The Effect of 5 Proteins On DPPSC (Dental Pulp Pluripotent Stem Cells) For Osteoblast Differentiation Proliferation In 3D

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Tesis Doctoral

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Que el trabajo de investigación titulado:

The effect of 5 proteins on DPPSC (dental pulp pluripotent stem cells) for osteoblast differentiation proliferation in 3D.

De la que es autora Punjamaporn Chatakun, se ha realizado bajo nuestra dirección en el del laboratorio de medicina regenerativa de la facultad de odontología para optar al grado de Doctora por la Universitat Internacional de Catalunya.

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1. ABBREVIATIONS

ADSCs: Adipose-derived stromal cells

ASCs: Adult human adipose-derived stem cells

ALP: Alkaline phosphatase

AP1: Activator protein 1

BMPs: Bone morphogenetic proteins

BMSC: Bone mesenchymal stem cell

BMSCs: Bone marrow stromal cells

BSP: Bone sialoprotein

CaP: Calcium phosphate

CRE: cAMP response element

DPSC: Dental pulp stem cell.

DPPSC: Dental pulp pluripotent stem cells

DPMSC: Dental pulp mesenchymal stem cells

ECM: Extracellular matrix

ERRalpha: Estrogen receptor-related receptor alpha

ESC: Embryonic stem cells

FGF: Fibroblast growth factor

FGF2: Fibroblast growth factor 2

FN: Fibronectin

FRE: Fibroblast growth factor 2 response element

FSK: Forskolin

GM-CSF: Granulocyte macrophage colony-stimulating factor

HA: Hydroxyapatite

HGF: Hepatocyte growth factor

HOK: Homeodomain protein-binding site

JNK: c-Jun NH2-Terminal Kinase

LPS: Lipopolysaccharide

MMP: Metalloproteinase

MSCs: Mesenchymal stem cell

OA: Osteoactivin

OC: Osteocalcin

OPN: Osteopontin

PDGF-AB: Platelet-derived growth factor-AB

PEA: Poly ethyl acrylate

PEMF: Pulsed electromagnetic fields

PKA: cAMP-dependent protein kinase

PLGA: Poly lactic-co-glycolic acid

PLLA; Poly L-lactic acid

PTH: Parathyroid hormone

ROK: Rho-associated protein kinase

TG: Tissue transglutaminase

TGF-BETA1: Transforming growth factor-beta1

TNC: Tenascin C

VEGF: Vascular endothelial growth factor

2. ABSTRACT

Bone-tissue engineering is a therapeutic target in the field of dental implant and orthopaedic surgery. It is therefore essential to find a microenvironment that enhances the growth and differentiation of osteoblasts both from mesenchymal stem cells (MSCs) and those derived from dental pulp (DPSC). The aim of this research is to determine the relationship among the proteins fibronectin (FN), bone morphogenetic protein 2 (BMP2) osteopontin (OPN), tenascin C (TNC) and bone sialoprotein (BSP) and their ability to coat different types of biomaterials and surfaces to enhance osteoblast differentiation. To evaluate the osteogenic capacities of dental pulp pluripotent stem cells (DPPSC) and dental pulp mesenchymal stem cells (DPMSC), DPPSC and DPMSC were cultured on bio-coating of 5 different types of proteins 20 days. We performed quantification of alkaline phosphatase and Ca^{2+} in the medium and RT-PCR with osteogenic marker (Col I, BMP2 and OCN). SEM images corresponding to OPN and TNC group were performed. After that we tested the strength of scaffold in all groups. The result represents DPPSC seeded on 3d scaffold coated with OPN has the highest osteoinduction. DPMSC seeded on 3d scaffold coated with BMP2 has highest osteoinduction. We suggest that DPMSC is more suitable for bone regeneration than DPPSC. However, for dental implant placement or trauma, we prefer trabecular bone with great blood supply, thus, bone regeneration from DPPSC may get a better result. DPPSC is a new strategy to regenerate bone. We are coming closer to provide bone regeneration by using DPPSC.

3. JUSTIFICATION

Bone augmentation is generally carried out using autogenous bone. The ideal bone graft incorporates three main elements: osteogenic, osteoinductive and osteoconductive. Autogenous bone is the gold standard. Osteogenic means stem cell or progenitors that are able to form new bone. Osteoinduction is the ability to stimulate bone's new formation. Osteoconduction is the ability to support osteogenesis in a vital bone. [1] The advantages of autogenous bone graft are the fast angiogenic from the surrounding original bone [2] and provide osteoblast.[3] Autogenous bone is not able to fulfill all requirements for bone regeneration. Discomfort and pain at the donor site of autogenous bone grafting are unavoidable and the donor sites are limited supply. [4] The large variability in bone defects, the huge biological complexity of bone, the high metabolic activity of bone and the necessitating vascularization motivate the development of new treatment strategies.[5] Tissue engineering is an alternative to conventional methods for reconstruction. Strategies to engineer bone tissue have been developed using 3 components: scaffold, growth factors, and stem cells. The existence of osteoblasts is important to develop and maintain the skeleton owing to the capable of secreting the structural proteins of bone.[6]

Stem cells are unspecialized cells. They are characterized by two unique properties in one cell: their high self-renewal activity and their multilineage differentiation potential, which make them an ideal source for cellular therapy and regenerative medicine. [7] Mesenchymal stem cell (MSCs) is one type of stem cells and can be isolated from a variety tissue, such as bone marrow, adipose tissue, dental pulp, etc. MSCs can differentiate into several types of cells, including fibroblasts, adipocytes, osteoblasts, chondrocytes and skeletal muscle cells.[8] Dental pulp is a highly vascularized tissue and is another type of MSCs. Dental Pulp Stem Cells or (DPSCs) are multipotent stem cells. DPSCs presented

parameters characterizing the osteoblastic phenotype, osteoblast-like cells, alkaline phosphatase (ALP), collagen I (Col I), osteopontin (OPN) and osteocalcin (OCN). So it may be a potential source of osteoblasts to be used for bone regeneration.[9] DPSCs show good adherence and bone tissue formation on microconcavity surface textures. SBP-DPSCs are a multipotent stem cell subpopulation of DPSCs. They can differentiate into osteoblasts. Synthesizing 3D woven bone tissue chips in vitro and that are capable to synergically differentiate into osteoblasts and endotheliocytes.[10]

The nature of the surface on which cells are cultured plays an important role in their ability to attach, proliferate, migrate and function.[11] Components of the extracellular matrix (ECM) are often used to coat glass or plastic surfaces to enhance cell attachment in vitro.[11] Interestingly, which kinds of proteins are able to coat with scaffolds for enhancing osteoblast attachment. The aim of this review is to conclude the relationship between five proteins (Fibronectin(FN), Bone morphogenetic protein(BSP), Osteopontin(OPN), Tenascin(TN) and Bone sialoproteins(BSP)) and osteoblast differentiation.

3.1 Mesenchymal stem cell

Mesenchymal stem cells (MSCs) are multipotent stem cells found in the bone marrow stromal cells and other organs. MSCs can differentiate into multiple nonhematopoietic cell lineages, including fibroblasts, adipocytes, osteoblasts, chondrocytes and skeletal muscle cells.[8] Prospective markers needed for distinguishing them from other cells and for monitoring lineage-specific differentiation is necessary for full therapeutic potential of hMSCs.[12] The markers used to identify multipotential hMSCs is CD105, CD166 and Stro-1.[13, 14] Foster et al [12] studied the dynamic changes undergone by the hMSC

membrane proteome before and after short-term osteoblast differentiation by identifying 463 proteins. They suggest the increased expression levels of 16 proteins known or implicated in cell adhesion (9 cell matrix adhesion proteins, 5 hnRNPs, versican, and tenascin) is important to osteoblast adhesion to the underlying matrix in the process of osteoblast differentiation. The contact with vitronectin and collagen I promote the osteogenic differentiation of hMSC, and the extracellular matrix contact alone may be sufficient to induce differentiation in these cells.[15] The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes 3 criterias to define human MSC.[16] First, it must be plastic-adherent when maintained in standard culture conditions. Second, it must be positive for CD105, CD73 and CD90, and negative negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules. Third, it can differentiate to lastic-adherent when maintained in standard culture conditions.[16] Recent study reported the expanded hMSC population expressed the human pluripotency surface markers SSEA-3, SSEA-4 and the transcription markers NANOG, OCT3/4 and SOX2.[17]

3.2 Dental pulp stem cell

Gronthos and colleagues firstly isolated stem cells from human dental pulp in 2000.[18] In 2005 Laino and colleague [19] isolated a selected subpopulation of DPSC called SBP-DPSCs. In vitro it can form the woven bone. The advantage of dental pulp stem cells include collecting easily, producing very low morbidity.[10] The limited hard tissue regeneration ability of dental pulp stromal cells questions their practical application for complete tooth regeneration.[20] Repeated cell passaging may explain the reduction of the osteogenic ability of both bone- and dentinal-derived stem cells.[20] Therefore, it is

essential to develop new cell culture methods to harvest the desired cell numbers while not obliterating the osteogenic potential.[20] Osteogenesis and angiogenesis mediated by human stromal stem cells from DPSCs may be regulated by distinct mechanisms, leading to the organization of adult bone tissue after stem cell transplantation.[21] CD34(+) cells obtained from dental pulp can be used for engineering bone, without the need for prior culture expanding procedures.[22] Gene expression in osteoblastoids from DPSCs is significantly different from that in osteosarcoma cells, suggesting differences in cell function and activity between these cells.[23] Pluripotential cells isolated from the pulp of human teeth expanded in vitro and differentiated into osteoblasts, chondrocytes, and adipocytes.[24] Human deciduous dental pulp is an approachable "niche" of stromal stem cells, and that it is an ideal source of osteoblasts, as well as of mineralized tissue, ready for bone regeneration, transplantation, and tissue-based clinical therapies.[25] DPSC and stem cells from human exfoliated deciduous teeth (SHED) are not only derived from a very accessible tissue resource but also capable of providing enough cells for potential clinical applications.[24] SHED show a spindle-shaped morphology, high proliferation rates, and collagen production, resulting in soft tissue formation.[26] In contrast, DPSC reduce proliferation, but exhibit an osteoblast-like phenotype, express osteoblast marker genes, and deposit mineral.[26]

DPSCs respond to ionizing radiation-induced damage by permanent cell cycle arrest in the G2 phase and by stress-induced premature senescence.[27] BMP2-transfected DPSCs effectively show mineralized tissue formation upon ectopic implantation.[28] STRO-1-selected dental pulp stem cells show effective hard tissue formation in vivo, and a short in vitro culture period and addition of BMP2 can enhance this effect.[29] STRO-1+ DPSCs consist of several interrelated subpopulations which can spontaneously differentiate into

odontoblasts, osteoblasts, and chondrocytes.[30] The differentiation capacity of these DPSCs changes during cell passaging, and DPSCs at the 9th passage restrict their differentiation potential to the osteoblast lineage in vivo.[30] The pilot study, tooth and bone constructs were prepared from third molar tooth tissue and iliac-crest bone marrow-derived osteoblasts showed small tooth structures. This tooth structure consisted of organized dentin, enamel, pulp, and periodontal ligament tissues, surrounded by new bone.[31]

In vitro study of mesenchymal stem cells derived from human dental pulp reported that surface microcavities appear to support a more vigorous osteogenic response of stem cells and should be used in the design of therapeutic substrates to improve bone repair and bioengineering applications in the future.[32] Subcultured dental pulp cells actively differentiate into odontoblast-like cells and induce calcification in an alginate scaffold.[33] Otaki et al showed cultured human adult dental pulp cells produced bone when transplanted into immunocompromised mice.[34] The dental pulp of the erupted molars contain a small population of multipotent cells, whereas the dental pulp of the unerupted molars does not contain multipotent cells but is enriched in osteo-dentinogenic progenitors engaged in the formation of coronal and radicular odontoblasts.[35] Dental follicle mesenchymal stem (DFMSCs) proliferated faster, contained cells larger in diameter, exhibited a higher potential to form adipocytes and a lower potential to form chondrocytes and osteoblasts, compared with dental pulp mesenchymal stem cells (DPMSCs).[36] Stem cells from deciduous teeth, dental pulp, and bone marrow with platelet-rich plasma (PRP) have the ability to form bone, and bone formation with deciduous teeth stem cells (DTSCs) might have the potential to generate a graft between a child and parent.[37] In vitro study exhibited Gangliosides play a more important role in

regulating the osteoblast-differentiation of hDPSCs compared to human adipose-derived stem cells (hADSCs).[38]

In vivo of patients with bilateral bone reabsorption of the alveolar ridge exhibited dental pulp stem/progenitor cells /collagen sponge biocomplex can completely restore human mandible bone defects and indicates that this cell population could be used for the repair and/or regeneration of tissues and organs.[39] In Matrigel(tm) DPSCs differentiated with osteoblast/osteocyte characteristics and connected by gap junction, and therefore formed calcified nodules with a 3D intercellular network.[40] Transplantation of human dental pulp cells-- expanded ex vivo in the presence of bFGF into immunocompromised mice -- revealed the formation of bone, cartilage, and adipose tissue.[41] DPSCs differentiated in collagen sponge actively secrete human type I collagen micro-fibrils and form calcified matrix containing trabecular-like structures.[40] DPSCs differentiated into osteoblasts, forming a biocomplex made of Biocoral, ECM and differentiated cells.[42] In vivo study reported that fibrin allows for the growth and differentiation of dental stem cells, can be inserted into small defects and thus appears to be a promising biomaterial for tissue regeneration in the oral cavity.[43] Another study showed that the tissue-engineered bone complex with nano-hydroxyapatite/collagen/poly(L-lactide) (nHAC/PLA), recombinant human bone morphogenetic protein 2 (rhBMP-2) and autologous DPSCs might be a better alternative to autologous bone for the clinical reconstruction of periodontal bone defects.[44] Similarly, osteoblast differentiation of DPSCs and bone morphogenetic protein production was obtained in a better and quicker way, when challenging stem cells with the LST surfaces.[45] Exogenous glucosamine (GlcN) can promote the osteogenic differentiation of human DPSCs, and the underlying mechanism involves a TGF-betar-dependent Smad signal pathway.[46] DPSCs express insulin growth

factor-binding protein 5 and can form mineralized matrix nodules that are a feature exclusive to osteoblasts.[9] Exposure of DPSC cultured in osteogenic medium to vascular endothelial growth factors (VEGF)-A165 for a similar period enhanced cell differentiation towards osteoblasts.[47] The combination of amniotic fluid stem cells (AFSCs) with DPSCs may provide a rich source of soluble proteins useful for bone engineering purposes. [48]

3.3 Dental pulp pluripotent stem cells

Very recently, a new stem population from the human dental pulp of third molars has been isolated and characterized.[49] These cells, termed dental pulp pluripotent stem cells (DPPSCs). These cells express pluripotency markers such as Oct-4, Lin-28, Sox-2, and Nanog, which are four factors whose induced expression alone is sufficient to revert human-differentiated cells to a pluripotential phenotype.[49] DPPSCs have been shown to differentiate to cells from the three embryonic layers: endoderm, mesoderm, and ectoderm, thus displaying a potency that was widely thought to be exclusive from embryonic stem (ES) cells and induced pluripotent stem (IPS) cells. [50]

3.4 Fibronectin

Fibronectin(FN) promote cell adhesion[51] and migration.[52] FN enhanced vascular calcification by promoting the osteoblastic differentiation of vascular smooth muscle cells via ERK signal pathway.[53] FN-mediated cell spreading and proliferation are dependent on surface energy and establish a new combinatorial approach for screening cell response to changes in surface energy.[54] FN as an adhesive glycoprotein and osteonectin as a counter-adhesive protein, are known to be involved in the early stages of osteogenesis. [55] FN adsorption is correlated to human osteoblast adhesion through morphology and actin cytoskeleton formation.[56] Cell morphology is more rounded when the degree of

FN fibrillogenesis on the substrate is lower.[56] The attachment, proliferation and morphology of pre-osteoblasts were significantly improved on the cyclic potentiodynamic polarization(CPP)-modified surface, which was attributed to the more open conformation of FN on the CPP-modified surface.[57] Type I collagen, FN and TN-C localized during embryonic osteogenesis in the dentary of mandibles and tibias.[58] bFGF increased Fn expression in rat osteoblasts via the FGFR2/PLCgamma2/PKCalpha/c-Src/NF-kappaB signaling pathway.[59] The mixture of FN and transglutaminase may prove to be a useful treatment for producing increased osteoblast differentiation on scaffolds.[60] Transglutaminase2-mediated crosslinking enhances the cell-adhesive properties of FN by increasing the molecular rigidity of FN in the extracellular matrix.[61] Evaluation of gene modified hESCs that were subsequently attached onto FN-coated gold nanoparticles revealed that the un-differentiation marker, Oct-4, was no longer present following electrical stimulation.[62] In vitro study exhibited a novel cel adhesion/survival mechanism in human osteoblasts requires association of FN bound tissue transglutaminase2 with the cell surface heparan sulphates in a transamidase independent manner.[63] Osteoblast FN affects osteoblasts function. This does not seem to be mediated by the RGD motif on FN. In contrast, liver-derived FN affects bone matrix properties without affecting osteoblast or osteoclast function.[64] The presence of the adsorbed FN layer on the calcium phosphate thin films improved MG63 osteoblast cell adhesion, proliferation and promoted early onset differentiation.[65] The study of the influence of deep pit on biological activity of FN, FN reorganisation takes places on the 29 and 45 nm deep pits surfaces, where enhanced late matrix production was found.[66]

FN monolayer coverage and the root mean square (rms) roughness are similar on --OH and --COOH terminated self-assembles monolayers with or without calcium phosphate

coating, higher levels of ALP activity, more actin cytoskeleton formation and more cell growth are obtained on --OH- and --COOH-terminated SAMs with calcium phosphate coating.[67] The study of the morphology of osteoblast on nano-grooved substrates showed that FN coating initially modulated cellular spreading, length, and orientation on all types of grooved surfaces.[68] After 24 h of culture, the cell morphology was not affected by FN coating on the 250-nm and 500-nm surfaces, while FN decreased cell alignment on the 90-nm surfaces.[68]

The thickness of this FN layer increased when the roughness of the underlying topography was increased, but not by more than half of the total maximum peak-to-valley distance.[70] In vitro attachment and proliferation of bone-forming cells on hydroxyapatite is significantly increased by pretreatment with FN/fetal calf serum, but this difference is less profound and not significant in vivo. [73] The study of the influence of two distinct nanophased hydroxyapatite ceramics on FN and osteonectin adsorption also reported that the osteoblast adhesion and metabolic activity seemed to be more sensitive to surfaces morphology and roughness than to the type of adsorbed proteins. [55] The presence of FN in the Dulbecco's phosphate-buffered saline solutions containing calcium chloride (DPBS) solutions delayed the formation and affected the morphology of the apatite. [74]

The FN-calcium phosphate composite layer formed on the hydroxyapatite is useful for the enhancement of the spreading and osteogenic differentiation of hMSCs in vitro. [75] FN incorporated into apatite deposited on the surface of titanium did not affect its biological activity in terms of promoting osteoblast adhesion. [74] FN reversibility does not seem to be dependent on the human serum albumin/FN adsorption mass ratio in solution,

suggesting that FN competitively adsorbs to TiO2 in a favorable conformation and does not suffers subsequent conformational changes allowing exchange with other FN molecules in solution. [76] The study absorption and conformation behavior of biotinylated FN on TiO surface found the conformational change of biotinylated FN on the streptavidin monolayer delivers a FN structure similar to the conformation inside the extracellular matrix and therefore explains the higher cell affinity for these surfaces.[77] Cells attached on FN-immobilized titanium at a higher rate than untreated titanium. The immobilization of FN on tresylated titanium promoted early matrix mineralization and bone formation. [78] The in vivo results showed that faster direct bone formation was seen for the fibronectin-Ti-acryl group compared to the Ti-acryl group.[79] The in vitro results showed that pFN significantly promoted BMSCs chemotaxis, however, had no effect on proliferation or differentiation.[79] The results indicate that pFN regulated chemotaxis of osteogenic cells and coating the implant with pFN enhanced earlier osseointegration.[79] Ti-6Al-7Nb possess a good potential to support SaOS-2 cells on spreading and FN and OPN synthesis, therefore, this material may be one of a candidate material used in implant dentistry.[80] Park et al evaluated the bone response around anodized titanium implants treated with FGF-FN fusion protein using the histomorphometric analysis and the removal torque test.[81] They showed that FGF-FN fusion protein coating on anodized implants may enhance osseointegration.[81]

PLLA substrates coated with FN and subsequently exposed to albumin exhibited the highest level of cell differentiation, as assayed via alkaline phosphatase activity. [82] The FN module III7-10 and extracellular domains 1 and 2 of cadherin 11 bio-inspired ceramic surface possesses enhanced functionality in adhesion, proliferation and ossification and may be a promising scaffold for tissue engineering. [83] The investigation of the effect of

two phases of Nitinol with plasma FN exhibited that FN improved cell proliferation in both phases, but the effect of FN coating was stronger on the austenite surface.[84] In both Nitinol phases, the proportion of cells in the G(1) phase was observed to grow in the presence of FN. This could indicate cell differentiation on Nitinol. [84]

Table 3.1: *Fibronectin (Keyword: fibronectin and osteoblast, 2006-2011 and published in English)*

Author	Type	Cell	Objective	Result
Hindié et al 2011	In vitro	MC3T3-E1 osteoblast like cells	To study MC3T3-E1 osteoblast-like cell behavior on silicon oxide and PLLA substrates with a focus on the influence of the adhesive protein fibronectin and the non-adhesive protein albumin adsorbed on the substrates.	PLLA substrates coated with fibronectin and subsequently exposed to albumin exhibited the highest level of cell differentiation, as assayed via alkaline phosphatase activity.
Langmuir et al 2011	In vitro	Osteoblasts	To study absorption and conformation behavior of biotinylated fibronectin on TiO surface	The conformational change of biotinylated fibronectin on the streptavidin monolayer delivers a FN structure similar to the conformation inside the ECM and therefore explains the higher cell affinity for these surfaces.
Wang et al 2011	In vitro	Osteoblasts	To investigate the involvement of cell surface receptors and their intracellular signalling molecules to further explore the pathway mediated by this novel TG-FN heterocomplex.	A novel cell adhesion/survival mechanism in human osteoblasts requires association of FN bound TG2 with the cell surface heparan sulphates in a transamidase independent manner.
Bentmann et al 2010	In vivo; transgenic mice		To identify the receptor involved in fibronectin effects on osteoblasts.	Osteoblast fibronectin affects osteoblasts function. This does not seem to be mediated by the RGD motif on fibronectin. In contrast, liver-derived fibronectin affects bone matrix properties without affecting osteoblast or osteoclast function.

Cairns et al 2010	In vitro	MG63 osteoblast-like cells	To investigate the role of surface topography in determining the nature of this cell-protein-surface interaction	The presence of the adsorbed FN layer on the CaP thin films improved MG63 cell adhesion, proliferation and promoted early onset differentiation.
Chen et al 2010	In vitro		To investigate the details of apatite nucleation and growth on the coating layer in Dulbecco's phosphate-buffered saline solutions containing calcium chloride (DPBS) or DPBS with fibronectin (DPBSF)	The presence of fibronectin in the DPBS solutions delayed the formation and affected the morphology of the apatite. Fibronectin incorporated into apatite deposited on the surface of titanium did not affect its biological activity in terms of promoting osteoblast adhesion.
González'García et al 2010	In vitro	MC3T3 osteoblast-like cells	To investigate the influence of deep pit on biological activity of FN	FN reorganisation, only takes places on the 29 and 45 nm deep pits surfaces, where enhanced late matrix production was found.
Pegueroles et al 2010	In vitro	MG63 osteoblast-like cells	To investigate the early events of bone matrix formation, and specifically the role of fibronectin (FN) in the initial osteoblast interaction and the subsequent organization of a provisional FN matrix on different rough titanium (Ti) surfaces	Osteoblasts deposit FN fibrils in a specific facet-like pattern that is organized within the secreted total matrix overlying the top of the samples. The thickness of this FN layer increased when the roughness of the underlying topography was increased, but not by more than half of the total maximum peak-to-valley distance.
Ribeiro et al 2010	In vitro	MC3T3-E1 osteoblast-like cells	To analyze the influence of two distinct nanophased HA ceramics, (HA725 and HA1000) on FN and osteonectin adsorption	FN as an adhesive glycoprotein and osteonectin as a counter-adhesive protein, are known to be involved in the early stages of osteogenesis. However, the osteoblast adhesion and metabolic activity seemed to be more sensitive to surfaces morphology and roughness than to the type of adsorbed proteins.
Rico et al 2010	In vitro	MC3T3-E1 osteoblast-like cells	To investigate the organization of a recombinant fragment of fibronectin (FNIII(7-10))	The bioavailability of specific cell adhesion domains, including RGD, within the molecules was higher on

			upon adsorption on this particular chemistry, PEA	PEA than on the control glass.
Zhang et al 2010	In vitro	hMSC	To investigate the effects of a bio-inspired ceramic surface modified with a novel recombinant protein on surface parameters and cell behavior	The fibronectin module III7-10 and extracellular domains 1 and 2 of cadherin 11 bio-inspired ceramic surface possesses enhanced functionality in adhesion, proliferation and ossification and may be a promising scaffold for tissue engineering.
Aamer et al 2009	In vitro	MC3T3-E1 osteoblasts -like cells	To report the impact of increasing iodine inclusion on the cell morphology (cell area and shape) of MC3T3-E1 osteoblasts on a series of homopolymers and discrete blend thin films of poly(desaminotyrosyl tyrosine ethyl ester carbonate), poly(DTE carbonate), and an iodinated analogue poly(I(2)-DTE carbonate)	Incorporation of iodine within the polymer backbone has a distinct impact on the way FN proteins adsorb to the surface and within the studied blend systems; the effect is composition dependent.
Ball et al 2009	In vitro		To explore the possibility of controlling cell interaction with biomaterials, tricalcium phosphate scaffolds were modified using the enzyme tissue transglutaminase (tTgase) in conjunction with fibronectin.	The mixture of fibronectin and transglutaminase may prove to be a useful treatment for producing increased osteoblast differentiation on scaffolds.
Forsprecher et al 2009	In vitro	MC3T3-E1 osteoblast	To test the effects of extracellular FN, before and after in vitro crosslinking and polymerization by TG2	TG2-mediated crosslinking enhances the cell-adhesive properties of FN by increasing the molecular rigidity of FN in the extracellular matrix.
Muhonen et al 2009	In vitro	MC3T3-E1 osteoblast	To investigate the effect of two phases of Nitinol with plasma fibronectin and studied if this modifies the proliferation and cell cycle of MC3T3-E1 osteoblasts.	Fibronectin improved cell proliferation in both phases, but the effect of fibronectin coating was stronger on the austenite surface. In both Nitinol phases, the proportion of cells in the G(1) phase was observed to grow in the presence of fibronectin. This could

				indicate cell differentiation on Nitinol.
Rico et al 2009	In vitro		To investigate the influence of surface chemistry and effect on osteoblast adhesion	FN adsorption is correlated to human osteoblast adhesion through morphology and actin cytoskeleton formation. Actin polymerization is in need of the formation of the protein network on the substrate's surface. Cell morphology is more rounded when the degree of FN fibrillogenesis on the substrate is lower.
Shahryari et al 2009	In vitro		To investigate the influence of an electrochemical surface passivation technique (cyclic potentiodynamic polarization, CPP) on the physico-chemical surface properties of SS316LS and its subsequent response to FN and pre-osteoblasts	The attachment, proliferation and morphology of pre-osteoblasts were significantly improved on the CPP-modified surface, which was attributed to the more open conformation of Fn on the CPP-modified surface. Thus, the CPP surface passivation method was demonstrated to yield a SS316LS surface of enhanced biocompatibility.
Toworfe et al 2009	In vitro	MC3T3-E1	To investigate the effect of silane self-assembled monolayer surfaces on apatite formation, fibronectin adsorption and osteoblast cell function	FN monolayer coverage and the root mean square (rms) roughness are similar on --OH and --COOH terminated self-assembles monolayers with or without CaP coating, higher levels of ALP activity, more actin cytoskeleton formation and more cell growth are obtained on --OH- and --COOH-terminated SAMs with CaP coating.
Tsai et al 2009	In vitro	MG-63 osteoblast-like cells	To investigate the morphology of osteoblast on nano-grooved substrates	FN coating initially modulated cellular spreading, length, and orientation on all types of grooved surfaces. After 24 h of culture, the cell morphology was not affected by FN coating on the 250-nm and 500-nm surfaces, while FN decreased

				cell alignment on the 90-nm surfaces.
Woo et al 2009	In vitro	hESCs	To investigate the effect of electronical stimulation on the differentiation of hESCs adhered onto fibronectin-coated gold nanoparticles	Evaluation of gene modified hESCs that were subsequently attached onto fibronectin-coated gold nanoparticles revealed that the un-differentiation marker, Oct-4, was no longer present following electrical stimulation.
Schönmeyr et al 2008	In vitro In vivo; mice	Bone-firming cells	To study the result of the treatment of HA scaffolds with FN and fetal calf serum	In vitro attachment and proliferation of bone-forming cells on hydroxyapatite is significantly increased by pretreatment with fibronectin/fetal calf serum, but this difference is less profound and not significant in vivo.
Sousa et al 2008	In vitro	MC3T3-E2 osteoblast	To assess the influence of the competitive preadsorption of human serum albumin (HSA) and human plasma FN from binary solutions and 10% plasma on MC3T3-E1 osteoblast adhesion and morphology on two types of TiO2 substrates	FN reversibility does not seem to be dependent on the HSA/FN adsorption mass ratio in solution, suggesting that FN competitively adsorbs to TiO2 in a favorable conformation and does not suffers subsequent conformational changes allowing exchange with other FN molecules in solution.
Jimbo et al 2007	In vitro, In vivo; mouse femur	BMSCs	To clarify the role of pFN during osseointegration.	The in vivo results showed that faster direct bone formation was seen for the FN-Ti-acryl group compared to the Ti-acryl group. The in vitro results showed that pFN significantly promoted BMSCs chemotaxis, however, had no effect on proliferation or differentiation. The results indicate that pFN regulated chemotaxis of osteogenic cells and coating the implant with pFN enhanced earlier osseointegration.
Pugdee et al 2007	In vitro	MC3T3-E2 osteoblast	To examine the cell attachment and gene expression of MC3T3-E1	Cells attached on FN-immobilized titanium at a higher rate than untreated

			cells on FN-immobilized titanium using GeneChip	titanium. The immobilization of FN on tresylated titanium promoted early matrix mineralization and bone formation.
Sogo et al 2007	In vitro	hMSCs	To investigate FN-calcium phosphate composite layer on HA	The FN-calcium phosphate composite layer formed on the HA is useful for the enhancement of the spreading and osteogenic differentiation of hMSCs in vitro.
Tang et al 2007	In vitro	Osteoblast	To show basic fibroblast growth factor stimulates fibronectin expression through phospholipase C gamma, protein kinase C alpha, c-Src, NF-kappaB, and p300 pathway in osteoblasts	bFGF increased Fn expression in rat osteoblasts via the FGFR2/PLCgamma2/PKCalpha/c-Src/NF-kappaB signaling pathway.
Deligianni et al 2006	In vitro	human osteoblasts	To investigate the contribution of fibronectin preadsorption to enhance osteoblasts' adhesion and strength on hydroxyapatite.	Fibronectin preadsorption increased the number of attached osteoblasts on smooth and rough hydroxyapatite substratum at 40% and 62% respectively, while it increased osteoblast attachment strength on the smooth and rough substratum at 165% and 73% respectively.
Ding et al 2006	In vitro	Vascular smooth muscle cells	To investigate the effect of FN on the calcification of vascular smooth muscle cells	FN enhanced vascular calcification by promoting the osteoblastic differentiation of vascular smooth muscle cells via ERK signal pathway.
Kennedy et al 2006	In vitro	Osteoblast	To study the effect of surface energy on fibronectin-mediated cell response	FN-mediated cell spreading and proliferation are dependent on surface energy and establish a new combinatorial approach for screening cell response to changes in surface energy.
Osathanon et al 2006	In vitro	SaOS-2 human osteoblast-like cells	To compare the early response of human osteoblast-like cells (SaOS-2) on commercially pure titanium (cpTi) and titanium-6-aluminium-7-niobium (Ti-6Al-7Nb)	Ti-6Al-7Nb possess a good potential to support SaOS-2 cells on spreading and fibronectin and osteopontin synthesis, therefore, this material may be one of a candidate material used in

				implant dentistry.
Park et al 2006	In vivo; rabbit tibiae		To evaluate the bone response around anodized titanium implants treated with FGF-FN fusion protein using the histomorphometric analysis and the removal torque test.	FGF-FN fusion protein coating on anodized implants may enhance osseointegration.

3.5 BMP2

Bone morphogenetic protein-2 (BMP-2) is strongly involved in the induction of osteoblast differentiation from mesenchymal cell precursors, as well as in enhancing bone matrix production by osteoblastic cells. [85] The observed functional redundancy of type II BMP receptors in osteoblasts is novel information about the BMP signaling pathway essential for initiating osteoblast differentiation.[86] The study of three different bone cell samples showed the possibility that BMP receptor-IB could be a therapeutic target for enhancing bone regeneration in vivo.[87] The nonautologous BMP2 gene-transfected stem cells are of potential utility for enhancement of bone repair and bone regeneration in vivo.[88] The sensitivity of cells to BMP2 would correlate with BMP receptor expression.[89] BMP2 stimulated osteoblastic markers faster and to a greater extent than Runx2.[90] Runx2-engineered cells did not utilize paracrine signaling via secreted osteogenic factors, in contrast to cells overexpressing BMP-2.[90] Activation of the PKA pathway may be one of key BMP-2-activated signaling events that lead to osteogenesis and that downregulation of PKIgamma may be prerequisite for the PKA activation during the osteoblastic differentiation of precursor cells.[91] BMP-2 induces Runx2-deficient cells to express markers related to osteoblast and chondroblast differentiation using a Runx2-independent pathway, but it failed to induce these cells to differentiate into bone-

forming osteoblasts and mature chondrocytes. [92] Runx2 may be important to regulate osterix (Osx) during osteoblast lineage progression. When Runx2 activity is blocked, it inhibits the BMP-2-mediated induction of Osx.[93] BMP-2-induced Osx expression is mediated by Dlx5. [94, 95]

Different mechanisms for BMP2/4- and BMP6/7-induced osteoblastic differentiation in primary hMSCs.[96] Osteogenic sensitivity of muscle progenitors and provide a mechanistic insight into the variable response of different cell lineages to BMP2.[89] BMP2 may regulate osteoblast function in part through modulation of the beta-catenin signaling.[97] BMP2 enhances dexamethasone/ascorbic acid/glycerolphosphate-induced osteogenic differentiation in mesenchymal bone marrow cells.[98] Both agents interact in various ways and can modify osteoblastic bone formation.[98] Pulsed electromagnetic fields (PEMF) enhances osteogenic effects of BMP2 on MSCs cultured on calcium phosphate substrates, suggesting that PEMF will improve MSC response to BMP2 in vivo in a bone environment.[99] The down-regulation of microRNAs-208 in BMP2-stimulated osteoblast differentiation is an important part of the regulatory machinery involved in early osteogenesis.[100] MC3T3-E1-clone 24 cells can be induced by BMP-2 to differentiate into mineralizing osteoblast cultures.[101] BMP-2 also stimulates Pi transport activity through a selective increase in expression of type III Pi transporters Pit-1. In MC3T3-E1 cells, this effect is mediated by the JNK pathway and plays an essential role in bone matrix calcification induced by BMP-2.[102] Nell-1 and BMP-2 synergistically enhanced osteogenic differentiation of myoblasts and phosphorylated the JNK MAPK pathway.[103] Glucocorticoids regulate BMP2 via a far-downstream domain, and activation of Smad, not ALP, best predicts the pro-mineralization potential of rhBMPs.[104] BMP-2 regulates osteoactivin expression through the Smad1 signaling

pathway. Osteoactivin protein acts as a downstream mediator of BMP-2 effects on osteoblast differentiation and function. The BMP2-induced osteoactivin transcription is differentially regulated by Dlx3, Dlx5 and Msx2 during osteoblast differentiation.[105] Simvastatin can promote osteoblast viability and differentiation via membrane-bound Ras/Smad/Erk/BMP2 pathway.[106] CYR61 up-regulates BMP-2 mRNA and protein expression, resulting in enhanced cell proliferation and osteoblastic differentiation through activation of the ∝(v)β(3) integrin/integrin-linked kinase/ERK signaling pathway.[107] PDZRN3 plays an important role in negative feedback control of BMP2-induced osteoblast differentiation in C2C12 cells through inhibition of Wnt-β-catenin signaling.[108] In vitro, the combined gene therapy with the human angiopoietin-1 gene (hAng-1) and hBMP2 using lentivirally co-transfected MSCs is feasible.[109] WISP-1 in preosteoblast has a positive influence on bone cell differentiation and function and may work by enhancing the effects of BMP-2 to increase osteogenesis through a mechanism potentially involving binding to integrin∝(5)β(1).[110] In vitro study by using bone marrow-derived mesenchymal stem cells were harvested from the iliac crest of three human donors and tuber coxae of three equine donors showed that genetically modified bone marrow-derived mesenchymal stem cells could be useful for cell-based delivery of BMPs to a site of bone formation.[111] The lipid microtube system is able to provide sustained delivery of biologically active BMP2 and thereby induce osteogenic differentiation.[112] The proteoglycan populations that are induced in C2C12 cells upon osteoblastic differentiation produced by BMP-2.[113] Specific levels of static stretching force increase cell proliferation and effectively stimulate the osteoblast differentiation of C2C12 cells in conjunction with BMP2 stimulation, thus indicating a synergistic interaction between mechanical strain and cytokine signaling.[114]

DeltaEF1 acts as a potent inhibitor of BMP-2-induced osteogenesis in vitro, in part, by differentially regulating the AP-1 signaling pathway.[115] Cells contacting 316L steel are exposed to increased concentrations of Ni which suffice to impair BMP2-induced ALP activity.[116] CCN3 exerts inhibitory effects on BMP2-induced osteoblast differentiation by its involvement of the BMP and Notch signaling pathways.[117] Heparin suppresses BMP2-BMP receptor binding, and inhibits BMP2 osteogenic activity in vitro.[118] Gremlin inhibits BMP2 signaling and activity, and does not have independent actions on ERK signaling in osteoblasts.[119] The effect of platelet-released supernatant (PRS) on human MSCs could be at least partially mediated by BMP-2. Activated autologous PRS could therefore provide an alternative to agents like recombinant bone growth factors by increasing osteoblastic differentiation of bone precursor cells at bone repair sites, although further studies are needed to fully support our observations.[120] Hypoxia enhances BMP2 expression in osteoblasts by an HIF-1alpha-dependent mechanism involving the activation of integrin-linked kinase /Akt(1L-6-hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate]) and mammalian target of rapamycin pathways.[121] Tanshinone IIA enhances the commitment of C2C12 cells into osteoblasts and their differentiation through synergistic cross talk between tanshinone IIA-induced p38 activation and BMP-2-induced Smad activation.[122] BMP2 down-regulation of PTHrP could facilitate terminal differentiation of osteoblasts.[85] PTH stimulated BMP-2 mRNA expression via the mevalonate pathway and ROK in osteoblastic MC3T3-E1 cells.[123] Adiponectin enhances BMP2 expression in osteoblastic cells, and AdipoR1 receptor, AMPK, p38 and NF-kappaB signaling pathways may be involved in increasing BMP-2 expression by adiponectin.[124] A signalling pathway linking BMP-2stimulated Nox4-derived physiological reactive oxygen species to BMP-2 expression and osteoblast

differentiation.[125] Ultrasound increased BMP2 expression in osteoblasts via the PI3K, Akt, c-Fos/c-Jun, and AP-1 signaling pathway.[126] High-power, low-level Nd:YAG laser increased osteoblast activity, very efficiently accelerating mineral deposition. Osteoinductive effect of laser is likely mediated by activation of BMP2-related signaling pathway.[127] Raise a possibility that PDL cells respond to BMP2 via a unique signaling pathway dependent on endoglin, which is involved in the osteoblastic differentiation and mineralization of the cells.[128] The addition of BMP2 had a beneficial effect in vitro, reducing the initial cytotoxicity of freshly mixed mineral trioxide aggregate (MTA). However, the pulp reaction to a combination of MTA and BMP2 was not significantly better than use of MTA alone.[129] BMP2 is the most suitable candidate for osteogenic stimulation of rat bone marrow cells when comparing with transforming growth factor beta-1 or COLLOSS E (a bone-derived collagen product containing a variety of naturally occurring growth factors).[130]

Increased BMP receptor-IB by TGF-beta1, FGF-2, and PDGF-AB significantly enhances BMP-2-induced osteogenic functions in vitro, suggesting that they might positively modulate bone formation by up-regulating BMPR-IB in vivo.[131] Vascular endothelial growth factor (VEGF) might enhance BMP2-induced bone formation through modulation of angiogenesis.[132] Mouse model reported that treatment with HGF prior to administration of BMP2 induced cellular proliferation of mouse embryonic fibroblasts and did not influence subsequent osteoblast differentiation induced by BMP2.[133] The number of responding cells or immature osteoblasts was increased by the supplementation of FGF2 in the early phase of the culture and that these cells can show osteoblastic differentiation, of which capability was augmented by BMP2 in the late phase.[134] In vitro study of MC3T3E1 osteoblast-like cells, FGF2 plays a critical role of

osteoblast growth in early fracture repair while BMP-2 is instrumental in stimulating mineralization.[135] In the same way, in vivo showed FGF2 increased the pool of committed osteoblasts by up-regulating the Cbfa1/Runx2 gene. The later stages of bone formation seemed to be induced by Cbfa1/Runx2-downstream factors such as BMP2, ALP, collagen type I, bone sialoprotein and osteocalcin.[136] The combination of collagen type I and exogenous rhBMP-2 did not ameliorate the bioactivity of hydroxyapatite calcified from red algae in the initial period of cultivation.[137] The investigation of bone healing upon administration of the growth factor BMP-2 embedded with Adult human adipose-derived stem cells (ASCs) in a locally applied fibrin matrix concluded that transplantation of ASC modulated the callus induction by BMP-2 to a normal volume.[138] In vivo of MC3T3-E1 pre-osteoblasts, a 3D scaffold with embedded growth factor-delivering microspheres exhibited that released BMP2 promoted bone formation.[139] The formation of the BMP-2/carboxymethylated dextran polymers grafted with high amounts of benzylamide(DMCB) complex may protect the protein from being inactivated. In rats in vivo, DMCB also stimulated ectopic calcification mediated by BMP-2. [140] The BMP2, BMP7 and a mixture of BMP2/BMP7 all promoted osteoblast growth on the collagen scaffold, with the mixture of BMP2/BMP7 enhancing the most growth. BMP2, BMP7 and the mixture of BMP2/BMP7 could promote bone regeneration via different mechanisms involving IL-6 and MMP inhibitors. [141] Physisorbed BMP2 is more active than diffusible BMP2. The current clinical practice of immobilizing BMPs on collagen type I scaffolds not only prolongs local delivery of the morphogen but could also enhance biological activity at the cellular level.[142] Cowan et al exhibited BMP2 (doses of 30 to 240 ng/mm) was grafted into 5 mm critical sized rat calvarial defects, where increased bone regeneration was observed in a dose dependent manner, with higher doses of BMP-2 inducing greater

bone area, volume, and density.[143] Adipose-derived stromal cells (ADSCs) modified by the BMP-2 gene can enhance the repair of critical-sized bone defects in large animals.[144] An enhancement in gene expression of BMP2 and VEGF can be achieved by an incorporation of BMP2 into the PLLA nanofibers.[145] The incorporation of BMP2 into PLLA-collagen type I nanofibers resulted in a decrease in diameter as well as pore sizes of the scaffold. Mesenchymal stem cells showed better adherence and a reduced proliferation on BMP-containing scaffolds.[146] The combination of PLLA scaffolds and BMP-2 increase bone regeneration in vivo better than PLLA only.[147] BMP2 linked to titanium surfaces can enhance the rate of bone healing as compared with untreated Ti surfaces.[148] One study shows that BMP-2 chondroitin sulfate nanocomplex effect in holding BMP-2 on appatite-coated Ti surface and osteoblast proliferation was faster in the Ti(C)-HA-BMP-2[149] Heparin enhanced BMP-2-induced osteogenesis on apatite-coated titanium without the loss of BMP-2 activity.[150] Grafting heparin and immobilizing BMP-2 on Ti surfaces inhibited inflammation and promoted osteoblast function.[151] Likewise, Gentamicin and bone morphogenic protein-2 (BMP-2)-delivering heparinized-titanium implant enhanced osteointegration.[152] Another study also exhibited that co-delivery of PDGF-BB and BMP-2 using heparinized-titanium enhanced of osteoblast function and osteointegration.[153] Tissue engineered bone complex with beta-TCP scaffold and BMP2 gene-modified bone marrow stromal cells could be used to promote mandibular repairing and bone regeneration.[154] Nanosilver of defined particle size (with a size of 20-40nm) -PLGA composite grafts have strong antibacterial properties, in vitro and in vivo cytotoxicity or negative effects on BMP-2 osteoinductivity, making it an ideal antimicrobial for bone regeneration in infected wounds. [155] A novel injectable drug delivery system consisting of starch-poly-epsilon-caprolactone microparticles induce

osteogenesis and reduce the amount of BMP-2 needed and allow more sustained osteogenic effects.[156]

Table 3.2: *Bone morphogenetic proteins 2 (Keyword: BMP2 and osteoblast, 2006-2011 and published in English).*

Author	Type	Cell	Objective	Result
Hughes-Fulford et al 2011	In vitro	MC3T3E1 osteoblast-like cells	To describe the sequential roles of FGF-2 in inducing gene expression, cell growth and BMP-2 in gene expression and mineralization of bone	The ability of FGF-2 to re-program a mineralizing gene expression profile to one of proliferation suggests that FGF-2 plays a critical role of osteoblast growth in early fracture repair while BMP-2 is instrumental in stimulating mineralization.
Kaewsrichan et al 2011	In vivo; nude mice	Marrow stromal cells	To develop culture conditions that permit a rapid increase in the number of marrow stromal cells while retaining or improving their potential for complete differentiation in vivo.	FGF2 increased the pool of committed osteoblasts by up-regulating the Cbfa1/Runx2 gene. The later stages of bone formation seemed to be induced by Cbfa1/Runx2-downstream factors such as BMP2, ALP, collagen type I, bone sialoprotein and OC.
Keibl et al 2011	In vivo; femur of male rat	ASCs	To investigate bone healing upon administration of the growth factor BMP-2 embedded with ASCs in a locally applied fibrin matrix.	Transplantation of ASC modulated the callus induction by BMP-2 to a normal volume.
Lee et al 2011	In vitro, In vivo	MC3T3-E1 pre-osteoblasts	To examine a 3D scaffold with embedded growth factor-delivering microspheres	Solid free-form fabrication scaffolds created by microstereolothography were superior to traditional scaffolds produced using a particulate leaching/gas foaming method. The scaffolds that released BMP2 promoted bone formation.
Liu et al 2011	In vitro	Marrow-derived mesenchymal stromal cells	To investigate the osteogenic and angiogenic effects of marrow-derived mesenchymal stromal cells when co-transfected (by means of lentivirus) the human angiopoietin-1 gene (hAng-1) and hBMPs	The combined gene therapy with hAng-1 and hBMP2 using lentivirally co-transfected MSCs is feasible.
Liu et al 2011	In vitro	Osteoblast	To investigate the role of type II BMP receptors in osteoblasts	The observed functional redundancy of type II BMP receptors in osteoblasts is novel information about the BMP signaling pathway

				essential for initiating osteoblast differentiation.
Ono et al 2011	In vivo; transgenic mice that overexpressed human WISP-1 in preosteoblasts		To determine the function of WISP-1 during osteogeneis, osteogenic bone marrow stromal cells	WISP-1 has a positive influence on bone cell differentiation and function and may work by enhancing the effects of BMP-2 to increase osteogenesis through a mechanism potentially involving binding to integrin α(5)β(1).
Schofer et al 2011	In vitro	Mesenchymal stem cell differentiation of osteoblasts	To analyze whether these processes can be remodeled in an artificial PLLA based nanofiber scaffold.	The incorporation of BMP2 into PLLA-collagen type I nanofibers resulted in a decrease in diameter as well as pore sizes of the scaffold. Mesenchymal stem cells showed better adherence and a reduced proliferation on BMP-containing scaffolds.
Singh et al 2011	In vitro	Osteoblast	To investigate homeodomain proteins regulate BMP-2-induced OA transcription during osteoblast differentiation.	The BMP2-induced OA transcription is differentially regulated by Dlx3, Dlx5 and Msx2 during osteoblast differentiation.
Carpenter et al 2010	In vitro	Bone marrow-derived mesenchymal stem cells were harvested from the iliac crest of three human donors and tuber coxae of three equine donors	to compare the effect of genetic modification of human and equine bone marrow-derived mesenchymal stem cells with BMP2 or -7 or BMP2 and -7 on their osteoblastogenic differentiation in the presence or absence of dexamethasone.	Genetically modified bone marrow-derived mesenchymal stem cells could be useful for cell-based delivery of BMPs to a site of bone formation.
Chen et al 2010	In vitro	Osteoblasts	To understand the events of osteoblast differentiation induced by statins	Simvastatin can promote osteoblast viability and differentiation via membrane-bound Ras/Smad/Erk/BMP2 pathway. Statins stimulate osteoblast differentiation in vitro and may be a promising drug for the treatment of osteoporosis in the future.
Honda et al 2010	In vitro	C2C12 mouse mesenchymal progenitor cells	To examine the potential role of PDZRN3 in the differentiation of C2C12 cells into osteoblasts	PDZRN3 plays an important role in negative feedback control of BMP2-induced osteoblast differentiation in C2C12 cells through inhibition of Wnt-β-catenin signaling.
Hsieh et al 2010	In vitro	osteoblast cells were harvested from 8-month old female Imprinting	To examine the molecular mechanisms of icariin by using primary osteoblast cell cultures obtained from adult mice	Icariin is a bone anabolic agent that may exert its osteogenic effects through the induction of BMP2 and nitric oxide synthesis, subsequently regulating Cbfa1/Runx2, OPG,

		Control Region mice		and RANKL gene expressions. This effect may contribute to its action on the induction of osteoblasts proliferation and differentiation, resulting in bone formation.
Huang et al 2010	In vitro	cultured osteoblasts	To investigate the effects of adiponectin on BMPs expression	Adiponectin enhances BMP2 expression in osteoblastic cells, and AdipoR1 receptor, AMPK, p38 and NF-kappaB signaling pathways may be involved in increasing BMP-2 expression by adiponectin.
Ishibashi et al 2010	In vitro	PDL cells, MC3T3-E1 osteoblastic cells	To analyze characteristics unique for PDL at a molecular leve	Raise a possibility that PDL cells respond to BMP2 via a unique signaling pathway dependent on endoglin, which is involved in the osteoblastic differentiation and mineralization of the cells.
Itoh et al 2010	In vitro	MC3T3-E1 cells, primary osteoblast cells	To investigate the role of microRNAs to BMP2	The down-regulation of microRNAs-208 in BMP2-stimulated osteoblast differentiation is an important part of the regulatory machinery involved in early osteogenesis.
Kawasaki et al 2010	In vivo; Mouse model		To analyze osteogenic properties of HGF, particularly during BMP2-induced bone formation.	Treatment with HGF prior to administration of BMP-2 induced cellular proliferation of mouse embryonic fibroblasts and did not influence subsequent osteoblast differentiation induced by BMP-2.
Kim et al 2010	In vitro	C2C12 cells	To demonstrate a stimulatory effect of tanshinone IIA isolated from the root of Salvia miltiorrhiza on the commitment of bi-potential mesenchymal precursor C2C12 cells into osteoblasts in the presence of BMP2	Tanshinone IIA enhances the commitment of C2C12 cells into osteoblasts and their differentiation through synergistic cross talk between tanshinone IIA-induced p38 activation and BMP-2-induced Smad activation.
Kim et al 2010	In vitro	MC3T3-E1 pre-osteoblasts	To investigate osteoblast responses to high-power laser and combined irradiation with BMP2 treatment	High-power, low-level Nd:YAG laser increased osteoblast activity, very efficiently accelerating mineral deposition. Osteoinductive effect of laser is likely mediated by activation of BMP2-related signaling pathway.
Ko et al 2010	In vitro, In vivo; Sprague-Dawley	MG-63 cells	To assess the cytotoxicity of mineral trioxide aggregate (MTA) and BMP2 and the response of	The addition of BMP2 had a beneficial effect in vitro, reducing the initial cytotoxicity of freshly mixed MTA.

	rats		rat pulp tissue to MTA and BMP2	However, the pulp reaction to a combination of MTA and BMP-2 was not significantly better than use of MTA alone.
Liu et al 2010	In vitro	MC3T3-E1-clone 24 cells	To determine that MC3T3-E1-clone 24 cells can be induced by BMP-2 to differentiate into mineralizing osteoblast cultures	1) JNK is required for phosphorylation of Smad1 by BMP-2 and subsequent activation of Smad1 signaling and osteoblast differentiation, 2) JNK1, but not JNK2, is required for BMP-2-induced formation of mineralized nodules, 3) JNK1 activation decreases binding of inhibitory Smad6 to the Type I BMP Receptor (BMPR-I) and reciprocally increases binding of Smad1, both observations that would increase responsiveness to BMP-2.
Mandal et al 2010	In vitro	mouse 2T3 pre-osteoblasts	To demonstrate a novel mechanism of BMP-2-induced osteoblast differentiation	A signalling pathway linking BMP-2stimulated Nox4-derived physiological reactive oxygen species to BMP-2 expression and osteoblast differentiation.
Seib et al 2010	In vitro	C2C12s	To investigate the role of ECM physisorbed BMPs in inducing the differentiation of resident mesenchymal stem cells into osteoblasts	Physisorbed BMP2 is more active than diffusible BMP2. The current clinical practice of immobilizing BMPs on collagen type I scaffolds not only prolongs local delivery of the morphogen but could also enhance biological activity at the cellular level.
Su et al 2010	In vitro	MC3T3-E1 osteoblasts, primary cultured osteoblasts	To investigate the interaction between CYR61 and BMP-2	CYR61 up-regulates BMP-2 mRNA and protein expression, resulting in enhanced cell proliferation and osteoblastic differentiation through activation of the α(v)β(3) integrin/integrin-linked kinase/ERK signaling pathway.
Tseng et al 2010	In vitro	osteoblastic cells MG-63, hFOB and bone marrow stromal cells M2-10B4	To investigate the effects of hypoxia exposure on BMP2 expression in cultured osteoblasts	Hypoxia enhances BMP2 expression in osteoblasts by an HIF-1alpha-dependent mechanism involving the activation of integrin-linked kinase /Akt (1L-6-hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate]) and mammalian target of rapamycin pathways.
Verrier et al 2010	In vitro	hMSCs	To test the effect of platelet-released	The effect of PRS on human MSCs could be at least partially

			supernatant (PRS) on human MSCs differentiation towards an osteoblastic phenotype	mediated by BMP-2. Activated autologous PRS could therefore provide an alternative to agents like recombinant bone growth factors by increasing osteoblastic differentiation of bone precursor cells at bone repair sites, although further studies are needed to fully support our observations.
Zhao et al 2010	In vivo; Mandibular defects in rats	bMSCs	To investigate the effects of mandibular defects repaired by a tissue engineered bone complex with beta-TCP and BMP2 gene-modified bone marrow stromal cells (bMSCs)	BMP2 regional gene therapy together with beta-TCP scaffold could be used to promote mandibular repairing and bone regeneration.
Zheng et al 2010	In vitro, In vivo; femoral defects	MC3T3-E1 pre-osteoblasts	To demonstrate that metallic nanosilver particles (with a size of 20-40nm)-PLGA composite grafts have strong antibacterial properties.	Nanosilver of defined particle size is bactericidal without discernable in vitro and in vivo cytotoxicity or negative effects on BMP-2 osteoinductivity, making it an ideal antimicrobial for bone regeneration in infected wounds.
Balmayor et al 2009	In vitro	osteo/myoblast cell line (C2C12)	To develope and test a novel injectable drug delivery system consisting of starch-poly-epsilon-caprolactone microparticles for inducing osteogenesis and requiring smaller amounts of BMP2.	Starch-poly-epsilon-caprolactone microparticles are suitable carriers for the incorporation and controlled release of glucocorticoids and growth factors. Specifically, they reduce the amount of BMP-2 needed and allow more sustained osteogenic effects.
Degat et al 2009	In vitro, In vivo; rats	C2C12 myoblasts	To explore the binding capacity of synthetic heparin-like dextran derivatives to rhBMP2	The formation of the BMP-2/carboxymethylated dextran polymers grafted with high amounts of benzylamide (DMCB) complex may protect the protein from being inactivated. In rats in vivo, DMCB also stimulated ectopic calcification mediated by BMP-2.
Hou et al 2009	In vitro	Osteoblast	To investigate the effect of ultrasound to BMP2 expression	Ultrasound increased BMP2 expression in osteoblasts via the PI3K, Akt, c-Fos/c-Jun, and AP-1 signaling pathway.
Johnson et al 2009	In vitro		To develop sustained release systems	The lipid microtube system is able to provide sustained delivery of biologically active BMP2 and thereby induce osteogenic differentiation.
Kim et al 2009	In vitro	C2C12 pluripotential myoblasts	To test mechanical stretching enhances osteoblast differentiation	Specific levels of static stretching force increase cell proliferation and effectively

			in distraction osteogenesis by means of interaction with BMP2 induced cytokine stimulation.	stimulate the osteoblast differentiation of C2C12 cells in conjunction with BMP2 stimulation, thus indicating a synergistic interaction between mechanical strain and cytokine signaling.
Liu et al 2009	In vitro	myoblastic murine cell lines, primary cells with osteoprogenitors	To test the sensitivity of cells to BMP2 would correlate with BMP receptor expression	Osteogenic sensitivity of muscle progenitors and provide a mechanistic insight into the variable response of different cell lineages to BMP2.
Schofer et al 2009	In vitro	hMSCs	To analyze the impact of PLLA nanofibers on VEGF and BMP2 gene expression during the time course of hMSC differentiation towards osteoblasts	The PLLA nanofibers have little effect on growth factor production. An enhancement in gene expression of BMP2 and VEGF can be achieved by an incorporation of BMP2 into the PLLA nanofibers.
Takase et al 2009	In vitro	Osteoblastic MC3T3-E1 cells	To examine if PTH affects BMP2 expression and to clarify its involvement of the mevalonate pathway	PTH stimulated BMP-2 mRNA expression via the mevalonate pathway and ROK in osteoblastic MC3T3-E1 cells
Zhang et al 2009	In vitro	primary osteoblasts , osteoblast precursor cell lines 2T3 and MC3T3-E1 cells	To investigate the effect of BMP-2 on beta-catenin signaling	BMP2 may regulate osteoblast function in part through modulation of the beta-catenin signaling.
Jäger et al 2008	In vitro	Bone marrow-derived human MSCs	To investigate interactions between dexamethasone and BMP2 for an osteoblastic differentiation of MSCs	BMP2 enhances dexamethasone/ascorbic acid/glycerolphosphate-induced osteogenic differentiation in mesenchymal bone marrow cells. Both agents interact in various ways and can modify osteoblastic bone formation.
Kanzaki et al 2008	In vitro	osteoblasst	To investigate the role of heparin in the biological activity of BMP	Heparin suppresses BMP2-BMP receptor binding, and inhibits BMP2 osteogenic activity in vitro.
Laflamme et al 2008	In vitro	osteoblasts	To evaluate the effect of BMP2 and BMP7 homodimers and a mixture of BMP2/BMP7 homodimers on osteoblast adhesion and growth following culture on a collagen scaffold	The BMP2, BMP7 and a mixture of BMP2/BMP7 all promoted osteoblast growth on the collagen scaffold, with the mixture of BMP2/BMP7 enhancing the most growth. BMP2, BMP7 and the mixture of BMP2/BMP7 could promote bone regeneration via different mechanisms involving IL-6 and MMP inhibitors.
Lavery et al	In vitro	hMSCs	To evaluate receptor	Different mechanisms for

2008			utilization by BMP-2, BMP-4, BMP-6, and BMP-7 in primary hMSCs	BMP2/4- and BMP6/7-induced osteoblastic differentiation in primary hMSCs.
Luppen et al 2008	In vitro		To better understand how glucocorticoids regulate BMPs	Glucocorticoids regulate BMP2 via a far-downstream domain, and activation of Smad, not ALP, best predicts the pro-mineralization potential of rhBMPs.
Schwartz et al 2008	In vitro	hMSCs	To test PEMF enhances osteogenesis of MSCs in the presence of an inductive stimulus like BMP2	PEMF enhances osteogenic effects of BMP2 on MSCs cultured on calcium phosphate substrates, suggesting that PEMF will improve MSC response to BMP2 in vivo in a bone environment.
Singhatanadgit et al 2008	In vitro	three different bone cell samples	To examine the effects of BMPR-IB knockdown on BMP-induced osteoblast-associated genes.	The possibility that BMP receptor-IB could be a therapeutic target for enhancing bone regeneration in vivo.
Samee et al 2008	In vitro, In vivo	Human periosteum-derived cells were transfected with BMP-2, VEGF, BMP-2 + VEGF	To evaluate the feasibility and efficacy of BMP2 and/or VEGF on periosteal cell differentiation to osteoblasts in vitro and ectopic bone formation in vivo.	VEGF might enhance BMP2-induced bone formation through modulation of angiogenesis.
Susperregui et al 2008	In vitro	pluripotent mesenchymal C2C12 cells	To test BMP2 rapidly down-regulated PTHrP gene expression through a transcriptional mechanism	BMP2 down-regulation of PTHrP could facilitate terminal differentiation of osteoblasts.
Van de Zande et al 2008	In vitro	osteoblast-like cells from rat bone marrow	To investigate the combined application of TGFbeta-1 and BMP2 to stimulate osteogenic expression in vitro.	BMP-2 is the most suitable candidate for osteogenic stimulation of rat bone marrow cells
Zanotti et al 2008	In vitro	ST-2 murine stromal cell lines, primary cultures of murine calvarial osteoblasts	To determine whether gremlin has direct effects in osteoblasts, independent of its BMP binding activity	Gremlin inhibits BMP-2 signaling and activity, and does not have independent actions on ERK signaling in osteoblasts.
Abdelmagid et al 2007	In vitro	osteoblast cultures	To examine the regulation of OA expression by BMP-2 and the role OA plays as a downstream mediator of BMP-2 effects in osteoblast function.	BMP-2 regulates OA expression through the Smad1 signaling pathway. OA protein acts as a downstream mediator of BMP-2 effects on osteoblast differentiation and function.
Cowan et al 2007	In vitro	C2C12 myoblasts were transduced with AdLacZ,	To show Nell-1 and BMP-2 synergistically enhanced osteogenic differentiation of myoblasts and phosphorylated the JNK	Osteochondral specificity of Nell-1 signaling and the potential therapeutic effects of enhanced BMP-2 action with coordinated Nell-1 delivery.

		AdNell-1, AdBMP-2, or AdNell-1+AdBMP-2 overexpression viruses	MAPK pathway.	
Cowan et al 2007	In vitro	MC3T3-E1 osteoblasts	To examine the utility of microcomputed tomography over conventional techniques in the evaluation of the BMP2 dose response effect in a 3D in vitro culture system and in an established calvarial defect model	The utility of microcomputed tomography analysis as a beneficial addition to existing techniques for objective evaluation of bone tissue engineering and regeneration.
Ding et al 2007	In vivo	MSCs	To investigate the encapsulation of BMP-2 gene-modified MSCs in alginate-poly-L-lysine microcapsules for the persistent delivery of BMP2 to induce bone formation.	The nonautologous BMP2 gene-transfected stem cells are of potential utility for enhancement of bone repair and bone regeneration in vivo.
Gersbach et al 2007	In vitro, Invivo	Osteoblast	To investigate the relative efficacy of different strategies for inducing osteoblastic differentiation	BMP2 stimulated osteoblastic markers faster and to a greater extent than Runx2. Runx2-engineered cells did not utilize paracrine signaling via secreted osteogenic factors, in contrast to cells overexpressing BMP-2
Li et al 2007	In vivo; Canine model		To investigate bone regeneration by implantation of ADSCs expressing BMP2	ADSCs modified by the BMP-2 gene can enhance the repair of critical-sized bone defects in large animals.
Liu et al 2007	In vitro	RD-C6 cells	To investigate the molecular mechanism underlying the differentiation of osteoblasts and chondroblasts	BMP-2 induces Runx2-deficient cells to express markers related to osteoblast and chondroblast differentiation using a Runx2-independent pathway, but it failed to induce these cells to differentiate into bone-forming osteoblasts and mature chondrocytes.
Maegawa et al 2007	In vitro	MSCs from rat bone marrow	To investigate the culture conditions that contributed to extensive osteoblastic differentiation	The number of responding cells or immature osteoblasts was increased by the supplementation of FGF-2 in the early phase of the culture and that these cells can show osteoblastic differentiation, of which capability was augmented by BMP-2 in the late phase.
Minamizato et al 2007	In vitro	MC3T3-E1 osteoblastic cells	To elucidate the role of CCN3/NOV in osteoblast differentiation	CCN3 exerts inhibitory effects on BMP-2-induced osteoblast differentiation by its

				involvement of the BMP and Notch signaling pathways.
Mölders et al 2007	In vitro	MC3T3-E1 cells	To analyze effects of the Ni-containing steel 316L and major metal constituents thereof on BMP2-induced alkaline phosphatase (ALP)	Cells contacting 316L steel are exposed to increased concentrations of Ni which suffice to impair BMP2-induced ALP activity. Zn2+, as a competitor of this inhibition, may help to restore normal osteoblastic function and bone development under these conditions.
Turhani et al 2007	In vitro	Osteosarcoma cells (SaOS-2)	To examine whether extracellular matrix compartments and osteoinductive factors could further ameliorate the bioactivity of the scaffold.	The combination of collagen type I and exogenous rhBMP-2 did not ameliorate the bioactivity of hydroxyapatite calcified from red algae in the initial period of cultivation.
Yang et al 2007	In vitro	murine pre-myoblast C2C12 cells	To investigate the role of deltalEF1 to BMP2	DeltaEF1 acts as a potent inhibitor of BMP-2-induced osteogenesis in vitro, in part, by differentially regulating the AP-1 signaling pathway.
Gutierrez et al 2006	In vivo	mouse myoblast cell line C2C12	To analyze and characterize the proteoglycan populations that are induced in C2C12 cells upon osteoblastic differentiation produced by BMP-2	The results are the first biochemical evidence and analysis for the effect of BMP-2 on the synthesis of proteoglycan during osteogenic conversion of myoblasts and suggest a role for decorin in cell response to BMP-2.
Seol et al 2006	In vitro	osteoblast-like MC3T3-E1 cells	To test BMP2 linked to titanium surfaces	Biochemical modifications of titanium surfaces can enhance the rate of bone healing as compared with untreated Ti surfaces.
Singhatanadgi t et al 2006	In vitro	Primary human bone cell	To examine the effects of TGF-beta1, FGF-2, and PDGF-AB on BMP receptor expression and BMP-2-mediated osteoblast functions	Increased BMP receptor-IB by TGF-beta1, FGF-2, and PDGF-AB significantly enhances BMP-2-induced osteogenic functions in vitro, suggesting that they might positively modulate bone formation by up-regulating BMPR-IB in vivo.
Suzuki et al 2006	In vitro	MC3T3-E1 osteoblast-like cells	To study the role of Pi transport in BMP-2-induced matrix calcification	BMP-2 also stimulates Pi transport activity through a selective increase in expression of type III Pi transporters Pit-1. In MC3T3-E1 cells, this effect is mediated by the JNK pathway and plays an essential role in bone matrix calcification induced by BMP-2.
Zhao et al 2006	In vitro	human MSCs, murine pre-myoblast	To test that PKA pathway is involved in osteogenesis.	Activation of the PKA pathway may be one of key BMP-2-activated signaling events that

		C2C12 cells		lead to osteogenesis and that downregulation of PKIgamma may be prerequisite for the PKA activation during the osteoblastic differentiation of precursor cells.
Zhu et al 2006	In vitro	C2C12 myoblastic cells	To investigate the result of Noggin to BMP2/7	'Fusion gene' construct led to the production of bioactive BMP2/7 heterodimers, which were not antagonized by Noggin as effectively as it to BMP homodimers. The weaker Noggin antagonism on BMP heterodimers compared to homodimers may contribute to increased osteogenic potency of heterodimers.

3.6 Osteopontin

Osteopontin (OPN) was initially isolated from bovine bone cortex. It has been cloned for the first time in 1986. Its genes share common expression in bone and tooth.[157] OPN has an important role in the effects of unloading-induced alterations of differentiation of bone marrow into osteoblasts and osteoclasts.[158] Similarly, Li et al exhibited that OPN, a matrix protein found in mineralized tissues and pivotal in modulating osteoclast functions, was present in increased concentrations in Nf1+/- osteoblasts. [159] Addition of OPN neutralizing antibody to Nf1+/- osteoblast conditioned media diminished the gain in bioactivity on osteoclast functions, including osteoclast migration and bone resorption.[159] Local feedback regulation by the bone matrix protein OPN also plays a significant role in the regulation of parathyroid hormone(PTH) actions.[160] OPN is an estrogen receptor-related receptor alpha (ERRalpha) target gene whose promoter is regulated by ERRalpha in a cell context-dependent manner and that a predicted silencing mutation in AF2 or a more flexible helix 12 increases ERRalpha transcriptional activity, effects with implications for ERRalpha as a therapeutic target in bone.[161] The specific

binding of OPN to collagen I may naturally orient OPN, thus influencing osteoblast adhesion.[162]

OPN deficiency enhanced the direct anabolic action of prostaglandin E receptor agonist locally injected onto the parietal bone in inducing new bone formation.[163] OPN inhibits mineralization in bone and urine.[157] Another study similarly reported that OPN acidic serine- and aspartate-rich motif inhibits mineralization by binding to hydroxyapatite in a phosphorylation-dependent manner. [164] The study in human fetal-osteoblast (hFOB 1.19) to explore the osteoblastic cellular response to physicochemical characteristics of fluoridated hydroxyapatite reported that sintered Fluoridated- calcined hydroxyapatite composites could enhance OPN and COL I gene expression after 6-day culture ($P \leq 0.05$).[165] Otherwise, sintered hydroxy fluorapatites composites inhibited the expression.[165] Sintered Fluoridated- calcined hydroxyapatite composites with both OH and OHF bands were bioactive bone graft materials.[165] The early human response to systemic endotoxemia boosts OPN levels and modifies bone biomarkers, indicating a decrease in the lytic activity of osteoclasts, accompanied by an increase in the activity of immature osteoblasts.[166]

In vivo study reported that the secretion of granulocyte macrophage colony-stimulating factor (GM-CSF) and OPN by immunocompetent cells such as macrophages and dendritic cells plays a role in the maturation of dendritic cells and the differentiation of odontoblasts, respectively, in the regenerated pulp tissue following tooth transplantation.[167] OPN promotes osteoblast and osteoclast adhesion, differentiation and function.[168, 169] The study to compare the cell binding ability of adsorbed BSP and OPN specifically bound to hydroxyapatite concluded that There is a preference for cell

binding to HA with adsorbed BSP as compared to OPN, but not to a statistically significant level.[170] Another study reported that OPN is more important than BSP for osteoblast adhesion to the collagen matrix.[171] In the MC3T3-E1/C4 osteoblastic cell, surfaces coated with oligomerized OPN and BSP promote cell adhesion better than surfaces coated with the monomeric form of the proteins. [172] The investigation the effect of inorganic pyrophosphate on osteoblast function and matrix mineralization found that inorganic pyrophosphate prevents mineralization in MC3T3-E1 osteoblast cultures by at least three different mechanisms that include direct binding to growing crystals, induction of OPN expression, and inhibition of tissue-nonspecific alkaline phosphatase activity.[173] Ti-6Al-7Nb possess a good potential to support SaOS-2 cells on spreading and fibronectin and OPN synthesis, therefore, this material may be one of a candidate material used in implant dentistry.[80] Preadsorption of osteopontin on the HA particles of a degradable PDLLA/HA composite enhances the composite osteoconductive properties when used as a coating on a commercial titanium implant but no differences in the gap.[174]

Table 3.3: *Osteopontin (Keyword: osteopontin and osteoblast, 2006-2011 and published in English)*

Author	Type	Cell	Objective	Result
Saito et al 2011	In vivo	Cells extracted-mouse molar	To clarify the expression of GM-CSF and OPN in the process of reparative dentin formation by allogenic tooth transplantation using in situ hybridization for OPN and immunohistochemistry for GM-CSF and OPN	The secretion of GM-CSF and OPN by immunocompetent cells such as macrophages and dendritic cells plays a role in the maturation of dendritic cells and the differentiation of odontoblasts, respectively, in the regenerated pulp tissue following tooth transplantation.
Addison et al 2010	In vitro	MC3T3-E1 osteoblast	To examine the role of OPN acidic serine- and aspartate-rich motif and its interaction with PHEX enzyme	OPN acidic serine- and aspartate-rich motif inhibits mineralization by binding to hydroxyapatite in a phosphorylation-dependent manner.
Grimm et al 2010	In vivo; healthy men		To investigate the changes in biochemical parameters of bone	The early human response to systemic endotoxemia boosts OPN levels and modifies bone

			turnover following human endotoxemia, an experimental model of self-limiting systemic infection and inflammation	biomarkers, indicating a decrease in the lytic activity of osteoclasts, accompanied by an increase in the activity of immature osteoblasts.
Wu et al 2010	In vitro	human fetal-osteoblast (hFOB 1.19)	To explore the osteoblastic cellular response to physicochemical characteristics of fluoridated hydroxyapatite	Sintered Fluoridated- calcined hydroxyapatite composites could enhance OPN and COL I gene expression after 6-day culture ($P \leq 0.05$). Otherwise, sintered hydroxy fluorapatites composites inhibited the expression.Sintered Fluoridated-calcined hydroxyapatite composites with both OH and OH···F bands were bioactive bone graft materials.
Li et al 2009	In vitro	Osteoblast	To identify whether haploinsufficiency of Nf1 (Nf1+/-) osteoblasts and their precursors secrete cytokines that have a central role	OPN, a matrix protein found in mineralized tissues and pivotal in modulating osteoclast functions, was present in increased concentrations in Nf1+/- osteoblasts. Addition of OPN neutralizing antibody to Nf1+/- osteoblast conditioned media diminished the gain in bioactivity on osteoclast functions, including osteoclast migration and bone resorption.
Bernards et al 2008	In vitro	MC3T3-E1 cell	To compare the cell binding ability of adsorbed BSP and OPN specifically bound to hydroxyapatite	There is a preference for cell binding to HA with adsorbed BSP as compared to OPN, but not to a statistically significant level
Bernards et al 2008	In vitro	MC3T3-E1 cell	To examine and compare the orientation of BSP under similar circumstances with OPN	OPN is more important than BSP for osteoblast adhesion to the collagen matrix
Ono et al 2008	In vivo; Parathyroid hormone receptor (PPR) transgenic mice		To examine the effects of deficiency of the bone matrix protein osteopontin (OPN) on the systemic effects of PTH specifically within osteoblastic cell lineages	Local feedback regulation by the bone matrix protein OPN also plays a significant role in the regulation of PTH actions.
Zirngibl et al 2008	In vitro	rat osteosarcoma ROS17/2.8 cells, non-osteoblastic (HeLa) cell lines	To investigate whether the transcriptional regulation by ERRalpha of the gene for OPN	OPN is an ERRalpha target gene whose promoter is regulated by ERRalpha in a cell context-dependent manner and that a predicted silencing mutation in AF2 or a more flexible helix 12 increases ERRalpha transcriptional activity, effects with implications for ERRalpha as a therapeutic target in bone.
Addison et al	In vitro	MC3T3-E1	To investigate the effect	Inorganic pyrophosphate

2007		osteoblast	of inorganic pyrophosphate on osteoblast function and matrix mineralization	prevents mineralization in MC3T3-E1 osteoblast cultures by at least three different mechanisms that include direct binding to growing crystals, induction of OPN expression, and inhibition of tissue-nonspecific alkaline phosphatase activity.
Ishijima et al 2007	In vitro	bone marrow cells obtained from hind limb bones of OPN-/- mice	To obtain further insight into the role of OPN in mediating mechanical stress effect on bone	OPN has an important role in the effects of unloading-induced alterations of differentiation of bone marrow into osteoblasts and osteoclasts.
Kato et al 2007	In vivo; wild-type mice		To test whether deficiency of OPN, a secreted phosphorylated protein, could modulate the effects of prostaglandin E receptor agonist	OPN deficiency enhanced the direct anabolic action of prostaglandin E receptor agonist locally injected onto the parietal bone in inducing new bone formation.
Liu et al 2007	In vitro	osteoblast MC3T3-E1	To attempt to control the orientation/conformation of bone OPN via its specific interactions with type I collagen	The specific binding of OPN to collagen I may naturally orient OPN, thus influencing osteoblast adhesion.
Osathanon et al 2006	In vitro	human osteoblast-like cells (SaOS-2)	To compare the early response of human osteoblast-like cells (SaOS-2) on commercially pure titanium (cpTi) and titanium-6-aluminium-7-niobium (Ti-6Al-7Nb)	Ti-6Al-7Nb possess a good potential to support SaOS-2 cells on spreading and fibronectin and OPN synthesis, therefore, this material may be one of a candidate material used in implant dentistry.

3.7 Tenascin

Tenascin (TN) is extracellular matrix proteins which secretes from different connective tissue cells. TN-C expression is regulated by mechanical stress. It shows highest expression in connective tissue surrounding tumors, in wounds and in inflamed tissues.[175] TN add to connective tissue function in many different ways.[175] TN-C seems to influence the function of other adhesion proteins such as adhesin and syndecans. By the way, it also have repulsive (anti-adhesive) interactions.[176-178] TN-C expression exhibits an interesting mechanism of activating by certain growth factors and through the application of mechanical stress to a tissue or to cells in culture.[179] TN

distribution changes with development and growth.[180] TN is important to osteoblast adhesion to the underlying matrix in the process of osteoblast differentiation.[12] In mouse study showed TN-W is expressed in osteoblasts at the edge of the developing bone domain prior to mineralization in mouse fetuses. TN-W is involved in osteoblast maturation (i.e. mineralization).[181] TN-W can accelerate the formation of new bone in a complex multicellular environment.[182] TN-W has no effect on the initial stage of osteogenesis in bone marrow cells.[183] TN-W is a novel marker of preosteoblasts in early stage of osteogenesis, and that TN-W inhibits cell proliferation and differentiation of preosteoblasts mediated by canonical Wnt signaling.[183] TN promote osteoblast differentiation.[6] It is associated with the process of cartilage development. During development, TN strongly express in the interface zone between components of the musculoskeletal element, such as myotendinous junctions and insertions of ligaments and tendons of bone.[184] In the mature bone, TN-C expression is seen at the pericellular space surrounding some osteocytes and to articular cartilage.[185] It is absent from the matrix surrounding proliferating and hypertrophic chondrocytes, but remains in a restricted distribution in peripheral epiphyseal cartilage.[185] TN-C is known to be induced in inflammation. The increase found in peri-implantitis was less than expected. In the context of peri-implantitis, TN-C might be a marker of bone remodelling rather than inflammation and infection.[186]

The study of Juhasz and colleague has shown that increased levels of TN, matrix metalloproteinase 9- positive cells (MMP-9), and proliferative activity of lesions and decreased levels of apoptosis. So they concluded that TN and MMP-9 can be key molecules of bone destruction during cholesteatoma progression.[187] Rat's tibias study of Sasano and colleague indicated that type I collagen, fibronectin and TN-C localized

during embryonic osteogenesis in the dentary of mandibles and tibias. TN-C was localized in the perichondral mesenchymal tissue. [58]

Table 3.4: *Tenascin (Keyword: tenascin and osteoblast, 2006-2011 and published in English).*

Author	Type	Sample	Objective	Result
Koch et al 2011	In vitro	Human osteoblasts (HOB-c)	To investigate the influence on adhesion and migration of human osteoblasts after bisphosphonate therapy	Bisphosphonates seem to inhibit human osteoblast adhesion and migration. The downregulation of the adhesive genes integrin aVb3 and tenascin C, which possibly even enhanced the antiadhesive effect by autocrine secretion, could be one of the molecular, intracellular reasons for the bisphosphonate induced osteonecrosis of the jaw.
Ozçakir – Tomruk et al 2011	In vitro		To determine the amount and concentration of tenascin-C in gingival crevicular fluid around teeth and in peri-implant sulcus fluid from healthy implants and implants with peri-implantitis, and to correlate it with matrix metalloproteinase-9 levels.	Tenascin C is known to be induced in inflammation. The increase found in peri-implantitis was less than expected. In the context of peri-implantitis, Tenascin C might be a marker of bone remodelling rather than inflammation and infection.
Juhász etal 2009	In vitro	Cholesteatoma tissue samples	To identify factors that could play important role during the invasion of the disease	TN and MMP-9 can be key molecules of bone destruction during cholesteatoma progression.
Mikura et al 2009	In vivo	mouse	To investigate the roles of tenascin-W in osteogenesis	Tenascin-W is expressed in osteoblasts at the edge of the developing bone domain prior to mineralization in mouse fetuses. Tenascin-W is involved in osteoblast maturation (i.e. mineralization).

Meloty-Kapella et al 2008	In vitro	avian osteoblasts	To examine possible roles for tenascin-W in osteogenesis	Tenascin-W can accelerate the formation of new bone in a complex multicellular environment.
Zhang et al 2008	In vitro	Rat BMSCs	To investigate the effect of uniaxial stretching on the orientation and biological functions of BMSC	Cyclic stretching promotes the synthesis of collagen types I and III and tenascin-C by the rat BMSC.
Kimura et al 2007	In vivo	ATDC5 osteo-chondroprogenitors	To identify a cDNA encoding mouse Tenascin-W (TN-W) upregulated by bone morphogenetic protein (Bmp)2	TN-W is a novel marker of preosteoblasts in early stage of osteogenesis, and that TN-W inhibits cell proliferation and differentiation of preosteoblasts mediated by canonical Wnt signaling.

3.8 Bone sialoprotein

Bone sialoprotein (BSP) is one of the major non-collagenous glycosylated phosphoproteins of the extracellular matrix in bone. [188] It is a mineralized tissue-specific protein expressed in differentiated osteoblasts that appears to function in the initial mineralization of bone.[189] The BSP nucleating motif may help nucleate an amorphous calcium phosphate cluster, which ultimately converts to hydroxyapatite crystal formation.[190] As one of the noncollagenous proteins in extracellular bone matrix, BSP promotes osteoclast adhesion, differentiation and function.[168, 191] BSP can inhibit human bone marrow stem cells proliferation and enhance their osteogenic differentiation and mineralization.[192] BSP can stimulate osteoblast differentiation through RGD-mediated cell interactions to promote mineralization.[193] BSP may serve as a matrix-associated signal directly promoting osteoblast differentiation resulting in the increased production of a mineralized matrix.[193] In the MC3T3-E1/C4 osteoblastic cell, surfaces coated with oligomerized OPN and BSP promote cell adhesion better than

surfaces coated with the monomeric form of the proteins. [172] However, previous in vitro study presented that OPN is more important than BSP for osteoblast adhesion to the collagen matrix.[171] There is a preference for cell binding to hydroxyapatite with adsorbed BSP as compared to OPN, but not to a statistically significant level.[170] In vivo study, BSP, but not OPN, plays a role in primary bone formation and mineralization of newly formed bone during the process of cortical bone healing.[194] Increased expression of BSP in osteoblast cells can increase expression of the osteoblast-related genes Runx2 and Osx as well as alkaline phosphatase and osteocalcin and increase matrix mineralization.[195] Nevertheless, the effects of BSP overexpression decreased osteoblast population and increased osteoclastic activity and leads to an uncoupling of bone formation and resorption, which in turn results in osteopenia and mild dwarfism in mice.[196] Likely, BSP deficiency impairs bone growth and mineralization, concomitant with dramatically reduced bone formation.[197] and the absence of BSP delays bone repair at least in part by impairing both new bone formation and osteoclast activity.[198] In mice study, lack of BSP affects both osteoclast formation and activity.[199] PDGF-BB stimulates human BSP transcription by targeting the CRE1, CRE2, AP1(3) and SSRE1 elements in the human BSP gene promoter.[200] IGF-I stimulate BSP transcription by targeting FRE and HOX elements.[201] FGF2 stimulates BSP gene transcription by targeting the FRE and AP1/GRE elements in the rat BSP gene promoter.[202] Sodium phosphate glass type 25 stimulates BSP transcription by targeting FRE and HOX elements in the proximal promoter of the rat BSP gene.[203] Butyric acid also increases the transcription of the BSP gene mediated through FRE in the rat BSP gene promoter, and induces osteoblast activity in the early stage of bone formation[204] Kaempferol increased BSP gene transcription mediated through inverted CCAAT, CRE, and FRE

elements in the rat BSP gene promoter, and could induce osteoblast activities in the early stage of bone formation.[205] P. gingivalis lipopolysaccharide increased BSP gene transcription mediated through CRE and FRE elements in the rat BSP gene promoter.[206] IL-11 stimulate BSP transcription by targeting CRE, FRE and HOX sites in the proximal promoter of the rat BSP gene.[189] Another study found that FSK and FGF2 stimulate BSP transcription in DU145 human prostate cancer cells by targeting the CRE1 and CRE2 elements in the human BSP gene promoter.[207] $Ca(OH)_2$ stimulates BSP transcription by targeting the CRE1, CRE2 and FRE elements in the human BSP gene promoter.[208] In the rat osteoblast-like ROS17/2.8 cells, CO22laser irradiation increases BSP transcription via FRE in the BSP gene promoter.[209]

By the way, Runx2 and HDAC3 repress BSP gene expression and that this repression is suspended upon osteoblastic cell differentiation.[210] Chlorpromazine suppresses BSP gene transcription through tyrosine and MAP kinase-dependent pathways and that the chlorpromazine effects are mediated by CRE and FRE elements in the proximal promoter of the BSP gene.[211] In the same group of researchers also reported that LPS suppresses BSP gene transcrition through protein kinase A and tyrosine kinase-dependent pathways and that the LPS effects are mediated through CRE and FRE elements in the proximal BSP gene promoter.[212] The study of the effect of BSP to osteogenic cell migration through basement membrane and collagen matrices showed that Pre-osteoblasts and their BMSC precursors may use MMP-2/BSP/integrin complexes to disrupt matrix barriers during migration to their final destinations in vivo.[213]

Modification of PCL/pHEMA surfaces with BSP significantly enhanced osteoblastic cell attachment and spreading, without compromising proliferation.[214] In contrast, BSP

coating of a variety of substrates is not directly associated with an enhancement of osteoprogenitor cell differentiation in vitro or in vivo, and that presentation of BSP on polymeric materials is not sufficient to prime BMSC functional osteoblastic differentiation in vivo.[215] The investigation to compare BSP as a surface-coating material against the major organic and inorganic components of bone, collagen type I and hydroxyapatite (TICER) exhibited that BSP precoating of the rough TICER implant surface enhanced the osteoinductive effect much more than did collagen precoating.[216] BSP is osteoinductive when coated onto femoral implants.[217] BSP has an enhancement of the osteoinductive effect both smooth and rough surface implant.[218]

Table 3.5: *Bone Sialoprotein (Keyword: Bone sialoprotein and osteoblast, 2006-2011 and published in English).*

Author	Type	Cell	Objective	Result
Forsprecher et al 2011	In vitro	MC3T3-E1/C4 osteoblastic cell	To examine effects of tissue transglutaminase - mediated crosslinking and oligomerization of OPN and BSP on osteoblast cell adhesion	Surfaces coated with oligomerized OPN and BSP promote MC3T3-E1/C4 osteoblastic cell adhesion significantly better than surfaces coated with the monomeric form of the proteins
Li et al 2011	In vitro	human prostate cancer DU145 cells	To investigate the effects of cAMP and FGF2 on BSP	FSK and FGF2 stimulate BSP transcription in DU145 human prostate cancer cells by targeting the CRE1 and CRE2 elements in the human BSP gene promoter
Sasaki et al 2011	In vitro	rat osteoblast-like ROS17/2.8 cells	To investigate the effects of CO(2) laser irradiation on BSP gene transcription	CO(2) laser irradiation increases BSP transcription via FRE in the rat BSP gene promoter
Wang et al 2011	In vitro	rat BSP gene promoter	To analyze the effects of IL-11 on the expression of the BSP gene in osteoblast-like cells	IL-11 stimulates BSP transcription by targeting CRE, FRE and HOX sites in the proximal promoter of the rat BSP gene. Moreover, phospho-CREB1, c-Fos, c-Jun, JunD, Fra2, Dlx5, Msx2, Runx2 and Smadl transcription factors appear to be key regulators of IL-11 effects on BSP transcription.
Wang et al 2011	In vitro	human osteoblast-like Saos2 cells	To detail the mechanism involved in the mineralization induced by Ca(OH)(2)	Ca(OH)(2) stimulates BSP transcription by targeting the CRE1, CRE2 and FRE elements in the human BSP gene promoter.
Xia et al 2011	In vitro	human BMSCs	To investigate the effects of recombinant human BSP on the proliferation	BSP is capable of inhibiting hBMSCs proliferation and enhancing their osteogenic

			and osteodifferentiation of human BMSCs	differentiation and mineralization in the presence of osteogenic medium
Boudiffa et al 2010	In vivo	mice	To investigate the effect of BSP deficiency to osteoclastogenesis and mineral resorption	Lack of BSP affects both osteoclast formation and activity
Chan et al 2010	In vitro	Murine preosteoblastic cell line(MC3T3-E1)	To evaluate modification of PCL/pHEMA surfaces with BSP	Modification of surfaces with BSP significantly enhanced osteoblastic cell attachment and spreading, without compromising proliferation.
Li et al 2010	In vitro	rat osteoblast-like ROS17/2.8 cells	To investigate the effects of P. gingivalis lipopolysaccharide on BSP transcription	0.1 microg/ml suppressed, and 0.01 microg/ml P. gingivalis lipopolysaccharide increased BSP gene transcription mediated through CRE and FRE elements in the rat BSP gene promoter
Monfoulet et al 2010	In vivo	mouse femur	To compare the roles of BSP and OPN in the repair process	BSP, but not OPN, plays a role in primary bone formation and mineralization of newly formed bone during the process of cortical bone healing
Schaeren et al 2010	In vivo In vitro	BMSC and nude mice	To test whether synthetic polymer-based porous scaffolds could support ectopic bone formation by human BMSC if coated with BSP	BSP coating of a variety of substrates is not directly associated with an enhancement of osteoprogenitor cell differentiation in vitro or in vivo, and that presentation of BSP on polymeric materials is not sufficient to prime BMSC functional osteoblastic differentiation in vivo
Wang et al 2010	In vitro	osteoblast-like ROS 17/2.8 cells	To investigate the effects of inorganic polyphosphate on BSP	Sodium phosphate glass type 25 stimulates BSP transcription by targeting FRE and HOX elements in the proximal promoter of the rat BSP gene
Yang et al 2010	In vitro	rat osteoblast-like ROS17/2.8 cells	To investigate the regulation of BSP transcription by butyric acid	Butyric acid increases the transcription of the BSP gene mediated through FRE in the rat BSP gene promoter, and induces osteoblast activity in the early stage of bone formation
Yang et al 2010	In vitro	rat osteoblast-like UMR106 cells	To investigate the regulation of BSP transcription by kaempferol	Kaempferol increased BSP gene transcription mediated through inverted CCAAT, CRE, and FRE elements in the rat BSP gene promoter, and could induce osteoblast activities in the early stage of bone formation
Gordon et al 2009	In vitro	primary rat bone osteoblastic cells	To determine the molecular mechanisms responsible for the BSP-mediated increase in osteoblastic	Increased expression of BSP in osteoblast cells can increase expression of the osteoblast-related genes Runx2 and Osx as well as alkaline phosphatase and

			differentiation	osteocalcin and increase matrix mineralization
Malaval et al 2009	In vivo	Mice femur	To report that absence BSP impair cortical defect repair	The absence of BSP delays bone repair at least in part by impairing both new bone formation and osteoclast activity
Mezawa et al 2009	In vitro	osteoblast-like Saos2 and ROS17/2.8 cells	To determine the molecular mechanisms PDGF regulation of human BSP gene transcription	PDGF-BB stimulates human BSP transcription by targeting the CRE1, CRE2, AP1(3) and SSRE1 elements in the human BSP gene promoter
Baht et al 2008	In vitro	-----	To investigate the nature of the BSP-collagen interaction and its role in HA nucleation	Optimal binding of rBSP requires collagen to be in a native, triple-helical structure, does not require the telopeptides, and is stabilized by hydrophobic interactions. Upon binding to collagen, rBSP displays an increase in nucleation potency, implying a co-operative effect of BSP and collagen in mineral formation.
Bernards et al 2008	In vitro	MC3T3-E1 cell	To compare the cell binding ability of adsorbed BSP and OPN specifically bound to hydroxyapatite	There is a preference for cell binding to HAP with adsorbed BSP as compared to OPN, but not to a statistically significant level
Bernards et al 2008	In vitro	MC3T3-E1 cell	To examine and compare the orientation of BSP under similar circumstances with OPN	OPN is more important than BSP for osteoblast adhesion to the collagen matrix
Graf et al 2008	In vitro	Osteoblast	To compare BSP as a surface-coating material against the major organic and inorganic components of bone, collagen type I and hydroxyapatite (TICER)	BSP precoating of the rough TICER implant surface enhanced the osteoinductive effect much more than did collagen precoating
Malaval et al 2008	In vivo	BSP(-/-) mice	To investigate the role of BSP in bone formation and osteoclastogenesis	BSP deficiency impairs bone growth and mineralization, concomitant with dramatically reduced bone formation
Takai et al 2008	In vitro	osteoblastic cell line ROS17/2.8	To report that AP1 binding site overlapping with glucocorticoid response element (GRE) AP1/GRE in the rat BSP gene promoter is another target of FGF2	FGF2 stimulates BSP gene transcription by targeting the FRE and AP1/GRE elements in the rat BSP gene promoter
Valverde et al 2008	In vivo	homozygous transgenic mouse	To determine the effects of BSP overexpression in bone metabolism	Overexpression of BSP decreased osteoblast population and increased osteoclastic activity and leads to an uncoupling of bone formation and resorption, which in turn results in osteopenia and

				mild dwarfism in mice.
Gordon et al 2007	In vitro	MC3T3E1 cell	To demonstrate that BSP can stimulate osteoblast differentiation through RGD-mediated cell interactions to promote mineralization	BSP may serve as a matrix-associated signal directly promoting osteoblast differentiation resulting in the increased production of a mineralized matrix
Lamour et al 2007	In vitro	osteoblast-like Saos2 cells	To investigate the role of Runx2 in the regulation of BSP expression	Runx2 and HDAC3 repress BSP gene expression and that this repression is suspended upon osteoblastic cell differentiation
Karadag et al 2006	In vitro	BMSCs and pre-osteoblasts	To investigate the effect of BSP to osteogenic cell migration through basement membrane and collagen matrices	Pre-osteoblasts and their BMSC precursors may use MMP-2/BSP/integrin complexes to disrupt matrix barriers during migration to their final destinations in vivo
Kato et al 2006	In vitro	osteoblast-like ROS 17/2.8 cells	To determine the molecular mechanisms involved in the suppression of bone formation	LPS suppresses BSP gene transcription through protein kinase A and tyrosine kinase-dependent pathways and that the LPS effects are mediated through CRE and FRE elements in the proximal BSP gene promoter
Nakajima et al 2006	In vitro	osteoblast-like ROS17/2.8 cells and rat stromal bone marrow cells (SBMC-D8)	To investigate the effect of chlorpromazine on BSP gene transcription	Chlorpromazine suppresses BSP gene transcription through tyrosine and MAP kinases-dependent pathways and that the chlorpromazine effects are mediated by CRE and FRE elements in the proximal promoter of the BSP gene
Nakayama et al 2006	In vitro	osteoblast-like Saos2 and rat stromal bone marrow (RBMC-D8) cells	To determine the molecular mechanism of IGF-I regulation of osteogenesis	IGF-I stimulates BSP transcription by targeting the FRE and HOX elements in the proximal promoter of BSP gene
Wang et al 2006	In vivo	rats	To investigate the potential role of BSP in more complex in vivo environments	BSP stimulates calcification and osteogenesis in a site-specific manner, and that local environment and the specificities of responding cells may play critical roles in the function of BSP in vivo

4. HYPOTHESIS

1. **H0:** DPMSC group has not faster osteogenesis than DPPSC group.**H1:** DPMSC group has faster osteogenesis than DPPSC group.

2. **H0:** Osteogenic capacities are not different between DPPSCs and DPMSCs pretreatment 3D scaffold with Fibronectin

   H1: Osteogenic capacities are different between DPPSCs and DPMSCs pretreatment 3D scaffold with Fibronectin

3. **H0:** Osteogenic capacities are not different between DPPSCs and DPMSCs pretreatment 3D scaffold with BMPs

   H1: Osteogenic capacities are different between DPPSCs and DPMSCs pretreatment 3D scaffold with BMPs

4. **H0:** Osteogenic capacities are not different between DPPSCs and DPMSCs pretreatment 3D scaffold with Osteopontin

   H1: Osteogenic capacities are different between DPPSCs and DPMSCs pretreatment 3D scaffold with Osteopontin

5. **H0:** Osteogenic capacities are not different between DPPSCs and DPMSCs pretreatment 3D scaffold with Tenascin

   H1: Osteogenic capacities are different between DPPSCs and DPMSCs pretreatment 3D scaffold with Tenascin

6. **H0:** Osteogenic capacities are not different between DPPSCs and DPMSCs pretreatment 3D scaffold with Bone sialoprotein

   H1: Osteogenic capacities are different between DPPSCs and DPMSCs pretreatment 3D scaffold with Bone sialoprotein

5. OBJECTIVE

General objectives:

The main objective of this work is to perform a comparative in vitro study to realize the osteogenesis of different proteins as Fibronectin, BMPs, Osteopontin, Tenascin, and Bone sialoprotein. Using two cell types as DPPSC and DPMSC. To determine which protein cultured of DPPSC has the highest osteoinduction and which protein cultured of DPMSC has the highest osteoinduction.

Specific objectives:

1. Compare the osteogenesis behavior between DPPSC and DPMSC.

2. Compare the osteogenic capacities with fibronectin between DPMSC and DPPSC in 3D.

3. Compare the osteogenic capacities with BMPs between DPMSC and DPPSC in 3D.

4. Compare the osteogenic capacities with Osteopontin between DPMSC and DPPSC in 3D.

5. Compare the osteogenic capacities with Tenascin between DPMSC and DPPSC in 3D.

6. Compare the osteogenic capacities with Bone sialoprotein between DPMSC and DPPSC in 3D.

6. METHODOLOGY

Experimental design

The same amount of DPPSC and DPMSC were cultured on bio-coating of 5 different types of proteins; Fibronectin, BMPs, Osteopontin, Tenascin, and Bone sialoprotein. All of them were differentiated into osteoblast medium 20 days. Throughout the study, the first indicator using, quantification of ALP and Ca^{2+} in the medium (markers of osteogenic capacity). This indicator was used for proving that DPPSC was differentiated to osteoblasts. The day 20, the SEM was performed to indicate quantity and quality of bone tissue that had been generated in OPN and TNC group (observation the osteogenic capacity), and the quantification and identification of specific RNA markers; OCN, BMP2 and Col I-(capacity of osteogenic gene expression). These were genetically indicated that these were real bone tissue. After that, we performed to do strength test of all scaffolds. (Fig 5.1)

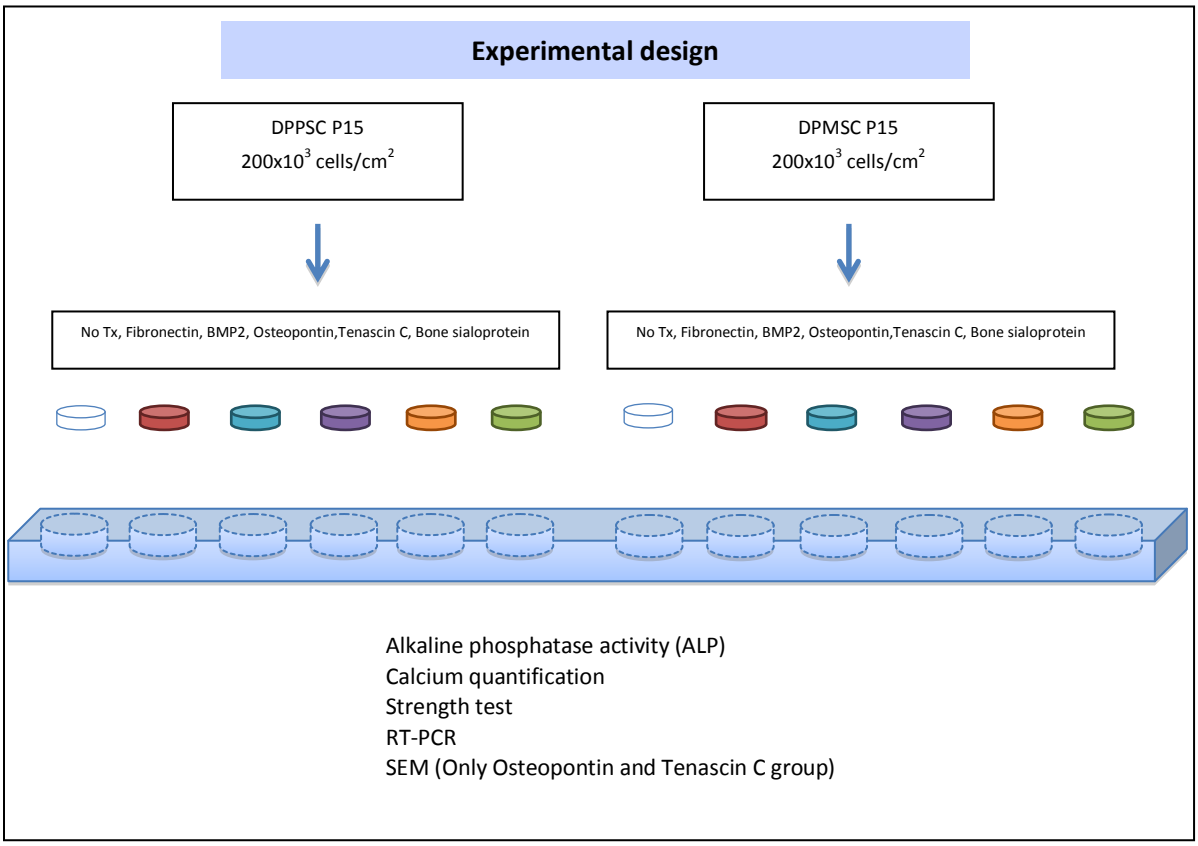


Fig 5.1: *Experimental design*

Ethics Committee

The study was conducted in accordance with the standards of the Ethics Committee of International University of Catalonia. A patient who agreed to participate voluntarily in this study was informed and the consent would be signed.

Search Strategy

For the purpose of this review was defined the overview of current researches in term of induced dental pulp stem cells to osteoblast. We conducted a PubMed electronic search between June 2006 and June 2011. We used the keyword including “Dental pulp stem cell” or “Dental pulp stem cells” and “Osteoblast”. We also restricted in English and original research. One author scanned the titles and abstracts. Inclusion criteria should be clearly present of technique of osteoblast differentiation. This review table contained with author, published year, type of study, type of cell, origin of cell and DPSCs medium.

Patient Selection

One healthy human third molar extracted for orthodontic and prophylactic reasons were selected from a healthy patient. The extraction procedure was kept simple to prevent tooth damage.

Primary cells obtained from human molar samples

Immediately after extraction, a third molar was washed using gauze soaked in 70% ethanol, followed by a wash with sterile distilled water. Tooth was cut around cementoenamel junction using cylindrical bur and was spitted into pieces using upper incisor forcep under sterile technique. Following, it was placed in falcon flask with sterile

PBS 1X. Then, it was delivered to the laboratory for isolating dental pulp stem cells (DPSCs). The pulp was extirpated by a sterile barbed broach no.15. After that, pulp tissue was digested in collagenase type I (3 mg/ml, SIGMA) during 60 minutes at 37 ºC. The pulp tissue was subsequently in the mechanical cellular separation by centrifuge for 10 minutes at 1,800 rpm. The cells were washed twice with PBS 1X and were centrifuged for 10 minutes at 1,800 rpm at room temperature. Afterwards, the number of cells was counted.

Cell culture

Cells were then cultured in a DPPSCs medium supplemented with 60% DMEM-low glucose (Sigma) and 40% MCDB-201 (Sigma) supplemented with 1X insulin-transferrin-selenium (ITS) (Sigma), 1X linoleic acid-bovine serum albumin (LA-BSA) (Sigma), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 100 units of penicillin/1000 units of streptomycin (PAA), 2% fetal bovine serum (Sigma), 10ng/ml hPDGFBB (R&D Systems), 10 ng/ml EGF (R&D Systems), 1000 units/ml hLIF (CHEMICON), 1X SITE supplement (Sigma), Chemically Defined Lipid Concentrate (Gibco), 0.8 mg/ml BSA (Sigma) and 55 µM β-mercaptoethanol (β-ME, Sigma). Cell suspensions were seeded into 150 cm^2 cell culture flask coated with 100 ng/ml human fibronectin. They were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37 ºC. During 3 weeks for primary culture, the culture medium was changed 2-3 times per week. Cells were passaged in a 4:1 when cell cultures were grown 80-100 cells/cm^2 (60% confluence).

For mesenchymal isolation, the cell suspensions were cultured in dental pulp mesenchymal stem cells (DPMSCs) medium containing Dulbecco's modified eagle (DME)-medium (Biochrom) containing 2 ng/ml basic fibroblast growth factor (bFGF) and 10%

fetal bovine serum (FBS, Hyclone). Cells suspensions were plated at a cell density of 3 x 10^6 cells/cm^2, the medium was replaced every 3 days until reached approximately 90% confluence and then disaggregated by adding PBS (Biochrom) and 0.05% trypsin containing ethylenediamine tetraacetic acid (EDTA; Biochrom). The next passage was seeded at 4,000 cells/cm^2 and incubated at 37 ºC and 5% CO_2.

Scaffolds preparation

All scaffolds used were fabricateded from the company Orla Proteins, all the proteins fibronectin, BMPs, osteopontin, tenascin C, y bone sialoprotein were treated with 10 ng/ml with crystal 3D scaffolds.

3D Culture of DPPSCs and DPMSCs

3D culture used Cell Carrier 3D glass scaffold (Orla). Almost proteins that we used in this study were coated on scaffold by manufacturing. For fibronectin group, 2 scaffolds were treated with 100 ng/ml hFN in a 5% CO_2 1 hour before culture. In short, in 24-well plates contained with 2 glass scaffolds with no treatment, 2 glass scaffolds treated with fibronectin, 2 glass scaffolds treated with BMP-2, 2 glass scaffolds treated with osteopontin, 2 glass scaffold treated with tenascin C and 2 glass scaffolds treated with bone sialoproteins. Cells were seeded in 24-well plates at a density of 2 x 10^3 cells per cm^2. The medium was changed every 2-3 days during 20 days. In our study, we did 3D culture 3 times.

Alkaline phosphatase activity (ALP)

During the 3D osteoblast differentiation of DPPSCs and DPMSCs, ALP activity was quantified at day 3, day 11 and day 20 by fluorometer using an Alkaline Phosphatase

Detection kit (Sigma-Aldrich, MO, USA), in accordance with the manufacturer's instructions.

Calcium quantification

During the 3D osteoblast differentiation of DPPSCs and DPMSCs, calcium quantification was quantified at day 3, day 11 and day 20 using lysis solutions contained in the Calcium Colorimetric Assay kit (Biovision, CA, USA) for the analysis of calcium accumulation, in accordance with the manufacturer's instructions. The total calcium was calculated using standard solutions and the absorbance at 575 nm was measured.

RNA Isolation and RT-PCR

Total RNA was isolated using Trizol (Invitrogen) from DPPSCs and DPMSCs. Total RNA (2 µg) was treated with DNAse I (Invitrogen) and was reverse-transcribed with M-MLV (Invitrogen) according to the manufacturer's protocol. The efficacy of cDNA was analyzed in different concentrations (1, 0.1, 0.01, 0.001, 0.0001 dilution) for all primers. Fifty nanograms of the cDNA samples were subjected to RT-PCR using SYBR Green Supermix (Bio-Rad Laboratories, Inc.) PCR cycling conditions were performed by one cycle with 2 minutes at 50^{o}C, 10 minutes at 95^{o}C, the amplification was performed in 40 cycles at 95^{o}C for 15 seconds, 55^{o}C for 1 minute, followed by 72^{o}C for 1 minute and melt curve 65^{o}C to 95^{o}C: increment 0.5^{o}C for 5 seconds. Amplification was performed in a CFX96 (Bio-Rad).

Expression levels for each gene of interest were calculated by normalizing against the housekeeping gene (GADPH) in all samples, and relative gene expression using $2^{\triangle\triangle Ct}$ Method. The primers used for RT-PCR were shown in Table 5.1.

Table 5.1 *Primer used for amplification*

Gene symbol	Forward primer	Reverse primer
COL I	TGCTGGCAAGAATGGCGATC	CTGTCTCAGCCTTGTCACCAC
BMP2	GCGGAAACGCCTTAAGTCCA	GTGGAGTTCAGATGATCAGC
OCN	CTCACACTCCTCGCCCTATT	GCTCCCAGCCATTGATACAG

Morphologic analysis

DPPSC and DPMSC were cultured for 20 days. The samples (DPPSC+OPN group, DPMSC+OPN group, DPPSC+TNC group and DPMSC+TNC group) were fixed in 1.5% glutaraldehyde and were then evaluated surface morphology using scanning electron microscope. (SEM, Faculty of Medicine, University of Barcelona).

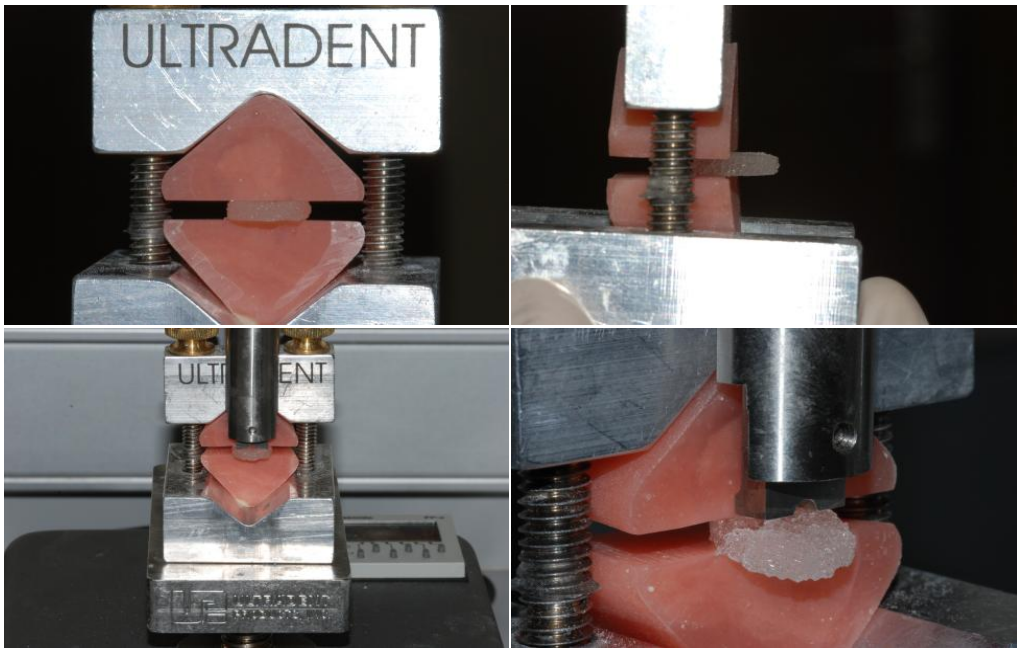


Fig 5.2 *Strength test*

Strength test

After 20 days, samples were tested strength of Orla glass scaffolds by using Ultradent testing jig (Ultradent Product, Inc., Utah, USA), and the shear strength was measured using a Universal testing machine (Model 6022, Instron Co., Canton, MA, USA). (Fig5.2)

Statistic analyses

Mean values and standard deviation were performed. Alkaline phosphatase activity and Calcium quantification of different proteins between DPPSC and DPMSC were examined by a multifactorial variance analysis (ANOVA). If ANOVA detected any significance, a series of Student *t*-tests were performed to establish treatment effects. In same way, results of strength tests were analyzed by Student *t*-tests. In all analyses, a P-value of 0.05 was considered to represent a statistical significance.

7. RESULTS

Cellular morphology of DPPSC and DPMSC

The change in morphology was followed at day 7, day 15 and day 20 for each of the cell populations by using the JuLi Smart Fluorescence Cell Imager microscope, using 10x magnifying lens.

The initial morphology of DPPSC is characterized by round symmetry, narrow cytoplasm with large nucleus in proportion to its size that is initially lower than in mesenchymal cells. These features are maintained within stated 30% to 50% confluence in culture. (Fig 6.1A) DPMSC is characterized by flat cells and the cytoplasm is elongated and the nucleus is round, microscopically visible. The confluence do not have any effect on the growth, for which passes is performed when displaying 60% confluence in culture medium for mesenchymal stem cell. (Fig 6.1B)

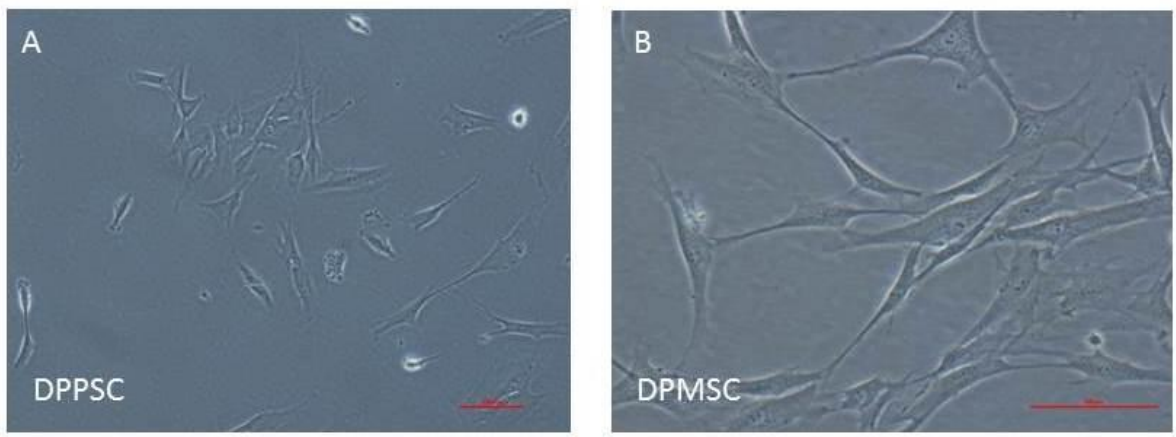


Fig 6.1: *Cellular morphology of DPPSC and DPMSC at P15*

1: FIbronectin (FN)

1-1: Alkaline phosphatase activity (ALP)

ALP of DPPSC in FN group was 97, 81, 118 for day 3, day 11 and day 20, respectively. ALP of DPMSC in FN group was 124, 98, 139 for day 3, day 11 and day 20, respectively.

ALP of DPPSC in control group (No treatment) was 118, 125, 112 for day 3, day 11 and day 20, respectively. ALP of DPMSC in control group was 171, 103, 132 for day 3, day 11 and day 20, respectively.

DPPSC seeded on 3D glass scaffold pretreatment with FN showed an increase in ALP activity until day 20. DPMSC seeded on 3D glass scaffold pretreatment with FN showed an increase in ALP activity until day 20. (Fig 6.3A)

1-2: Calcium quantification

Calcium quantification of DPPSC in FN group was 0.018 Ug/Ul, 0.030 Ug/Ul, and 0.026 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in FN group was 0.026 Ug/Ul, 0.038 Ug/Ul, and 0.036 Ug/Ul for day 3, day 11 and day 20, respectively.

Calcium quantification of DPPSC in control group was 0.031 Ug/Ul, 0.023 Ug/Ul, and 0.025 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in control group was 0.037 Ug/Ul, 0.031 Ug/Ul, and 0.027 Ug/Ul for day 3, day 11 and day 20, respectively.

On day 20, quantification of calcium of FN group showed a 4% increase in the calcium deposition in DPPSC and 33.3% in DPMSC, with respect to control. (Fig 6.3B)

1-3: RT-PCR

RT-PCR of DPPSC in FN group was 8.20, 12.94 and 13.63 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in FN group was 44.30, 0.46 and 25.72 for Col I, BMP2 and OCN, respectively.

RT-PCR of DPPSC in control group was 12.30, 6.20 and 1.40 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in control group was 4.20, 4.10 and 0.08 for Col I, BMP2 and OCN, respectively.

These results showed the high level expression of Col I and OCN in differentiated of DPMSC than DPPSC. The expression of BMP2 of DPMSC is lower than DPPSC. (Fig 6.2B)

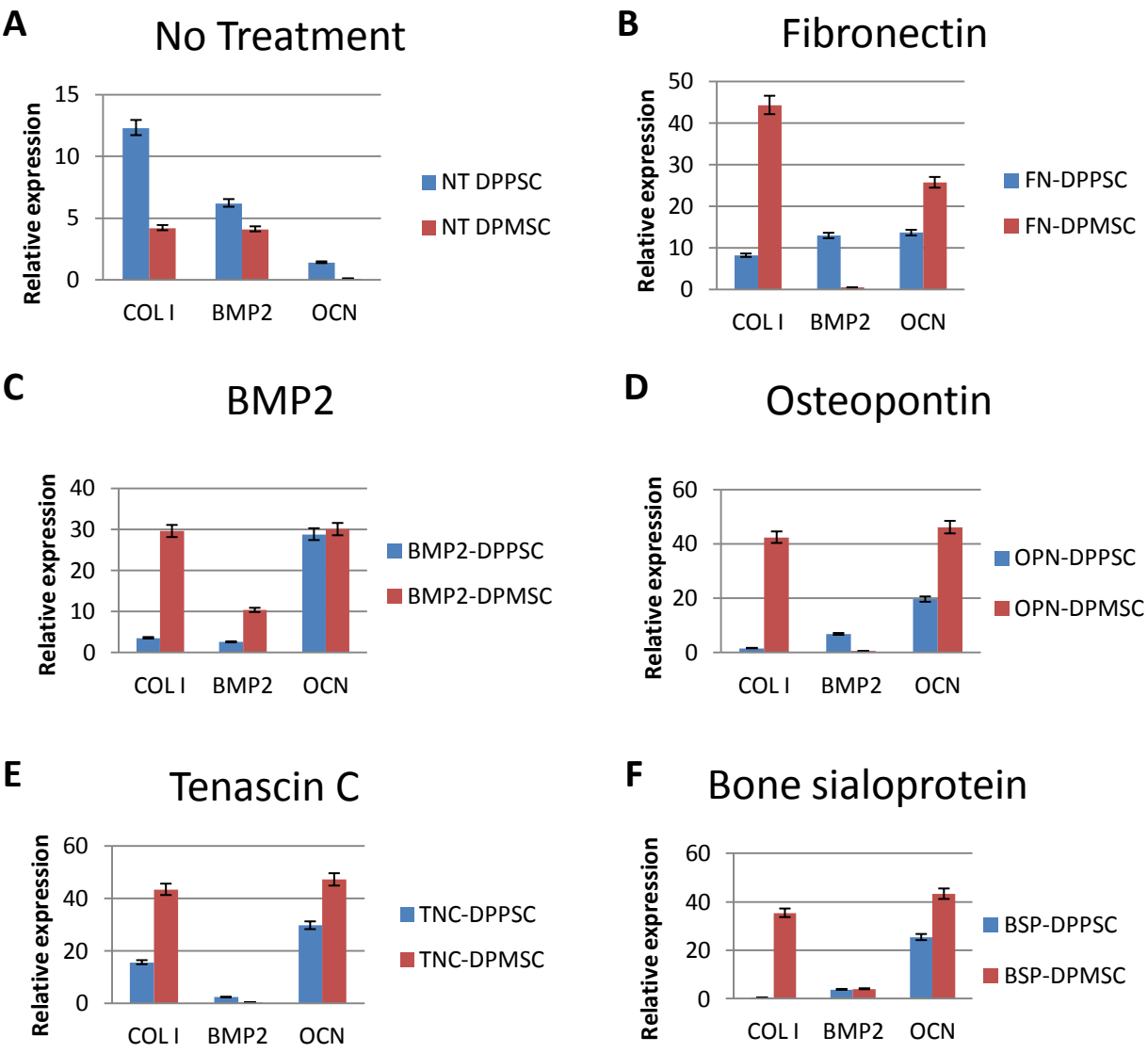


Fig 6.2: *The RT Real time - PCR results based on triplicate measurements (relative expression) for each sample. Values were normalized for GAPDH. RT-PCR of no treatment (A), fibronectin (B), BMP2 (C), osteopontin (D), tenascin C (E), bone sialoprotein (F).*

1-4: Strength test

Strength test of DPPSC and DPMSC in FN group was 7.4 MPa and 16.2 MPa, respectively. Strength test of DPPSC and DPMSC in control group was 3.1 MPa and 5.1 MPa, respectively. Strength test of 3D glass scaffold before cell culture was 1.8 MPa.

They presented higher fracture toughness in both scaffolds (DPMSC and DPPSC groups), respect to a control group. We found that DPMSC group has greater strength than DPPSC group. (Fig 6.3C)

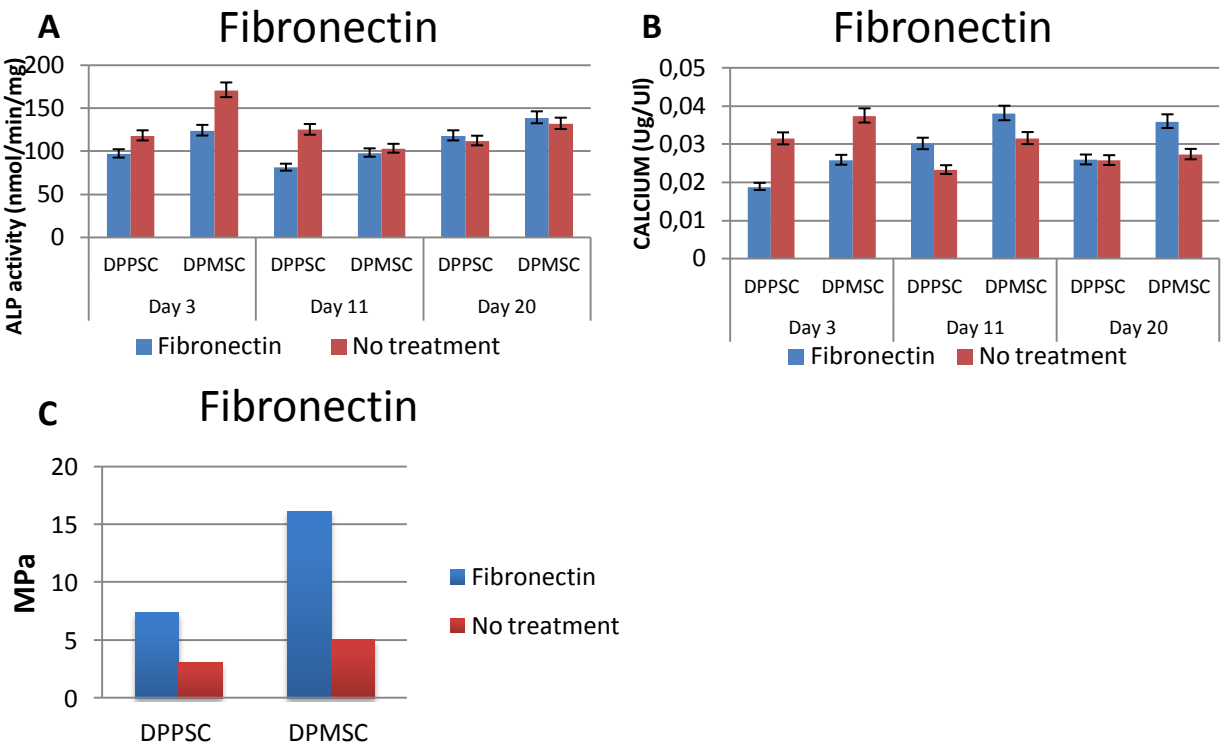


Fig 6.3: *ALP of fibronectin (A), Calcium quantification of fibronectin (B), Strength test of fibronectin (C)*

2: BMP2

2-1: Alkaline phosphatase activity (ALP)

ALP of DPPSC in BMP2 group was 115, 87, 134 for day 3, day 11 and day 20, respectively.

ALP of DPMSC in BMP2 group was 139, 101, 138 for day 3, day 11 and day 20, respectively.

ALP of DPPSC in control group was 118, 125, 112 for day 3, day 11 and day 20, respectively. ALP of DPMSC in control group was 171, 103, 132 for day 3, day 11 and day 20, respectively.

DPPSC seeded on 3D glass scaffold pretreatment with BMP2 showed an increase in ALP activity until day 20. DPMSC seeded on 3D glass scaffold pretreatment with BMP2 showed peak ALP activity at day 3. (Fig 6.4A)

2-2: Calcium quantification

Calcium quantification of DPPSC in BMP2 group was 0.039 Ug/Ul, 0.031 Ug/Ul, and 0.029 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in BMP2 group was 0.040 Ug/Ul, 0.048 Ug/Ul, and 0.037 Ug/Ul for day 3, day 11 and day 20, respectively.

Calcium quantification of DPPSC in control group was 0.031 Ug/Ul, 0.023 Ug/Ul, and 0.025 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in control group was 0.037 Ug/Ul, 0.031 Ug/Ul, and 0.027 Ug/Ul for day 3, day 11 and day 20, respectively.

On day 20, quantification of calcium of BMP2 group showed a 12% increase in the calcium deposition in DPPSC and 37% in DPMSC, with respect to control. (Fig 6.4B)

2-3: RT-PCR

RT-PCR of DPPSC in BMP2 group was 3.54, 2.55 and 28.75 for Col I, BMP2 and OCN,

respectively. RT-PCR of DPMSC in BMP2 group was 29.52, 10.32 and 29.98 for Col I, BMP2 and OCN, respectively.

RT-PCR of DPPSC in control group was 12.30, 6.20 and 1.40 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in control group was 4.20, 4.10 and 0.08 for Col I, BMP2 and OCN, respectively.

These results showed the level expression of Col I, BMP2 and OCN in differentiated of DPMSC higher than DPPSC. (Fig 6.2C)

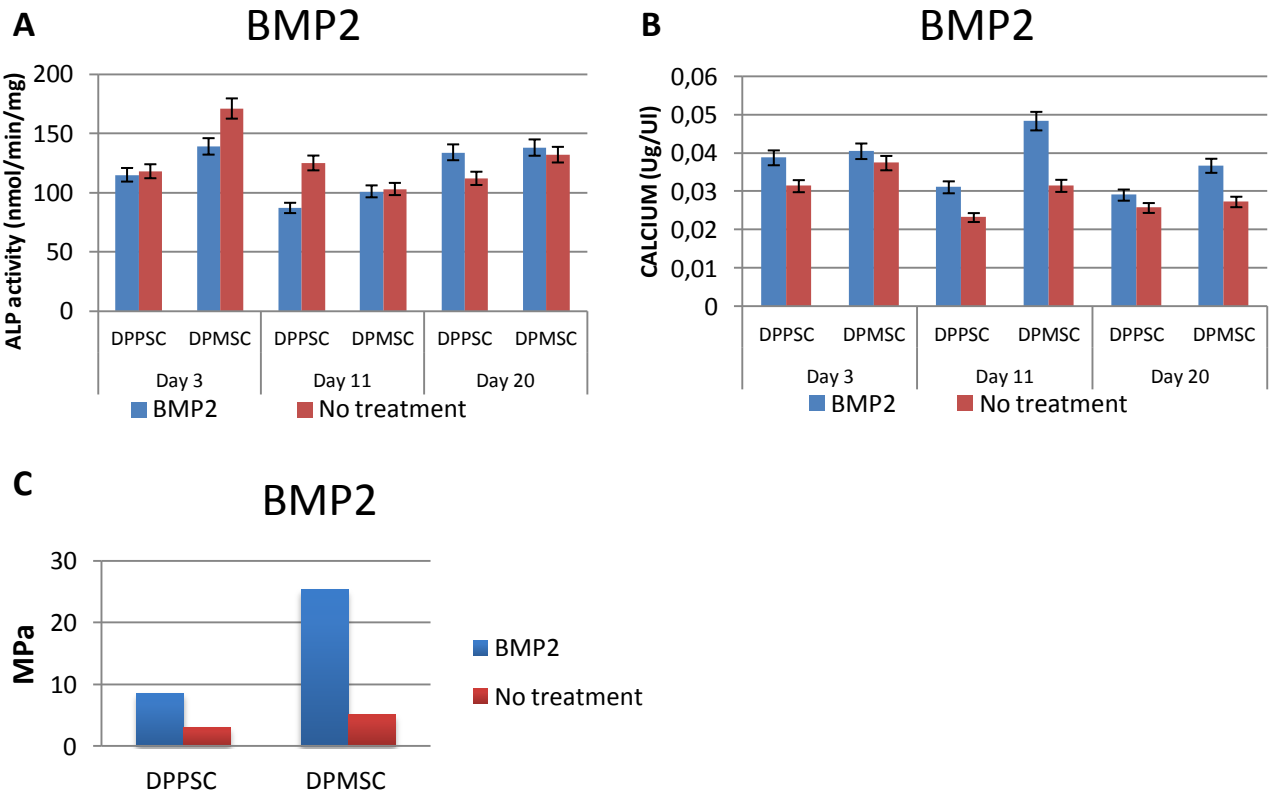


Fig 6.4 *ALP of BMP2 (A), Calcium quantification of BM P2 (B), Strength test of BMP2 (C)*

2-4: Strength test

Strength test of DPPSC and DPMSC in BMP2 group was 8.6 MPa and 25.4 MPa, respectively. Strength test of DPPSC and DPMSC in control group was 3.1 MPa and 5.1

MPa, respectively.

They presented higher fracture toughness in both scaffolds (DPMSC and DPPSC groups), respect to a control group. We found that DPMSC group has greater strength than DPPSC group. (Fig 6.4C)

3: Osteopontin

3-1: Alkaline phosphatase activity (ALP)

ALP of DPPSC in OPN group was 126, 95, 125 for day 3, day 11 and day 20, respectively. ALP of DPMSC in OPN group was 156, 99, 113 for day 3, day 11 and day 20, respectively.

ALP of DPPSC in control group was 118, 125, 112 for day 3, day 11 and day 20, respectively. ALP of DPMSC in control group was 171, 103, 132 for day 3, day 11 and day 20, respectively.

DPPSC seeded on 3D glass scaffold pretreatment with OPN showed peak ALP activity at day 3 and day 20. DPMSC seeded on 3D glass scaffold pretreatment with OPN showed peak ALP activity at day 3. (Fig 6.5A)

3-2: Calcium quantification

Calcium quantification of DPPSC in OPN group was 0.036 Ug/Ul, 0.038 Ug/Ul, and 0.048 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in OPN group was 0.041 Ug/Ul, 0.026 Ug/Ul, and 0.037 Ug/Ul for day 3, day 11 and day 20, respectively.

Calcium quantification of DPPSC in control group was 0.031 Ug/Ul, 0.023 Ug/Ul, and 0.025 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in control group was 0.037 Ug/Ul, 0.031 Ug/Ul, and 0.027 Ug/Ul for day 3, day 11 and day 20, respectively.

On day 20, quantification of calcium of OPN group showed an 88% increase in the calcium deposition in DPPSC and 37% in DPMSC, with respect to control. (Fig 6.5B)

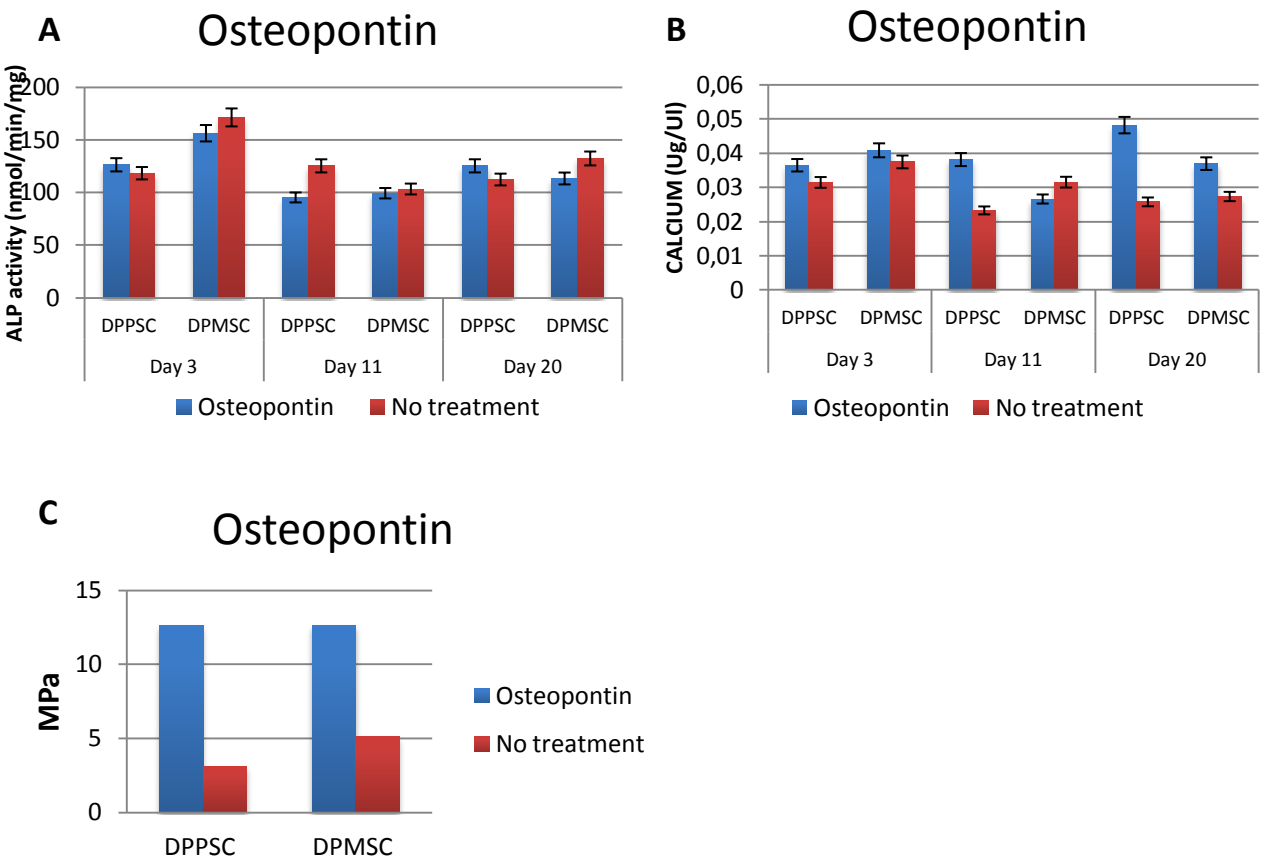


Fig 6.5 *ALP of Osteopontin (A), Calcium quantification of Osteopontin (B), Strength test of Osteopontin (C)*

3-3: RT-PCR

RT-PCR of DPPSC in OPN group was 1.57, 6.79 and 19.60 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in OPN group was 42.41, 0.50 and 46.11 for Col I, BMP2

and OCN, respectively.

RT-PCR of DPPSC in control group was 12.30, 6.20 and 1.40 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in control group was 4.20, 4.10 and 0.08 for Col I, BMP2 and OCN, respectively.

These results showed higher level expression of Col I and OCN in differentiated of DPMSC than DPPSC. The expression of BMP2 of DPMSC is lower than DPPSC. (Fig 6.2D)

3-4: Strength test

Strength test of DPPSC and DPMSC in OPN group was 12.6 MPa and 12.6 MPa, respectively. Strength test of DPPSC and DPMSC in control group was 3.1 MPa and 5.1

MPa, respectively.

They presented higher fracture toughness in both scaffolds (DPMSC and DPPSC groups), respect to a control group. We found that DPMSC group has similar strength with DPPSC group. (Fig 6.5C)

4: Tenascin C

4-1: Alkaline phosphatase activity (ALP)

ALP of DPPSC in TNC group was 127, 101, 139 for day 3, day 11 and day 20, respectively. ALP of DPMSC in TNC group was 177, 93, 104 for day 3, day 11 and day 20, respectively.

ALP of DPPSC in control group was 118, 125, 112 for day 3, day 11 and day 20, respectively. ALP of DPMSC in control group was 171, 103, 132 for day 3, day 11 and day 20, respectively.

DPPSC seeded on 3D glass scaffold pretreatment with TNC showed an increase in ALP activity until day 20. DPMSC seeded on 3D glass scaffold pretreatment with TNC showed peak ALP activity at day 3. (Fig 6.6A)

4-2: Calcium quantification

Calcium quantification of DPPSC in TNC group was 0.026 Ug/Ul, 0.060 Ug/Ul, and 0.025 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in TNC group was 0.036 Ug/Ul, 0.057 Ug/Ul, and 0.035 Ug/Ul for day 3, day 11 and day 20, respectively.

Calcium quantification of DPPSC in control group was 0.031 Ug/Ul, 0.023 Ug/Ul, and 0.025

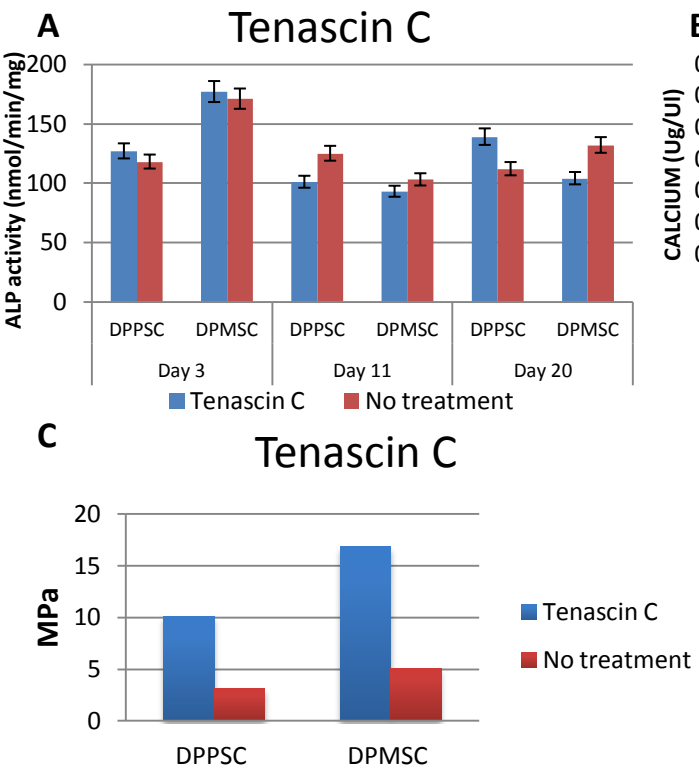
Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in control group was 0.037 Ug/Ul, 0.031 Ug/Ul, and 0.027 Ug/Ul for day 3, day 11 and day 20, respectively.

On day 20, quantification of calcium of TNC group showed a 0% increase in the calcium deposition in DPPSC and 29.6% in DPMSC, with respect to control. (Fig 6.6B)

4-3: RT-PCR

RT-PCR of DPPSC in TNC group was 15.58, 2.28 and 29.67 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in TNC group was 43.37, 0.32 and 47.17 for Col I, BMP2 and OCN, respectively.

RT-PCR of DPPSC in control group was 12.30, 6.20 and 1.40 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in control group was 4.20, 4.10 and 0.08 for Col I, BMP2 and OCN, respectively.



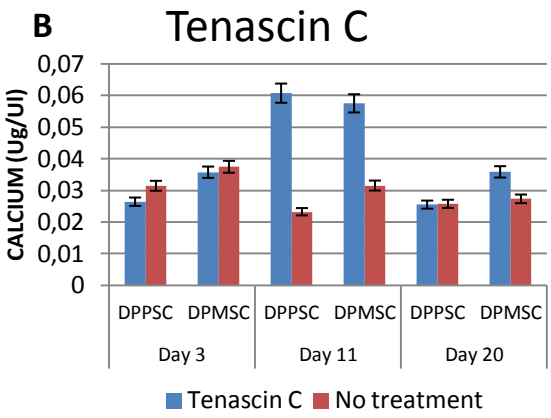


Fig 6.6 *ALP of Tenascin C (A), Calcium quantification of Tenascin C (B), Strength test of Tenascin C (C)*

These results showed higher level expression of Col I and OCN in differentiated of DPMSC than DPPSC. The expression of BMP2 of DPMSC is lower than DPPSC. (Fig 6.2E)

4-4: Strength test

Strength test of DPPSC and DPMSC in TNC group was 10.1 MPa and 16.8 MPa, respectively.

Strength test of DPPSC and DPMSC in control group was 3.1 MPa and 5.1 MPa, respectively.

They presented higher fracture toughness in both scaffolds (DPMSC and DPPSC groups), respect to a control group. We found that DPMSC group has greater strength than DPPSC group. (Fig 6.6C)

5: Bone sialoprotein

5-1: Alkaline phosphatase activity (ALP)

ALP of DPPSC in BSP group was 135, 102, 141 for day 3, day 11 and day 20, respectively. ALP of DPMSC in BSP group was 165, 93, 97 for day 3, day 11 and day 20, respectively.

ALP of DPPSC in control group was 118, 125, 112 for day 3, day 11 and day 20, respectively. ALP of DPMSC in control group was 171, 103, 132 for day 3, day 11 and day 20, respectively.

DPPSC seeded on 3D glass scaffold pretreatment with BSP showed an increase in ALP activity until day 20. DPMSC seeded on 3D glass scaffold pretreatment with BSP showed

peak ALP activity at day 3. (Fig 6.7A)

5-2: Calcium quantification

Calcium quantification of DPPSC in BSP group was 0.034 Ug/Ul, 0.053 Ug/Ul, and 0.035 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in BSP group was 0.034 Ug/Ul, 0.029 Ug/Ul, and 0.038 Ug/Ul for day 3, day 11 and day 20, respectively.

Calcium quantification of DPPSC in control group was 0.031 Ug/Ul, 0.023 Ug/Ul, and 0.025 Ug/Ul for day 3, day 11 and day 20, respectively. Calcium quantification of DPMSC in control group was 0.037 Ug/Ul, 0.031 Ug/Ul, and 0.027 Ug/Ul for day 3, day 11 and day 20, respectively.

On day 20, quantification of calcium of BSP group showed a 40% increase in the calcium deposition in DPPSC and 40.7% in DPMSC, with respect to control. (Fig 6.7B)

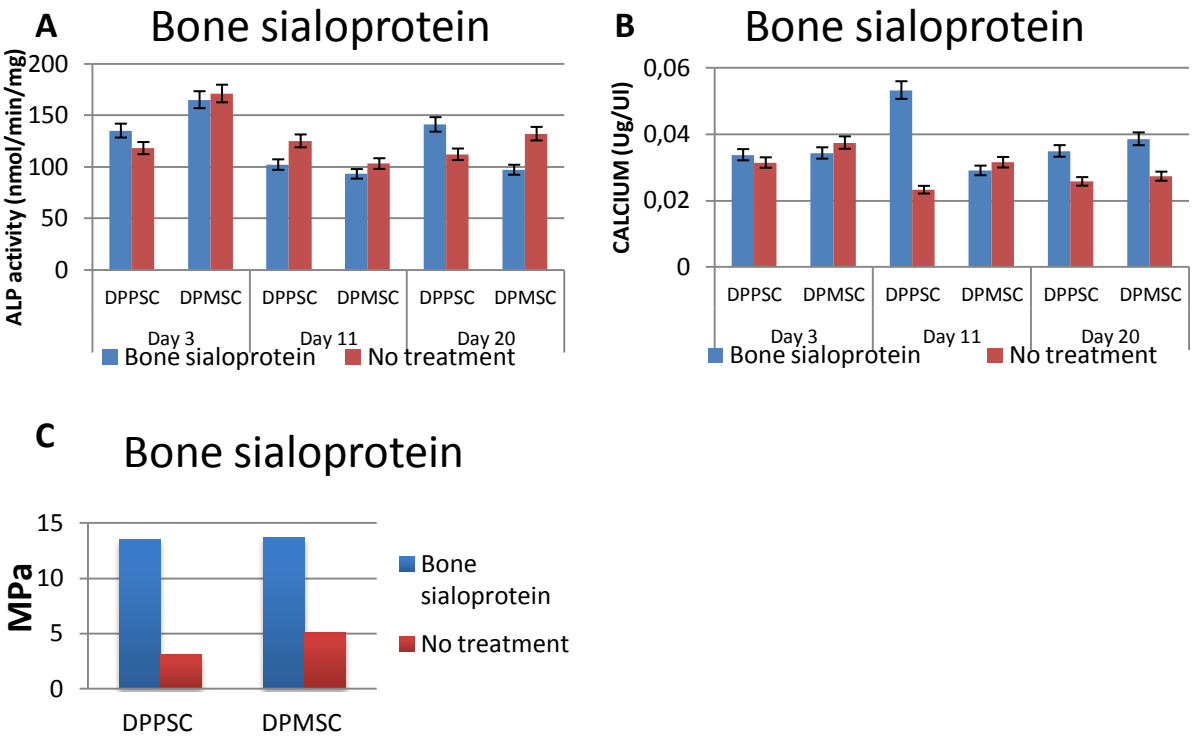


Fig 6.7 *ALP of Bone sialoprotein (A), Calcium quantification of Bone sialoprotein (B), Strength test of Bone sialoprotein (C)*

5-3: RT-PCR

RT-PCR of DPPSC in BSP group was 0.37, 3.77 and 25.39 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in BSP group was 35.41, 4.03 and 43.31 for Col I, BMP2 and OCN, respectively.

RT-PCR of DPPSC in control group was 12.30, 6.20 and 1.40 for Col I, BMP2 and OCN, respectively. RT-PCR of DPMSC in control group was 4.20, 4.10 and 0.08 for Col I, BMP2 and OCN, respectively.

These results showed higher level expression of bone markers (Col I, BMP2, OCN) in differentiated of DPMSC than DPPSC. DPMSC group show higher expression of bone marker than DPPSC group. (Fig 6.2F)

5-4: Strength test

Strength test of DPPSC and DPMSC in BSP group was 13.5 MPa and 13.7 MPa, respectively. Strength test of DPPSC and DPMSC in control group was 3.1 MPa and 5.1 MPa, respectively.

They presented higher fracture toughness in both scaffolds (DPMSC and DPPSC groups), respect to a control group. We found that DPMSC group has greater strength than DPPSC group. (Fig 6.7C)

Scanning electron microscope

We observed that osteoblast attachs and spreads on all specimens. SEM photographs of DPPSC+OPN, DPMSC+OPN and DPPSC+TNC show extensive matrix and cellular networking on 3D glass scaffold. (Fig 6.8A1-2, Fig 6.8B1-2, Fig 6.8C1-2) All specimens showed cortical structure like human osteoblast. (Red arrow, Fig 6.8A1, B1, C1, D1) We also observed the bundle of fibers with associated spherical structures, which is calcium phosphate nodule. (Fig 6.8A3-6, Fig 6.8B3-6, Fig 6.8C3-6) We observed calcium phosphate nodule (Blue arrow) in range size estimates varied from 1 to 2.5 µm. SEM photographs of DPMSC+TNC formed multilayers on the surface of 3d glass scaffold. (Fig 6.8D1-4) We observed calcium phosphate nodules aggregate at the surface and inside of the cell band. (Fig 6.8D5-8)

Statistic analysis

ALP activity

DPPSC seeded on 3D glass scaffold pretreatment with different proteins showed ALP activities significantly peak at day 20. ($P<0.0001$) ALP activity of DPPSC was 119.67±13.275 at day 3, 97.33±16.255 at day 11 and 128.22±21.109 at day 20. ALP activity of DPPSC was significantly different between day 3/11 and day 11/20. DPMSC

seeded on 3D glass scaffold pretreatment with different proteins showed ALP activities significantly peak at day 3. (P<0.0001) ALP activity of DPMSC was 155.33±20.457 at day 3, 97.72±7.411 at day 11 and 120.61±23.301 at day 20. ALP activity of DPMSC was significantly different among day 3, day 11 and day 20.When ALP activity on DPPSC and DPMSC were not significantly different. (Mean of ALP of DPPSC and DPMSC were 115.07±21.377 and 124.56±29.959, respectively)

Calcium quantification

Calcium quantification on DPPSC and DPMSC were not signifinatly different (mean of calcium level of DPPSC and DPMSC were 0.028±0.07 and 0.030±0.006). In DPPSC, there are significant difference between No tx/BMP2, No tx/OPN, No tx/BSP, FN/BMP2, FN/OPN, FN/BSP and OPN/TNC. DPPSC seeded on scaffold pretrement with OPN showed the highest calcium level. In DPMSC, there are significant difference between No tx/FN, No tx BMP2, FN/BMP2, BMP2/OPN, BMP2/ TNC and BMP2/BSP. DPMSC seeded on scaffold pretrement with BMP2 show the highest calcium level.

Strength test

When 12 cultured scaffolds and scaffold without culture were compared, significant difference was observed (P=0.036) and mean was 12.092±5.95. Strength of scaffolds cultured with DPMSC were significantly higher than DPPSC group (P=0.047; one tailed) Mean of DPPSC and DPMSC group were 9.2±3.7 and 14.96±6.6, respectively.

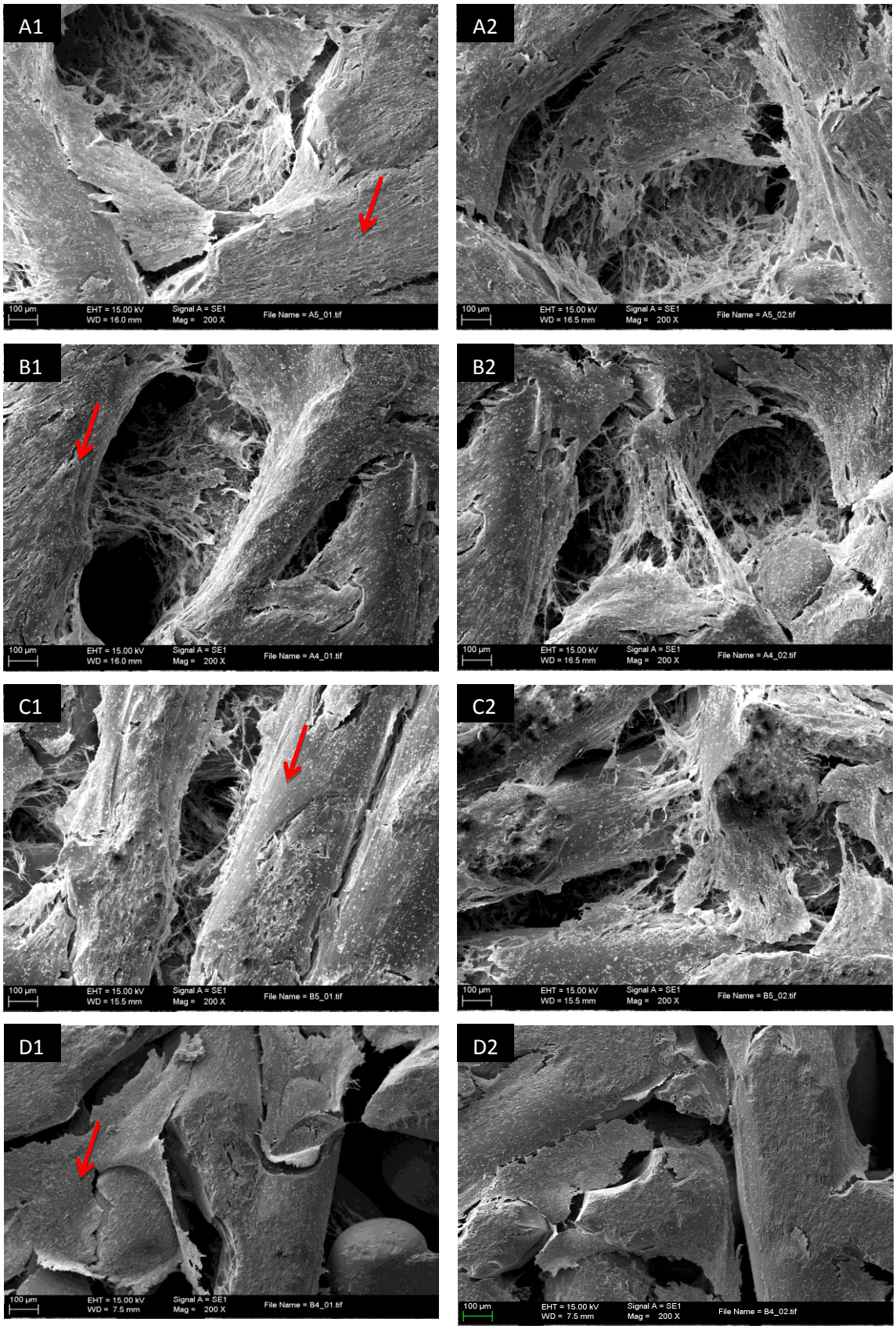


Fig: 6.8 SEM (200x) of DPPSC+OPN (A), SEM of DPMSC+OPN (B), SEM of DPPSC+TNC (C) and SEM of DPMSC+TNC (D)

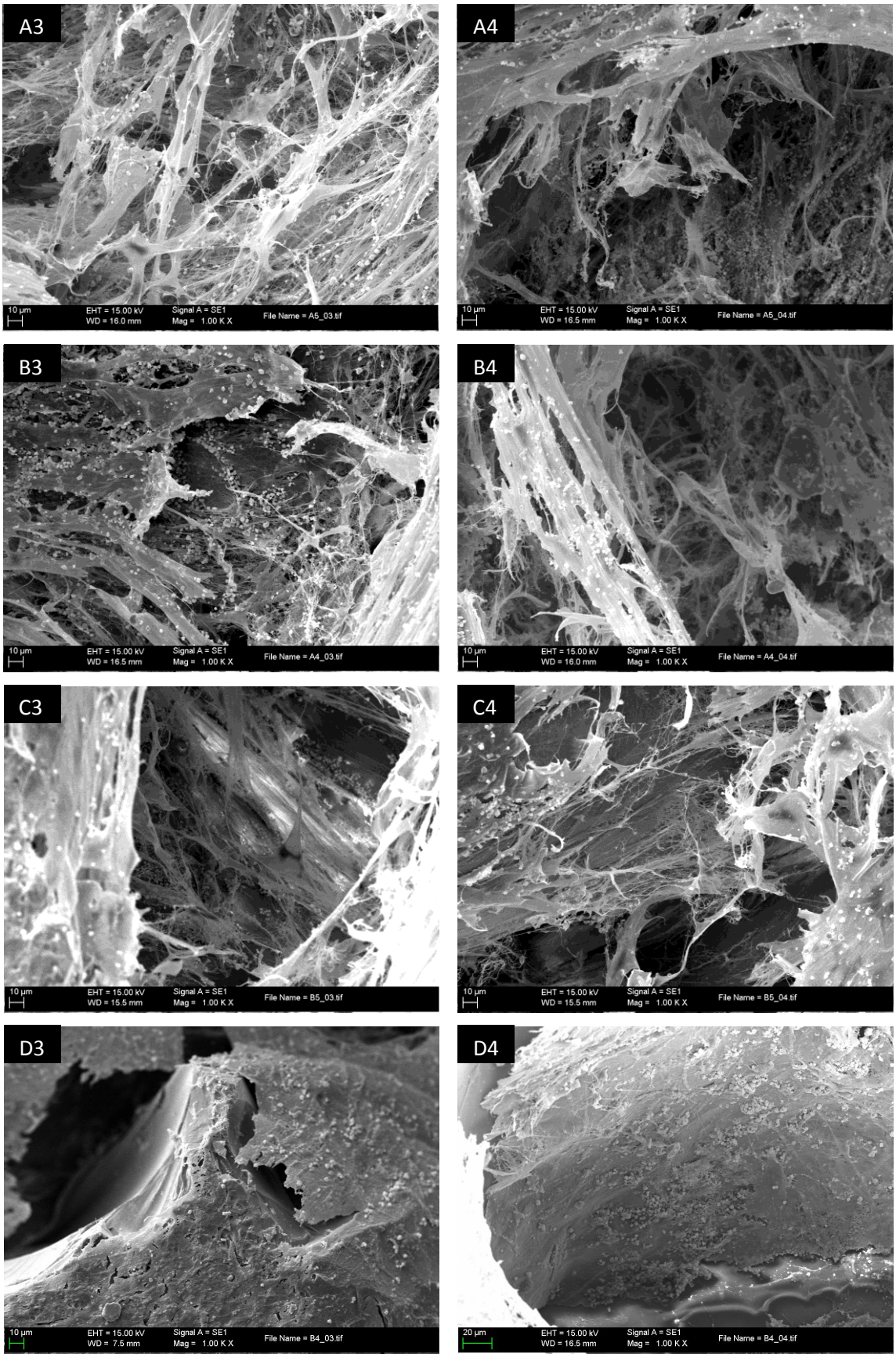


Fig: 6. 8 (Continue) SEM (1000x) of DPPSC+OPN (A), SEM of DPMSC+OPN (B), SEM of DPPSC+TNC (C) and SEM of DPMSC+TNC.

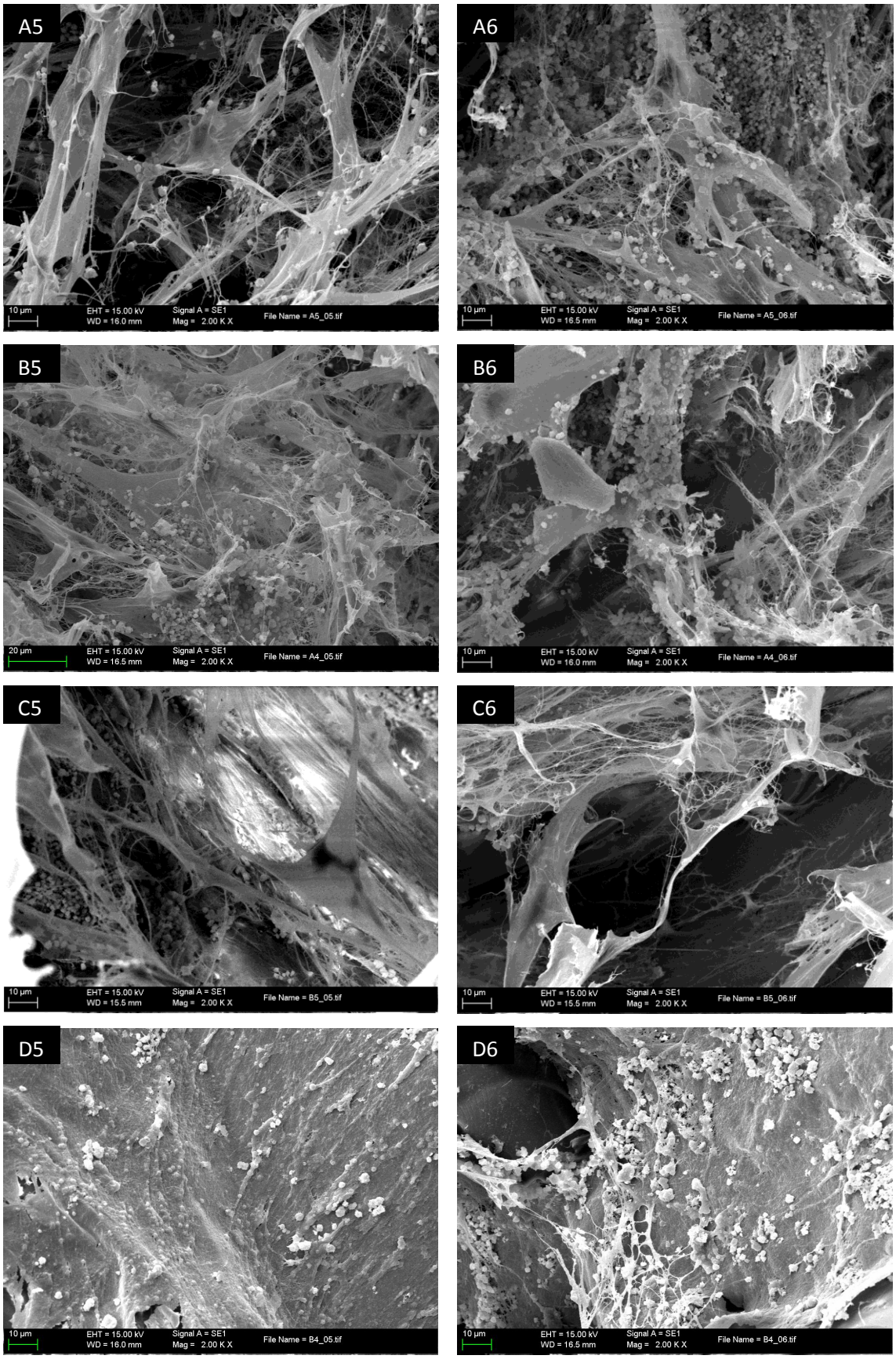


Fig: 6.8 (Continue) SEM (2000x) of DPPSC+OPN (A), SEM of DPMSC+OPN (B), SEM of DPPSC+TNC (C) and SEM of DPMSC+TNC (D)

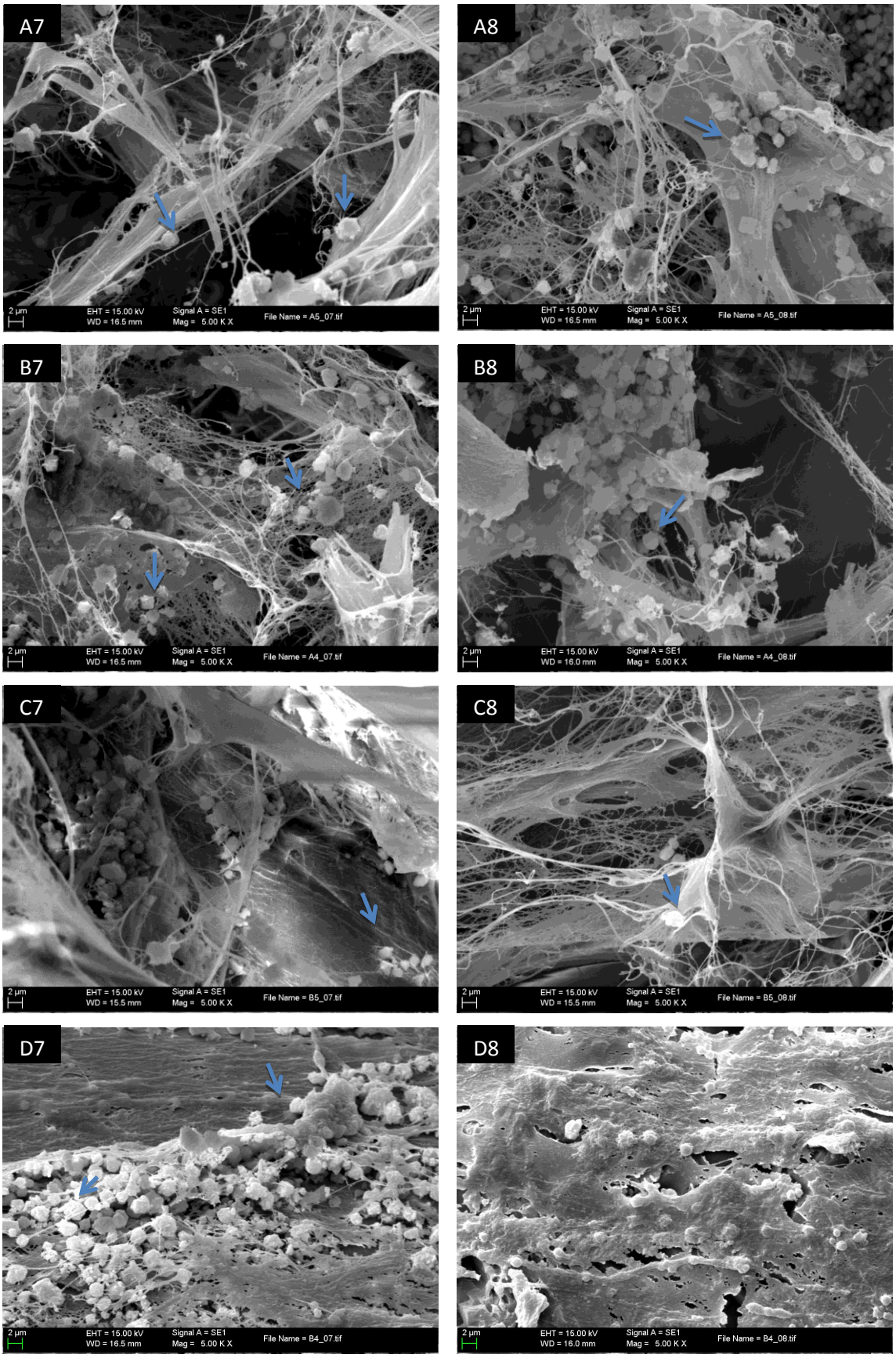


Fig: 6.8 (Continue) SEM (5000x) of DPPSC+OPN (A), SEM of DPMSC+OPN (B), SEM of DPPSC+TNC (C) and SEM of DPMSC+TNC (D)

8. DISCUSSION

Osteogenic capacity of DPPSC and DPMSC in 3D

In this study, and for completing our main objective, the different pieces were characterized (bio-coating, DPPSC and DPMSC) and joined them in a comparative study, through the evaluation of the osteogenic capacity of DPPSC, we also tried to find out what bio-coating and/or surface treatment could promote greater osseointegration. In addition, the development and protocol of this comparative study, the high capacity of DPPSC would be demonstrated, and thus, as the standardization of this study would help in future research.

The osteogenic capacity of DPPSC served in ability to adhesion, proliferation and bone differentiation, and for that reason, a number of specific indicators were used in order to prove that these characteristics were fulfilled in our comparative study.

The activity of ALP and calcium were considered to be good markers/indicators of osteoblast activity. Because for osteoblastic differentiation of DPPSC, DPPSC previously combine with surface or bio-coatings, had had adhere, proliferate and mature enough to differentiate. Furthermore, quantification of both indicators also helped us to know that surface or biocoating was being able to encourage greater osseointegration, the greater osteoblast activity and greater bone formation.

To understand the interpretation and the action of ALP and calcium in the medium, we must first refresh the biology of osteoblast. Thereof, beyond being synthesizers and releasing the ECM, the mineralization thanks to the production vesicles accumulator of Ca^{2+} and PO_4^{2-} , and prior stimulation enzyme of ALP and pyrophosphatase (accumulated in specific areas of the plasma membrane),which causing to release Ca^{2+} y PO_4^{2-} to concentrate on specific points of the matrix called the center of nucleation. So that, mineral salts (calcium phosphate, and HA) are precipitate more easily, and then continue

the calcification until being a proper tissue bone (osteoblast are now called osteocytes).

Alkaline phosphatase activity

Alkaline phosphatase activity (ALP) is used to assess early differentiation activity of osteoblastic cells.[219] After the onset of mineralization, ALP activity level will decrease before a mature mineralized matrix is formed.[220] In vitro study, ALP activity was significantly higher at 2 and 4 days on the bioactive glass with 46.1 mol % silica content (45S5 Bioglass) cultures human primary osteoblast-like cells. [221]

In our study, bone-specific alkaline phosphatase (ALP) activity was detected from supernatant at day 3, day 11 and day 20. DPPSC seeded on 3D glass scaffold pretreatment with fibronectin, bone morphogenic protein 2, osteopontin, tenascin c and bone sialoprotein exhibited an increase in ALP activity until day 20. ALP activity in DPPSC increases over time in culture. Similarly, ALP assay of DPMSC seeded on scaffold pretreatment with fibronectin showed similar pattern of expression with DPPSC group. On the contrary, DPMSC seeded on 3D glass scaffold pretreatment with bone morphogenic protein 2, osteopontin, tenascin c and bone sialoprotein exhibited peak ALP activity at day 3. These results are in agreement with that DPMSC+BMP2, DPMSC+OPN, DPMSC+TNC and DPMSC+BSP have early onset of osteoblast differentiation. DPPSC seeded on 3D glass scaffold pretreatment with different proteins showed ALP activities significantly peak at day 20, DPMSC showed significantly peak at day 3.

Calcium quantification

Calcium was estimated as a dependent variable factors, cell type, biomaterial surface, day treatment and time in which were quantified (day 3, day 11 and day 20). For this in vitro study, low levels in the supernatant were indicative of mineralization of the extracellular matrix. On day 20, quantification of calcium in DPPSC group showed a 4%, 12%, 88%, 0%

and 40% for FN, BMP2, OPN, TNC and BSP, respectively. Quantification of calcium in DPMSC group showed a 33.3%, 37%, 37%, 29.6% and 40.7% for FN, BMP2, OPN, TNC and BSP, respectively.

Cell type, interesting data found that cell would present initial differentiation and reflected the lowest values at day 20 in the DPPSC. DPMSC represented Fisher's medium, was corresponded a late stage of differentiation or mineralization, where the highest concentration of calcium is stored in the extracellular matrix. The DPPSC did not present at the initial differentiation and was at an early stage of differentiation in vitro, exhibited low levels in the supernatant and can understand that this population had a better adaptation to the bone differentiation process. Finally calcium quantification on DPPSC and DPMSC were not signifinatly different.

RT-PCR

In this study, osteoblast differentiation marker including Col I, BMP2 and OCN were investigated using real-time RT-PCR. BMP2 is a marker at early stages of osteogenesis, whilst, Col I and OCN are found at later stages of osteogenesis. [222] These results showed the high level expression of bone markers (Col I, BMP2, OCN) in differentiated of DPMSC than DPPSC. It may be caused by different cell amount of DPPSC were used in this experiment and the differentiation medium was also different. The distribution of osteoblast differentiation markers, COL I and OCN in DPMSC group, were high. It may be caused by DPMSC group are at the late stage of osteogenesis. DPPSC group has not reach the late stage of osteogenesis.

Strength test

They presented higher fracture toughness in both scaffolds (DPMSC and DPPSC groups), respect to a control group. We observed that DPMSC group was significantly greater

strength than DPPSC group. It could be explained that the size of cell of DPPSC is much smaller than DPMSC and the root of DPPSC migrated into center of scaffold, instead of, many cells of DPMSC were on superficial. [50] We found the lowest strength in DPPSC+FN group was 7.4 MPa and the highest strength in DPMSC+BMP2 was 25.4 MPa. The yield strength of human bone is in range of 0.6 to 17.5 MPa.

Scanning electron microscope

All specimens showed to adhere and proliferate well on 3D glass scaffold. We observed that cells distinctly seed inside scaffold and also deposited mineralized matrix. Interestingly, on the 3D glass scaffold pretreatment with tenascin c, DPMSC has greater proliferation. This observation is supported by the finding that tenascin c actively stimulate osteogenic differentiation of DPMSC and has good adhere to scaffold. However, DPMSC seeded on scaffold pretreatment with OPN has denseness of cellular network similar with DPPSC+OPN and DPPSC+TNC. Strength test of this study has also proved that DPMSC+TNC has the highest strength when comparing with DPMSC+OPN, DPPSC+TNC and DPPSC+OPN. Mineralized nodule formation is a phenotypic marker for the last stage of mature osteoblast.[219]

Among 5 proteins, we can conclude that DPPSC seeded on 3d scaffold coated with OPN has the highest osteoinduction. DPMSC seeded on 3d scaffold coated with BMP2 has highest osteoinduction

This finding could conclude that DPMSC is more suitable for bone regeneration than DPPSC. However, for dental implant placement or trauma, we prefer trabecular bone with great blood supply, thus, bone regeneration from DPPSC may get a better result.

9. CONCLUSION

1. DPMSC group is at the late stage of osteogenesis before DPPSC group.
2. Pre-treatment surface of scaffold with FN, DPPSC showed an increase in ALP activity until day 20. DPMSC also showed an increase in ALP activity until day 20. Quantification of calcium showed a 4% and 33.3% increase in DPPSC and DPMSC, respectively. DPMSC group has greater strength with DPPSC group. The result of RT-PCR exhibited that DPMSC group is at the late stage of osteogenesis before DPPSC group.
3. Pre-treatment surface of scaffold with BMP2, DPPSC showed an increase in ALP activity until day 20. DPMSC showed peak ALP activity at day 3. Quantification of calcium showed a 12% and 37% increase in DPPSC and DPMSC, respectively. DPMSC group has greater strength than DPPSC group. The result of RT-PCR exhibited that DPMSC group is at the late stage of osteogenesis before DPPSC group.
4. Pre-treatment surface of scaffold with OPN, DPPSC showed peak ALP activity at day 3 and day 20. DPMSC showed peak ALP activity at day 3. Quantification of calcium showed a 88% and 37% increase in DPPSC and DPMSC, respectively. DPMSC group has similar strength with DPPSC group. The result of RT-PCR exhibited that DPMSC group is at the late stage of osteogenesis before DPPSC group. The SEM shows DPPSC+OPN and DPMSC+OPN look similar.
5. Pre-treatment surface of scaffold with TNC, DPPSC showed an increase in ALP activity until day 20. DPMSC showed peak ALP activity at day 3. Quantification of calcium showed a 0% and 29.6% increase in DPPSC and DPMSC, respectively. DPMSC group has similar strength with DPPSC group. The result of RT-PCR exhibited that DPMSC

group is at the late stage of osteogenesis before DPPSC group. The SEM shows DPPSC+TNC has denseness of cellular network less than DPMSC+TNC.

6. Pre-treatment surface of scaffold with BSP, DPPSC showed an increase in ALP activity until day 20. DPMSC showed peak ALP activity at day 3. Quantification of calcium showed a 40% and 40.7% increase in DPPSC and DPMSC, respectively. DPMSC group has similar strength with DPPSC group. The result of RT-PCR exhibited that DPMSC group is at the late stage of osteogenesis before DPPSC group.

10. PROPOSAL FOR FUTURE RESEARCH

Further studies are necessary to assess the outcome of surface modification of biomaterials and titanium with these proteins, which could lead to selective therapeutic strategies in the field of dental implant and orthopaedic surgery. The combination among these proteins for getting maximum osteoblast growth is also interesting. Understanding the molecular mechanisms of cell interactions and their regulation by surrounding matrix cues has important implications for biotechnology (production of cells and cell products) and medicine (tissue engineering, prosthetic implants, cancer and developmental biology). It will provide the basis for fabrication of scaffolds bearing biomimetics and/or therapeutics to facilitate tight control of cell phenotype in a target tissue.

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12. RESUMEN

La ingeniería de tejido óseo es un objetivo terapéutico en el campo de la cirugía de implante dental y ortopédica. Por lo tanto, es esencial encontrar un microambiente que mejore el crecimiento y la diferenciación ósea, tanto a partir de células madre mesenquimales (MSC) como de los derivados de pulpa dentaria (DPSC). El objetivo de esta investigación es determinar la relación entre las proteínas de la fibronectina (FN), la proteína morfogenética ósea (BMP-2), la osteopontina (OPN), la tenascina (TN) y la sialoproteína ósea (BSP), así como determinar su capacidad para recubrir diferentes tipos de biomateriales y superficies para mejorar la diferenciación de osteoblastos. Para evaluar la capacidad osteogénica de las células madre pluripotentes de la pulpa dental (DPPSC) y de las células madre mesenquimales de la pulpa dental (DPMSC), se cultivaron estos tipos de células en bio-capas de 5 clases diferentes de proteínas durante 20 días. Se analizó la cuantificación de calcio (Ca^{2+}), la actividad de fosfatasa alcalina (ALP) y la reacción en cadena de la polimerasa cuantitativa con transcripción inversa (RT-PCR) de marcadores óseos como Col I, BMP-2 y OCN. Se han tomado imágenes de microscopía electrónica (SEM) correspondientes a los grupos OPN y TNC. También se ha realizado la medición de la resistencia a la fractura de los andamios de todos los grupos. El resultado obtenido en esta tesis confirma que las DPPSC, cultivadas en un andamio cubierto con OPN, presentan una mayor osteoinducción que el resto de las muestras. También demuestra que las DPMSC, cultivadas en un andamio cubierto con BMP-2, presentan una osteoinducción todavía mayor. De este modo, sugerimos que las DPMSC son más adecuadas para la regeneración ósea que las DPPSC. Sin embargo, para la colocación de implantes dentales o para tratar casos de trauma, es preferible emplear hueso trabecular con gran suministro de sangre. En definitiva, la regeneración ósea de las DPPSC puede obtener un mejor resultado. Las DPPSC se han convertido en una nueva estrategia para

regenerar hueso. Nos estamos acercando a proporcionar la regeneración ósea mediante las DPPSC.

Introducción

El aumento de hueso se lleva a cabo generalmente utilizando hueso autógeno. Sin embargo, el hueso autógeno no es capaz de cumplir con todos los requisitos para la regeneración ósea por dos motivos principales: las molestias y el dolor en la zona donante del injerto óseo autógeno son inevitables y las zonas donantes son cantidades limitadas.

Las células madre son células no especializadas. Se caracterizan por presentar dos propiedades únicas en una misma célula: su alta actividad de autorrenovación y su potencial de diferenciación multilinaje. Estas propiedades las convierte en una fuente ideal para la terapia celular y la medicina regenerativa. La naturaleza de la superficie sobre la que se cultivan las células desempeña un papel importante en su capacidad para unir, proliferar, migrar, así como para la función que van a desarrollar. Habitualmente se utilizan componentes de la matriz extracelular (ECM) en la capa de vidrio o en superficies de plástico para mejorar la unión de las células *in vitro.*

La FN promueve la adhesión celular y la migración. Esta proteína mejoró la calcificación vascular mediante la promoción de la diferenciación osteoblástica de las células del músculo liso vascular a partir de la señal obtenida a través de la vía ERK. Se sabe que la FN, como una glicoproteína adhesiva, y la osteonectina, como una proteína de contra-adhesivo, están implicadas en las primeras etapas de la osteogénesis. La capa de material compuesta de fosfato de calcio y FN formada sobre la hidroxiapatita es útil para la mejora de la diferenciación osteogénica y la difusión de las células madre mesenquimales humanas (hMSC) *in vitro.*

La BMP-2 está fuertemente implicada en la inducción de la diferenciación de los osteoblastos a partir de precursores de células mesenquimales, así como en la mejora de la producción de matriz ósea por células osteoblásticas. El estudio de tres muestras de células óseas diferentes mostró la posibilidad de que el receptor de BMP-IB podría ser una diana terapéutica para la mejora de la regeneración ósea *in vivo.* Las células madre transfectadas con genes no autólogos BMP-2 son de utilidad potencial para la mejora de la reparación del hueso y la regeneración ósea *in vivo.*

Inicialmente la OPN se aisló de la corteza del hueso bovino. La OPN tiene un rol importante en los efectos de las inducidas por descarga alteraciones de la diferenciación de médula ósea en los osteoblastos y osteoclastos. La respuesta humana a principios de la endotoxemia sistémica aumenta los niveles de OPN y modifica los biomarcadores óseos, lo que indica una disminución en la actividad lítica de los osteoclastos, acompañada de un aumento en la actividad de los osteoblastos inmaduros.

La TN es una proteína de la ECM que segrega diferentes células del tejido conectivo. La tenascina C (TNC) parece influir en la función de otras proteínas de adhesión, tales como la adhesina y los syndecans, pero también presenta interacciones repulsivas (antiadhesivas). En el hueso maduro, la TNC se observa en el espacio pericelular que rodea a algunos osteocitos y al cartílago articular.

La BSP es una de las principales fosfoproteínas glicosiladas no colágenas de la ECM en el hueso. Es una proteína específica de tejido mineralizado que se expresa en osteoblastos diferenciados y que parece funcionar en la mineralización inicial del hueso. La BSP puede inhibir la proliferación de células madre de médula ósea humana y mejorar su diferenciación osteogénica y su mineralización. En la célula osteoblástica MC3T3

E1/C4, superficies recubiertas con oligomerizado de OPN y BSP, promueve la adhesión celular mejor que las superficies recubiertas con la forma monomérica de las proteínas.

Curiosamente, qué tipos de proteínas son capaces de capa con andamios para mejorar la unión de osteoblastos. El objetivo de este estudio es comparar la capacidad de 5 proteínas: FN, BSP, OPN, TN y BSP, y la diferenciación de los osteoblastos.

Material y método

Durante 20 días se cultivó la misma cantidad de DPPSC y DPMSC sobre los diferentes biorrecubrimientos. Para ello se han usado 5 tipos de proteínas: FN, BSP-2, OPN, TN y BSP, junto con un medio de diferenciación ósea. A lo largo del estudio, los primeros indicadores utilizados, la cuantificación de ALP y Ca^{2+} en el medio (marcadores de la capacidad osteogénica), sirvieron para demostrar que las DPPSC se diferenciaban de los osteoblastos. Una vez llegado el día 20, el SEM sirvió para poder indicar la cantidad y la calidad del tejido óseo que se había generado en la OPN y la TNC (observación de la capacidad osteogénica). La cuantificación e identificación de marcadores específicos del ácido ribonucleico (ARN), OCN, BMP-2 y COL I (expresión genética de la capacidad osteogénica) sirvió para poder indicar que en realidad esos tejidos eran genéticamente tejido óseo. Después de eso, se realizó la prueba de resistencia de todos los andamios (fig. 5.1, página 52).

Comité de Ética

El estudio se realizó en conformidad con las normas del Comité de Ética de la Universidad Internacional de Cataluña.

Selección de pacientes

Se seleccionaron terceros molares humanos sanos, extraídos por razones de ortodoncia y profilácticos. Se llevó a cabo un procedimiento simple de extracción para evitar dañar los dientes.

Células primarias obtenidas a partir de muestras de molares humanos

Inmediatamente después de la extracción, se lavó un tercer molar con una gasa empapada en etanol al 70 %, seguido de otro lavado con agua destilada estéril. A continuación se extirpó la pulpa. Después de que el tejido de la pulpa se digiriese en 3mg/ml de colagenasa tipo I (Sigma) durante 60 minutos a 37 C. El tejido de la pulpa fue posteriormente objeto de la separación celular mecánica por centrifugación durante 10 minutos a 1800 rpm. Se lavaron dos veces las células con una solución de tampón fosfato salino (PBS) y se centrifugaron durante 10 minutos a 1800 rpm a temperatura ambiente. Después se contó el número de células.

Cultivo de células

Las células se cultivaron en un medio DPPSC, suplementado con un 60 % de *Eagle Modificado de Dulbecco* (DMEM, Sigma) bajo en glucosa y un 40 % de MCDB-201 (Sigma), suplementado con una combinación de insulina-transferrina-selenio (ITS,)Sigma), una dosis de ácido linoleico y albúmina de suero bovino (LA-BSA,)Sigma), 10^{-9} M de dexametasona (Sigma), 10^{-4} M de ácido ascórbico 2-fosfato (Sigma), 100 unidades de penicilina, 1000 unidades de estreptomicina (PAA), 2 % de suero fetal bovino (Sigma), 10 ng/ml de hPDGFBB (R&D Systems), 10 ng/ml de EGF (R&D Systems), 1000 uds./ml de hLIF (CHEMICON), un suplemento SITE (Sigma), concentrado de lípidos químicamente definidos (Gibco), 0,8 mg/ml de BSA (Sigma) y 55 mM de β-mercaptoetanol (β-ME, Sigma). Las suspensiones celulares se sembraron en un frasco de cultivo de 150 cm^2 de

células recubiertas con FN humana (100 ng/ml). Se incubaron en una atmósfera humidificada de 5 % de CO_2 y 95 % de aire a 37 C. Durante 3 semanas de cultivo primario, el medio de cultivo se cambió de 2 a 3 veces por semana. Las células se pasaron en un 4:1 cuando los cultivos celulares alcanzaron 80-100 células/cm^2 (60 % de confluencia).

Para el aislamiento de las DPMSC, las mismas muestras de las células se cultivaron en un medio de cultivo que contiene DMEM medio (Biochrom), 2 ng/ml de factor de crecimiento de fibroblastos básico ml (bFGF) y 10 % de suero fetal bovino (FBS, Hyclone). Las suspensiones celulares se cultivaron en placas a una densidad celular de 3 x 10^6 células/cm^2. El medio se reemplazó cada 3 días hasta que alcanzó aproximadamente el 90 % de confluencia y luego se desagregó mediante la adición de PBS (Biochrom) y 0,05 % de tripsina, que contiene ácido etilendiaminotetraacético (EDTA, Biochrom). El siguiente paso fue cultivar 4000 células/cm^2 e incubar a 37 °C y 5 % de CO_2.

Cultura 3D de DPPSCs y DPMSCs

Se han utilizado andamios 3D de vidrio (Orla). Todos los andamios utilizados los trató la empresa Orla con las 5 proteínas. La empresa no dio a conocer (las concentraciones de las proteínas ni la técnica de aplicación. Se cultivaron las DPPSC y DPMSC (2 x 10^6 células/cm^2) sobre los diferentes andamios en placas de 24 pocillos con medio de diferenciación ósea. El medio se cambió cada 2 o 3 días durante 20 días. Se han utilizado andamios sin tratamiento como controles y. se ha realizado la diferenciación de cada muestra por triplicado.

Actividad de la ALP

Durante la diferenciación de osteoblastos 3D de las DPPSC y DPMSC, se cuantificó la actividad de la ALP los días 3, 11 y 20 a través del fluorómetro. Para ello se utilizó un kit

de detección de ALP (Sigma-Aldrich, MO, EE. UU.), de acuerdo con las instrucciones del fabricante.

Cuantificación de Ca^{2+}

Durante la diferenciación de osteoblastos 3D de las DPPSC y DPMSC, se cuantificó el Ca^{2+} los días 3, 11 y 20. Para el análisis de la acumulación de Ca^{2+} se emplearon soluciones de lisis, que contienen Ca^{2+} en el kit de ensayo colorimétrico (Biovision, CA, EE. UU.),de acuerdo con las instrucciones del fabricante.

Aislamiento de ARN y RT-PCR

El ARN total se aisló de las DPPSC y las DPMSC utilizando Trizol (Invitrogen). El ARN total (2 μg) se trató con ADNasa I (Invitrogen) y se transcribió de forma inversa con M-MLV (Invitrogen), de acuerdo con el protocolo del fabricante. La eficacia de ADNc se analizó en diferentes concentraciones (1, 0,1, 0,01, 0,001, 0,0001 dilución) para todos los *primers*. Se utilizó SYBR Green Supermix (Bio-Rad Laboratories, Inc.) para analizar 50 μg de las muestras de cDNA mediante RT-PCR.

Primer usado para la amplificación

Símbolo genético	***Primer* directo**	***Primer* reverso**
COL I	TGCTGGCAAGAATGGCGATC	CTGTCTCAGCCTTGTCACCAC
BMP-2	GCGGAAACGCCTTAAGTCCA	GTGGAGTTCAGATGATCAGC
OCN	CTCACACTCCTCGCCCTATT	GCTCCCAGCCATTGATACAG

Análisis morfológico

Se cultivaron las DPPSC y las DPMSC durante 20 días. Se fijaron las muestras (DPPSC + OPN, DPMSC + OPN, DPPSC + TNC y DPMSC + TNC) en el 1,5 % de glutaraldehído y luego se evaluó la morfología de la superficie con un microscopio electrónico de barrido (SEM, Facultad de Medicina, Universidad de Barcelona).

Prueba de la fuerza

Después de 20 días, se probó la fuerza de los andamios de las muestras. Para ello se usó Ultradent testing jig (Ultradent Product, Inc., UT, EE. UU.). La resistencia al cizallamiento se midió con una máquina de ensayo universal (modelo 6022, Instron Co., Canton, MA, EE. UU.) (fig. 5.2, página 57).

Análisis estadísticos

Se midieron los valores medios y la desviación estándar. La actividad de ALP y de Ca^{2+} y la cuantificación de proteínas diferentes entre las DPPSC y las DPMSC se analizaron mediante un análisis de varianza multifactorial (ANOVA). En los casos en los que ANOVA detectó significación, se realizaron una serie de pruebas de la *t* de Student para determinar los efectos del tratamiento. Los resultados de las pruebas de resistencia se obtuvieron a partir de los análisis realizados por las pruebas de la *t* de Student. En todos los análisis, se consideró un valor P de 0,05 para representar una significación estadística.

Resultados

Morfología celular de las DPPSC y las DPMSC

Los cambios en la morfología se estudiaron los días, 7, 15 y 20 para cada una de las poblaciones celulares a través del microscopio JuLi Smart Fluorescence Cell Imager, con una lente de 10 aumentos. La morfología inicial de las DPPSC se caracteriza por una simetría redonda y, un citoplasma estrecho con un núcleo grande en proporción a su tamaño y que, en principio, es inferior al observado en las DPMSC. Estas características se mantienen si se respeta del 30 al 50 % de confluencia en el cultivo (fig 6.1A, página 59). Las DPMSC se caracterizan morfológicamente por ser células planas con un citoplasma alargado y un núcleo redondo y visible al microscopio. La confluencia no afecta el

crecimiento, para lo cual el pase se realiza cuando presentan un 60 % de confluencia en cultivo con medio mesenquimal (fig. 6.1B, página 59).

Actividad de la ALP

Las DPPSC se sembraron en el pretratamiento con andamios 3D de vidrio con FN, BMP-2, TNC y BSP. Mostraron un aumento en la actividad de la ALP hasta el día 20, excepto el grupo OPN que mostró un aumento en la actividad de la ALP hasta el día 3 y el día 20. Las DPMSC se sembraron en el pretratamiento con andamios 3D de vidrio con BMP-2, OPN, TNC y BSP. Mostraron un aumento en la actividad de la ALP hasta el día 3, excepto el grupo FN que mostró un aumento en la actividad de la ALP hasta el día 20 (fig 6.3A, página 62; Fig 6.4A, página 64; fig 6.5A, página 66; fig. 6.6A, página 68; fig 6.7A, página 70).

Cuantificación de Ca^{2+}

El día 20 el grupo DPPSC la cuantificación de calcio (con respecto al control) del grupo FN, BMP2, OPN, TNC y BSP mostró un aumento del 4 %, 12 %, 88 %, 0 % y 40 % en el depósito de Ca^{2+}, respectivamente. Grupo DPMSC en el día 20, la cuantificación de Ca^{2}del grupo FN mostró un aumento del 33,3 %, 37 %, 37 %, 29,6 % y 40,7 % en el depósito de Ca^{2}, respectivamente. (fig 6.3B, página 62; fig 6.4B, página 64; fig. 6.5B, página 66; fig. 6.6B, página 68; fig. 6.7B, página 70).

RT-PCR

El nivel de expresión de Col I y OCN de las DPMSC es mayor que el de las DPPSC y la expresión de BMP-2 de las DPMSC es menor que las DPPSC en el grupo FN, OPN y TNC. El nivel de expresión de Col I, BMP-2 y OCN de las DPMSC es mayor que el de las DPPSC en el grupo No tx, BMP-2 y BSP (fig. 6.2, página 61).

Prueba de fuerza en el andamio

El resultado de la prueba de fuerza en los andamios 3D de vidrio antes del cultivo de células fue de 1,8 MPa. En el caso de la prueba de fuerza de las DPPSC en el grupo No tx, FN, BMP-2, OPN, TNC y BSP los resultados fueron de 3,1 MPa, 7,4 MPa, 8,6 MPa, 12,6 MPa, 10,1 MPa y 13,5 MPa, respectivamente. Los resultados de la prueba de fuerza de las DPMSC en el grupo No tx, FN, BMP2, OPN, TNC y BSP fueron de 5,1 MPa, 16,2 MPa, 25,4 MPa, 12,6 MPa, 16,8 MPa y 13,7 MPa, respectivamente (fig. 6.3C, página 62; fig. 6.4C, página 64; fig. 6.5C, página 66; fig. 6.6C, página 68; fig. 6.7C, página 70).

Microscopio electrónico de barrido

Hemos observado que los osteoblastos se adjuntaron y se extendieron en todas las muestras. Las fotografías SEM de DPPSC + OPN, DPMSC + OPN y DPPSC + TNC presentaron un matriz extenso y redes celulares en los andamios 3D de vidrio (fig. 6.8A1-2, fig. 6.8B1-2 y fig. 6.8C1-2, página 72). Todas las muestras mostraron la estructura cortical como osteoblastos humanos (flecha roja, fig. 6.8A1, B1, C1 y D1, página 72). También se observó el envoltorio de fibra con estructuras esféricas asociadas, que es un nódulo de fosfato de calcio (fig, 6.8A3-6, fig. 6.8B3-6 y fig. 6.8C3-6, página 73, 74). Hemos observado el nódulo de fosfato de calcio (flecha azul) y hemos descubierto que en el rango de estimación del tamaño variaba de 1 a 2,5 μm. Las fotografías de SEM de DPMSC + TNC muestran cómo se formaron multicapas en la superficie de los andamios 3D de vidrio (fig. 6.8D1-4, página 72, 73). Se observó que los nódulos de fosfato de calcio también aparecieron en la superficie y en el interior de la banda celular (fig. 6.8D5-8, página 74-75).

Análisis estadístico

Actividad de la ALP

Las DPPSC sembradas durante el pretratamiento en el andamio 3D de vidrio con diferentes proteínas mostraron el pico de actividad de la ALP el día 20 (P<0,0001). La actividad de la ALP de las DPPSC fue de 119,67±13,275 el día 3; 97,33±16,255, el día 11 y 128,22±21,109, el día 20. La actividad de las ALP de las DPPSC fue significativamente diferente entre los días 3 y 11 y los días 11 y 20. Las DPMSC sembradas durante el pretratamiento en el andamio 3D con diferentes proteínas mostraron el pico de actividad de la ALP el día 3 (P<0,0001). La actividad de la ALP de las DPMSC fue de 155,33±20,457 el día 3; 97,72±7,411, el día 11 y 120,61±23,301,el día 20. La actividad de la ALP en las DPMSC fue significativamente diferente entre los días 3, 11 y 20. La actividad de la ALP comparada entre las DPPSC y las DPMSC no fue significativamente diferente. Las medias de la ALP en las DPPSC y las DPMSC fueron de 115,07±21,377 y 124,56±29,959, respectivamente.

Cuantificación de Ca^{2+}

La cuantificación de Ca^{2+} en las DPPSC y las DPMSC no presenta diferencias significativas entre ellas. La media del nivel de Ca^{2+} en las DPPSC y las DPMSC fue de 0,028±0,07 y 0,030±0,006, respectivamente. Existen diferencias considerables en el caso de los grupos de las DPPSC:,No tx/BMP2, No tx/OPN, No tx/BSP, FN/BMP2, FN/OPN, FN/BSP y OPN/TNC. Las DPPSC sembradas durante el pretratamiento en el andamio con OPN presentó el nivel de calcio más alto. También se pueden observar diferencias entre los grupos de las DPMSC: No tx/FN, No tx BMP2, FN/BMP2, BMP2/OPN, BMP2/ TNC y BMP2/BSP. Las DPMSC sembradas durante el pretratamiento en andamio con BMP-2 presentó el nivel de calcio más alto.

Prueba de fuerza en el andamio

Se compararon los 12 andamios cultivados y los andamios sin cultivos y se observaron diferencias considerables (valor P=0,036). La media fue de 12,092±5,95. La fuerza de los andamios cultivados con las DPMSC fue significativamente mayor que las del grupo DPPSC.(valor P=0,047; test de una cola). Las medias del grupo DPPSC y el grupo DPMSC fueron de 9,2±3,7 y 14,96±6,6, respectivamente.

Conclusión

1.° Las DPPSC tienen una capacidad osteogénica.

2.° Las DPPSC ese pueden considerar una población celular nueva debido a su diferente comportamiento respecto a las DPMSC.

3.° Las DPMSC cultivadas en andamio tratado con FN mostraron un patrón similar de expresión al del grupo DPPSC.

4.° Las DPMSC cultivadas en andamios con BMP-2, OPN, TNC y BNP experimentó un pico de actividad de ALP en el día 3. Estos resultados muestran que las DPMSC cultivadas con estas proteínas tienen un inicio temprano de la diferenciación de los osteoblastos en comparación con las DPPSC.

5.° Los niveles de expresión (RT-PCR) de los marcadores de osteoblastos, como el Col I, BMP-2 y la OCN, indicaron un nivel más alto al utilizar las DPMSC con todas las proteínas.

6.° Las DPMSC presentaron una mayor resistencia a la fractura respecto a las DPPSC. Se podría explicar que el tamaño de célula de DPPSC es mucho más pequeño que las DPMSC y la raíz de DPPSC migra hacia el centro del andamio, en lugar de, muchas células de DPMSC estaban en superficiales.

7.° Todas las muestras tendieron a adherirse y proliferar en el andamio 3D de vidrio. Hemos observado que las células claramente sembrado dentro de andamio y también

depositamos matriz mineralizada. Curiosamente en el pretratamiento con andamios 3D de vidrio con TNC, se observó que las DPMSC tienen mayor proliferación. Esta observación se apoya apoyada en el hallazgo de que la TNC estimula activamente la diferenciación osteogénica de las DPMSC y tiene buena adhesión al andamio. Sin embargo, las DPMSC sembradas en el pretratamiento con andamios con OPN tienen una densidad de la red celular similar con DPPSC + OPN y DPPSC + TNC.

8.° La formación de nódulos mineralizados es un marcador fenotípico para la última etapa de los osteoblastos maduros.

9.° Las DPPSC sembradas en andamios 3D cubiertos con OPN presentan la mayor osteoinducción. Las DPMSC sembradas en andamios 3D cubiertos con BMP-2 presentan una mayor osteoinducción. Las DPMSC son las más adecuadas para la regeneración ósea de las DPPSC. Sin embargo, para la colocación de implantes dentales o trauma, se prefiere hueso trabecular con gran suministro de sangre. En definitiva, la regeneración ósea con DPPSC puede obtener un mejor resultado.

13. APPENDIX

CEIC Comitè Ètic d'Investigació Clínica
Clínica Universitària d'Odontologia
Universitat Internacional de Catalunya

CARTA APROVACIÓ ESTUDI PEL CEIC

Número de l'estudi:B-27-EFP-09
Versió del protocol: 1.1
Data de la versió: 17-01-11
Títol: "Evaluación de la capacidad regenerativa de células madre de la pulpa dental"

Sant Cugat del Vallès, 27 de gener de 2011

Dr. Maher Al-Atari

Referència:

Benvolgut Doctor,

Els membres del CEIC de la Clínica Universitària d'Odontologia, li agraeixen l'aportació científica en el camp de la investigació i la presentació del Protocol en aquest Comité per a la seva avaluació.

Valorades les noves aportacions realitzades a l'estudi, sol.licitades pel nostre CEIC, el passat dia 17 de gener de 2011, li comuniquem que el dictamen final ha sigut FAVORABLE.

Quedem a la seva disposició per a qualsevol dubte o aclaració al respecte.

Atentament,

Sra. Immaculada Puga
Presidenta CEIC