocysts, at least in rodents.[183] Relatively few experiments have been performed to date to explore and confirm the biology of these cells.

**3.6.1.3.2. e) Induced pluripotent stem cells (iPS)**

Fibroblasts and other adult somatic cells can be directly reprogramated into an induced pluripotent status by transfecting 4 pluripotent transcription factors. OCT3/4, SOX2, c-Myc, and Klf4. These cells, named induced pluripotent stem cells, once cultured in embryonic stem cell specific medium with basic fibroblast growth factor, can generate cells with large nuclei and scant cytoplasm which grow in suspension resembling embryonic stem cells. IPS express characteristic embrionary stem cell genes such as OCT3/4, SOX2 and NANOG and are able to differentiate into any of the three germ layers *in vitro*. They also show high telomerase activity and teratoma formation *in vivo*.[184] For drug development, human iPS cells may facilitate to generate panels of immortalized cell lines that more closely reflect the genetic diversity of a population. They also may make possible to generate cell lines from individuals predisposed to specific diseases, avoiding the controversial of using human embryonic stem cells.

IPS may be obtained from other tissues as well, such as cord blood and adult blood CD34+ cells,[185] bone marrow mononuclear cells,[186] neural stem cells (NSC) and adipose stem cells.[187]

#### 3.6.1.3.2. f) Cancer stem cells

It was discovered more than 15 years ago, that only a tiny population of leukemia cells could transmit the cancer from one experimental animal to another. More remarkably, the cells had a property previously seen only in stem cells: the ability to produce an exact copy of themselves each time they divide, thereby maintaining the ability to reproduce in perpetuity. This property of these cells, called cancer stem cells, might be what make the disease so hard to eradicate by conventional treatments. Radiation and many chemotherapeutic drugs wipe out dividing cells, but stem cells remain in a quiescent state the most of the time, and they may survive cancer treatment.[188]

These cells show low proliferative rates, high self-renewing capacity, propensity to differentiate into actively proliferating tumour cells, resistance to chemotherapy or radiation, and they are often characterized by elevated expression of the stem cell surface marker CD133.[189] Cancer stem cells also express high doses of vascular endothelial growth factor, what promotes angiogenesis and supports tumor growth.

Cancer stem cells are believed to arise from oncogenic mutations in normal tissue stem cells or progenitor cells, and they can spread in the body driving to metastasis. This is caused by highly active genes involved in cell migration and tissue invasion. Some ways of treatment are to directly kill the cells, i.e. using drug parthenolide, block their spread, i.e. antibody binding to CD44, or inducing them to lose their "stemness" and differentiate into non-renewing cells.[188] Bone morphogenetic proteins inhibit the tumorigenic properties of human glioblastoma stem cells in this fashion.[190]

### 3.6.2. Stem cell niche

In absence of specific and unique markers that would allow for a proper identification of MSC *in vivo*, a histological localization of these cells is virtually impossible to identify, and is clearly lacking. These cells reside in places termed niches, comprised of complex mixtures of extracellular cues delivered by support cells in close proximity. Stem cells in niches are in close contact with stromal support cells, providing short-range signals via soluble factors and cell-cell communication, as well as biochemical and mechanical signals from the surrounding

extracellular matrix. Intriguinly, blood vessels are often found near niches, suggesting they serve as conduit to attract circulating cells and to transport long-range signals. The key functions of the niches are to keep stem cells in an undifferentiated state and to regulate theirs self-renewal. Loss of contact with the niche or disruption of the structure means a loss of stem cell function. An adult stem cell in its niche can undergo four different fates: it can remain quiescent, undergo self-renewal divisions that result in two daughter stem cells (symmetric divisions), self-renewal divisions that result in one daughter stem cell and one differentiated cell (asymmetric divisions), or divisions in the absence of self-renewal resulting in two differentiated progeny. These fates are tightly regulated by the niche, in order to assure an adequate size of the stem cell pool during homeostasis and regeneration. In development and regeneration adult stem cells in the niche must increase in numbers. Mammalian niches have been identified in multiple self-renewing tissues including the skin (bulge region of the hair follicle), intestine (in the epithelium), brain (subventricular zone), bone marrow (on the endosteal surface and near blood vessels) and muscle (beneath the muscle fiber basal lamina).[191]

An extensive literature has pointed to pericytes as a potential source of MSC.[192-195] MCAM/CD146 (+) subendothelial cells isolated from the human bone marrow stroma adhere to the plastic *in vitro* and are clonogenic; moreover, they self-renew, at least *in vitro*, and they can generate bone and a hematopoietic supportive microenvironment in subcutaneous transplants in mice. This is the only cell population in bone marrow that is both clonogenic *in vitro* and capable of transferring a hematopoietic microenvironment in subcutaneous transplants.[196] These cells, which reside in the wall of the sinusoidal blood vessels of the bone marrow, are also positive for angiopoietin-1 a critical regulator of vascular remodeling. The findings by Sacchetti and colleagues (2007) represent the first rigorous attempt to histologically localize and phenotypically define MSC-like cells, or at least a subpool of this population. Notably, a recent paper by Crisan and colleagues (2008) suggests that multipotent MSC with perivascular localization exist in numerous human organs.[197] Whether the vascular setting provides a true niche for pericytic MSC-like cells and is the main source of MSC *in vivo*, remains to be established. In this regard, however, it is important to note that an increasing amount of evidence has recently linked angiogenesis to osteoblastogenesis, suggesting that blood vessels could be a source of osteoprogenitors or of MSC with osteogenic potential.[198] A possible implication of these exciting findings is that MSC could be

the skeletal stem cells that contribute to the physiological processes of bone modeling and remodeling *in vivo.*[195]

Another extremely important but yet unanswered question in regard to MSC and their site of origin is whether the bone periosteal compartment, which is critical for fracture repair, is also a source of MSC and whether this periosteal population shares significant similarities with the MSC isolated from bone marrow.[195]

Bone and medullary hematopoietic environment, which consists of special stromal cells capable of supporting hematopoiesis, plays a crucial role in supporting and regulating hematopoietic stem cells. All the bone marrow cavities of newborn mammals contain functionally active hematopoietic tissue, which is supported by full-value hematopoietic microenvironment referred to as red bone marrow. This hematopoietic microenvironment is progressively replaced by mesenchymal cells that accumulate lipid drops, and the hematopoietic tissue gradually disappears converting into fat-containing tissue referred to as yellow or fatty bone marrow. In contrast, although the hematopoietic microenvironment in cancellous bones also acquires some fat-accumulating cells, it continues to support hematopoietic tissue permanently. Yellow bone marrow transplanted to irradiated or old recipients can produce ectopic bones and hematopoietic microenvironment, what confirms that yellow bone marrow contains mesenchymal progenitor cells capable of producing hematopoietic microenvironment under particular conditions.[199]

The osteoprogenitor CD146+ cells found in the bone marrow stroma, which express angiopoietin-1, consistently with their nature as subendothelial cells (mural cells/ pericytes), participate in hematopoiesis and are physically associated with blood vessel walls *in vivo.* There is a complex interplay of osteogenesis and hematopoiesis in bone development, physiology and disease, rooted by the unique functional interplay of two systems of progenitor/stem cells that takes place in the bone marrow environment at specific sites.[200] The CD146+ cells contribute to hematopoietic niche *in vivo* and form capillaries once transplanted with endothelial cells.[201]

### 3.6.3. Scaffolds

#### 3.6.3.1. Definition and types

The scaffold is another key element effective in good and fast bone regeneration. An ideal scaffold should be three dimensional and highly porous, with an interconnected pore network to allow cell growth and flow transport of nutrients and metabolic waste. Besides this, it should be biocompatible and bioresorbable, with a controllable degradation or resorption rate to match cell/tissue growth *in vitro* and *in vivo*. Finally, it should possess a suitable surface chemistry to allow cell attachment, proliferation and differentiation, and be capable of osteoinduction and osteoconduction.[202] Physiologically, an ideal bone grafting material should provide osteogenicity, osteoinductivity, and osteoconductivity for new bone formation.[203] Degradation and absorption of the scaffold are crucial in functional tissue engineering. Ideally, the rate of scaffold degradation should mirror the rate of tissue formation, and the resulting products of degradation should be non-toxic, as the scaffold itself. Absorbable scaffolds used in tissue engineering are divided in natural polymers, synthetic polymers, inorganic materials containing calcium/phosphate (Ca/P), and composite materials. Natural polymers, from human, animal or vegetal origin, include proteins such as collagens, gelatin, fibrin, and silk fibroin, and polysaccharides such as hyaluronic acid, alginate, chondroitin sulfate, chitosan, and chitin. Synthetic polymers include poly(α-hydroxyacids), poly(ε-caprolactone) (PCL), poly(propylene fumarates), poly(carbonates), poly(phospha-zenes), and poly (anhydrides). They are bioabsorbable but present the major drawback that the by-products from their degradation might provoke an undesirable reaction in the body. Hydroxyapatite and β-tricalcium phosphate are among the inorganic materials containing Ca/P, naturally found in bone in the structure of apatite. Coral contains Ca/P as well, but has become difficult to obtain due to environmental regulations. Finally, composite materials combine inorganic and organic materials, combining the mechanical properties and osteoconductivity of calcium phosphates with bioabsorbable polymers.[82,204]

#### 3.6.3.2. Properties

Ceramics have been widely used in the biomedical engineering and bone substitution/regeneration field. They can be from natural (e.g., coralline hydroxylapatite)

origin or synthetic origin such as synthetic hydroxyapatite or ß-TCP. Due to their osteoconductivity and osteoinductivity they are promising scaffolds for bone tissue engineering applications, and support attachment, proliferation, and promote differentiation of osteoprogenitor cells.[205,206,207] However, these materials have some major drawbacks such as brittleness and low mechanical stability, which prevent their use in the regeneration of large bone defects.[208,209] Furthermore, due to factors that happen *in vivo* such as osteoclastic activity, their degradation/dissolution rates are difficult to anticipate. This could be a problem because if it degrades too fast it will compromise the mechanical stability of the construct, which is low by itself. At the same time, this would dramatically increase the extracellular concentrations of Ca/P, which can cause cellular death. As an alternative to these materials there are biodegradable polymers.

Polylactic acid and polylactic-coglycolide copolymers (PLGA) are synthetic absorbable polymers which support proliferation and differentiation of osteoprecursor cells.[210,211] Polylactic acid, however, possesses low mechanical properties and contains a hydrophobic surface which is not conductive to cell attachment.[212] Poly(ε-caprolactone) is a synthetic absorbable polymer with a good biocompatibility, processability, and mechanical properties.[213,214] This material, however, has a high hydrophobicity and low degradability *in vivo*.[215] PLGA are synthetic absorbable polymers with a higher cellular adhesion than PLAc surfaces,[216] which promote growth and differentiation of osteoprogenitor cells. The disadvantages of PLGA are the low mechanical properties, harmful degradation products, and hydrophobicity of the material.[217] Collagen, on the contrary, is a natural polymer that supports cell growth, proliferation and differentiation.[218] Type I collagen is the major organic component of bone matrices and a major regulator of cell adhesion and osteogenic differentiation.[219] The main disadvantage of this material is the poor mechanical strength, what makes necessary to add polyglicolic acid (PGA) fibers to increase resistance to compression of the material and to increase cellular attachment.[220,221] Hyluronic acid is a natural biodegradable polymer that possess a minimal immunogenicity and promotes differentiation of osteoprogenitor cells.[222] However, its low mechanical properties make this scaffold not recommended for reconstruction of large bone defects. Fibrin scaffolds are composed of a cross-linked fibrin network and enhance angiogenesis, cell attachment and proliferation and promote osteogenesis.[223] On the contrary, fibrin scaffolds present a mild angiogenic effect when combined with ASC, and an inflamatory response with monocytic

infiltration is observed after the use.[224] Other materials for bone tissue engineering are bioactive glass with silica-based surface and silk. Bioactive glass is osteoinductive and osteostimulative, however is not indicated for large size defects due to its brittleness.[225] Silk fibroin materials offer impressive mechanical properties, biodegradability, biocompatibility, and versatility in processing for biomedical applications. Silk degrades very slowly, what promotes more homogenous growth of cells and new tissue formation, and is indicated as scaffold in cases where slow tissue ingrowth is desirable. However, use of silk presents some problems, such as potential sensitization to silk fibroin resulting in an allergic response upon exposure, and aborted proteolytic attack by macrophages leading to encapsulation.[226,227]

The creation of pores in the scaffold is crucial, because it determines the transport of molecules such as oxygen and nutrients to the cells, and facilitates tissue ingrowth, allowing migration of cells through the scaffold. The optimal pore size for cell migration, adhesion and matrix deposition is dependent on the cell type. Generally, the optimal pore size ranges 100-500 μm and the porosity is above 90%. High porosity enables maximal conversion of cells and tissue invasion, together with conduits that facilitate blood vessel formation.

Different techniques have been developed to design porous scaffolds, such as solvent casting, salt or particulate leaching, gas foaming with pressurized carbon dioxide, melt molding, temperature-induced phase separation, membrane lamination, forging, injection molding, pressing, and inkjet printing, among others. A simple method is to use absorbable fibers as a starting material, because fibers can yield a wide range of porous products including woven or knitted cloth, web, mesh, felt, and fleece.[228] There is an interesting technique, called electrospinning, which uses an electrical charge to create nanofibers from liquid that resemble the extracellular matrix (Fig. 11). During this process, the fiber composition, fiber diameter, and fiber cross-linking can be manipulated to alter the macroscale architecture and material properties of the scaffold.[229] These fibers allow cell attachment, proliferation, and mineralization of osteoprogenitor cells.[230]

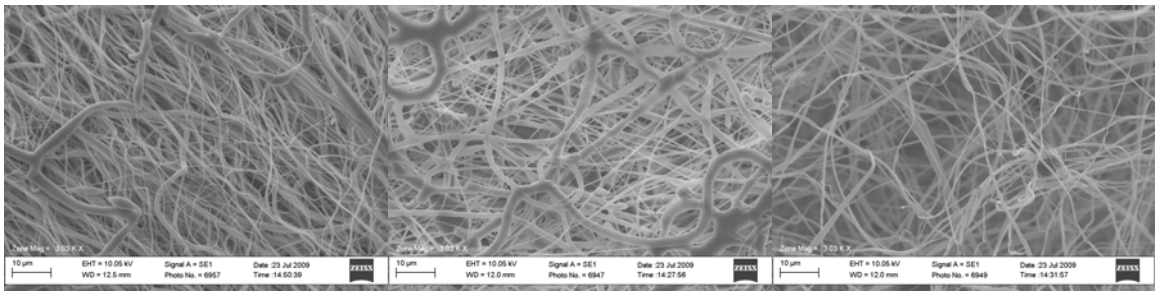


**Fig. 11: Scanning electron microscope (SEM) images of synthetic biodegradable polymers by the technique of electrospinning. A:** Poly(ε-caprolactone)**. B:** Poly(ε-caprolactone) combined with polylactic-coglycolide copolymers (PLGA)**. C:** PLGA combined with silk fibers. (E. Farré. Virginia Commonwealth University. 2009).

### 3.6.4. Growth factors, cytokines and hormones in bone

A critical issue of using growth factors is to chose what agent to use, what dose (the volume delivery), the site (focal delivery) and matrix. Besides this, it is important to chose the adequate type of delivery: bolus release, near bolus release or a sustained released.[231]

#### 3.6.4.1. Bone Morphogenetic Proteins (BMPs)

The osteogenetic chemical components of the matrix of bone, dentin, and other hard tissues that are deinsulated by demineralization and associated intimately with collagen fibrils, are termed the bone morphogenetic proteins.[232] Bone morphogenetic proteins are members of the Transforming Growth Factor-β superfamily and are essential in embryonic development and bone cell differentiation.[233] BMPs are expressed in bone cells as well as a wide number of soft tissues. BMPs were first discovered in demineralized bone matrix and responsible to promote endochondral bone induction.[47] In a study using BMPs derived from bovine teeth, a 71% to 83% bone formation was induced after implantation in the quadriceps muscle pouch of mice.[234] The process of tissue differentiation initiated by close contact and mutual interaction of cell populations of diverse origins is called induction.

In a study by Takagi and co-workers (1982), trephine defects in the adult rat skull 0.8 cm in diameter, which do not spontaneously heal, were filled with BMPs. The defects healed not only by bony ingrowth from the trephine rim, but also by proliferation of perivascular mesenchymal-type cells of the dura mater. Under the influence of BMPs, these dural pericytes differentiated into chondroid and woven bone. Between three and four weeks

postimplantation, sinusoids formed and the woven bone remodelled into lamellar bone. BMPs induced new bone formation in the underlying dura mater and completed repair of the defect.[235]

BMP-2, -3, -4, -6 and -7 are related with bone-inductive activity. BMP-2 acts as a chemoatractant for osteoblasts. BMP-2, BMP-4 and BMP-7 are responsible for endochondral bone induction, and increase proliferation and osteogenic differentiation of cells.[236] BMP-7 provokes an increase in CBFA1 transcription factor, regulating the genes that code for bone matrix proteins. BMP-2 and BMP-7 are being used clinically to accelerate bone healing and to create new bone.[23]

TGF-α is produced by malignant cells and activated macrophages and has a mitogenic effect on fibroblasts and osteogenic cells, stimulating proliferation and decreasing differentiation of the osteoprogenitor cells. TGF-α also stimulates osteoclast bone resorption and is responsible of the bone resorption associated with certain neoplasms.

TGF-β regulates growth and differentiation of many cell types, and has stimulative effects on cells of mesenchymal origin. This growth factor is entrapped in the bone matrix, and after release by osteoclasts exerts a paracrine effect, increasing pre-osteoblast number. Besides this, TGF-β acts as an autocrine factor, increasing synthesis of collagen, alkaline phosphatase and osteopontin in osteoblasts. TGF-β stimulates synthesis of prostaglandin and platelet-derived growth factor (PDGF) by osteoblasts, potent stimulators of bone formation. TGF-β also inhibits matrix degradation by autocrine-negative regulation of osteoclasts and by downregulation of osteoclast differentiating factor/RANKL. There is evidence of a synergic effect with BMP-7 to induce ectopic bone formation.[236]

#### 3.6.4.2. Tumor necrosis factor (TNF)

A member of the tumor necrosis factor is osteoprotegerin, also known as osteoclastogenesis inhibitory factor, a cytokine that may inhibit the production of osteoclasts. Osteoprotegerin has been used experimentally to decrease bone resorption in women with postmenopausal osteoporosis and in patients with lytic bone metastasis.[237]

### 3.6.4.3. Colony stimulating factor (CSF)

This growth factor controls hematopoiesis and pool of osteoclast precursors. Monocyte colony-stimulating growth factor (CSF-1) regulates monocytes proliferation and promotes preosteoclast differentiation.[236]

### 3.6.4.4. Granulocyte colony-stimulating factor (G-CSF)

This growth factor has various functions, including proliferation and differentiation of hematopoietic stem cells and endothelial progenitor cells, as well as mobilization of progenitor cells. G-CSF promotes bone formation through neovascularization via angiogenesis/vasculogenesis, and it has been able to regenerate bone on a critical size deffect in rabbit in combination with a hydrogel.[238]

### 3.6.4.5. Basic fibroblast growth factor (bFGF)

Basic fibroblast growth factor increases proliferation and differentiation of osteogenic cells and enhances bone formation. Besides this, bFGF increases expression of TGF-ß in osteogenic cells. A mediated effect of TGF-ß in the osteogenic response to bFGF has been proposed.[236]

### 3.6.4.6. Interleukins (IL)

Interleukins are cytokines secreted by active lymphocytes and macrophages in high levels, as well as by other cells such as fibroblasts and keratinocytes. IL-1 is a potent stimulator of osteoclasts and is involved in bone resorption. IL-2 and parathyroid hormone stimulate osteoclastic activity. IL-6 and IL-11 increase preosteoclasts and bone resorption. In contrast, IL-4 IL-10 and IL-13 decrease bone resorption .[236]

### 3.6.4.7. Insulin-like growth factor (IGF)

The insulin-like growth factors-1 and -2 are polypeptide hormones structurally related to insulin. IGF-1 is synthesized by the liver under the influence of growth hormone, and also locally in the skeleton. IGF-1 and IGF-2 regulate embryonic growth and can promote

osteoclast diferentiation, being involved in bone resorption and bone remodeling.[239] Besides this, they promote proliferation and differentiation of osteoblast-like cells.[240]

### 3.6.4.8. Glucocorticoids

Gluocorticoids such as cortisol, prednisone, dexamethasone and aldosterone, are a class of steroids hormones used chronically for treatment of rheumatic diseases. Glucocorticoids affect bone by a decrease in bone formation, mediated by osteoblast and osteocyte apoptosis, together with increased bone resorption, and their prolonged exposure leads to osteoporosis. Prevention of glucocorticoids-induced osteoporosis begins with non-pharmacological lifestyle interventions such as increased weight-bearing physical activity, smoking cessation, mitigating the risk of falls, and moderating alcohol/caffeine intake. Sufficient calcium intake and vitamin D or calcitriol are also the first steps to preserve bone.[241] In contrast, some studies show that glucocorticoids at physiological levels may stimulate proliferation and differentiation of osteoprogenitor cells.[242,243] In ASC a predominat adipogenic effect has been observed in a dose dependent manner[244] ASC with lower concentration of dexamethasone show higher osteoblast differentiation than with higher concentration of this glucocorticoid.[245]

### 3.6.4.9. Hepatocyte growth factor (HGF) and macrophage colony-stimulating factor

Both growth factors activate bone resorption. Circulating osteoclast precursors are capable of differentiating into osteoclasts in the presence of hepatocyte growth factor and macrophage colony-stimulating factor.[246] HGF has also been reported to have mitogenic, angiogenic, antiapoptotic, and antifibrotic effects.[247] This angiogenic growth factor is secreted by undifferentiated adipose stem cells, together with vascular endothelial growth factor, and contribute to their regenerative capabilities.[248]

### 3.6.4.10. Leptin

Leptin is a hormone produced by fat cells, and acts through the central nervous system to regulate bone mass. This hormone has a positive effect on proliferation and differentiation of osteoblasts and chondrocytes, and inhibits osteoclast formation. Leptin deficiency is associated with reduced linear growth, reduced cortical bone mass and reduced trabecular bone in the femora, where huge adipocytes occupy much of the marrow space.[249] Leptin,

together with parathyroid hormone and sex steroids, exerts an effect on bone mass. Leptin has a positive control on bone mass and this could explain the protective effects of obesity to osteoporosis. Parathyroid hormone favors bone resorption and steroid hormones are involved in bone resorption.[250]

### 3.6.4.11. Sex steroids

Sex steroids such as estrogen exert an anabolic effect on bones by stimulating osteoblast proliferation and differentiation and decreasing IL-6 transcription. A drop in the hormone estrogen produced in menopause can result in postmenopausal osteoporosis, produced by a rapid bone loss and increase in bone resorption and trabecular thinning. Estrogens induce osteoprotegerin expression in human osteoblast cells, and can counteract the development of osteoporosis, and also influences the skeleton through increasing production of IGF-1, BMP-6, and TGF-β, which results in osteoblast formation and increased osteoclast apoptosis.[251] Treatment with estrogen in ovariectomized rats has showed impaired alveolar bone healing after tooth extraction.[252]

### 3.6.4.12. Epidermal growth factor (EGF)

Epidermal growth factor is a key molecule to regulate cell growth and differentiation, and acts as a potent mitogen for many cell types such as keratinocytes and epithelial cells. This growth factor interacts with EGF receptors located in the dental follicle and in alveolar bone, thereby playing an important role in tooth development. EGF also promotes osteogenic cell proliferation and osteoblast differentiation, and is involved in bone remodelling and regeneration.[253] EGF added to medium of culture can promote osteogenic differentiation of ASC *in vitro*, and once injected subcutaneously into nude mice, in presence of ASC and biphasic calcium phosphate granules, induces the formation of a highly vascularized mineralized woven bone displaying numerous osteoblasts, osteocytes, and osteoclasts.[254]

### 3.6.4.13. Platelet-derived growth factor (PDGF)

Platelet-derived growth factor is a heparin-binding family of polypeptide growth factors targeting mesoderm-derived cells. This growth factor, also expressed by ASC and endothelial

cells, is involved in angiogenesis and promotes wound healing.[255] PDGF also acts as a chemotactic and mitogen factor on osteoblastic cells and increases bone matrix production.[256]

### 3.6.4.14. Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor is a glycoprotein that shares homology to PDGF and acts as an angiogenic factor, controlling the proliferation, migration, specialization and survival of vascular endothelial cells. VEGF also mobilizes endothelial progenitor cells from bone marrow and enhances neovascularization in ischemic limb.[257] Other important functions of VEGF are to play a role in tooth formation, tooth eruption, and bone resorption.[258,259] VEGF induces alkaline phosphatase activity in primary osteoblasts and enhances responsiveness to parathyroid hormone.[260]

Vascular endothelial growth factor is an essential mediator of endochondral bone development, and, in addition, exerts multiple non-vascular functions by acting directly upon the involved bone cells. This growth factor is essential to coordinate bone vascularization, cartilage morphogenesis, and ossification during endochondral bone formation.[261]

### 3.6.4.15. Prostaglandins (PG)

Prostaglandins are cytokines produced in bone in response to inflammation, injury and mechanical stress such as orthodontic movements. Prostaglandins $E_1$ ($PGE_1$) and prostaglandins $E_2$ ($PGE_2$) are potent stimulators of bone formation. $PGE_1$ and $PGE_2$ stimulate osteoblast cells to produce vascular endothelial growth factor, that acts as mitogen for endothelial cells. The role of osteogenic cells in coordinating vascular proliferation ensures an adequate blood supply for bone formation.[262] For osteoclast formation, two molecules are esential: colony stimulating factor-1 and RANKL, also referred to as osteoclast differentiation factor. $PGE_2$ also acts on osteoblasts to facilitate the differentiation of hematopoietic cells into osteoclasts, by increasing the secretion of RANKL on the cell surface of osteoblasts and cell-to-cell interaction with osteoclast progenitors.[263] Prostaglandins, together with interleukins, have short half-lives (2-3 minutes) and their effects are local and short-acting.

## 3.7. Adipose Tissue

Macroscopically, at least 5 different types of adipose tissue exist: bone marrow, brown, mammary, mechanical, and white, with each performing a distinct biological function. In the bone marrow, adipose tissue occupies space no longer required for hematopoiesis, and serves as an energy reservoir and cytokine source for osteogenic and hematopoietic events. Brown adipose tissue (BAT) is thermogenic, generating heat through the expression of a unique uncoupling protein, also known as thermogenin. Whereas brown adipose tissue is found around the major organs (heart, kidney, aorta and gonads) in the newborn infant, it disappears as humans mature. Mammary adipose tissue provides nutrients and energy during lactation and is regulated partially by pregnancy-associated hormones. Mechanical adipose depots such as the retroorbital and palmar fat pads, provide support to the eye, hand, and other critical structures. Finally, white adipose tissue (WAT) serves to store energy and provide insulation. There is an emerging role of white and other adipose tissues as an important endocrine organ. Adipose secretion of adiponectin, leptin, resistin, and other adipokines exerts systemic physiological and pathological effects on the organism.[69]

### 3.7.1. Adipose tissue development and morphology

- Embryonic development

Adipocytes are considered to be derived from mesenchyme, although recent evidence points out that neural crest stem cells are able to differentiate into adipocytes in culture, and could have a ectodermal origin likewise the bones and muscles of the skull and face, derived specifically from the neural crest.[264]

The embryonic development of human adipose tissue takes place at the beginning of the second third of the gestation period in various sites (buccal, neck, shoulder, gluteal, perirenal) and remains after birth. Initially, the aggregation of a dense mass of mesenchymal cells is associated with the organization of a vascular structure. Primitive fat cell clusters are then formed, adjacent to capillaries. In the last stage, growth of adipose tissue is mainly due to an increase in size of fat cell clusters surrounded by mesenchyme, which condenses rapidly to form septa among the clusters, process tightly coordinated by angiogenesis. Fat cell clusters are crucial, and adipocytes are important modulators of this activity, secreting TGF-β, $PGE_2$

and monobutyrin (1-butyrylglycerol), lipid that stimulates angiogenesis and vasodilation of microvascular beds.[265]

- Cellular composition

Adipose tissue, as bone marrow, is derived from embrionary mesoderm and is composed of loose connective tissue containing a heterogeneous population of cells. The primary cellular component for adipose tissue is a collection of lipid filled cells known as adipocytes that are held in place by collagen fibers. Cytoplasm in the mature adipocyte contains approximately 90% lipid. Other cellular components contained in adipose tissue are stromal vascular cells, including blood cells, vascular cells such as endothelial (progenitor) cells, smooth muscle cells and pericytes, preadipocytes, fibroblasts, and adipose stem cells. Adipocytes represent between one third and two thirds of the total number of cells in adipose tissue, and more than 90% of the tissue volume.[266]

- Regional differences in fat tissue growth and funcional diferences among fat depots

The transcriptional profile of adipose tissue varies between depots. Primary preadipocytes from various depots in the body show distinct global transcriptional profile, even after culturing, adding support to the idea that adipocytes in different depots may exert unique effects on body makeup and metabolism. Factors extrinsic to preadipocytes and adipocytes, including hormones, drugs such as sex steroids, glucocorticoids, human immunodeficiency virus protease inhibitors or thiazolidinediones, vascular supply, anatomic constraints, innervation, and presence of other cell types (e.g., macrophages or endothelial cells), likely contribute to fat depot-specific characteristics. Also contribute regional differences in expression of receptors, signaling, effector, and processing pathway components, that may have effects on chemokine production in the body. [267]

### 3.7.2. Physiology of the adipose tissue

- White adipose tissue (WAT) & brown adipose tissue (BAT)

Individual adipocytes are divided in brown or white. Brown adipocytes are mitochondria rich (responsible for its signature brown color) and lipid poor in comparison with white

adipocytes, which contain a single large lipid filled organelle. White adipocytes are spherical, thereby allowing for maximal volume within minimal space, with a diameter of around 100 μm, but which can be sometimes more than 200 μm, according to depot site. The lipid droplet containing triglycerides occupies the majority of intracellular space, compressing the cytoplasm and nucleus into a thin visible rim. White adipocytes exist as isolated cells within loose conjunctive tissue and bone marrow or as a collection of cells grouped together to form white adipose tissue. WAT represents around 15% to 20% of the weight of non-obese adults and up to 50% in obese individuals. The brown adipocyte is multilocular, with a polygonal shape and a centrally placed nuclei. Brown adipocytes are relatively smaller than white adipocytes, ranging from 20 to 40 μm. White and brown adipocytes have different functions in the body: brown fat expends energy and white fat has a storage function. Brown adipocytes express many genes in common with white adipocytes. They both express the master transcriptional regulator peroxisome proliferator-activated receptor gamma (PPARγ), which is necessary and suficient to induce adipocyte differentiation, and is also implicated in the transcription of a group of adipogenesis-specific transcripts involved in the adipogenesis. Mature adipocytes in WAT and BAT are also marked by the presence of markers of terminal differentiation (such as Glut4 and fatty-acid synthase), and insulin-regulated glucose uptake and metabolism. In addition, WAT is characterized by the presence of leptin, whereas BAT is distinguished by the existence of the uncoupling protein-1.[268] Through uncoupling protein-1 activity, BAT burns off eaten or stored fat to generate heat, keeping the organism warm and slim. Until recently, it was thought that in humans brown fat occurred only in newborns. But accumulating evidence suggests that adults can retain metabolically active brown fat.[269] Recent evidence points out that not only BAT can produce uncoupling protein-1. A potential for conversion of muscle stem cells into brown adipocytes expressing uncoupling protein-1 has been proven also in humans, showing that uncoupling protein production is not restricted to brown adipocytes.[270]

- White adipose tissue and brown adiposse tissue have a different vascular and nerve supply and a different physiology

All brown and white adipose depots are irrigated by nerves and blood vessels. Collections of white adipocytes comprise fat lobules, each of which is supplied by an arteriole and surrounded by connective tissue septae. An individual adipocyte is supplied by an adjacent

capillary, and it is additionally associated with a glycoprotein layer as well as reticular fibrils, fibroblasts, mastocytes, and macrophages. WAT and BAT are both innervated by the noradrenergic fibers from the sympathetic nervous system. Noradrenergic fibers in WAT are mostly confined to the capillary wall, whereas in BAT they directly interface the plasma membrane of brown adipocytes via ‘‘incidental’’ synapses. Although the development of white adipocytes begins in the embryonic stage, it is not until shortly after birth that the majority of the differentiation process occurs. The WAT has been identified as an endocrine organ and, in humans, it can be found mainly in two sites, each with unique anatomic, metabolic, endocrine, paracrine, and autocrine properties: visceral, or surrounding organs, and subcutaneous. The subcutaneous adipose tissue is further divided into two distinct layers: the superficial subcutaneous adipose tissue and deep subcutaneous adipose tissue. Excessive visceral or gut fat, composed of retroperitoneal fat (“behind the peritoneum”), omental fat (adipose in a sheet of connective tissue hanging as a flap originating at the stomach and draping the intestines), and mesenteric fat (adipose in the sheets of connective tissue holding the intestines in their looping structure), has been shown to be a risk factor for metabolic complications, markedly increasing the risk of hyperlipidemia, diabetes, and cardiovascular disease, while subcutaneous beds appear protective against these sequelae. In addition to the subcutaneous and visceral compartments, WAT is also found in small amounts around other organs, such as the heart, kidney, and genitalia. In humans, development of brown adipocytes begins at the 20th week of pregnancy and continues until shortly after birth, at which time BAT comprises 1% of body weight. In neonates and newborn children, BAT can be found in several areas, including the interscapular region, surrounding blood vessels, muscles in the neck, in the axillae, along the great vessels, trachea, esophagus at the thoracic inlet, and around the abdominal aorta, pancreas, adrenal glands, and kidneys. In small mammals such as rodents BAT persists throughout its lifespan. In large mammals and humans, however, brown adipocytes from BAT depots undergo a morphologic transformation, rapidly accumulating lipids, become unilocular, and lose the characteristic ultrastructural and molecular properties, including mitochondria lose. As a consequence, there are no discrete collections of BAT that may be found in the adult.[271,272,273] However, some studies point substantial metabolic activity in BAT located in the neck, adrenal glands, supraclavicular, mediastinal and paraspinal areas of adult humans,[274,275] showing a heterogeneous morphology with a mixture of brown and white adipose tissue cells by biopsy.[276]

While WAT is not as highly vascularized as its brown counterpart, each fat cell in WAT is in contact with at least one capillary, providing a vascular network that allows continued growth of the tissue. White adipose cells store fat primarily as tryglycerides, in a single large droplet. They are distributed throughout the body, are highly vascularized owing to high metabolic activity, exhibit numerous receptors for different hormones that influence accumulation and release of lipid, and secret hormone leptin to increase lipid metabolism and to inhibit appetite. Brown adipose cells are smaller than white adipose cells and store lipid as multiple droplets, and are best developed in hibernating animals. In newborns and animals emerging from hibernation, generate body heat. The heat production by BAT is regulated by the sympathetic nervous system, which releases norepinephrine to promote hydrolysis of lipids.[277]

- Phenotype of adipose tissue is variable: plasticity of the adipose tissue

The anatomy of the adipose tissue is variable. Transformation of the phenotype can occur by cold and warm exposure, pregnancy and lactation, hypertrophy and hyperplasia by overweight and obesity, and hypoplasia by caloric restriction and fasting.

Adipose organ of cold-exposed mice changes the color into a more brown phenotype, indicating a change of white adipocytes into brown adipocytes. Noradrenergic fibers play a central role in this phenomenon, mainly acting on $\beta_3$-adrenoreceptors of adipocytes in a reversible manner. Capillary density also increases as a result of increase in production of vascular endothelial growth factor from brown adipocytes. During pregnancy and lactation the anatomy of mammary glands changes with progressive reduction of adipocytes and formation of milk-secreting epithelial glands. This phenomenon is reversible and at the end of lactation adipocytes are reappearing. In pregnancy, adipocytes undergo a delipidation process and after lactancy lipid refilling process occurs. All this suggests an adipoepithelial transdifferentiation. When the energy balance becomes positive, adipocytes undergo hypertrophy followed by hyperplasia of adipose organ, what results in overweight and obesity. In obese animals the morphology of brown adipocytes gradually changes into a morphology of white adipocytes, accompanied by activation of the leptin gene. On the other hand, when the energy balance becomes negative, in caloric restriction and fasting, the morphology of the adipose organ changes with a variable amount of slimmed cells present in white adipose tissue. Vasculogenesis and neurogenesis is also found in WAT of fasted

animals, and in chronic caloric restriction the reduction in size of adipocytes is homogeneously distributed.[278]

Brown adipose tissue has emerged as an independent organ with specific protein expression patterns and unique purpose. The mitochondrial protein uncoupling protein-1, expressed exclusively in BAT, is responsible for mediating the transfer of energy from food into heat. Uncoupling protein-1 activity, triggered in response to adrenergic signaling via the sympathetic nervous system, is up-regulated in rodents, hibernators, and large mammal and human neonates whenever extra heat is needed such as during episodes of cold exposure, during the postnatal period, entry into a febrile state, and arousal from hibernation. BAT not only provides an important adaptive mechanism for acute body temperature regulation, but also functions as protection against obesity.

The retinoblastoma protein, which is required for spontaneous white adipogenesis, acts as a molecular switch that determines whether adipocyte precursor cells follow a path leading to differentiation into white or brown adipocytes.[273]

### 3.7.3. Biology of adipose tissue

For many years the dogma claimed that the number of adipocytes is fixed during childhood and remains constant throughout life. According to this model, changes in size of adipose tissue could only be achieved by modulation of adipocyte volume, depending on storage of triglycerides (lipogenesis) and mobilization of fatty acids (lipolysis). This model does not hold true any longer. Adipose tissue contains a large pool of precursor cells or MSC, known as adipose stem cells, which modulate the adipocyte number throughout life.

#### 3.7.3.1. Adipose stem cells (ASC), history and definition

It was discovered in 2001 by Zuk and co-workers that adipose tissue contains a fibroblast-like population of cells that possess mesenchymal stem cell characteristics, i.e. they grow adhered on culture plastic, exhibit potent self-renewal capacity, and maintain clonogenicity and multipotency after expansion, and termed this population processed lipoaspirate cells (PLA). Previous reports had demonstrated the capability of preadipocytes to exhibit an osteogenic

potential.[279] Nowadays, it has been described the extraction of ASC from multiple mammalian species besides humans, including rats, rabbits, dogs, pigs, and horses,[280-283] as well as the capacity of these cells to differentiate into the adipogenic, [284] osteogenic, [285,286] chondrogenic,[287,288] myogenic,[289] and there is increasing evidence of their pluripotent potential to give rise to cells from other germ layers such as cardiac muscle, neuronal cells, hematopoietic cells, endothelial cells or hepatocytes.[290] As it happens frequently in rapidly developing scientific fields, stem cells from adipose tissue have been named with a very wide variety of terms. The cells isolated from the digestion of the adipose tissue with collagenase have been termed adipose stem/stromal cells (ASCs), adipose-derived adult stem (ADAS) cells, adipose-derived stromal cells (ADSCs), adipose mesenchymal stem cells (AdMSCs), PLA, and preadipocytes. To avoid the confusion in this field by the use of multiple nomenclatures, the researchers who participated in the annual conference of the International Federation of Adipose Therapeutics and Science Society (Pittsburgh, 2004) reached a consensus, adopting the designation of adipose stem cells.

#### 3.7.3.2. Localization of ASC

There are several adipose depots all around the body, and each one situated in a specific niche. There are differences in ASC regarding fat depot as well as type of adipose tissue. ASC from white adipose tissue and brown adipose tissue differ in number and phenotype. In fact, the number of adult stem cells that can be found in the WAT is higher than that found in BAT, and the cells from WAT grow faster than the cells isolated from BAT.[291] Further, there are significant differences in the surface markers found in stem cells obtained from white or brown adipose tissue, which suggests the possible existence of two different stem cell populations, corresponding to cells isolated from these two different types of fat tissue.[273] WAT shows also a higher osteogenic differentiation potential than BAT.[292] Differences have also been found when comparing ASC isolated from subcutaneous WAT with ASC from visceral WAT and also when different subcutaneous depots were considered. Studies on ASC isolated from visceral and subcutaneous adipose tissue shown that stem cells isolated from subcutaneous fat show a higher proliferation rate but a lower differentiation capacity, especially regarding the osteogenic differentiation as compared to the stem cells isolated from visceral fat tissue.[293,291] Although the mechanism is unknown, it probably could be due to the different histologic characteristics of adipose tissues due to different anatomical sites; visceral

adipose tissue is containing more vascular supplies and less fibrous encapsulation compared with subcutaneous adipose, what might contribute to the different populations of osteoprogenitor cells isolated from these adipose tissues, resulting in different potentials of osteogenesis. More fibrous tissue mixed in subcutaneous adipose tissue results in more populations of fibroblasts within adipose-derived stromal cells than that from visceral adipose, what may decrease the osteogenic differentiation potentials of adult stem cells if mixed with dermal fibroblasts. This indicates that the population of heterogeneous cells critically influences the differentiation functions of osteoprogenitor cells. This finding probably could also explain why subcutaneous ASC show lower osteogenic differentiation potential than that of cells from visceral tissue.[294] Significant differences have also been observed comparing the chondrogenic differentiation potential of ASC isolated from fibrous synovium, adipose synovium, and subcutaneous fat. The cells isolated from fibrous synovium and fat synovium show the highest chondrogenic and osteogenic differentiation potentials than ASC isolated from subcutaneous fat tissue.[295] There are differences in the ASC population even when the cells are isolated from two different anatomical regions of the same type of adipose tissue. ASC from subcutaneous fat tissue harvested from the hip have a higher osteogenic potential than ASC harvested from the abdomen.[296]

#### 3.7.3.3. Isolation of ASC

The first isolation method of cells from the adipose stromal vascular fraction was optimized by the pioneer work of Rodbell in the far 60s,[297] that reported isolation of adipocytes from the SVF using collagenase enzyme digestion. This method was posteriorly used by Zuk and co-workers (2001) to isolate the adult stem cells from fat tissue. Collagenase digestion is still the basis of most methods used nowadays for this purpose, followed by a natural selection of the cells based on the propertty of ASC to adhere to the plastic surface of tissue culture flasks. ASC contain different subpopulations of cells, each with a proper marker expression and differentiation potential, therefore it is important to establish a clear and optimized isolation method to obtain purified and viable ASC populations.[298] A centrifugation speed higher than 3,000g result in ASC damage, while no significant difference is seen between 400xg and 1,200xg.[299] Another important issue to take into account is the harvest method to obtain the cells. Lipoaspiration surgery does not affect the ASC, and apparently only the lipocytes are damaged, as these are cells of bigger dimensions that are affected by the mechanical stress

applied. However, the fine minced tissue fragments are more homogeneous and smaller than excision, allowing a more efficient enzymatic digestion and could result in a higher yield of ASC.[300]

### 3.7.3.4. Characterization of ASC phenotype

ASC express HLA-ABC, CD29, CD49e, CD51, CD90 (42) and show a variable expression of CD49d, CD9, CD34, CD105 and CD166.[301] ASC are negative to immunologically relevant surface antigens such as major histocompatibility complexes (MHC)-II (HLA-DR), CD40, CD40L, CD80 and CD86, and inhibit lymphocyte proliferation induced by allogenic cells. ASC remain negative to these markers after osteogenic and chondrogenic differentiation.[302,303] These immunoprivileged characteristic makes these cells available for cell replacement therapies in human leukocyte antigen (HLA) incompatible hosts. Besides this, their immunomodulatory effect could make suitable as alternative to use of immunosuppressants, to prevent organ rejection after liver transplantation, avoiding the toxic side effects of these drugs.[304]

The presence or absence of Stro-1 is controversial, likewise in bone marrow. While some studies report their presence,[71] other studies report the absence of this marker.[27] Expression of characteristic markers enhances during cell passage[26] and becomes stable after passage 2. ASC also express molecules typicall embrionary such as OCT4 and Nodal[305] which are crucial for the migration of embryonic precursors in development. There is till a lack of consensus of characteristic markers of ASC.

### 3.7.3.5. Mesenchymal stem cell sources: bone marrow versus adipose tissue

The bone marrow has been the most widely used source of MSC for tissue engineering and other cell-based therapies. Adipose tissue, as bone marrow, is a mesodermally derived organ, and therefore stem cells derived from these two tissues share common characteristics such as their proliferative and differentiation potential. Their phenotype is quite similar; however, they differ in expression of CD54, expressed in higher levels in ASC, and in CD49d and CD34, which are only expressed in ASC. BM MSC express CD106, marker not detected in ASC.[306] The differentiation potential of these cells is not always identical. While some studies

report there is no difference between ASC and BM MSC regarding the chondrogenic potential,[307,308] some other recent articles[309,310,311] claim that the BM MSC show a higher chondrogenic differentiation potential, demonstrated by a higher expression of the genes correlated to the chondrogenic lineage and a higher synthesis of glycosaminoglycans. However, these contradictory results might be explained by the different culturing conditions, as well as many other variables such as the age of the donor or serum used. Besides this, one should also consider that ASC may need a different medium composition or a different cocktail of growth factors, because these cells are not identical to BM MSC, as seen by their surface marker expression, and therefore could require different culture conditions. The same occurs regarding osteogenic differentiation, showing controversial results with respect to MSC comparison from both sources.[312,313] ASC and BM MSC show differences in gene expression comparing differentiation pathways.[314,315] ASC have different niche and could need a different chemical stimuli. Furthermore, the anatomical site of origin should also been taken into account because ASC from different sites have different responses to differentiation, differently from what occurs in BM MSC that have the same niche. ASC promote vascularization in isquemic nude mice, showing a higher angiogenic potential than BM MSC.[36]

#### 3.7.3.6. Stromal vascular fraction (SVF) and their potential

The SVF, obtained after digestion with collagenase and centrifugation to separate the floating adipocytes, consists of a heterogeneous cell population, including circulating blood cells, fibroblasts, pericytes, and endothelial cells, as well as adipocyte progenitors.[24] Initial enzymatic liberation of ASC yields a mixture of stromal and vascular cells (referred to as the stromal–vascular fraction (SVF), indicating a spatial proximity. Several studies have examined the surface marker profiles of the ASC population within the SVF, and have observed that the total nonendothelial population is highly enriched for CD34+ cells with absence of CD45, characteristic marker of hematopoietic cells,[66] being the CD34 expression rapidly downregulated after culture.[34] The stromal cells derived from subcutaneous adipose tissue showing CD34+ marker expression are thought to serve structurally and functionally as pericytes within adipose tissue, supported by their location in the vessel at the interface between endothelium and adipocytes and their ability to support the vascular structure. ASC could play an important role linking adipose tissue parenchymal mass with provision of its

vascular supply.[70] Human SVF cells contain a population of cells with characteristic of EPC, which are positive for CD34, CD133 and the drug efflux pump ABCG2 possess endothelial colony forming ability *in vitro*, and are able to induce angiogenesis in a hindlimb ischemia model *in vivo*.[91] Angiogenesis can occur directly or through release of factors such as IGF-1, HGF-1 and VEGF secreted by CD34+ cells. SVF also contains a population of CD45+ cells able to give rise to variable types of colonies such as erythroid burst-forming units and granulocyte, erythroid, macrophage, and megakaryocyte–colony-forming units, likewise HSC derived from bone marrow. HSC derived from the SVF are capable of long-term reconstitution of hematopoiesis *in vivo*.[317]

Use of freshly isolated ASC could have immediate clinical applications avoiding the need of expansion, without losing their properties. Fresh ASC improve cardiac function when transplanted directly into the hearts of mice subjected to an acute myocardial infarction.[318] The heterogeneous population of SVF, containing ASC besides EPC and HSC, are able to improve left ventricular function *in vivo*, once implanted in hearts with impaired cardiac function.[319,320] SVF has also been used to ameliorate immune-mediated synovial inflammation and joint deterioration caused by rheumatoid arthritis. After SVF transplantation, a patient reported considerable resolution of her joint pain and stiffness, and disappearance of pain or symptoms by the third day after treatment. Physical exam at this time showed no joints effusions in hands, wrists or feet, and a decrease of a rheumatoid factor at year of treatment.[321] Another disabling autoimmune disease that at present has no treatment is multiple sclerosis. In this disease the central nervous system is affected and often progresses to physical and cognitive disability. In a case report three patients presenting multiple sclerosis were infused with SVF, showing no side effects. Balance and coordination improved dramatically over a period of several weeks, and a patient showing multiple severe tonic spasms reported a significant improvement of his cognition and almost complete reduction of the spasticity in his extremities after treatment. Neurological evaluation revealed an intact cranial nerve (II-XII) function and normal motor function without any atrophy or fasciculations, and intact sensory and cerebellar functions.[322] SVF also induced new bone formation in rat calvarial defects. The fact that SVF alone induced more new bone formation than implanted mixed with a Copolymer P(L/DL)LA, could be due to the induction of inflammatory or foreign body reaction by these scaffolds, that might have an inhibitory effect on bone regeneration by stem cells.[323] In this study, the use of a demineralized bone matrix in

combination with the SVF showed more bone formation, what indicates that choice of the scaffold is a critical issue for bone tissue engineering. SVF has also been used in over 160 patients with multiple sclerosis as part of a medical procedure, and reports of benefit have been published with no adverse effects reported.[322] Additionally, autologous SVF administration has been used commercially in over 3000 race horses for post-injury acceleration of healing, with published efficacy data in a double-blind canine osteoarthritis trial.[281] Autologous SVF is currently being used in clinical trials for post infarct remodeling, ischemic heart failure, and type I diabetes.[324]

### 3.7.3.7. Clinical trials with ASC

If cells are going to be transplanted into a patient, then as part of the ethical review process it is crucial to demonstrate aseptic and clean processing of the cells, to avoid any risk of infection and cross-contamination. The good laboratory practice and good manufacturing practice ensures the quality and safety of these cells. However, this requires high costs to build up laboratories dedicated exlusively for clinical application of these cells.[325] Most therapies require several small-scale studies (clinical trials) recruiting voluntary patients, before any approval is given to the product to enter to the clinic fully.

ASC were first used to stimulate bone repair in critical calvarial defects[326] and to heal chronic fistulas in Crohn's disease with fistulas unresponsive to medical treatment.[327] In a a phase II multicenter, randomized controlled trial, fistula healing was observed in 71% of patients.[328] There are two currently ongoing clinical trials being carried to test the efficiency of freshly isolated ASC with the Celution™ system (Cytori Therapeutics, San Diego, CA, USA) in both acute miocardial infarction and chronic myocardial ischemia patients: the APOLLO and the PRECISE. These are prospective, double-blind, randomized, placebo-controlled, sequential dose-escalation trials being carried out in the Hospital General Universitario Gregorio Marañón (Madrid, Spain) in collaboration with the Thoraxcenter (Erasmus MC Rotterdam. The Netherlands). For sinus floor elevation there is already an ongoing clinical trial using this system with freshly isolated ASC (ACTA-VU University Medical Center Amsterdam, The Netherlands). An interesting point is the low expression of MHC-I and MHC-II, expressed in only 1% of ASC, and therefore, these cells could behave as universal donor cells and could be used for autologous as well as for allogenic transplantation.[329] The Celution™ system allows

to isolate and concentrate stem cells and regenerative cells automatically from adipose tissue without losing their characteristics and properties.[63]

### 3.7.4. Endocrine and metabolic functions of adipose tissue

Traditionally, adipose tissue was considered as a passive organ playing a metabolic role in total energy homeostasis. Its function was restricted to the storage of excess of energy as triglycerides, and its release according to the need in the form of fatty acids. Now, there has been a paradigm shift and it becomes increasingly clear that adipose tissue is an endocrine organ, secreting a wide range of hormones and other factors. Adipokines released by adipose tissue include cytokines, growth and angiogenic factors, and proteins involved in vascular and glucose homeostasis and in lipid metabolism. They establish interplay with molecules secreted by other tissues such as brain, skeletal muscle, bone, and vascular system. This cross-talk can lose its balance in a pathological condition, such as the development of obesity and even by a rapid weight loss, and can contribute to the development of obesity-related disorders, particularly type-2 diabetes and cardiovascular disease.[330]

Adipose tissue is considered as the largest endocrine gland because it produces free fatty acids, hormones, growth factors, and cytokines. Growing adipocytes produce a dozen angiogenic factors including leptin, VEGF, FGF-2, HGF, IGF, TNF-α, TGF-β, placental growth factor, VEGF-C, resistin, tissue factor, neuropeptide Y, heparin-binding epidermal growth factor, monobutyrin and angiopoietins. ASC secrete high levels of a number of angiogenic factors including VEGF, HGF, granulocyte macrophage colony stimulating factor, FGF-2, and TGF-β. Recruitment of inflammatory cells also significantly contributes to adipose neovascularization. For example, activated macrophages produce potent angiogenic factors such as TNF-α, VEGF, FGF-2, IL-1b, IL-6, and IL-8.[331] The biologically active peptides and proteins secreted by the adipose tissue are collectively termed "adipokines". The main adipokines are adiponectin, leptin and resistin and these play a central role in body homeostasis, through regulation of food intake and energy balance, insulin action, lipid and glucose metabolism, angiogenesis, vascular remodeling, and the regulation of blood pressure and coagulation.[332]

Pituitary hormones may trigger and/or modulate directly or indirectly the formation of mature fat cells. Growth hormone deficiency and possibly hypothyroidism are accompanied by hypoplasia and hypertrophy of subcutaneous fat tissue. Glucocorticoids such as dexamethasone have been long known to increase adipose tissue mass via their hypertrophic effect, controlling terminal differentiation of human adipose precursor cells. Steroid sex hormones play a role in the development of hyperplastic adipose tissue. Low levels of testosterone in men or low levels of female sex hormones with increased levels of testosterone in women or estrogens in men have been associated with increased visceral fat mass. After menopause, as estrogen levels decline, women develop increased visceral adiposity. A variety of cytokines including TNF-α, IL-1, and many other proinflammatory molecules have been found to suppress fat cell differentiation. In addition, several growth factors are potent inhibitors of adipogenesis, including PDGF, FGF, and EGF. TGF-α and TGF-β have been shown to inhibit adipogenesis *in vitro*.[265]

### 3.8. Buccal fat pad (BFP)

The oral cavity contains a mass of specialized fatty tissue which is distinct from subcutaneous fat, named buccal fat pad or Bichat's fat pad. This encapsuled adipose tissue was first identified more than 300 years ago by the german anatomist and surgeon Lorenz Heister, which named this mass of adipose tissue Molar Gland or "glandula molaris" and painted this gland in the Compendium Anatomicum with several latin editions in the XVIII century.[333] The histologic adipose origin and anatomic position was described in the begin of 1800 by the French anatomist Xavier Bichat.[92] Ranke noted how it prevented the cheeks from collapsing in the sucking infant and named it 'saug polster' or sucking pad in 1884.[334] This encapsulated mass of adipose tissue that fills the masticatory spaces is a remanent tissue, which serves for mastication and sucking during childhood. Buccal fat pad has an aesthetic function contributing to the 'fullness' of the cheeks. Removal of the fat pad may produce a change in the facial contour, reducing cheek fullness, highlighting the malar eminences and giving a more sculptured look to the face.[335]

### 3.8.1. Anatomy

The buccal fat pad is the fat tissue that stays in the profound facial spaces. Its body lies behind the zygomatic arch. Dissection of BFP shows that the adipose tissue mass can be divided in three lobes: anterior, intermediate, and posterior, according to the structure of the lobar envelopes, the formation of the ligaments, and the source of the nutritional vessels. The anterior lobe is triangular, and is located below the zygoma, extending to the front of the buccinator, maxilla, and the deep space of the quadrate muscle of the upper lip and major zygomatic muscle. It is composed of connective tissue septa that separate the fat tissue into smaller masses. The anterior lobe joins with the intermediate and posterior lobes by loose connective tissue. The intermediate lobe lies in the space around the posterior lobe, lateral maxilla, and anterior lobe. It is a membrane-like structure with thin fat tissue in adults and a large mass in children, and is separated from the posterior lobe with a membranous septum. The posterior lobe of the buccal fat pad exists throughout life in humans. The posterior lobe is divided into 4 extensions: the buccal, pterygoid, pterygo-palatine, and temporal extensions (superficial and profound).[336]

The buccal fat pad consists of a main body, accounting for approximately 30% of the total BFP, which rests on the posterior maxilla and the buccinator muscle, and protrudes anterolaterally to the masseter muscle above the parotid duct (Fig. 12A,B).[337] From there four bodies extend:

1) The buccal extension travels inferiorly to the parotid duct on top of the buccinator muscle, limited anteriorly by the facial vessels.
2) The pterygoid extension goes medially of the ramus of the mandible to lie laterally to the pterygoid muscles, packing the lingual nerve and mandibular neurovascular bundle.
3) The deep temporal extension travels underneath the zygomatic arch to lie on top of the temporalis muscle and underneath the deep layer of the deep temporal fascia, as well as extending downwards behind the lateral orbital wall towards the sphenoid.
(4) The pterygopalatine extension wraps around the posterior maxilla and runs into the pterygomaxillary fissure encapsulating the maxillary neurovascular bundle, and also extends upwards through the infraorbital fissure.

Unlike past reports, the superficial temporal fat pad lies in between the two layers of the temporal fascia and is not in continuity with the buccal fat pad and as such should be considered a separate entity.[338]

The buccal fat pad is fixed by six ligaments to the maxilla, posterior zygoma, inner and outer rim of the infraorbital fissure, temporalis tendon, or buccinator membrane. These ligaments are: the maxillary ligament, the posterior zygomatic ligament, the medial infraorbital fissure ligament, the lateral infraorbital fissure ligament, the temporalis tendon ligament, and the the buccinator ligament. Several nutritional vessels exist in each lobe and in the subcapsular vascular plexus forms. The buccal and deep temporal branches of the maxillary artery, transverse facial branches of the superficial temporal artery, and branches of the facial artery provide the high blood supply of this tissue.[336]

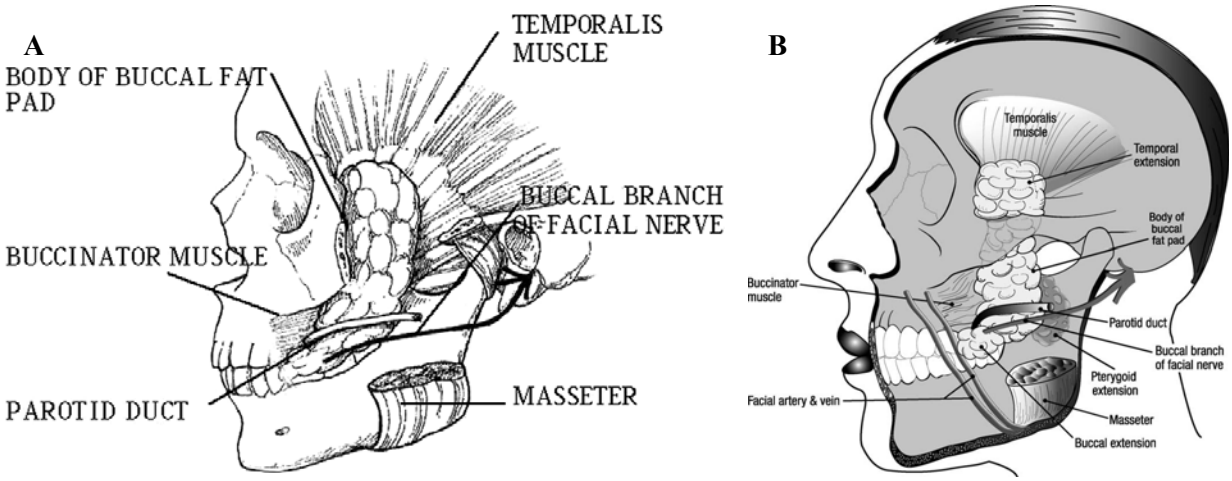



**Fig. 12: Location of the buccal fat pad (BFP) in a frontal section with related structures.** A) Muscle related structures (modified from Matarsso A. Ann Plast Surg. 1991). B) Main body and extensions of buccal fat pad (from Amin MA. Br J Oral Maxillofac Surg. 2004).

### 3.8.2. Physiology

Although the physiology of buccal fat tissue is not totally clarified, it is known that plays an important role in masticatory function especially in the infant during sucking. Its size diminishes as the infant grows with the accompanying growth of the surrounding facial structures.[337] In the adult, the BFP resembles orbital fat in appearance and function.[339]

It is suspected that the embrionary origin differs from subcutaneous adipose tissue and can be considered a marker of visceral fat.[340] The buccal process is located superficially within the cheek and is partially responsible for the cheek contour. There is no volume difference in BFP between men and women. However, trauma can alter buccal fat pad volume considerably. The BFP seems to have its own mechanism of lipolysis, independent of the subcutaneous fat, and is important to note that it differs anatomically and physiologically from the subcutaneous fat, illustrated by the fact that it is uninfluenced by adiposity or severe wasting.[341] This is shown by the fact that the size of the buccal fat pad is constant among different individues regardless of the overall body weight and fat distribution. Even cachectic patients with little subcutaneous fat have BFP that are of normal weight and volume.[337] However, there is a link between facial fat accumulation and abdominal fat accumulation, and suggest a possible correlation between facial fat and insulin resistance. In Cushing's syndrome, moon facies and increased visceral fat develop in these two adipose-tissue depots, and changes after treatment are similar, suggesting that the adipose tissue in these two anatomically separate depots have similar metabolic properties.[342] Microcirculation in BFP shows a network similar to other zones of white adipose tissue such as in the abdomen.

### 3.8.3. Uses

In the late 70s it was reported the first use of BFP, using the BFP as a pedicled graft to close oral defects in 4 cases with complete success.[343] In the begin of 80s it was reported the use of BFP as a free graft for closure of intra-oral defects.[344]

Since then, BFP has been widely used for oral surgery with broad success. The special clinical importance of BFP is given by several reasons. There is good vascularization, ease of access, proximity between the donor site and the recipient site and the donor site is concealed. Surgery has no influence on either its appearane or function. Therefore this fat mass becomes an attractive graft to repair soft defects of bucal mucosa caused by malignant tumors, achieving complete repair of epithelia and mucosa and no sign of infection.[345] It has also successfully been used to repair intra-oral defects in the maxilla, retromandibular area, check, and oral commissure caused by maxillary cysts, oroantral communications, tumors, or posttraumatic defects. BFP, when fully epithelialized, is not affected by postoperative

radiation therapy. However, its use as a pedicled flap in previously irradiated patients is not recommended; attempts to close the defect with BFP result in complete failure.[61,94,346,347,348]

Oroantral communications are a common complication in dentoalveolar and maxillofacial surgery often seen after complicated tooth extractions, but also after tumor resection of the maxilla, cyst removal, sinus grafting, osteonecrosis, trauma or peri-implantitis, and are related with severe complications such as persistent sinusitis. BFP is widely used for closure of these defects with successful results.[62,348] It has also successfully been used for the treatment of periodontal defects[93,349] and combined with autogenous bone and Bio-Oss for sinus floor elevation.[350] The rich blood supply of BFP promotes rapid neovascularization of the grafted material and provides excellent results.[347] Another reported use is for vocal cord augmentation, achieving improvement of phonation in 90% of all patients. BFP is obtained with a simple intraoral incision and no sign of complication such as hematoma at the fat collection site is seen in any case.[351]

Because of its rich blood supply, it can be considered as an ideal pedicled graft and reliable tool for reconstructing defects in the maxillary region. In particular, the body and buccal process can be easily reached through the oral cavity, and are therefore available for reconstructive procedures. The easy mobilization of the buccal fat pad, and its excellent blood supply and minimal donor site morbidity, has made it an ideal flap not only for closure of oroantral fistulas and hard palate defects, but also for soft palate defects and coverage of bone augmentation procedures, together with their easeness of use, allowing to reconstruct defects in older patients quickly under local anesthesia.[352] BFP may be also removed by aesthetical reasons during a face-lift procedure to achieve a more angled contour emphasizing the malar bone, and to minimize facial aging signs. BFP produces an excess of fat in the cheek area, and is associated together with atrophy of the malar area with the classic aging signs.[353]

The use of buccal fat pad is an easy procedure, well tolerated and has an uncomplicated harvest technique, with a minimal incision using infiltrative anesthesia, which is commonly used by dentists; this implies a minimal morbidity for the patient. Because this, BFP could represent an interesting source of stem cells of easy access for tissue engineering.

# Chapter 4 HYPOTHESIS

**Study 1: Buccal fat pad, an oral access-source of human adipose stem cells (ASC) with potential for osteochondral tissue engineering**

Hypothesis 1:

Null hypothesis ($H_0$) in this study is the absence of ASC in buccal fat pad with potential to differentiate to osteoblasts, chondrocytes and adipocytes.

Alternative hypothesis ($H_1$) in this study is the presence of ASC in buccal fat pad with potential to differentiate to osteoblasts, chondrocytes and adipocytes.

Hypothesis 2:

Null hypothesis ($H_0$) in this study is that buccal fat pad is a source of ASC with the same characteristics than ASC from subcutaneous abdominal adipose tissue.

Alternative hypothesis ($H_1$) in this study is that buccal fat pad is a source of ASC with different characteristics than ASC from subcutaneous abdominal adipose tissue.

**Study 2: Osteogenic differentiation of bone morphogenetic protein-2 (BMP-2) induced human ASC seeded on biphasic calcium phosphate and β-tricalcium phosphate scaffolds**

Hypothesis 1:

Null hypothesis ($H_0$) in this study is that BMP-2 does not affect ASC attachment on different types of calcium phosphate scaffolds.

Alternative hypothesis ($H_1$) in this study is that BMP-2 affects ASC attachment on different types of calcium phosphate scaffolds.

Hypothesis 2:

Null hypothesis ($H_0$) in this study is that BMP-2 does not affect ASC proliferation dependent on the type of calcium phosphate scaffolds.

Alternative hypothesis ($H_1$) in this study is that BMP-2 affects ASC proliferation dependent on the type of calcium phosphate scaffolds.

Hypothesis 3:

Null hypothesis ($H_0$) in this study is that BMP-2 does not stimulate osteogenic differentiation of ASC dependent on the type of calcium phosphate scaffolds.

Alternative hypothesis ($H_1$) in this study is that BMP-2 stimulates osteogenic differentiation of ASC dependent on the type of calcium phosphate scaffolds.

# Chapter 5 MATERIALS & METHODS

# 5.1. STUDY 1: BUCCAL FAT PAD, AN ORAL ACCESS-SOURCE OF HUMAN ADIPOSE STEM CELLS WITH POTENTIAL FOR OSTEOCHONDRAL TISSUE ENGINEERING

## 5.1.1. ASC isolation and characterization

### 5.1.1.1. Tissue preparation and cell isolation/expansion

Subcutaneous abdominal adipose tissue (SC) and buccal fat pad were obtained from healthy individuals undergoing elective abdominal and orthognatic surgery procedures in different Hospitals (Hospital Clinic de Barcelona and Hospital Sant Joan de Déu, Barcelona, Spain). The age of the patients submitted to abdominal surgery was 19-57 years (n=4), and the age of the patients undergoing oral surgery 17-40 years (n=10). All patients were in good health, and no diabetes or other systemic complications were reported. The informed consent and experimental protocols in this study were reviewed and approved by the Ethical Committee from the International University of Catalonia in Sant Cugat (Barcelona, Spain). Sample tissues were processed according to a modification of a previously reported procedure.[27] Raw oral fat tissue (16 ± 4.1 ml) and subcutaneous fat tissue (22 ± 12.2 ml) (mean ± SD) were washed several times with sterile phosphate buffered saline (PBS), minced into small pieces, and treated with 0.075% collagenase I (Sigma, St. Louis, MO, USA) for 60min at 37ºC. After incubation, adipose tissue was centrifugated at 400xg for 10min to separate the adipocytes and lipid droplets from the SVF.

Cell pellets were resuspended in red blood cell lysis buffer (8.2 g/l $NH_4Cl$, 0.84 g/l $NaHCO_3$, 0.37 g/l disodium EDTA, pH 7.4) and incubated for 10min at room temperature. After centrifugation SVF were resuspended in Dulbbeco's Modified Eagle's medium (DMEM; Sigma) low glucose (lg) containing 10% fetal bovine serum (FBS) and 100 units/ml antibiotics/antimycotics solution. Suspended cells were passed through a 100 µm cell strainer (BD Biosciences, San Jose, CA, USA), cells were counted, and their viability was assesed with Trypan Blue exclusion. Cells were seeded at 5 x $10^3$ cells/$cm^2$ in 100 mm tissue culture dishes and maintained in a humidified incubator at 37ºC and 5% $CO_2$.

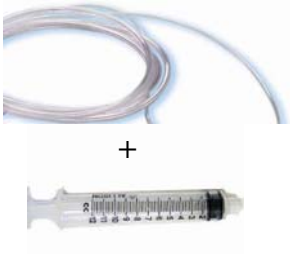
Sample tissues from BFP were processed in four ways in order to develop an efficient protocol of ASC isolation for future immediate clinical applications. #1 minced adipose tissue

was digested for 60min at 37ºC with collagenase I and centrifuged at 400xg for 10min to separate the SVF containg the ASC from the floating adipocytes and further filtered; #2: minced adipose tissue was passed 5 times through a plastic cannula connected to two syringes to be mechanically digested and centrifuged and filtered as previously described; #3: enzymatic and mechanical disgregation of tissue was omitted and instead minced tissue was centrifuged and filtered as previously described; #4: the last protocol consisted in omitting the enzymatic digestion and mechanic disgregation and centrifugation of the tissue, adipose tissue was passed through a filter of 100 μm and 40 μm. The concentration and incubation with collagenase was also studied, analyzing if a reduction to half concentration affected cell proliferation, as well as a reduction in time incubation to 30 minutes (Fig. 13).

Minced adipose tissue from buccal fat pad (BFP)

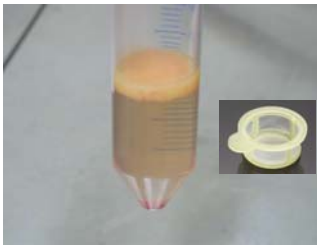
#1 #2 #3 #4

Enzymatic digestion with collagenase I



Mechanical disgregation with cannula connected to two syringes

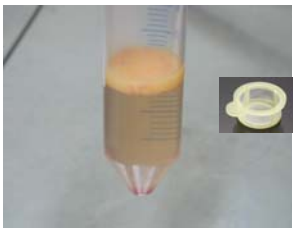
Centrifugation to separate adipocytes from SVF



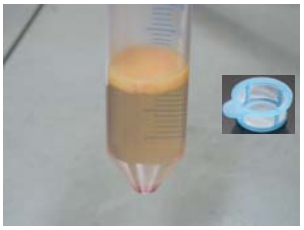
Filtration through a 100 µm cell strainer

Centrifugation to separate adipocytes from SVF

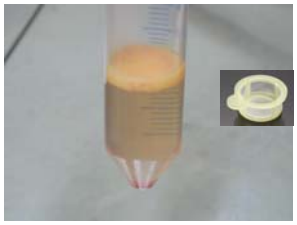
Centrifugation to separate adipocytes from SVF



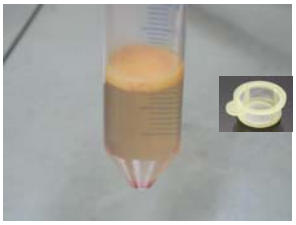
Filtration through a 100 µm cell strainer



Filtration through a 40 µm cell strainer



Filtration through a 100 µm cell strainer



Filtration through a 100 µm cell strainer

**Fig. 13: The protocols used in the study to optimize ASC isolation.** Protocol #1 is the standard protocol of ASC isolation.

After 3-4 days individual cell colonies were visible upon microscopic examination. The initial adherent cell population, referred to as passage 0 (P0), as well as after several passages (up to one month of culture), was analyzed by flow cytometry. After one week of culture adherent cells from BFP (n=5) and abdominal fat (n=4) were counted, and cell number per gram of adipose tissue calculated. Cells from BFP in P2 (n=3) were induced to differentiate in adipogenic medium (AM), osteogenic medium (OM), or chondrogenic medium (CM) for several weeks. Differentiation media consisted of DMEM high glucose (hg) and lineage-specific media supplementation as described[27] (Table 5).

| Medium | Medium | Fetal Bovine Serum, % | Supplements |
|---|---|---|---|
| Control | DMEM lg | 10% | 100 units/ml antibiotics/ antimycotics |
| AM | DMEM hg | 10% | 0.5mM isobutylmethylxantine, 1µM dexamethasone, 10µM insulin, 200 µM indomethacin, 100 units/ml antibiotics/ antimycotics |
| OM | DMEM hg | 10% | 0.01 µM 1,25-dihydroxy-vitamin $D_3$, 50 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 100 units /ml antibiotics/antimycotics |
| CM | DMEM hg | 1% | 6.25 µg/ml insulin, 10 ng/ml TGFβ1, 50 nM ascorbate-2-phosphate, 100 units/ml anti-biotics/antimycotics |

**Table 5: Composition of the lineage-specific differentiation medium.** AM, adipogenic medium; OM, osteogenic medium; CM, chondrogenic medium; DMEM lg, dulbbeco's modified Eagle's medium low glucose; DMEM hg, dulbbeco's modified Eagle's medium high glucose.

### 5.1.1.2. Flow cytometry

Flow cytometry was performed with fresh adipose tissue from BFP (n=5) and passaged ASC obtained from BFP (n=4) and SC (n=4) from patients undergoing elective abdominal surgery. For flow cytometry analysis cells were resuspended at $10^6$ cells/ml in control medium. 50 ml of the cell suspension containing 3-5 x $10^5$ cells were incubated with 5 ml primary monoclonal antibodies (MAbs) directed against the following antigens and coupled to either

phycoerythrin (PE), fluorescein isothiocyanate (FITC) or peridinin-chlorophyll-protein complex (PerCP) for 30min at 4ºC: CD14[PE], CD34[PerCP], CD45[PerCP], CD29[PerCP], CD73[PE], SSEA[PE], CD90[FITC], CD146[FITC], HLA-DR[FITC], CD105[FITC]. All MAbs were purchased from BD Biosciences, except CD105 and CD146, which were purchased from R&D Systems (Minneapolis, MN, USA) and eBioscience (San Diego, CA, USA) respectively.

All samples were washed in PBS containing 2% FBS, fixed with Cell-Fix (BD Biosciences) and incubated on ice for 30min. Samples were then analyzed by FACScan flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Aliquots of cells incubated with isotype-matched mouse anti-human immunoglobulins (IgGs) served as negative control. Data was analyzed using CellQuest software and FACSDIVA for SVF (Becton Dickinson Immunocytometry Systems).

### 5.1.2. ASC differentiation

#### 5.1.2.1. ASC differentiation medium and seeding

##### 5.1.2.1.1. Osteogenic differentiation

Osteogenic medium was composed of DMEM hg (Sigma), 50 µM ascorbate-2-phosphate (Sigma), 10 mM B-glycerolphosphate (Sigma) and 0.01 µM 1,25-dihydroxyvitamin $D_3$ (Sigma). For alkaline phosphatase assay 18 x $10^4$ cells were seeded in 100 mm tissue culture dishes and maintained in osteogenic medium for 3 weeks. For Alizarin Red stain, cells were seeded at 5 x $10^4$ cells/$cm^2$ cells in 24 multi-wells and induced to differentiate in osteogenic medium for 3 weeks. For immunofluorescence cells were seeded at 2 x $10^4$ cells/well in 24 multi-wells and induced to differentiate in osteogenic medium for 22 days. For real-time PCR cells were seeded at 1 x $10^5$ cells/well in 6 multi-wells and induced to differentiate in osteogenic medium up to 21 days.

Undifferentiated ASC served as controls. Human osteogenic sarcoma cells (SAOS-2) were obtained from the American Type Culture Collection (ATCC HTB85; Manassas, VA, USA) and cultured in McCoy's 5ª medium (Gibco BRL, Grand Island, NY, USA) containing 15% FBS and used as osteogenic control.

#### 5.1.2.1.2. Adipocyte differentiation

Adipogenic medium consisted of DMEM hg supplemented with 0,5 mM isobutylmethylxantine (Sigma), 1 µM dexamethasone (Sigma), 10 µM insulin (Sigma) and 200 µM indomethacin (Sigma). For Oil Red staining cells were seeded at 2 x $10^4$ cells/well in 24 multi-wells and induced to differentiate in adipogenic medium for 15 days. For real-time PCR cells were seeded at 1 x $10^5$ cells/well in 6 multi-wells and induced to differentiate in adipogenic medium up to 21 days.

Undifferentiated ASC served as controls. Fresh adipose tissue was obtained from BFP as well as from excised abdominal adipose tissue from patients and used as adipogenic control.

#### 5.1.2.1.3. Chondrogenic differentiation

Chondrogenic medium consisted of DMEM hg supplemented with 6.25 µg/ml insulin (Sigma), 50 nM ascorbate-2-phosphate (Sigma) and 10 ng/ml TGFB-1 (Peprotech, London, UK). For Toluidine Blue staining cells were seeded at 2 x $10^4$ cells/well in 24 multi-wells following the micromass technique culture provided by Zuk and colleagues (2001) and induced to differentiate in chondrogenic medium for 28 days. For immunofluorescence cells were seeded at 2 x $10^4$ cells/well in 24 multi-wells and induced to differentiate in chondrogenic medium for 32 days. For real-time PCR cells were seeded at 1 x $10^5$ cells/well in 6 multi-wells and induced to differentiate in chondrogenic medium up to 14 days. Undifferentiated ASC served as controls. Chondrocytes derived from human nucleus pulposus were used as chondrogenic control.

### 5.1.2.2. ASC differentiation assays

#### 5.1.2.2.1. Immunohistochemistry

Cells were rinsed twice with PBS and fixed with 3% paraformaldehyde for 20min at room temperature. Thereafter, cells were incubated in 100 mM $NH_4Cl$ in PBS for 10min to quench autofluorescence. Non-specific binding was blocked by incubating the cells in 1% BSA in PBS containing 0.2% Triton X-100 for 15min at room temperature. Osteogenically stimulated cells and undifferentiated ASC were incubated for 1h with a mouse anti-human osteocalcin monoclonal antibody (R&D Systems) diluted 1:100 in PBS with 1% BSA, and anti-mouse

Alexa 546 secondary antibody was used at a concentration of 1:500. Chondrogenically stimulated and undifferentiated ASC cells were pre-treated with pepsin solution (Zymed Laboratories, San Francisco, CA, USA) for 10min at 37ºC and incubated for 1h with a mouse anti-human type II collagen antibody (Acris antibodies, Hiddenhausen, Germany) diluted 1:5 in PBS with 1% BSA. Cells were conjugated with anti-mouse Fluorescein secondary antibody diluted 1:500 (Chemicon International, Temecula, CA, USA).

#### 5.1.2.2.2. Histology

Differentiated and undifferentiated ASC were processed using the following histological assays: for adipogenesis, Oil Red O stain (Sigma) was used to detect intracellular lipid accumulation according to standard protocols. Cells were counterstained with Mayer Hematoxylin Eosin solution (Sigma). For osteogenesis, Alizarin Red solution (Millipore, Billerica, MA, USA) was used to detect mineral deposition according to the manufacturer's instructions. For chondrogenesis, Toluidine Blue (Panreac, Barcelona, Spain) was used to assess proteoglycan extracellular matrix according to standard protocols. Stained slides were examined by microscopy.

#### 5.1.2.2.3. Alkaline phosphatase (ALP) activity assay

Triplicate ASC cultures were induced to differentiate in osteogenic medium for up to 3 weeks. At 7 and 21 days, cells were washed twice with PBS, and lysed in 10mM Tris-HCl (pH 8.2) containing 2mM $MgCl_2$ and 0.05% Triton X-100 at 4ºC for 1h. The lysates were centrifuged at 200xg for 10 min at 4ºC, and submitted to two freeze-thaw cycles. The lysates were cleared by centrifugation at 11,300xg for 30min (4ºC) and incubated for 1min in pre-warmed alkaline phosphatase solution provided by Linear Chemicals (Barcelona, Spain). P-nitrophenolate release was determined spectrophotometrically at 405 nm. Protein concentration in the supernatant was analyzed using the BioRad Protein Assay (Bio-Rad laboratories GmbH, München, Germany) with BSA as standard. Undifferentiated ASC served as negative control and SAOS-2 cells as positive control.

#### 5.1.2.2.4. Quantitative real-time PCR

Gene expression of peroxisome proliferating receptor gamma (PPARγ) was quantified in adipogenically stimulated cell cultures. Total cellular RNA was extracted from SC, BFP, and ASC cultured in adipogenic medium for 3, 9, and 21 days, using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 2 µg of RNA was reverse-transcribed using M-MLV Reverse transcriptase (Invitrogen). Undifferentiated ASC and human adipose tissue from SC and BFP served as control. The expression of PPARγ and the osteogenic genes CBFA1 and osteonectin (SPARC) were quantified in ASC cultured in osteogenic medium for 7, 14 (CBFA1, SPARC, PPARγ), and 21 days (PPARγ). Undifferentiated ASC and SAOS-2 cells were used as control. The expression of PPARγ and SOX9 was analyzed in ASC cultured for 5 days (PPARγ) and 14 days (SOX9, PPARγ) in chondrogenic medium. Undifferentiated ASC and chondrocytes derived from human nucleus pulposus were used as chondrogenic control. Real-time polymerase chain reactions (real-time PCR) were performed using 2.5 µl cDNA and SYBR Green Supermix (Bio-Rad Laboratories, Inc.). Triplicate reactions were performed using primer pairs for PPARγ,[354] CBFA1, osteonectin, and SOX9. The oligonucleotide sequence of the primers is listed in Table 6. Specific transcription levels were normalized to the housekeeping genes human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA 18S (18S), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ). The target and reference genes were amplified in separate wells. All reactions were performed in triplicate. In each run the reaction mixture without the cDNA was used as negative control. A standard curve was performed with PPARγ primers ($y = -3.79x + 425.4$, $r^2 = 0.999$), SOX9 primers ($y = -3.725x + 411.71$, $r^2 = 0.999$), CBFA1 primers ($y = -3.8111x + 419.45$, $r^2 = 0.999$), osteonectin primers ($y = -3.027x + 341.11$, $r^2 = 0.982$), 18S primers ($y= -3.3923x + 373$, $r^2 = 0.999$), YWHAZ primers ($y= -2.95x + 332.51$, $r^2 = 0.999$), and GAPDH primers ($y = -3.36x + 379.17$, $r^2 = 0.998$). Gene expression levels are presented as the fold-increase-over-control (undifferentiated ASC are control).

| Gene | | Oligonucleotide sequence | size, base pairs |
|---|---|---|---|
| PPARγ | Forward | 5′-TGAATGTGAAGCCCATTGAA-3′ | 161 |
| | Reverse | 5′-CTGCAGTAGCTGCACGTGTT-3′ | |
| SOX9 | Forward | 5′-CCCAACGCCATCTTCAAGG -3′ | 242 |
| | Reverse | 5′-CTGCTCAGCTCGCCGATGC -3′ | |
| CBFA1 | Forward | 5′-ATGCTTCATTCGCCTCACAAAC -3′ | 156 |
| | Reverse | 5′-TTTGATGCCATAGTCCCTCTTT -3′ | |
| SPARC | Forward | 5′-CTGTCCAGGTGGAAGTAGG -3′ | 233 |
| | Reverse | 5′-GTGGCAGGAAGAGTCGAAG -3′ | |

**Table 6: The primers used for real-time PCR.** PPARγ, peroxisome proliferator-activated receptor gamma; SOX9, sex reversal Y-related high-mobility group box protein; CBFA1, core binding factor-alpha1; SPARC, osteonectin.

### 5.1.3. Statistical analysis

Average values were expressed as mean ± SD, or mean ± SEM as showed in figure legend. ALP and real-time PCR was expressed as mean ± SEM.

Unpaired t-tests (Mann-Whitney U-test) were performed for comparison between marker expression differences from two different sources of ASC, for ALP activity analysis and for PPARγ, CBFA1, SPARC and SOX9 gene expression analysis in ASC from BFP cultured in osteogenic, chondrogenic and adipogenic medium. The analyses were performed using a statistical software package SPSS version 17.0 (SPSS Inc., Chicago, USA). A probability value $<0.05$ was considered to denote statistical significance.

## 5.2. STUDY 2: OSTEOGENIC DIFFERENTIATION OF BMP-2 INDUCED HUMAN ADIPOSE STEM CELLS SEEDED ON BIPHASIC CALCIUM PHOSPHATE AND β-TRICALCIUM PHOSPHATE SCAFFOLDS

### 5.2.1. ASC isolation and culture on different scaffolds

Adipose tissue was harvested from abdomen of healthy women undergoing elective abdominal wall correction in Tergooiziekenhuizen Hilversum Hospital, The Netherlands (n=9, age 23-32 years old). The study has been approved by the Ethical Comittee of the VU Medisch Centrum, Amsterdam, The Netherlands and an informed consent is obtained from all patients. Isolation of ASC from the stromal vascular fraction was performed according to a modification of a previously described procedure.[72]

#### 5.2.1.1. ASC isolation/expansion

Adipose tissue obtained by resection was cut into small pieces, and enzymatically digested with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) for 45min at 37ºC in phosphate-buffered saline (PBS) with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) under intermittent shaking. A single cell suspension was obtained by filtration through a 100 µm mesh filter. After thorough washing with PBS with 1% BSA a Ficoll density centrifugation step (1.077 g/ml ficoll, 280 ± 15 mOsm; Lymphoprep, Axis-Shield, Oslo, Norway) was performed to separate contaminating erythrocytes from the SVF. After centrifugation at 600xg for 10 min, the SVF pellet containing the ASC was resuspended in expansion medium composed of Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) containing 10% FBS (Gibco) supplemented with 500 µg/ml streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA), 500 µg/ml penicillin (Sigma-Aldrich), and 2.5 µg/ml amphotericin B (Fungizone, Gibco). Cell viability was assessed using the trypan blue exclusion assay. Viable, large cells were counted, using light microscopy, and immediately cultured on the different scaffolds or resuspended in Cryoprotective medium (Freezing Medium, BioWhittaker, Cambrex, Verviers, Belgium), frozen under "controlled rate" conditions in a Kryosave (HCl Cryogenics BV., Hedel, The Netherlands), and stored in liquid nitrogen until further use.

### 5.2.1.2. ASC culture, and seeding on scaffolds

Freshly isolated and freshly frozen and thawed ASC were treated for 15 minutes with or without 10 ng/ml BMP-2 (Peprotech, London, UK) at 37°C. Single cell suspensions were seeded at $1x10^5$ cells per 25 mg of scaffold and cultured in Costar® Transwell® containers (Corning Life Sciences, Lowell, MA, USA) in 12 well plates (Greiner Bio-One, Kremsmuenster, Austria), containing expansion medium, with 25 mg of scaffold with cells per well. Four different scaffolds were used (Table 7): 1) Straumann® BoneCeramic (Straumann, Basel, Switzerland), a porous BCP with 60% HA and 40% ß-TCP (BCP 60/40), 2) Straumann® BoneCeramic, a porous BCP with 20% HA and 80% ß-TCP (BCP 20/80), 3) Ceros® TCP (Mathys, Bettlach, Switzerland), a porous ß-TCP with particle size 0.5-0.7 mm (ß-TCP<0.7mm), and 4) Ceros® TCP, a porous ß-TCP with particle size 0.7-1.4 mm (ß-TCP>0.7mm) (Fig. 14).

| Scaffold | Composition | Particle size (µm) | Porosity (%) | Pore width (µm) | Quantity/unit |
|---|---|---|---|---|---|
| Straumann® BoneCeramic BCP 60/40 | 60% HA/40% ß-TCP | 500-1000 | 90 | 500-1000 | 0.5g |
| Straumann® BoneCeramic BCP 20/80 | 20% HA/80% ß-TCP | 500-1000 | 90 | 500-1000 | 0.5g |
| Ceros® TCP β-TCP | 100% ß-TCP | 500-700 | 60 | 100-500 | 0.5g |
| Ceros® TCP β-TCP | 100% ß-TCP | 700-1400 | 60 | 100-500 | 0.5g |

**Table 7: Composition of the different scaffolds used in the study,** with particle size, porosity, pore width and quantity per unit. HA, hydroxyapatite; ß-TCP, ß-tricalcium phosphate; BCP, biphasic calcium phosphate.

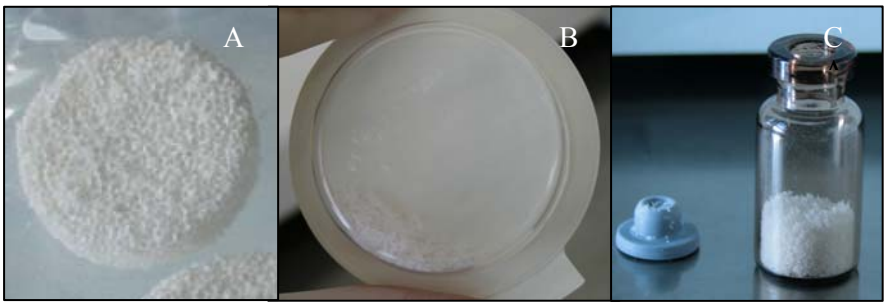



**Fig. 14: Different types of calcium phosphate. A)** Discs of pure HA showing the porosity of the material. **B)** Ceros® TCP large particles used in this study. **C)** Biphasic calcium phosphate containing 20% HA and 80% ß-TCP used in this study. HA, hydroxyapatite; ß-TCP, ß-tricalcium phosphate.

### 5.2.2. ASC Attachment and proliferation on different scaffolds

Freshly isolated ASC, either or not pre-treated with BMP-2, were seeded onto the different scaffolds and allowed to attach for 30 minutes. Then ASC-seeded scaffolds were washed with PBS, and DNA concentration was determined as a measure for cell number to evaluate the number of cells attached. ASC attachment was also analyzed using the depletion colony-forming unit fibroblasts assay (depl CFU-F), to determine the percentage of non-attached cells on the different scaffolds. To assess if BMP-2 affects the frequency of ASC, the CFU-F assay was performed on tissue culture plastic. For proliferation analysis, the scaffolds with cells were cultured in expansion medium for 4, 14 and 21 days in 5% $CO_2$ in air at 37°C in a humidified atmosphere. After 4, 14 and 21 days, the DNA concentration was determined as a measure for cell number to evaluate the proliferation profiles.

#### 5.2.2.1. Colony-forming unit fibroblasts (CFU-F) assay and CFU-F depletion assays (depl CFU-F)

The CFU-F assay was performed to assess if the frequency of ASCs in the SVF of adipose tissue was affected by BMP-2, as described elsewhere.[73] The CFU-F assay is a biologic assay reflecting functional viability of ASC. A total of $1x10^3$ and $1x10^4$ ASC were seeded in 6-well plates (Greiner Bio-One). After 14 days of culture CFU-F were fixed in 4% paraformaldehyde for 10min and stained with a 0.2% toluidine blue solution in borax buffer (pH 12) for 1min. The number of colonies was counted using a light microscope at 100x magnification. The percentage of CFU-F per total number of ASC seeded was calculated. Presence of ≥10 cells was considered a colony.

ASC attachment to scaffolds was determined using the depl CFU-F assay as described elsewhere.[74] Non-attached cells, obtained by washing the scaffolds with PBS, were collected, and tested for CFU-F frequencies at 10-fold higher densities as normal CFU-F seeding densities, since it was established that seeding at normal densities resulted in no colonies.

#### 5.2.2.2. CyQUANT DNA proliferation assay

Immediately after ASC seeding and attachment, and after 4, 14, and 21 days post-attachment, the amount of DNA was quantified using the CyQUANT cell proliferation assay kit (Molecular Probes/Invitrogen, Carlsbad, CA, USA). Cells were washed with PBS, and stored at −80°C prior to analysis as recommended by the manufacturer. A reference standard curve was used. To quantify the amount of DNA, 200 µl of the CyQUANT GR dye/cell lysis buffer was added to the samples and incubated for 4 min at room temperature, protected from light. Then fluorescence was measured using a fluorescein filter set with filters for 480 nm excitation and 520 nm emision.

### 5.2.3. Osteogenic differentiation of ASC on different scaffolds

Osteogenic differentiation was assessed by seeding fresh ASC either or not treated with BMP-2 at $1x10^5$ cells per 25 mg scaffold material. Cells cultured in monolayer served as control to assess ALP activity and gene expression using RT-PCR. For osteogenic differentiation, ASC (either or not treated with BMP-2), were cultured up to 21 days in DMEM supplemented with 10% FBS, antibiotics, 10 mM β-glycerol phosphate (Sigma) and 50 µg/ml ascorbic acid (Merck, Darmstadt, Germany). If cells were seeded on calcium phosphate scaffolds, addition of β-glycerol phosphate was omitted from the culture medium. Medium was changed twice a week. ALP activity was determined at 4, 14, and 21 days of culture, and mRNA gene expression analysis was performed at 14 days as described below.

#### 5.2.3.1. ALP activity

ALP activity was measured after culturing the ASC seeded onto the scaffolds for 4, 14 and 21 days. Cells were lysed with distilled water, and ALP activity and protein content were determined. To determine ALP activity, p-nitrophenyl-phosphate (Merck) at pH 10.3 was used according to manufacturer's instructions. ALP activity was expressed as micromole per microgram of protein in the cell layer. The amount of protein was determined by using a BCA Protein Assay reagent Kit (Pierce, Rockford, Ill., USA), and the absorbance was read at 540 nm with a microplate reader (Bio-Rad Laboratories GmbH, München, Germany).

#### 5.2.3.2. Quantitative real-time PCR

Cells from several donors were collected using lysis solution reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was separated from DNA with RNA isolation Assay NucleoSpin® (Macherey-Nagel, Düren, Germany) and stored to -80ºC prior to assay. The cDNA synthesis was performed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, USA) in a thermocycler (GeneAmp® PCR System 9700; PE Applied Biosystems, Foster City, CA, USA) with 0.1 µg total RNA in a 20-µL reaction mix containing VILO™ Reaction Mix and SuperScript® Enzyme Mix. The cDNA was stored at -20ºC prior to real-time PCR. Real-time PCR reactions were performed using the SYBR® Green reaction kit (Roche Laboratories, IN, USA) according to the manufacturer's instructions in a LightCycler® (Roche Diagnostics). The target and reference genes were amplified in separate wells. In each run the reaction mixture without the cDNA was used as negative control. Primers used for real-time PCR were from Invitrogen. For quantitative real-time PCR, the values of relative target gene expression were normalized for relative YWHAZ and ubiquitin (UB) housekeeping genes expression. Real-time PCR will be used to assess and compare between the different scaffolds the gene expression of the following genes: CBFA1, COL1, ALP, ON, OPN, OCN, and PPARγ. Human primary osteoblasts were used as positive controls. Gene expression was compared between ASC seeded on tissue culture plastic and on different scaffolds with or without BMP-2 treatment.

### 5.2.4. Statistical analysis

Average values for ASC seeded onto the scaffolds and on tissue culture plastic were expressed as mean ± S.E.M (n=6-9).

Paired student's t-tests were used to compare data groups of colony-forming unit fibroblasts seeded on tissue culture plastic and for proliferation and ALP activity assays of ASC on scaffolds. ANOVA two-way analysis of variance tests were used to measure differences between groups regarding ASC attachment. A probability value $<0.05$ was considered to denote statistical significance. A probability value $<0.001$ was considered statistically highly significant. The analysis was performed using SPSS 17.0 (SPSS Inc.) and GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, USA).

# Chapter 6 RESULTS

## 6.1. STUDY 1: BUCCAL FAT PAD, AN ORAL ACCESS-SOURCE OF HUMAN ADIPOSE STEM CELLS WITH POTENTIAL FOR OSTEOCHONDRAL TISSUE ENGINEERING

### 6.1.1. ASC isolation

Collagenase digestion is still the basis in the methods used to isolate ASC from adipose tissue. We aimed our first part of the study to develop an optimized and efficient protocol of ASC isolation, in order to be able to obtain and apply the ASC in the same surgical procedure, for immediate clinical applications.

#### 6.1.1.1. Enzymatic digestion protocol is effective to isolate ASC from buccal fat pad

*In vitro* ASC isolation protocols were carried out in four ways as follows: #1: adipose tissue was enzymatically digested and afterwards was centrifuged and filtered; #2: adipose tissue was mechanically disgregated through a cannula connected to two syringes and further centrifuged and filtered; #3: adipose tissue was centrifuged and filtered; #4: adipose tissue was filtered several times.

Spindled-shape cells characteristic of ASC were observed in the protocol using collagenase digestion after 3 days of culture (Fig. 15A). Only round-shaped non-adherent cells were observed in the other protocols that did not include the enzymatic digestion step (Fig 15B-D).

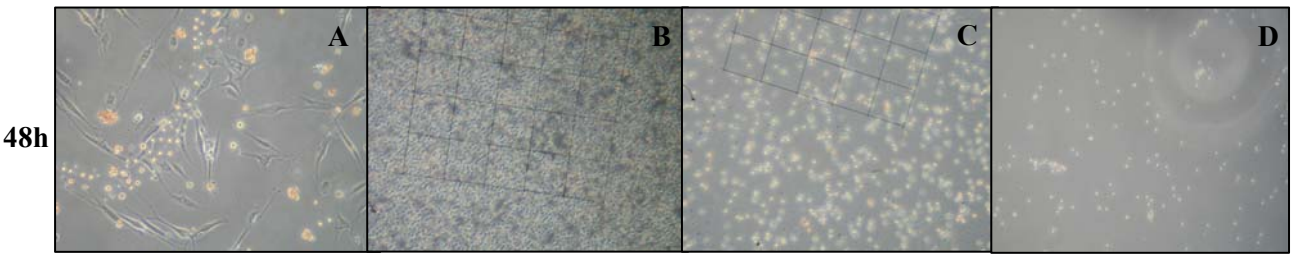



**Fig. 15: Adipose stem cells (ASC) isolation after 48h culture.** Presence of spindled-shaped adherent cells were observed on culture dishes after ASC isolation using protocol #1. **A)**. Spindled-shaped adherent cells were absent on culture dishes using protocols #2 **B)**, #3 **C),** and #4 **D)**. See matherials and methods for further description of the protocols. 100x magnification.

After 4 days of culture, we observed more adherent cells with ASC morphology after collagenase digestion, indicating the reliability of the protocol (Fig. 16A). No adherent cells

with ASC morphology were observed in the other protocols excluding the collagenase digestion step (Fig. 16B-D).

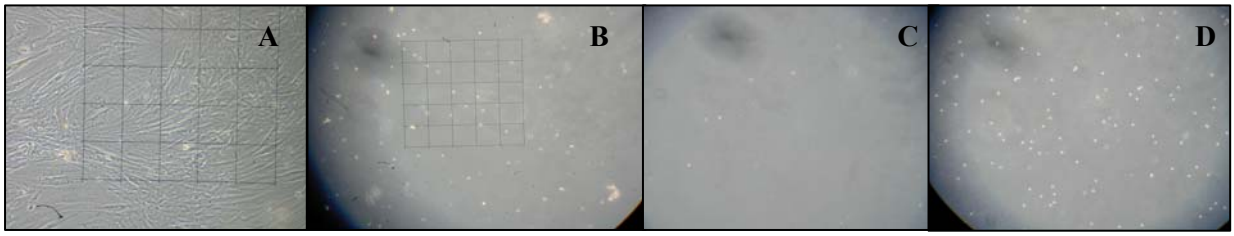



**Fig. 16: Adipose stem cells (ASC) isolation after 4 days culture. A)** Spindled-shape cells characteristic of ASC were observed on culture dishes after collagenase digestion, centrifugation and passing the cells through a cell strainer. **B,C,** and **D)** No presence of spindled-shaped cells was observed in the other protocols omitting the collagenase digestion step. 100x magnification.

### 6.1.1.2. Reduction of 30 minutes collagenase digestion does not affect ASC cell proliferation

After 24h of culture adherent spindled-shaped cells were numerous on culture dishes (Fig. 17A). A similar number of ASC was observed after reducing the collagenase digestion to 30min (Fig. 17B). Some non-adherent cells, characteristic of hematopoietic cells, were observed as well (arrows), but they disappeared after time in culture. ASC presence was similar in both cell cultures (Fig. 18).

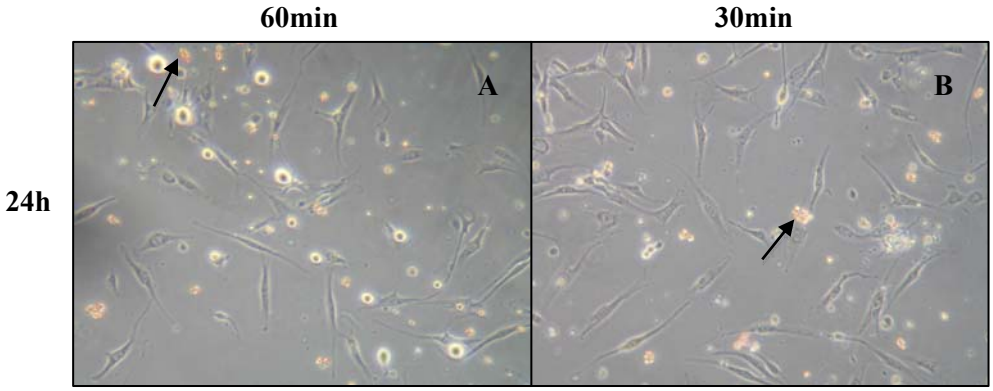



**Fig. 17: Collagenase digestion protocol of 60min and 30min after 24h of culture. A)** After collagenase digestion of 60 min, some adherent cells with adipose stem cells (ASC) characteristics were present on culture dishes, as well as some non adherent cells. **B)** After reduction of collagenase digestion to 30min, adherent cells with fibroblast morphology, characteristic of ASC were observed. Floating round cells were also observed, characteristic of hematopoietic cells (arrows). 100x magnification.

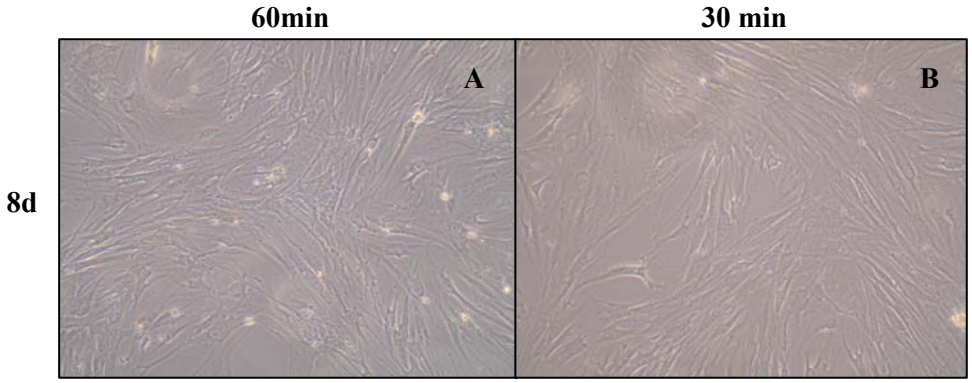



**Fig. 18: Collagenase digestion protocol of 60min and 30min after 8 days culture. A,B)** Similar number of adipose stem cells (ASC) was observed after 60 and 30 minutes of collagenase digestion, indicating that a lower incubation time with collagenase could be performed. 100x magnification.

### 6.1.1.3. Reduction of collagenase concentration by 50% seems to affect ASC proliferation

After reducing the collagenase concentration by 50%, we observed a lower number of adherent cells after 24 hours (Fig. 19) and after 8 days of culture (Fig. 20). A reduction of time and concentration affects cell number and ASC profile, and seems to affect the progeny of stem cells inducing an adipogenic commitment (Fig. 21).

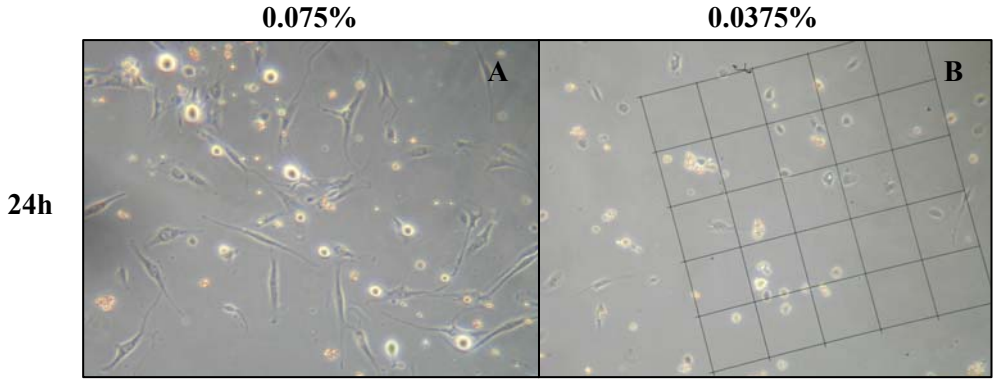



**Fig. 19: Collagenase digestion protocol 0.075% and 0.0375 after 24h culture. A,B)** A lower adipose stem cells (ASC) cell number was observed when reducing the collagenase concentration to a half. 100x magnification.

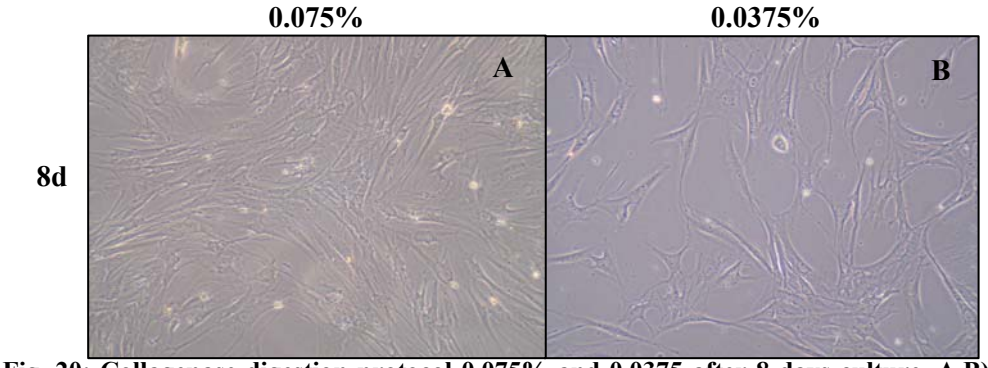



**Fig. 20: Collagenase digestion protocol 0.075% and 0.0375 after 8 days culture. A,B)** Lower number of adipose stem cells (ASC) was observed after reducing the collagenase concentration to a half. 100x magnification.

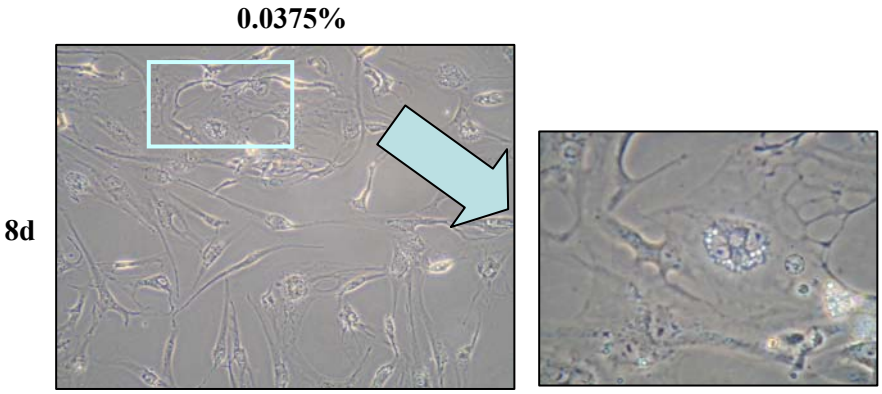



**Fig. 21: Adipose stem cells (ASC) obtained using collagenase digestion at 0.0375% showing spontaneous adipogenic differentiation of ASC.** We observed presence of adipocytes after reducing collagenase concentration to a half, indicating loss of potential of ASC. 100x magnification.

### 6.1.2. ASC characterization

#### 6.1.2.1. Buccal fat pad contains stem cells with ASC characteristics

To study if BFP contains stem cells with ASC characteristics, BFP from healthy patients were extirpated and SVF isolated and cultured. Freshly isolated and cultured ASC were analyzed for cell morphology and membrane marker profile to observe whether ASC derived from BFP share characteristics with ASC from another fat depot. After one week of culture adherent cells from abdominal fat (n=4) and BFP (n=5) were counted and cell number per gram of adipose tissue calculated.

#### 6.1.2.2. Morphology of cultured ASC from two different sources

We wanted to study the stem cells derived from an oral fat depot after several passages and compare them with stem cells from another fat depot. We compared the cultured ASC derived from subcutaneous adipose tissue with passaged ASC derived from BFP. We first determined that cells isolated from BFP were similar to cells derived from subcutaneous fat. After digestion by collagenase and separation of the adipocytes by centrifugation, we cultured both cell types with the culture medium.

Some adherent cells were observed 48h after seeding under a light microscope examination (Fig. 22, images above). Apart from these cells, few not adhered red cells were observed in

culture (marked in arrows). Cells from both tissues remained in a quiescent phase (dormant) during 2-4 days, whereafter they began to multiply rapidly, approaching confluence as a monolayer of large flat cells. Both BFP and SC cells showed similar morphology, i.e. they were spindle shaped. After 7 days of culture (Fig. 22, images below), ASC from both types of fat tissue were showing a homogeneous fibroblast-like morphology, characteristic of ASC. At this time point, cells reached 90% confluence and were passaged.

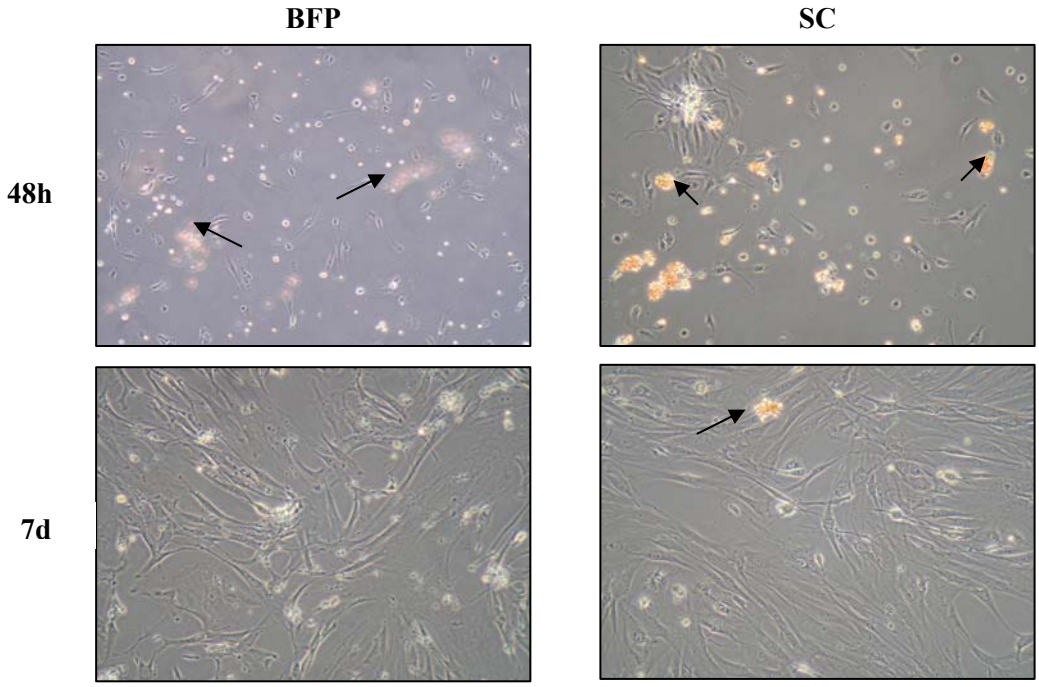



**Fig. 22: Comparison of morphology of adipose stem cells (ASC) derived from buccal fat pad (BFP) or subcutaneous abdominal adipose tissue (SC).** Morphology of cells isolated from BFP or SC, observed by microscopy at 48h and 7 days. The morphology of both ASC from BFP and SC is similar. At 48h, there is abundant presence of hematopoietic cells (arrows) and fibroblast-like cells. The population after 7 days of culture is homogenous and hematopoietic cells are scarce or lacking. Magnification 100x.

### 6.1.2.3. Comparison between cell surface marker profile ASC from buccal fat pad and ASC from subcutaneous abdominal adipose tissue (SC)

To evaluate the surface marker profile, cells were stained with a panel of antibodies for marker expression to define the percentage of ASC in BFP and SC, following the minimal criteria of marker expression to define MSC.[14]

Cultured ASC from BFP and SC were positive for CD73, CD90, and CD105, and negative for the hematopoietic markers CD45, CD19, CD14, and HLA-DR (Table 8). Cells were also positive for CD29, a characteristic marker of ASC,[355] and negative for CD146, a marker expressed in endothelial and vascular smooth muscle cells.[63]

Some cross-contaminating populations were found in initial passaged ASC from BFP (Fig. 23A) and SC (Fig. 23B), but they disappeared with further passaging (Fig. 23, right images). A CD146+CD29+ population was observed in initial passaged ASC from BFP, but not from SC. CD146 is characteristic of endothelial progenitor cells which are likely found in the rich microvasculature within this adipose tissue.

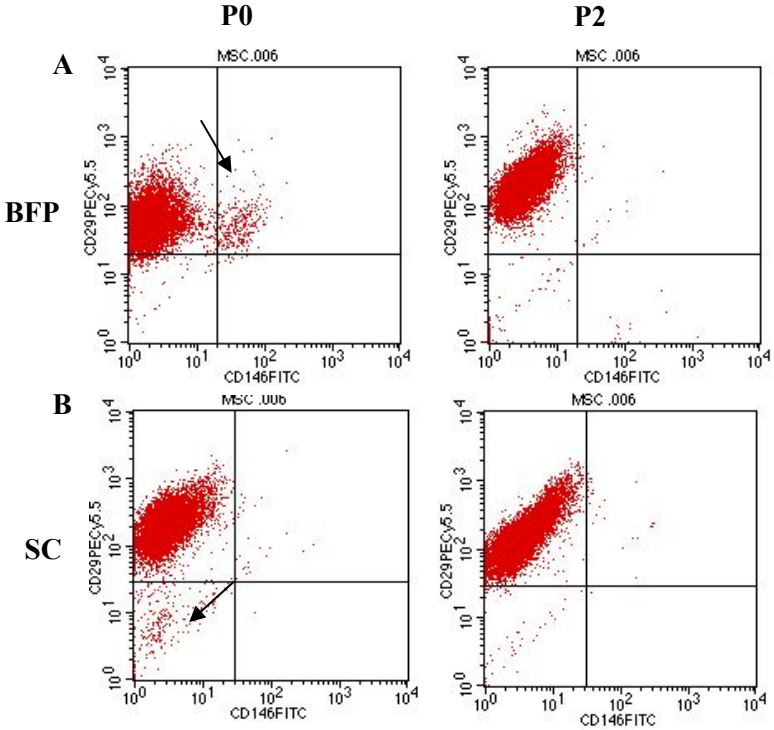



**Fig. 23: Contaminant population present in adipose tissue. A)** Dot plot images from flow cytometry analysis at passage 0 (P0) and passage 2 (P2) from adipose stem cells (ASC) derived from buccal fat pad (BFP). **B)** Dot plot images from flow cytometry analysis at passage 0 (P0) and passage 2 (P2) from ASC derived from subcutaneous abdominal adipose tissue (SC). Some contaminant populations (arrows) were present at P0, which represents cells following 7 days of culture on plastic, but disappeared at P2 revealing the purification of culture with successive passaging.

After one month of culture of ASC from BFP (P5), more than 98% of the cells expressed the characteristic mesenchymal stem cell markers CD105, CD73, and CD29 according to previous studies.[32,14] On the contrary, we observe a low expression of the hematopoietic markers CD45, CD19, CD14 and HLA-DR and also for the endothelial markers SSEA and CD146, at week and after two weeks of culture (Table 8). According to previous studies, expression of characteristic markers of MSC enhances during cell passage and becomes stable after P2.[26] Around 30% of cells were expressing CD34 at P0, but the expression decreased after 2 weeks of culture.

| Marker | P0 | P2 | P5 | n |
|---|---|---|---|---|
| CD105 | 89.1± 13.6 | 99.5 ± 0.8 | 99.1± 0.2 | n=3 |
| CD90 | 75.2 ± 18.3 | 80.8 ± 12 | 83.4 ± 18.3 | n=4 |
| CD73 | 84.7 ± 16.4 | 97.2 ± 1.3 | 99.1 ± 0.4 | n=4 |
| CD29 | 96.4 ± 2.4 | 98.6 ± 0.9 | 99.3* | n=3 |
| CD34 | 32.1 ± 20.1 | 12.7 ± 1.4 | 7.5 ± 4.5 | n=3 |
| CD45 | 1.3 ± 1.3 | 0.4 ± 0.5 | 0.5 ± 0.6 | n=4 |
| CD14 | 1.6 ± 2.2 | 6.6 ± 7.4 | 0.5 ± 0.3 | n=4 |
| HLA-DR | 2 ± 2.3 | 1.1 ± 1.4 | 0.9 ± 1 | n=3 |
| CD19 | 0.9 ± 0.9 | 1.4 ± 2 | 1.9 ± 2.2 | n=3 |
| CD146 | 1.1 ± 1.4 | 0.3 ± 0.1 | 0.1* | n=3 |
| SSEA | 1.8 ± 1.7 | 0.4 ± 0.1 | 0.1* | n=3 |

**Table 8: Percentage of stem cell and hematopoietic and endothelial marker expression from adipose stem cells (ASC) derived from buccal fat pad (BFP) at several passages in culture.** Results are shown as mean ± SD of the percentage of cells expressing a marker. Cells were analyzed at 7 days of culture (P0), passage 2 (P2) and passage 5 (P5) of culture. For CD29, CD146 and SSEA marker expression at P5 cells of one patient were analyzed (*).

ASC from BFP (marked in blue) and ASC from SC (in red) showed a similar stem cell marker expression at P0 and P2 of culture (Fig. 24A). The high expression of CD73, CD90, CD105, and CD29 is characteristic of ASC, and it is observed at one week after seeding in both sources of ASC (P0). After two weeks of culture of ASC (P2), percentage of cells expressing markers characteristics of ASC increased in both sources of ASC, showing a similar expression of stem cell markers. However, we observe a different expression of the marker CD34 at week of culture (P0) (Fig. 24B). ASC derived from BFP have a higher expression of this marker (32.1 ± 20.1) compared to ASC derived from subcutaneous adipose tissue, which express CD34 in lower percentage (24.7 ± 9.5). At P2 we observe a significant difference of CD34 expression (Mann-Whitney test, $p<0.05$) between both sources. The expression is 5 times higher in ASC derived from BFP than ASC derived from subcutaneous adipose tissue (12.4 ± 1.7 vs 2.5 ± 0.8). Although CD34 expression declines with time in culture, still remains 7.5% of expression in ASC derived from BFP at month of culture. These

differences in CD34 expression might be attributed to the higher blood supply of the buccal fat pad, which might contribute to the heterogeneity of the cell population found in this tissue.

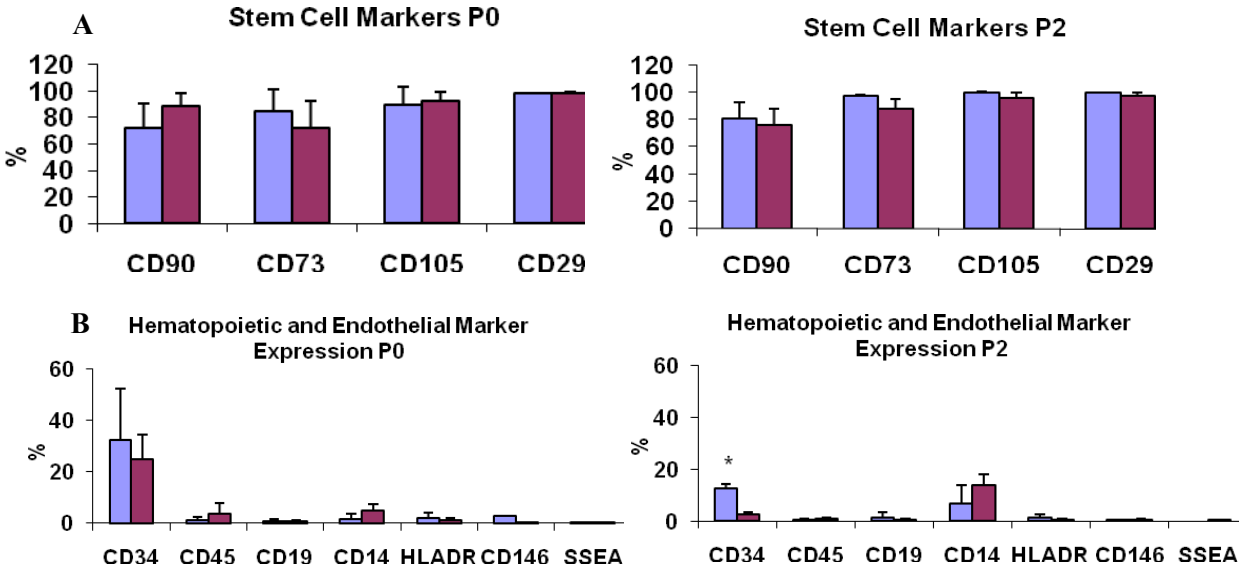



**Fig. 24: Comparison of phenotype of adipose stem cells (ASC) derived from buccal fat pad (BFP) or subcutaneous abdominal adipose tissue. (SC)** Surface marker profile of ASC from BFP in blue (n=4) and ASC from SC in red (n=4) was analyzed by flow cytometry. **A)** Stem cell marker expression at P0 and P2. **B)** Hematopoietic and endothelial marker expression was similar in the two different fat tissue sources, except CD34 expression, which was significantly higher in ASC from BFP than in ASC from SC at P2. * Significant difference of surface marker expression between BFP and SC ($p<0.05$).

### 6.1.2.4. Comparison of ASC yield from buccal fat pad and subcutaneous abdominal adipose tissue

Cell yield from adipose tissue obtained from patients undergoing elective oral or abdominal surgery was analyzed (Table 9). The mean age was 19.6 years for patients undergoing oral surgery (n=5, range 18-22 years), and 43.3 years for patients undergoing abdominal surgery (n=4, range 19-57 years). The mean yield of ASC obtained from BFP after one week of culture was 513 ± 227 x $10^3$ cells per gram of tissue, and the mean yield of ASC from abdominal adipose tissue was 253 ± 56 x $10^3$ cells per gram of adipose tissue. This result shows more ASC in BFP than in SC, although differences are not significative due to high patient variability. The number of ASC obtained from SC was similar to the number of ASC observed in another study.[26]

| Pacient | Source | Age (years) | Volume of fat (ml) | Weight of fat (g) | Cell number | Days of culture | Cell number/ g | X ± SEM |
|---|---|---|---|---|---|---|---|---|
| Man | BFP | 18 | 14 | 12 | 3,800,000 | 7 | 316,667 | |
| Man | BFP | 20 | 23 | 18.2 | 6,615,000 | 7 | 363,462 | |
| Woman | BFP | 22 | 16 | 13.6 | 11,679,999 | 7 | 858,823 | 513 ± |
| Woman | BFP | 20 | 15 | 14.5 | 9,075,000 | 7 | 625,862 | 227 |
| Woman | BFP | 18 | 12.5 | 11.9 | 4,780,000 | 7 | 401,681 | |
| Woman | SC | 19 | 14 | 12.2 | 2,339,000 | 7 | 191,721 | |
| Woman | SC | 47 | 36 | 29 | 9,045,000 | 7 | 311,897 | 253 ± |
| Woman | SC | 50 | 16 | 13.2 | 2,940,000 | 7 | 222,727 | 256 |
| Woman | SC | 57 | 5.6 | 5.5 | 1,579,999 | 7 | 287,273 | |

**Table 9: Cell number at P0 obtained from two different sources of adipose tissue: buccal fat pad (BFP) and subcutaneous abdominal adipose tissue (SC).** Cell number is expressed per gram of adipose tissue after one week of culture.

### 6.1.2.5. SVF from BFP is a rich source of ASC.

Adipose tissue can be split in two different fractions, i.e. one fraction containing mature adipocytes, and another fraction, SVF, containing a heterogeneous cell population. To our knowledge, the SVF from BFP has not yet characterized. To determine the percentage of ASC present in this fraction we characterized the SVF from 5 patients. The co-expression of different markers was determined by flow cytometry to quantify the cell population representing fresh ASC. Patients were aged between 19 and 29 years, and 60% were female. SVF from BFP contained a mixture of cells, as shown by representative dot plots from the different patients and percentages of marker co-expression (Fig. 25B).

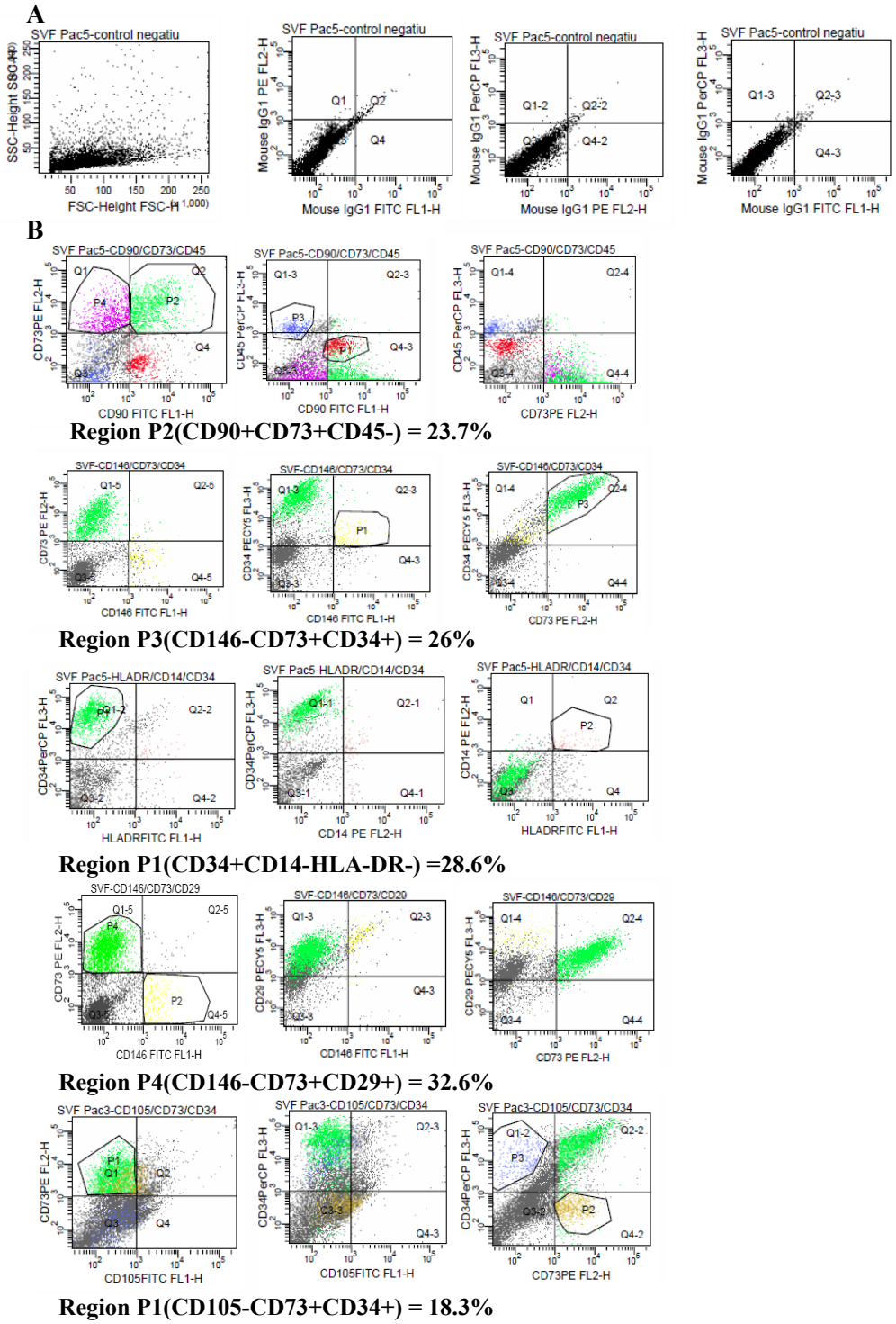



**Fig. 25: Flow cytometry of fresh adipose stem cells (ASC) obtained from buccal fat pad (BFP), analyzed by FACS DIVA software**. **A)** General population and isotype controls of different fluorochromes. Samples were analyzed by flow cytometry using forward scatter (FSC) to detect cell size, and side scatter (SSC) to detect granularity. **B)** Mixed population of cells observed in the stromal vascular fraction (SVF), analyzing co-expression of 3 characteristic markers from 3 representative patients. Cells expressing characteristic markers of fresh ASC are marked in green. Endothelial cells, hematopoietic cells, fibroblasts, and endothelial progenitor cells/vascular smooth muscle cells are marked respectively in purple, blue, red, and yellow.

The SVF contains a high percentage of cells (21-38%) which express CD90, CD73, CD29, and CD34, and which do not express the hematopoietic and endothelial lineage markers CD45, CD19, CD14, HLA-DR, and CD146 (Table 10). In addition, this population of cells does express CD34, but not CD105, as reported previously.[67,68] This marker profile is characteristic of fresh ASC, while culture of ASC on plastic plates increases CD105 expression and reduces CD34 expression. The expression of the CD34 by ASC, contrasts with bone marrow MSC, which lack expression of this marker.[69] CD34+ cells stimulate angiogenesis, and they are involved in neovascularization processes that facilitate healing of ischemic tissues.[70]

Taken together, these results demonstrate that SVF from BFP is a rich source of ASC (~30%), similar to other sources of adipose tissue, and this makes them good candidates for clinical applications.

| Marker profile | % ASC in SVF (Mean ± SD) | # Patients |
|---|---|---|
| CD90+/CD73+/CD45- | 27.2 ± 13.9 | 4 |
| CD90+/CD73+/CD34+ | 21.5 ± 13.9 | 5 |
| CD105-/CD73+/CD34+ | 21.3 ± 14.3 | 5 |
| CD105-/CD19-/CD34+ | 38.1 ± 18.3 | 2 |
| HLA-DR-/CD14-/CD34+ | 26.5 ± 2.9 | 2 |
| CD146-/CD73+/CD34+ | 21.4 ± 6.4 | 2 |
| CD146-/CD73+/CD29+ | 23.9 ± 12.2 | 2 |

**Table 10. Mean percentage of adipose stem cells (ASC) in the stromal vascular fraction (SVF) from buccal fat pad (BFP), analyzed by flow cytometry.** Samples (n=5) were incubated with 9 markers and analyzed for co-expression of 3 cell surface markers by flow cytometry.

### 6.1.3. Multilineage differentiation potential of ASC derived from buccal fat pad in vitro

#### 6.1.3.1. Osteogenic differentiation

To assess the osteogenic potential of ASC, ALP, an early marker of osteoblastic differentiation, was determined after 0, 7, and 21 days of culture in OM. SAOS-2 cells were used as a positive control.

After 1 week of culture, ASC changed their morphology from spindle shaped to more polygonal shaped, which was accompanied by an increase in ALP activity up to day 21 (Fig. 26). ASC cultured in osteogenic medium showed a 2.5-fold increase in ALP activity at day 7, and a significant increase in ALP activity of 16.5-fold at day 21 of culture compared to control (Mann-Whitney test, $p<0.05$).

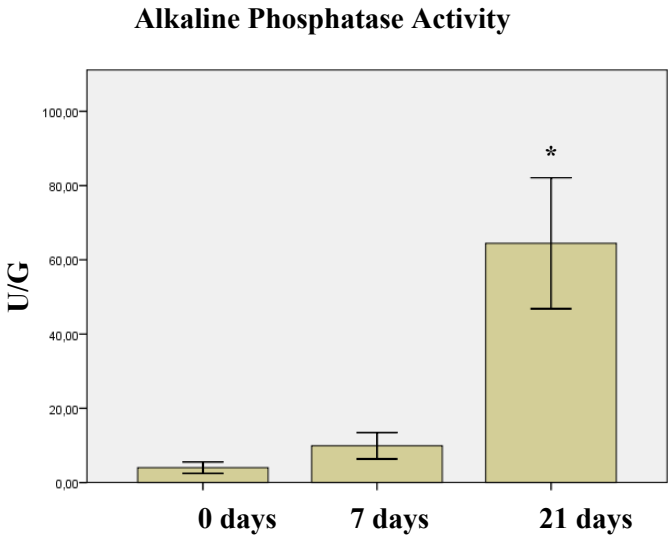



**Fig. 26: Alkaline phosphatase activity of adipose stem cells (ASC) from buccal fat pad (BFP)**. Graphical representation of alkaline phosphatase (ALP) activity in units per gram (U/G) of ASC at 0, 7, and 21 days of osteogenic induction. Values are mean ± SEM. ALP activity of SAOS-2 cells (positive control) was 672.7 U/G ± 125.5 (results not shown) and ALP activity of ASC was 3.9 U/G ± 1.2 at 0 days, 9.9 U/G ± 3.5 at 7 days and 64.4 U/G ± 17.6 at 21 days of osteogenic differentiation. * Significant difference of ALP activity between 0 days and 21 days ($p<0.05$).

Areas of high granular density appeared and multiple layers of ASC were formed after 2 weeks, which increased with time in culture. These areas were stained intensely with Alizarin Red, indicating calcification of the extracellular matrix (Fig. 27B). ASC grown in control medium did not stain with Alizarin Red (Fig. 27A). Alizarin Red staining was not seen in undifferentiated ASC at visual examination, (Fig. 27C), but it was clearly visible after culture in OM (Fig. 27D), as well as in SAOS-2 cells (Fig. 27E).

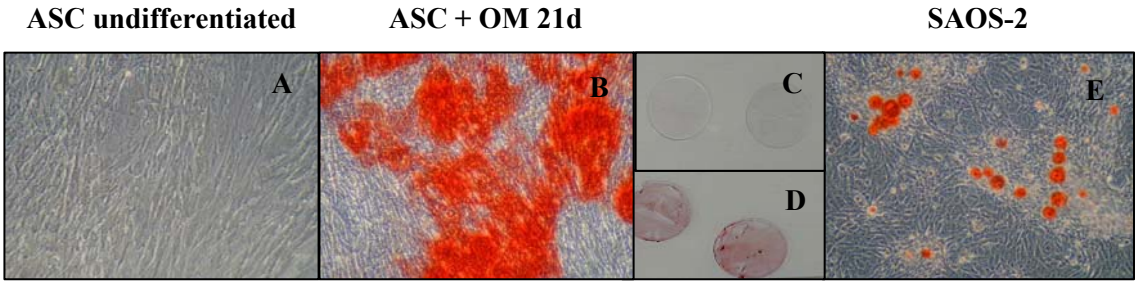



**Fig. 27: Matrix calcification of adipose stem cells (ASC) from buccal fat pad (BFP) showed by Alizarin Red staining of ASC from BFP**. Alizarin Red-stained ASC in osteogenic medium (OM) **(B)** and in control medium **(A)** at 100x magnification. Cells were seeded at $5x10^4$ cells/well. Red nodules were visible in differentiated ASC **(D)** but absent in undifferentiated ASC **(C)**. SAOS-2 cells served as positive controls **(E)**.

Protein expression of OCN, a marker characteristic of mature osteoblasts and osteocytes, was determined by immunofluorescence. ASC cultured in OM, but not undifferentiated ASC, showed OCN expression (Fig. 28B), as did SAOS-2 cells (positive control) (Fig. 28C).

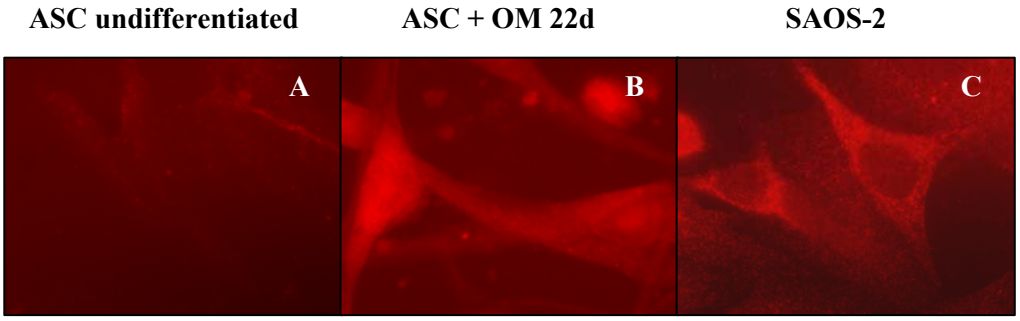



**Fig. 28: Osteocalcin expression by adipose stem cells (ASC) from buccal fat pad (BFP), showed by osteocalcin immunohistochemistry.** Cells were seeded at $2x10^4$ cells/well. ASC cultured in osteogenic medium (OM) **(B)** but not in control medium **(A)** for 22 days showed osteocalcin immunostaining. A characteristic osteocalcin staining was observed in SAOS-2 cells used as positive control **(C)**. Magnification 400x. Osteocalcin primary antibody was used at a concentration of 1:10, and Alexa 546 secondary antibody at a concentration of 1:500.

During the differentiation process, the expression of the osteogenic genes CBFA1 and SPARC increased by 8 fold and 2 fold respectively at day 14 of culture (Fig. 29A,B).

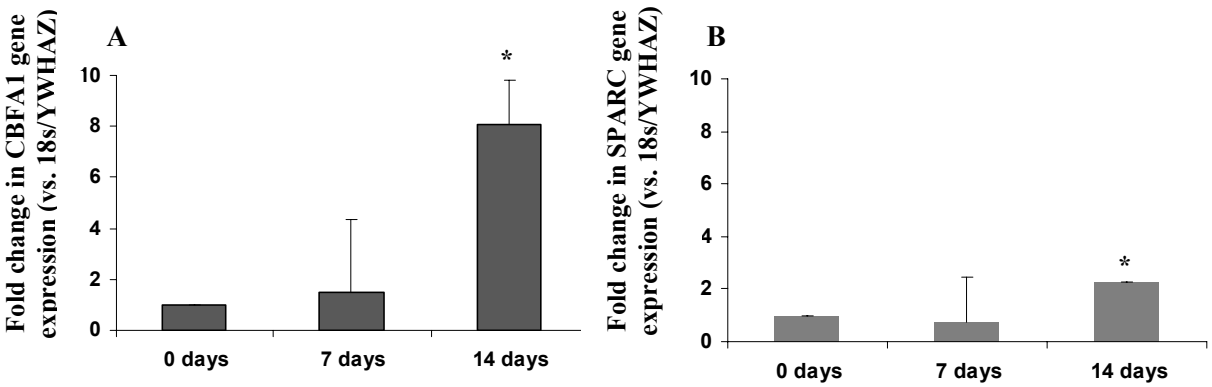



**Fig. 29: Osteogenic gene expression of adipose stem cells (ASC) from buccal fat pad (BFP) quantified by real-time PCR. A)** CBFA1 gene expression of ASC from BFP at 0, 7 and 14 days of osteogenic differentiation. **B)** SPARC gene expression of ASC from BFP at 0, 7 and 14 days in osteogenic medium. Gene expression levels were normalized to 18S and YWHAZ using the normalization factor and expressed relative to non-induced ASC. Values are mean ± SEM, n=3. * Significant difference of gene expression between 0 days and 14 days ($p<0.05$).

On the other hand, we did not observe a decrease in gene expression of the adipocyte marker PPARγ until day 21 (Fig. 30). SAOS-2 cells were used as positive control. These data demonstrate that ASC from BFP are capable of osteogenic differentiation *in vitro*.

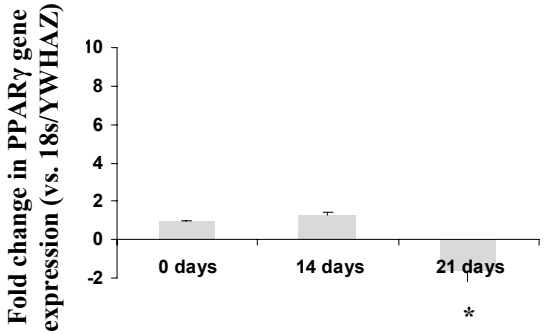



**Fig. 30: PPARγ gene expression of adipose stem cells (ASC) from buccal fat pad (BFP) quantified by real-time PCR.** ASC from BFP were cultured in control medium and osteogenic medium for up to 3 weeks. Gene expression levels were normalized to 18S and YWHAZ using the normalization factor and expressed relative to non-induced ASC. Values are mean ± SEM, n=3. * Significant difference of gene expression between 0 days and 21 days ($p<0.05$).

### 6.1.3.2. Adipogenic differentiation

After adipogenic induction of ASC, we observed intracellular lipid vacuoles (Fig. 31A), which increased in size and number during culture (Fig. 31B).

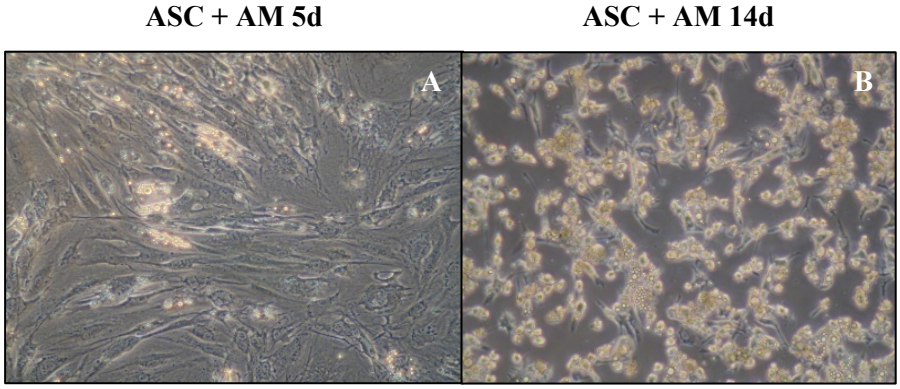



**Fig. 31: Adipogenic differentiation of adipose stem cells (ASC) from buccal fat pad (BFP).** Cells were cultured with adipogenic medium (AM) and analyzed under microscopy at day 5 **(A)** and day 14 **(B)** at 100x magnification. Lipid vacuoles were observed within the cells. The size of lipid vacuoles did increase with time in culture.

Oil Red staining revealed multiple intracellular lipid filled droplets in ASC cultured in AM (Fig. 32B), but not in undifferentiated ASC (Fig. 32A).

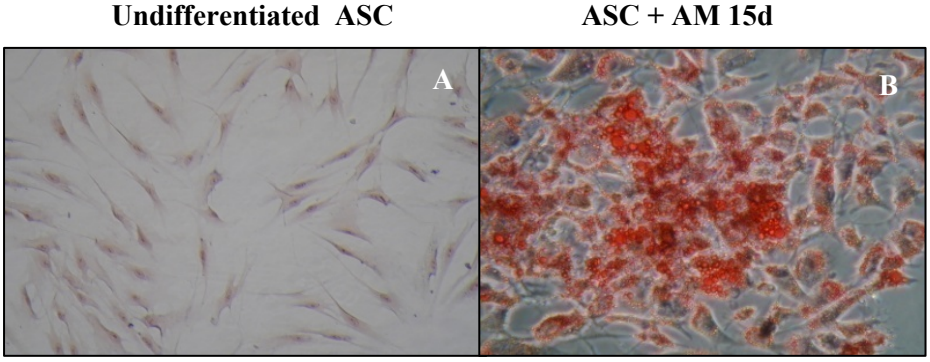



**Fig. 32: Oil Red stain to detect lipid vacuoles characteristic of adipocytes.** At day 15 cells were stained with Oil Red and analyzed using a microscope. Lipid vacuoles stained intense red in adipose stem cells (ASC) grown in adipogenic medium (AM) **(B)**, but no lipid vacuoles were present in undifferentiated ASC **(A).**

The expression levels of the specific adipocyte marker PPARγ and GAPDH were measured by quantitative real-time PCR using the comparative $C_t$ method (Fig. 33). PPARγ expression increased during culture, reaching ~4 fold induction compared to undifferentiated ASC. Whole adipose tissue samples served as positive control.

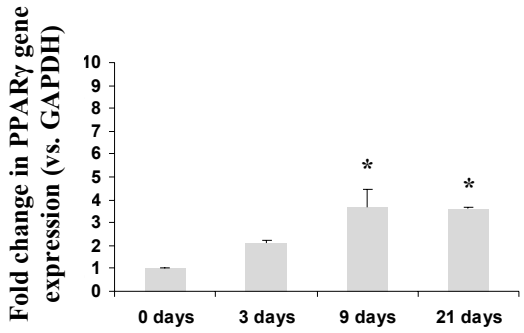



**Fig. 33: PPARγ gene expression of adipose stem cells (ASC) from buccal fat pad (BFP) quantified by real-time PCR.** PPARγ gene expression levels measured by real-time PCR in undifferentiated ASC and ASC cultured in adipogenic medium (AM) during 3, 9, and 21 days. Expression levels are represented as fold increase in comparison with undifferentiated controls, after normalization to the housekeeping gene GAPDH. Whole subcutaneous adipose tissue and BFP samples were used as positive controls, which showed an increase in PPARγ expression compared to undifferentiated ASC of 28 ± 4.4 and 66 ± 7.8 fold respectively. * Significant difference of gene expression between 0 days and 9 days, and between 0 and 21 days ($p<0.05$).

### 6.1.3.3. Chondrogenic differentiation

ASC from BFP synthesized cartilage matrix molecules, and produced an extracellular matrix characteristic of chondrocytes when grown in CM. After ~5 days of culture in CM, ASC changed their morphology to more spheroid-shaped and the round morphology increased after time in culture (Fig. 34A). ASC under control conditions did not show Toluidine Blue staining at pH 2, which is specific for sulfated proteoglycans in cartilage matrix (Fig. 34B). The number of nodules, which stained positive with Toluidine Blue (Fig. 34C) increased during 4 weeks of culture in CM (data not shown). Toluidine Blue-stained purple nodules were not seen in undifferentiated ASC (Fig. 34D), but they were clearly visible after culture in CM (Fig. 34E).

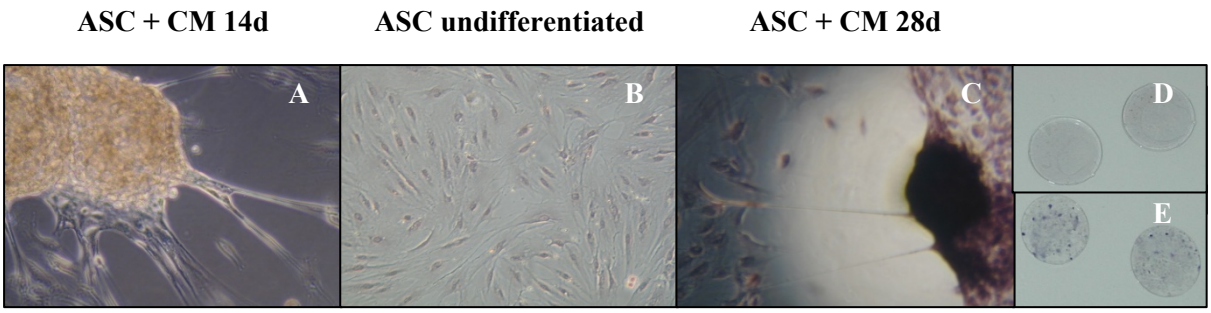



**Fig. 34: Toluidine Blue staining to detect a proteoglycan matrix characteristic of chondrocytes.** Micromasses were observed, and their number increased with time in culture. **A)** Adipose stem cells (ASC) at 14 days of chondrogenic differentiation. At day 28 of differentiation ASC were stained with Toluidine Blue for the presence of proteoglycan matrix. Intense blue nodules were observed in ASC induced to differentiate **(C)**, but these nodules were not observed in ASC cultured in control medium **(B)**. Magnification 100x. Coverslips stained with Toluidine Blue showed violet nodules from ASC differentiated with chondrogenic medium (CM) **(E)**. There was no staining in the coverslips with ASC grown in control medium **(D)**.

Immunohistochemical analysis did not show expression of collagen type II in undifferentiated cells (Fig. 35A), but it was expressed by cells grown in micromass in CM after 4 weeks (Fig. 35B).

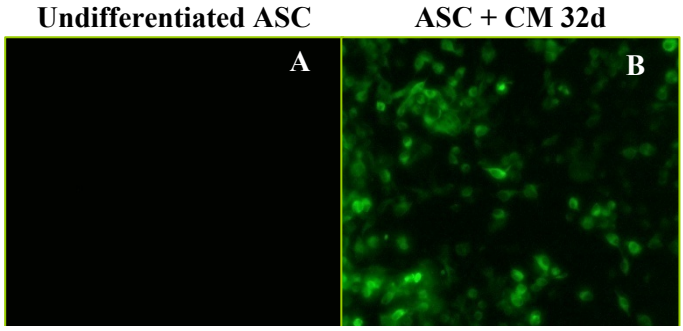



**Fig. 35:** Immunohistochemistry for Collagen II showed staining in adipose stem cells (ASC) cultured with chondrogenic medium (CM) for 32 days **(B)**, not present in ASC cultured in control medium **(A)**. Magnification of 100x. Collagen II primary antibody was used at a concentration of 1:5, and Fluorescein secondary antibody at a concentration of 1:500.

The expression of the master chondrogenic factor SOX9 seemed to increase by 3.4 fold in ASC grown in CM compared to undifferentiated ASC at day 14 (Fig. 36). Expression of the adipocyte gene PPARγ decreased during chondrogenic differentiation (Fig. 37) indicating the chondrogenic commitment of the cells.

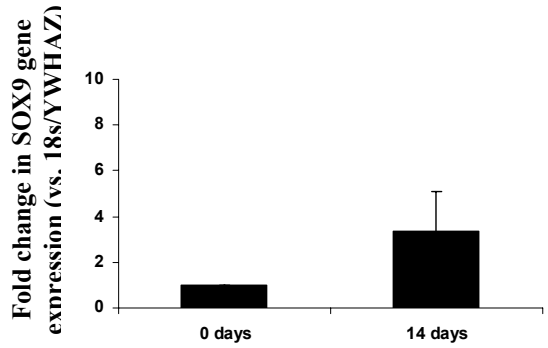



**Fig 36: SOX9 gene expression of adipose stem cells (ASC) from buccal fat pad (BFP).** SOX9 gene expression levels were measured by real-time PCR in ASC cultured in control medium and chondrogenic medium up to 14 days. Expression levels are represented as fold increase in comparison with levels found in undifferentiated cells, and normalized by using 18S and YWHAZ as housekeeping genes. Cells from nucleus pulposus were used as the control.

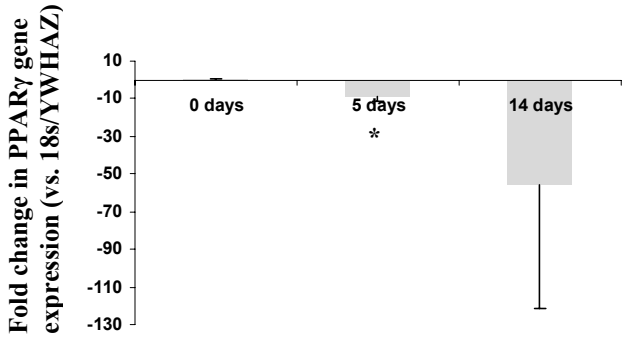



**Fig. 37: PPARγ gene expression of adipose stem cells (ASC) from buccal fat pad (BFP).** PPARγ gene expression levels measured by real-time PCR in undifferentiated ASC and ASC cultured in chondrogenic medium during 5 and 21 days. Expression levels are represented as fold increase in comparison with undifferentiated controls, after normalization to the housekeeping gene 18S and YWHAZ. * Significant difference of gene expression between 0 days and 5 days ($p<0.05$).

## 6.2. STUDY 2: OSTEOGENIC DIFFERENTIATION OF BMP-2 INDUCED HUMAN ADIPOSE STEM CELLS SEEDED ON BIPHASIC CALCIUM PHOSPHATE AND β-TRICALCIUM PHOSPHATE SCAFFOLDS

### 6.2.1. Effect of BMP-2 on attachment and proliferation of ASC on different scaffolds

To determine if a short stimulation of 15min with BMP-2 affects the frequency of mesenchymal stem cells from adipose tissue, the colony-forming unit fibroblasts assay was performed after seeding BMP-2 treated or non treated ASC on tissue culture plastic. Results were expressed in percentage of CFU-F (n=9). Cell attachment was determined measuring the concentration of DNA, to evaluate the number of cells attached after seeding BMP-2 treated or non treated ASC onto the different scaffolds (n=6). Depletion CFU-F assay was also performed on the pooled flow-through/washings collected after washing BMP-2 treated or non treated ASC seeded on scaffolds and allowed to attach for 30min (n=6), to determine the number of non-attached ASC, from the different scaffolds (n=6). Cell proliferation of BMP-2 treated or non treated ASC cultured on BCP and β-TCP was quantified and compared by measuring DNA concentration at day 4, day 14, and day 21 of culture (n=6).

#### 6.2.1.1. BMP-2 increases CFU-F and does not affect cell attachment on different scaffolds

The number of toluidin blue-positive colonies (CFU-F) was counted 14 days after seeding freshly isolated and freshly frozen and thawed ASC, either incubated or not with BMP-2, on tissue culture plastic.

Colonies were present at 14 days of culture which stained positively by Toluidine Blue staining, the so-called CFU-F (Fig. 38).

**Fig. 38: Colony-forming unit fibroblasts stained with Toluidine Blue after two weeks of culture.** 10X magnification.

The frequency of the CFU-F was on average 0.08% ± 0.007 (mean ± SEM; Table 11), and after a short incubation with BMP-2 of 15min was almost two-fold higher (0.15% ± 0.004).

| CFU | Donors (n) | % CFU-F (mean ± SEM) | |
|---|---|---|---|
| | | -BMP-2 | +BMP-2 |
| CFU-F | 9 | 0.08 ± 0.007 | 0.15 ± 0.004 |
| Depl CFU-F BCP 60/40 | 6 | 0.01 ± 0.001 | 0.02 ± 0.002 |
| Depl CFU-F BCP 20/80 | 6 | 0.01 ± 0.002 | 0.02 ± 0.001 |
| Depl CFU-F ß-TCP<0.7mm | 6 | 0.01 ± 0.002 | 0.02 ± 0.001 |
| Depl CFU-F ß-TCP>0.7mm | 6 | 0.02 ± 0.001 | 0.02 ± 0.001 |

**Table 11: Colony-forming unit fibroblasts (CFU-F) and depletion colony-forming unit fibroblasts assays (Depl CFU-F).** Bone morphogenetic protein-2 (BMP-2) increases adipose stem cell (ASC) frequency on tissue culture plastic, as shown by CFU-F assay. BMP-2 treatment does not affect ASC attachment on different scaffolds, shown by Depl CFU-F assay. BCP 60/40, bicalcium phosphate 60% hydroxyapatite and 40% ß-tricalcium phosphate; BCP 20/80, bicalcium phosphate 20% hydroxyapatite and 80% ß-tricalcium phosphate; ß-TCP<0.7mm, ß-tricalcium phosphate with particle size of 0.5-0.7 mm; ß-TCP>0.7mm, ß-tricalcium phosphate with particle size of 0.7-1.4 mm.

This shows that a short stimulation of 15 min with BMP-2 increases significantly the CFU-F frequency of ASC seeded on tissue culture plastic after 2 weeks of culture (Fig. 2, $p<0.001$). (Fig. 39, $p<0.001$).

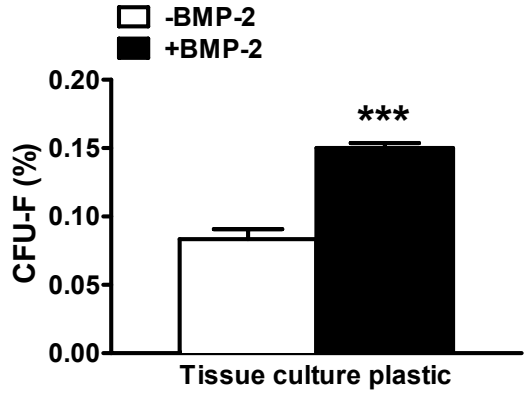



**Fig. 39:** Effect of bone morphogenetic protein-2 (BMP-2) on the frequency of adipose stem cells (ASC) in adipose tissue. BMP-2 treatment of freshly isolated ASC significantly increased the percentage of Toluidine Blue-stained colonies, characteristic of ASC. ***p<0.001. CFU-F, colony-forming unit fibroblasts.

ASC either or not pre-treated with BMP-2 from several donors were seeded onto biphasic calcium phosphate as well as β-tricalcium phosphate. After 30min of seeding DNA was quantified and expressed in nanograms (ng). We found that regarding the attachment, no significant differences were detected between the scaffolds after BMP-2 treatment (Fig. 40, black bars). A higher cell number appears to be found on BCP than on ß-TCP.

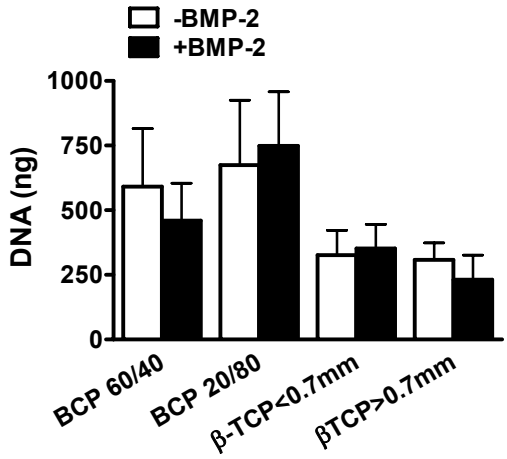



**Fig. 40: Effect of bone morphogenetic protein-2 (BMP-2) on adipose stem cells (ASC) attachment on different scaffolds.** CyQUANT DNA assay was performed to quantify the number of ASC on the different biomaterials. Graphic bars show the amount of DNA in ng at day 0, after 30min of seeding without BMP-2 stimulation (white bars), and after BMP-2 stimulation (black bars). No statistic significances were observed between both groups. BCP 60/40, biphasic calcium phosphate 60% hydroxyapatite and 40% ß-tricalcium phosphate; BCP 20/80, biphasic calcium phosphate 20% hydroxyapatite and 80% ß-tricalcium phosphate; ß-TCP<0.7mm, ß-tricalcium phosphate with particle size of 0.5-0.7 mm; ß-TCP>0.7mm, ß-tricalcium phosphate with particle size of 0.7-1.4 mm. BCP, biphasic calcium phosphate; ß-TCP; ß-tricalcium phosphate.

This validates our methods to use the SVF after is frozen, and it shows that the freezing method does not affect the proliferation and osteogenic differentiation of adipose stem cells from the stromal vascular fraction, as previously observed.[74] Regarding the effect of BMP-2 on the attachment of fresh ASC to the scaffolds, we found that it is similar with freshly frozen ASC (data not shown).

The collection of washing steps after seeding on the scaffolds and allowing the cells to attach 30min was collected and seeded on tissue culture plastic, to measure the CFU-F by the depletion CFU-F assay (Fig. 41). Cell attachment was unaffected by BMP-2. More CFU-F were observed from washing steps collected from ß-TCP than from BCP before and after treatment, indicating more adhered cells in BCP ($p<0.05$).

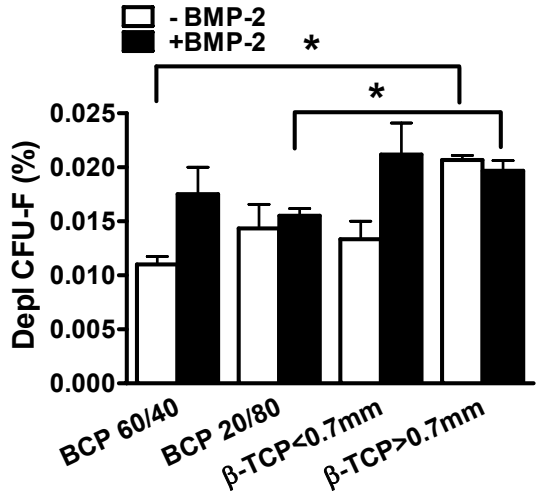



**Fig. 41: Effect of bone morphogenetic protein-2 (BMP-2) on adipose stem cells (ASC) attachment as determined by using the colony-forming unit fibroblasts depletion assay (Depl CFU-F); with BMP-2 stimulation (black bars) and without BMP-2 stimulation (white bars).** Cell attachment to biomaterials was unaffected by BMP-2. More CFU-F were observed from washing steps collected from ß-TCP than from BCP before and after treatment. *p<0.05. BCP 60/40, biphasic calcium phosphate 60% hydroxyapatite and 40% ß-tricalcium phosphate; BCP 20/80, biphasic calcium phosphate 20% hydroxyapatite and 80% ß-tricalcium phosphate; ß-TCP<0.7mm, ß-tricalcium phosphate with particle size of 0.5-0.7 mm; ß-TCP>0.7mm, ß-tricalcium phosphate with particle size of 0.7-1.4 mm. BCP, biphasic calcium phosphate; ß-TCP; ß-tricalcium phosphate.

### 6.2.1.2. BMP-2 affects ASC proliferation dependent on the carrier

ASC cultured on BCP and β-TCP were quantified and compared by DNA concentration at day 4, day 14, and day 21 of culture as fold increase of BMP-2 treated ASC versus non-treated ASC. At day 21, DNA concentration of stem cells seeded on BCP 60/40 was significantly higher after BMP-2 treatment, with an increase of 2.4 ± 0.5 fold versus non-treated cells (Fig. 42, $p<0.05$). DNA content of stem cells seeded on BCP 20/80, ß-TCP<0.7mm, and on ß-TCP>0.7mm after BMP-2 treatment was incremented 1.5 ± 0.6, 1.1 ± 0.09, and 1.9 ± 0.8 fold respectively. Therefore BMP-2 affects ASC proliferation on the biomaterial dependent on the carrier.

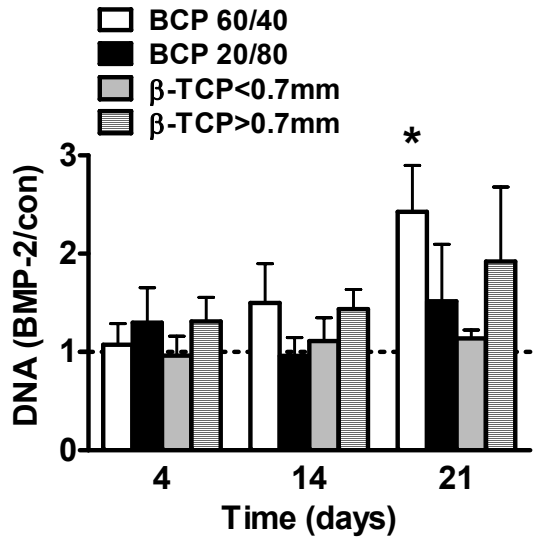



**Fig. 42: Bone morphogenetic protein-2 (BMP-2) increases adipose stem cells (ASC) proliferation dependent on the scaffold at 21 days of culture.** BMP-2 significantly stimulated DNA content of stem cells seeded on BCP 60/40 at day 21. *p<0.05. DNA content is expressed as BMP-2-treated-over-untreated control. 1 means no effect by BMP-2. BCP 60/40, biphasic calcium phosphate 60% hydroxyapatite and 40% ß-tricalcium phosphate; BCP 20/80, biphasic calcium phosphate 20% hydroxyapatite and 80% ß-tricalcium phosphate; ß-TCP<0.7mm, ß-tricalcium phosphate with particle size of 0.5-0.7 mm; ß-TCP>0.7mm, ß-tricalcium phosphate with particle size of 0.7-1.4 mm; con, control. BCP, biphasic calcium phosphate; ß-TCP; ß-tricalcium phosphate.

### 6.2.2. Effect of BMP-2 on osteogenic differentiation of ASC on different scaffolds

To investigate whether 15min treatment with BMP-2 on human ASC cultured on different calcium phosphate carriers is enough to undergo osteogenic differentiation of ASC *in vitro*, we measured ALP activity (n=6) and expression of genes associated with osteogenesis after 14 days of culture (n=2).

#### 6.2.2.1. BMP-2 induces ALP activity dependent on the carrier

Early differentiation of mesenchymal stem cells into immature osteoblasts is characterized by ALP enzyme activity, which is expressed by MSC as early as 4 days after induction, and maximum levels are observed after 14 days of osteogenic induction.[75]

ALP activity of ASC cultured on four different calcium phosphate carriers was measured using an ELISA method and we expressed the results as fold increase in BMP-2 treated cells

versus non-treated cells. The ALP activity of ASC cultured on BCP 60/40 scaffold increased throughout the entire culture period, being significantly higher at day 21 of culture with 1.9 ± 0.5 fold increase versus non-treated cells (Fig. 43, white bars, $p<0.05$). ASC seeded on BCP 20/80 showed an increase in ALP activity of 1.6 ± 0.9 fold at day 21 of culture. ASC seeded on ß-TCP<0.7mm and on ß-TCP>0.7mm showed an increase in ALP activity of 2.6 ± 1.5 fold and 1.2 ± 0.4 fold respectively.

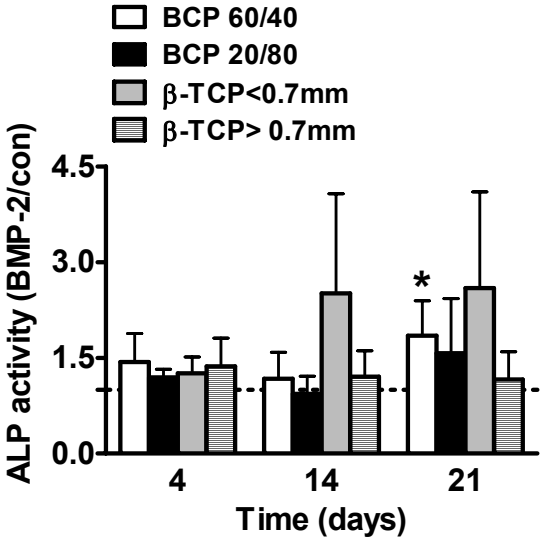



**Fig. 43: Bone morphogenetic protein-2 (BMP-2) increases alkaline phosphatase (ALP) activity dependent on the scaffold at 21 days of culture.** BMP-2 significantly stimulated ALP activity in adipose stem cells seeded on BCP 60/40 at day 21. *p<0.05. ALP activity is expressed as BMP-2-treated-over-untreated control, and is normalized for protein. 1 means no effect of BMP-2. BCP 60/40, biphasic calcium phosphate 60% hydroxyapatite and 40% ß-tricalcium phosphate; BCP 20/80, biphasic calcium phosphate 20% hydroxyapatite and 80% ß-tricalcium phosphate; ß-TCP<0.7mm, ß-tricalcium phosphate with particle size of 0.5-0.7 mm; ß-TCP>0.7mm, ß-tricalcium phosphate with particle size of 0.7-1.4 mm; con, control. BCP, biphasic calcium phosphate; ß-TCP; ß-tricalcium phosphate.

### 6.2.2.2. BMP-2 induces osteogenic gene expression dependant on the carrier

To further confirm the differentiation of BMP-2 treated ASC on different calcium phosphate carriers, mRNA expression of genes associated with osteogenesis such as CBFA1, Col1, ALP, ON, OPN, and OCN was examined by real-time PCR after 14 days of culture (Fig. 44A-F).

ASC cultured on tissue culture plastic without BMP-2 seemed to express only CBFA1, Col1, and ON mRNA (Fig. 44A,B,D, white bars). After BMP-2 treatment, the expression of the osteogenic genes CBFA1, Col1, and ON seemed to increase (Fig. 44A,B,D, black bars) on tissue culture plastic at day 14 of culture. BMP-2 treatment seemed to increase ALP mRNA gene expression as well, with an increase of 3 fold in ASC cultured on tissue culture plastic (Fig. 44C, black bar). ASC cultured on BCP 60/40 scaffolds without BMP-2 seemed to express only CBFA1, Col1, ALP and ON mRNA (Fig. 44A-D, white bars). BMP-2 treatment seemed to increase the expression of the osteogenic genes CBFA1, Col1, and ON mRNA (Fig. 44A,B,D, black bars). OPN and OCN gene expression seemed to increase in BMP-2 treated ASC seeded on BCP 60/40 as well (Fig. 44E,F, black bars). On the contrary, BMP-2 treated ASC cultured on ß-TCP scaffolds showed no increase in osteogenic genes, except OCN gene expression, detected in BMP-2 treated ASC seeded on ß-TCP>0.7mm (Fig. 44F, black bar).

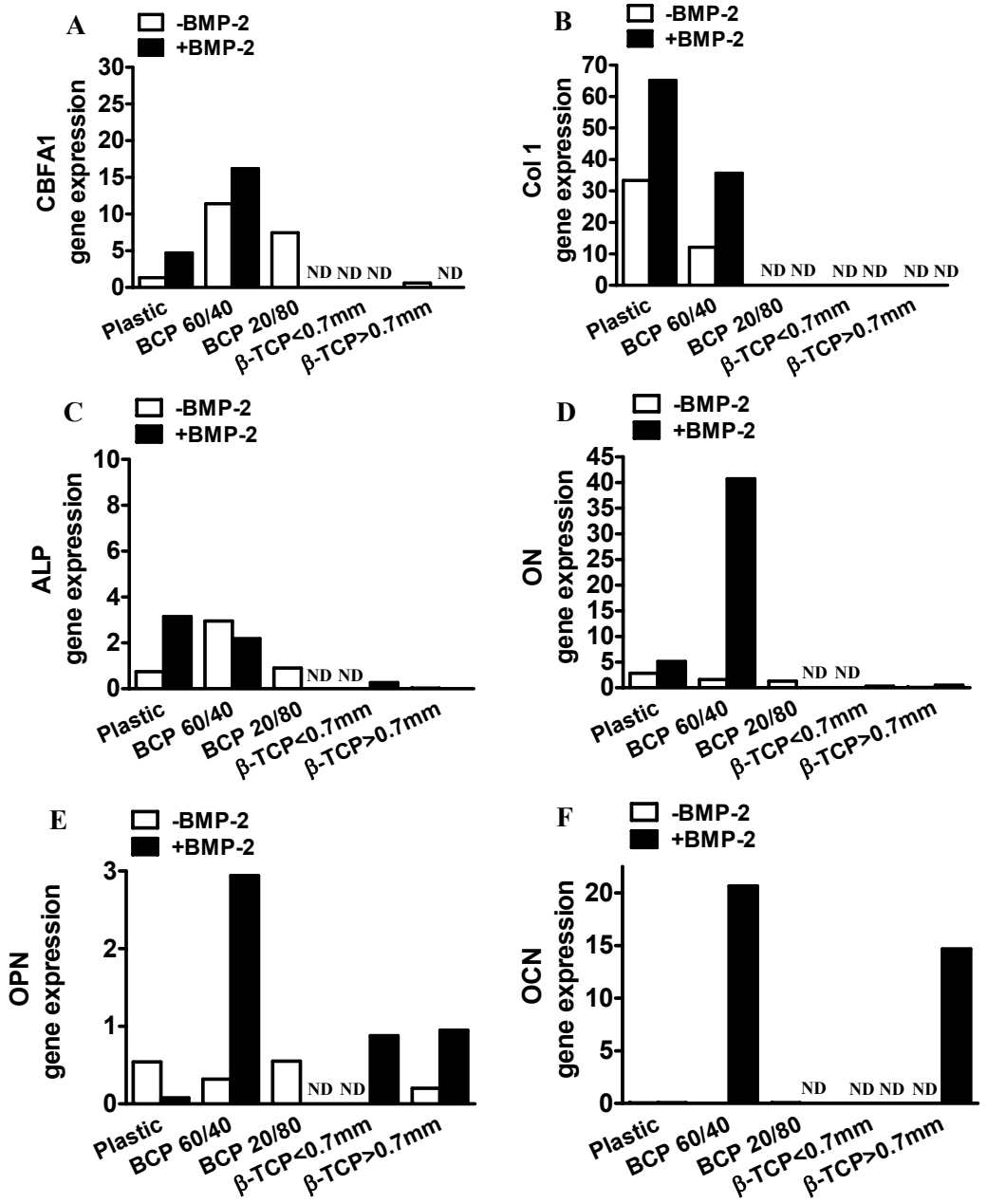



**Fig. 44: Bone morphogenetic protein (BMP-2) seems to increase osteogenic gene expression in ASC seeded on BCP 60/40 at 14 days of culture.** Freshly isolated ASC, either or not treated with BMP-2, were seeded on tissue culture plastic and on different scaffolds. **A)** CBFA1 gene expression. **B)** Col1 gene expression. **C)** ALP gene expression. **D)** ON gene expression. **E)** OPN gene expression. **F)** OC gene expression. Expression levels are represented as fold increase in comparison with undifferentiated ASC, after normalization to the housekeeping genes UB and YWHAZ. CBFA1, core binding factor-alpha1; COL1, collagen I; ALP, alkaline phosphatase; ON, osteonectin; OPN, osteopontin; OCN, osteocalcin; UB, ubiquitin; BCP 60/40, biphasic calcium phosphate 60% hydroxyapatite and 40% ß-tricalcium phosphate; BCP 20/80, biphasic calcium phosphate 20% hydroxyapatite and 80% ß-tricalcium phosphate; ß-TCP<0.7mm, ß-tricalcium phosphate with particle size of 0.5-0.7 mm; ß-TCP>0.7mm, ß-tricalcium phosphate with particle size of 0.7-1.4 mm; ND, not detected. BCP, biphasic calcium phosphate; ß-TCP; ß-tricalcium phosphate.

Expression of the adipogenic gene PPARγ was also measured at 14 days of culture. This transcription factor has been shown to be critical in promoting adipogenesis and inhibiting osteogenesis.[76] PPARγ mRNA gene expression seemed to decrease on ASC cultured on tissue culture plastic and on different calcium phosphate carriers, indicating the osteogenic commitment of the cells (Fig. 45).

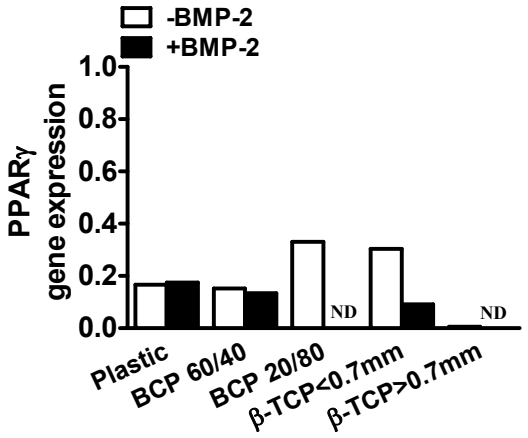



**Fig. 45: Adipogenic gene expression decreased at 14 days of culture, indicating the osteogenic commitment.** Freshly isolated ASC, either or not treated with bone morphogenetic protein (BMP-2), were seeded on tissue culture plastic and on different scaffolds. PPARγ gene expression levels are represented as fold increase in comparison with undifferentiated ASC, after normalization to the housekeeping genes UB and YWHAZ. UB, ubiquitin; BCP 60/40, biphasic calcium phosphate 60% hydroxyapatite and 40% ß-tricalcium phosphate; BCP 20/80, biphasic calcium phosphate 20% hydroxyapatite and 80% ß-tricalcium phosphate; ß-TCP<0.7mm, ß-tricalcium phosphate with particle size of 0.5-0.7 mm; ß-TCP>0.7mm, ß-tricalcium phosphate with particle size of 0.7-1.4 mm; ND, not detected. BCP, biphasic calcium phosphate; ß-TCP; ß-tricalcium phosphate.

To resume, ASC seeded on a bicalcium phosphate 60/40 after BMP-2 stimulation showed higher ALP activity at 21 days of culture. BMP-2 treatment seemed to upregulate CBFA-1, Col1, ON, OPN, and OCN mRNA gene expression in ASC seeded on this biomaterial (Fig. 44A-F, black bars). Therefore BMP-2 seemed to affect ASC osteogenic differentiation dependent on the carrier.

# Chapter 7 DISCUSSION

**Buccal fat pad, a rich source of adipose stem cells**

Buccal fat pad is a mass of encapsulated adipose tissue that has shown a high plasticity, being able to reconstruct orofacial defects as well as gingival defects in maxillofacial surgery. Therefore we aimed to study the biologic characteristics of this tissue to explain its excellent properties. Adult stem cells are present in various organs and develop important functions in tissue maintenance and homeostasis.[356] In our study we aimed to assess the presence of ASC in BFP using flow cytometry, to compare the number of ASC present in BFP with that in adipose tissue obtained from another fat depot (subcutaneous abdominal adipose tissue), to compare the cell marker expression in BFP with that in subcutaneous abdominal adipose tissue, and to determine the multipotent potential of ASC from BFP *in vitro.* One major goal of tissue engineering medicine is to find a source that can provide an adequate number of stem cells for clinical application which implies minimal morbidity. Adipose tissue holds great promise in regenerative medicine, i.e. it is available in large quantities as waste material, and it contains more progenitor cells giving rise to different cell populations than bone marrow.[357] Unlike subcutaneous fat, BFP is a specialized mass of adipose tissue considered an ideal flap for oral surgery, since it is easy to harvest and reliable, it contains a rich blood supply, and its harvesting causes minimal donor site morbidity and low complication.[352,358] The size of BFP appears to be similar among different persons and independent of their body weight and fat distribution.[337] Patients with little subcutaneous fat have BFP with normal weight and volume. The results of our study are in agreement with these published results, showing that the size of BFP is not related to a person's general adiposity.

Our results showed that BFP contains a high proportion of cells with ASC characteristics, and can be easily isolated and expanded *in vitro*. Approximately 30% of SVF from BFP express characteristic markers of fresh ASC, i.e. CD90, CD73, CD29, and CD34.[63,305] The percentage of fresh ASC from BFP obtained in our study (~30%) is slightly higher than that obtained from abdominal subcutaneous adipose tissue (~22%).[359] In addition, the occurrence of MSC in bone marrow tissue is <0.01%,[10] which makes BFP an excellent source of fresh ASC, avoiding the need for *in vitro* cell expansion to obtain a high enough cell number for bone tissue engineering.

**Phenotypical differences between fresh ASC and passaged ASC from buccal fat pad**

CD34 expression is characteristic of fresh ASC,[70] and its expression decreases with passaging

in ASC. The expression of CD34, a molecule generally recognized as a marker of hematopoietic stem cells, as well as microvascular endothelial cells and fibroblast like dendritic cells, in these cells contrasts with MSC from bone marrow, which lack expression of this marker (Table 12).[69]

| | CD73 | CD90 | CD105 | CD45 | CD34 | CD19 | CD14 | HLA-DR |
|---|---|---|---|---|---|---|---|---|
| **MSC** | + | + | + | - | - | - | - | - |
| **ASC (BFP)** | + | + | + | - | ++- | - | - | - |
| **ASC (SC)** | + | + | + | - | +- | - | - | - |
| **Fresh ASC (BFP)** | + | + | - | - | + | - | - | - |
| **Fresh ASC (SC)** | + | + | - | - | + | - | - | - |
| **HSC** | + | + | + | - | + | - | - | - |
| **M/MØ** | - | - | +- | + | - | - | + | + |
| **B cell** | + | - | - | + | - | + | - | + |
| **T cell** | + | - | - | + | - | - | - | + |
| **EC** | + | + | + | - | + | - | - | - |
| **Fibroblasts** | - | + | - | - | +- | - | - | - |
| **HC** | - | - | - | + | - | - | - | - |
| **Epithelial cells** | + | - | - | - | - | - | - | - |

**Table 12: Surface marker expression of different cell populations.** MSC, mesenchymal stem cells; ASC (BFP); adipose stem cells from buccal fat pad; ASC (SC); adipose stem cells from subcutaneous abdominal adipose tissue; HSC, hematopoietic stem cells; M, monocytes; MØ, macrophages; EC, endothelial cells; HC, hematopoietic cells; +-, surface marker expression <5%; ++-, surface marker expression 10-15%.

CD34+ cells are capable of stimulating angiogenesis, and they are involved in neovascularization processes that facilitate healing of ischemic tissues.[65,66] The *in vitro* expansion of cells from SVF causes changes in the phenotype and characteristics of these cells such as loss of CD34 expression.[72] We hypothesize that CD34 expression might be related to differences in cell properties and differentiation potential. Freshly isolated ASC from BFP lack expression of CD105, but expression of this marker increases rapidly after seeding, as has been described by others using ASC from other fat depots.[68,360] ASC also lack expression of CD146, a characteristic marker of endothelial cells as well as vascular smooth muscle cells.[63] In our study we found a small population of CD146+ cells in the first passages (up to P2) of ASC from BFP. The presence of this CD146+ contaminating population, as well as the presence of CD34+ cells, might be due to the highly enriched blood vessel supply in BFP.[197] This could be related to the excellent wound healing properties of BFP as a pedicled graft in oral surgery for treatment of oroantral communications,[62,348] maxillary defects,[205] oral submucous fibrosis,[361] and vocal cord defects.[351] Expression of CD146 may be correlated

with a higher osteogenic potential, as the osteogenic potential of CD146 expressing BM MSC is high compared with placenta-derived MSC which do not express CD146. Expression of pericyte markers by ASC from BFP may also contribute to increases in microvascular density.[362] All this makes them good candidates for clinical applications. ASC were negative to characteristic hematopoietic markers such as CD45, CD19, CD14 and HLADR. The low expression of HLADR in passaged ASC (lower than 1%) makes them suitable for autologous and allogenic transplantation.[363] HLADR corresponds to one of the antigens of HLA class II involved in transplantation immune responses and its low expression makes ASC an interesting tool for *in vivo* applications without the need of immunosuppressive drug intake.[364] ASC have been successfully used in humans for the treatment of severe refractory acute graft-versus-host disease without side effects.[101]

**Phenotypical differences between ASC from buccal fat pad and ASC from subcutaneous abdominal adipose tissue**

It was expected that differences in the site of origin of the adipose tissue is related to different ratios of cell types such as epithelial progenitor cells, fibroblasts, pericytes, myoblasts, etc. We evaluated differences between subcutaneous abdominal adipose tissue and BFP, a mass of adipose tissue considered a marker of visceral fat.[60] We observed differences between BFP and subcutaneous adipose tissue regarding CD34 expression. This difference might be attributed to the higher blood supply of visceral tissue, which might contribute to the heterogeneity of the cell population, that in turn might increase the osteogenic potential.[293] The CD34+ cells obtained from dental germ pulp also have the ability to differentiate to pre-osteoblasts, form bone nodules after implantation in mice, and show angiogenic potential.[365] We found higher CD34 expression in passaged ASC from BFP compared with passaged ASC from subcutaneous adipose tissue, which could make them more suitable for bone engineering therapies.

**Cell yield differences between ASC from buccal fat pad and ASC from subcutaneous abdominal adipose tissue**

After one week of culture, we observed that the number of ASC derived from BFP was 2 fold higher than the number of ASCderived from SC, which suggests that BFP is a better source of mesenchymal stem cells.On the other hand, this difference might be due to the differences in

age of the patients.[83,366] In the two groups we studied, the average age of the group donating BFP was 19.6 years (range 18-22 years), and that of the group donating SC was 43.3 years (range 19-57 years). This shows that the mean age was two-fold higher in the group of SC. This two-fold difference in the patient age might have an effect on the different cell number found. However, the only young patient of the group of SC aged 19 years old had the lowest number of ASC. Our results are in agreement with other studies that did not find differences between age and cell proliferation, but did find significantly differences between different adipose tissue depots.[74,296] These differences might also be attributed to the intrinsic characteristics of the patients or to other particular properties of the adipose source. Other studies have also shown differences between subcutaneous and visceral adipose tissue depots, showing a higher proliferation rate but a lower differentiation capacity in ASC from subcutaneous fat, especially regarding the osteogenic differentiation, as compared to the stem cells isolated from visceral fat tissue.[291,293] This could be due to the different histologic characteristics of adipose tissues due to different anatomical sites; visceral adipose tissue is containing more blood vessels and less fibrous encapsulation, in comparison with subcutaneous adipose, which might contribute to different populations of osteoprogenitor and therefore different osteogenic potential. It has also been observed that the presence of more fibrous tissue in the subcutaneous adipose tissue may result in more populations of fibroblasts within adipose-derived stromal cells than in visceral adipose, and this mixture may decrease the osteogenic differentiation potentials of the adult stem cells. This probably could also explain why subcutaneous ASC show lower osteogenic differentiation potential than that of cells from visceral tissue, which show less presence of fibroblastic cells.[294] This indicates that the population of heterogeneous cells critically influences the differentiation functions of the osteoprogenitor cells, regulating their indifferentiated state and inducing them to differentiate, like occurs in a niche.

Differentiation and proliferation of stem cells might be stimulated by the presence of other cell populations, such as osteoblasts and mesenchymal stem cells which promote hematopoietic stem cell proliferation and differentiation, as also occurs in the niche *in vivo*, where hematopoietic stem cell proliferation and differentiation is tightly controlled by physical contact with mesenchymal stem cells and osteoblasts, together with various growth factors and other factors such as angiopoietin, Ca+2 ions, or CXCL12. Besides the presence of these cells, perivascular cells could also play an important role in proliferation of

HSC.[175,176] We observed a higher cell number in ASC from BFP than in ASC from subcutaneous abdominal adipose tissue at one week of culture, which could be due to a higher cell number present in this tissue or to the presence of other cell populations such as CD146+ cells. CD146 is a marker characteristic of endothelial progenitor cells,[63] which might stimulate ASC cell proliferation and increase the differentiation potential of ASC. We did not observe CD146+ cells in subcutaneous abdominal adipose tissue after flow cytometry analysis as observed in other studies,[367] showing that different cell sources may have different cell populations. This could have also an effect on cell differentiation. Other studies have shown significant differences when comparing the chondrogenic differentiation potential of ASC isolated from fibrous synovium, adipose synovium, and subcutaneous fat, showing ASC from fibrous and fat synovium higher chondrogenic and osteogenic differentiation potential than from subcutaneous fat tissue.[295] Differences in the ASC population are found even when the cells are isolated from two different anatomical regions of the same type of adipose tissue; ASC from subcutaneous fat tissue obtained from the hip show higher osteogenic potential than ASC harvested from the abdomen.[296]

**Multipotent differentiation potential of ASC from BFP**

When ASC from BFP were cultured in AM for two weeks, more than 90% of the cells accumulated lipid vacuoles and expressed the characteristic adipogenic gene PPARγ, which is considered a master regulator of adipogenic differentiation.[368] However, we observed constitutive expression of this adipocyte gene in non-induced ASC, similar as in other studies.[369] Osteogenesis occurred in the presence of OM within one week as shown by increased ALP activity. 1,25-Dihydroxyvitamin $D_3$ plays an important role in bone formation and maturation by increasing $Ca^{2+}$ concentration at the mineralization site. It promotes early differentiation of progenitor cells to osteoblastic commitment. We, as well as others,[370] found that the combination of 1,25-dihydroxyvitamin $D_3$ with ascorbate-2-phosphate and β-glycerophosphate induces matrix calcification as shown by Alizarin Red staining and osteocalcin expression. With use of dexamethasone, a different effect has been observed in a dose dependent manner in ASC,[244] using a lower concentration of dexamethasone has been shown to commite cells to osteoblast lineage, on contrast with higher concentration of dexamethasone, that induces the cells to the adipogenic lineage.[245] Osteocalcin is an osteogenic marker indicative of the final mature stages of osteoblastic differentiation and is expressed when mineralization begins.[123] In addition to the increased ALP activity and matrix

calcification, the expression of the osteogenic genes CBFA1 and osteonectin confirmed osteogenic differentiation of ASC from BFP. No increase in PPARγ was observed during culture. After exposing ASC to CM for 4 weeks, we observed Toluidine Blue-stained nodules indicative of a proteoglycan matrix characteristic for cartilage. Expression of collagen II, a marker believed to be specific for articular cartilage, was observed by immunohistochemistry in differentiated ASC. Increased expression of the master chondrogenic factor SOX9 was observed in ASC from BFP at 14 days of culture, followed by decreased expression of the adipogenic marker PPARγ. ASC could represent a valuable tool for cartilage repair that might overcome the limited intrinsic repair capacity of this avascular connective tissue, which most orthopaedic patients complain about. Flow cytometry analysis of marker expression in ASC before differentiation showed low expression of hematopoietic markers CD45, CD19, CD14 and HLADR, as well as low expression of endothelial markers CD146 and SSEA, which demonstrates that cross-contamination with hematopoietic or endothelial cells did not occur.[371,372]

Our results did not show a decrease in the adipocyte gene PPARγ until day 21 in ASC from BFP induced to differentiate to adipocytes. On the contrary, PPARγ gene expression was downregulated as soon as 5 days after chondrogenic differentiation in ASC from BFP. It has been demonstrated that fat amb bone are related, as observed by the increase of adipocytes in bone marrow, accompanied by decrease in bone mass in bone diseases such as osteoporosis. Bone marrow contains adipocytes and trabecular bone cells that can also express the adipose phenotype, showing the hability to transdifferentiate of these cells, which is an important mechanism in the pathogenesis of osteoporosis.[116] Hormones, glucocorticoids such as dexamethasone, and growth factors play an important role in modulating the differentiation process of the progenitor cells. In our study, we used high doses of dexamethasone and insulin to induce adipogenic differentiation. The deficiency of the hormon leptin is associated with reduced linear growth, reduced cortical bone mass and reduced trabecular bone in the femora, where huge adipocytes occupy much of the marrow space.[249] This hormone is produced by fat cells to regulate bone mass through proliferation and differentiation of osteoblasts and chondrocytes. All this shows the inverse relationship between adipocytes and osteoblasts and their importance in the rol of bone formation.

We observed that osteogenic and chondrogenic differentiation was accompanyed by a decrease in PPARγ gene expression, which confirmed the commitment of the cells to the osteogenic and chondrogenic lineage, and the inverse relationship between the adipogenic and the osteogenic and chondrogenic lineage.

The ideal source of stem cells for tissue engineering would be one that can be easily harvested, and contains a high number of expandable stem cells.[373] This study shows that SVF isolated from BFP contains ~30% ASC with high expression of the angiogenic marker CD34, and therefore might be an interesting source for bone tissue engineering where blood vessel formation is crucial. Besides this, adipose stem cells secrete a variety of angiogenic growth factors, such as hepatocyte growth factor, vascular endothelial growth factor, and platelet derived growth factor,[248,255] that contribute to their regenerative capabilities and angiogenic potential, making them interesting candidates for bone tissue engineering, where formation of blood vessels to supply oxygen and nutrients to the cells is essential.[135] Under appropriate conditions, ASC from BFP differentiate to chondrocytes, osteoblasts or adipocytes, suggesting that BFP can be a rich alternative source of stem cells, readily available for clinical application in the field of tissue engineering for the repair of bone and cartilage defects.

**Use of polyamine spermine and growth factors such as BMPs to stimulate the osteogenic differentiation of ASC**

Besides the appropriate source of stem cells, the use of the adequate type of scaffold, with adequate pore size, porosity and composition, which resorbs in a gradual manner allowing simultaneous replacement by new tissue, and osteoinductive growth factors that stimulate cell differentiation are crucial for bone tissue engineering. It is important what agent to use, which dose (the volume delivery), which site (focal delivery) and which delivery matrix. Besides this, it is important to chose the adequate type of delivery, i.e. sustained or bolus release. It has been shown previously that spermine, a polyamine coumpound, is involved in bone and cartilage development. The polyamine spermine has been implicated in the response of ASC to mechanical loading and a short incubation of 30min is enough to induce osteogenic differentiation of freshly isolated ASC.[374] However, the FDA has not yet approved the use of spermine or other polyamines for clinical application.[375] Therefore we aimed in the second study to use the growth factor BMP-2 instead of spermine as an osteoinductive factor, to improve the osteogenic differentiation of ASC. Bone growth factors such as BMP-2 are

available as FDA approved recombinant human proteins and may be used for immediate clinical applications, in combination with stem cells and scaffolds, to accelerate bone healing and to create new bone.[50,51,236,376] There are many studies showing that BMP-2 induces bone formation *in vitro* and i*n vivo*. In the study of Miyazaki (2008) progenitor cells overexpressing BMPs promoted more bone formation that bone precursor cells no overexpressing BMPs, producing more ALP and calcified extracellular matrix stained by von Kossa staining.[54] ASC combined with BMP-2 induced abundant posterolateral spinal fusion with evidence of bone formation at 4 weeks. In another case study, surgical implantation of recombinant human bone morphogenetic protein-2 (rhBMP-2) was applied combined with 85% tricalcium phosphate and 15% hydroxyapatite for reconstruction after complete surgical resection of an active juvenile ossifying fibroma in a 9 year old boy.[57] Implantation of rhBMP-2 within the resulting periosteal chamber induces migration of these stem cells into the implant, with subsequent proliferation and differentiation into osteoblasts. This mechanism known as osteoinduction, is followed by an intense vascular ingrowth from the surrounding soft tissues. The initial postoperative 3D CT scan at 6 weeks showed bone filling in the defect from edge to edge, with stabilization of the regenerated bone volume after 24 weeks shown by 3D CT scan. A short treatment of 10 ng/ml of BMP-2 has also been shown to induce an osteogenic phenotype. In other studies,[377] fifteen minutes of BMP-2 treatment significantly up-regulated ALP activity and CBFA1 gene expression by 1.8-fold at 4 days of culture. On the contrary, four days of continuous treatment with either 10 ng/ml BMP-2 or BMP-7 did not affect CBFA1 gene expression; therefore a stimulation of 15min was showed to be enough to promote an osteogenic phenotype in ASC. That is in line with results found in our study, where we also observed an increase in CBFA1 and ALP gene expression after BMP-2 treatment, together with an increase in Col1 and ON gene expression. The increase in CBFA1, ALP and ON after seeding ASCs on BCP 60/40 was similar or higher than after seeding the cells on tissue culture plastic. An increase in OPN and OCN gene expression was observed as well. OPN and OCN are mature markers of osteoblast differentiation, showing the terminal differentiation of ASC seeded on BCP 60/40, while expression of these late markers was not observed in ASC seeded on tissue culture plastic after 14 days of culture in osteogenic medium, suggesting that seeding BMP-2 treated ASC on BCP 60/40 may accelerate the osteogenic differentiation of ASC. The results obtained in our study correlate with the results known from literature, showing a higher osteogenic differentiation after applying BMP-2 in different manners on osteoprogenitor cells[52-55,59,378-382] and/or

osteoconductive scaffolds[50-55,379,380,381] (Table 13 and table 14). In a study in rats comparing BMP-2 overexpressing ASC cells (I), ASC alone (II), collagen-ceramic carrier alone (III) and collagen-ceramic carrier containing 20 μg/ml BMP-2 (IV), better bone healing was observed in groups I and IV by histological and histomorphometrical analysis, with similar results between both groups. This shows that BMP-2-producing human adipose-derived mesenchymal stem cells seeded in a collagen–ceramic carrier can heal critically sized femoral defects in athymic rats.[52] In another study comparing PLGA/HA scaffolds alone (I), BMP-2-loaded scaffolds (II), scaffolds with undifferentiated ASC (III), and BMP-2-loaded scaffolds with undifferentiated ASC (IV) demonstrated higher osteogenic differentiation *in vitro* and *in vivo* in group IV.[378] Results showed significantly higher calcium deposition and more bone formation in BMP-2-loaded scaffolds than in scaffolds without BMP-2; furthermore, osteogenic differentiation increased significantly after seeding ASC on these BMP-2-loaded scaffolds. Real-time PCR analysis showed expression of osteocalcin, terminal marker of osteoblast differentiation, in group IV, which was not present when the scaffolds were not incubated with BMP-2 (Table 13). This correlated with our findings, where we observed osteocalcin gene expression in BMP-2 treated ASC seeded on BCP 60/40, and lack of osteocalcin gene expression on non-treated ASC seeded on these calcium phosphate scaffolds.

It has been shown that a short incubation of only 15-30 min with BMP-2 could enhance the osteogenic differentiation of ASC and osteoblasts *in vitro,*[49,59] and increase the bone forming ability of scaffolds *in vivo.*[50,51,379] In our study, we tested the effect of this short incubation on ASC seeded on different calcium phosphate scaffolds in a lower concentration. We observed a higher osteogenic response of BMP-2 treated ASC seeded on BCP 60/40, higher than in BMP-2 treated ASC seeded on tissue culture plates, similarly to another study.[380] This study reported that medium supplementation with BMP-2 at a concentration of 100 ng/ml significantly increased ALP activity, collagen, ALP, and osteocalcin gene expression on β-TCP carriers, without affecting the cell viability[380] (Table 14). However, we did not observe an increase after seeding these cells on β-TCP carriers in our study. The porosity of the scaffolds is similar between the studies (60% our study vs. 75±10% Ling-Ling et al), so we believe that the different incubation periods between the studies (15 min our study vs. 14d Ling-Ling et al), could affect the osteogenic differentiation of ASC seeded on these β-TCP scaffolds. On the contrary, an only 15min incubation with BMP-2 seems enough to stimulate ALP activity and osteogenic gene expression on ASC seeded on the BCP 60/40, which

suggests a higher osteogenic potential of bicalcium phosphate scaffolds consisting of a mixture of 60% HA and 40% β-TCP. Other studies have also shown that medium supplementations of BMP-2 have increased the osteogenic response of ASC *in vitro*[53,381,382] and improved bone healing *in vivo.*[54,55,381]

| Reference | BMP-2 delivery | Type of study | Concentration | Carrier | Analysis | Healing time of bone defects |
|---|---|---|---|---|---|---|
| Choi SH et al.[50] | Material incubation (30min) | *In vivo* (dogs) | 0.2 mg/ml | Collagen | Hm, Rx | 8w (periodontal) |
| Smith DM et al.[51] | Material incubation (15min) | *In vivo* (rabbits) | 0.43 mg/ml | Collagen | Histology, Rx | 6w (large calvarial) |
| Dragoo JL et al.[53] | Transduction | *In vivo* (mice) | - | ASC+ collagen | Histology, Rx | 6w (hind limb) |
| Miyazaki MM et al.[54] | Transduction/ incubation | *In vivo* (rats) | -/10 µg/ml | ASC+ collagen, MSC+ collagen | Histology, Rx | 8w (spinal fusion) |
| Mesimaki K et al.[55] | Material incubation (48h) | Clinical study | 12 mg/ml | ASC+β-TCP | Rx | 8w (maxillar) |
| He D, Genecov et al.[379] | Material incubation (15min-2h) | *In vivo* (dogs) | 0.11 mg/ml, 0.21 mg/ml, 0.43 mg/ml | Collagen+ BCP | Histology, Hm | 16w (large size cranial) |
| Peterson B et al.[52] | Transduction | *In vivo* (rats) | - | ASC+ collagen-ceramic | Histology, Rx, Hm, Bm | 8w (critical sized femoral) |
| Jeon O et al.[378] | Material incubation (1h) | *In vivo* (mice) | 1 µg/ml | ASC+PLGA/HA | Histology, RT-PCR | 8w (dorsal) |
| Ling-Ling MD et al.[380] | Medium supplemented (4, 7, and 28d) | *In vivo* (mice) | 100 ng/ml | ASC+β-TCP | Hm, histology | 8w (dorsal) |
| Cowan CM et al.[381] | Medium supplemented (4w) | *In vitro*, *In vivo* (mice) | 200 ng/ml | ASC, MSC+ PLGA | Histology, Rx | 2w (critical size calvarial) |

**Table 13: Characteristics of *in vivo* study models using BMP-2 for osteogenic differentiation.** Rx, radiographic; Hm, histomorphometry; Bm, Biomechanical; RT-PCR, real-time PCR.

| Reference | BMP-2 delivery | Type of study | Concentration | Carrier | Analysis |
|---|---|---|---|---|---|
| Emes Y et al.[49] | Medium supplemented (24-48h) | *In vitro* | 1-1000 ng/ml | Osteoblasts | ALP, $Ca^{+2}$ |
| Dragoo JL et al.[53] | Transduction/ medium supplemented (4 and 7d) | *In vitro* | -/1 µg/ml<br>- | ASC, MSC | ALP, RT-PCR, WB, histology |
| Mesimaki K et al.[55] | Material incubation (48h) | *In vitro*, *in vivo* (clinical study) | 12 mg/ml | ASC+β-TCP | ALP, histology, RT-PCR |
| Knippenberg M et al.[59] | Cell incubation (15min) | *In vitro* | 10 ng/ml | Fresh ASC | ALP, RT-PCR |
| Maegawa N et al.[382] | Medium supplemented (12d) | *In vitro* | 100 ng/ml | MSC | ALP, RT-PCR, histology |
| Jeon O et al.[378] | Material incubation (1h) | *In vitro* *in vivo* (mice) | 1 µg/ml | ASC+PLGA/HA | ALP, $Ca^{+2}$, RT-PCR, histology |
| Ling-Ling MD et al.[380] | Medium supplemented (4, 7, and 28d) | *In vitro, in vivo* (mice) | 100 ng/ml | ASC+ β-TCP | ALP, $Ca^{+2}$, RT-PCR, histology, SEM |
| Cowan CM et al.[381] | Medium supplemented (4w) | *In vitro*, *In vivo* (mice) | 200 ng/ml | ASC, MSC+ PLGA | Histology |
| Our study | Cell incubation of 15min | *In vitro* | 10 ng/ml | Fresh ASC+β-TCP, fresh ASC +BCP | ALP, RT-PCR |

**Table 14: Characteristics of *in vitro* study models using BMP-2 for osteogenic differentiation.** ALP, alkaline phosphatase activity; RT-PCR, real-time PCR; WB, Western Blot; SEM, scanning electron microscope.

**Advantages to use a short stimulation of only 15min with BMP-2 versus other methods to apply BMP-2**

After having observed that a short stimulation of only 15min with the growth factor BMP-2 triggers adipose stem cells into osteogenic differentiation, we speculated that ASC triggered for 15min with BMP-2 before seeding onto a calcium phosphate carrier in a one-step surgical procedure could stimulate bone regeneration after maxillary sinus elevation. The objective of the second study was therefore to investigate whether a short incubation of 15min with BMP-2 affects the attachment, proliferation and differentiation profiles of ASC seeded on biphasic calcium phosphate and ß-tricalcium phosphate, most commonly used as allogenic bone grafts in clinical applications. The low dose of BMP-2 used in our study avoids the concerns of bone overgrowth which implies risks at the site of application, caused by the high dosis of growth factor used in clinical studies of 0.9–2 mg/ml carrier,[383,384] as well as the high costs of using these concentrations.[385] Moreover, there are concerns regarding local inflammatory effects. An *ex vivo* incubation with growth factors at a million-fold lower concentration for only 15min could easily be applied in the operating room for immediate clinical applications of the progenitor cells. Autologous stem cells could be isolated, triggered with growth factors, and subsequently used for bone or cartilage regeneration in the patient in the same operation.

There are several methods to apply BMP-2 to cells. One method to apply BMP-2 is by cell transfection.[386,387] However, BMP-2 transfected cells present some disadvantages. The vectors transfected cannot integrate into the host genome, and protein production by the transfected cells is thus limited to 3 weeks in an immunocompromised animal model. Furthermore, adenoviral vectors usually retain their ability to synthesize adenoviral proteins, which stimulate the host immune response.[388] Host immunity destroys the transduced cells and reduces the effect of transgene expression. Modifying MSC genetically enhances osteogenesis and spinal fusion by both paracrine and autocrine responses. However, the safety of viral vectors and the side effects of prolonged BMP-2 production need to be elucidated before adopting this technology clinically.[54] Other approaches are the addition of BMP-2 to culture media[382] or make inductive scaffolds that are able to release BMP-2 in a sustained manner.[378,380] We aimed to develope an alternative to retroviral therapies and what are called "intelligent scaffolds", allowing induction of the cells to differentiate into the osteogenic lineage, and which can be applied to the patient avoiding cell culture, which is time consuming, expensive, and carries risks of cell contamination and cell loss.

**Increase in cell proliferation after BMP-2 treatment**

The frequency of CFU-F was significantly higher after BMP-2 treatment. This is in agreement with other studies, showing that the treatment with doses ranging from 10 ng/ml to 0.1 mg/ml of human recombinant BMP-2 promotes the proliferation of MSC *in vivo* and *in vitro* and increases the levels of hematopoietic cytokines in MSC, which may contribute to the improvement of hematopoietic function and bone formation.[389] A MSC cell line transfected with BMP-2 have also shown increased proliferation.[390] BMP-2 also significantly increases mRNA expression and protein levels of interleukins, i.e. IL-6, IL-7, IL-11, and granulocyte colony-stimulating factor, monocyte colony-stimulating factor, and stem cell factor, which contribute to bone forming and bone remodeling by increasing stem cell proliferation.[236,381,391] We observed an increase in ASC proliferation after BMP-2 treatment as well. This is in contrast with another study with bone marrow MSC, which did not show increased proliferation after treatment with recombinant BMP-2, but showed that BMP-2 treatment did affect endothelial progenitor cells.[392] This suggests that different cell populations and/or different sources of mesenchymal stem cells may respond differently to this growth factor. Although adipose stem cells are mesenchymal stem cells, as bone marrow MSC, they differ in expression of stem cell markers such as CD54, expressed in higher levels in ASC, CD49d and CD34, which are only expressed in ASC, and in CD106, a marker not detected in ASC but expressed in BM MSC. These differences in stem cell marker expression could cause the different response of ASC and BM MSC to BMP-2 treatment.[306]

**Advantages to use the SVF from adipose tissue and clinical implications**

Most patients have an adequate supply of adipose tissue that can be safely be made available by liposuction. Therefore adipose tissue may be the ideal source of MSC for tissue engineering therapies, containing a high number of adipose stem cells readily available for immediate applications.[371]

Adipose tissue, after collagenase digestion and centrifugation, is separated into two fractions: one fraction composed of floating adipocytes and lipid droplets, and another fraction containing the stromal vascular fraction, a heterogeneous cell population containing fibroblasts, circulating blood cells, pericytes endothelial progenitor cells, pluripotent vascular progenitor cells, and adipose tissue-derived stem cells,[27] with potential for osteochondral tissue engineering.[254,300] For clinical applications it would be advantageous to use the whole

SVF for transplantation, thereby avoiding *in vitro* selection and/or expansion steps, which are time consuming, costly, and carry the risk of contamination. Additionally, the harvesting of human fat by liposuction is an easy and safe procedure, unlike the harvesting of human bone marrow which is associated with donor site morbidity.[21] Most studies have been performed on cultured adipose-derived stem cells,[68,393] but before being used clinically in patients, they must overcome prohibitively expensive good manufacturing practice production facilities, and FDA approval in the USA, and European Agency for the Evaluation of Medicinal Products approval for Europe is required before use, which is time and money consuming.[325] These limitations hurdle to envisage the use of cultured stem cells for clinical use. However, using uncultured adipose stem cells in the form of the stromal vascular fraction would overtake these restrictions.[371,394]

Because the SVF is composed of freshly isolated uncultured cells, it may be safely applied clinically without any ethical restriction, and is a rich source of stem cells, containing one stem cell per $10^3$ nucleated cells, which makes it an interesting alternative to bone marrow–derived stem cells.[24] Furthermore, the SVF has been shown earlier to promote bone regeneration *in vivo*.[323,395]

Interestingly, MSC in SVF act coordinated with blood cells such as endothelial cells and hematopoietic cells, regulating their undifferentiated state, like occurs in a perivascular niche.[396] These cells secrete factors that increase proliferation of progenitor cells, such as adipose stem cells, and enhance the osteogenic potencial of ASC.[397]

Use of freshly isolated ASC could have immediate clinical applications, avoiding the need of expansion, without losing their properties. Vasculogenesis and osteogenesis may be improved by a synergic action of MSC with hematopoietic stem cells present in this fresh SVF, by increasing vascular number and diameter and ectopic mineralization when co-transplanted on calcium phosphate scaffolds. Angiogenesis is the common roadblock in tissue regeneration, and is critical for bone tissue engineering.[64] In previous studies we observed that fresh ASC show a higher expression of CD34, and culture of ASC leads to a downregulation of CD34 expression while CD34 expression is totally lost after several passages of culture. The marker CD34, which is also expressed in hematopoietic and endothelial stem cells, is related with angiogenesis and could have implications in bone tissue engineering. The heterogeneous

population of SVF from BFP, containing ASC besides endothelial progenitor cells and hematopoietic stem cells as shown in our study by flow cytometry, might be applied successfully to improve defects caused by trauma or disease *in vivo.* It has been observed that the SVF from adipose tissue is able to improve left ventricular function *in vivo* once implanted in hearts with impaired cardiac function,[319] and to induce new bone formation in rat calvarial defects.[323] In this study in rat calvarial defects, the fact that SVF alone induces more new bone formation than SVF implanted mixed with a copolymer P(L/DL)LA could be due to the induction of inflammatory or foreign body reaction by these polymer scaffolds, that may have an inhibitory effect on bone regeneration by stem cells. In this study, the use of a demineralized bone matrix in combination with SVF showed increased bone formation than using SVF combined with a copolymer P(L/DL)LA, indicating that the choice of the scaffold is a critical issue for bone tissue engineering.

**Scaffolds in bone tissue engineering**

It is well known that scaffolds, growth factors, and mesenchymal stem cells are the three major factors of tissue engineering. The scaffold is another key element, and should be effective in fast bone regeneration, resulting in high quality bone. An ideal scaffold should be three dimensional and highly porous, with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste, as well as biocompatible and bioresorbable with a controllable degradation or resorption rate to match cell/tissue growth *in vitro* and *in vivo*. It also should have a suitable surface chemistry and composition for cell attachment proliferation and differentiation, and be capable of osteoinduction and osteoconduction.[82,202] BMP-2 treated ASC would provide to the scaffold osteogenicity, osteoinductivity, and osteoconductivity, and therefore would be ideal physiologically to promote new bone formation.[203] Degradation and absorption of the scaffold are crucial in functional tissue engineering. Ideally, the rate of scaffold degradation should mirror the rate of tissue formation, and the resulting products of degradation should be non-toxic, as the scaffold itself. Absorbable scaffolds used in tissue engineering are divided in natural polymers, synthetic polymers, inorganic materials containing Ca/P (ceramics), and composite materials. Composite materials combine inorganic and organic materials, combining the mechanical properties and osteoconductivity of calcium phosphates with bioabsorbable polymers.[82]

Ceramics have been widely used in the biomedical engineering and bone substitution/regeneration field. They can be from natural (e.g., bovine-derived hydroxyapatite or coralline hydroxylapatite) origin or synthetic origin, such as synthetic hydroxyapatite or ß-TCP.[205] (Table 15). Due to their osteoconductive properties, they are promising scaffolds for bone tissue engineering applications, and support attachment, proliferation and promote differentiation of osteoprogenitor cells.[205,206,207] However, these materials have some major drawbacks such as brittleness and low mechanical stability, which prevent their use in the regeneration of large bone defects.[208,209] Furthermore, due to factors *in vivo* such as osteoclastic activity, their degradation/dissolution rates are difficult to predict. This could be a problem because if it degrades too fast it will compromise the mechanical stability of the construct, which is low by itself. As an alternative to these materials, there are biodegradable polymers. However, polymers present some drawbacks such as low degradability *in vivo*,[215] low mechanical properties,[220,222] and harmful degradation products and hydrophobicity of the material,[212,217] which can cause an inmflammatory response in the organism and aborted proteolytic attack by macrophages leading to encapsulation.[224,226] Advantages of using ceramics such as β-TCP is that supports cell in-growth and promotes the osteogenic differentiation of osteoprogenitor cells.[42,380] However, unlike HA, β-TCP reabsorbs rapidly, but this does not occur in a 1:1 ratio, and often is less bone produced as compared with the volume of β-TCP reabsorbed.[41,398] Biodegradation of β-TCP can occur either by the activity of the surrounding osteoclats or by chemical dissolution by tissue fluids.[399] Combining the bioactive properties of HA with the good bioresorbability of β-TCP may solve the above-mentioned problems.

| Scaffold | Type | Advantages | Disadvantages |
|---|---|---|---|
| **Hydroxyapatite (HA)** | Synthetic ceramics | Support attachment, proliferation and differentiation of MSC. It is gradually degraded through cellular phago-cytosis and extracellular dissolution processes.[205,206,207] | Poor resorption properties.[207,208] |
| **ß-Tricalcium phosphate (ß-TCP)** | Synthetic ceramics | Support attachment, proliferation and differentiation of MSC.[205,286,380,401] | Brittleness and slow degradation rates. Low fracture strength makes them not suitable for load-bearing areas.[208,209] |
| **Calcium carbonate (coral)** | Natural ceramics | Matched with bone morphologically and mechanically. Integrated with host bone. [204] | Quick degradation. Brittle and low mechanical stability.[85] |
| **Polylactic acid (PLAc)** | Synthetic absorbable polymers | Support proliferation and differentiation of osteoprecursor cells.[210,211] | Low mechanical properties. Hydrophobic surface, difficults cell attachment.[212] |
| **Polyglycolic acid (PGA)** | Synthetic absorbable polymers | Support cell proliferation and osteogenic differentiation[211,20,221] | Quick absorption of the scaffold in vivo.[228] |
| **Poly(ε-caprolactone) (PCL)** | Synthetic absorbable polymers | Good biocompatibility and processability.[213,214] | High hydrophobicity and low degradability in vivo.[215] |
| **Polylactic-coglycolide copolymers (PLGA)** | Synthetic absorbable polymers | Higher cellular adhesion than PLA surfaces. Support proliferation and differentiation of osteoprecursor cells.[216] | Low mechanical properties, Harmful degradation products and hydrophobicity.[217] |
| **Collagen (bovine or mineralized)** | Natural biodegradable polymers | Support cell growth, proliferation and differentiation.[218] Type I collagen is the major organic component of bone matrices and a major regulator of cell adhesion and osteogenic differentiation.[219] | Poor mechanical strength,necessary to add PGA fibers to increase resistance to compression and attachment.[220,221] |
| **Bioactive glass (silica-based surface)** | Glass-ceramic | Promotes growth and differentiation of osteoprogenitor cells.[225] | Brittle in a larger size.[86] |
| **Hyaluronic Acid** | Natural biodegradable polymers | Minimal immunogenicity Chemotactic when combined with appropriate agents.[82,222] | Scaffolds with low mechanical properties.[222] |
| **Silk** | Natural biodegradable polymers | Biocompatiility. Slow degradability, useful in biodegradable scaffolds in which a slow tissue ingrowth is desirable.[227] | Potential sensitization to silk fibroin resulting in an allergic response upon exposure. Aborted proteolytic attack by macrophages leading to encapsulation.[226,227] |
| **Fibrin** | Natural biodegradable polymers | Enhances angiogenesis, cell attachment, and proliferation. Promotes osteogenesis.[223] | Mild angiogenic effect combined with ASC. Monocytic infiltration observed.[224] |

**Table 15: Comparison of properties between scaffolds for bone tissue engineering.**

**Advantages to use a scaffold with a mixture of HA and β-TCP (BCP)**

Recent research has introduced the use of a biphasic calcium phosphate containing a mixture of 60% HA and 40% β-TCP that shows accelerate bone formation *in vivo*[39,400] and facilitates the proliferation and osteogenic differentiation of progenitor cells due to their high surface area and three-dimensional hierarchical porosity.[401,402] Therefore we decided to study the effect of a short BMP-2 treatment, which could fit in a one-step surgery, on ASC seeded on a biomaterial combining HA with β-TCP, and compare it with β-TCP alone, which is widely used for clinical applications as bone allograft material. We have shown that biphasic calcium phospates of 60% HA and 40% β-TCP induces significantly higher cell proliferation and osteogenic differentiation of BMP-2 treated adipose stem cells. This suggests that this scaffold could be successfully used for bone tissue engineering. HA is a major component of bones and teeth and provides strength and resistance to compression, and the roughness of the surface may improve cell attachment of osteoprogenitor cells. The composition of HA, with a high content of Ca/P, may also improve the osteogenic differentiation of these cells. Scaffolds containing HA such as BCP 60/40 combined with BMP-2 treated ASC, could be applied *in vivo* to heal critical size bone defects and to stimulate bone regeneration after maxillary sinus elevation.

**Osteogenic differentiation potential of BMP-2-treated ASC seeded on different calcium phosphate scaffolds**

The sequence of bone development consists of three phases, i.e. proliferation with matrix secretion, matrix maturation, and matrix mineralization.[393,403] Each phase is characterized by the expression of different types of osteogenic genes. The proliferation phase comprises cell growth, maturation, and the development of the extracellular matrix. Osteoblast differentiation is believed to be under the control of the transcription factor CBFA1. This transcription factor performs a dominant and nonredundant role in osteoblast differentiation and controls bone formation.[124] CBFA1 mRNA was expressed at a higher level in BMP-2 treated ASC on tissue culture plastic and on BCP 60/40, than in non-treated ASC on tissue culture plastic as well as on BCP 60/40 (Fig. 44A). Osteoblasts are derived from mesenchymal stem cells in the mesenchyme or in the marrow stroma. Once committed to the osteoblast lineage, osteoblast precursor cells proliferate and differentiate into preosteoblasts and then into mature osteoblasts. After cell growth and maturation, the extracellular matrix is formed. The principal components of this bone matrix are anorganic hydroxyapatite and

organic collagen, mainly Col1 protein; this is the matrix on which future mineralization will take place and provides the mechanical properties to bone. Col1 mRNA was expressed at a much higher level in BMP-2 treated ASC than in non-treated ASC on tissue culture plastic as well as on BCP 60/40 (Fig. 44B), suggesting that a short stimulation with BMP-2 stimulates the initiation of extracellular matrix deposition by ASC, as previously shown.[377] The cessation of cell growth leads to the matrix maturation stage or second phase of bone development, which induces expression of alkaline phosphatase and specialized bone proteins such as osteonectin and osteopontin, which render the osteoid competent for mineral deposition. The membrane-bound enzyme ALP is abundantly expressed in early bone formation, and its expression level appears to be correlated with increased bone formation.[75] ALP was expressed in ASC seeded on tissue culture plastic and on BCP 60/40 (Fig. 44C). ON is expressed in ASC on tissue culture plastic and on BCP 60/40 (Fig. 44D). OPN is known to be an early bone marker with a bimodal expression pattern, with an early expression during the proliferative phase and a later expression after the initial mineralization of the extracellular matrix.[53,404] OPN is expressed by BMP-2 treated ASC seeded on BCP 60/40 and on β-TCP<0.7mm and >0,7mm, but is not observed on tissue culture plastic (Fig. 44E), similar to another study.[378] Osteocalcin is secreted in later stages of osteoblast differentiation and led to matrix mineralization by calcium deposition, the third stage in bone development.[393] OCN expression was observed in ASC seeded on BCP 60/40 and β-TCP>0.7mm after BMP-2 induction, but was not observed in ASC on tissue culture plastic (Fig. 44F). This late bone marker is only secreted by osteoblasts[405] and also signals terminal osteoblast differentiation.[406] It is therefore used as one of the primary markers to recognize terminally differentiated osteoblasts. Finally, donwregulation of the adipocytic gene PPARγ (Fig. 45) confirms the osteogenic commitment of the cell. PPARγ is expressed early in adipocyte differentiation and its deficiency has been correlated with increased osteoblastogenesis from bone marrow progenitors, both *in vivo* and *ex vivo*.[76] All these results show that a short incubation with a low concentration of BMP-2 of 10ng/ml for 15min was enough to stimulate bone extracellular matrix deposition, matrix maturation, osteogenic terminal differentiation, and matrix mineralization of ASC seeded on BCP 60/40, as shown by increased expression of CBFA1, ALP, Col1, ON, OPN and OCN after BMP-2 treatment, and this short treatment could fit in a one-step surgery for immediate bone tissue engineering applications. These results are in agreement with other studies that have also shown higher gene expression of osteogenesis-related markers such as osteopontin, osteocalcin, and collagen type I, and

increased mineralization after BMP-2 treatment of ASC.[386] These findings demonstrate that BMP-2 can enhance osteogenic differentiation of ASC seeded on biphasic calcium phosphate carriers. BCP contains HA, with high biocompatibility and osteoconductivity, and therefore it is widely used as a bone substitute material. Scaffolds with HA can incorporate and modulate the delivery of molecular signals controlling cellular functions, promoting the proliferation and attachment of osteoprogenitor cells.[407] We have observed higher proliferation of BMP-2 treated ASC and cell attachment on scaffolds containing a 60% of HA than on β-TCP alone. HA particles have been shown to act as a controlled release carrier of growth factors and after BMP-2 treatment could provide a sustained release of the growth factor, which would be beneficial for bone tissue engineering.[408] Besides this, HA particles have shown higher bone formation than β-TCP particles once implanted with MSC *in vivo*.[205]

**Advantages of a low-dose short incubation with BMP-2**

Other alternatives to gene transfection[386] and BMP-2 delivery by scaffolds[378] are *ex vivo* incubation of the cells with the growth factor.[382] We have shown that a short incubation with a low concentration (10 ng/ml) of BMP-2 might stimulate proliferation and osteogenic differentiation on BCP scaffolds *in vitro*, and does not affect attachment of ASC on BCP or β-TCP scaffolds. Therefore this procedure could be easily fitted in a one-step surgical procedure, where the stromal vascular fraction from the patient would be obtained, processed, treated with BMP-2, seeded on the carrier and transferred to the patient, like an autologous tissue engineered bone graft. Currently clinical studies for bone tissue engineering using BMP-2 and BMP-7 on carrier materials are using high doses of the growth factors, which raises concerns for bone overgrowth and clinical problems as swelling.[383,384,409,410] This side effect has been shown to be dose-dependent.[411] Since BMP-2 is rapidly cleared, one mechanism for extended potentiation of systemic BMP-2-mediated effects is the production of anti-BMP-2 antibodies. Since these antibodies might have unknown effects, the FDA cautions the use in women for 1 year before pregnancy. An *ex vivo* short incubation with BMP-2 at a million-fold lower concentration would overcome this side dose-related effect, and could easily be fitted in a one-step procedure, in which the cells are harvested and implanted during the same surgical procedure to the patient. After the SVF has been isolated from resection or liposuction material, differentiation of the ASC down to the osteogenic lineage should be induced preferably in a short period of time (15-30min) to fit the one-step surgical procedure in clinical settings, using the cells within 2-3 hour.[2] This avoids risk of cell

contamination and expensive costs of the good manufacture practice proceedings. BMPs are a promising tool to trigger the osteogenic differentiation of ASC, which could be used to heal critical size defects in bone. Furthermore, the use of exogenous approaches avoids the problems associated with retroviral therapies or use of the "intelligent scaffolds".[43,59]

**Advantages of using scaffolds with an adequate porosity and pore size for bone tissue engineering**

Ideally, for bone tissue engineering the scaffold should be resorbed at a rate commensurate with new bone formation. Coral scaffold is reabsorbed rapidly, i.e. within weeks,[85] which makes synthetic HA and tricalcium-phosphate-hydroxyapatite interesting alternatives to this natural scaffold. For bone tissue engineering, the appropriate pore size is very important as well. In pioneering studies it has been shown that pore sizes less than 15–50 μm result in fibrovascular ingrowth, while pore sizes of 50–150 μm favour osteoid formation, and pore sizes larger than 150 μm favour mineralized bone ingrowth.[87] Hydroxyapatite scaffolds with pore size of 500 μm have shown optimal production of bone *in vivo* in comparison with scaffolds with pore size of 200 μm, which did not show any signs of bone formation.[412] Therefore we have chosen scaffolds with pore size not less than 100 μm for β-TCP, and not less than 500 μm for biphasic calcium phosphate scaffolds containing HA. Furthermore, the presence of pores favours blood vessel formation which is crucial for bone tissue engineering, and therefore we chose scaffolds with high porosity (60-90%). Angiogenesis is considered as top priority of tissue engineering and is a common and vital barrier in tissue regeneration. To regenerate a tissue over 200 μm exceeds the capacity of nutrient supply and waste removal from the tissue, and it requires an intimate supply of vascular networks. All this leads to use of angiogenic growth factors or transplantation of proangiogenic cells, such as endothelial progenitor cells, in combination with scaffolds, but this presents several inconveniences, such as cost of cytokines, potential citotoxicity and suboptimal migration in large constructs, and neovasculature fails to resemble native, multilayered mature microvessels.[413] The potential of ASC to stimulate angiogenesis holds interesting promises to the field of tissue engineering.

In our first study we validate in the hypothesis 1 the $H_{1,}$ the presence of ASC in buccal fat pad with potential to differentiate to osteoblasts, chondrocytes and adipocytes, and in the hypothesis 2 the $H_{1,}$ that buccal fat pad is a source of ASC with different characteristics than ASC from subcutaneous abdominal adipose tissue. Although the phenotypic differences

regarding CD34 marker expression have been shown stadistically significance with $p<0.05$, the differences in cell number per gram of adipose tissue are not stadistically significant ($p=0.05$). In the second study we validated in the hypothesis 1 the $H_{0,}$ that BMP-2 does not affect ASC attachment dependent on the type of calcium phosphate scaffolds, in the hypothesis 2 the $H_{1,}$ that BMP-2 affects ASC proliferation dependent on the type of calcium phosphate scaffolds, and in the hypothesis 3 the $H_{1,}$ that BMP-2 stimulates osteogenic differentiation of ASC dependent on the type of calcium phosphate scaffolds.

It is important to mention that in the first study the potency of the stadistical analysis is low. The limited size of the sample leads us to use conservative measurements for comparison of ASC yield from BFP and from SC. We observed a tendency that suggests differences in BFP regarding cell yield that could be confirmed with a higher sample size. In the second study we did not observe differences using BMP-2 regarding the type of calcium phosphate scaffolds, by measuring the amount of DNA from the seeded scaffolds, although a tendency to a higher cell adhesion is noted in BCP compared to β-TCP that could be confirmed with a higher sample size. This showed a possibly statistical significance between these scaffolds, confirmed by the indirect measuring of cell attachment in the CFU-F depletion assay, which showed a higher number of ASC non-attached in β-TCP scaffolds.

**Summary**

In summary, BFP, a mass of encapsulated adipose tissue found bimaxillary in the oral cavity, has been shown to contain a rich population of adipose stem cells besides other cell populations, such as endothelial progenitor cells and hematopoietic stem cells. These ASC found in BFP showed higher angiogenic marker expression, and ability to differentiate to osteoblasts, chondrocytes, and adipocytes. Furthermore, the number of ASC present in this tissue after one week of culture was two-fold higher than in abdominal subcutaneous adipose tissue, which makes BFP an interesting source of mesenchymal stem cells readily available for dentists and oral surgeons. A short incubation of only 15min with a $10^6$ fold dilution of the clinically used concentration of the growth factor BMP-2, i.e. 10 ng/ml, could be enough to stimulate osteogenic differentiation of ASC seeded on biphasic calcium phosphate scaffolds composed of 60% HA and 40% β-TCP. This scaffold showed an optimal attachment, proliferation, and osteogenic response of seeded BMP-2-treated ASC, and could be applied for bone regeneration after maxillary sinus elevation. The short incubation of only 15min

could easily fit in a one-step surgery, where the cells from the patient could be harvested, incubated with BMP-2, seeded on the scaffold and immediately applied to the patient in the same surgical procedure.

# Chapter 8 CONCLUSIONS

**Study 1: Buccal fat pad, an oral access-source of human adipose stem cells (ASC) with potential for osteochondral tissue engineering.**

**1)** We developed a protocol for efficient and rapid isolation of ASC from buccal fat pad that includes a minimum of 30 min of collagenase digestion at a 0.075% concentration. The reduction to a half of the concentration or their elimination affects ASC isolation.

**2)** ASC from buccal fat pad express characteristic markers of mesenchymal stem cells such as CD73, CD90 and CD105 and are negative for hematopoietic markers such as CD45, CD14, CD19 and HLA-DR, according to the minimal criteria of marker expression postuled by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Stem Cell Research in 2006.

**3)** The number of ASC obtained per gram of adipose tissue from buccal fat pad obtained at week of culture is approximately of 500 x $10^3$ cells per gram of adipose, two times higher than the number of adipose stem cells found in subcutaneous abdominal adipose tissue.

**4)** Around a 30% of cells from stromal vascular fraction showed ASC characteristics analyzed by flow cytometry. We observed a heterogeneous population of cells after analyzing the stromal vascular fraction by flow cytometry.

**5)** ASC from buccal fat pad show higher expression of the angiogenic marker CD34, which could make them interesting candidates for bone tissue engineering.

**6)** ASC from buccal fat pad are able to differentiate *in vitro* to osteogenic, chondrogenic and adipogenic lineage after the appropriate inductive stimuli.

**Study 2: Osteogenic differentiation of bone morphogenetic protein-2 (BMP-2) induced human ASC seeded on biphasic calcium phosphate and β-tricalcium phosphate scaffolds.**

**1)** BMP-2 increases ASC cell proliferation.

**2)** BMP-2 does not affect cell attachment of ASC seeded on biphasic calcium phosphate scaffolds and β-tricalcium phosphate scaffolds.

**3)** BMP-2 increases cell proliferation of ASC seeded on biphasic calcium phosphate scaffolds of 60% hydroxyapatite and 40% β-tricalcium phosphate, but does not increase cell proliferation of ASC seeded in β-tricalcium phosphate.

**4)** BMP-2 increases osteogenic differentiation of ASC seeded on biphasic calcium phosphate scaffolds of 60% hydroxyapatite and 40% β-tricalcium phosphate, but does not increase osteogenic differentiation of ASC seeded in β-tricalcium phosphate.

# Chapter 9 FUTURE DIRECTIONS

Buccal Fat Pad is an ASC rich tissue, as analyzed by Flow Cytometry analysis of the fresh SVF showing around a 30% of ASC present in this tissue, and the higher cell number of ASC observed at week of culture, in comparison with SC, which presented a lower cell number per gram of adipose tissue.

The phenotype of these cells has been widely characterized by flow cytometry, studying the expression of CD29, CD73, CD90, C105, characteristic markers of ASC, and the expression of CD14, CD19, HLA-DR, CD45, CD34, SSEA-1, CD146 and STRO-1, characteristic markers of other cell populations that could be present in this heterogeneous tissue. However, it would be interesting to study more in detail the changes of marker expression observed after plating the cells, and if they could have differences in the properties of the cells as well.

In the first study we observed that the SVF of adipose tissue contains a heterogeneous cell population. It would be interesting to delucidate if this SVF would have another clinical input than a pure fraction of ASC, because it contains other cell populations such as endothelial progenitor cells and hematopoietic cells, and this could avoid the need of co-cultures to mimic the natural niche *in vivo*. It would be interesting to use this SVF, because could be used for immediate clinical applications as one step surgery, and could avoid the need of expansion, with the time and laboratory cost that it involves.

It would be interesting also to compare different age population groups, to observe if there are differences in the cell yield regarding the senescence of induvidues. We compared two different aged groups, and would be interesting to compare similar age groups as well, to observe if the aging of individues is related to a lower stem cell number, because there is controversial regarding this issue. It would be ideal to use adipose tissue from BFP and from subcutaneous abdominal adipose tissue obtained from the same patient.

Another important aspect to study is to choose the right biomaterial to use for clinical applications. This biomaterial should be biocompatible, allowing the ASC attachment, proliferation, and osteogenic differentiation, and therefore promoting an adequate bone growth and mineralization, resembling the original tissue. This biomaterial should be degraded gradually while the new bone is forming, without inflammation and formation of fibrous tissue. This biomaterial, besides being biocompatible, should have a right pore size to

allow an optimal vascularization to the new forming tissue, which is a critical process in bone tissue engineering.

Another key issue is the need to add growth factors, such as BMP-2, that promote cell proliferation and osteogenic differentiation of these cells *in vivo*. It is important to determine the adequate dose, method of delivery and application time for tissue engineering applications. We have observed in the second study, that a short incubation of 15min with BMP-2 is enough to promote proliferation, as well as an osteogenic response, on adipose stem cells seeded on calcium phosphate scaffolds. It would be interesting to reproduce these experiments *in vivo* for future immediate clinical applications in one-step surgery. This short incubation would avoid the cell expansion in laboratories, with the high costs and time involved, and would avoid the risk of contamination of these cells. BMP-2 is commercially available and approved by the FDA as safe medical device, and BMP-2 treatment has been shown to not affect attachment to these scaffolds.

Finally, in a clinical setting quantitative image processing of three-dimensional bone structures is critical in the context of bone quality assessment. Introduced in the early 1970s, computed tomography or CT has become a widely used as imaging technique in the medical field. Several projection images of a specimen are taken under different angular positions, using X-ray radiation, which are registered using a gas or a solid-state detector. The projections are then used to reconstruct the specimen numerically on a computer, for the most part by performing conventional filtered backprojection. By this means, 3D data of the object is retrieved in a non- destructive manner. By introducing desktop scanners for micro-computed tomography (μCT).[414] Recently, μCT has become a standard for bone morphometry using new 3D methods for the morphometric quantification of trabecular bone without model assumptions, and is being used in the context of investigating different diseases and their treatment such as osteoporosis, osteoarthritis, genetics, dental research and implants, tissue engineering, and scaffoldss, and also for the validation of other techniques aimed at investigating bone microstructure in a more clinical setting.[415] It would be important in a clinical setting when using adult stem cells in combination of scaffolds, assessing the quality of new bone formed with reliable methods.

Because the mechanical properties of bone depend not only on bone density, but also on microstructure and ultrastructure, is important for the clinic to develop techniques in bone imaging that assess the density and the trabecular and cortical macrostructure, microstructure and ultrastructure of bones using fully non destructive measurement techniques, therefore avoiding the use of invasive biopsies. With histology and radiology aside, another critical parameter of success in bone regeneration studies is function, to know if the bone generated will perform the task for which it was engineered. Three-dimensional bioresorbable models of osseous defects may someday be obtained, and serve as BMP-2 delivery systems, for off-the-shelf replacement of craniofacial boney defects. Adipose-derived stem cells might be primed *ex vivo* with BMP-2 before implantation in bony defects, thus ending the morbidity associated with autografts harvest and this may play a critical role in the advancement of craniofacial surgery. Although further study is required to allow for the responsible use of this technology in the clinical setting, the results are promising and very favorable.

# Chapter 10 ABBREVIATIONS

| | |
|---|---|
| ADAS | Adipose-derived Adult Stem |
| AdMSCs | Adipose Mesenchymal Stem Cells |
| ADSCs | Adipose-derived Stromal Cells |
| ALP | Alkaline Phosphatase |
| AM | Adipogenic Medium |
| ASC | Adipose Stem Cells |
| ASCs | Adipose Stem/Stromal Cells |
| BAT | Brown Adipose Tissue |
| BCP | Biphasic Calcium Phosphate |
| bFGF | Basic Fibroblast Growth Factor |
| BFP | Buccal Fat Pad |
| BM | Bone Marrow |
| BM MSC | Bone marrow mesenchymal stem cells |
| BMP | Bone Morphogenetic Protein |
| BSA | Bovine Serum Albumin |
| BSP | Bone Sialoprotein |
| β-TCP | β-Tricalcium Phosphate |
| Ca/P | Calcium/Phosphate |
| CBFA1 | Core binding factor-alpha1 |
| CFU-F | Colony-forming Unit Fibroblasts |
| CM | Chondrogenic Medium |
| Col | Collagen |
| CSF | Colony Stimulating Factor |
| CSF-1 | Monocyte colony-stimulating growth factor |
| CTGF | Connective Tissue Growth Factor |
| DMEM | Dulbbeco's Modified Eagle's medium |
| EC | Endothelial Cells |
| EGF | Epidermal Growth Factor |
| EPC | Endothelial Progenitor Cells |
| ES | Embryonic Stem Cells |
| FBS | Fetal Bovine Serum |
| FDA | Food and Drug Administration |
| FGF | Fibroblast Growth Factor |

| | |
|---|---|
| FITC | Fluorescein Isothiocyanate |
| FSC | Forward Scatter |
| G-CSF | Granulocyte Colony-Stimulating Factor |
| HA | Hydroxyapatite |
| Hg | High glucose |
| HGF | Hepatocyte Growth Factor |
| HLA | Human Leukocyte Antigen |
| HSC | Hematopoietic Stem Cells |
| IFATS | International Fat Applied Technology Society |
| IGF | Insulin-like Growth Factor |
| IgGs | Immunoglobulins |
| IL | Interleukins |
| iPS | Induced Pluripotent Stem Cells |
| ISCT | International Society for Cellular Therapy |
| Lg | Low glucose |
| MAbs | Monoclonal Antibodies |
| MAPC | Multipotent Progenitor Cells |
| MHC | Major Histocompatibility Complexes |
| MMP | Matrix Metalloproteinases |
| MSC | Mesenchymal Stem Cells |
| OCN | Osteocalcin |
| OM | Osteogenic Medium |
| ON | Osteonectin |
| OPN | Osteopontin |
| OPG | Osteoprotegerin |
| PBS | Phosphate Buffered Saline |
| PCL | Poly(ε-caprolactone) |
| PDGF | Platelet-Derived Growth Factor |
| PE | Phycoerythrin |
| PerCP | Peridinin-chlorophyll-protein Complex |
| PGA | Polyglycolic acid |
| PGE | Prostaglandins |
| PLA | Processed Lipoaspirate Cells |

| | |
|---|---|
| PLAc | Polylactic Acid |
| PLGA | Polylactic-coglycolide copolymers |
| PPARγ | Peroxisome Proliferator-Activated Receptor gamma |
| RANK | Receptor Activator of Nuclear Kappa B |
| RANKL | Receptor Activator of Nuclear Kappa B Ligand |
| RGD | Arg-Gly-Asp |
| SAOS-2 | Human Osteogenic Sarcoma Cells |
| SC | Subcutaneous Abdominal Adipose Tissue |
| SOX9 | Sex reversal Y-related high-mobility group box protein |
| SSC | Side Scatter |
| SVF | Stromal Vascular Fraction |
| TCP | β-Tricalcium Phosphate |
| TERMIS | Tissue Engineering and Regenerative Medicine International Society |
| TGF | Transforming Growth Factor |
| TNF | Tumor Necrosis Factor |
| UB | Ubiquitin |
| VEGF | Vascular Endothelial Growth Factor |
| WAT | White Adipose Tissue |

# Chapter 11 REFERENCES

1. Termis.org. Baltimore: Tissue Engineering & Regenerative Medicine International Society; [cited 3 August 2010]. Available at http://www.termis.org/.

2. Helder MN, Knippenberg M, Klein-Nulend J, Wuisman PI. Stem cells from adipose tissue allow challenging new concepts for regenerative medicine. Tissue Eng. 2007 Aug;13(8):1799-1808.

3. Vacanti CA. The history of Tissue Engineering. J Cell Mol Med. 2006 Jul-Sep;10(3):569-576.

4. Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol. 2005 Jun;166(6):1781-1791.

5. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, et al. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J. 2007 May;21(7):1345-1357.

6. Wagers AJ, Weissman IL. Plasticity of adult stem cells. Cell. 2004 Mar;116(5):639-648.

7. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol. 1966 Dec;16(3):381-390.

8. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991 Sep;9(5):641-650.

9. Pittenger MF. Mesenchymal stem cells from adult bone marrow. Methods Mol Biol. 2008;449:27-44.

10. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr;284(5411):143-147.

11. Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res. 1998 Mar;16(2):155-162.

12. Koga H, Muneta T, Nagase T, Nimura A, Ju YJ, Mochizuki T, et al. Comparison of mesenchymal tissues-derived stem cells for *in vivo* chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit. Cell Tissue Res. 2008 Aug;333(2):207-215.

13. Chen CH, Wei HJ, Lin WW, Chiu I, Hwang SM, Wang CC, et al. Porous tissue grafts sandwiched with multilayered mesenchymal stromal cell sheets induce tissue regeneration for cardiac repair. Cardiovasc Res 2008. Oct;80(1):88-95.

14. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-317.

15. Arthur A, Zannettino A, Gronthos S. The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. J Cell Physiol. 2009 Feb;218(2):237-245.

16. Levy YS, Bahat-Stroomza M, Barzilay R, Burshtein A, Bulvik S, Barhum Y, et al. Regenerative effect of neural-induced human mesenchymal stromal cells in rat models of Parkinson's disease. Cytotherapy. 2008;10(4):340-352.

17. Psaltis PJ, Zannettino AC, Worthley SG, Gronthos S. Concise review: mesenchymal stromal cells: potential for cardiovascular repair. Stem Cells. 2008 Sep;26(9):2201-2210.

18. Fu X, Li H. Mesenchymal stem cells and skin wound repair and regeneration: possibilities and questions. Cell Tissue Res. 2009 Feb;335(2):317-321.

19. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. 2007 Nov;25(11):2739-2749.

20. Nishimori M, Yamada Y, Hoshi K, Akiyama Y, Hoshi Y, Morishima Y, et al. Health-related quality of life of unrelated bone marrow donors in Japan. Blood. 2002 Mar;99(6):1995-2001.

21. Kennedy GA, Morton J, Western R, Butler J, Daly J, Durrant S. Impact of stem cell donation modality on normal donor quality of life: a prospective randomized study. Bone Marrow Transplant. 2003 Jun;31(11):1033-1035.

22. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem. 1997 Feb;64(2):278-294.

23. Caplan AI. Why are MSCs therapeutic? New data: new insight. J Pathol. 2009 Jan;217(2):318-324.

24. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001 Apr;7(2):211-22.

25. Aust L, Devlin B, Foster SJ, Halvorsen YD, Hicok K, du Laney T, et al. Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy. 2004;6(1):7-14.

26. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells. 2006 Feb;24(2):376-385.

27. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-4295.

28. Cousin B, Andre M, Arnaud E, Penicaud L, Casteilla L. Reconstitution of lethally irradiated mice by cells isolated from adipose tissue. Biochem Biophys Res Commun. 2003 Feb;301(4):1016-1022.

29. Fraser JK, Schreiber R, Strem B, Zhu M, Alfonso Z, Wulur I, et al. Plasticity of human adipose stem cells toward endothelial cells and cardiomyocytes. Nat Clin Pract Cardiovasc Med. 2006 Mar;3 Suppl 1:S33-37.

30. Xu Y, Liu Z, Liu L, Zhao C, Xiong F, Zhou C, et al. Neurospheres from rat adipose-derived stem cells could be induced into functional Schwann cell-like cells *in vitro*. BMC Neurosci. 2008 Feb;9:21.

31. Trottier V, Marceau-Fortier G, Germain L, Vincent C, Fradette J. IFATS collection: Using human adipose-derived stem/stromal cells for the production of new skin substitutes. Stem Cells 2008 Oct;26(10):2713-2723.

32. Lee J, Han DJ, Kim SC. *In vitro* differentiation of human adipose tissue-derived stem cells into cells with pancreatic phenotype by regenerating pancreas extract. Biochem Biophys Res Commun. 2008 Oct;375(4):547-551.

33. Aurich H, Sgodda M, Kaltwasser P, Vetter M, Weise A, Liehr T, et al. Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue *in vitro* promotes hepatic integration *in vivo*. Gut. 2009 Apr;58(4):570-581.

34. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation. 2004 Mar;109(10):1292-1298.

35. Rubina K, Kalinina N, Efimenko A, Lopatina T, Melikhova V, Tsokolaeva Z, et al. Adipose Stromal Cells Stimulate Angiogenesis via Promoting Progenitor Cell Differentiation, Secretion of Angiogenic Factors, and Enhancing Vessel Maturation. Tissue Eng Part A. 2009 Aug;15(8):2039-2050.

36. Kim Y, Kim H, Cho H, Bae Y, Suh K, Jung J. Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. Cell Physiol Biochem. 2007;20(6):867-876.

37. Ellis E. Surgical reconstruction of defects of the jaws. Peterson LJ, Ellis E, Hupp J, Tucker M, eds. Contemporary Oral and Maxillofacial Surgery. Philadelphia: Ed Mosby. 2003. pp. 646- 651.

38. Petite H, Viateau V, Bensaïd W, Meunier A, de Pollak C, Bourguignon M, et al. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-963.

39. Frenken JW, Bouwman WF, Bravenboer N, Zijderveld SA, Schulten EA, ten Bruggenkate CM. The use of Straumann Bone Ceramic in a maxillary sinus floor elevation procedure: a clinical, radiological, histological and histomorphometric evaluation with a 6-month healing period. Clin Oral Implants Res. 2010 Feb;21(2):201-208.

40. Lee JH, Jung UW, Kim CS, Choi SH, Cho KS. Histologic and clinical evaluation for maxillary sinus augmentation using macroporous biphasic calcium phosphate in human. Clin Oral Implants Res. 2008 Aug;19(8):767-771.

41. Zijderveld SA, Zerbo IR, van den Bergh JP, Schulten EA, ten Bruggenkate CM. Maxillary sinus floor augmentation using a beta-tricalcium phosphate (Cerasorb) alone compared to autogenous bone grafts. Int J Oral Maxillofac Implants. 2005 May-Jun;20(3):432-440.

42. Zerbo IR, Bronckers AL, de Lange G, Burger EH. Localisation of osteogenic and osteoclastic cells in porous beta-tricalcium phosphate particles used for human maxillary sinus floor elevation. Biomaterials. 2005 Apr;26(12):1445-1451.

43. Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, et al. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol. 2004 May;22(5):560-567.

44. Yoon E, Dhar S, Chun DE, Gharibjanian NA, Evans GR. *In vivo* osteogenic potential of human adipose-derived stem cells/poly lactide-co-glycolic acid constructs for bone regeneration in a rat critical-sized calvarial defect model. Tissue Eng. 2007 Mar;13(3):619-627.

45. Cui L, Liu B, Liu G, Zhang W, Cen L, Sun J, et al. Repair of cranial bone defects with adipose derived stem cells and coral scaffold in a canine model. Biomaterials. 2007 Dec;28(36):5477-5486.

46. Hicok KC, Du Laney TV, Zhou YS, Halvorsen YD, Hitt DC, Cooper LF, et al. Human adipose-derived adult stem cells produce osteoid *in vivo*. Tissue Eng. 2004 Mar-Apr;10(3-4):371-380.

47. Urist MR. Bone formation by autoinduction. Science. 1965 Nov;150(698):893-899.

48. Gautschi OP, Frey SP, Zellweger R. Bone morphogenetic proteins in clinical applications. ANZ J Surg. 2007 Aug;77(8):626-631.

49. Emes Y, Aybar B, Vural P, Saral NY, Atalay B, Kaya AS, et al. Effects of bone morphogenetic proteins on osteoblast cells: vascular endothelial growth factor, calcium, inorganic phosphate, and nitric oxide levels. Implant Dent. 2010 Oct;19(5):419-427.

50. Choi SH, Kim CK, Cho KS, Huh JS, Sorensen RG, Wozney JM, et al. Effect of recombinant human bone morphogenetic protein-2/absorbable collagen sponge

(rhBMP-2/ACS) on healing in 3-wall intrabony defects in dogs. J Periodontol. 2002 Jan;73(1):63-72.

51. Smith DM, Afifi AM, Cooper GM, Mooney MP, Marra KG, Losee JE. BMP-2-based repair of large-scale calvarial defects in an experimental model: regenerative surgery in cranioplasty. J Craniofac Surg. 2008 Sep;19(5):1315-1322.

52. Peterson B, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P, et al. Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. Tissue Eng. 2005 Jan-Feb;11(1-2):120-129.

53. Dragoo JL, Choi JY, Lieberman JR, Huang J, Zuk PA, Zhang J, et al. Bone induction by BMP-2 transduced stem cells derived from human fat. J Orthop Res. 2003 Jul;21(4):622-629.

54. Miyazaki M, Zuk PA, Zou J, Yoon SH, Wei F, Morishita Y, et al. Comparison of human mesenchymal stem cells derived from adipose tissue and bone marrow for ex vivo gene therapy in rat spinal fusion model. Spine (Phila Pa 1976). 2008 Apr;33(8):863-869.

55. Mesimäki K, Lindroos B, Törnwall J, Mauno J, Lindqvist C, Kontio R, et al. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. Int J Oral Maxillofac Surg. 2009 Mar;38(3):201-209.

56. Li RH and Wozney JM. Delivering on the promise of bone morphogenetic proteins. Trends Biotechnol. 2001 Jul;19(7):255-265.

57. Chao M, Donovan T, Sotelo C, Carstens MH. In situ osteogenesis of hemimandible with rhBMP-2 in a 9-year-old boy: osteoinduction via stem cell concentration. J Craniofac Surg. 2006 May;17(3):405-412.

58. Smoljanovic T, Janjanin S, Bojanic I. Avoiding unanticipated adverse effects of recombinant human bone morphogenetic protein-2 therapy in craniofacial surgery with experiences from spinal applications. J Craniofac Surg. 2009 Sep;20(5):1626.

59. Knippenberg M, Helder MN, Zandieh Doulabi B, Wuisman PI, Klein-Nulend J. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. Biochem Biophys Res Commun. 2006 Apr;342(3):902-908.

60. Sierra-Johnson J, Johnson BD. Facial fat and its relationship to abdominal fat: a marker for insulin resistance? Med Hypotheses. 2004;63(5):783-786.

61. Alkan A, Dolanmaz D, Uzun E, and Erdem E. The reconstruction of oral defects with buccal fat pad. Swiss Med Wkly. 2003 Aug;133(33-34):465-470.

62. Abuabara A, Cortez AL, Passeri LA, de Moraes M, and Moreira RW. Evaluation of different treatments for oroantral/oronasal communications: experience of 112 cases. Int J Oral Maxillofac Surg 2006 Feb;35(2):155-158.

63. Lin K, Matsubara Y, Masuda Y, Togashi K, Ohno T, Tamura T, et al. Characterization of adipose tissue-derived cells isolated with the Celution system. Cytotherapy. 2008;10(4):417-426.

64. Moioli EK, Clark PA, Chen M, Dennis JE, Erickson HP, Gerson SL, et al. Synergistic actions of hematopoietic and mesenchymal stem/progenitor cells in vascularizing bioengineered tissues. PLoS One. 2008;3(12):e3922.

65. Madonna R, and De Caterina R. *In vitro* neovasculogenic potential of resident adipose tissue precursors. Am J Physiol Cell Physiol. 2008 Nov;295(5):C1271-1280.

66. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation. 2004 Feb;109(5):656-663.

67. Tapp H, Hanley EN Jr, Patt JC, Gruber HE. Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. Exp Biol Med. 2009 Jan;234(1):1-9.

68. Yoshimura K, Shigeura T, Matsumoto D, Sato T, Takaki Y, Aiba-Kojima E, et al. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol. 2006 Jul;208(1):64-76.

69. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res. 2007 May;100(9):1249-1260.

70. Traktuev DO, Merfeld-Clauss S, Li J, Kolonin M, Arap W, Pasqualini R, et al. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res. 2008 Jan 4;102(1):77-85.

71. Lin G, Garcia M, Ning H, Banie L, Guo YL, Lue TF, et al. Defining stem and progenitor cells within adipose tissue. Stem Cells Dev. 2008 Dec;17(6):1053-1063.

72. Varma MJ, Breuls RG, Schouten TE, Jurgens WJ, Brontkes HJ, Schuurhuis GJ, et al. Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells. Stem Cells Dev.2007;16:91-104.

73. Jurgens WJ, Oedayrajsingh-Varma MJ, Helder MN, Zandiehdoulabi B, Schouten TE, Kuik DJ, et al. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell Tissue Res. 2008 Jun;332(3):415-426.

74. Jurgens WJ, Kroeze RJ, Bank RA, Ritt MJ, Helder MN. Rapid attachment of adipose stromal cells on resorbable polymeric scaffolds facilitates the one-step surgical procedure for cartilage and bone tissue engineering purposes. J Orthop Res. 2011.

75. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. J Cell Biochem. 1997 Feb;64(2):295-312.

76. Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung UI, Kubota N, et al. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest. 2004 Mar;113(6):846-855.

77. Haseltine WA. The Emergence of Regenerative Medicine: A New Field and a New Society. E-biomed: The Journal of Regenerative Medicine. 2001 June,2(4):17-23.

78. Langer R, Vacanti JP. Tissue engineering. Science. 1993 May;260(5110):920-926.

79. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: a review of current technology and applications. J Appl Biomater. 1991 Fall;2(3):187-208.

80. Crichlow RJ, Andres PL, Morrison SM, Haley SM, Vrahas MS. Depression in orthopaedic trauma patients. Prevalence and severity. J Bone Joint Surg Am. 2006 Sep;88(9):1927-1933.

81. Starr AJ, Smith WR, Frawley WH, Borer DS, Morgan SJ, Reinert CM, et al. Symptoms of posttraumatic stress disorder after orthopaedic trauma. Bone Joint Surg Am. 2004 Jun;86-A(6):1115-1121.

82. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. Macromol Biosci. 2004 Aug 9;4(8):743-765.

83. Huibregtse BA, Johnstone B, Goldberg VM, Caplan AI. Effect of age and sampling site on the chondro-osteogenic potential of rabbit marrow-derived mesenchymal progenitor cells. J Orthop Res. 2000 Jan;18(1):18-24.

84. Steenhuis P, Carr KM, Pettway GJ, Ignelzi MA, Jr. Osteogenic and Adipogenic Cell Fractions Isolated from Postnatal Mouse Calvaria. Cells Tissues Organs. 2008; Dec 190(3):150-157.

85. Guillemin G, Meunier A, Dallant P, Christel P, Pouliquen JC, Sedel L. Comparison of coral resorption and bone apposition with two natural corals of different porosities. J Biomed Mater Res. 1989 Jul;23(7):765-779.

86. Haimi S, Suuriniemi N, Haaparanta AM, Ellä V, Lindroos B, Huhtala H, et al. Growth and osteogenic differentiation of adipose stem cells on PLA/bioactive glass and PLA/beta-TCP scaffolds. Tissue Eng Part A. 2009 Jul;15(7):1473-1480.

87. Hulbert SF, Young FA, Mathews RS, Klawitter JJ, Talbert CD, Stelling FH. Potential of ceramic materials as permanently implantable skeletal prostheses. J Biomed Mater Res. 1970 Sep;4(3):433-456.

88. Griffith LG, Naughton G. Tissue engineering--current challenges and expanding opportunities. Science. 2002 Feb; 295(5557):1009-1014.

89. Kanczler JM, Oreffo RO. Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater. 2008 May 2;15:100-114.

90. Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun. 2002 Jun 7;294(2):371-379.

91. Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R, Bouloumie A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation. 2004 Jul 20;110(3):349-355.

92. Shoja MM, Tubbs RS, Loukas M, Shokouhi G, Ardalan MR. Marie-Francois Xavier Bichat (1771-1802) and his contributions to the foundations of pathological anatomy and modern medicine. Ann Anat. 2008 Nov 20;190(5):413-420.

93. El Haddad SA, Abd El Razzak, MY, and El Shall M. Use of pedicled buccal fat pad in root coverage of severe gingival recession defect. J Periodontol. 2008 Jul;79(7):1271-1279.

94. Martin-Granizo R, Naval L, Costas A, Goizueta C, Rodriguez F, Monje F, et al. Use of buccal fat pad to repair intraoral defects: review of 30 cases. Br J Oral Maxillofac Surg. 1997 Apr;35(2):81-84.

95. Stein JI, Greenberg AM. Maxilllary sinus grafting and osseointegration surgery. Greenberg AM. Prein J, editors. Craniomaxillofacial reconstructive and corrective bone surgery. New York: Springer; 2002. pp. 179-180.

96. Lemoli RM, Bertolini F, Cancedda R, De Luca M, Del Santo A, Ferrari G, et al. Stem cell plasticity: time for a reappraisal?. Haematologica. 2005 Mar;90(3):360-381.

97. Schmidt A, Ladage D, Schinköthe T, Klausmann U, Ulrichs C, Klinz FJ, et al. Basic fibroblast growth factor controls migration in human mesenchymal stem cells. Stem Cells. 2006 Jul; 24(7):1750-1758.

98. Jung S, Sen A, Rosenberg L, Behie LA. Identification of growth and attachment factors for the serum-free isolation and expansion of human mesenchymal stromal cells. Cytotherapy. 2010 Sep;12(5):637-657.

99. Cancedda R, Bianchi G, Derubeis A, Quarto R. Cell therapy for bone disease: a review of current status. Stem Cells. 2003;21(5):610-619.

100. Jorgensen C, Gordeladze J, Noel D. Tissue engineering through autologous mesenchymal stem cells. Curr Opin Biotechnol. 2004 Oct;15(5):406-410.

101. Fang B, Song Y, Liao L, Zhang Y, Zhao RC. Favorable response to human adipose tissue-derived mesenchymal stem cells in steroid-refractory acute graft-versus-host disease. Transplant Proc. 2007 Dec;39(10):3358-3362.

102. Bronner-Fraser M. Neural crest cell formation and migration in the developing embryo. FASEB J. 1994 Jul;8(10):699-706.

103. Dupin E, Creuzet S, Le Douarin NM. The contribution of the neural crest to the vertebrate body. Adv Exp Med Biol. 2006;589:96-119.

104. Le Douarin NM, Creuzet S, Couly G, Dupin E. Neural crest cell plasticity and its limits. Development. 2004 Oct;131(19):4637-4650.

105. Royce PM, Steinmann B, editors. Bone pathology. New York: Wiley-Liss; 2002. pp. 16-18.

106. Caceci T. Connective tissues: cartilage and bone; [cited 3 September 2009]. Available at http://education.vetmed.vt.edu/Curriculum/VM8054/Labs/Lab8/ Lab8.htm.

107. Safadi FF, Barbe MF, Abdelmagid SM, Rico MC, Aswad RA, Litvin, J et al. Bone structure, development and bone biology. Khurana JS, editor. Bone pathology. Philadelphia: Humana-Press; 2009. pp. 16-18.

108. The Skeletal System. Marieb EN, editor. Essentials of Human Anatomy & Physiology. 7th ed. San Francisco: Benjamin Cummings; 2002. pp. 117-122.

109. Cancellous bone [internet page]. London: Encyclopædia Britannica Online; [updated 15 Aug. 2010, cited 30 Aug. 2010]. Available at http://www.britannica.com/EBchecked/ topic/92222/cancellous-bone>.

110. Roberts WE. Metabolism and biomechanics in orthodontic practice. Graber TM, Vanarsdall RL, editors. Orthodontics: current principles and techniques. 3rd ed. St. Louis: Mosby. 2000; p. 204.

111. Compact bone [internet page]. London: Encyclopædia Britannica Online; [updated 15 Aug. 2010, 30 Aug. 2010]. Available at http://www.britannica.com /EBchecked/topic/ 92222/cancellous-bone.

112. Zoetis T, Tassinari MS, Bagi C, Walthall K, Hurtt ME. Species comparison of postnatal bone growth and development. Birth Defects Res B Dev Reprod Toxicol. 2003 Apr;68(2):86-110.

113. Mosekilde L. Vertebral structure and strength *in vivo* and *in vitro*. Calcif Tissue Int. 1993;53 Suppl 1:S121-5; discussion S125-126.

114. Connective tissue. Watkins J, editor. Structure and function of the musculoskeletal system. 2nd ed. Champaign: Human Kinetiks; 2010. pp. 90-99.

115. Bones and Skeletal Tissues. Marieb EN, Hoehn K, editors. Human Anatomy & Physiology. 7th ed. San Francisco: Pearson International; 2007. pp. 176-182.

116. Nuttall ME, Patton AJ, Olivera DL, Nadeau DP, Gowen M. Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteopenic disorders. J Bone Miner Res. 1998 Mar;13(3):371-382.

117. Bone: Structure, Function, Growth, and Remodeling. Fitzgerald RH, Kaufer H, Malkani AL, editors. Orthopaedics. St Louis: Mosby; 2002. pp. 150-154.

118. Bone structure, Development and Bone Biology. Khurana JS, editor. Bone Pathology. 2nd ed. New York: Humana Press; 2009. pp. 11-13.

119. Papanicolaou SE, Phipps RJ, Fyhrie DP, Genetos DC. Modulation of sclerostin expression by mechanical loading and bone morphogenetic proteins in osteogenic cells. Biorheology. 2009;46(5):389-399.

120. Westbroek I, van der Plas A, de Rooij KE, Klein-Nulend J, Nijweide PJ. Expression of serotonin receptors in bone. J Biol Chem. 2001 Aug 3;276(31):28961-28968.

121. Everts V, Delaissé JM, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P, et al. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res. 2002 Jan;17(1):77-90.

122. Bone mechanics handbook. Cowin SC, editor. 2nd ed. Boca Raton: CRC; 2001.

123. Fernández-Tresguerres-Hernández-Gil I, Alobera-Gracia MA, del-Canto-Pingarrón M, Blanco-Jerez L. Physiological bases of bone regeneration I. Histology and physiology of bone tissue. Med Oral Patol Oral Cir Bucal. 2006 Jan 1;11(1):E47-51.

124. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell. 1997 May 30;89(5):747-754.

125. Anderson JM. The cellular cascades of wound healing. Davies JE, editor. Bone engineering. Toronto: Em squared incorporated; 2000. pp. 82-83.

126. Parfitt AM. The cellular basis of bone turnover and bone loss: a rebuttal of the osteocytic resorption-bone flow theory. Clin Orthop Relat Res. 1977;(127):236-247.

127. Vatsa A, Smit TH, Klein-Nulend J. Extracellular NO signalling from a mechanically stimulated osteocyte. J Biomech. 2007;40 Suppl 1:S89-95.

128. Schneider P, Meier M, Wepf R, Müller R. Towards quantitative 3D imaging of the osteocyte lacuno-canalicular network.Bone. 2010 Nov;47(5):848-858.

129. Skeletal anatomy in densitometry. Bonnick SL, editor. Denton: Humana Press. Bone densitometry in clinical practice; 2009. p. 73.

130. Hill PA. Bone remodelling. Br J Orthod. 1998 May;25(2):101-107.

131. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci USA. 1999;96(7):3540–3545.

132. Everts V, Delaissé JM, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P, et al. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res. 2002 Jan;17(1):77-90.

133. Fernández-Tresguerres-Hernández-Gil I, Alobera-Gracia MA, del-Canto-Pingarrón M, Blanco-Jerez L. Physiological bases of bone regeneration II. The remodelling process. Med Oral Patol Oral Cir Bucal. 2006 Mar 1;11(2):E151-157.

134. Díaz-Romeral-Bautista M, Manchón-Miralles A, Asenjo-Cabezón J, Cebrián-Carretero JL, Torres-García-Denche J, Linares-García-Valdecasas R. Autogenous calvarium bone grafting as a treatment for severe bone resorption in the upper maxilla: a case report. Med Oral Patol Oral Cir Bucal. 2010 Mar 1;15(2):e361-365.

135. Trueta J. The role of the vessels in osteogenesis. Trauma and bone growth. J Bone J Surg. 1963;45B:402-409.

136. Urken ML, Buchbinder D. Oromandibular Reconstruction. Urken ML, editor. Multidisciplinary Head and Neck. Reconstruction: A Defect-Oriented Approach. Philadelphia: Lippincott-Williams; 2010. pp. 2-3.

137. Lind M, Bünger C. Factors stimulating bone formation. Eur Spine J. 2001 Oct;10 Suppl 2:S102-109.

138. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors. 2004 Dec;22(4):233-241.

139. Estrada KD, Lyons, KM. BMP signaling in skeletogenesis. Bronner F, Farach-Carson MC, Roach HI, editors. Bone and development. London: Springer; 2010. pp. 125-127.

140. Stemcells.nih.gov. The National Institutes of Health resource for stem cell research; [updated 20 July 2010, cited 10 August 2010]. Available at http://stemcells.nih.gov/info/ basics/basics1.asp nih.

141. Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC. Review: ex vivo engineering of living tissues with adult stem cells. Tissue Eng. 2006 Nov;12(11):3007-3019.

142. Adams GB, Martin RP, Alley IR, Chabner KT, Cohen KS, Calvi LM, et al. Therapeutic targeting of a stem cell niche. Nat Biotechnol. 2007 Feb;25(2):238-243.

143. Bianco P, Gehron Robey P. Marrow stromal stem cells. J Clin Invest. 2000 Jun;105(12):1663-1668.

144. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981 Jul 9;292(5819):154-156.

145. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998 Nov 6;282(5391): 1145-1147.

146. Martin MJ, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med. 2005 Feb;11(2):228-232.

147. Stem cell culture shock. Nat Methods. 2005 Mar;2(3):153.

148. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, et al. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J. 2007 May;21(7):1345-1357.

149. Amariglio N, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, et al. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med. 2009 Feb 17;6(2):e1000029.

150. ClinicalTrials.gov. National Institutes of Health; [updated 23 August 2010, 29 August 2010]. Available at http://clinicaltrials.gov.

151. Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo*. Science. 1999 Jan 22;283(5401):534-537.

152. Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. Science. 2000 Dec 1;290 (5497):1779-1782.

153. Quesenberry PJ, Colvin G, Dooner G, Dooner M, Aliotta JM, Johnson K. The stem cell continuum: cell cycle, injury, and phenotype lability. Ann N Y Acad Sci. 2007 Jun;1106:20-29.

154. Graham JJ, Foltz WD, Vaags AK, Ward MR, Yang Y, Connelly KA, et al. Long-term tracking of bone marrow progenitor cells following intracoronary injection post-myocardial infarction in swine using MRI. Am J Physiol Heart Circ Physiol. 2010 Jul;299(1):H125-133.

155. Assmus B, Rolf A, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, et al. Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction. Circ Heart Fail. 2010 Jan;3(1):89-96.

156. Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, et al. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. Stem Cell Rev. 2009 Dec;5(4):378-386.

157. Kim JH, Jee MK, Lee SY, Han TH, Kim BS, Kang KS, et al. Regulation of adipose tissue stromal cells behaviors by endogenic Oct4 expression control. PLoS One. 2009 Sep 24;4(9):e7166.

158. Ohishi M, Schipani E. Bone marrow mesenchymal stem cells. J Cell Biochem. 2010 Feb 1;109(2):277-282.

159. Petrakova KV, Tolmacheva AA, Fridenshtein A. Bone formation occuring in bone marrow transplantation in diffusion chambers. Biull Eksp Biol Med. 1963 Dec;56:87-91.

160. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 1970 Oct;3(4):393-403.

161. Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell. 2008 Apr 10;2(4):313-319.

162. Kolf C, Cho E, Tuan R. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Research and Therapy. 2007;9(1):204.

163. Caplan AI. New era of cell-based orthopedic therapies. Tissue Eng Part B Rev. 2009 Jun;15(2):195-200.

164. Bernardo ME, Locatelli F, Fibbe WE. Mesenchymal stromal cells. Ann N Y Acad Sci. 2009 Sep;1176:101-117.

165. Young HE, Mancini ML, Wright RP, Smith JC, Black AC Jr, Reagan CR, et al. Mesenchymal stem cells reside within the connective tissues of many organs. Dev Dyn. 1995 Feb;202(2):137-144.

166. Yoo KH, Jang IK, Lee MW, Kim HE, Yang MS, Eom Y. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. Cell Immunol. 2009;259(2):150-156.

167. Prockop DJ. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). Clin Pharmacol Ther. 2007 Sep;82(3):241-243.

168. Ortiz LA, Dutreil M, Fattman C, Pandey AC, Torres G, Go K, et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. Proc Natl Acad Sci USA. 2007; 104: 11002–11007.

169. Minguell J.J, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. Exp Biol Med (Maywood). 2006; 231: 39–49.

170. Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proc Natl Acad Sci USA. 2006; 103: 17438–17443.

171. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol. 2007; 213: 341–347.

172. Zimmerlin L, Donnenberg VS, Pfeifer ME, Meyer EM, Péault B, Rubin JP, et al. Stromal vascular progenitors in adult human adipose tissue. Cytometry A. 2010 Jan;77(1):22-30.

173. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997 Feb 14;275(5302):964-967.

174. Miñana MD, Carbonell-Uberos F, Mirabet V, Marín S, Encabo A. IFATS collection: Identification of hemangioblasts in the adult human adipose tissue. Stem Cells. 2008 Oct;26(10):2696-2704.

175. Kiel MJ, Morrison SJ. Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol. 2008 Apr;8(4):290-301.

176. De Barros AP, Takiya CM, Garzoni LR, Leal-Ferreira ML, Dutra HS, Chiarini LB, et al. Osteoblasts and bone marrow mesenchymal stromal cells control hematopoietic

stem cell migration and proliferation in 3D *in vitro* model. PLoS One. 2010 Feb 8;5(2):e9093.

177. Becker AJ, McCulloch EA, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 1963 Feb 2;197:452-454.

178. Nowell PC, Hirsch BE, Fox DH, Wilson DB. Evidence for the existence of multipotential lympho-hematopoietic stem cells in adult rat. J Cell Physiol. 1970 Apr;75(2):151-158.

179. Ratajczak MZ. Phenotypic and functional characterization of hematopoietic stem cells. Curr Opin Hematol. 2008 Jul;15(4):293-300.

180. Lynch L, O'Donoghue D, Dean J, O'Sullivan J, O'Farrelly C, Golden-Mason L. Detection and characterization of hemopoietic stem cells in the adult human small intestine. J Immunol. 2006 May 1;176(9):5199-5204.

181. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. Science. 2001 Nov 30;294(5548):1933-1936.

182. Isscr.org. Illinois: International Society for Stem Cell Research; [cited 25 August 2010]. Available at http://www.isscr.org/public/adultstemcells.htm.

183. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002 Jul 4;418(6893):41-49.

184. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007 Nov 30;131(5):861-872.

185. Ye Z, Zhan H, Mali P, Dowey S, Williams DM, Jang YY, et al. Human iPS from blood cells of healthy donors and patients with acquired blood disorders. Blood. 2009 Dec 24;114(27): 5473-5480.

186. Kunisato A, Wakatsuki M, Kodama Y, Shinba H, Ishida I, Nagao K. Generation of iPS by efficient reprogramming of adult bone marrow cells. Stem Cells Dev. 2010 Feb;19(2):229-238.

187. Tat PA, Sumer H, Jones KL, Upton K, Verma PJ. The efficient generation of induced pluripotent stem (iPS) cells from adult mouse adipose tissue-derived and neural stem cells. Cell Transplant. 2010;19(5):525-536.

188. Marx J. Molecular biology. Cancer's perpetual source?. Science. 2007 Aug 24;317(5841): 1029-1031.

189. Neuzil J, Stantic M, Zobalova R, Chladova J, Wang X, Prochazka L, et al. Tumour-initiating cells vs. cancer 'stem' cells and CD133: what's in the name?. Biochem Biophys Res Commun. 2007 Apr 20;355(4):855-859.

190. Piccirillo SG, Reynolds BA, Zanetti N, Lamorte G, Binda E, Broggi G, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature. 2006 Dec 7;444(7120):761-765.

191. Lutolf MP, Blau HM. Control of adult stem cell function in bioengineered artificial niches. Polak J, ed. Tissue engineering. London: Imperial College Press; 2008. p.177-181.

192. Crisan M, Deasy B, Gavina M, Zheng B, Huard J, Lazzari L, et al. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. Methods Cell Biol. 2008;86:295-309.

193. Dennis J.E, Charbord P. Origin and differentiation of human and murine stroma. Stem Cells. 2002;20(3):205-214. Jones E, McGonagle D. Human bone marrow mesenchymal stem cells *in vivo*. Rheumatology (Oxford). 2008 Feb;47(2):126-131.

194. Da Silva Meirelles L, Caplan AI, Nardi NB. In search of the *in vivo* identity of mesenchymal stem cells. Stem Cells. 2008 Sep;26(9):2287-2299.

195. Mödder UI, Khosla S. Skeletal stem/osteoprogenitor cells: current concepts, alternate hypotheses, and relationship to the bone remodeling compartment. J Cell Biochem. 2008 Feb 1;103(2):393-400.

196. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell. 2007 Oct 19;131(2):324-336.

197. Crisan M, Yap S, Casteilla L, Chen C.W, Corselli M, Park T.S, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2008 Sep 11;3(3):301-313.

198. Wang Y, Wan C, Deng L, Liu X, Cao X, Gilbert SR, et al. The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. J Clin Invest. 2007 Jun;117(6):1616-1626.

199. Gurevitch O, Slavin S, Resnick I, Khitrin S, Feldman A. Mesenchymal progenitor cells in red and yellow bone marrow. Folia Biol (Praha). 2009;55(1):27-34.

200. Bianco P, Robey PG, Saggio I, Riminucci M. "Mesenchymal" stem cells in human bone marrow (skeletal stem cells) - a critical discussion of their nature, identity, and significance in incurable skeletal disease. Hum Gene Ther. 2010. Sep;21(9):1057-1066.

201. Bianco P. A system of self-renewing tissue specific progenitors and their microvascular niche. International Society for Stem Cell Research 7th Annual Meeting; 8-11 July; Barcelona.

202. Burg KJ, Porter S, Kellam JF. Biomaterial developments for bone tissue engineering. Biomaterials. 2000 Dec;21(23):2347-2359.

203. Li J, Wang HL. Common implant-related advanced bone grafting complications: classification, etiology, and management. Implant Dent. 2008 Dec;17(4):389-401.

204. Ge Z, Jin Z, Cao T. Manufacture of degradable polymeric scaffolds for bone regeneration. Biomed Mater. 2008 Jun;3(2):022001.

205. Matsushima A, Kotobuki N, Tadokoro M, Kawate K, Yajima H, Takakura Y, et al. *In vivo* osteogenic capability of human mesenchymal cells cultured on hydroxyapatite and on beta-tricalcium phosphate. Artif Organs. 2009 Jun;33(6):474-481.

206. Guarino V, Causa F, Netti PA, Ciapetti G, Pagani S, Martini D, et al. The role of hydroxyapatite as solid signal on performance of PCL porous scaffolds for bone tissue regeneration. J Biomed Mater Res B Appl Biomater. 2008 Aug;86B(2):548-557.

207. Chen Y, Cho MR, Mak AF, Li JS, Wang M, Sun S. Morphology and adhesion of mesenchymal stem cells on PLLA, apatite and apatite/collagen surfaces. J Mater Sci Mater Med. 2008 Jul;19(7):2563-2567.

208. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials. 2005 Sep;26(27):5474-5491.

209. Blattert TR, Jestaedt L, Weckbach A. Suitability of a calcium phosphate cement in osteoporotic vertebral body fracture augmentation: a controlled, randomized, clinical trial of balloon kyphoplasty comparing calcium phosphate versus polymethylmethacrylate. Spine (Phila Pa 1976). 2009 Jan 15;34(2):108-114.

210. Jäger M, Feser T, Denck H, Krauspe R. Proliferation and osteogenic differentiation of mesenchymal stem cells cultured onto three different polymers *in vitro*. Ann Biomed Eng. 2005 Oct;33(10):1319-1332.

211. Wang L, Dormer NH, Bonewald LF, Detamore MS. Osteogenic differentiation of human umbilical cord mesenchymal stromal cells in polyglycolic acid scaffolds. Tissue Eng Part A. 2010 Jun;16(6):1937-1948.

212. Di Bella C, Farlie P, Penington AJ. Bone regeneration in a rabbit critical-sized skull defect using autologous adipose-derived cells. Tissue Eng Part A. 2008 Apr;14(4):483-490.

213. Fabbri P, Bondioli F, Messori M, Bartoli C, Dinucci D, Chiellini F. Porous scaffolds of polycaprolactone reinforced with in situ generated hydroxyapatite for bone tissue engineering. J Mater Sci Mater Med. 2010 Jan;21(1):343-351.

214. Abbah SA, Lam CX, Hutmacher DW, Goh JC, Wong HK. Biological performance of a polycaprolactone-based scaffold used as fusion cage device in a large animal model of spinal reconstructive surgery. Biomaterials. 2009 Oct;30(28):5086-5093.

215. Sung HJ, Meredith C, Johnson C, Galis ZS. The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. Biomaterials. 2004 Nov;25(26):5735-5742.

216. El-Amin SF, Lu HH, Khan Y, Burems J, Mitchell J, Tuan RS, et al. Extracellular matrix production by human osteoblasts cultured on biodegradable polymers applicable for tissue engineering. Biomaterials. 2003 Mar;24(7):1213-1221.

217. Hao W, Pang L, Jiang M, Lv R, Xiong Z, Hu YY. Skeletal repair in rabbits using a novel biomimetic composite based on adipose-derived stem cells encapsulated in collagen I gel with PLGA-beta-TCP scaffold. J Orthop Res. 2010 Feb;28(2):252-257.

218. Domaschke H, Gelinsky M, Burmeister B, Fleig R, Hanke T, Reinstorf A, et al. *In vitro* ossification and remodeling of mineralized collagen I scaffolds. Tissue Eng. 2006 Apr;12(4): 949-958.

219. Hao W, Hu YY, Wei YY, Pang L, Lv R, Bai JP, et al. Collagen I gel can facilitate homogenous bone formation of adipose-derived stem cells in PLGA-ß-TCP scaffold. Cells Tissues Organs. 2008;187(2):89-102.

220. Hiraoka Y, Kimura Y, Ueda H, Tabata Y. Fabrication and biocompatibility of collagen sponge reinforced with poly(glycolic acid) fiber. Tissue Eng. 2003 Dec;9(6):1101-1112.

221. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Tabata Y. Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic Acid) fiber. Tissue Eng. 2005 Sep-Oct;11(9-10):1476-1488.

222. Manferdini C, Guarino V, Zini N, Raucci MG, Ferrari A, Grassi F, et al. Mineralization behavior with mesenchymal stromal cells in a biomimetic hyaluronic acid-based scaffold. Biomaterials. 2010;31(14):3986-3996.

223. Ahmed TA, Dare EV, Hincke M. Fibrin: a versatile scaffold for tissue engineering applications. Tissue Eng Part B Rev. 2008 Jun;14(2):199-215.

224. Follmar KE, Prichard HL, DeCroos FC, Wang HT, Levin LS, Klitzman B, et al. Combined bone allograft and adipose-derived stem cell autograft in a rabbit model. Ann Plast Surg. 2007 May;58(5):561-565.

225. Haimi S, Moimas L, Pirhonen E, Lindroos B, Huhtala H, Räty S, et al. Calcium phosphate surface treatment of bioactive glass causes delay in early osteogenic differentiation of adipose stem cells. J Biomed Mater Res A. 2009 Nov;91(2):540-547.

226. Kim HJ, Kim UJ, Kim HS, Li C, Wada M, Leisk GG, et al. Bone tissue engineering with premineralized silk scaffolds. Bone. 2008 Jun;42(6):1226-1234.

227. Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen J, et al. Silk-based biomaterials. Biomaterials. 2003 Feb;24(3):401-416.

228. Scope of Tissue Engineering. Ikada Y, editor. Tissue Engineering: Fundamentals and Applications. Oxford: Elsevier Ltd; 2006. pp. 25-26.

229. Newton D, Mahajan R, Ayres C, Bowman JR, Bowlin GL, Simpson DG. Regulation of material properties in electrospun scaffolds: Role of cross-linking and fiber tertiary structure. Acta Biomater. 2009 Jan;5(1):518-529.

230. McCullen SD, Zhu Y, Bernacki SH, Narayan RJ, Pourdehymi B, Gorga RE, et al. Electrospun composite poly(L-lactic acid)/tricalcium phosphate scaffolds induce proliferation and osteogenic differentiation of human adipose-derived stem cells. Biomed Mater. 2009 Jun;4(3):035002.

231. Wikesjo UM. Comparative study of BMPs and Delivery strategy in Peri-implant settings. International Association for Dental Research (IADR) General Session; 2010 Jul 14-17; Barcelona.

232. Urist MR, Strates BS. Bone morphogenetic protein. J Dent Res. 1971 Nov-Dec;50(6):1392-1406.

233. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. Bone Morphogenetic Proteins: A critical review. Cell Signal. 2011 Apr;23(4):609-620.

234. Kawai T, Urist MR. Bovine tooth-derived bone morphogenetic protein. J Dent Res. 1989 Jun;68(6):1069-1074.

235. Takagi K, Urist MR. The reaction of the dura to bone morphogenetic protein (BMP) in repair of skull defects. Ann Surg. 1982 Jul;196(1):100-109.

236. Garant PR. Influence of growth factors and cytokines on bone cells. Garant PR, editor. Oral Cells Tissues. Illinois: Quintessence; 2006. pp. 213-215.

237. Schoppet M, Preissner KT, Hofbauer LC. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arterioscler Thromb Vasc Biol. 2002 Apr 1;22(4):549-553.

238. Ishida K, Matsumoto T, Sasaki K, Mifune Y, Tei K, Kubo S, et al. Bone Regeneration Properties of Granulocyte Colony-Stimulating Factor via Neovascularization and Osteogenesis. Tissue Eng Part A. 2010 Oct;16(10):3271-3284.

239. Fukuoka H, Aoyama M, Miyazawa K, Asai K, Goto S. Hypoxic stress enhances osteoclast differentiation via increasing IGF2 production by non-osteoclastic cells. Biochem Biophys Res Commun. 2005 Mar 25;328(4):885-894.

240. Hulley P, Russell G, Croucher P. Growth factors. Seibel MJ, Robins SP, Bilezikian J, editors. Dynamics of bone and cartilage metabolism. 2nd ed. San Diego: Academic Press; 2006. p. 99-100.

241. Schett G, Saag KG, Bijlsma JW. From bone biology to clinical outcome: state of the art and future perspectives. Ann Rheum Dis. 2010 Aug;69(8):1415-1419.

242. Walsh S, Jordan GR, Jefferiss C, Stewart K, Beresford JN. High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors *in vitro*: relevance to glucocorticoid-induced osteoporosis. Rheumatology (Oxford). 2001 Jan;40(1):74-83.

243. Lee SY, Lim J, Khang G, Son Y, Choung PH, Kang SS, et al. Enhanced ex vivo expansion of human adipose tissue-derived mesenchymal stromal cells by fibroblast growth factor-2 and dexamethasone. Tissue Eng Part A. 2009 Sep;15(9):2491-2499.

244. Malladi P, Xu Y, Yang GP, Longaker MT. Functions of vitamin D, retinoic acid, and dexamethasone in mouse adipose-derived mesenchymal cells. Tissue Eng. 2006 Jul;12(7): 2031-2040.

245. De Girolamo L, Sartori MF, Albisetti W, Brini AT. Osteogenic differentiation of human adipose-derived stem cells: comparison of two different inductive media. Tissue Eng Regen Med. 2007 Mar-Apr;1(2):154-157.

246. Knowles HJ, Athanasou NA. Canonical and non-canonical pathways of osteoclast formation. Histol Histopathol. 2009 Mar;24(3):337-346.

247. Bell LN, Cai L, Johnstone BH, Traktuev DO, March KL, RV Considine. A central role for hepatocyte growth factor in adipose tissue angiogenesis, Am J Physiol Endocrinol Metab. 2008 Feb;294(2):E336–344.

248. Iwashima S, Ozaki T, Maruyama S, Saka Y, Kobori M, Omae K, et al. Novel culture system of mesenchymal stromal cells from human subcutaneous adipose tissue. Stem Cells Dev. 2009 May;18(4):533-543.

249. Reid IR. Fat and bone. Arch Biochem Biophys. 2010. Nov 1;503(1):20-27.

250. Ducy P, Schinke T, Karsenty G. Science. The osteoblast: a sophisticated fibroblast under central surveillance. 2000 Sep;289(5484):1501-1504.

251. Islander U, Jochems C, Lagerquist MK, Forsblad-d'Elia H, Carlsten H. Estrogens in rheumatoid arthritis; the immune system and bone. Mol Cell Endocrinol. 2011 Mar 15;335(1):14-29.

252. Luvizuto ER, Queiroz TP, Dias SM, Okamoto T, Dornelles RC, Garcia IR Jr, et al. Histomorphometric analysis and immunolocalization of RANKL and OPG during the alveolar healing process in female ovariectomized rats treated with oestrogen or raloxifene. Arch Oral Biol. 2010 Jan;55(1):52-59.

253. Laflamme C, Curt S, Rouabhia M. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. Arch Oral Biol. 2010 Sep;55(9):689-701.

254. Elabd C, Chiellini C, Massoudi A, Cochet O, Zaragosi LE, Trojani C, et al. Human adipose tissue-derived multipotent stem cells differentiate *in vitro* and *in vivo* into osteocyte-like cells. Biochem Biophys Res Commun. 2007 Sep 21;361(2):342-348.

255. Keerl S, Gehmert S, Gehmert S, Song YH, Alt E. PDGF and bFGF modulate tube formation in adipose tissue-derived stem cells. Ann Plast Surg. 2010 Apr;64(4):487-490.

256. Schwarz F, Ferrari D, Podolsky L, Mihatovic I, Becker J. Initial pattern of angiogenesis and bone formation following lateral ridge augmentation using rhPDGF and guided bone regeneration: an immunohistochemical study in dogs. Clin Oral Implants Res. 2010 Jan;21(1): 90-99.

257. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived EPC. EMBO J. 1999 Jul 15;18(14):3964-3972.

258. Miwa Y, Fujita T, Sunohara M, Sato I. Immunocytochemical localization of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 of the human deciduous molar tooth germ development in the human fetus. Ann Anat. 2008;190(3):246-251.

259. Wise GE. Cellular and molecular basis of tooth eruption. Orthod Craniofac Res. 2009 May;12(2):67-73.

260. Deckers MM, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Löwik CW. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology. 2000 May;141(5):1667-1674.

261. Maes C, Carmeliet G. Vascular and non vascular roles of VEGF in bone development. Ruhrberg C, editor. VEGF in development. New York: Springer & Landes Bioscience; 2008. pp. 79-88.

262. Harada S, Nagy JA, Sullivan KA, Thomas KA, Endo N, Rodan GA, et al. Induction of vascular endothelial growth factor expression by prostaglandin E2 and E1 in osteoblasts. J Clin Invest. 1994 Jun;93(6):2490-2496.

263. Fujita D, Yamashita N, Iita S, Amano H, Yamada S, Sakamoto K. Prostaglandin E2 induced the differentiation of osteoclasts in mouse osteoblast-depleted bone marrow cells. Prostaglandins Leukot Essent Fatty Acids. 2003 May;68(5):351-358.

264. Billon N, Iannarelli P, Monteiro MC, Glavieux-Pardanaud C, Richardson WD, Kessaris N, et al. The generation of adipocytes by the neural crest. Development. 2007 Jun;134(12):2283-2292.

265. Ailhaud G, Grimaldi P, Négrel R. Cellular and molecular aspects of adipose tissue development. Annu Rev Nutr. 1992;12:207-233.

266. Van Harmelen V, Skurk T, Hauner H. Primary culture and differentiation of human adipocyte precursor cells. Methods Mol Med. 2005;107:125-135.

267. Tchkonia T, Lenburg M, Thomou T, Giorgadze N, Frampton G, Pirtskhalava T, et al. Identification of depot-specific human fat cell progenitors through distinct expression profiles and developmental gene patterns. Am J Physiol Endocrinol Metab. 2007 Jan;292(1):E298-307.

268. Gesta S, Tseng YH, Kahn CR. Developmental origin of fat: tracking obesity to its source. Cell. 2007 Oct;131(2):242-256.

269. Cannon B, Nedergaard J. Developmental biology: Neither fat nor flesh. Nature. 2008 Aug;454(7207):947-948.

270. Vettor R, Milan G, Franzin C, Sanna M, De Coppi P, Rizzuto R, et al. The origin of intermuscular adipose tissue and its pathophysiological implications. Am J Physiol Endocrinol Metab. 2009.

271. Cook A, Cowan C, Adipose StemBook. The Stem Cell Research Community, ed; [updated March 31, 2009, cited 18 July 2010]. Available at http://www.stembook.org.

272. Zeve D, Tang W, Graff J. Fighting fat with fat: the expanding field of adipose stem cells. Cell Stem Cell. 2009 Nov;5(5):472-481.

273. Avram AS, Avram MM, James WD. Subcutaneous fat in normal and diseased states: 2. Anatomy and physiology of white and brown adipose tissue. J Am Acad Dermatol. 2005 Oct;53(4):671-683.

274. Van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009 Apr;360(15):1500-1508.

275. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009 Apr;360(15):1509-1517.

276. Cinti S. Transdifferentiation properties of adipocytes in the Adipose Organ. Am J Physiol Endocrinol Metab. 2009.

277. Connective tissue. Atlas of histology with functional correlations. Eroschenko VP, editor. Philadelphia: Lippincott Williams & Wilkins; 2008. pp. 67-68.

278. Cinti S. Plasticity of the adipose organ, Leff T, Granneman JG, editors. Adipose tissue in health and disease. Malden: Wiley-Blackwell; 2010. pp. 55-61.

279. Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, et al. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng. 2001 Dec;7(6):729-741.

280. Torres FC, Rodrigues CJ, Stocchero IN, Ferreira MC. Stem cells from the fat tissue of rabbits: an easy-to-find experimental source. Aesthetic Plast Surg. 2007 Sep-Oct;31(5):574-578.

281. Black LL, Gaynor J, Gahring D, Adams C, Aron D, Harman S, et al. Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. Vet Ther. 2007 Winter;8(4):272-284.

282. Huang T, He D, Kleiner G, Kuluz J. Neuron-like differentiation of adipose-derived stem cells from infant piglets *in vitro*. J Spinal Cord Med. 2007;30 Suppl 1:S35-40.

283. Pascucci L, Mercati F, Marini C, Ceccarelli P, Dall'Aglio C, Pedini V, et al. Ultrastructural morphology of equine adipose-derived mesenchymal stem cells.Histol Histopathol. 2010 Oct;25(10):1277-1285.

284. Stillaert FB, Di Bartolo C, Hunt JA, Rhodes NP, Tognana E, Monstrey S, et al. Human clinical experience with adipose precursor cells seeded on hyaluronic acid-based spongy scaffolds. Biomaterials. 2008 Oct;29(29):3953-3959.

285. Giusta MS, Andrade H, Santos AV, Castanheira P, Lamana L, Pimenta AM, et al. Proteomic analysis of human mesenchymal stromal cells derived from adipose tissue undergoing osteoblast differentiation. Cytotherapy. 2010 Jul;12(4):478-490.

286. Marino G, Rosso F, Cafiero G, Tortora C, Moraci M, Barbarisi M, et al. Beta-tricalcium phosphate 3D scaffold promote alone osteogenic differentiation of human adipose stem cells: *in vitro* study. J Mater Sci Mater Med. 2010 Jan;21(1):353-363.

287. Estes BT, Diekman BO, Gimble JM, Guilak F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc. 2010 Jul;5(7):1294-1311.

288. Pilgaard L, Lund P, Duroux M, Fink T, Ulrich-Vinther M, Søballe K, et al. Effect of oxygen concentration, culture format and donor variability on *in vitro* chondrogenesis of human adipose tissue-derived stem cells. Regen Med. 2009 Jul;4(4):539-548.

289. Di Rocco G, Iachininoto MG, Tritarelli A, Straino S, Zacheo A, Germani A, et al. Myogenic potential of adipose-tissue-derived cells. J Cell Sci. 2006 Jul;119(Pt 14):2945-2952.

290. Fraser JK, Wulur I, Alfonso Z, Hedrick MH. Fat tissue: an underappreciated source of stem cells for biotechnology. Trends Biotechnol. 2006 Apr;24(4):150-154.

291. Tchkonia T, Giorgadze N, Pirtskhalava T, Thomou T, DePonte M, Koo A, et al. Fat depot-specific characteristics are retained in strains derived from single human preadipocytes. Diabetes. 2006 Sep;55(9):2571-2578.

292. Prunet-Marcassus B, Cousin B, Caton D, André M, Pénicaud L, Casteilla L. From heterogeneity to plasticity in adipose tissues: site-specific differences. Exp Cell Res. 2006 Apr;312(6):727-736.

293. Peptan IA, Hong L, Mao JJ. Comparison of osteogenic potentials of visceral and subcutaneous adipose-derived cells of rabbits. Plast Reconstr Surg. 2006 Apr;117(5):1462-1470.

294. Lennon DP, Haynesworth SE, Arm DM, Baber MA, Caplan AI. Dilution of human mesenchymal stem cells with dermal fibroblasts and the effects on *in vitro* and *in vivo* osteochondrogenesis. Dev Dyn. 2000 Sep;219(1):50-62.

295. Mochizuki T, Muneta T, Sakaguchi Y, Nimura A, Yokoyama A, Koga H, et al. Higher chondrogenic potential of fibrous synovium- and adipose synovium-derived cells

compared with subcutaneous fat-derived cells: distinguishing properties of mesenchymal stem cells in humans. Arthritis Rheum. 2006 Mar;54(3):843-853.

296. Fraser JK, Wulur I, Alfonso Z, Zhu M, Wheeler ES. Differences in stem and progenitor cell yield in different subcutaneous adipose tissue depots. Cytotherapy. 2007;9(5):459-467.

297. Rodbell M. Metabolism of isolated fat cells. I. Effect of hormones on glucose metabolism and lipolysis. J Biol Chem. 1964 Feb;239:375-380.

298. Rada T, Reis RL, Gomes ME. Distinct Stem Cells Subpopulations Isolated from Human Adipose Tissue Exhibit Different Chondrogenic and Osteogenic Differentiation Potential. Stem Cell Rev. 2011 Mar;7(1):64-76.

299. Kurita M, Matsumoto D, Shigeura T, Sato K, Gonda K, Harii K, et al. Influences of centrifugation on cells and tissues in liposuction aspirates: optimized centrifugation for lipotransfer and cell isolation. Plast Reconstr Surg. 2008 Mar;121(3):1033-1043.

300. Rada T, Reis RL, Gomes ME. Adipose tissue-derived stem cells and their application in bone and cartilage tissue engineering. Tissue Eng Part B Rev. 2009 Jun;15(2):113-125.

301. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol. 2001 Oct;189(1):54-63.

302. Niemeyer P, Kornacker M, Mehlhorn A, Seckinger A, Vohrer J, Schmal H, et al. Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation *in vitro*. Tissue Eng. 2007 Jan;13(1):111-121.

303. Technau A, Froelich K, Hagen R, Kleinsasser N. Adipose tissue-derived stem cells show both immunogenic and immunosuppressive properties after chondrogenic differentiation. Cytotherapy. 2011 Mar;13(3):310-317.

304. Wan CD, Cheng R, Wang HB, Liu T. Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model. Hepatobiliary Pancreat Dis Int. 2008 Feb;7(1):29-33.

305. Peroni D, Scambi I, Pasini A, Lisi V, Bifari F, Krampera M, et al. Stem molecular signature of adipose-derived stromal cells. Exp Cell Res. 2008 Feb 1;314(3):603-615.

306. Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, et al. Multipotential differentiation of adipose tissue-derived stem cells. Keio J Med. 2005 Sep;54(3):132-141.

307. Winter A, Breit S, Parsch D, Benz K, Steck E, Hauner H, et al. Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells. Arhritis Rheum. 2003 Feb;48(2):418-429.

308. De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, et al. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs. 2003;174(3):101-109.

309. Diekman BO, Rowland CR, Lennon DP, Caplan AI, Guilak F. Chondrogenesis of adult stem cells from adipose tissue and bone marrow: induction by growth factors and cartilage-derived matrix. Tissue Eng Part A. 2010 Feb;16(2):523-533.

310. Afizah H, Yang Z, Hui JH, Ouyang HW, Lee EH. A comparison between the chondrogenic potential of human bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs) taken from the same donors. Tissue Eng. 2007 Apr;13(4):659-666.

311. Mehlhorn AT, Niemeyer P, Kaiser S, Finkenzeller G, Stark GB, Südkamp NP, et al. Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. Tissue Eng. 2006 Oct;12(10): 2853-2862.

312. Peng L, Jia Z, Yin X, Zhang X, Liu Y, Chen P, et al. Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. Stem Cells Dev. 2008 Aug;17(4): 761-773.

313. Lin L, Shen Q, Wei X, Hou Y, Xue T, Fu X, et al. Comparison of osteogenic potentials of BMP4 transduced stem cells from autologous bone marrow and fat tissue in a rabbit model of calvarial defects. Calcif Tissue Int. 2009 Jul;85(1):55-65.

314. Liu TM, Martina M, Hutmacher DW, Hui JH, Lee EH, Lim B. Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages. Stem Cells. 2007 Mar;25(3):750-760.

315. Noël D, Caton D, Roche S, Bony C, Lehmann S, Casteilla L, et al. Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. Exp Cell Res. 2008 Apr;314(7):1575-1584.

316. Kim Y, Kim H, Cho H, Bae Y, Suh K, Jung J. Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia.Cell Physiol Biochem. 2007;20(6):867-876.

317. Han J, Koh YJ, Moon HR, Ryoo HG, Cho CH, Kim I, et al. Adipose tissue is an extramedullary reservoir for functional hematopoietic stem and progenitor cells. Blood. 2010 Feb;115(5):957-964.

318. Bai X, Yan Y, Song YH, Seidensticker M, Rabinovich B, Metzele R, et al. Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. Eur Heart J. 2010 Feb;31(4):489-501.

319. Schenke-Layland K, Strem BM, Jordan MC, Deemedio MT, Hedrick MH, Roos KP, et al. Adipose tissue-derived cells improve cardiac function following myocardial infarction. Surg Res. 2009 May;153(2):217-223.

320. Valina C, Pinkernell K, Song YH, Bai X, Sadat S, Campeau RJ, et al. Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. Eur Heart J. 2007 Nov;28(21):2667-2677.

321. Ichim TE, Harman RJ, Min WP, Minev B, Solano F, Rodriguez JP, et al. Autologous stromal vascular fraction cells: a tool for facilitating tolerance in rheumatic disease. Cell Immunol. 2010;264(1):7-17

322. Riordan NH, Ichim TE, Min WP, Wang H, Solano F, Lara F, et al. Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. J Transl Med. 2009 Apr;7:29.

323. Rhee SC, Ji YH, Dhong ES, Park SH, Yoon ES. *In vivo* evaluation of mixtures of uncultured freshly-isolated adipose-derived stem cells and demineralized bone matrix for bone regeneration in a rat critically-sized calvarial defect model. Stem Cells Dev. 2011 Feb;20(2):233-242.

324. ClinicalTrials.org; [updated 28 June 2008, cited 20 August 2010] Available at http://www.clinicaltrials.gov /ct2/show/NCT00703599.

325. Bosse R, Singhofer-Wowra M, Rosenthal F, Schulz G. Good manufacturing practice production of human stem cells for somatic cell and gene therapy. Stem Cells. 1997;15 Suppl 1:275-280.

326. Lendeckel S, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, et al. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. J Craniomaxillofac Surg. 2004 Dec;32(6):370-373.

327. García-Olmo D, García-Arranz M, García LG, Cuellar ES, Blanco IF, Prianes LA, et al. Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy. Int J Colorectal Dis. 2003 Sep;18(5):451-454.

328. Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, et al. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. Dis Colon Rectum. 2009 Jan;52(1):79-86.

329. Sanz-Ruiz R, Fernández-Santos E, Domínguez-Muñoa M, Parma R, Villa A, Fernández L, et al. Early translation of adipose-derived cell therapy for cardiovascular disease. Cell Transplant. 2009;18(3):245-254.

330. Baptista LS, da Silva KR, da Pedrosa CS, Claudio-da-Silva C, Carneiro JR, Aniceto M, et al. Adipose tissue of control and ex-obese patients exhibit differences in blood vessel content and resident mesenchymal stem cell population. Obes Surg. 2009 Sep;19(9):1304-1312.

331. Cao Y. Angiogenesis modulates adipogenesis and obesity. J Clin Invest. 2007 Sep;117(9): 2362-2368.

332. Athyros VG, Tziomalos K, Karagiannis A, Anagnostis P, Mikhailidis DP. Should adipokines be considered in the choice of the treatment of obesity-related health problems? Curr Drug Targets. 2010 Jan;11(1):122-135.

333. Marzano UG. Lorenz Heister's "molar gland". Plast Reconstr Surg. 2005 Apr;115(5):1389-1393.

334. Ranke H. Ein saugpolster in der menschlichen backe. Arch Pathol Anat Physiol Klin Med. 1884;97:527-529.

335. Tostevin PJM, Ellis H. The buccal fat pad: a review. Clin Anat. 1995;8(6):403-406.

336. Zhang HM, Yan YP, Qi KM, Wang JQ, Liu ZF. Anatomical structure of the buccal fat pad and its clinical adaptations. Plast Reconstr Surg. 2002 Jun;109(7):2509-2518.

337. Stuzin JM, Wagstrom L, Kawamoto HK, Baker TJ, Wolfe SA. The anatomy and clinical applications of the buccal fat pad. Plast Reconstr Surg. 1990 Jan;85(1):29-37.

338. Stuzin JM, Wagstrom L, Kawamoto HK, Wolfe SA. Anatomy of the frontal branch of the facial nerve: the significance of the temporal fat pad. Plast Reconstr Surg. 1989 Feb;83(2):265-271.

339. Samman N, Cheung LK, Tideman H. The buccal fat pad in oral reconstruction. Int J Oral Maxillofac Surg. 1993 Feb;22(1):2-6.

340. De Wijn RS, van der Heijden EP, Kon M. On lipoma of the buccal fat pad: report of two cases and review of the literature. J Plast Reconstr Aesthet Surg. 2009 Jan;62(1):28-35.

341. Jackson IT. Anatomy of the buccal fat pad and its clinical significance. Plast Reconstr Surg. 1999 Jun;103(7):2059-60; discussion 2061-2063.

342. Levine JA, Ray A, Jensen MD. Relation between chubby cheeks and visceral fat. N Engl J Med. 1998 Dec 24;339(26):1946-1947.

343. Egyedi P. Utilization of the buccal fat pad for closure of oro-antral and/or oro-nasal communications. J Maxillofac Surg. 1977 Nov;5(4):241-244.

344. Neder A. Use of buccal fat pad for grafts. Oral Surg Oral Med Oral Pathol. 1983 Apr;55(4):349-350.

345. Fan L, Chen G, Zhao S, Hu J. Clinical application and histological observation of pedicled buccal fat pad grafting. Chin Med J (Engl). 2002 Oct;115(10):1556-1559.

346. Rapidis AD, Alexandridis CA, Eleftheriadis E, Angelopoulos AP. The use of the buccal fat pad for reconstruction of oral defects: review of the literature and report of 15 cases. J Oral Maxillofac Surg. 2000 Feb;58(2):158-163.

347. Liversedge RL, Wong K. Use of the buccal fat pad in maxillary and sinus grafting of the severely atrophic maxilla preparatory to implant reconstruction of the partially or completely edentulous patient: technical note. Int J Oral Maxillofac Implants. 2002 May-Jun;17(3):424-428.

348. Poeschl PW, Baumann A, Russmueller G, Poeschl E, Klug C, Ewers R. Closure of oroantral communications with Bichat's buccal fat pad. J Oral Maxillofac Surg. 2009 Jul;67(7):1460-1466.

349. Kumari BN, Thiagarajan R, Narayanan V, Devadoss P, Mammen B, Emmadi P. A new technique for root coverage using buccal fat pad--a short case report. Quintessence Int. 2010 Jul-Aug;41(7):547-549.

350. Hassani A, Khojasteh A, Alikhasi M, Vaziri H. Measurement of volume changes of sinus floor augmentation covered with buccal fat pad: a case series study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009 Mar;107(3):369-374.

351. Tamura E, Fukuda H, Tabata Y, Nishimura M. Use of the buccal fat [corrected] pad for vocal cord augmentation. Acta Otolaryngol. 2008 Feb;128(2):219-224.

352. Baumann A, Ewers R. Application of the buccal fat pad in oral reconstruction. J Oral Maxillofac Surg. 2000 Apr;58(4):389-392; discussion 392-393.

353. Tapia A, Ruiz-de-Erenchun R, Rengifo M. Combined approach for facial contour restoration: treatment of malar and cheek areas during rhytidectomy. Plast Reconstr Surg. 2006 Aug;118(2):491-497.

354. Janjanin S, Djouad F, Shanti RM, Baksh D, Gollapudi K, Prgomet D, et al. Human palatine tonsil: a new potential tissue source of multipotent mesenchymal progenitor cells. Arthritis Res Ther. 2008;10(4):R83.

355. Harting M, Jimenez F, Pati S, Baumgartner J, Cox C Jr. Immunophenotype characterization of rat mesenchymal stromal cells. Cytotherapy. 2008;10(3):243-253.

356. Bajada S, Mazakova I, Richardson JB, Ashammakhi N. Updates on stem cells and their applications in regenerative medicine. J Tissue Eng Regen Med. 2008 Jun;2(4):169-183.

357. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and differentiation. Methods. 2008 Jun;45(2):115-120.

358. Amin MA, Bailey BM, Swinson B, Witherow H. Use of the buccal fat pad in the reconstruction and prosthetic rehabilitation of oncological maxillary defects. Br J Oral Maxillofac Surg. 2005 Apr;43(2):148-154.

359. Lin CS, Xin ZC, Deng CH, Ning H, Lin G, Lue TF. Defining adipose tissue-derived stem cells in tissue and in culture. Histol Histopathol. 2010 Jun;25(6):807-815.

360. Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. Stem Cells. 2007 Apr;25(4):818-827.

361. Mehrotra D, Pradhan R, Gupta S. Retrospective comparison of surgical treatment modalities in 100 patients with oral submucous fibrosis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009 Mar;107(3):e1-10.

362. Pilz GA, Ulrich C, Ruh M, Abele H, Schäfer R, Kluba T, et al. Human term placenta-derived mesenchymal stromal cells are less prone to osteogenic differentiation than bone marrow-derived mesenchymal stromal cells. Stem Cells Dev. 2011 Apr; 20(4):635-646.

363. Amos PJ, Shang H, Bailey AM, Taylor A, Katz AJ, Peirce SM. IFATS collection: The role of human adipose-derived stromal cells in inflammatory microvascular remodeling and evidence of a perivascular phenotype. Stem Cells. 2008 Oct;26(10):2682-2690.

364. Yañez R, Lamana ML, García-Castro J, Colmenero I, Ramírez M, Bueren JA. Adipose tissue-derived mesenchymal stem cells have *in vivo* immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells. 2006 Nov;24(11):2582-2591.

365. Graziano A, d'Aquino R, Laino G, Proto A, Giuliano MT, Pirozzi G, et al. Human CD34+ stem cells produce bone nodules *in vivo*. Cell Prolif. 2008 Feb;41(1):1-11.

366. Schipper BM, Marra KG, Zhang W, Donnenberg AD, Rubin JP. Regional anatomic and age effects on cell function of human adipose-derived stem cells. Ann Plast Surg. 2008 May;60(5):538-544.

367. Zheng B, Cao B, Li G, Huard J. Mouse adipose-derived stem cells undergo multilineage differentiation *in vitro* but primarily osteogenic and chondrogenic differentiation *in vivo*. Tissue Eng. 2006 Jul;12(7):1891-1901.

368. Morrison RF, Farmer SR. Hormonal signaling and transcriptional control of adipocyte differentiation. J Nutr. 2000 Dec;130(12):3116S-3121S.

369. Flynn LE, Prestwich GD, Semple JL, Woodhouse KA. Proliferation and differentiation of adipose-derived stem cells on naturally derived scaffolds. Biomaterials. 2008 Apr;29(12): 1862-1871.

370. Gupta A, Leong DT, Bai HF, Singh SB, Lim TC, Hutmacher DW. Osteo-maturation of adipose-derived stem cells required the combined action of vitamin D3, beta-glycerophosphate, and ascorbic acid. Biochem Biophys Res Commun. 2007 Oct;362(1):17-24.

371. Astori G, Vignati F, Bardelli S, Tubio M, Gola M, Albertini V, et al. "*In vitro*" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. J Transl Med. 2007 Oct;5:55.

372. Anjos-Afonso F, Bonnet D. Nonhematopoietic/endothelial SSEA-1+ cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. Blood. 2007 Feb;109(3):1298-1306.

373. De Ugarte DA, Ashjian PH, Elbarbary A, Hedrick MH. Future of fat as raw material for tissue regeneration. Ann Plast Surg. 2003 Feb;50(2):215-219.

374. Tjabringa GS, Vezeridis PS, Zandieh-Doulabi B, Helder MN, Wuisman PI, Klein-Nulend J. Polyamines modulate nitric oxide production and COX-2 gene expression in response to mechanical loading in human adipose tissue-derived mesenchymal stem cells. Stem Cells. 2006 Oct;24(10):2262-2269.

375. Tjabringa GS, Zandieh-Doulabi B, Helder MN, Knippenberg M, Wuisman PI, Klein-Nulend J. The polymine spermine regulates osteogenic differentiation in adipose stem cells.J Cell Mol Med. 2008 Sep-Oct;12(5A):1710-1717.

376. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. Bone Morphogenetic Proteins: A critical review. Cell Signal. 2011 Apr;23(4):609-620.

377. Knippenberg M, Helder MN, Doulabi BZ, Bank RA, Wuisman PI, Klein-Nulend J. Differential effects of bone morphogenetic protein-2 and transforming growth factor-beta1 on gene expression of collagen-modifying enzymes in human adipose tissue-derived mesenchymal stem cells. Tissue Eng Part A. 2009 Aug;15(8):2213-2225.

378. Jeon O, Rhie JW, Kwon IK, Kim JH, Kim BS, Lee SH. *In vivo* bone formation following transplantation of human adipose-derived stromal cells that are not differentiated osteogenically. Tissue Eng Part A. 2008 Aug;14(8):1285-1294.

379. He D, Genecov DG, Herbert M, Barcelo R, Elsalanty ME, Weprin BE, et al. Effect of recombinant human bone morphogenetic protein-2 on bone regeneration in large defects of the growing canine skull after dura mater replacement with a dura mater substitute. J Neurosurg. 2010 Feb;112(2):319-328.

380. E LL, Xu LL, Wu X, Wang DS, Lv Y, Wang JZ, et al. The interactions between rat-adipose-derived stromal cells, recombinant human bone morphogenetic protein-2, and beta-tricalcium phosphate play an important role in bone tissue engineering. Tissue Eng Part A. 2010 Sep;16(9):2927-2940.

381. Cowan CM, Aalami OO, Shi YY, Chou YF, Mari C, Thomas R. Bone morphogenetic protein 2 and retinoic acid accelerate *in vivo* bone formation, osteoclast recruitment, and bone turnover. Tissue Eng. 2005 Mar-Apr;11(3-4):645-645.

382. Maegawa N, Kawamura K, Hirose M, Yajima H, Takakura Y, Ohgushi H. Enhancement of osteoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2). J Tissue Eng Regen Med. 2007 Jul-Aug;1(4):306-313.

383. Samartzis D, Khanna N, Shen FH, An HS. Update on bone morphogenetic proteins and their application in spine surgery. J Am Coll Surg. 2005 Feb;200(2):236-234.

384. Burkus JK. Surgical treatment of the painful motion segment: matching technology with indications. Spine (Phila Pa 1976). 2005 Aug;30(16 Suppl):S7-15.

385. Perri B, Cooper M, Lauryssen C, Anand N. Adverse swelling associated with use of rh-BMP-2 in anterior cervical discectomy and fusion: adiscectomy and fusion: a case study. Spine J. 2007 Mar-Apr;7(2):235-239.

386. Lee SJ, Kang SW, Do HJ, Han I, Shin DA, Kim JH, et al. Enhancement of bone regeneration by gene delivery of BMP2/Runx2 bicistronic vector into adipose-derived stromal cells. Biomaterials. 2010 Jul;31(21):5652-5659.

387. Hao W, Dong J, Jiang M, Wu J, Cui F, Zhou D. Enhanced bone formation in large segmental radial defects by combining adipose-derived stem cells expressing bone morphogenetic protein 2 with nHA/RHLC/PLA scaffold. Int Orthop. 2010 Dec;34(8):1341-1349.

388. Nayak S, Herzog RW. Progress and prospects: immune responses to viral vectors. Gene Ther. 2010 Mar;17(3):295-304.

389. Liu SB, Hu PZ, Hou Y, Li P, Cao W, Tian Q. Recombinant human bone morphogenetic protein-2 promotes the proliferation of mesenchymal stem cells *in vivo* and *in vitro*. Chin Med J (Engl). 2009 Apr;122(7):839-843.

390. Lou J, Xu F, Merkel K, Manske P. Gene therapy: adenovirus-mediated human bone morphogenetic protein-2 gene transfer induces mesenchymal progenitor cell proliferation and differentiation *in vitro* and bone formation *in vivo*. J Orthop Res. 1999 Jan;17(1):43-50.

391. Tachi K, Takami M, Zhao B, Mochizuki A, Yamada A, Miyamoto Y, et al. Bone morphogenetic protein 2 enhances mouse osteoclast differentiation via increased levels of receptor activator of NF-κB ligand expression in osteoblasts. Cell Tissue Res. 2010 Nov;342(2):213-220.

392. Raida M, Heymann AC, Günther C, Niederwieser D. Role of bone morphogenetic protein 2 in the crosstalk between endothelial progenitor cells and mesenchymal stem cells. Int J Mol Med. 2006 Oct;18(4):735-739.

393. Hattori H, Sato M, Masuoka K, Ishihara M, Kikuchi T, Matsui T, et al. Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cell source. Cells Tissues Organs. 2004;178(1):2-12.

394. Vidal MA, Kilroy GE, Lopez MJ, Johnson JR, Moore RM, Gimble JM. Characterization of equine adipose tissue-derived stromal cells: adipogenic and

osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. Vet Surg. 2007 Oct;36(7): 613-622.

395. Lendeckel S, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, et al. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. J Craniomaxillofac Surg. 2004 Dec;32(6):370-373.

396. Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010 Aug;466(7308):829-834.

397. Saleh FA, Whyte M, Ashton P, Genever PG. Regulation of Mesenchymal Stem Cell Activity by Endothelial Cells. Stem Cells Dev. 2010.

398. Zerbo IR, Zijderveld SA, de Boer A, Bronckers AL, de Lange G, ten Bruggenkate CM, et al. Histomorphometry of human sinus floor augmentation using a porous beta-tricalcium phosphate: a prospective study. Clin Oral Implants Res. 2004 Dec;15(6):724-732.

399. Lu JX, Gallur A, Flautre B, Anselme K, Descamps M, Thierry B. Comparative study of tissue reactions to calcium phosphate ceramics among cancellous, cortical, and medullar bone sites in rabbits. J Biomed Mater Res. 1998 Dec;42(3):357-367.

400. Friedmann A, Dard M, Kleber BM, Bernimoulin JP, Bosshardt DD. Ridge augmentation and maxillary sinus grafting with a biphasic calcium phosphate: histologic and histomorphometric observations. Clin Oral Implants Res. 2009 Jul;20(7):708-714.

401. Saldaña L, Sánchez-Salcedo S, Izquierdo-Barba I, Bensiamar F, Munuera L, Vallet-Regí M, et al. Calcium phosphate-based particles influence osteogenic maturation of human mesenchymal stem cells. Acta Biomater. 2009 May;5(4):1294-1305.

402. Guha AK, Singh S, Kumaresan R, Nayar S, Sinha A. Mesenchymal cell response to nanosized biphasic calcium phosphate composites. Colloids Surf B Biointerfaces. 2009 Oct;73(1):146-151.

403. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al. Progressive development of the rat osteoblast phenotype *in vitro*: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol. 1990 Jun;143(3):420-430.

404. Yao KL, Todescan R Jr, Sodek J. Temporal changes in matrix protein synthesis and mRNA expression during mineralized tissue formation by adult rat bone marrow cells in culture. J Bone Miner Res. 1994 Feb;9(2):231-240.

405. Tracy RP, Andrianorivo A, Riggs BL, Mann KG. Comparison of monoclonal and polyclonal antibody-based immunoassays for osteocalcin: a study of sources of variation in assay results. J Bone Miner Res. 1990 May;5(5):451-461.

406. Malaval L, Modrowski D, Gupta AK, Aubin JE. Cellular expression of bone-related proteins during *in vitro* osteogenesis in rat bone marrow stromal cell cultures. J Cell Physiol. 1994 Mar;158(3):555-572.

407. Marot D, Knezevic M, Novakovic GV. Bone tissue engineering with human stem cells. Stem Cell Res Ther. 2010 May 4;1(2):10.

408. Matsumoto T, Okazaki M, Inoue M, Yamaguchi S, Kusunose T, Toyonaga T, et al. Hydroxyapatite particles as a controlled release carrier of protein. Biomaterials. 2004 Aug;25(17):3807-3812.

409. Edwards RB 3rd, Seeherman HJ, Bogdanske JJ, Devitt J, Vanderby R Jr, Markel MD. Percutaneous injection of recombinant human bone morphogenetic protein-2 in a calcium phosphate paste accelerates healing of a canine tibial osteotomy. Bone Joint Surg Am. 2004 Jul;86-A(7):1425-1438.

410. Shields LB, Raque GH, Glassman SD, Campbell M, Vitaz T, Harpring J, et al. Adverse effects associated with high-dose recombinant human bone morphogenetic protein-2 use in anterior cervical spine fusion. Spine (Phila Pa 1976). 2006 Mar;31(5):542-547.

411. Smith DM, Cooper GM, Mooney MP, Marra KG, Losee JE. Bone morphogenetic protein 2 therapy for craniofacial surgery. J Craniofac Surg. 2008 Sep;19(5):1244-1259.

412. Kühne JH, Bartl R, Frisch B, Hammer C, Jansson V, Zimmer M. Bone formation in coralline hydroxyapatite. Effects of pore size studied in rabbits. Acta Orthop Scand. 1994 Jun;65(3): 246-252.

413. Colton CK. Implantable biohybrid artificial organs. Cell Transplant. 1995 Jul-Aug;4(4):415-436.

414. Rüegsegger P, Koller B, Müller R. A microtomographic system for the nondestructive evaluation of bone architecture. Calcif Tissue Int. 1996 Jan;58(1):24-29.

415. Müller R. Hierarchical microimaging of bone structure and function. Nat Rev Rheumatol. 2009 Jul;5(7):373-381.

# Chapter 12 APPENDIX

**INFORMED CONSENT APPROVED BY ETHICAL COMMITTEE FROM UIC**

Universitat Internacional de Catalunya

La Comisión Científica del Departamento de Odontología de la Universitat Internacional de Catalunya, CERTIFICA, que

El presente estudio de investigación, titulado: "Estudio del potencial osteogénico de células mesenquimales adultas derivadas de tejido humano adiposo", cuyo investigador principal es la alumna de Doctorado, Dña. Elisabeth Farre Guash, y cuyo tutor es el Dr. Carles Martí,

Cumple los siguientes requisitos para llevarse a cabo en la CUO:

-Aprobación del Comité de Ética del Departamento de Odontología, conforme se consideran garantizados los derechos del paciente, previa revisión del consentimiento informado y hoja de información al paciente.

-Aprobación del protocolo, conforme se adecuan los materiales y métodos a los objetivos previstos.

-Aprobación de la ampliación del Seguro de la Clínica específico para este estudio. Al tratarse éste, de un estudio observacional, no requiere seguro específico.

-Aprobación económica del presupuesto de dicho estudio: no hay prestación económica prevista en este estudio.

Para que así conste, lo firmo en Sant Cugat del Vallés, a 15 de abril de dos mil siete.

Universitat Internacional de Catalunya

Dr. Miquel Cortada Colomer
Director de la Comisión Científica

DEPARTAMENT D'ODONTOLOGIA

## HOJA DE INFORMACION AL PACIENTE

***TITULO***: "Estudio del potencial osteogénico de células mesenquimales adultas derivadas de tejido humano".

Se ha solicitado su participación en un estudio de investigación. Antes de decidir si desea participar es importante que comprenda en qué consistirá el estudio.

**¿Cuál es el objetivo del estudio?**

El objetivo es encontrar una fuente óptima de células mesenquimales adultas para estudiar su capacidad de transformarse en tejido óseo. Es un estudio de laboratorio con material de rechazo con lo cual no precisamos la colaboración activa del paciente a nivel control postoperatorio y seguimiento.

*Dentro del plan de tratamiento que hemos diseñado para usted en el Área de Cirugía Ortognática de la Universidad Internacional de Catalunya* se precisa utilizar la bola adiposa de Bichat, material que se rechaza, con el objetivo de llevar a cabo nuestra investigación. Se agradecería su colaboración para permitirnos analizar en profundidad esta estructura anatómica.

Para completar el estudio se necesita comparar los resultados con un grupo control que serán aquellos pacientes sometidos a una lipectomía y se analizará el material de rechazo (grasa).

**¿Cómo será el postoperatorio?**

Sin diferencias en relación a la cirugía de esta intervención. Puesto que es un material de rechazo no implica ningún acto quirúrgico extra y el postoperatorio será el mismo que el que implica la cirugía a realizar.

**¿Qué le pedimos a usted?**

Colaboración en este proyecto de investigación entregando sus muestras de tejido adiposo que se desecharían a nuestro laboratorio para el estudio del potencial de diferenciación ósea de estas células.

En caso de dudas diríguese a Dr Carles Martí los miércoles en el teléfono 935042000 o los otros días a Dra Elisabet Farré en el mismo número.

**HOJA DE CONSENTIMIENTO INFORMADO**

_____

***TITULO***: "Estudio del potencial osteogénico de células mesenquimales adultas derivadas de tejido humano adiposo".

Yo, Sr/a……..

- He recibido información verbal acerca del estudio anterior y he leído la información escrita que se adjunta.
- He comprendido lo que se me ha explicado.
- He podido comentar el estudio y realizar preguntas al profesional responsable.
- Doy mi consentimiento para tomar parte en el estudio y asumo que mi participación es totalmente voluntaria.
- Entiendo que podré retirarme en cualquier momento sin que ello afecte a mi futura asistencia médica.

Mediante la firma de este formulario de consentimiento informado, doy mi consentimiento para que mis datos personales se puedan utilizar como se ha descrito en este formulario de consentimiento.

Entiendo que recibiré una copia de este formulario de consentimiento informado.

Firma del paciente. Fecha de la firma.

**PATIENT INFORMATION FROM VRIJE UNIVERSITEIT VAN AMSTERDAM MEDISCHE CENTRUM (VUMC)**

Geachte mevrouw/mijnheer,

Bij u wordt binnenkort een operatieve ingreep verricht waarbij vetweefsel vrijkomt. Middels deze brief vragen wij u om medewerking aan zeer belangrijk wetenschappelijk onderzoek door toestemming te geven dat een kleine hoeveelheid van het vrijgekomen vetweefsel wordt gebruikt voor wetenschappelijk onderzoek.

In het VU medisch centrum (VUmc) wordt veel aandacht besteed aan onderzoek naar nieuwe behandelingsmethoden en geneesmiddelen. Dit is het geval voor hart en vaat ziekten, en dan voornamelijk naar het afsterven van hartspiercellen na een hartinfarct, en ook voor ziekten van het bot en kraakbeen, ontstaan door bijvoorbeeld slijtage of ongevallen. Als samenwerkende afdelingen Pathologie, Cardiologie, Orale Celbiologie en de Plastische Chirurgie houden wij ons intensief bezig met het ontwikkelen van nieuwe behandelingsmethoden voor mensen met ziekten van het hart en het bewegingsapparaat.

**Stamcellen in wetenschappelijk onderzoek**
Het gebruik van stamcellen (ongerijpte cellen die meerdere celtypen kunnen worden) voor klinische doeleinden staat de laatste jaren steeds meer in de belangstelling. De reden hiervoor is dat deze cellen nog de mogelijkheid hebben om verschillende weefsels te vormen met een uiteindelijke klinische toepassing.

Recent onderzoek heeft aangetoond dat in menselijk vetweefsel veel stamcellen aanwezig zijn die zich kunnen ontwikkelen tot (hart)spiercellen, vetcellen, bot en kraakbeen. Dit biedt nieuwe mogelijkheden voor nieuwe therapieën voor ziekten als artritis, hernia en hartinfarcten. In onze laboratoria in het VUmc wordt onderzocht hoe deze cellen het beste geïsoleerd en gebruikt kunnen worden voor de nieuwe behandelingsmethodes. Het zwaartepunt van ons onderzoek richt zich op de vorming van nieuwe hartspiercellen, bot en kraakbeen uit deze stamcellen.

Met behulp van deze brief willen wij u informatie geven over het doel van het onderzoek en de te gebruiken onderzoeksprocedure. Met behulp van deze informatie kunt u beslissen og u vetweefsel wilt afstaan voor dit belangrijke wetenschappelijke onderzoek.

**Verschillende doelen van het onderzoek**
In onze laboratoria worden de stamcellen geïsoleerd uit het vetweefsel en deze cellen worden gestimuleerd tot de vorming van nieuwe weefsels. Enkele doelen van de verschillende projecten zijn:

- Optimalisatie van de isolatieprocedure van de stamcellen uit het vetweefsel
- Het verbeteren van de stimulatie van de stamcellen zodat ze nog beter de verschillende weefsels vormen
- Er zal onderzocht worden of deze cellen uiteindelijk in de kliniek toegepast kunnen worden

**Wat betekent meedoen voor u?**

Als u aan het onderzoek deelneemt zal een kleine hoeveelheid van het vrijgekomen vetweefsel dat tijdens de ingreep vrijkomt verzonden worden naar het VUmc en daar zullen de stamcellen geïsoleerd worden uit dit weefsel. Er zal geen extra vet worden afgenomen en ook geen extra ingreep plaatsvinden. Omdat er alleen gebruik wordt gemaakt van het vetweefsel dat vrijkomt en geen extra ingreep plaatsvindt, zitten er aan dit verzoek geen extra bijwerkingen of nadelen voor u.

Er is ook geen voordeel voor u persoonlijk als u besluit mee te doen met dit onderzoek. Uw deelname is echter wel van groot wetenschappelijk belang voor het ontwikkelen van nieuwe

behandelingsmethoden en geneesmiddelen. Door uw deelname kunnen toekomstige patiënten met aandoeningen aan het hart, bot of kraakbeen, mogelijk wel heel erg gebaat zijn.

**Vertrouwelijkheid van uw gegeven**
De gegevens die in het kader van het onderzoek over u verzameld worden zullen vertrouwelijk behandeld worden. Ook zullen ze op aparte formulieren ingevuld worden, waar alleen een nummer voorkomt en niet uw naam of andere persoonlijke gegevens als uw adres.

**Vrijwilligheid van deelname**
Om dit onderzoek te kunnen doen hebben we uw toestemming nodig om het vrijgekomen vetweefsel te mogen gebruiken. Uw beslissing om wel of niet mee te werken aan dit onderzoek is vanzelfsprekend niet van invloed op de aandacht en zorg die de artsen en verpleegkundigen voor u hebben.

**Ondertekening van het toestemmingsformulier (informed consent)**
Als u besluit mee te werken, vragen wij u het toestemmingsformulier te ondertekenen dat aan u overhandigd wordt. Hiermee bevestigt u uw voornemen om bij het de operatie vrijgekomen vetweefsel aan dit onderzoek af te staan.

Met vriendelijke groet,

De afdelingen Pathologie, Orale Celbiologie, Cardiologie en Plastische Chirurgie van het VU medisch centrum

**INFORMED CONSENT FROM FROM VRIJE UNIVERSITEIT VAN AMSTERDAM MEDISCHE CENTRUM (VUMC)**

Bij u wordt binnenkort in ons centrum een operatieve ingreep verricht waarbij vetweefsel vrijkomt. Ons centrum verleent medewerking aan onderzoek van het VU medisch centrum (VUmc) waarvoor vetweefsel nodig is. Voor u persoonlijk zijn er geen voor- of nadelen verbonden aan het beschikbaar stellen van het vrijgekomen vetweefsel voor dit zeer belangrijke wetenschappelijk onderzoek. Door dit formulier te ondertekenen verleend u toestemming om het vetweefsel beschikbaar te stellen.

Ik verklaar hierbij op voor mij duidelijke wijze, mondeling en/of schriftelijk, te zijn ingelicht over de aard, methode en doel van het onderzoek. Mijn vragen zijn naar tevredenheid beantwoord. De schriftelijke informatie, behorend bij deze verklaring, is aan mij overhandigd.

Ik stem geheel vrijwillig in het deelname aan dit onderzoek. Ik behoud daarbij het recht deze instemming weer in te trekken zonder dat ik daarvoor een aanwijsbare reden dien op te geven.

Ik heb geen bezwaar tegen het geanonimiseerd bewaren van eventueel overblijvende cellen en/of vetweefsel voor nader wetenschappelijk onderzoek.

Naam: …… Plaats en datum: ……

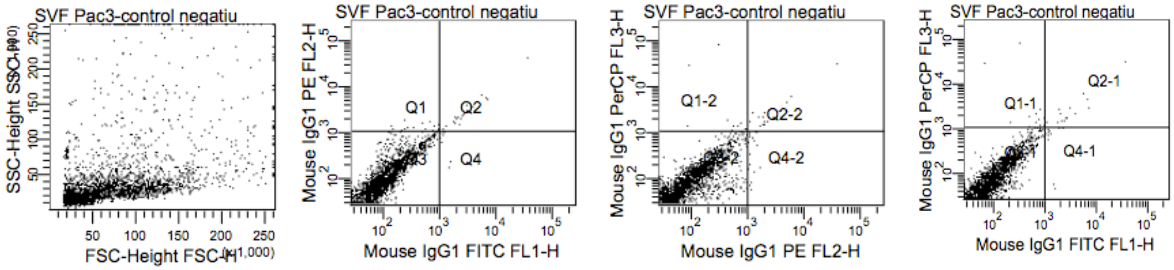
Handtekening: ……

**Patientgegevens (anoniem)**

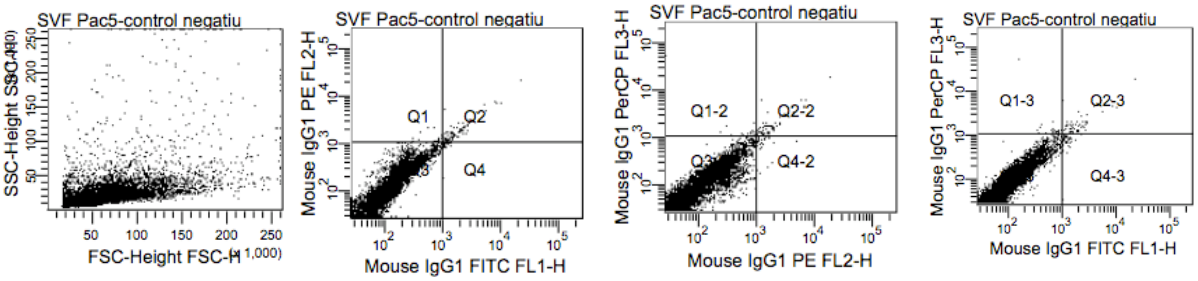
Geboortedatum: ……
Geslacht: m / v
Lengte: ……
Gewicht: ……
Roken: ja / nee
Medicijngebruik: ……
……
……
……

## ISOTYPE CONTROLS OF FRESH STROMAL FRACTION (SVF) FROM BUCCAL FAT PAD BY FLOW CYTOMETRY

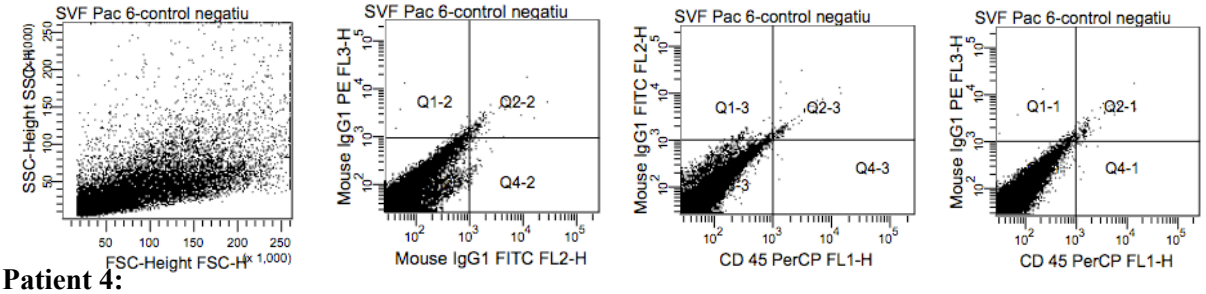
**Patient 1:**



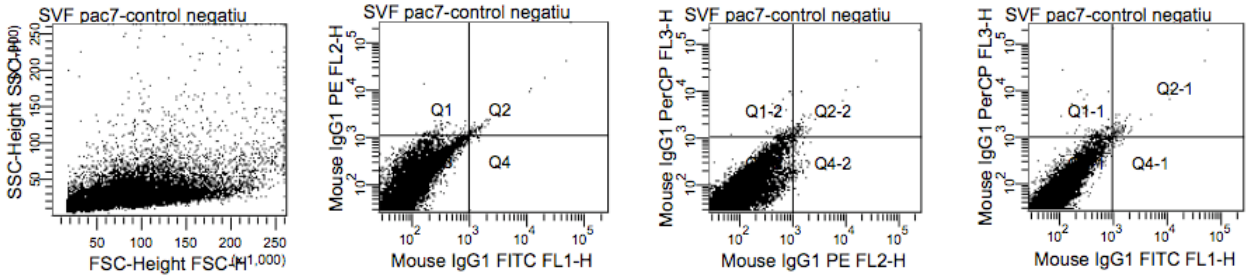

**Patient 2:**



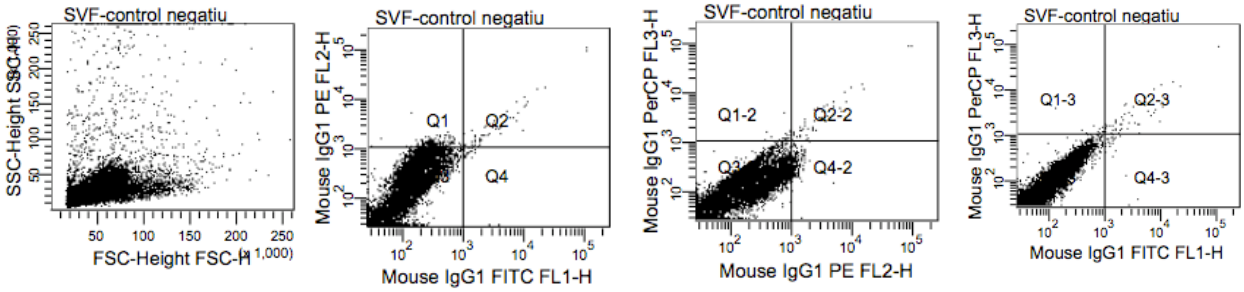

**Patient 3:**




**Patient 4:**

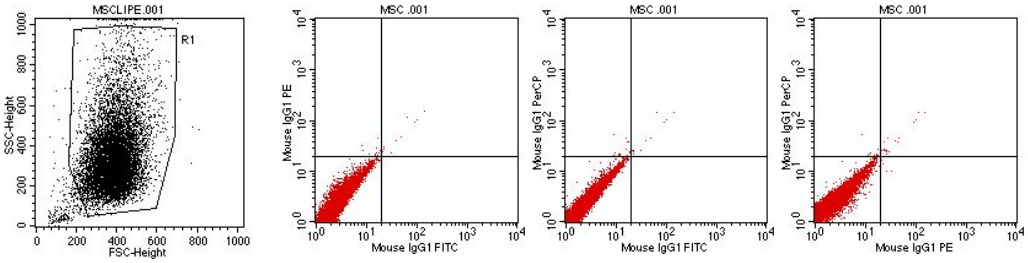



**Patient 5:**

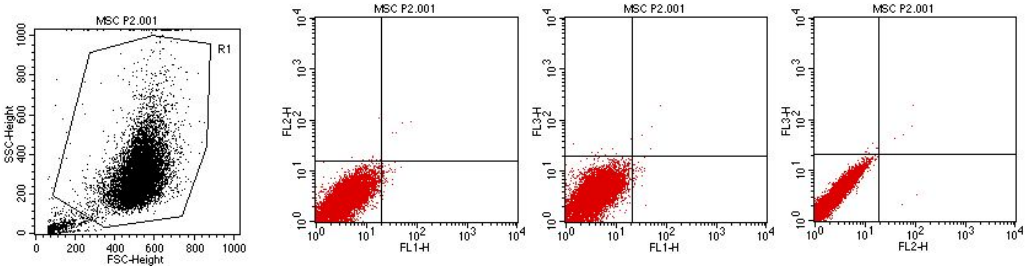


## ISOTYPE CONTROLS OF ADIPOSE STEM CELLS FROM SUBCUTANEOUS ABDOMINAL ADIPOSE TISSUE

**Passage 0:**

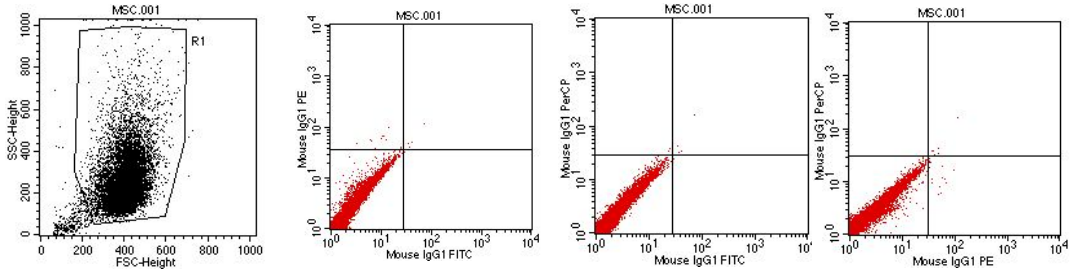



**Passage 2:**

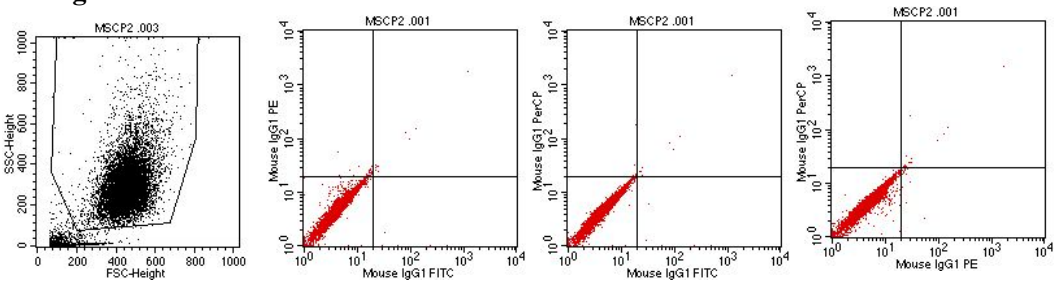



## ISOTYPE CONTROLS OF ADIPOSE STEM CELLS FROM ASC FROM BUCCAL FAT PAD

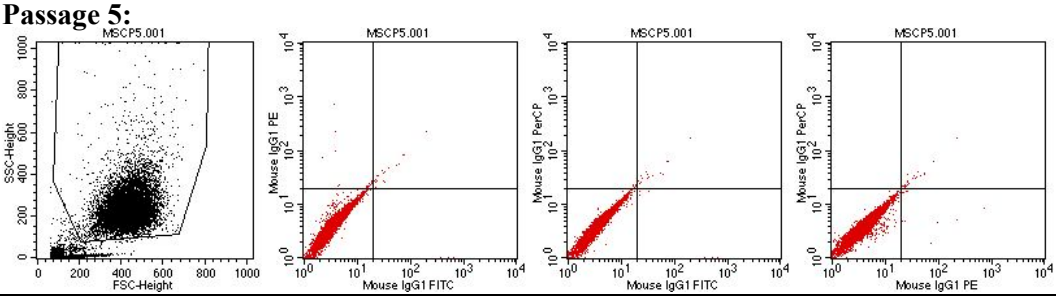
**Passage 0:**




**Passage 2:**




**Passage 5:**



TISSUE ENGINEERING: Part C
Volume 16, Number 5, 2010

DOI: 10.1089/ten.tec.2009.0487

# Buccal Fat Pad, an Oral Access Source of Human Adipose Stem Cells with Potential for Osteochondral Tissue Engineering: An *In Vitro* Study

Elisabet Farré-Guasch, D.D.S., M.D.,[1,2] Carles Martí-Pagès, M.D., FEBOMS, Ph.D.,[1,3]
Federico Hernández-Alfaro, D.D.S., M.D., F.E.B.O.M.S., Ph.D.,[1,4]
Jenneke Klein-Nulend, Ph.D.,[5] and Núria Casals, Ph.D.[2,6]

Stem cells offer an interesting tool for tissue engineering, but the clinical applications are limited by donor-site morbidity and low cell number upon harvest. Recent studies have identified an abundant source of stem cells in subcutaneous adipose tissue. Adipose stem cells (ASCs) present in adipose tissue are able to differentiate to several lineages and express multiple growth factors, which makes them suitable for clinical application. Buccal fat pad (BFP), an adipose-encapsulated mass found in the oral cavity, could represent an easy access source for dentists and oral surgeons. The stromal vascular fraction obtained from fresh BFP-derived adipose tissue and passaged ASCs were analyzed to detect and quantify the percentage of ASCs in this tissue. Here we show that BFP contains a population of stem cells that share a similar phenotype with ASCs from abdominal subcutaneous fat tissue, and are also able to differentiate into the chondrogenic, adipogenic, and osteogenic lineage. These results define BFP as a new, rich, and accessible source of ASCs for tissue engineering purposes.

## Introduction

TISSUE ENGINEERING IS an emerging field that allows regeneration with restitution of lost tissues, combining the principles of bioengineering, cell transplantation, and biomaterial engineering. There is increasing biological knowledge regarding human development that will likely allow new future therapies to satisfy the clinical patient's needs.

In the late 1960s Friedenstein *et al.* demonstrated that the mesenchymal stroma from human bone marrow contains a population of cells that proliferate when cultured on plastic and differentiate to cell lineages derived from the mesoderm, such as chondrocytes and osteoblasts.[1] Later, these precursor spindle-shaped cells are referred to as mesenchymal stem cells (MSCs),[2] and shown to differentiate to several lineages *in vitro*[3] and *in vivo*,[4–6] making these cells promising candidates for mesodermal defect repair. However, the clinical use of MSCs provides several problems, such as pain associated with the harvest procedure, complexity of the technique, and low cell number upon purification, especially in old donors.[7] This makes an *ex vivo* expansion step necessary to obtain therapeutic cell doses, which is time consuming and expensive, and contains the risk of cell contamination and cell loss.

Adipose tissue represents a promising source of MSCs available in large quantities, which does not require the use of general anesthesia and results in minimal patient discomfort. Further, adipose tissue yields higher numbers of MSCs than bone marrow, which could avoid cell expansion.[8] The nomenclature of MSCs from adipose tissue varies widely, but the consensus reached at the Second Annual International Fat Applied Technology Society Meeting (2004, Pittsburgh, PA) was to use the term "adipose stem cells" (ASCs). ASCs are able to differentiate to multiple mesenchymal tissue cell types, such as osteoblasts, chondrocytes, adipocytes, myocytes, cardiomyocytes, and endothelial cells, and there is growing evidence suggesting that they can also give rise to cells from other lineages, such as ectoderm and endoderm.[9–12] In addition, adipose tissue secretes a variety of angiogenic and antiapoptotic growth factors that makes fat a promising source for reconstructive surgery.[13] ASCs have

[1]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry and [2]Department of Basic Sciences, Faculty of Medicine and Health Sciences, International University of Catalonia, Barcelona, Spain.
[3]Hospital Clínic de Barcelona, Barcelona, Spain.
[4]Institute of Maxillofacial Surgery and Implantology, Teknon Medical Center, Barcelona, Spain.
[5]Department of Oral Cell Biology, Research Institute MOVE, ACTA-University of Amsterdam and VU University Amsterdam, Amsterdam, The Netherlands.
[6]CIBER Institute of Physiopathology of Obesity and Nutrition (CB06/03), Instituto de Salud Carlos III, Madrid, Spain.

been used successfully in several experimental studies in the bone tissue engineering field.[14–17] Therefore, adipose tissue is an interesting source for cell-based therapy.

The oral cavity contains a mass of specialized fatty tissue, buccal fat pad (BFP) or Bichat's fat pad, that is distinct from subcutaneous fat.[18] The easy accessibility and rich vascularization make this fat mass an attractive graft, which has been used widely in oral surgery for the repair of bone and periodontal defects.[19–22] The harvesting of BFP is a noncomplicated procedure, which requires a minimal incision with local anesthesia, and causes minimal donor-site morbidity.

This study demonstrates that BFP is a rich and accessible source of stem cells that express the characteristic markers of ASCs and that can differentiate to chondrocytes, osteoblasts, and adipocytes.

## Materials and Methods

### *Tissue preparation and cell isolation/expansion*

Subcutaneous abdominal adipose tissue (SC) and BFP were obtained from healthy individuals undergoing elective abdominal and orthognathic surgery procedures in different Hospitals (Hospital Clínic and Hospital Sant Joan de Déu, Barcelona, Spain). The age range of the patients submitted to abdominal surgery was 19–57 years ($n=4$), and the age range of the patients undergoing oral surgery was 17–40 years ($n=10$). All patients were in good health, and no diabetes or other systemic complications were reported. The informed consent and experimental protocols in this study were reviewed and approved by the Ethics Committee of the Universitat Internacional de Catalunya in Sant Cugat (Barcelona, Spain). Sample tissues were processed according to a modification of a previously reported procedure.[23] Raw oral fat tissue ($16 \pm 4.1$ mL) and subcutaneous fat tissue ($22 \pm 12.2$ mL) (mean ± standard deviation [SD]) were washed several times with sterile phosphate-buffered saline (PBS), minced into small pieces, and treated with 0.075% collagenase I (Sigma, St. Louis, MO) for 60 min at 37°C. After incubation, adipose tissue was centrifuged at 400 *g* for 10 min to separate the adipocytes and lipid droplets from the stromal vascular fraction (SVF).

Cell pellets were re-suspended in red blood cell lysis buffer (8.2 g/L $NH_4Cl$, 0.84 g/L $NaHCO_3$, and 0.37 g/L disodium ethylenediaminetetraacetic acid, pH 7.4) and incubated for 10 min at room temperature. SVF cells were resuspended in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 100 units/mL antibiotics/antimycotics solution. Suspended cells were passed through a 100 µm cell strainer (BD Biosciences, Palo Alto, CA), cells were counted, and their viability was assessed with Trypan Blue exclusion. Cells were seeded at $5\times10^3$ cells/cm$^2$ in 100 mm tissue culture dishes and maintained in a humidified incubator at 37°C and 5% $CO_2$. After 3–4 days individual cell colonies were visible upon microscopic examination. The initial adherent cell population, referred to as passage 0 (P0), as well as after several passages (up to 1 month of culture), was analyzed by flow cytometry. After 1 week of culture adherence, cells from BFP ($n=5$) and abdominal fat ($n=4$) were counted, and cell number per gram of adipose tissue was calculated. Cells from BFP in P2 ($n=3$) were induced to differentiate in the adipogenic medium (AM), osteogenic medium (OM), or chondrogenic medium (CM) for several weeks. Differentiation media consisted of high-glucose Dulbecco's modified Eagle's medium and lineage-specific medium supplementation as described[23] (Supplemental Table S1, available online at www.liebertonline.com).

Human osteogenic sarcoma cells (SAOS-2) were obtained from the American Type Culture Collection (ATCC HTB85, Manassas, VA) and cultured in McCoy's 5ª medium (Gibco BRL, Grand Island, NY) containing 15% FBS and used as osteogenic control. Fresh adipose tissue was obtained from BFP as well as from excised abdominal adipose tissue from patients and used as adipogenic control. Chondrocytes derived from human nucleus pulposus were used as chondrogenic control.

### *Flow cytometry*

Flow cytometry was performed with fresh adipose tissue from BFP ($n=5$), and passaged ASCs were obtained from BFP ($n=4$) and SC ($n=4$) from patients undergoing elective abdominal surgery. For flow cytometry analysis, cells were resuspended at $10^6$ cells/mL in the control medium. Fifty microliters of the cell suspension containing $3–5\times10^5$ cells was incubated with 5 µL primary monoclonal antibodies (MAbs) directed against the following antigens and coupled to phycoerythrin (PE), fluorescein isothiocyanate (FITC), or peridinin–chlorophyll–protein complex (PerCP) for 30 min at 4°C: CD14[PE], CD34[PerCP], CD45[PerCP], CD29[PerCP], CD73[PE], SSEA[PE], CD90[FITC], CD146[FITC], HLA-DR[FITC], and CD105[FITC]. All MAbs were purchased from BD Biosciences, except CD105 and CD146, which were purchased from R&D Systems (Minneapolis, MN) and eBioscience (San Diego, CA), respectively.

All samples were washed in PBS containing 2% FBS, fixed with Cell-Fix (BD Biosciences), and incubated on ice for 30 min. Samples were then analyzed by FACScan flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA). Aliquots of cells that were incubated with isotype-matched mouse anti-human IgGs served as negative control. Data were analyzed using CellQuest software and FACSDIVA for SVF (Becton Dickinson Immunocytometry Systems).

### *Immunohistochemistry*

Cells were rinsed twice with PBS and fixed with 3% paraformaldehyde for 20 min at room temperature. Thereafter, cells were incubated in 100 mM $NH_4Cl$ in PBS for 10 min to quench autofluorescence. Nonspecific binding was blocked by incubating the cells in 1% bovine serum albumin (BSA) in PBS containing 0.2% Triton X-100 for 15 min at room temperature. Osteogenically stimulated cells and undifferentiated ASCs were incubated for 1 h with a mouse anti-human osteocalcin MAb (R&D Systems) diluted 1:100 in PBS with 1% BSA, and anti-mouse Alexa 546 secondary antibody was used at a concentration of 1:500. Chondrogenically stimulated and undifferentiated ASCs were pretreated with pepsin solution (Zymed Laboratories, San Francisco, CA) for 10 min at 37°C and incubated for 1 h with a mouse anti-human type II collagen antibody (Acris antibodies, Hiddenhausen, Germany) diluted 1:5 in PBS with 1% BSA. Cells were conjugated with anti-mouse fluorescein secondary antibody diluted 1:500 (Chemicon International, Temecula, CA).

*Histology*

Differentiated and undifferentiated ASCs were processed using the following histological assays: for adipogenesis, Oil Red O stain (Sigma) was used to detect intracellular lipid accumulation according to standard protocols. Cells were counterstained with Mayer hematoxylin and eosin solution (Sigma). For osteogenesis, Alizarin Red solution (Millipore, Billerica, MA) was used to detect mineral deposition according to the manufacturer's instructions. For chondrogenesis, Toluidine Blue (TB) (Panreac, Barcelona, Spain) was used to assess proteoglycan extracellular matrix according to standard protocols. Stained slides were examined by microscopy.

*Alkaline phosphatase activity assay*

Triplicate ASC cultures were induced to differentiate in OM for up to 3 weeks. At 7 and 21 days, cells were washed twice with PBS, and lysed in 10 mM Tris-HCl (pH 8.2) containing 2 mM $MgCl_2$ and 0.05% Triton X-100 at 4°C for 1 h. The lysates were centrifuged at 200 *g* for 10 min at 4°C, and submitted to two freeze–thaw cycles. The lysates were cleared by centrifugation at 11,300 *g* for 30 min (4°C) and incubated for 1 min in prewarmed alkaline phosphatase (ALP) solution provided by Linear Chemicals (Barcelona, Spain). *p*-Nitrophenolate release was determined spectrophotometrically at 405 nm. Protein concentration in the supernatant was analyzed using the BioRad Protein Assay (Bio-Rad Laboratories, München, Germany) with BSA as standard. Undifferentiated ASCs served as negative control and SAOS-2 cells as positive control.

*Quantitative real-time polymerase chain reaction*

Gene expression of peroxisome proliferating receptor gamma (PPARγ) was quantified in adipogenically stimulated cell cultures. Total cellular RNA was extracted from SC, BFP, and ASCs cultured in AM for 3, 9, and 21 days, using Trizol reagent (Invitrogen, Carlsbad, CA), and 2 μg of RNA was reverse-transcribed using M-MLV Reverse transcriptase (Invitrogen). Undifferentiated ASCs and human adipose tissue from SC and BFP served as control. Expression of PPARγ and the osteogenic genes core-binding factor alpha subunit 1 (CBFA1) and osteonectin (SPARC) was quantified in ASCs cultured in OM for 7, 14 (CBFA1, SPARC, and PPARγ), and 21 days (PPARγ). Undifferentiated ASCs and SAOS-2 cells were used as control. Expression of PPARγ and SOX9 was analyzed in ASCs cultured for 5 days (PPARγ) and 14 days (SOX9 and PPARγ) and in CM. Undifferentiated ASCs and chondrocytes derived from human nucleus pulposus were used as chondrogenic control. Real-time polymerase chain reactions (PCRs) were performed using 2.5 μL cDNA and SYBR Green Supermix (Bio-Rad Laboratories). Triplicate reactions were performed using primer pairs for PPARγ,[24] CBFA1, osteonectin, and SOX9. The oligonucleotide sequence of the primers is listed in Supplemental Table S2 (available online at www.liebertonline.com). Specific transcription levels were normalized to the housekeeping genes human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal RNA 18S (*18S*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*). The target and reference genes were amplified in separate wells. All reactions were performed in triplicate. In each run the reaction mixture without the cDNA was used as negative control. A standard curve was performed with PPARγ primers ($y = -3.79x + 425.4$, $r^2 = 0.999$), SOX9 primers ($y = -3.725x + 411.71$, $r^2 = 0.999$), CBFA1 primers ($y = -3.8111x + 419.45$, $r^2 = 0.999$), osteonectin (SPARC) primers ($y = -3.027x + 341.11$, $r^2 = 0.982$), 18S primers ($y = -3.3923x + 373$, $r^2 = 0.999$), YWHAZ primers ($y = -2.95x + 332.51$, $r^2 = 0.999$), and GAPDH primers ($y = -3.36x + 379.17$, $r^2 = 0.998$). Gene expression levels are presented as the fold-increase over control (undifferentiated ASCs are control).

*Statistical analysis*

Average values were expressed as mean ± SD. ALP and real-time PCR was expressed as mean ± standard error of the mean. Marker expression differences between two different sources of ASCs were analyzed statistically using a nonparametric Mann–Whitney *U*-test. Differences were considered significant if $p < 0.05$.

## Results

*BFP contains progenitor cells with ASC characteristics*

To study if BFP contains progenitor cells with ASC characteristics, BFPs from healthy patients were extirpated and SVF was isolated. Freshly isolated and cultured SVF was analyzed for cell morphology and membrane marker profile to observe whether the cells derived from BFP share characteristics with ASCs from another fat depot. After 1 week of culture, adherent cells from abdominal fat ($n = 4$) and BFP ($n = 5$) were counted, and cell number per gram of adipose tissue was calculated.

*Morphology*

Some adherent cells were observed 48 h after seeding. Nonadherent cells disappeared over time in culture (Fig. 1A). Cells from BFP and SC remained in a quiescent phase (dormant) during 2–4 days; afterward, they began to multiply rapidly, approaching confluence as a monolayer of large flat cells. Both BFP and SC cells showed similar morphology; that is, they were spindle shaped. After 7 days of culture, both BFP and SC cells exhibited a more fibroblast-like morphology characteristic of ASCs. At this time point, cells reached 90% confluence and were passaged.

*Cell surface marker profile*

To evaluate the surface marker profile, cells were stained with a panel of antibodies for marker expression to define the percentage of ASCs in BFP and SC, following the minimal criteria of marker expression to define MSCs.[25] Cultured ASCs from BFP and SC were positive for CD73, CD90, and CD105, and negative for the hematopoietic markers CD45, CD19, CD14, and HLADR (Table 1). Cells were also positive for CD29, a characteristic marker of ASCs,[26] and negative for CD146, a marker expressed in endothelial and vascular smooth muscle cells.[27] Some cross-contaminating populations were found in initial passaged ASCs from BFP and SC, but they disappeared with further passaging (Fig. 1B). A $CD146^+CD29^+$ population was observed in initial passaged ASCs from BFP, but not SC. CD146 is characteristic of endothelial progenitor cells, which are likely found in the rich microvasculature within this adipose tissue.

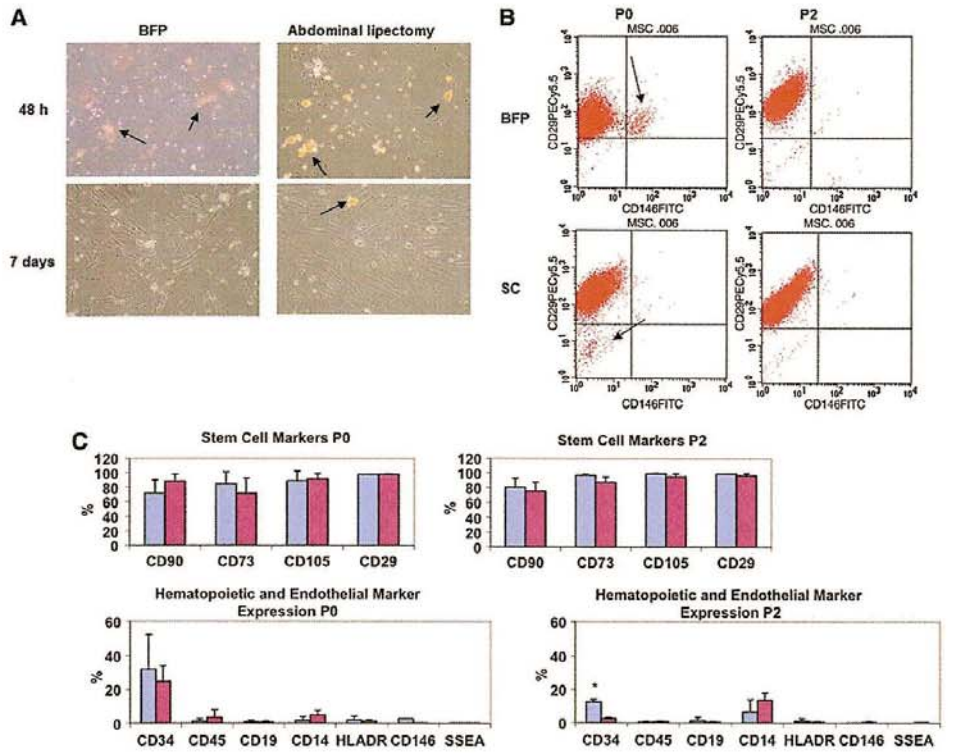



**FIG. 1.** Comparison of morphology and phenotype of ASCs derived from BFP or SC. (**A**) Morphology of cells isolated from BFP or SC, observed by microscopy at 48 h and 7 days. The morphology of ASCs from BFP and SC is similar. At 48 h, there is abundant presence of hematopoietic cells (arrows) and fibroblast-like cells. The population after 7 days of culture is homogenous, and hematopoietic cells are scarce or lacking. Magnification, 100×. (**B**) Dot plot images from flow cytometry analysis at P0 and P2 from ASCs derived from BFP ($n=4$) or SC ($n=4$). Some contaminant populations (arrows) were present at P0, which represents cells after 7 days of culture on plastic, but disappeared at P2, revealing the purification of culture with successive passaging. (**C**) Surface marker profile of ASCs from BFP (light grey; blue, online) and ASCs from SC (dark grey; red, online). Cell surface markers were analyzed by flow cytometry at P0 and P2. Stem cell marker expression and hematopoietic and endothelial marker expression showed a similar phenotype in the two different fat tissue sources. *Significant difference of surface marker expression between BFP and SC ($p<0.05$). ASCs, adipose stem cells; BFP, buccal fat pad; SC, subcutaneous abdominal adipose tissue; P, passage. Color images available online at www.liebertonline.com/ten.

After 1 month of culture of ASCs from BFP (P5), more than 98% of the cells expressed the characteristic MSC markers CD105, CD73, and CD29, and less than 2% cells expressed the hematopoietic markers CD45, CD19, CD14, and HLADR, according to previous studies.[28] ASCs from BFP showed some expression of CD34 (32%), but this declined with time in culture to 7.5% at 1 month of culture. Table 1 shows the marker expression percentage of ASCs from BFP, as mean ± SD measured at several passages. Figure 1C shows the comparison between marker expression of ASCs from BFP and SC at P0 and P2. Marker expression was similar for BFP and SC, except CD34 expression, which was much higher in ASCs from BFP at P2 ($p<0.05$) than in ASCs from SC.

*Cell yield*

Cell yield from adipose tissue obtained from patients undergoing elective oral or abdominal surgery was analyzed (Table 2). The mean age was 19.6 years for patients undergoing oral surgery ($n=5$, range 18–22 years), and 43.3 years for patients undergoing abdominal surgery ($n=4$, range 19–57 years). The mean yield of ASCs obtained from BFP after 1 week of culture was $513 \pm 227 \times 10^3$ cells per gram of tissue, and the mean yield of ASCs from abdominal adipose tissue was $253 \pm 56 \times 10^3$ cells per gram of adipose tissue. This result shows more ASCs in BFP than in SC; however, it did not reach significance due to high patient variability. The num-

TABLE 1. PERCENTAGE OF STEM CELL AND HEMATOPOIETIC AND ENDOTHELIAL MARKER EXPRESSION FROM ADIPOSE STEM CELLS DERIVED FROM BUCCAL FAT PAD AT SEVERAL PASSAGES IN CULTURE

| Marker | P0 | P2 | P5 | n |
|---|---|---|---|---|
| CD105 | 89.1 ± 13.6 | 99.5 ± 0.8 | 99.1 ± 0.2 | 3 |
| CD90 | 75.2 ± 18.3 | 80.8 ± 12 | 83.4 ± 18.3 | 4 |
| CD73 | 84.7 ± 16.4 | 97.2 ± 1.3 | 99.1 ± 0.4 | 4 |
| CD29 | 96.4 ± 2.4 | 98.6 ± 0.9 | 99.3[a] | 3 |
| CD34 | 32.1 ± 20.1 | 12.7 ± 1.4 | 7.5 ± 4.5 | 3 |
| CD45 | 1.3 ± 1.3 | 0.4 ± 0.5 | 0.5 ± 0.6 | 4 |
| CD14 | 1.6 ± 2.2 | 6.6 ± 7.4 | 0.5 ± 0.3 | 4 |
| HLADR | 2 ± 2.3 | 1.1 ± 1.4 | 0.9 ± 1 | 3 |
| CD19 | 0.9 ± 0.9 | 1.4 ± 2 | 1.9 ± 2.2 | 3 |
| CD146 | 1.1 ± 1.4 | 0.3 ± 0.1 | 0.1[a] | 3 |
| SSEA | 1.8 ± 1.7 | 0.4 ± 0.1 | 0.1[a] | 3 |

Results are shown as mean ± standard deviation of the percentage of cells expressing a marker. Cells were analyzed at 7 days of culture (P0), passage 2 (P2), and passage 5 (P5) of culture.
[a]For CD29, CD146, and SSEA, marker expression at P5 cells of one patient were analyzed.

ber of ASCs obtained from SC was similar as observed in another study.[29]

### *SVF from BFP is a rich source of ASCs*

Adipose tissue can be split in two different fractions, that is, one fraction containing mature adipocytes and another fraction, SVF, containing a heterogeneous cell population. To our knowledge, the SVF from BFP has not yet characterized. To determine the percentage of ASCs present in this fraction, we characterized the SVF from five patients. Coexpression of different markers was determined by flow cytometry to quantify the cell population representing fresh ASCs. Patient age was between 19 and 29 years, and 60% were women. SVF from BFP contained a mixture of cells, as shown by representative dot plots from the different patients and percentages of marker coexpression (Fig. 2B, C). The SVF contains a high percentage of cells (21%–38%) that express CD90, CD73, CD29, and CD34, and which do not express the hematopoietic and endothelial lineage markers (CD45, CD19, CD14, HLADR, and CD146). In addition, this population of cells does express CD34, but not CD105, as reported previously.[30,31] This marker profile is characteristic of fresh ASCs, whereas culture of ASCs on plastic plates increases CD105 expression and reduces CD34 expression.[30,31] Taken together, these results demonstrate that SVF from BFP is a rich source of ASCs (~30%), similar to other sources of adipose tissue.

### *Multilineage differentiation potential of ASCs derived from BFP* in vitro

Osteogenesis. To assess the osteogenic potential of ASCs, ALP, an early marker of osteoblastic differentiation, was determined after 0, 7, and 21 days of culture in OM. SAOS-2 cells were used as a positive control. After 1 week of culture, ASCs changed their morphology from spindle shaped to more polygonal shaped, which was accompanied by an increase in ALP activity up to day 21 (Fig. 3A). ASCs cultured in OM showed a 2.5-fold increase in ALP activity at day 7, and a 16.5-fold increase at day 21. Areas of high granular density appeared and multiple layers of ASCs were formed after 2 weeks. These areas were stained intensely with Alizarin Red, indicating calcification of the extracellular matrix (Fig. 3BII). ASCs grown in the control medium did not stain with Alizarin Red (Fig. 3BI).

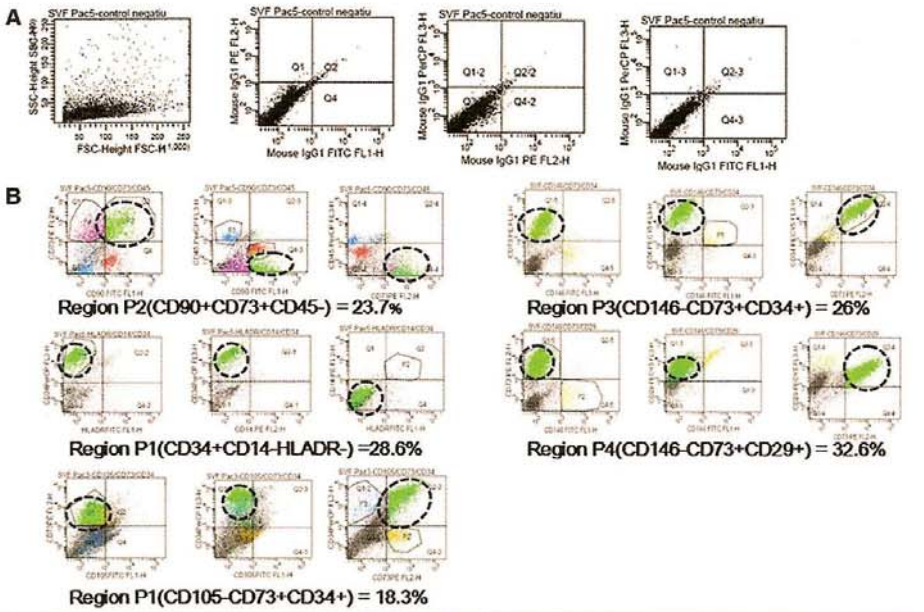
Protein expression of osteocalcin, a marker characteristic of mature osteoblasts and osteocytes, was determined by immunofluorescence. ASCs cultured in OM, but not undifferentiated ASCs, showed osteocalcin expression (Fig. 3CI, CII), as did SAOS-2 cells (positive control) (Fig. 3CIII). During the differentiation process, expression of the osteogenic genes *CBFA1* and osteonectin (*SPARC*) increased by eight- and twofold, respectively, at day 14 of culture. On the other hand, we did not observe a decrease in gene expression of the adipocyte marker PPARγ until day 21 (Fig. 3D). SAOS-2 cells were used as positive control. These data demonstrate that ASCs from BFP are capable of osteogenic differentiation *in vitro*.

Adipogenesis. After adipogenic induction of ASCs, we observed intracellular lipid vacuoles, which increased in size and number during culture (Fig. 4A). Oil Red staining revealed multiple intracellular lipid filled droplets in ASCs cultured in AM, but not in undifferentiated ASCs. The expression levels of the specific adipocyte marker PPARγ and GAPDH were measured by quantitative real-time (RT)-PCR

TABLE 2. CELL NUMBER AT P0 OBTAINED FROM TWO DIFFERENT SOURCES OF ADIPOSE TISSUE: BUCCAL FAT PAD AND SUBCUTANEOUS ABDOMINAL ADIPOSE TISSUE

| Patient | Source | Age (years) | Volume of fat (mL) | Weight of fat (g) | Cell number | Days of culture | Cell number/g |
|---|---|---|---|---|---|---|---|
| Man | Buccal fat pad | 18 | 14 | 12 | 3,800,000 | 7 | 316,667 |
| Man | Buccal fat pad | 20 | 23 | 18.2 | 6,615,000 | 7 | 363,462 |
| Woman | Buccal fat pad | 22 | 16 | 13.6 | 11,679,999 | 7 | 858,823 |
| Woman | Buccal fat pad | 20 | 15 | 14.5 | 9,075,000 | 7 | 625,862 |
| Woman | Buccal fat pad | 18 | 12.5 | 11.9 | 4,780,000 | 7 | 401,681 |
| Woman | Abdominal lipectomy | 19 | 14 | 12.2 | 2,339,000 | 7 | 191,721 |
| Woman | Abdominal lipectomy | 47 | 36 | 29 | 9,045,000 | 7 | 311,897 |
| Woman | Abdominal lipectomy | 50 | 16 | 13.2 | 2,940,000 | 7 | 222,727 |
| Woman | Abdominal lipectomy | 57 | 5.6 | 5.5 | 1,579,999 | 7 | 287,273 |

Cell number is expressed per gram of adipose tissue after 1 week of culture.




C

| Marker profile | % ASCs in SVF (Mean ± SD) | # Patients |
|---|---|---|
| CD90+/CD73+/CD45- | 27.2 ± 13.9 | 4 |
| CD90+/CD73+/CD34+ | 21.5 ± 13.9 | 5 |
| CD105-/CD73+/CD34+ | 21.3 ± 14.3 | 5 |
| CD105-/CD19-/CD34+ | 38.1 ± 18.3 | 2 |
| HLADR-/CD14-/CD34+ | 26.5 ± 2.9 | 2 |
| CD146-/CD73+/CD34+ | 21.4 ± 6.4 | 2 |
| CD146-/CD73+/CD29+ | 23.9 ± 12.2 | 2 |

**FIG. 2.** Flow cytometry of fresh ASCs obtained from BFP, analyzed by FACS DIVA software. (**A**) General population and isotype controls of different fluorochromes. Samples were analyzed by flow cytometry using FSC and SSC. (**B**) Mixed population of cells observed in SVF, analyzing coexpression of three characteristic markers from three representative patients. Cells expressing characteristic markers of fresh ASCs are marked by a dotted circle. Endothelial cells, hematopoietic cells, fibroblasts, and endothelial progenitor cells/vascular smooth muscle cells are marked respectively in purple, blue, red, and yellow (online version). (**C**) Mean percentage of ASCs in the SVF from BFP, analyzed by flow cytometry ($n = 5$). Samples were incubated with nine markers and analyzed for coexpression of three cell surface markers by flow cytometry. FSC, forward scatter; SSC, side scatter; BFP, buccal fat pad; SVF, stromal vascular fraction. Color images available online at www.liebertonline.com/ten.

using the comparative $C_t$ method (Fig. 4B). PPARγ expression increased during culture, reaching approximately four-fold induction compared to undifferentiated ASCs. Whole adipose tissue samples served as positive control.

Chondrogenesis. ASCs from BFP synthesized cartilage matrix molecules, and produced an extracellular matrix characteristic for chondrocytes when grown in CM. After ~5 days of culture in CM, ASCs changed their morphology to more spheroid shaped (Fig. 5AI). ASCs under control conditions did not show TB staining at pH 2, which is specific for sulfated proteoglycans in cartilage matrix (Fig. 5AII). The number of nodules that stained positive with TB (Fig. 5AIII) increased during 4 weeks of culture in CM (data not shown). TB-stained purple nodules were not seen in undifferentiated ASCs (Fig. 5AIV), but they were clearly visible after culture

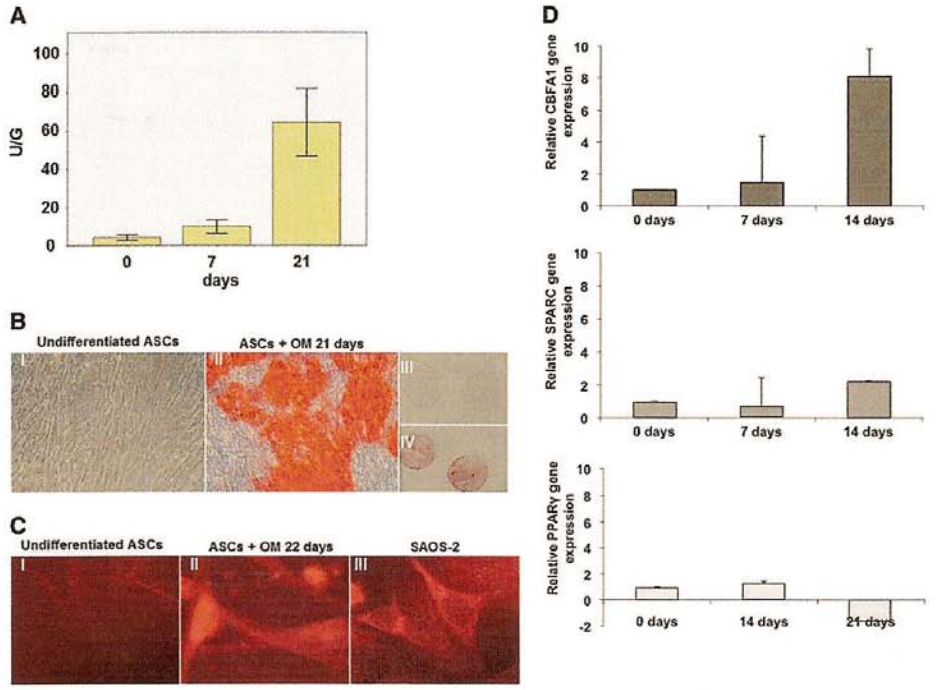



**FIG. 3.** Osteogenic differentiation of ASCs from BFP. (**A**) Graphical representation of ALP activity in units per gram (U/G) of ASCs at 0, 7, and 21 days of osteogenic induction. Values are mean ± SEM. ALP activity of SAOS-2 cells (positive control) was 672.7 U/G ± 125.5 (results not shown), and ALP activity of ASCs was 3.9 U/G ± 1.2 at 0 day, 9.9 U/G ± 3.5 at 7 days, and 64.4 U/G ± 17.6 at 21 days of osteogenic differentiation. (**B**) Alizarin Red–stained ASCs in OM (**II**) and in the control medium (**I**) at 100× magnification. Cells were seeded at $5\times10^4$ cells/well. Red nodules were visible in differentiated ASCs (**IV**) but absent in undifferentiated ASCs (**III**). (**C**) Osteocalcin immunohistochemistry. Cells were seeded at $2\times10^4$ cells/well. ASCs cultured in OM but not in the control medium (**I**) for 22 days showed osteocalcin immunostaining (red color, **II**). **III** shows the characteristic osteocalcin staining of SAOS-2 cells (positive control). Magnification, 400×. Osteocalcin primary antibody was used at a concentration of 1:10, and Alexa 546 secondary antibody at a concentration of 1:500. (**D**) Gene expression of CBFA1, SPARC, and PPARγ was quantified by real-time PCR in ASCs cultured in the control medium and OM for up to 3 weeks. Gene expression levels were normalized to 18S and YWHAZ using the normalization factor and expressed relative to noninduced ASCs. Values are mean ± SEM, $n = 3$. ASCs, adipose stem cells; ALP, alkaline phosphatase; OM, osteogenic medium; CBFA1, core-binding factor alpha subunit 1; SPARC, osteonectin; PPARγ, peroxisome proliferating receptor gamma; 18S, ribosomal RNA; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; PCR, polymerase chain reaction; SEM, standard error of the mean. Color images available online at www.liebertonline.com/ten.

in CM (Fig. 5AV). Immunohistochemical analysis did not show expression of collagen type II in undifferentiated cells (Fig. 5BI), but it was expressed by cells grown in micromass in CM after 4 weeks (Fig. 5BII). Expression of the master chondrogenic factor SOX9 was increased by 3.4-fold in ASCs grown in CM compared to undifferentiated ASCs at day 14 (Fig. 5C). Expression of the adipocyte gene PPARγ decreased during chondrogenic differentiation (Fig. 5C).

## Discussion

Adult stem cells are present in various organs and develop important functions in tissue maintenance and homeostasis.[32] One major goal of tissue engineering medicine is to find a source that can provide an adequate number of stem cells for clinical application that implies minimal morbidity. Adipose tissue holds great promise in regenerative medicine; that is, it is available in large quantities as waste material, and it contains more progenitor cells giving rise to different cell populations than bone marrow.[33] Unlike subcutaneous fat, BFP is a specialized mass of adipose tissue considered an ideal flap for oral surgery; since it is easy to harvest and reliable, it contains a rich blood supply, and its harvesting causes minimal donor-site morbidity and low complication.[34,35] The size of BFP appears to be similar among different persons and independent of their body weight and fat distribution.[36] Patients with little subcutaneous fat have BFP with normal weight and volume. The results of our study are

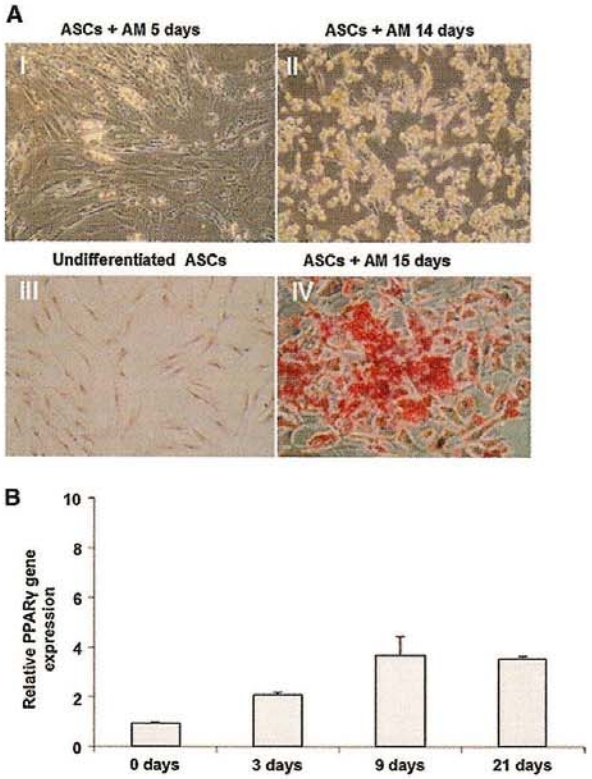



**FIG. 4.** Adipogenic differentiation of ASCs from BFP. (**A**) Oil Red stain to detect lipid vacuoles. Cells were cultured with AM and analyzed under microscopy at day 5 (**I**) and day 14 (**II**) at 100× magnification. Lipid vacuoles were observed within the cells. The size of lipid vacuoles did increase with time in culture. At day 15 cells were stained with Oil Red and analyzed using a microscope. Lipid vacuoles stained intense red in ASCs grown in AM (**IV**), but no lipid vacuoles were present in undifferentiated ASCs (**III**). (**B**) PPARγ gene expression levels measured by real-time PCR in undifferentiated ASCs and ASCs cultured in AM during 3, 9, and 21 days. Expression levels are represented as fold increase in comparison with undifferentiated controls, after normalization to the housekeeping gene human glyceraldehyde-3-phosphate dehydrogenase. Whole subcutaneous adipose tissue and BFP samples were used as positive controls, which showed an increase in PPARγ expression compared to undifferentiated ASCs of 28 ± 4.4-fold and 66 ± 7.8-fold, respectively. ASCs, adipose stem cells; BFP, buccal fat pad; AM, adipogenic medium; PPARγ, peroxisome proliferating receptor gamma. Color images available online at www.liebertonline.com/ten.

in agreement with published results showing that the size of BFP is not related to a person's general adiposity.[36]

In this study we aimed to assess the presence of ASCs in BFP using flow cytometry, to compare the number of ASCs present in BFP with that in adipose tissue obtained from another fat depot (SC), to compare the cell marker expression in BFP with that in SC, and to determine the multipotent potential of ASCs from BFP *in vitro*. Our results showed that BFP contains a high proportion of cells with ASC characteristics that can be easily isolated and expanded *in vitro*. Approximately 30% of SVF from BFP express characteristic markers of fresh ASCs, that is, CD90, CD73, CD29, and CD34.[27,37] The percentage of ASCs from BFP obtained in our study (~30%) is slightly higher than that obtained from abdominal subcutaneous adipose tissue (~22%).[38] In addition, the occurrence of MSC in bone marrow tissue is <0.01%,[3] which makes BFP an excellent source of fresh ASCs, avoiding the need for *in vitro* expansion, high costs, and contamination risk.

CD34 expression is characteristic of fresh ASCs,[39] and its expression decreases with passaging in ASCs. Expression of CD34 in these cells contrasts with MSCs from bone marrow, which lacks expression of this marker.[40] $CD34^+$ cells are capable of stimulating angiogenesis, and they are involved in neovascularization processes that facilitate healing of ischemic tissues.[41,42] The *in vitro* expansion of cells from SVF causes changes in the phenotype and characteristics of these cells such as loss of CD34 expression.[43] We hypothesize that CD34 expression might be related to differences in cell properties and differentiation potential. Freshly isolated ASCs from BFP lack expression of CD105, but expression of this marker increases rapidly after seeding, as has been described by others using ASCs from other fat depots.[30,44] ASCs also lack expression of CD146, a characteristic marker of endothelial cells as well as vascular smooth muscle cells.[27] In our study we found a small population of $CD146^+$ cells in the first passages (up to P2) of ASCs from BFP. The presence of this $CD146^+$ contaminating population, as well as the presence of $CD34^+$ cells, might be due to the highly enriched blood vessel supply in BFP.[45] This could be related to the excellent wound healing properties of BFP as a pedicled graft in oral surgery for treatment of oroantral communications,[46,47] maxillary defects,[34] oral submucous fibrosis,[48] and vocal cord defects.[49]

It was expected that differences in the site of origin of the adipose tissue is related to different ratios of cell types such as epithelial progenitor cells, fibroblasts, pericytes, myoblasts, and the like. We evaluated differences between SC and BFP, a mass of adipose tissue considered a marker of visceral fat.[18]

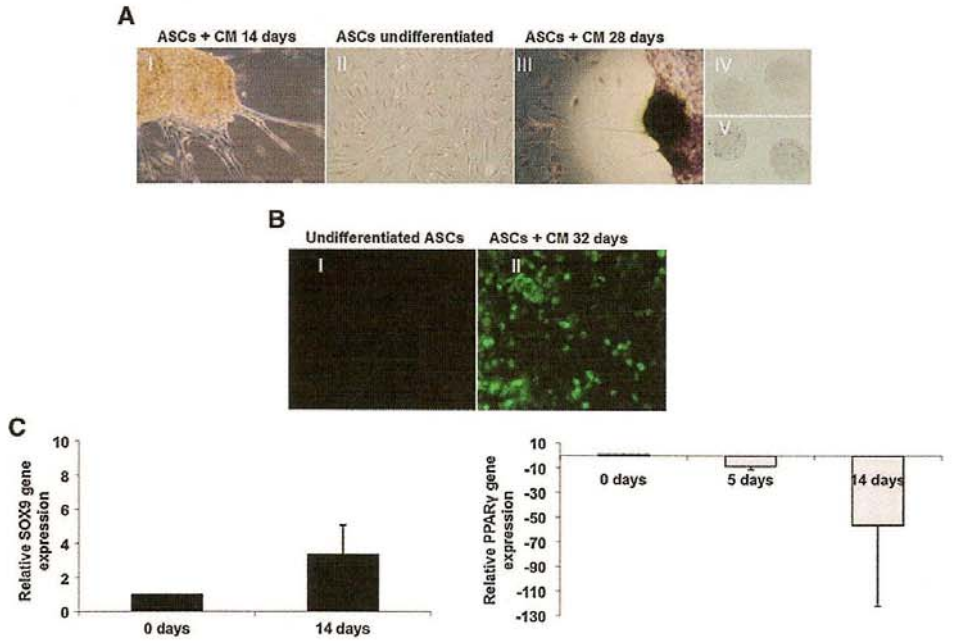



**FIG. 5.** ASCs from BFP cultured in CM. (**A**) Micromasses were observed, and their number increased with time in culture. **I** shows ASCs with CM at 14 days of chondrogenic differentiation. At day 28 of differentiation ASCs were stained with Toluidine Blue for the presence of proteoglycan matrix. Intense blue nodules were observed in ASCs induced to differentiate (**III**), but these nodules were not observed in ASCs cultured in the control medium (**II**). Magnification, 100×. Coverslips stained with Toluidine Blue showed violet nodules from ASCs differentiated with CM (**V**). There was no staining in the coverslips with ASCs grown in the control medium (**IV**). (**B**) Immunohistochemistry for collagen II showed staining in ASCs cultured with CM for 32 days (**II**), not present in ASCs cultured in the control medium (**I**). Magnification, 100×. Collagen II primary antibody was used at a concentration of 1:5, and fluorescein secondary antibody at a concentration of 1:500. (**C**) SOX9 and PPARγ gene expression levels measured by real-time PCR in ASCs cultured in the control medium and CM during 5 and 14 days. Expression levels are represented as fold increase in comparison with levels found in undifferentiated cells, and normalized by using 18S and YWHAZ as housekeeping genes. The bars represent the mean ± SEM. Cells from nucleus pulposus were used as the control. ASCs, adipose stem cells; CM, chondrogenic medium; PPARγ, peroxisome proliferating receptor gamma; 18S, ribosomal RNA; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide. Color images available online at www.liebertonline.com/ten.

We observed differences between BFP and subcutaneous adipose tissue regarding CD34 expression. This difference might be attributed to the higher blood supply of visceral tissue, which might contribute to the heterogeneity of the cell population, which in turn might increase the osteogenic potential.[50] The $CD34^+$ cells obtained from dental germ pulp also have the ability to differentiate to preosteoblasts, form bone nodules after implantation in mice, and show angiogenic potential.[51] We found higher CD34 expression in passaged ASCs from BFP compared with passaged ASCs from subcutaneous adipose tissue, which could make them more suitable for bone engineering therapies.

After 1 week of culture, we observed that the number of BFP-derived ASCs was twofold higher than the number of ASCs in SC. This difference might be due to the differences in age of the patients,[52] although this difference might also be attributed to the intrinsic characteristics of the patients or to other particular properties of the adipose source.[53]

When ASCs from BFP were cultured in AM for 2 weeks, more than 90% of the cells accumulated lipid vacuoles and expressed the characteristic adipogenic gene PPARγ, which is considered a master regulator of adipogenic differentiation.[54] However, we observed constitutive expression of this adipocyte gene in noninduced ASCs, similar as in other studies.[55] Osteogenesis occurred in the presence of OM within 1 week as shown by increased ALP activity. 1,25-Dihydroxyvitamin $D_3$ plays an important role in bone formation and maturation by increasing $Ca^{2+}$ concentration at the mineralization site. It promotes early differentiation of progenitor cells to osteoblastic commitment. We, as well as others,[56] found that the combination of 1,25-dihydroxyvitamin $D_3$ with ascorbate-2-phosphate and β-glycerophosphate induces

matrix calcification as shown by Alizarin Red staining and osteocalcin expression. Osteocalcin is an osteogenic marker indicative of the final mature stages of osteoblastic differentiation and is expressed when mineralization begins.[57] In addition to the increased ALP activity and matrix calcification, expression of the osteogenic genes *CBFA1* and osteonectin confirmed osteogenic differentiation of ASCs from BFP. No increase in PPARγ was observed during culture. After exposing ASCs to CM for 4 weeks, we observed TB-stained nodules indicative of a proteoglycan matrix characteristic for cartilage. Expression of collagen II, a marker believed to be specific for articular cartilage, was observed by immunohistochemistry in differentiated ASCs. Increased expression of the master chondrogenic factor SOX9 was observed in ASCs from BFP at 14 days of culture, followed by decreased expression of the adipogenic marker PPARγ. ASCs could represent a valuable tool for cartilage repair that might overcome the limited intrinsic repair capacity of this avascular connective tissue, which most orthopedic patients complain about. Flow cytometry analysis of marker expression in ASCs before differentiation showed low expression of hematopoietic markers CD45, CD19, CD14, and HLADR, as well as low expression of endothelial markers CD146 and SSEA, which demonstrates that cross-contamination with hematopoietic or endothelial cells did not occur.[58,59]

The ideal source of stem cells for tissue engineering would be one that can be easily harvested, and contains a high number of expandable stem cells.[60] This study shows that SVF isolated from BFP contains ~30% ASCs with high expression of the angiogenic marker CD34. Under appropriate conditions, ASCs from BFP differentiate to chondrocytes, osteoblasts, or adipocytes, suggesting that BFP can be a rich alternative source of stem cells, readily available for clinical application in the field of tissue engineering for the repair of bone and cartilage defects.

**Acknowledgments**

We wish to extend our thanks to the surgical staff of the Hospital Clínic and Hospital Sant Joan de Déu for facilitating the donation of human adipose tissue for this study. We also thank the staff of the Flow Cytometry research core, in Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona (UAB), for their assistance with the flow cytometry studies, and the Skeletal Tissue Engineering Group Amsterdam (STEGA) at the VU University Amsterdam, The Netherlands, for their excellent support with the real-time PCR analysis. This work was funded by the Faculty of Dentistry, International University of Catalonia. E. Farré Guasch received a fellowship from International University of Catalonia.

**Disclosure Statement**

No competing financial interests exist.

**References**

1. Friedenstein, A.J., Piatetzky-Shapiro, I.I., and Petrakova, K.V. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol **163,** 381, 1966.
2. Caplan, A.I. The mesengenic process. Clin Plast Surg **213,** 429, 1994.
3. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143, 1999.
4. Bruder, S.P., Kurth, A.A., Shea, M., Hayes, W.C., Jaiswal, N., and Kadiyala, S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res **16,** 155, 1998.
5. Koga, H., Muneta, T., Nagase, T., Nimura, A., Ju, Y.J., Mochizuki, T., and Sekiya, I. Comparison of mesenchymal tissues-derived stem cells for *in vivo* chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit. Cell Tissue Res **333,** 207, 2008.
6. Chen, C.H., Wei, H.J., Lin, W.W., Chiu, I., Hwang, S.M., Wang, C.C., Lee, W.Y., Chang, Y., and Sung, H.W. Porous tissue grafts sandwiched with multilayered mesenchymal stromal cell sheets induce tissue regeneration for cardiac repair. Cardiovasc Res **80,** 88, 2008.
7. Nishimori, M., Yamada, Y., Hoshi, K., Akiyama, Y., Hoshi, Y., Morishima, Y., Tsuchida, M., Fukuhara, S., and Kodera, Y. Health-related quality of life of unrelated bone marrow donors in Japan. Blood **99,** 1995, 2002.
8. Aust, L., Devlin, B., Foster, S.J., Halvorsen, Y.D., Hicok, K., du Laney, T., Sen, A., Willingmyre, G.D., and Gimble, J.M. Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy **6,** 7, 2004.
9. Rada, T., Reis, R.L., and Gomes, M.E. Adipose tissue-derived stem cells and their application in bone and cartilage tissue engineering. Tissue Eng B **15,** 113, 2009.
10. Trottier, V., Marceau-Fortier, G., Germain, L., Vincent, C., and Fradette, J. IFATS collection: using human adipose-derived stem/stromal cells for the production of new skin substitutes. Stem Cells **26,** 2713, 2008.
11. Aurich, H., Sgodda, M., Kaltwasser, P., Vetter, M., Weise, A., Liehr, T., Brulport, M., Hengstler, J.G., Dollinger, M.M., Fleig, W.E., and Christ, B.E. Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue *in vitro* promotes hepatic integration *in vivo*. Gut **58,** 480, 2008.
12. Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Osaki, M., Kawamata, M., Kato, T., Okochi, H., and Ochiya, T. IFATS collection: *in vivo* therapeutic potential of human adipose tissue mesenchymal stem cells after transplantation into mice with liver injury. Stem Cells **26,** 2705, 2008.
13. Rubina, K., Kalinina, N., Efimenko, A., Lopatina, T., Melikhova, V., Tsokolaeva, Z., Sysoeva, V., Tkachuk, V., and Parfyonova, Y. Adipose stromal cells stimulate angiogenesis via promoting progenitor cell differentiation, secretion of angiogenic factors, and enhancing vessel maturation. Tissue Eng A **15,** 2039, 2009.
14. Cowan, C.M., Shi, Y.Y., Aalami, O.O., Chou, Y.F., Mari, C., Thomas, R., Quarto, N., Contag, C.H., Wu, B., and Longaker, M.T. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol **22,** 560, 2004.
15. Cui, L., Liu, B., Liu, G., Zhang, W., Cen, L., Sun, J., Yin, S., Liu, W., and Cao, Y. Repair of cranial bone defects with adipose derived stem cells and coral scaffold in a canine model. Biomaterials **28,** 5477, 2007.
16. Hicok, K.C., Du Laney, T.V., Zhou, Y.S., Halvorsen, Y.D., Hitt, D.C., Cooper, L.F., and Gimble, J.M. Human adipose-derived adult stem cells produce osteoid *in vivo*. Tissue Eng **10,** 371, 2004.
17. Yoon, E., Dhar, S., Chun, D.E., Gharibjanian, N.A., and Evans, G.R. *In vivo* osteogenic potential of human adipose-

derived stem cells/poly lactide-co-glycolic acid constructs for bone regeneration in a rat critical-sized calvarial defect model. Tissue Eng **13,** 619, 2007.
18. Sierra-Johnson, J., and Johnson, B.D. Facial fat and its relationship to abdominal fat: a marker for insulin resistance? Med Hypotheses **63,** 783, 2004.
19. Abuabara, A., Cortez, A.L., Passeri, L.A., de Moraes, M., and Moreira, R.W. Evaluation of different treatments for oroantral/oronasal communications: experience of 112 cases. Int J Oral Maxillofac Surg **35,** 155, 2006.
20. Alkan, A., Dolanmaz, D., Uzun, E., and Erdem, E. The reconstruction of oral defects with buccal fat pad. Swiss Med Wkly **133,** 465, 2003.
21. Martin-Granizo, R., Naval, L., Costas, A., Goizueta, C., Rodriguez, F., Monje, F., Muñoz, M., and Diaz, F. Use of buccal fat pad to repair intraoral defects: review of 30 cases. Br J Oral Maxillofac Surg **35,** 81, 1997.
22. El Haddad, S.A., Abd El Razzak, M.Y., and El Shall, M. Use of pedicled buccal fat pad in root coverage of severe gingival recession defect. J Periodontol **79,** 1271, 2008.
23. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell **13,** 4279, 2002.
24. Janjanin, S., Djouad, F., Shanti, R.M., Baksh, D., Gollapudi, K., Prgomet, D., Rackwitz, L., Joshi, A.S., and Tuan, R.S. Human palatine tonsil: a new potential tissue source of multipotent mesenchymal progenitor cells. Arthritis Res Ther **10,** R83, 2008.
25. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D.J., and Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy **8,** 315, 2006.
26. Harting, M., Jimenez, F., Pati, S., Baumgartner, J., and Cox, C., Jr. Immunophenotype characterization of rat mesenchymal stromal cells. Cytotherapy **10,** 243, 2008.
27. Lin, K., Matsubara, Y., Masuda, Y., Togashi, K., Ohno, T., Tamura, T., Toyoshima, Y., Sugimachi, K., Toyoda, M., Marc, H., and Douglas, A. Characterization of adipose tissue-derived cells isolated with the Celution system. Cytotherapy **10,** 417, 2008.
28. Lee, J., Han, D.J., and Kim, S.C. *In vitro* differentiation of human adipose tissue-derived stem cells into cells with pancreatic phenotype by regenerating pancreas extract. Biochem Biophys Res Commun **375,** 547, 2008.
29. Mitchell, J.B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z.E., Kloster, A., Di Halvorsen, Y., Storms, R.W., Goh, B., Kilroy, G., Wu, X., and Gimble, J.M. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells **24,** 376, 2006.
30. Yoshimura, K., Shigeura, T., Matsumoto, D., Sato, T., Takaki, Y., Aiba-Kojima, E., Sato, K., Inoue, K., Nagase, T., Koshima, I., and Gonda, K. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol **208,** 64, 2006.
31. Tapp, H., Hanley, E.N., Jr., Patt, J.C., and Gruber, H.E. Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. Exp Biol Med (Maywood) **234,** 1, 2009.
32. Bajada, S., Mazakova, I., Richardson, J.B., and Ashammakhi, N. Updates on stem cells and their applications in regenerative medicine. J Tissue Eng Regen Med **2,** 169, 2008.
33. Bunnell, B.A., Flaat, M., Gagliardi, C., Patel, B., and Ripoll, C. Adipose-derived stem cells: isolation, expansion and differentiation. Methods **45,** 115, 2008.
34. Amin, M.A., Bailey, B.M., Swinson, B., and Witherow, H. Use of the buccal fat pad in the reconstruction and prosthetic rehabilitation of oncological maxillary defects. Br J Oral Maxillofac Surg **43,** 148, 2005.
35. Baumann, A., and Ewers, R. Application of the buccal fat pad in oral reconstruction. J Oral Maxillofac Surg **58,** 389; discussion 392, 2000.
36. Stuzin, J.M., Wagstrom, L., Kawamoto, H.K., Baker, T.J., and Wolfe, S.A. The anatomy and clinical applications of the buccal fat pad. Plast Reconstr Surg **85,** 29, 1990.
37. Peroni, D., Scambi, I., Pasini, A., Lisi, V., Bifari, F., Krampera, M., Rigotti, G., Sbarbati, A., and Galiè, M. Stem molecular signature of adipose-derived stromal cells. Exp Cell Res **314,** 603, 2008.
38. Lin, G., Garcia, M., Ning, H., Banie, L., Guo, Y.L., Lue, T.F., and Lin, C.S. Defining stem and progenitor cells within adipose tissue. Stem Cells Dev **17,** 1053, 2008.
39. Traktuev, D.O., Merfeld-Clauss, S., Li, J., Kolonin, M., Arap, W., Pasqualini, R., Johnstone, B.H., and March, K.L. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res **102,** 77, 2008.
40. Gimble, J.M., Katz, A.J., and Bunnell, B.A. Adipose-derived stem cells for regenerative medicine. Circ Res **100,** 1249, 2007.
41. Planat-Benard, V., Silvestre, J.S., Cousin, B., Andre, M., Nibbelink, M., Tamarat, R., Clergue, M., Manneville, C., Saillan-Barreau, C., Duriez, M., Tedgui, A., Levy, B., Pénicaud, L., and Casteilla, L. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation **109,** 656, 2004.
42. Madonna, R., and De Caterina, R. *In vitro* neovasculogenic potential of resident adipose tissue precursors. Am J Physiol Cell Physiol **295,** C1271, 2008.
43. Varma, M.J., Breuls, R.G., Schouten, T.E., Jurgens, W.J., Bontkes, H.J., Schuurhuis, G.J., van Ham, S.M., and van Milligen, F.J. Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells. Stem Cells Dev **16,** 91, 2007.
44. Schaffler, A., and Buchler, C. Concise review: adipose tissue-derived stromal cells—basic and clinical implications for novel cell-based therapies. Stem Cells **25,** 818, 2007.
45. Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., Norotte, C., Teng, P.N., Traas, J., Schugar, R., Deasy, B.M., Badylak, S., Buhring, H.J., Giacobino, J.P., Lazzari, L., Huard, J., and Péault, B. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell **3,** 301, 2008.
46. de Moraes, E.J. Closure of oroantral communication with buccal fat pad flap in zygomatic implant surgery: a case report. Int J Oral Maxillofac Implants **23,** 143, 2008.
47. Abuabara, A., Cortez, A.L., Passeri, L.A., de Moraes, M., and Moreira, R.W. Evaluation of different treatments for oroantral/oronasal communications: experience of 112 cases. Int J Oral Maxillofac Surg **35,** 155, 2006.
48. Mehrotra, D., and Pradhan, R., Gupta, S. Retrospective comparison of surgical treatment modalities in 100 patients with oral submucous fibrosis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod **107,** e1, 2009.

49. Tamura, E., Fukuda, H., Tabata, Y., and Nishimura, M. Use of the buccal fat [corrected] pad for vocal cord augmentation. Acta Otolaryngol **128,** 219, 2008.
50. Peptan, I.A., Hong, L., and Mao, J.J. Comparison of osteogenic potentials of visceral and subcutaneous adipose-derived cells of rabbits. Plast Reconstr Surg **117,** 1462, 2006.
51. Graziano, A., d'Aquino, R., Laino, G., Proto, A., Giuliano, M.T., Pirozzi, G., de Rosa, A., di Napoli, D., and Papaccio, G. Human CD34+stem cells produce bone nodules *in vivo*. Cell Prolif **41,** 1, 2008.
52. Schipper, B.M., Marra, K.G., Zhang, W., Donnenberg, A.D., and Rubin, J.P. Regional anatomic and age effects on cell function of human adipose-derived stem cells. Ann Plast Surg **60,** 538, 2008.
53. Jurgens, W.J., Oedayrajsingh-Varma, M.J., Helder, M.N., Zandiehdoulabi, B., Schouten, T.E., Kuik, D.J., Ritt, M.J., and van Milligen, F.J. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell Tissue Res **332,** 415, 2008.
54. Morrison, R.F., and Farmer, S.R. Hormonal signaling and transcriptional control of adipocyte differentiation. J Nutr **130,** 3116S, 2000.
55. Flynn, L.E., Prestwich, G.D., Semple, J.L., and Woodhouse, K.A. Proliferation and differentiation of adipose-derived stem cells on naturally derived scaffolds. Biomaterials **29,** 1862, 2008.
56. Gupta, A., Leong, D.T., Bai, H.F., Singh, S.B., Lim, T.C., and Hutmacher, D.W. Osteo-maturation of adipose-derived stem cells required the combined action of vitamin D3, beta-glycerophosphate, and ascorbic acid. Biochem Biophys Res Commun **362,** 17, 2007.
57. Fernandez-Tresguerres-Hernandez-Gil, I., Alobera-Gracia, M.A., del-Canto-Pingarron, M., and Blanco-Jerez, L. Physiological bases of bone regeneration I. Histology and physiology of bone tissue. Med Oral Patol Oral Cir Bucal **11,** E47, 2006.
58. Astori, G., Vignati, F., Bardelli, S., Tubio, M., Gola, M., Albertini, V., Bambi, F., Scali, G., Castelli, D., Rasini, V., Soldati, G., and Mocetti, T. "In vitro" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. J Transl Med **5,** 55, 2007.
59. Anjos-Afonso, F., and Bonnet, D. Nonhematopoietic/endothelial SSEA-1+cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. Blood **109,** 1298, 2007.
60. De Ugarte, D.A., Ashjian, P.H., Elbarbary, A., and Hedrick, M.H. Future of fat as raw material for tissue regeneration. Ann Plast Surg **50,** 215, 2003.

Address correspondence to:
*Núria Casals, Ph.D.*
*Department Basic Sciences*
*International University of Catalonia*
*C/Josep Trueta s/n, 08190*
*Sant Cugat, Barcelona*
*Spain*

*E-mail:* ncasals@csc.uic.es

*Received: July 15, 2009*
*Accepted: January 15, 2010*
*Online Publication Date: February 23, 2010*

**-TO BE SUBMITTED-**

# Osteogenic Differentiation of BMP-2 Induced Human Adipose Stem Cells Seeded on Biphasic Calcium Phosphate and β-tricalcium Phosphate Scaffolds.

J.R. Overman[1,2*], E. Farré-Guasch[3,4*], M.N. Helder[5], C.M. ten Bruggenkate[2], E.A.J.M. Schulten[2], J. Klein-Nulend[1]

[1] Dept Oral Cell Biology, ACTA-University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Amsterdam, The Netherlands

[2] Dept Oral and Maxillofacial Surgery, ACTA-VU University Medical Center, Research Institute MOVE, Amsterdam, The Netherlands

[3] Dept Oral and Maxillofacial Surgery, Faculty of Dentistry, International University of Catalonia, Sant Cugat, Barcelona, Spain

[4] Dept Basic Sciences, Faculty of Medicine and Health Sciences, International University of Catalonia, Sant Cugat, Barcelona, Spain

[5] Dept Orthopaedics, VU University Medical Center, Research Institute MOVE, Amsterdam, The Netherlands

***Shared first authorship**, Overman J.R. and Farré-Guasch E. contributed equally to this manuscript.

Short title: Osteogenic Differentiation of BMP-2 Induced Human Adipose Stem Cells

Sponsor: Grant support by ZonMw, project number 116001009.

Correspondence to:
Jenneke Klein-Nulend, PhD
Dept Oral Cell Biology
ACTA-VU University Amsterdam
Research Institute MOVE
Gustav Mahlerlaan 3004
1081 LA Amsterdam
The Netherlands
tel +31 205980881

e-mail: j.kleinnulend@acta.nl

**ABSTRACT**

Adipose stem cells (ASCs) constitute a promising tool for tissue engineering approaches. Earlier we showed that treatment for only 15 min with bone morphogenetic protein-2 (BMP-2) induces osteogenesis in ASCs. This study investigates whether this treatment affects attachment, proliferation, and/or differentiation profiles of ASCs seeded on biphasic calcium phosphate (BCP) or β-tricalcium phosphate (ß-TCP).

Human ASCs were treated ± BMP-2 (10 ng/ml) for 15 min, seeded on BCP 60/40 or BCP 20/80 (60%/40% or 20%/80% hydroxyapatite/ß-TCP respectively), or ß-TCP granules sized <0.7mm or >0.7mm, and cultured for 3 weeks. ASC attachment and proliferation rates were determined. Osteogenic differentiation was determined by measuring alkaline phosphatase (ALP) activity, and gene expression of the osteogenic markers cbfa1/runx2, collagen-I, ALP, osteonectin, osteopontin, and osteocalcin.

ASC attachment seemed higher on BCP than ß-TCP, and unaffected by BMP-2. After 3 weeks, BMP-2 upregulated DNA content on BCP 60/40 by 2.4-fold compared to untreated cells, whereas on BCP 20/80 and ß-TCP >0.7mm the upregulation was 1.5 and 1.9-fold respectively. ALP activity was increased by 1.9-fold in cells seeded on BCP 60/40. BMP-2 upregulated ALP activity in cells on BCP 20/80 by 1.6-fold, and on ß-TCP<0.7mm by 2.6-fold, but had no effect after seeding on ß-TCP>0.7mm. BMP-2 seemed to upregulate all osteogenic genes studied in ASCs seeded on BCP 60/40.

In conclusion, 15 min BMP-2 treatment did not affect cell attachment to BCP or ß-TCP scaffolds, but increased cell proliferation and osteogenic differentiation of ASCs seeded on BCP biomaterials. Therefore 15 min BMP-2 treated ASCs seeded on BCP scaffolds may improve bone regeneration.

Keywords: adipose stem cells, osteogenic differentiation, BMP-2, biphasic calcium phosphate, ß-tricalcium phosphate, hydroxyapatite, scaffold, bone tissue engineering

## 1. Introduction

Use of autograft, or patient's own bone, is the golden standard for bone tissue engineering. It is the only type of bone graft supplying living bone cells, and has osteogenic and osteoinductive properties. Since autograft is retrieved from the same individual, the tissue is recognized as "self" and the immune system it does not provoke an immunological response (Ellis, 2003). Although

there are many advantages to using autologous bone, there are major drawbacks associated to the harvesting, such as limited bone source, donor-site morbidity, risk of infection, and the surgery may cause post-operative continuous pain, hypersensitivity, pelvic instability, and paresthesia. Use of allografts, xenografts, or biosynthetic substitutes eliminates these drawbacks associated with autologous bone harvesting (Meijer *et al*., 2007). Biosynthetic substitutes such as β-tricalcium phosphate (β-TCP), hydroxyapatite (HA), and mixtures of HA/β-TCP (biphasic calcium phosphates; BCP) have been successfully used as a bone graft substitute, because of their good biocompatibility and chemical composition, which resemble the composition of the natural bone matrix (Frenken *et al*., 2010; Lee *et al*., 2008; Zerbo *et al*., 2005; Zijderveld *et al*., 2005). Nevertheless, the inertness of these materials and the lack of osteogenic properties results in a slower rate of new bone formed (Petite *et al*., 2000, Zerbo *et al*., 2004).

Autologous adult mesenchymal stem cells provide new and innovative tools in tissue engineering. These cells maybe used to restore or replace tissues or organs. Bone marrow is a common source for mesenchymal stem cells, but only available in limited amounts (Pittenger *et al*.,1999). However, recently adipose tissue has been described as an alternative source for adult stem cells, which show mesenchymal markers (Lee *et al*., 2004; Zuk *et al*., 2001). Zuk *et al*. (2002) demonstrated that adipose tissue-derived mesenchymal stem cells (ASCs) can differentiate towards the adipogenic, chondrogenic, myogenic, neurogenic, and osteogenic lineage. BMP-2 has been shown to induce osteogenic differentiation in bone marrow-derived mesenchymal stem cells and ASCs *in vitro* and *in vivo* (Dragoo *et al*., 2003; Yamagiwa *et al*., 2001). BMP-2 transfected ASCs induce bone formation *in vivo* SCID mouse assay, and it stimulates healing of critical size defects in rats and rabbits (Dragoo *et al*., 2003; Peterson *et al*, 2005; Smith *et al*., 2008). In the bone tissue engineering field, ASCs have been used successfully to repair critical size calvarial defects in animals (Cowan *et al*., 2004; Cui *et al*., 2007; Yoon *et al*., 2007) as well as in a 7 year-old-girl (Lendeckel *et al*., 2004).

BMP-2 is available as FDA approved recombinant human protein and provides the possibility of immediate clinical use. Short treatment with BMP-2 could be of clinical importance especially if freshly isolated stem cells could be stimulated with BMP-2 during one surgical procedure and directly implanted in the patient (Helder *et al*., 2007). The delivery method and concentration of BMP-2 is of utmost importance for tissue engineering. The high doses of BMP-2 used in clinical studies present some adverse effects such as bone overgrowth and swelling (Chao *et al*., 2006; Smoljanovic *et al*., 2009). We have shown earlier that ex vivo incubation with BMP-2 at a million-fold lower concentration than used in clinical studies and applied for only 15 min is enough to stimulate osteogenic differentiation of ASCs (Knippenberg *et al*., 2006). Whether this

short incubation with BMP-2 also affects the attachment, proliferation, and differentiation of ASCs is yet unknown.

Different ceramics such as HA, β-TCP, or biphasic calcium phosphate (BCP) ceramics have been widely used in tissue engineering. β-TCP and HA support cell in-growth and promote osteogenic differentiation of mesenchymal stem cells (LL *et al*., 2010; Matsushima *et al*., 2009; Zerbo *et al*., 2005). Recently a BCP containing a mixture of 60% HA and 40% β-TCP has been shown to accelerate bone formation *in vivo* (Frenken *et al*., 2010; Friedmann *et al*., 2009). It facilitates proliferation and osteogenic differentiation of progenitor cells due to its high surface area and three-dimensional hierarchical porosity (Guha *et al*., 2009; Saldaña *et al*., 2009). The pore size is very important for bone tissue engineering, since large pore sizes promote neo-vascularization and favour mineralized bone ingrowth, whereas smaller pore sizes (90-120) primarily induce endochondral bone formation (Kuboki *et al*., 2001). HA scaffolds with pore size of 500 μm have shown optimal bone formation *in vivo* in comparison with scaffolds with pore size of 200 μm, which did not show any signs of bone formation (Kühne *et al*., 1994). Furthermore, high porosity favours blood vessel formation which is crucial for bone tissue engineering. Therefore for our study we used biomaterials with high porosity (60-90%), and β-TCP scaffolds with pore size larger than 100 μm, and BCP scaffolds with pore size larger than 500 μm.

The aim of our study was to investigate whether a short 15 min BMP-2 treatment affects the attachment, proliferation, and/or differentiation profiles of ASCs seeded on 4 different scaffolds: BCP 60/40 (Straumann® BoneCeramic; 60% HA and 40% ß-TCP), BCP 20/80 (Straumann® BoneCeramic; 20% HA and 80% ß-TCP), ß-TCP with particle size <0.7mm (Ceros® TCP), and ß-TCP with particle size >0.7mm (Ceros® TCP). The outcome of this study will bring us closer to the overall goal, which is to develop a tissue engineering approach for bone regeneration within a one-step-surgical procedure, using fresh autologous adipose stem cells in combination with a calcium phosphate scaffold.

## 2. Materials and Methods

### 2.1 Donors

Adipose tissue was harvested from the abdomen of nine healthy women (age: 23-32 years) undergoing elective abdominal wall correction in Tergooiziekenhuizen Hilversum Hospital, The Netherlands. The Ethical Review Board of the VU Medical Center, Amsterdam, The Netherlands, approved the protocol. Informed consent was obtained from all patients.

**2.2. ASC isolation**

Human adipose tissue was obtained by resection. ASCs were isolated from the resection material as described earlier with minor modifications (Varma *et al*., 2007). Adipose tissue was cut into small pieces, and enzymatically digested with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) for 45 min at 37ºC in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) under intermittent shaking. A single cell suspension was obtained by filtration through a 100 µm mesh filter. After thorough washing with PBS with 1% BSA a Ficoll density centrifugation step (1.077 g/ml ficoll, 280 ± 15 mOsm; Lymphoprep, Axis-Shield, Oslo, Norway) was performed to remove remaining erythrocytes from the SVF. After centrifugation at 600xg for 10 min, the resulting SVF pellet containing the ASCs was resuspended in expansion medium composed of Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) containing 10% fetal bovine serum (Gibco) supplemented with 500 µg/ml streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA), 500 µg/ml penicillin (Sigma-Aldrich), and 2.5 µg/ml amphotericin B (Gibco). Cell viability was assessed using the trypan blue exclusion assay. Cells were counted, using light microscopy, and immediately cultured on the different scaffolds or resuspended in Cryoprotective medium (Freezing Medium, BioWhittaker, Cambrex, Verviers, Belgium), frozen under "controlled rate" conditions in a Kryosave (HCl Cryogenics BV., Hedel, The Netherlands), and stored in liquid nitrogen until further use.

**2.3. ASC seeding on scaffolds and culture**

Freshly isolated or freshly cryopreserved and thawed ASCs were either or not incubated for 15 min with 10 ng/ml BMP-2 (Peprotech, London, UK) at 37°C. Single cell suspensions were seeded at $1x10^5$ cells per 25 mg of scaffold and cultured in Costar® Transwell® containers (Corning Life Sciences, Lowell, MA, USA) in 12 well plates (Greiner Bio-One, Kremsmuenster, Austria), containing expansion medium, with 25 mg of scaffold containing the cells per well. Four different scaffolds were used (Table 1): 1) Straumann® BoneCeramic (Straumann, Basel, Switzerland), a porous BCP with 60% HA and 40% ß-TCP (BCP 60/40), 2) Straumann® BoneCeramic, a porous BCP with 20% HA and 80% ß-TCP (BCP 20/80), 3) Ceros® TCP (Mathys, Bettlach, Switzerland), a porous ß-TCP with particle size 0.5-0.7 mm (ß-TCP<0.7mm), and 4) Ceros® TCP, a porous ß-TCP with particle size 0.7-1.4 mm (ß-TCP>0.7mm).

| Scaffold | Composition | Particle size (µm) | Porosity (%) | Pore width (µm) |
|---|---|---|---|---|
| Straumann® BoneCeramic BCP 60/40 | 60% HA/ 40% ß-TCP | 500-1000 | 90 | 500-1000 |
| Straumann® BoneCeramic BCP 20/80 | 20% HA/ 80% ß-TCP | 500-1000 | 90 | 500-1000 |
| Ceros® TCP β-TCP | 100% | 500-700 | 60 | 100-500 |
| Ceros® TCP β-TCP | 100% | 700-1400 | 60 | 100-500 |

**Table 1: Characteristics of the different scaffolds used.** HA, hydroxyapatite; ß-TCP, ß-tricalcium phosphate; BCP, biphasic calcium phosphate.

### 2.4. ASC attachment and proliferation on different scaffolds

ASCs, either or not incubated with BMP-2, were seeded onto the different scaffolds and allowed to attach for 30 minutes. Then ASC-seeded scaffolds were washed with PBS, and DNA concentration was determined as a measure for cell number to evaluate the number of cells attached. The scaffolds with cells were cultured in expansion medium in transwell containers up to 21 days in 5% CO2 in air at 37°C in a humidified atmosphere. After 4, 14 and 21 days, DNA concentration was determined as a measure for cell number to evaluate the proliferation profiles.

#### 2.4.1. Colony-forming unit fibroblasts (CFU-F) assay and CFU-F depletion assay

CFU-F assay was performed to assess if the frequency of ASCs in the SVF of adipose tissue was affected by BMP-2, as described elsewhere (Jurgens *et al*., 2008). A total of 1x103 and 1x104 cells were seeded in 6-well plates (Greiner Bio-One). After 14 days of culture CFU-F were fixed in 4% paraformaldehyde and stained with a 0.2% toluidine blue solution in borax buffer (pH 12) for 1 min. A colony was defined as ≥ 10 cells. The number of colonies was counted using light microscope at 100x magnification. The percentage of CFU-F per total number of ASCs seeded was calculated.

ASC attachment to the scaffolds was determined using the CFU-F depletion assay as described elsewhere (Jurgens *et al*., 2011). Non-attached cells, obtained by washing the scaffolds with PBS, were collected, and tested for CFU-F frequencies at 10-fold higher densities as normal CFU-F

seeding densities, since it was established that seeding at normal densities did not result in colony formation.

### 2.4.2. CyQUANT DNA proliferation assay

Immediately after ASC seeding and attachment, and after 4, 14, and 21 days of culture, the amount of DNA was quantified using the CyQUANT cell proliferation assay kit (Molecular Probes/Invitrogen, Carlsbad, CA, USA). Cells were washed with PBS, and stored at −80°C prior to analysis as recommended by the manufacturer. A reference standard curve was used. To quantify the amount of DNA, 200 µl of the CyQUANT GR dye/cell lysis buffer was added to the samples and incubated for 4 min at room temperature, protected from light. Then fluorescence was measured using a fluorescein filter set with filters for 480 nm excitation and 520 nm emission.

## 2.5. Osteogenic differentiation of ASCs on different biomaterials

Osteogenic differentiation was assessed by seeding fresh ASCs either or not incubated with BMP-2 at 1x105 cells per 25 mg scaffold material. Cells cultured in monolayer served as control to assess alkaline phosphatase (ALP) activity and gene expression using RT-PCR. ASCs (either or not treated with BMP-2), were cultured up to 21 days in DMEM supplemented with 10% fetal bovine serum, antibiotics, 10 mM β-glycerol phosphate (Sigma) and 50 µg/ml ascorbic acid (Merck, Darmstadt, Germany). If cells were seeded on calcium phosphate scaffolds, addition of β-glycerol phosphate was omitted from the culture medium. Medium was changed twice a week. ALP activity was determined at 4, 14, and 21 days of culture, and mRNA gene expression analysis was performed at 14 days as described below.

### 2.5.1. ALP activity

To assess the osteoblastic phenotype of ASCs cultured on scaffolds with and without 15 min of BMP-2 treatment, ALP activity was measured after 4, 14 and 21 days. Cells cultured on scaffolds were lysed with distilled water, and ALP activity and protein content were determined. As substrate p-nitrophenyl-phosphate (Merck) at pH 10.3 was used to determine ALP activity, according to the method described by Lowry *et al*. (1995). The absorbance was read at 410 nm. ALP activity was expressed as micromole per microgram of cellular protein. The amount of protein was determined by using a BCA Protein Assay reagent Kit (Pierce, Rockford, Ill., USA), and the absorbance was read at 540 nm with a microplate reader (Bio-Rad Laboratories GmbH, München, Germany).

**2.5.2. Quantitative Real-Time PCR**

Total cellular RNA was extracted from ASCs from 2 donors cultured on tissue culture plastic and different scaffolds for 14 days, using either RNA isolation Assay NucleoSpin® (Macherey-Nagel, Düren, Germany) or Trizol® reagent (Invitrogen, Carlsbad, USA) according to manufacturer's instructions, and stored at -80ºC prior to assay. The cDNA synthesis was performed in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City, CA, USA), using either SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) with 0.1 µg total RNA in a 20 µl reaction mix containing VILO™ Reaction Mix and SuperScript® Enzyme Mix, or 2 µg total RNA in a 20 µl reaction mix M-MLV Reverse transcriptase® (Invitrogen). cDNA was stored at -20ºC prior to real-time PCR. Real-time PCR reactions were performed using 2.5 µl cDNA and SYBR® Green Supermix (Roche Laboratories, IN, USA) according to the manufacturer's instructions in a LightCycler® (Roche Diagnostics). The target and reference genes were amplified in separate wells. All reactions were performed in triplicate. In each run the reaction mixture without the cDNA was used as negative control. Primers used for real-time PCR were from Invitrogen. For quantitative real-time PCR, the values of relative target gene expression were normalized for relative YWHAZ and ubiquitin (UB) housekeeping gene expression. Real-time PCR was used to assess expression of the following genes: cbfa1/runx2 (CBFA1), collagen 1 (COL1), alkaline phosphatase (ALP), osteonectin (ON), osteopontin (OPN), osteocalcin (OCN), and peroxisome proliferator-activated receptor gamma (PPARγ). Human primary osteoblasts were used as positive controls. Gene expression was compared between cells seeded on the different scaffolds with or without BMP-2 treatment.

**2.6. Statistical analysis**

Data was obtained from 6-9 donors. All data was expressed as mean ± SEM. Differences in proliferation and ALP activity between groups were tested with Student's two tailed t-test. ANOVA two-way analysis of variance was used to compare attachment data between groups. Differences were considered significant if $p<0.05$. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, USA) and GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, USA).

## 3. Results

### 3.1. BMP-2 increases ASC frequency and does not affect cell attachment on different scaffolds

Freshly isolated BMP-2 treated or nontreated ASCs, as well as freshly frozen ASCs from several donors, were seeded onto BCP and β-TCP (n=6). After 30 min of seeding, DNA was quantified. No significant differences in cell attachment were detected between the scaffolds after BMP-2 treatment (Fig. 1A). However, a higher cell number seemed to be present on BCP than on ß-TCP. The washing steps after cell attachment were collected, and seeded on tissue culture plastic to measure the CFU-F by using the depletion CFU-F assay (n=6) (Fig. 1B). Cell attachment was unaffected by BMP-2. More CFU-F were observed from washing steps collected from ß-TCP than from BCP indicating that more cells adhered to BCP than ß-TCP ($p<0.05$).

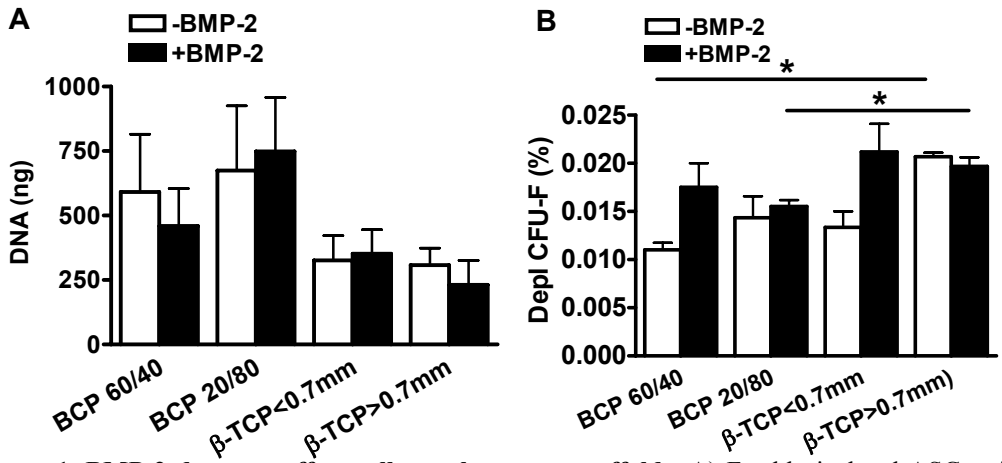



**Figure 1. BMP-2 does not affect cell attachment to scaffolds.** A) Freshly isolated ASCs, either or not treated with BMP-2, were seeded on four different scaffolds, allowed to attach for 30 min and the concentration of DNA was determined. The four different scaffolds were: 1) Straumann® BoneCeramic, a porous BCP with 60% HA and 40% ß-TCP (BCP 60/40), 2) Straumann® BoneCeramic, a porous BCP with 20% HA and 80% ß-TCP (BCP 20/80), 3) Ceros® TCP, a porous ß-TCP with particle size 0.5-0.7 mm (ß-TCP<0.7mm), and 4) Ceros® TCP, a porous ß-TCP with particle size 0.7-1.4 mm (ß-TCP>0.7mm). B) BMP-2 did not affect the number of non-attached ASCs to different scaffolds. More non-attached ASCs were collected from ß-TCP>0.7mm than from BCP 60/40. More BMP-2 treated non-attached ASCs were collected from ß-TCP>0.7mm than from BCP 20/80. *Significant difference between scaffolds, $p<0.05$. CFU-F, colony-forming unit fibroblasts; Depl CFU-F, colony-forming unit fibroblasts obtained by using the CFU-F depletion assay; BMP-2, bone morphogenetic protein-2; BCP, biphasic calcium phosphates; β-TCP, β-tricalcium phosphate.

To determine if a short stimulation of 15 min with BMP-2 affects the frequency of mesenchymal progenitors from adipose tissue, functional viability of ASCs was determined. The number of toluidin blue-positive colonies (CFU-F) was counted 14 days after seeding freshly isolated ASCs, treated with or without BMP-2, on tissue culture plastic (n=9). BMP-2 treatment increased by ~2-fold the percentage of CFU-F per total number of ASCs seeded. This shows that a short stimulation of 15 min with BMP-2 increases significantly the CFU-F frequency of ASCs seeded on tissue culture plastic after 2 weeks of culture ($p<0.001$; Fig. 2).

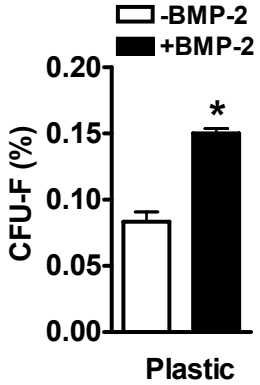



**Figure 2. Effect of BMP-2 on the frequency of ASCs in adipose tissue.** A) BMP-2 treatment of freshly isolated ASCs increased % of CFU-F, reflecting the number of viable ASCs in adipose tissue. *Significant effect of BMP-2, $p<0.001$. CFU-F, colony-forming unit fibroblasts; BMP-2, bone morphogenetic protein-2; Plastic, tissue culture plastic.

### 3.2. BMP-2 affects ASC proliferation dependent on the scaffold

ASCs cultured on BCP and β-TCP were quantified and DNA concentration was compared at day 4, day 14, and day 21 of culture, and results were expressed as fold increase of BMP-2 treated ASCs versus non-treated ASCs (n=6). At day 21, DNA concentration of stem cells seeded on BCP 60/40 was significantly higher after BMP-2 treatment, with an increase of 2.4 ± 0.5 fold versus non-treated cells ($p<0.05$; Fig. 3). DNA content of stem cells seeded on BCP 20/80, ß-TCP<0.7mm, and on ß-TCP>0.7mm after BMP-2 treatment seemed to increase by 1.5 ± 0.6 fold, 1.1 ± 0.09 fold, and 1.9 ± 0.8 fold, respectively. Therefore BMP-2 increases ASC proliferation on the biomaterial dependent on the scaffold.

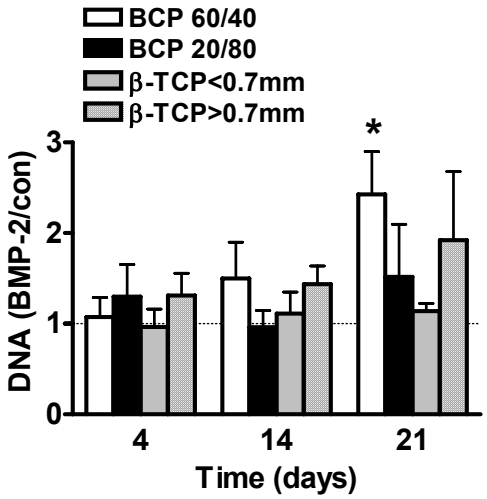



**Figure 3. BMP-2 increases ASC proliferation dependent on the scaffold at 21 days of culture.** Freshly isolated ASCs, either or not treated with BMP-2, were seeded on four different scaffolds: 1) Straumann® BoneCeramic, a porous BCP with 60% HA and 40% ß-TCP (BCP 60/40), 2) Straumann® BoneCeramic, a porous BCP with 20% HA and 80% ß-TCP (BCP 20/80), 3) Ceros® TCP, a porous ß-TCP with particle size 0.5-0.7 mm (ß-TCP<0.7mm), and 4) Ceros® TCP, a porous ß-TCP with particle size 0.7-1.4 mm (ß-TCP>0.7mm). BMP-2 significantly stimulated DNA content of stem cells seeded on BCP 60/40 at day 21. * $p<0.05$. DNA content is expressed as BMP-2-treated-over-untreated control. 1 means no effect by BMP-2. BMP-2, bone morphogenetic protein-2; BCP, biphasic calcium phosphates; β-TCP, β-tricalcium phosphate; con, control.

### 3.3. BMP-2 induces ALP activity dependent on the scaffold

To investigate whether 15 min treatment with BMP-2 on human ASCs cultured on different calcium phosphate scaffolds is enough to undergo osteogenic differentiation *in vitro*, we measured ALP activity after 4, 14 and 21 days of culture.

ALP activity of ASCs cultured on four different calcium phosphate scaffolds was measured using an ELISA method, and we expressed the results as fold increase in BMP-2 treated cells versus non-treated cells (n=6). The ALP activity of ASCs cultured on BCP 60/40 scaffold increased throughout the entire culture period, being significantly higher at day 21 of culture with $1.9 \pm 0.5$ fold increase versus non-treated cells ($p<0.05$; Fig. 4).

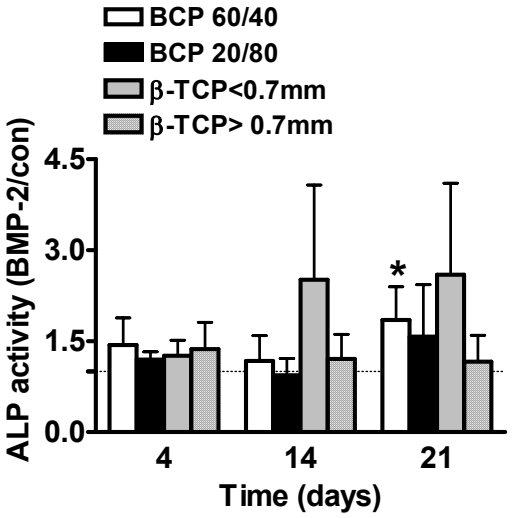



**Figure 4. BMP-2 increases ALP activity dependent on the scaffold at 21 days of culture.** Freshly isolated ASCs, either or not treated with BMP-2, were seeded on four different scaffolds: 1) Straumann® BoneCeramic, a porous BCP with 60% HA and 40% ß-TCP (BCP 60/40), 2) Straumann® BoneCeramic, a porous BCP with 20% HA and 80% ß-TCP (BCP 20/80), 3) Ceros® TCP, a porous ß-TCP with particle size 0.5-0.7 mm (ß-TCP<0.7mm), and 4) Ceros® TCP, a porous ß-TCP with particle size 0.7-1.4 mm (ß-TCP>0.7mm). BMP-2 significantly stimulated ALP activity in ASCs seeded on BCP 60/40 at day 21. * $p<0.05$. ALP activity is expressed as BMP-2-treated-over-untreated control. 1 means no effect of BMP-2. ALP, alkaline phosphatase activity; BMP-2, bone morphogenetic protein-2; BCP, biphasic calcium phosphates; β-TCP, β-tricalcium phosphate; con, control.

### 3.4. BMP-2 induces osteogenic gene expression dependent on the scaffold

To further confirm the differentiation of BMP-2 treated ASCs on different calcium phosphate scaffolds, mRNA expression of genes associated with osteogenesis such as cbfa1/runx2 (CBFA1), collagen 1 (COL1), alkaline phosphatase (ALP), osteonectin (ON), osteopontin (OPN), and osteocalcin (OCN) was examined by RT-PCR after 2 weeks of culture (Fig. 5A-F). ASCs cultured on tissue culture plastic without BMP-2 seemed to express only CBFA1, COL1, and ON mRNA (Fig. 5A,B,D). After BMP-2 treatment, the expression of the osteogenic genes CBFA1, COL1, and ON seemed to increase on tissue culture plastic at day 14 (Fig. 5A,B,D). BMP-2 treatment seemed to increase ALP mRNA gene expression in tissue culture plastic (Fig. 5C). ASCs cultured on BCP 60/40 scaffolds without BMP-2 expressed CBFA1, COL1, ALP and ON mRNA (Fig. 5A-D). BMP-2 treatment seemed to increase the expression of the osteogenic genes CBFA1, COL1, ALP and ON mRNA (Fig. 5A-D). OPN and OCN gene expression seemed to increase in BMP-2 treated ASCs seeded on BCP 60/40 as well (Fig. 5E,F). On the contrary, BMP-2 treated ASCs cultured on ß-TCP scaffolds did not show an increase in osteogenic genes,

except OCN gene expression, detected in BMP-2 treated ASCs seeded on ß-TCP>0.7mm (Fig. 5F).

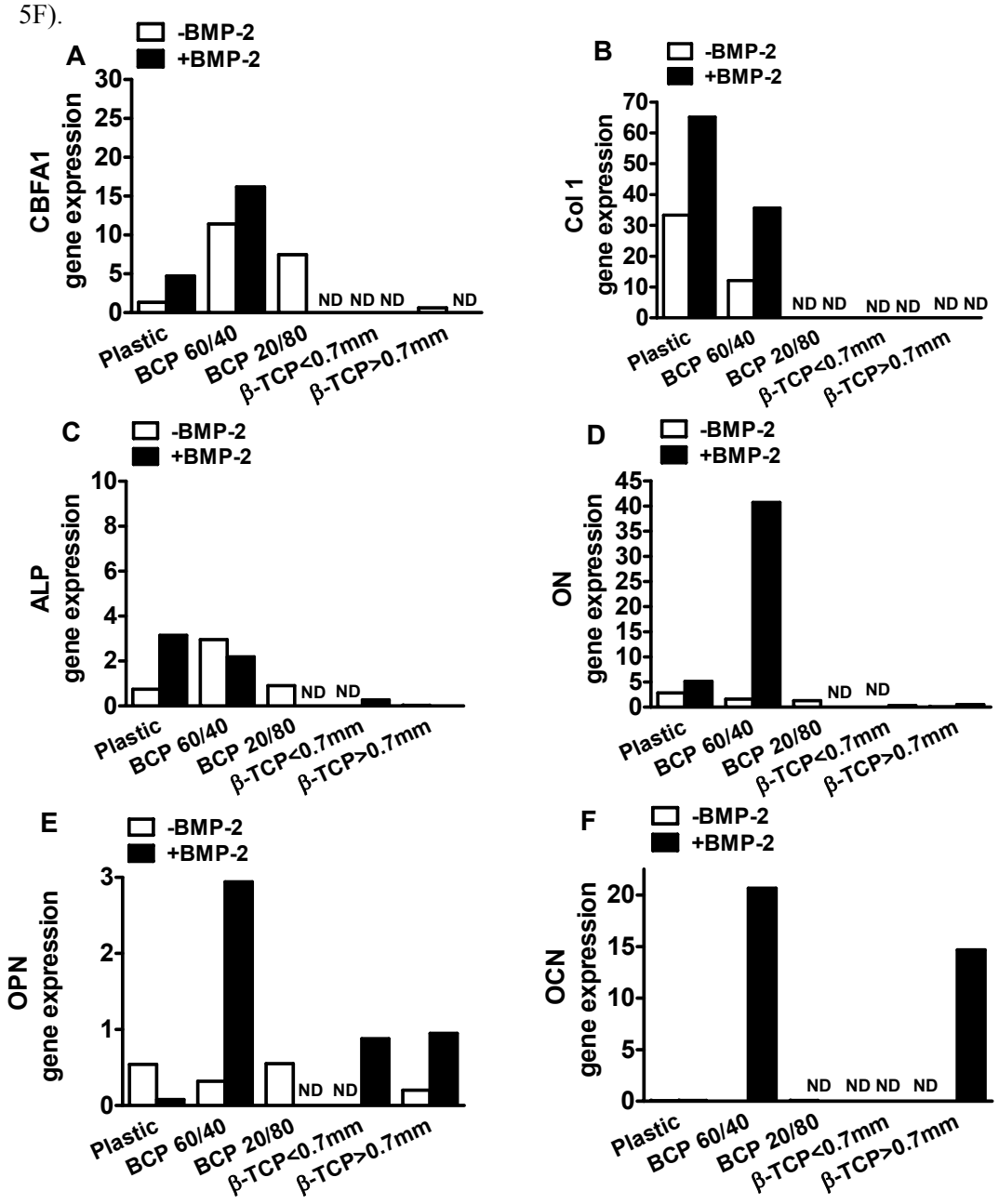



**Figure 5. BMP-2 seems to increase osteogenic gene expression in ASCs seeded on BCP 60/40 at 14 days of culture.** Freshly isolated ASCs, either or not treated with BMP-2, were seeded on tissue culture plastic and on four different scaffolds: 1) Straumann® BoneCeramic, a porous BCP with 60% HA and 40% ß-TCP (BCP 60/40), 2) Straumann® BoneCeramic, a porous BCP with 20% HA and 80% ß-TCP (BCP 20/80), 3) Ceros® TCP, a porous ß-TCP with particle size 0.5-0.7 mm (ß-TCP<0.7mm), and 4) Ceros® TCP, a porous ß-TCP with particle size 0.7-1.4 mm (ß-TCP>0.7mm).

**Figure 5 continuation.** A) CBFA1 gene expression. B) COL1 gene expression. C) ALP gene expression. D) ON gene expression. E) OPN gene expression. F) OC gene expression. Expression levels are represented as fold increase in comparison with undifferentiated ASCs, after normalization to the housekeeping genes UB and YWHAZ. CBFA1, cbfa1/runx2; COL1, collagen I; ALP, alkaline phosphatase; ON, osteonectin; OPN, osteopontin; OCN, osteocalcin; UB, ubiquitin; BMP-2, bone morphogenetic protein-2; BCP, biphasic calcium phosphates; β-TCP, β-tricalcium phosphate; ND, not detected.

PPARγ mRNA gene expression seemed to decrease on ASCs cultured on tissue culture plastic and on different calcium phosphate scaffolds, indicating the osteogenic commitment of the cells, and was not affected by BMP-2 treatment (Fig. 6).

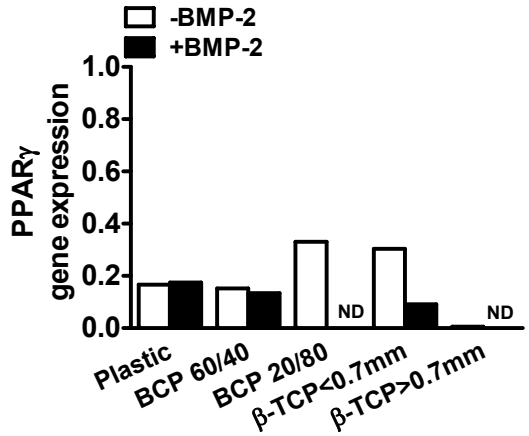



**Figure 6. BMP-2 does not affect adipogenic gene expression in ASCs seeded on BCP 60/40, BCP 20/80, ß-TCP<0.7mm, and ß-TCP>0.7mm after 14 days of culture.** PPARγ gene expression was measured in ASCs seeded on plastic, BCP, and β-TCP. Expression levels are represented as fold increase in comparison with undifferentiated ASCs, after normalization to the housekeeping genes UB and YWHAZ. PPARγ, peroxisome proliferator-activated receptor gamma; UB, ubiquitin; BMP-2, bone morphogenetic protein-2; BCP, biphasic calcium phosphates; β-TCP, β-tricalcium phosphate; ND, not detected.

## 4. Discussion

For bone tissue engineering, the appropriate source to obtain the stem cells is crucial, as well as the adequate type of scaffold, optimal pore size, porosity, and composition, and osteoinductive growth factors for stimulation of stem cell differentiation. It is important what agent to use, which dose (the volume delivery), which site (focal delivery), and which delivery matrix. In this study we investigated whether short BMP-2 treatment affects attachment, proliferation, and/or differentiation profiles of ASCs seeded on BCP or ß-TCP. We found that ASC attachment seemed higher on BCP than ß-TCP. Short 15 min BMP-2 treatment did not affect cell attachment

to BCP or ß-TCP scaffolds, but increased cell proliferation and osteogenic differentiation of ASCs seeded on BCP biomaterials. Therefore 15 min BMP-2 treated ASCs seeded on BCP scaffolds may improve bone regeneration.

Nowadays, the growth factor BMP-2 in combination with cells and/or scaffolds gain more and more significance for their use in bone tissue engineering. BMP-2 is available as FDA approved recombinant human proteins and may be used for immediate clinical application in combination with stem cells and scaffolds to accelerate bone healing and to create new bone (Smith *et al*., 2008). Earlier we found that BMP-2 treatment for only 15 min and at a concentration as low as 10 ng/ml is capable of inducing osteogenic differentiation in ASCs (Knippenberg *et al*., 2006). Fifteen minutes of BMP-2 treatment up-regulated ALP activity and CBFA1 gene expression in ASCs at 4 days of culture. We also observed an increase in gene expression of the early osteogenic differentiation markers CBFA1 and ALP after BMP-2 treatment, together with an increase in COL1 and ON gene expression. The increase in CBFA1, ALP, and ON after seeding ASCs on BCP 60/40 scaffolds was similar or higher than after seeding the cells on tissue culture plastic. An increase in gene expression of the mature markers of osteoblast differentiation OPN and OCN was observed as well, which shows that ASCs seeded on BCP 60/40 are terminally differentiated, while expression of these late markers was not observed in ASCs seeded on tissue culture plastic after 14 days of culture in osteogenic medium. This suggests that seeding BMP-2 treated ASCs on BCP 60/40 may accelerate osteogenic differentiation of ASCs. These results agree with other published observations showing increased osteogenic differentiation of mesenchymal cells after applying BMP-2 in different manners (Dragoo *et al*., 2003; Knippenberg *et al*., 2006; Peterson *et al*., 2005) and/or using osteoconductive scaffolds (LL *et al*., 2010; Smith *et al*., 2008). One study observed highest osteogenic differentiation *in vitro* and *in vivo* by BMP-2 loaded scaffolds with undifferentiated ASCs when compared to PLGA/HA scaffolds alone, BMP-2-loaded scaffolds, and scaffolds with undifferentiated ASCs (Jeon *et al*., 2008). Expression of OCN, a terminal marker of osteoblast differentiation, was observed in BMP-2 loaded scaffolds with undifferentiated ASCs, which was not present when the scaffolds were not incubated with BMP-2. This correlates with our findings, where we observed OCN gene expression in BMP-2 treated ASCs seeded on BCP 60/40, and lack of OCN gene expression on non-treated ASCs seeded on these calcium phosphate scaffolds. Expression of the adipogenic gene PPARγ was decreased after 14 days of culture. This transcription factor is critical in promoting adipogenesis and inhibiting osteogenesis (Akune *et al*., 2004). Therefore the observed PPARγ down-regulation shows the osteogenic commitment of ASCs seeded on the calcium phosphate scaffolds.

We observed that BMP-2 increased ASC proliferation. This is in contrast with observations on bone marrow mesenchymal stem cells (MSCs), which did not show a change in proliferation after treatment with recombinant BMP-2, but it did show an effect of BMP-2 on endothelial progenitor cell proliferation (Raida *et al*., 2006). This suggests that different cell populations and/or different sources of mesenchymal stem cells may respond differently to this growth factor. Although adipose stem cells are MSCs as are bone marrow MSCs, they differ in expression of stem cell markers. These differences in stem cell marker expression could be responsible for the different response of ASCs and bone marrow MSCs to BMP-2 treatment (Strem *et al*., 2006).

Incubation with recombinant BMP-2 at a low concentration of 10 ng/ml for 15 min has been shown to affect osteogenic differentiation of ASCs *in vitro* (Knippenberg *et al*., 2006). Another study reported that medium supplementation with BMP-2 at a concentration of 100 ng/ml significantly increased ALP activity, COL1, ALP, and OCN gene expression of MSCs on β-TCP scaffolds, without affecting the cell viability (LL *et al*. 2010). Others also showed that incubation with BMP-2 increased the osteogenic response of ASCs *in vitro* (Dragoo *et al*., 2003) and improved bone healing *in vivo* (Jeon *et al*., 2008, Mesimaki *et al*., 2009). The low dose of BMP-2 used in our study diminishes the risk of bone overgrowth at the site of application, caused by the high dosis of growth factor used in clinical studies of 0.9–2 mg/ml scaffold, as well as the high costs of using these concentrations (Perri *et al*., 2007; Samartzis *et al*., 2005). Moreover, there are concerns regarding local inflammatory effects caused by these high concentrations. An ex vivo incubation of autologous stem cells with growth factors at a million-fold lower concentration for only 15 min could be easily applied in the operating room for immediate clinical applications of the progenitor cells. Autologous stem cells could be isolated, triggered with growth factors, and subsequently used for bone or cartilage regeneration in the patient in the same operation (Helder *et al*., 2007).

We have shown that BCP of 60% HA and 40% β-TCP induces significantly higher cell proliferation and osteogenic differentiation of BMP-2 treated ASCs. This suggests that this scaffold could be successfully used for bone tissue engineering. HA is a major component of bones and teeth and provides strength and resistance to compression, and can incorporate and modulate the delivery of molecular signals controlling cellular functions, thereby promoting the proliferation and attachment of osteoprogenitor cells (Marot *et al*., 2007). The composition of HA, with a high content of calcium phosphate, may also improve the osteogenic differentiation of these cells. HA particles have shown higher bone formation than β-TCP particles once implanted with MSC *in vivo* (Matsushima *et al*., 2009). Scaffolds containing HA such as BCP 60/40 combined with BMP-2 treated ASCs, could be applied *in vivo* to heal critical size bone defects

and to stimulate bone regeneration after maxillary sinus elevation. A short treatment of 15 min with BMP-2 could be easily applied in one step surgery procedures, where autologous stem cells are isolated, treated with the growth factor, seeded immediately on scaffolds and applied to the patient in the same surgical procedure.

## 5. Acknowledgments

We wish to extend our thanks to the surgical staff of Tergooiziekenhuizen Hilversum Hospital, The Netherlands, for kindly providing the human adipose tissue for this study. We also thank the technical staff of the Department of Oral Cell Biology, ACTA-University of Amsterdam and VU University Amsterdam, for their assistance, and the Skeletal Tissue Engineering Group Amsterdam (STEGA) at the VU University Amsterdam for their support of this study.

## 6. Author disclosure statement

No competing financial interests exist.

## 7. References

Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung UI, Kubota N, et al. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest. 2004 Mar;113(6):846-855.

Chao M, Donovan T, Sotelo C, Carstens MH. In situ osteogenesis of hemimandible with rhBMP-2 in a 9-year-old boy: osteoinduction via stem cell concentration. J Craniofac Surg. 2006 May;17(3):405-412.

Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, Quarto N, Contag CH, Wu B, Longaker MT. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol. 2004 May;22(5):560-567.

Cui L, Liu B, Liu G, Zhang W, Cen L, Sun J, Yin S, Liu W, Cao Y. Repair of cranial bone defects with adipose derived stem cells and coral scaffold in a canine model. Biomaterials. 2007 Dec; 28(36):5477-5486.

Dragoo JL, Choi JY, Lieberman JR, Huang J, Zuk PA, Zhang J, et al. Bone induction by BMP-2 transduced stem cells derived from human fat. J Orthop Res. 2003 Jul;21(4):622-629.

E LL, Xu LL, Wu X, Wang DS, Lv Y, Wang JZ, et al. The interactions between rat-adipose-derived stromal cells, recombinant human bone morphogenetic protein-2, and beta-tricalcium phosphate play an important role in bone tissue engineering. Tissue Eng Part A. 2010 Sep;16(9):2927-2940.

Ellis E. Surgical reconstruction of defects of the jaws. Peterson LJ, Ellis E, Hupp J, Tucker M, eds. Contemporary Oral and Maxillofacial Surgery. Philadelphia: Ed Mosby. 2003. pp. 646-651.

Frenken JW, Bouwman WF, Bravenboer N, Zijderveld SA, Schulten EA, ten Bruggenkate CM. The use of Straumann Bone Ceramic in a maxillary sinus floor elevation procedure: a clinical, radiological, histological and histomorphometric evaluation with a 6-month healing period. Clin Oral Implants Res. 2010 Feb;21(2):201-208

Friedmann A, Dard M, Kleber BM, Bernimoulin JP, Bosshardt DD. Ridge augmentation and maxillary sinus grafting with a biphasic calcium phosphate: histologic and histomorphometric observations. Clin Oral Implants Res. 2009 Jul;20(7):708-714.

Guha AK, Singh S, Kumaresan R, Nayar S, Sinha A. Mesenchymal cell response to nanosized biphasic calcium phosphate composites. Colloids Surf B Biointerfaces. 2009 Oct;73(1):146-151.

Helder MN, Knippenberg M, Klein-Nulend J, Wuisman PI. Stem cells from adipose tissue allow challenging new concepts for regenerative medicine. Tissue Eng. 2007 Aug;13(8):1799-1808.

Jeon O, Rhie JW, Kwon IK, Kim JH, Kim BS, Lee SH. *In vivo* bone formation following transplantation of human adipose-derived stromal cells that are not differentiated osteogenically. Tissue Eng Part A. 2008 Aug;14(8):1285-1294.

Jurgens WJ, Kroeze RJ, Bank RA, Ritt MJ, Helder MN. Rapid attachment of adipose stromal cells on resorbable polymeric scaffolds facilitates the one-step surgical procedure for cartilage and bone tissue engineering purposes. J Orthop Res. 2011.

Jurgens WJ, Oedayrajsingh-Varma MJ, Helder MN, Zandiehdoulabi B, Schouten TE, Kuik DJ, et al. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell Tissue Res. 2008 Jun;332(3):415-426.

Knippenberg M, Helder MN, Zandieh Doulabi B, Wuisman PI, Klein-Nulend J. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. Biochem Biophys Res Commun. 2006 Apr 14;342(3):902-908.

Kuboki Y, Jin Q, and Takita H. Geometry of carriers controlling phenotypic expression in BMP-induced osteogenesis and chondrogenesis. J Bone Joint Surg Am. 2001;83-A Suppl 1 (Pt 2):S105-S115.

Kühne JH, Bartl R, Frisch B, Hammer C, Jansson V, Zimmer M. Bone formation in coralline hydroxyapatite. Effects of pore size studied in rabbits. Acta Orthop Scand. 1994 Jun;65(3): 246-252.

Lee JH, Jung UW, Kim CS, Choi SH, Cho KS. Histologic and clinical evaluation for maxillary sinus augmentation using macroporous biphasic calcium phosphate in human. Clin Oral Implants Res. 2008 Aug;19(8):767-771.

Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, Bae YC, and Jung JS. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem. 2004; 14(4-6):311-324.

Lendeckel S, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, et al. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. J Craniomaxillofac Surg. 2004 Dec;32(6):370-373.

Marot D, Knezevic M, Novakovic GV. Bone tissue engineering with human stem cells. Stem Cell Res Ther. 2010 May 4;1(2):10.

Matsushima A, Kotobuki N, Tadokoro M, Kawate K, Yajima H, Takakura Y, et al. *In vivo* osteogenic capability of human mesenchymal cells cultured on hydroxyapatite and on beta-tricalcium phosphate. Artif Organs. 2009 Jun;33(6):474-481.

Meijer GJ, de Bruijn JD, Koole R, van Blitterswijk CA. Cell-based bone tissue engineering. PloS Med. 2007 Feb;4(2):39.

Perri B, Cooper M, Lauryssen C, Anand N. Adverse swelling associated with use of rh-BMP-2 in anterior cervical discectomy and fusion: adiscectomy and fusion: a case study. Spine J. 2007 Mar-Apr;7(2):235-239.

Peterson B, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P, Lieberman JR. Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue, Tissue Eng. 2005 Jan-Feb;11(1-2):120-129.

Petite H, Viateau V, Bensaïd W, Meunier A, de Pollak C, Bourguignon M, et al. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-963.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr;284(5411):143-147.

Raida M, Heymann AC, Günther C, Niederwieser D. Role of bone morphogenetic protein 2 in the crosstalk between endothelial progenitor cells and mesenchymal stem cells. Int J Mol Med. 2006 Oct;18(4):735-739.

Saldaña L, Sánchez-Salcedo S, Izquierdo-Barba I, Bensiamar F, Munuera L, Vallet-Regí M, et al. Calcium phosphate-based particles influence osteogenic maturation of human mesenchymal stem cells. Acta Biomater. 2009 May;5(4):1294-1305.

Samartzis D, Khanna N, Shen FH, An HS. Update on bone morphogenetic proteins and their application in spine surgery. J Am Coll Surg. 2005 Feb;200(2):236-234.

Smith DM, Afifi AM, Cooper GM, Mooney MP, Marra KG, Losee JE. BMP-2-based repair of large-scale calvarial defects in an experimental model: regenerative surgery in cranioplasty. J Craniofac Surg. 2008 Sep;19(5):1315-1322.

Smoljanovic T, Janjanin S, Bojanic I. Avoiding unanticipated adverse effects of recombinant human bone morphogenetic protein-2 therapy in craniofacial surgery with experiences from spinal applications. J Craniofac Surg. 2009 Sep;20(5):1626.

Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, et al. Multipotential differentiation of adipose tissue-derived stem cells. Keio J Med. 2005 Sep;54(3):132-141.

Varma MJ, Breuls RG, Schouten TE, Jurgens WJ, Bontkes HJ, Schuurhuis GJ, et al. Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells. Stem Cells Dev.2007;16:91-104.

Yamagiwa H, Endo N, Tokunaga K, Hayami T, Hatano H, Takahashi HE. *In vivo* bone-forming capacity of human bone marrow-derived stromal cells is stimulated by recombinant human bone morphogenetic protein-2.J Bone Miner Metab. 2001;19(1):20-28.

Yoon E, Dhar S, Chun DE, Gharibjanian NA, Evans GR. *In vivo* osteogenic potential of human adipose-derived stem cells/poly lactide-co-glycolic acid constructs for bone regeneration in a rat critical-sized calvarial defect model. Tissue Eng. 2007 Mar;13(3):619-627.

Zerbo IR, Bronckers AL, de Lange G, Burger EH. Localisation of osteogenic and osteoclastic cells in porous beta-tricalcium phosphate particles used for human maxillary sinus floor elevation. Biomaterials. 2005 Apr;26(12):1445-1451.

Zerbo IR, Zijderveld SA, de Boer A, Bronckers AL, de Lange G, ten Bruggenkate CM, et al. Histomorphometry of human sinus floor augmentation using a porous beta-tricalcium phosphate: a prospective study. Clin Oral Implants Res. 2004 Dec;15(6):724-732.

Zijderveld SA, Zerbo IR, van den Bergh JP, Schulten EA, ten Bruggenkate CM. Maxillary sinus floor augmentation using a beta-tricalcium phosphate (Cerasorb) alone compared to autogenous bone grafts. Int J Oral Maxillofac Implants. 2005 May-Jun;20(3):432-440.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001 Apr;7(2):211-222.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-429.