Lorena de Oñate Monje

TESI DOCTORAL UPF / 2015

DIRECTOR DE LA TESI

Dra. Núria Montserrat (PSCs and activation of endogenous tissue programs for organ regeneration, IBEC)

DEPARTAMENT DE CIÈNCIES EXPERIMENTALS

I DE LA SALUT

A mi hermana

AKNOWLEDGMENTS

En primer lugar quisiera agradecer a mi Directora de Tesis la Dra. Núria Montserrat. Principalmente quiero destacar su capacidad de trabajo sobrehumana y el nivel insospechable de esfuerzo que ha realizado en estos meses de recta final, por tirar de mí y hacer que esto saliera adelante finalmente. Gracias Núria por seguir apostando por mi trabajo, mantenerme en tu equipo y permitirme ver de cerca cómo vas creciendo en tu carrera porque, éstas y muchas otras cosas han contribuido "muy significativamente" durante estos años a formarme como la persona que soy hoy en día.

En segundo lugar, quisiera agradecer al Dr. Juan Carlos Izpisúa que me aceptara para formar parte de su grupo de Investigación en el CMRB y que sin dudarlo, más adelante, me diera la oportunidad de realizar la estancia en el Salk Institute que tanto significado ha dado a esta Tesis a todos los niveles.

Gracias a Keiichiro Suzuki y Ying Gu por compartirme sus conocimientos, supervisar mis experimentos y permitirme formar parte de su equipo durante mi estancia en el Salk Institute, que tan buen recuerdo me deja y gracias también a todos los compañeros que allí encontré. Emi, April, Rupa, Jun, CJ, Ken y Yuta no podríais hacer sentir mejor a un recién llegado.

Muchas gracias a Elena Garreta por la ayuda de estos meses. Ha sido como quien a mitad de vía ferrata, cuando el asunto se complica, carga con tu mochila a la espalda para que sigas adelante.

Gracias a Cristina Eguizábal por involucrarme en los experimentos de su proyecto y confiar en mi criterio y mi trabajo. Lo aprecio de veras.

Carolina y Laia son las mejores compañeras de trabajo con las que uno podría encontrarse jamás. Gracias por la ayuda pero sobre todo vuestra confianza y amistad que me ha dado equilibrio estos años.

También me gustaría agradecer a algunos compañeros del CMRB; en especial a Toni, Alba y Anuska, por ser tan tiernos conmigo, darme ánimos y seguir confiando en mis posibilidades. A Marc por salvarme siempre en el último minuto informáticamente

hablando, y a todos los demás; Lola, Eva, Rubén, Ergin, Julián, Erika y Borja por estar siempre dispuestos a ayudar.

También debo agradecer a la Dra.Anna Veiga, tutora UPF de esta tesis y directora del Banco de Líneas Celulares, donde aprendí las bases del cultivo de células madre gracias a Yoli, Bego y por supuesto la Dra. Rita Vassena, que junto con Montserrat Barragán, me dieron las herramientas necesarias al principio del camino para sacar adelante los experimentos.

Finalmente quiero agradecer el apoyo incondicional de mi familia que aún sin acabar de comprender el porqué me dedico a ésto, siguen dándome ánimos y sintiéndose orgullosos y todo. Os quiero.

ABSTRACT

Probably, the gain in organ complexity and cell function has led to a decrease in healing capacities in the adult mammalian heart. In an effort to generate new venues for the generation of functional cardiac cells, in the present work we have explored the possibility to manipulate cell fate and plasticity making use of different technologies.

First, and taking advantage of somatic cell reprogramming we have generated induced pluripotent stem cells (iPSCs) from umbilical cord derived mesenchymal stem cells (ucMSCs), an amenable source of stem cells in the clinical setting. In a very simple manner we have converted ucMSCs into iPSCs with either four transcription factors (OCT4, SOX2, KLF4 and c-MYC) or two transcription factors (OCT4 and SOX2). Second, by a cell conversion approach, we have been able to produce cells expressing cardiac-related markers at the protein level from human post-natal dermal fibroblasts. Our protocol induces cell de-differentiation by the overexpression of OCT4 and SOX2 pluripotent-related transcription factors first, and the specific meso-cardiac transcriptional factor GATA4 afterwards. Finally, in order to define new conditions for the generation of cardiac-like cells from either pluripotent stem cells or somatic cells, we have developed a reporter cell line for the cardiac gene alpha Myosin Heavy Chain (*MYH6*) by means of TALEN and CRISPR/CAS9 genome editing technologies. In this manner we have been able to explore culture conditions promoting cardiac differentiation from human embryonic stem cells. The work presented here shows different approaches aiming to generate cardiac-like cells with an impact in Regenerative Medicine.

RESUM DE TESI

És probable que l'adquisició de complexitat tant en els òrgans, com en les funcions cel·lulars al llarg de l'evolucióhagi portat a la disminució de les capacitats d'autocuració del cor de mamífers adults. Per tal de generar noves plataformes per la generació de cèl·lules cardíaques funcionals, en el treball exposat a continuació, hem explorat la possibilitat de manipular tant el destí com la plasticitat cel·lular fent servir diferents tecnologies.

En primer lloc, aprofitant la reprogramació somàtica, hem generat cèl·lules mare pluripotents induïdes (iPSCs) a partir de cèl·lules mare mesenquimals derivades de sang de cordó umbilical (ucMSCs), una font fàcilment accessible de cèl·lules mare en l'entorn clínic. De manera senzilla, hem aconseguit convertir ucMSCs en iPSCs amb quatre (OCT4, SOX2, KLF4 i c-MYC) o amb tan sols dos factors de transcripció (OCT4 i SOX2). En segon lloc, mitjançant una estratègia de conversió cel·lular, hem aconseguit produir cèl·lules que expressen marcadors relacionats amb teixit cardíac a nivell proteic a partir de fibroblasts dèrmics post-natals d'origen humà. El nostre protocol indueix en una primera fase la de-diferenciació dels fibroblasts mitjançant la sobre expressió primer dels factors de pluripotència OCT4 i SOX2, i després del factor de transcripció específic per mesoderm cardíac GATA4. Finalment, amb l'objectiu de definir noves condicions per la generació de cèl·lules cardíaques a partir de cèl·lules mare pluripotents o cèl·lules somàtiques hem enginyat una línia cel·lular reportera pel gen cardíac que codifica per la cadena pesant de la miosina (alpha myosin heavy chain o MYH6) mitjançant les tècniques d'edició gènica TALEN i CRISPR/CAS9. D'aquesta manera hem estat capaços d'explorar diferents condicions de cultiu per promoure la diferenciació cardíaca a partir de cèl·lules mare pluripotents d'origen embrionari.

El treball aquí exposat, mostra diferents estratègies dissenyades amb la intenció de generar cèl·lules cardíaques amb un cert impacte en la futura Medicina Regenerativa.

PREFACE

The work presented in this doctoral thesis was supported by the *Ministerio de Ciencia e Innovación* and was carried out between the "Center of Regenerative Medicine of Barcelona (CMR[B]), and the "Gene Expression laboratory", both lead by Prof. Dr. Juan Carlos Izpisúa Belmonte at the Salk Institute (La Jolla, CA. USA) and the "Pluripotent stem cells and activation of endogenous tissue programs for organ regeneration Group" leaded by Dr. Núria Montserrat at the Institute for Bioengineering of Catalonia (IBEC) in Barcelona, Spain. This thesis has been supervised by Dr. Núria Montserrat.

This dissertation is an original and unpublished work.

The content of this thesis provides novel insights in methodologies for the reprogramming of human somatic cells to induced pluripotent stem cells and valuable tools for generation of cardiac cells *in vitro* for future Regenerative Medicine purposes.

TABLE OF CONTENTS

ACKNOWLEDGMENTS **I**
THESIS ABSTRACT **III**
RESUM DE LA TESI **V**
PREFACE **VI**

I.INTRODUCTION

1. REGENERATIVE MEDICINE **1**
1.1. Cell therapy: restoring organ function with cells 2
1.2. Cell sources in Regenerative Medicine 3
1.2.1 Stem Cells 3
1.2.1.1. Embryonic Stem Cells (ESCs) 6
1.2.1.2. Induced Pluripotent Stem Cells (iPSCs) 8
1.2.1.2.1 Induced Pluripotent Stem Cells (iPSCs): looking for the perfect cell source to be reprogrammed 9
1.2.1.2.2. Induced Pluripotent Stem Cells (iPSCs): looking for the safer technology to be applied 10
1.2.2 Adult Stem Cells 13
1.2.3 Somatic Cells 14

2. HEART CELL THERAPY **16**
2.1. Cardiomyocytes generation 16
2.1.1. Molecular networks that regulate cardiac cell fate 16
2.1.2. Cardiac differentiation from PSCs 21
2.1.3. Cell conversion and transdifferentiation to cardiac fate. 25
2.2. Cardiac Tissue Engineering 27

3. STRATEGIES FOR GENE EDITING IN PSCS **30**
3.1. Gene Transduction 30
3.1.1. Integrative Methods 31
3.1.1.1.Random Integration methods 31
A) Random integration methods with viral vectors 31
B) Random integration methods without viral vectors 32

3.1.1.2. Specific site integration methods 33
3.1.2. Non-Integrative Methods 35
3.1.2.1. Non-Integrative Methods with viral vectors 37
3.2. Gene Targeting and Editing 37
3.2.1 Recombineering Methods for genome editing 38
3.2.1.1 Engineered DNA nucleases 40
3.2.1.1.1. Meganucleases 40
3.2.1.1.2. Zinc Finger Nucleases (ZFN) 40
3.2.1.2. Transcription activator-like effectors nucleases (TALEN) 41
3.2.1.3 Clustered regularly interspaced short palindromic repeats (CRISPR)/CAS9 42
3.3. Reporter Cell lines 46
3.3.1. Cardiac Reporter Cell lines. 47

II. SPECIFIC AIMS **51**

III. MATERIALS AND METHODS
III.A. MATERIALS **57**
1. Bacterial strains **57**
2. Cell lines **57**
3. Plasmid and Viral Vectors **58**
4. Antibodies **59**
5. Oligonucleotides **61**
6. Reagents **64**
III.B. METHODS **67**
1. GENERAL PROTOCOLS **67**
1.1. Molecular Biology and Biochemistry techniques 67
1.1.1. Plasmid Cloning 67
1.1.1.1. Traditional Cloning 67
1.1.1.2. In-Fusion® cloning method 68
1.1.2. Transformation of DNA plasmids using Heat (A) or Electro (B) shock methods 69

1.1.3. Extraction of Nucleic Acids (DNA and RNA) from biological samples 73
1.1.4. Polymerase Chain Reaction (PCR) 74
1.1.5. Sanger Sequencing 75
1.2. Biohistochemistry and Imaging 75
1.2.1. General Immunostaining and image capitation 75
1.3. General Cell Culture and maintaining 76
1.4. Fluorescent Activated Cell Analysis and Sorting (FACS) 79
2. REPROGRAMMING OF HUMAN UCMSCs 80
2.1. ucMSCs derivation and characterization 80
2.1.1. Isolation of ucMSCs 80
2.1.2. Differentiation potential in ucMSCs 81
2.1.2.1.Differentiation towards Osteogenic fate 81
2.1.2.2. Differentiation towards Chondrogenic fate 81
2.1.2.3. Differentiation towards Adipogenic fate 81
2.2. Somatic cell reprogramming 82
2.2.1. Generation of retroviral supernatants 82
2.2.2. Retroviruses transduction to ucMSCs 83
2.2.3. Generation and culture of hiPSCs from ucMSCs 83
2.3. Molecular characterization of iPSCs derived from ucMSCs 83
2.3.1. qPCR analysis 83
2.3.2. Immunophenotyping analysis by Fluorescent Activated
Cell sorting (FACS) 84
2.4. *In vitro* differentiation of iPSCs derived from ucMSCS 84
2.4.1. iPSCs in vitro differentiation into endoderm 84
2.4.2. iPSCs in vitro differentiation into mesoderm 84
2.4.3. iPSCs in vitro differentiation into ectoderm 85
3. GENERATION OF CARDIOMYOCYTE-LIKE CELLS *in vitro* 86
3.1. Induction of cardiac differentiation by the Embryoid Body (EB) method 86
3.2. Chemical induction of cardiac differentiation from ES[4] and reporter ES[4]
targeted in the *MYH6* locus 86
3.3. Direct conversion of human fibroblasts (HFFs) towards cardiac-like Cells 87
3.3.1. Retroviral based plasmid construction 87

3.3.2. Two-step transdifferentiation protocol from HFFs towards cardiac fate. 88

4. GENE TARGETING AND knock-in BY TRANSCRIPTION ACTIVATOR-LIKE EFFECTORS NUCLEASES (TALEN) AND CLUSTERED REGULARLY INTERESPACED SHORT PALINDROMIC REPEATS (CRISPR)/CAS9 89

4.1. Design and Construction of TALENs 89

4.1.1. TALEN monomer library construction 89

4.1.2. TALEN target site selection 90

4.1.3. Construction of custom 20-bp targeting TALENs 90

4.1.4. Verification of the correct TALEN repeats assembly 91

4.2. CRISPR/CaS9 design and construction: guiding RNAs (gRNAs) 91

4.3. MYH6 Donor Vector (d-vector) design and construction 91

4.4. Functional Assay: Surveyor Assay 92

4.5. ES[4] cell transfection with (TALEN) and (CRISPR)/CAS9 93

4.5.1. Electroporation 93

4.5.2.Nucleofection 94

4.6. Determination of *MYH6* targeting and copy number integration in transfected ES[4] cells 94

4.6.1. Selection of targeted ES[4] by antibiotic treatment 94

4.6.2. PCR clone selection: identification of ES[4] clones targeted in *MYH6* locus by TALEN and CRISPR/CAS9 technologies 95

4.6.3 Southern Blot Analysis: determination of MYH6 copy number in ES[4] lines engineered by TALEN and CRISPR/CAS9 technologies 96

IV. RESULTS CHAPTER 1

1.GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS WITH OCT4 AND SOX2 99

1.1. Phenotype and purity of ucMSCs 100

1.2. ucMSCs can be differentiated towards bone, adipose and cartilage fate. 102

1.3.Reprogramming of ucMSC and characterization of pluripotency signatures in ucMSC-iPSC lines 103

1.4. Reprogramming of ucMSC*: in vitro* differentiation potential of ucMSC-iPS-2F cell lines 107

IV. RESULTS CHAPTER 2

2. CONVERSION OF DERMAL POST-NATAL FIBROBLASTS INTO CARDIAC-LIKE CELLS 111

2.1 Differentiation of hESCs towards cardiac like cells with ascorbic acid promotes the expression of cardiac-related markers. 112

2.2. Exposure of post-natal dermal fibroblasts to OCT4 and SOX2 promotes the expression of mesodermal and cardiac related genes. 114

2.3. Coupling first phase of mesodermal induction by OCT4 and SOX2 with GATA4, promotes cardiac features in human post-natal dermal fibroblasts. 119

IV. RESULTS CHAPTER 3

3. GENERATION OF HUMAN REPORTER CELL LINES FOR ALPHA MYOSIN HEAVY CHAIN (α−MHC) (MYH6) GENE: DEVELOPING TOOLS FOR CARDIAC DIFFERENTIATION 125

3.1. α–MHC (MYH6) expression during cardiac differentiation from hESCs 125

3.2. *Knock in* on the *α-MHC(MYH6)* locus in hESCs by TALEN and CRISPR/Cas9 technologies 127

3.2.1. TALEN and CRISPR/Cas9 design, construction and functional test 128

3.2.2. Targeting of *MYH6* locus in ES[4] line 130

3.2.3. Clone selection 132

3.2.4. Assessment of reporter transgene integration by PCR screening 133

3.2.5 Assessment of reporter transgene copy number integration and location by Southern Blot 135

3.3. Characterization of TALEN ES[4]_MYH6_mCherry cell line 136

3.3.1 Undifferentiated TALEN ES[4] MYH6_mCherry cell line shows stable karyotype 137

3.3.2. Undifferentiated TALEN ES[4] MYH6_mCherry cell line expresses pluripotency-related genes 138

3.3.3 TALEN ES[4] MYH6_mCherry cell line differentiate *in vitro* towards the three germ layers of the embryo 140

3.4. TALEN ES[4] MYH6_mCherry cell line as a tool for guiding cardiac differentiation 143

3.5. Our new protocol for cardiac derivation sustains TALEN ES[4] MYH6_mCherry - and ES[4]- cell lines differentiation toward cardiac-like cells. 148

3.6. TALEN ES[4] MYH6_mCherry derived cardiomyocytes expressed cardiac related markers by immunofluorescence 150

3.7. TALEN ES[4] MYH6_mCherry derived cardiomyocytes recapitulate cardiac features when co-cultured in human cardiac matrices 152

V. DISCUSSION 157

VI. CONCLUSIONS 171

VII. BIBLIOGRAPHY 175

VIII. APPENDIX.I. SUPLEMENTARY FIGURES 211

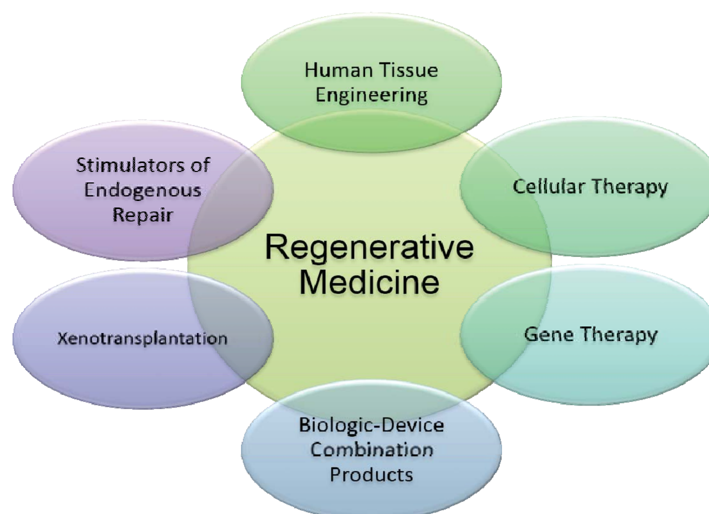
VIII. APPENDIX.II. LIST OF ABBREVIATIONS 241

VIII. APPENDIX.III. PUBLICATIONS 245

I. INTRODUCTION

1. REGENERATIVE MEDICINE

The term "Regenerative Medicine" was first found in a paper on hospital administration published by Leland Kaiser in 1992. Writing about future technologies, Kaiser defined "a new branch of medicine that attempt to change the course of chronic disease and in many instances, to regenerate tired and failing organ systems" (LR. 1992). Regenerative Medicine comprise multiple research areas, therefore can be defined as "an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause, including congenital defects, disease ortrauma" (Daar & Greenwood 2007). There are a wide variety of technologies that can contribute to achieve this goal. Some methodologies consisting on cell therapy, tissue engineering, gene therapy and biomedical engineering techniques, as well as more traditional treatments involving pharmaceuticals, biologics and devices. Overall Regenerative Medicine is the key new discipline with true potential to change the field of current health care from traditional reactive to preventative and restorative health care



style.**Figure.1**: **Regenerative Medicine technologies.** Since Regenerative Medicine is an interdisciplinary field of research and clinical applications, there are a wide variety of technologies that contribute to restore the loss of function of damaged organs or tissues. The set diagram represents principal disciplines and research areas implicated in the progress and success of Regenerative Medicine within the last three decades.

Degenerative diseases in humans are characterized by loss or malfunction of specific cell types. Often, replacement of the whole organ is the unique treatment in clinics (i.e; cardiac severe pathologies). Unfortunately, for most of the pathologies involving organs, transplant is the unique possibility of treatment. Nevertheless donor supplies are still scarce for clinical demand. In this regard, cell therapy is one of the most important approaches for tissue regeneration, and in some cases a promising and feasible alternative to whole organ transplantation.

1.1. Cell therapy: restoring organ function with cells

Cell therapy consists on transplantation of functional cells, with autologous or heterologous origin, already differentiated or capable to differentiate to the specific cell type affected during the development of the disease of study. In this regard, blood transfusions are good examples of first successful cell therapy treatments. It was in 1818 when Dr. James Blundell performed the first successful reported transfusion of human blood to a patient for the treatment of postpartum hemorrhage.

Figure 2: **First cell therapy approach in humans.** First successful blood transfusion in humans performed by Dr. James Blundell in 1828 for the treatment of a womans postpartum hemorrhage. Picture from *Blundell, J.: Experiments on the Transfusion of Blood by the Syringe. M. Chir. Tr. 9: 56-92, 1828 (London).*

Since that moment, other successful treatments have been demonstrated along the last century in the blood field. Since 1968 allogeneic bone marrow transplantation has become a well-established protocol and it is still the treatment of choice to treat many

blood disorders nowadays including anemias (Sebastiano et al. 2011; Wagner et al. 2007; Yoshida et al. 2014), leukemias (Keating et al. 2013), lymphomas (Appelbaum et al. 2015) and rare immunodeficiency diseases (Griffith et al. 2014).

1.2. Cell sources in Regenerative Medicine

Regenerative Medicine has traditionally stood in the use of Cell Therapy as one out of the most useful methodologies for tissue repair. The different cell types used for this purpose can be classified depending on their developmental origin, differentiation potential and intrinsic characteristics. Based on such criteria cells are traditionally classified as detailed below.

1.2.1. Stem Cells

Stem cell (SC) term appears in the scientific literature in the late 19th century as an answer to fundamental questions in embryology related to the continuity of the germ plasm and the origin of blood. It was later on, in early 1960s, with the experiments performed by using hematopoietic cells (Becker, A. J., McCulloch, E. A. & Till 1963; Till, J. E., & McCulloch 1961; Till, J. E., McCulloch, E. A. & Siminovitch 1964), that demonstration of the existence of a certain group of cells capable of replicating themselves nearly indefinitely, and giving rise to specialized cells, was referred as "stem cell". SC is the current term to define those unspecialized cells that are present in any tissue of the body and have the ability to proliferate or self-renew for long periods giving rise to the same type of cell of origin. Interestingly, Stem Cells (SCs) also show the capability to differentiate into a more specialized cell type within the same developmental program. In this regard, SCs are classified depending on their differentiation potential. During mammalian embryo development, SCs gradually lose their stem cell identity and become progressively differentiated to fulfill required specific functions in each tissue from the adult body.

SCs that are able to differentiate to any type of cell present in an organism are called **totipotent stem cells**. As such, totipotent stem cells only exist after egg fertilization

(with sperm) being present only in the zygote. Later on, during embryo development, totipotent stem cells will give rise to any cell type from the three germ layers of the embryo (ectoderm, mesoderm and endoderm) and to cells of the cytotrophoblast layer of the placenta (SJ 1977). **Pluripotent stem cells** (PSCs), do only exist in the inner cell mass (ICM) from ple-implantation embryos (*day 5* to *day 6* during embryo development in humans), and refer to cells capable to differentiate to any cell type derived from the three different germ layers; that means, cells derived from ectoderm (epidermal tissues and nervous system), mesoderm (muscle, blood, bone, etc) and endoderm (gastrointestinal tract, lungs, etc). **Multipotent or progenitor cells** (PCs) have differentiation potential to multiple but tissue-specific cell types. These cells can be found in the adulthood and are responsible of regeneration or repair of certain tissues. Hematopoietic stem cells (HSCs) are multipotent stem cells capable to differentiate to themselves (to self-renew), and into several specialized blood cell types including lymphocytes, monocytes, neutrophils, etc. However, HSCs cannot differentiate to bone cells, for example (Eaves 2015).

There exist lower degrees of potency, referred as: **oligopotency** and **bipotency** or **unipotency**. Examples of these categories are lymphoid SCs, that are oligopotent SCs that can give rise to B an T cells from blood (Dzierzak & Speck 2008). Bipotent or unipotent stem cells commonly named “precursor cells”, are limited to differentiate to a certain cell type like the case of hepatoblasts, that can give rise just to hepatocytes or cholangiocytes (Bogert & LaRusso 2007; Lemaigre & Zaret 2004).

During the last years, intense research has tried to identify which are the key molecular and cellular events regulating SCs properties and capacities, as well as differentiation potential. Nowadays it is well known that, under certain controlled conditions, SCs can be induced to become functional tissue- or organ-specific cells.

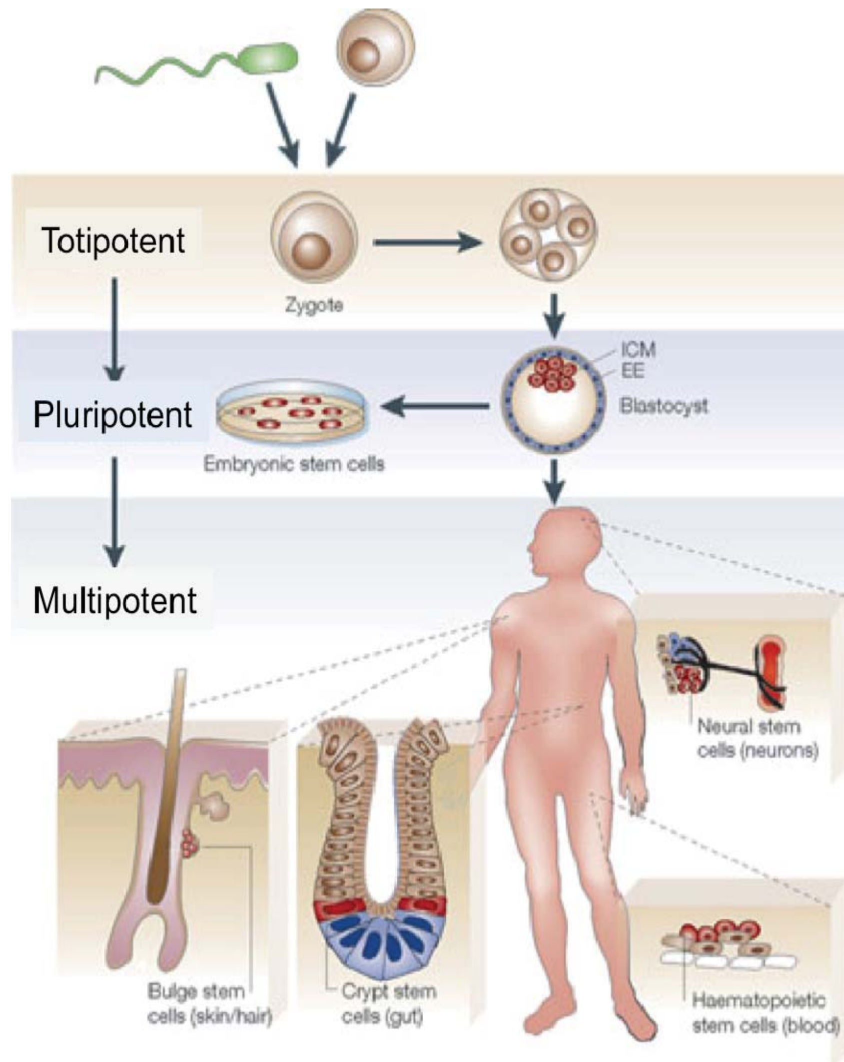


Figure 3: **The stem cell hierarchy**. The totipotent zygote formed by the fusion of egg and sperm divides to form the inner cell mass (ICM) and the extra-embryonic (EE) tissue of the blastocyst. When isolated from the blastocyst *in vitro*, the cells of the ICM can be maintained in culture as embryonic stem cell (ESC) lines. During the development of the embryo, the pluripotent stem cells in the ICM become increasingly restricted in their lineage potential and generate tissue-specific, multipotent stem cells. Adapted from *Eckfeldt et al., 2005.*

Interestingly, pioneer experiments performed in different animal species, showed that transfer of nuclei from SCs and/or adult somatic cells into enucleated oocytes, could lead to the formation of cells from the three germ layers of the embryo, and more important, to the formation of entire living animals (GURDON JB, ELSDALE TR 1958; Gurdon 2013; Gurdon & Byrne 2005; Hochedlinger & Jaenisch 2002; Wilmut et al. 1997). Thus, those primordial experiments set the basis for further experiments, which demonstrated that SC identity and potential can be fully achieved under exceptional conditions. Overall and despite the nature of the cell type of study, limited plasticity or intrinsic nature, hundreds of studies have demonstrated the possibility to change fate and destiny of initial SCs populations (i.e: by using chemically defined media promoting SCs differentiation, by using viral vectors to express corrected genes for gene therapy). In this regard and within the last decades SCs therapies have become real possibilities for patient treatment in clinics.

1.2.1.1. Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) are pluripotent stem cells (PSCs) derived from the ICM of a mammal embryo at the blastocyst stage, first isolated and cultured from mice by Evans, Kaufmann and Martin in 1981 (Evans MJ 1981). Later, Thompson demonstrated that same approaches could be transferred into human embryos (Thomson 1998). In both species ESCs have been shown to be cultured indefinitely without losing their proliferative and developmental potential. These features make ESCs a promise of unlimited cell supply for basic research or cell therapy, and a patient-specific resource of SCs capable to give rise *in vitro* to almost all possible tissues compromised during disease progression. In the last thirty years intense research has demonstrated that ESCs can differentiate *in vitro* to large number of somatic cell types. Since the approval of research with human ESCs (hESCs) in 2001 and Presidents' Barack Obama Executive Order in 2009 (Bethesda, MD: National Institutes of Health 2009), scientists world-wide have been able to differentiate hESCs to muscle cells (Huang et al. 2006; Hwang et al. 2014), cardiomyocytes (Kehat et al. 2001; Xu 2002; He et al. 2003), neurons (Zhang et al. 2001; Tabar et al. 2005; Pankratz et al. 2007; Elkabetz et al. 2008), hepatocytes (Touboul et al. 2010; Brolén et al. 2010; Yildirimman et al. 2011; Greenhough et al. 2010) endothelial (Wang et al. 2007; Levenberg et al. 2007), platelets

(Lu et al. 2011; Pick 2013; Takayama et al. 2010) retinal cells (Osakada et al. 2009; Nakano et al. 2012) among many other cell specific types. The timing and efficiencies of the reported protocols differ depending on cell type to be obtained, and for that reason exhaustive research improving differentiation methods have being carried out. Although progresses in the field have been quite impressive, still the key factors that control the differentiation of hESCs to determined specific cell types remain unravel (e.g: kidney cells).

Interestingly, different authors have demonstrated that hESCs differs from other animal-derived ESCs more than initially expected, raising important questions related to the intrinsic nature of these cells. Theoretically, one would expect that the basic molecular mechanisms that govern pluripotency would be conserved in the different philia of the animal kingdom, and indeed, it has been widely demonstrated that ESCs derived from different animal species conserve the same patterns of expression for many genes related with ESCs nature (J Cai et al. 2010). However, hESCs and mouse ESCs (mESCs) show important differences with regard of the specific signalling pathways regulating self-renewal. For example, Austin Smith´s group described that BMPs in combination with LIF promote self-renewal and undifferentiated state in mESCs (Ying et al. 2015). However, BMPs are major factors promoting rapid differentiation in hESCs (Xu et al. 2002). Another interesting difference is the high propensity of hESCs to be derived into trophoblasts compared with mESCs (Xu et al. 2002; Schulz et al. 2008). In this regard the exact molecular mechanisms governing pluripotency have become one of the key central questions of developmental biology.

Eventhough still remain many unsolved questions, transplantation of differentiated ESCs has been carried out offering promising results in animal models. Studies in the rat model have demonstrated that rat ESCs are able to develop into functional dopaminergic neurons after transplantation in a rat model of Parkinson Disease (Bjorklund et al. 2002); also that direct transplantation of rat ESCs improves cardiac function in post-infarcted animals (Min et al. 2002). Another study demonstrates that gerbils ESCs enhance healing of tympanic membrane perforations (von Unge et al. 2003). In the human setting it has been reported that the injection of hESCs enhances the re-closure capacity of spinal open neural tube defects in the chick (Lee et al. 2004), and very recently, hESCs have been described to restore vision parameters when

transplanted in the human eye (Schwartz, Hubschman, et al. 2015; Schwartz, Regillo, et al. 2015).

On the other hand, immune rejection after ESC transplantation has been observed in different animal models (Pearl et al. 2012; Swijnenburg et al. 2008). Therefore, basic and translational research is focused in the possibility to develop novel strategies for ESCs transplantation avoiding issues related with immune rejection, providing clinically translatable strategies for inducing ESCs tolerance after transplantation. In this particular regard, Yuqiong Pang and colleagues have very recently demonstrated the engraftment of mESCs and their *in vitro* derivates in allogeneic recipients previously conditioned with fractioned total lymphoid irradiation (TLI) and anti-thymocyte serum (ATS) or TLI plus regulatory T cells (Treg) (Pan et al. 2015).

1.2.1.2. Induced Pluripotent Stem Cells (iPSCs)

In 2006 Professor Shinya Yamanka and colleagues (Takahashi & Yamanaka 2006) showed for the first time, that introducing different transcription factors related with mESC biology, the epigenetic status of mouse embryonic derived fibroblasts (MEFs) could be reverted to pluripotency. In particular, the Japanese team induced the ectopic expression of a subset of specific transcription factors: Oct3/4, octamer-binding transcription factor 4 (also known as Pou5f1, POU domain class 5 transcription factor 1); Sox2, (SRY [sex determining region Y]-box 2); Klf4, Kruppel-Like Factor 4 and c-Myc (V-Myc Avian Myelocytomatosis Viral Oncogene Homolog), generating in a period of only thirty days, cells that were identical to mouse ESCs (mESCs) in terms of self-renewal capacity, expression of endogenous pluripotency-related factors, and *in vivo* and *in vitro* differentiation potential to give rise to cells belonging to the three germ layers of the embryo (ectoderm, mesoderm, endoderm). This discovery was awarded with the Nobel Price of Medicine in 2012 to Professor Shinya Yamanaka. The generated cells were referred as Induced Pluripotent Stem Cells (iPSCs), since their pluripotent state was artificially forced *in vitro*. The same group, could demonstrate that the same methodology was able to convert human fibroblast towards human iPSCs (hiPSCs) by means of the expression of four transcription factors: OCT3/4, SOX2, KLF4 and c-MYC (Takahashi et al. 2007).

The resetting of the whole transcriptome and epigenome of a somatic cell towards a pluripotent state was previously achieved by somatic cell nuclear transfer (SCNT) (GURDON JB, ELSDALE TR 1958) and cell fusion (Blau et al. 1983). Although SCNT and cell fusion offer several advantages in front of iPS technology (i.e: rapid and deterministic), still investigation of SCNT and cell fusion is difficult, requiring large amounts of oocytes. In contrast, in the transcription-factor mediated reprogramming, the factors that drive the process are known, making the process easily modulated and easier to follow. Although iPS methodology shows many advantages in front of the technologies described before, in many cases the reprogramming method is still time consuming and inefficient, generating iPSCs that vary widely in their developmental potential. In this regard, in the last years tremendous efforts have been done in order to identify the best cell source to be reprogrammed, as well as the best methodology to be applied.

1.2.1.2.1. Induced Pluripotent Stem Cells (iPSCs): looking for the perfect cell source to be reprogrammed

Different reports have demonstrated that iPSCs can be generated from a variety of somatic cell types other than fibroblasts originally reprogramed by Yamanaka. Seminal studies in the field of human reprogramming took into account the easy access to the original cell source to be reprogrammed. In this regard Giorgetti and Cai (Giorgetti 2009; Jinglei Cai et al. 2010) demonstrated independently, the possibility to reprogram cord blood stem cells (positive for the expression of CD133 surface marker, CD133+ cells) towards iPSCs. Later on other authors aimed to reprogram other easy-amenable cell types, such as peripheral blood mononuclear cells (PB-MNC) (Kunisato et al. 2010), and B-lymphocytes (Hanna et al. 2008). In the same manner common efforts described the possibility to reprogram other SCs characterized for their multipotent differentiation potential and adherent growth, such as mesenchymal stem cells (MSCs) (Kocaefe et al. 2010; Park et al. 2008; Sun, Panetta, et al. 2009; Jinglei Cai et al. 2010; Niibe et al. 2011).

In the quest of the best cell type to be reprogrammed other groups bet for cells expressing any of the Yamanaka factors endogenously, aiming to reduce the ectopic

over-expression of any of them. Following this hypothesis keratinocytes from skin or hair have been demonstrated to be reprogrammed in the absence of c-MYC (Aasen et al. 2008); CD133+ cord blood stem cells could be converted into iPSCs in the absence of KLF4 and c-MYC (Giorgetti et al. 2009); and neural stem cells were successfully reprogrammed in the absence of KLF4, SOX2 and c-MYC (J. B. Kim et al. 2009).

On the other side, epigenetic memory have been identified as a major barrier for somatic reprogramming (Morris et al. 2015; Nashun et al. 2015) and a limiting factor when differentiating iPSCs (Maria C. N. Marchetto, Gene W. Yeo, Osamu Kainohana, Martin Marsala, Fred H. Gage 2009; Kim et al. 2011; Polo et al. 2010). In this regard, Kim and coleagues demonstrated advantage on hematopoietic differentiation of hiPSC generated from cord blood cells when compared to hiPSCs generated from keratinocytes (Kim et al. 2011). Interestingly, iPSCs have been generated from cells arising from the three germ layers of the embryo such as: liver and stomach cells (Aoi et al. 2008), hepatocytes (Liu et al. 2010), pancreatic cells (Stadtfeld et al. 2008) or human renal proximal tubular cells (Montserrat et al. 2012), among others.

1.2.1.2.2. Induced Pluripotent Stem Cells (iPSCs): looking for the safer technology to be applied

In the last years intense research has been focused in the development of strategies aiming to derivate iPSCs with safe vectors (DNA carriers) sustaining the expression of Yamanaka factors during the early days of reprograming (up to 10 days in the human setting). Generally speaking these methods can be divided into **integrative** and **non-integrative** approaches **(Figure 4).**

Integrative methods integrate randomly copies of exogenous DNA sequences from the factors of interest. This strategy has been extensively used during the first years in the reprogramming field, being retroviral vectors the DNA carriers of choice (Takahashi et al. 2007; Takahashi & Yamanaka 2006; Park et al. 2008; Chang et al. 2009). Of note, this methodology enables for high efficiencies when reprogramming cells of human origin (around 0.001-1.1%). Although seminal studies have focused in the use of retroviral or lentiviral vectors for the expression of Yamanaka factors, the possibility of transgene expression after cell transplantation precludes their use in the clinical setting.

Thus, in the last years common efforts have been done in order to define safer strategies ensuring somatic reprogramming avoiding random transgene integration in the host cell.

Non-integrative methods involve transient expression of factors without the insertion of the selected factors in the genome of interest. Those novel strategies have been explored for iPSCs generation and include the use of adenovirus (Stadtfeld et al. 2008; Zhou & Freed 2009), polycistronic plasmid mediated transfections (Si-Tayeb et al. 2010), tricistronic episome mediated transfection (Okita et al. 2008) and excisable-transgene constructs (Kaji et al. 2009; Woltjen et al. 2009; Belay et al. 2010). Moreover, somatic cells have been demonstrated to be up to 1000% efficiently reprogrammed by means of a DNA-free system consisting on the use of synthetic messenger ribonucleic acids (mRNAs) (Warren et al. 2010) or micro RNAs (miRNAs) (Anokye-Danso et al. 2011; Miyoshi et al. 2015). Other DNA-free systems are based on the use of recombinant proteins (Zhou et al. 2009; D. Kim et al. 2009) or RNA based-virus such as SendaiVirus (SeV) (Fusaki et al. 2009). Other methods take advantage from the use of chemical compounds replacing Yamanaka factors (Lyssiotis et al. 2009; Hou et al. 2013); and/or boosting reprogramming efficiencies when combined with different combinations of the original Yamanaka´s cocktail (Hayes & Zavazava 2013). The use of chemical compounds replacing transcription factor-mediated reprogramming is envisioned as the safer method to generate iPSC in a clinical context, however the impact of chemical compounds on genome stability remains to be studied.

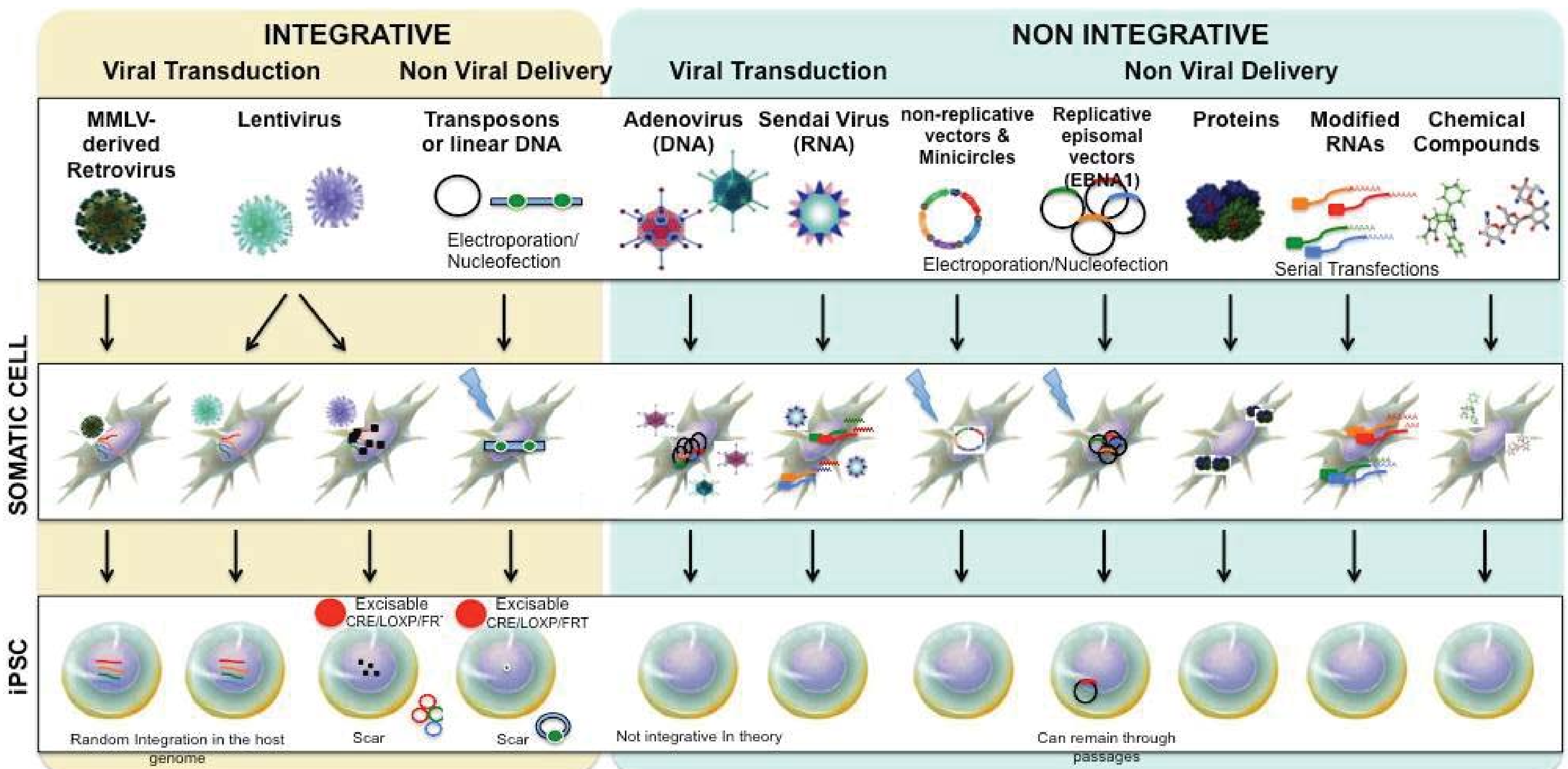


Figure 4: Methods to reprogram somatic cells into induced pluripotent stem cells (iPSCs). Representative flow diagram summarizing the different reprogramming methods developed so far. The possible delivery methods are divided into Viral or Non-Viral. For each approach, the design of the delivery system is shown on top; spine-shaped cells illustrate intermediate stages after inducing the expression of pluripotent-related factors; round-shaped cells represent final stages after reprogramming. More information at *Introduction 3. STRATEGIES FOR GENE EDITING IN PSCs.*

1.2.2. Adult Stem Cells.

Adult stem cells (ASCs) are undifferentiated cells that can be found as residents in a tissue or organ that possess multipotent-related properties; that means that ASCs can renew themselves and differentiate through all-major specialized cell types of the tissue or organ in which they are located. Their major function consists on repairing and maintaining different tissues or organs in the body.

First studies located two different types of ASCs in bone marrow. One population was called hematopoietic stem cells (HSCs), the progenitor cells of each different type of blood cells. The second population referred as bone marrow stromal cells [or mesenchymal stem cells, MSCs] was discovered later, and adopted its name for its ability to generate bone, cartilage and fat cells that support formation of blood and connective tissues (Grigoriadis et al. 1988). Later on, other studies demonstrated that dividing cells with SC properties could be localized in two different regions of the brain in rats (ALTMAN & DAS 1965). In this regard, at the end of 1990s several authors agreed that the adult brain contain SCs able to regenerate three major cell types of this organ: astrocytes, oligodendrocytes and neurons (Cameron & McKay 1998; Temple & Alvarez-Buylla 1999; Kuhn & Svendsen 1999; Gage 2000).

Overall ASCs have been identified to date in many different tissues, including: brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium and testis. These cells usually reside in specific areas of the tissue, called “stem cell niche”. ASCs may remain without dividing for long periods of time until they are activated by specific signals after injury. Thus, research in ASCs has generated hope and excitement in Regenerative Medicine. Examples of potential applications of ASCs include the already current treatment of bone marrow (BM) transplant done since 40 years ago. An interesting treatmen in this field is the use of BM derived autologous suspensions of both, hematopoietic and MSCs depleted from erythrocytes and lymphocytes for traumatic spinal cord injury. Also, BM mesenchymal stem cells (BMMSCs) exposed to melatonin treatment have been suggested as a potential treatment for chronic myocardial ischemia (Roncalli et al. 2011). In the same line BMMSCs derived from CD133+ cells have been described to improve heart function in patients with ischemic heart disease after myocardial infarct (MI) (Jeevanantham et al. 2013). MSCs derived from adipose tissue (aMSCs) have been

widely used for treating degenerative arthritis (Zhou et al. 2011), osteoarthritis (Michalek et al. 2015; Kristjánsson & Honsawek 2014) or articular cartilage defects (Seo & Na 2011; Ahmed & Hincke 2014). Interestingly, after beta-tricalcium phosphate treatment (one of the main combustion products of bone), aMSCs improve the bone structural and functional defects (Lu et al. 2015). Nevertheless the molecular mechanisms explaining such improvement remain to be defined.

Although ASCs could represent an ideal cell source for Regenerative Medicine purposes they still show a limited capacity to be expanded *in vitro*. Moreover, in some cases ASCs are not amenable from living tissues (e.g: brain stem cells). However some ASCs (e.g: aMSCs, umbilical cord MSCs, etc) present interesting properties making possible their use albeit their major inconveniences, as cell plasticity. In this regard different groups have referred to ASCs as "major plastic cells", since they can be easily converted into other cell types requiring limited changes in environmental conditions and their transcriptome state. Cell plasticity from ASCs has been already demonstrated by several authors that prove that ASCs under certain conditions (i.e: treatment with demethylating agents, over-expression of lineage transcription factors) can be converted into different cell types, like cardiac-like cells or skeletal muscle cells (reviewed in (Poulsom et al. 2002)). Such characteristics provide new insights in the use of ASCs for treating degenerative diseases.

1.2.3. Somatic Cells

Differentiated and specialized cells in the body of an organism are called somatic or vegetative cells. Somatic cells are able to replicate themselves giving rise to cells with same properties as the originally diving one. However the capacity to self-renew from somatic cells is very limited, thus compromising their use in *in vitro* and *in vivo* studies.

Nowadays, several somatic cell types for cell transplantation can be successfully obtained *in vitro* by means of ESCs or iPSCs differentiation and transplanted to the host with remarkable success. A study performed in a Parkinsonian mouse model showed improvement in animal´s behaviour after transplantation of dopaminergic neurons differentiated from ESCs *in vitro* (Nishimura et al. 2003). In agreement, anatomical and functional recovery was shown in mice models of brain damage when transplanting

neural cells derived from ESCs (Chiba et al. 2004). Other interesting works have demonstrated the possibility to transplant pancreatic-islet like cells derived from mESCs (Kim et al. 2003).

Recently various innovative therapies involving direct introduction of somatic cells into humans have been proposed. Implantation of somatic cells can be done with two different purposes; as an *in vivo* source of molecular species such as enzymes, cytokines or coagulation factors, for example, that may help tissue natural repair; or implantation of manipulated cell populations such as hepatocytes, myoblasts or pancreatic islet cells intended to engraft and perform a complex biological function as local cells (Rangappa et al. 2010; Noguchi 2010; Schmitz et al. 2010). The clearest example of somatic cell treatment is blood transfusion, in which specialized blood cell types are directly substituted with exactly same somatic cell types. Several possible treatments have came up during the last years, some of them recognized by the Committee for Advanced Therapies (CAT) as somatic cell therapy medicinal products. In this regard the major area in which experimental approaches are closer to clinics is cancer. Somatic cells such as lymphoid effector cells specific against tumor-cells are possible to use in an autologous immunotherapy product for treatment of cancer, derived from the patient's blood. Briefly, lymphocytes and monocytes are isolated and using 5-aza-2'-deoxycytidine some cells are induced to express cancer antigens that specifically activate lymphocytes. These activated lymphocytes are next expanded *ex vivo* and the resulting cell preparation is administered to the patient (Wachowska et al. 2014). A similar approach with tumor-infiltrating lymphocytes (TIL) has been proposed for the treatment of stage III melanoma. For other diseases such as diabetes, mixture of porcine pancreatic β cells embedded in an alginate matrix are useful as heterologous treatment in human (Tatarkiewicz et al. 2001).

Recently proposed methods bet for gene therapy approaches directly in somatic cells rather than transplantation, an alternative and promising field known as "somatic cell gene therapy".

2. HEART CELL THERAPY

Regenerative Medicine relies today in two main pilars. First, tissue engineering or fabrication of fine-tuned tissues by means of the use of autologous cells in conjunction with scaffolds, and second, tissue repair by means of cell therapy or transplantation *(detailed extensively in the previous* section*).*

Cardiovascular disease remains a leading cause of death worldwide. Medical therapy, mechanical device implantation and surgical intervention are current therapeutic approaches, however their success is still limited. When cardiovascular function is severely compromised, a whole heart transplant is mandatory, but in most of the cases, donor shortage remains the major roadblock to overcome this issue.

It has been extensively described that following MI, endogenous cardiac fibroblasts, which account for more than half of the cells in the heart, proliferate and synthesize extracellular matrix, leading to fibrosis and heart failure. Unfortunately for the adult human being, cardiac regenerative capacity demonstrated in other organisms such as zebra fish is far from our reality (Poss et al. 2003; Jopling et al. 2010). As terminally differentiated cardiomyocytes in humans have almost null regenerative capacity following injury, development of cardiac regenerative therapy is highly desired. Thus, different approaches to obtain functional cardiomyocytes able to be transplanted in dysfunctional heart tissue are in development in many laboratories worldwide.

2.1. Cardiomyocytes generation

2.1.1. Molecular networks that regulate cardiac cell fate

In most animal species, early step and determinant gene decisions take place in only a few hours during embryo development. Unfortunately, decoding the signals leading to cardiac development in such a tight window difficults their proper analysis. hESCs or hiPSCs offer the possibility of study development switches *in vitro,* and systematically monitorize events occurring at different stages during cardiac development. Actually, both hESCs and iPSCs (referred as pluripotent stem cells-PSCs) offer the possibility to recapitulate in a Petri dish those determinant events that take place *in vivo*.

The heart is the first functional organ to form in vertebrate embryos. Analyses of genetic lineage tracing found that 2 distinct mesodermal heart fields with a common origin contribute to develop the heart, the first heart field (FHF) and the second heart field (SHF) (Buckingham et al. 2005). The FHF derives from cells in the anterior lateral plate mesoderm that forms a crescent shape at approximately embryonic (E) day 7.5 in the mouse, corresponding roughly to week 2 of human gestation. Then, by E8 in mice or the third week of gestation in humans, these cells join to the ventral midline by forming a primitive heart tube, consisting on two layers of cells separated by extracellular matrix for reciprocal signaling; the external layer with myocardial cells and internal with endocardial cells (Srivastava 2006a). Afterwards, those cells continuously migrate in a temporally and spatially specific manner and ultimately form a functional heart with a 4 chambers structure by E9.5 to E14.5 in mice, and week 4 to 8 in humans. In **Figure 5**, an illustration of cardiac morphogenesis in mammals is shown. Both heart fields appear to be regulated by complex positive and negative signaling networks involving members of the bone morphogenetic protein (BMP), sonic hedgehog (SHH), fibroblast growth factor (FGF), WNT pathway, and Notch proteins. Part of those signals arise from adjacent endoderm, although their precise nature and role remain unknown (Zaffran & Frasch 2002)). Among those signaling networks, GATA binding-protein 4 (*GATA4*); myocyte enhancer factor 2C (*MEF2C*); heart and neural crest derivatives expressed 2 (*HAND2*), NK2 homeobox 5 (*NKX2.5*) and T-box family members as *Brachyury* and *TBX5* control expression of cardiac genes and direct the specification and differentiation of cardiac myocytes.

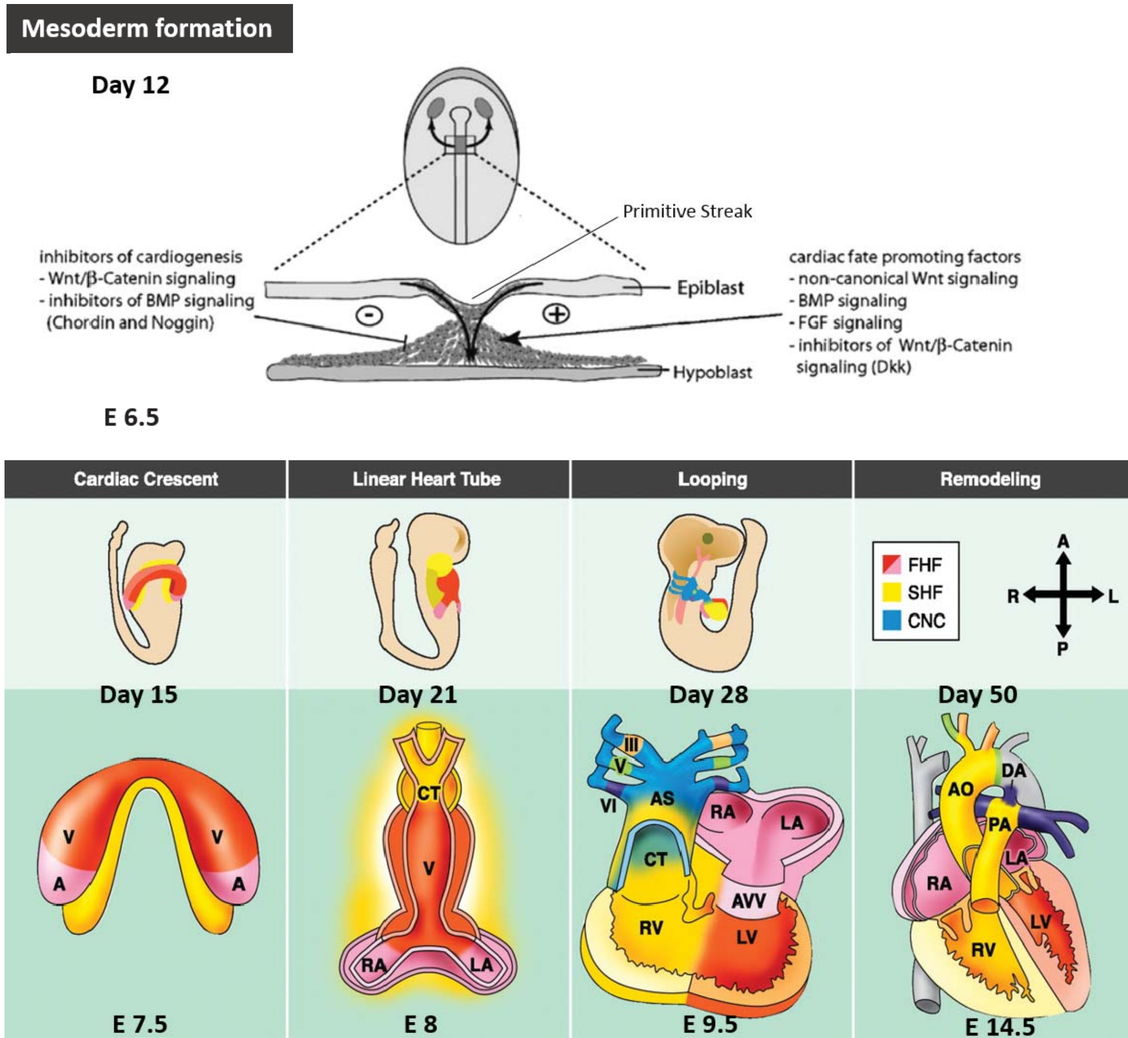


Figure 5: **Development of the mammal heart at embryonic stages**. Mesoderm formation takes place at day 12 in human, and day 6.5 in mice (E 6.5). Specification of myocardial precursor cells starts during their ingression through the anterior primitive streak region and becomes completed within the heart-forming fields. Extracellular growth factors are involved in this process. Cardiac fate promoting factors are released as BMP: Bone Morphogenetic Protein, FGF: Fibroblast Growth Factor, or DKK: Dickkopf. Besides, inhibitors of cardiogenesis are released as Noggin, to delimitate the formation of the cardiac crescent. At day 15 (E 7.5), the anterior portions of the heart-forming fields have merged to form the cardiogenic crescent. At this stage, the primary or First Heart Field (FHF) can be distinguished, with future ventricles (in red) and atriums (in pink). The Secondary Heart Field (SHF) can be distinguished too (in yellow). At day 21 or E 8.0, the linear heart tube has formed by progressive merging of the paired primary (red) and secondary (yellow) heart fields. CT: Conotruncal, V: Ventricle, RA: future Right Atrium, LA: future Left Atrium. At day 28 (E 9.5), cardiac looping is in progress and the formation of the endocardial cushions is initiated which includes specification, delamination, epithelial-mesenchymal transition (EMT) and proliferation. Mesenchymal cells form the cardiac valves from the conotruncal (CT) and Atrioventricular Valve (AVV) segments, which divide into separate left- and right-sided valves.

Neural crest cells populate the bilaterally symmetric aortic arch arteries (III, IV, and VI) and AS: Aortic Sac,that together contribute to specific segments of the mature aortic arch. The four chambers are preformed but not separated yet. RV, Right Ventricle and LV, Left Ventricle. Finally, a cross section of the heart at day 50 (E 14.5) is shown. PAS: primary atrial septum, VS: muscular anlage of the ventricular septum. The conduction system is formed by: the Ao: Aorta and DA: Ductus Arteriosus among others. Figure adapted from (Srivastava 2006b; Brade et al. 2006).

- *Early cardiac specification.*

During embryo development, several studies have shown that as early as cells enter through the cranial primitive streak, they are exposed to signalling factors expressed in and around the node, including retinoic acid (RA) and FGFs, that can induce the expression of *NKX2.5* locally (Twal et al. 1995; Niederreither et al. 1997; Lough & Sugi 2000; Lopez-Sanchez et al. 2002; Alsan & Schultheiss 2002).

Afterwards, the transcription factor (TF) MESP1 (mesoderm posterior 1 homolog), downstream FGF receptor 1 (FGFR1), is the earliest indicator of cardiogenic mesoderm specification, and it is expressed at the onset of gastrulation E6.5 to E7.5 (Saga et al. 2000). MESP1 triggers the expression of some cardiac specification factors including: NKX2.5, Insulin gene enhancer protein 1 (ISL-1*)*, and myocardin (MYOCD). In all animal models studied so far, ISL-1 is required for proliferation and survival of SHF progenitors, and maybe for their migration into the heart (Cai et al. 2008). Its early expression depends on canonical WNT signalling through β-Catenin, and also on FGF8, required for proliferation of early cardiac progenitors (Lin et al. 2007; Cohen et al. 2007; Ai et al. 2007; Kwon et al. 2007a). In this regard it has been described that WNT signalling also inhibits cardiac progenitor specification (Ueno et al. 2007). Additionally, pathways controlling endothelial and hematopoietic specification can contribute to the restriction of myocardial progenitor specification as Retinoic Acid (RA) signalling, that imposes the posterior limit of the early cardiac crescent (Schoenebeck et al. 2007).

- *Cardiomyocyte specification*

Transition from proliferative cardiac progenitors to differentiated cardiomyocytes requires both, downregulation of pro-proliferative signals and upregulation of

differentiation pathways. BMP signaling is required for cardiomyocyte differentiation, more precisely, to maintain expression of TBX20, a TF which has been described to exert a dual role during cardiac specification: it acts as a positive effector of cardiomyocyte differentiation and also as a repressor of *ISL-1* as cardiac progenitors enter into the heart (Yang et al. 2006; Mandel et al. 2010; Walters et al. 2001; Shi et al. 2000). Sonic Hedgehog (*SHH*) signaling also has dual function because is required for proliferation of SHF progenitors, and also to prevent differentiation from the SHF cells (Dyer & Kirby 2009).

For the differentiation of myocardial progenitors, down-regulation of WNT/ β-Catenin pathway is required, backwards before, when β-Catenin promotes proliferation of the SHF progenitors (Cohen et al. 2007; Ai et al. 2007; Kwon et al. 2007a). β-Catenin regulates expression of WNT11, which stimulates a non-canonical WNT pathway that controls critical aspects of cell polarity as myocardial cells differentiate (Zhou et al. 2007; Phillips et al. 2005).

Serum Response factor (SRF) is responsible of the regulation of sarcomeric genes and excitation-contraction coupling factors. Ablation of *SRF* in the *NKX2.5* expression domain results in failure to initiate cardiac beating (Niu et al. 2007). It has been demonstrated that SRF interacts with GATA4 and NKX2.5 to regulate cardiac genes (Zhao et al. 2005; Chen et al. 2006). Moreover, SRF regulates expression of some micro RNAs. Micro RNAs (miRs) are small non-coding RNAs conserved through evolution, that have post-transcriptional function affecting mRNA translation and stability, thus playing critical roles in heart development (Van Rooij et al. 2008; Callis & Wang 2008). SRF regulates expression of miR-1 and miR-133, which are derived from a common precursor transcript.

Finally, hypoxia seems to play an important role on myofibrillogenesis. Ablation of hypoxia-inducible factor 1 alpha (HIF1alpha) in differentiating myocytes results in lack of differentiation because the contractile apparatus cannot be assembled in its absence (Krishnan et al. 2008).

In summary, it seems that in order to promote cardiomyocyte differentiation it is necessary to down-regulate β-Catenin, Shh, BMP and non-canonical WNT signaling; and at the same time to stimulate SRF expression, that together with NKX2.5, and

GATA factors regulate myofibrillar gene expression. Also the induction of cardiac related microRNAs seems to be determinant to promote cell cycle withdrawal. Finally hypoxic conditions seem also to contribute to myocyte differentiation. Overall some of the conditions detailed here have been used to develop protocols aiming to generate cardiomyocyte-like cells from human PSCs.

In the last decades elegant molecular and genetic studies using ESCs and animal models have disclosed the TFs and miRs that determine cardiac cell fate. However, intense research still aims to elucidate which TFs and/or miRs conform the molecular network that establishes and maintains the cell fate of mature CMs, and more importantly, that can induce a new cardiac cell fate from terminally differentiated non-muscle related cells, such as human fibroblasts (Fu & Srivastava 2015).

2.1.2. Cardiac differentiation from PSCs

PSCs offer an unprecedented opportunity for the establishment of reproducible protocols for the generation of high yields of human cardiomyocytes. Moreover, their intrinsic properties lead to the derivation of different cardiac cells during the onset of differentiation (from proliferating cardiac precursors to finally matured cardiomyocytes).

The definition of protocols for cardiac differentiation from hPSCs has progressed considerably in the last 10 years. There are several methods reported to date that can trigger functional cardiomyocyte production in the dish. The most reproducible and efficient strategies involve activation and inhibition of the four main signalling pathways detailed above in a stage-specific manner and under chemically defined culture conditions. These strategies try to reactivate key developmental steps that occur during heart development in the early embryo.

First *in vitro* cardiac differentiation method described was based on the co-culture of hESCs with mouse visceral endoderm-like stromal cells (END-2) (Mummery et al. 2003). The amount of generated cardiomyocytes with this strategy was reported to be low, and cells were mostly resembling cardiomyocytes with ventricular-like properties. However, this protocol provided early insights into methods for improving

differentiation efficiencies by adding L-ascorbic acid (Passier et al. 2005) or removing insulin treatment between day 0 and 4 during the differentiation process (Freund et al. 2008).

A second approach consists on the induction of embryoid bodies (EBs) formation from hPSCs grown in suspension. Later EBs differentiate upon adhesion between day 4 (d 4) and d 7 to contracting outgrowths in the presence of fetal bovine serum (FBS) (Zwi et al. 2009). This protocol has been further improved by the use of some chemical additives as BMP4 during d 0-d 4 (Takei et al. 2009), WNT3A during d 0-d 2 (Tran et al. 2009; Bu et al. 2009) or inhibition at d4-d6 of the same WNT pathway by IWR-1 inhibitor (Ren et al. 2011). Another inhibitor that enhances cardiac differentiation is the MAP kinase inhibitor -SB203580 (Xu et al. 2008), specifically between d 4 and d 6 during the differentiation process (Gaur et al. 2010). More specifically, some factors can improve cardiac differentiation by enhancing cardiac progenitor cell proliferation: WNT3A (Bu et al. 2009), G-CSF (Shimoji et al. 2015), and L-Ascorbic acid (Cao et al. 2011). In the same manner other factors have been identified as potential candidates that enhance hPSC-derived cardiomyocyte proliferation, such as Insulin-like Growth Factors (IGFs)- IGF1 and IGF2 (McDevitt et al. 2005).

Other laboratories have developed protocols using serum free conditions with optimized BMP4, FGF2, Activin A and other factors as VEGFA and DKK1 combined with hypoxic culture conditions (5% oxygen) (Kattman et al. 2011; Yang et al. 2008; Willems et al. 2011). Moreover, to overcome differences in differentiation efficiencies related to EB size heterogeneity, and standardized chemical concentrations, a so-called “spin EB” method has been developed. This consists in determine the number of starting PSCs that are induced to aggregate into EB by centrifugation in V shaped cell culture plates (Burridge et al. 2007; Ng et al. 2005; Burridge et al. 2011; Elliott et al. 2011a).

Although END-2 conditioned medium protocols give rise to cardiomyocyte differentiation with efficiencies near 70% (Xu et al. 2008), and EB-related methods generate up to 60% of differentiated cardiomyocytes (Burridge et al. 2007; Yang et al. 2008), still these protocols are time consuming and technically complex. For this reason protocols based on the use of PSCs cultured as monolayers have been adapted for the generation of cardiac-like cells. This simple method allows PSCs to differentiate under

fully defined conditions without evolving the formation of EBs, deriving in the generation of cardiac homogeneous populations in larger amounts. In this regard, Laflamme and colleagues were pioneers developing such approach (Laflamme et al. 2007), and lately several other groups have improved their technique by combination of specific factors related to the cardiac specification pathway mentioned above (Paige et al. 2010). Also other studies have reported the use of a Matrigel® overlay one day *prior* differentiation in order to enhance epithelial to mesenchymal transition (EMT) (Uosaki et al. 2011). Moreover, as with EBs based protocols, it has been demonstrated that inhibition of WNT signalling pathway applied from d3 during differentiation is highly effective (Hudson et al. 2011). For this reason Lian X. and colleagues developed a growth factor- free method in where the WNT signalling pathway is first activated from d0 to d1, and subsequently inhibited from d3 to d5 (Lian et al. 2012a).

Together, the data reported with these methodologies show that four major signalling pathways are involved in early cardiac differentiation from hPSCs with temporal windows: BMP, TGFB/Activin /Nodal, WNT and FGF (**Figure 6**).

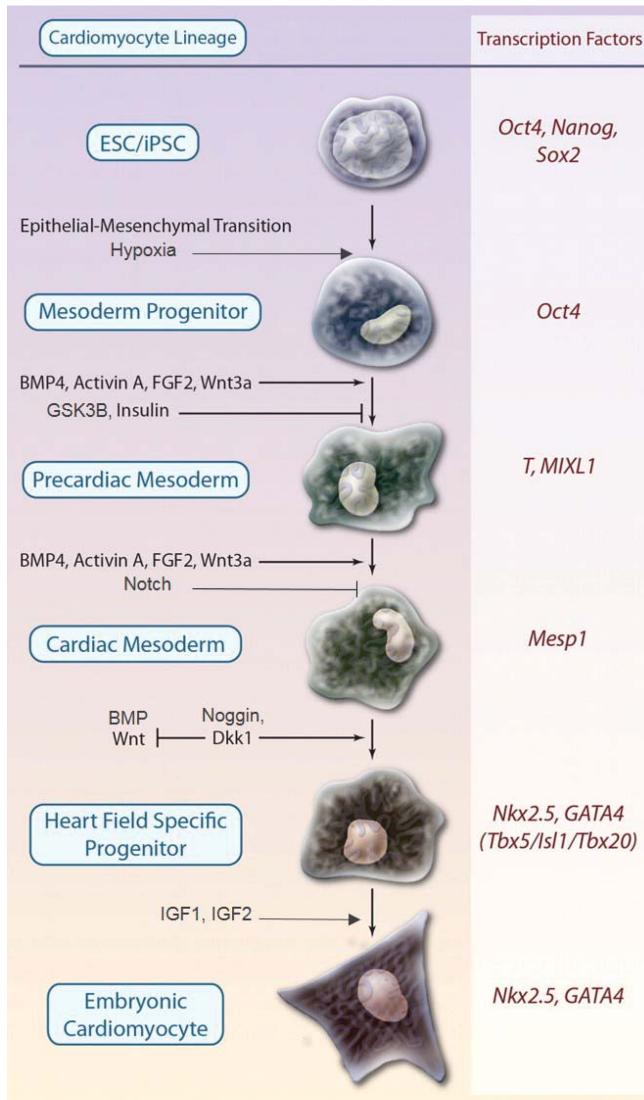


Figure 6: **Schematic of most important Factors involved in hPSC Cardiac Differentiation**. Factors that influence the progression through each of the major steps of hPSC cardiomyogenesis. Data shown are derived from available data from developmental biology models that have been directly assessed and proved functional in hPSC cardiac differentiation, a long with knowledge gained directly from hPSC differentiation. Some of the best characterized signaling pathways responsible for the sequential transitions in cell fate are shown on the left. On the right, the most important transcription factors involved in cardiac cell identity among others. Adapted from (Mummery et al. 2012).

Nevertheless, *in vitro* differentiation has several well-known bottlenecks such as incomplete programming and intrinsic variability from the starting PSCs (Osafune et al. 2008). Importantly current protocols described to differentiate PSCs to cardiomyocytes seem to give rise to immature or embryonic-like cardiomyocytes, rather than adult specialised cardiac myocytes. Characterization and functional studies have been performed to confirm that cardiac-like cells generated from iPSCs possess a fetal-like morphology (Cao et al. 2012), immature electrophysiological function (Davis et al. 2012), and embryonic related ion channel expression (Beqqali et al. 2006). Differentiation protocols are thus still aiming to generate high yields of mature cardiomyocytes. Moreover, protocols for the generation of pure populations of cardiac-cells (i.e. atrial, ventricular or peacemaker cells) are still missing.

2.1.3. Cell conversion and transdifferentiation to cardiac fate.

For a long time, differentiation process was considered to be one-way road with cell stages evolution resembling downfalls along the valleys of the "epigenetic landscape " proposed by Waddington (Waddington 1957). In parallel, it was also believed that unnecessary genetic information becomes deleted in cells 'committed' to a specific fate or state. This classical theory is known as Weismann barrier, described by Weismann in 1893 (Weismann 1893). Subsequent basic studies, however, not only suggested that 'committed' cells retain all genetic information, but that they could also change their fate in response to specific stimuli. Experiments performed in *Drosophila melanogaster*, in which cells from the imaginal discs of pupae stage were serially transplanted into the abdomen of an adult fly, demonstrated that explanted cells were surprisingly plastic, as confirmed by the formation of leg or head structures from cells originally poised to give rise to genital structures (Gehring 1967). Similarly, other studies proved that those cells could give rise to wings after transplantation (E. 1966). A similar study was performed in chick embryos demonstrating that neural crest cells from quails, were able to differentiate to bone, cartilage and connective tissue when transplanted in specific locations instructed to become bone-related tissues (Le Lièvre & Le Douarin 1975). Those preliminar experiments together with the performance of somatic cell nuclear transfer (SCNT), demonstrated unequivocally that the identity of differentiated cells can be fully reversed to any stage (Gurdon & Byrne 2005; Gurdon 2013; Gurdon & Melton 2008; Hochedlinger & Jaenisch 2002; Wilmut et al. 1997).

Following these first studies, alternative methods for cardiomyocyte generation have been developed. One of the very earliest mobiles to develop protocols for cardiomyocytes generation stands in the need to avoid long time consuming and expensive protocols. On the other hand the use of hPSCs for cardiomyocytes differentiation still carries the possibility of teratoma formation after the injection of heterogeneous populations containing undifferentiated PSCs. In this regard, those major reestrictions were efficiently solved by Efe, et al. in 2011 (Efe et al. 2011), who exposed MEFs to OCT4, SOX2, KLF4 and c-MYC ectopic expression followed with a short treatment of Jak/STAT pathway inhibitors preventing iPSCs derivation. By means of this strategy Efe and colleagues produced cardiac-like cells in a period of 12 days. Moreover, this approach avoided the need to fully reprogram the original differentiated cells, generating cardiac cells from intermediate de-differentiated cells expressing

reprogramming factors before being fully converted into PSCs. Although this approach would lead to the generation of patient-specific cardiac cells, still the efficiency of the process is reported to be low. Moreover, is still not known if transgene expression could be reactivated after transplantation.

Lineage conversion or transdiferentiation aims to convert terminally differentiated cells (cell A) into a different somatic cell type (cell B). Transdifferentiation takes advantage from the existence of lineage-associated TFs. In this regard lineage-associated TFs are characterized for the establishment and maintenance of cellular identity during development by driving the expression of cell type-specific genes while repressing the expression of other tissue related factors (Graf & Enver 2009; Feng et al. 2008). Lineage conversion was first described in 1986 by Lassar and colleagues (Lassar et al. 1986), who reported that the exposure of fibroblasts to the de-methylating agent 5-azacytidine together with the ectopic expression of Myo cDNAs, more precisely MyoD, sufficed for their conversion into myoblast-like cells (Davis et al. 1987). Subsequently, Davis identified MyoD as the “master regulator” of skeletal muscle formation (Davis et al. 1987), and other authors reported that the same factor sufficed for the generation of muscle specific cells from other somatic sources (Lassar et al. 1989; Dekel et al. 1992; Weintraub et al. 1990; Choi et al. 1990; Tapscott et al. 1988; Hopwood et al. 1989).

These initial experiments demonstrated that in some particular cases, specific TFs could directly change cell identity, without the need to generate iPSCs to be further differentiated into the cell type of interest. So far, any laboratory has identified the specific TF promoting the direct conversion of any somatinc type into cardiomyocytes. Indeed, some of the “master regulators“ related with cardiomyocyte identity as Mesp1, Nkx2.5 and Isl-1, were not required for the reprograming of mouse fibroblasts into beating cells; in fact authors used Gata4, Tbx5 and Baf60c (also called Smarcd3) transcription factors (Takeuchi & Bruneau 2009). Later Ieda and colleagues successfully reprogrammed cardiac fibroblasts into cardiomyocyte-like cells by exogenous expression of Gata4, Mef2c and Tbx5, referred as GMT cocktail (Ieda et al. 2010). Interestingly Ieda and colleagues screened for the ectopic expression of 14 different genes related with cardiac development together with epigenetic remodeling factors. In that particular work cardiac fibroblasts isolated from alpha myosin Heavy chain (*α-Mhc*)-GFP transgenic mice were used in order to monitor the process *in vitro*. Later, the original number of factors was reduced to the three factors (Gata4, Mef2c and

Tbx5) giving rise to 20 % of positive GFP spontaneous beating cells in a period of 4-5 weeks. Later, the same cocktail was used *in vivo* by Qian L 2012, who converted in mice, adult cardiac fibroblasts into functional cardiomyocytes (L. Qian et al. 2012). Moreover, the addition of Hand2 to the GMT cocktail enhanced cardiomyocyte generation *in vitro* and heart repair after transplantation in mice (Song et al. 2012).

By that time, several new approaches came up. One study reported successful direct reprogramming of cardiac fibroblasts *in vivo* and *in vitro* by using miRs; specifically miR-1, -133 and -208 and 499 (Jayawardena et al. 2012). All together these findings have encouraged the scientific community to test similar approaches in the human setting, realizing that GMT does not suffice to cardiomyocyte generation from human fibroblasts. However, interesting results have been reported in human by addition of MESP1 and MYOCD in the GMT cocktail (Wada et al. 2013); or MESP1 and ESRRG (Estrogen-related receptor gamma) (Fu et al. 2013). Also by replacing MEF2C from GMT cocktail with HAND2 together with MYOCD, miR-1 and miR-133 (Nam et al. 2013).

Overall the different reports published up to now reveal the impact of the cell of origin to be reprogrammed into cardiomyocytes in order to assess reproducibility and robust results. In this regard, it seems that cardiac fibroblasts are more efficient than adult fibroblasts for this purpose. Although promising results have been achieved in the field, none of discussed protocols is 100% efficient, neither reproducible, preventing the translation of these findings into the clinical context.

2.2. Cardiac Tissue Engineering

Recently, Chong (Chong et al. 2014) reported that directed cardiac differentiation emulating developmental cues from PSCs, generated cardiomyocytes efficiently; and more importantly, that transplantation of hES cell-derived cardiomyocytes can remuscularize substantial amounts of the infarcted areas in the monkey heart. Although this study reveals important findings for the scientific community, authors also reported that ventricular arrhythmic complications are observed after hES-derived cardiomyocyte transplantation.

Lately, several groups have also described the possibility to differentiate hPSCs in the presence of heart scaffolds of cadaveric origin (Scarritt et al. 2015). This approach represents an invaluable platform for the derivation of cardiac like cells profiting the specific signals from the cardiac extracellular matrix (ECM). Tissue engineering approaches have been used to design synthetic and natural three dimensional (3D) scaffold materials to mimic the structural, biochemical, and mechanical properties of the stem cell niche (Peerani & Zandstra 2010; Di Nardo et al. 2011). Synthetic and natural scaffolds have been designed to provide support for cardiac muscle growth and allow myofibroblasts to rebuild their native ECM. Natural scaffolds based on ECM proteins such as fibrin and collagens have been used to form hydrogels for musculo-skeletal tissue engineering (Liao & Zhou 2009; Walters & Stegemann 2014; Tamayol et al. 2013). Commercially available ECM substitutes such as Matrigel® hydrogels are also showing promising results in the differentiation of PSCs towards cardiomyocytes (Gu et al. 2014). However, current ECM protein-based scaffolds are limited by their immune rejection and scaling up technologies. Synthetic scaffolds, which can be fabricated with ideal architectures at the nanoscale, pore sizes and mechanical properties, represent an advantageous solution to mimic the 3D ECM microenvironment. Technologies such as electrospinning, which allows organizing the polymers into thin sheets of fibrous meshes, are promising in this field (Aviss et al. 2010; Mertens et al. 2014). Recently, it has been proved the reprogramming of mouse fibroblasts onto cardiomyocyte-like cells on polyethylene glycol (PEG) hydrogels functionalized with laminin and RGD peptides (Smith et al. 2013). This opens new perspectives toward the use of custom-engineered synthetic scaffolds in the differentiation of PSCs to muscle cells. Finally, the use of acellularized tissue scaffolds is also being explored in cardiac regeneration (Sánchez et al. 2015). The last offer a native ECM with the optimal biochemical and mechanical properties for cell culture and preserve the architectural features of the tissue. Their use as a matrix supporting the commitment of cardiac muscle cells has been recently reported, thus showing the potential of this approach (Oberwallner et al. 2014). In this line several biopolymers have been developed and used for this purpose such as: collagen (Chimenti et al. 2011), alginate and gelatine (Rosellini et al. 2009), chitosan (Wang et al. 2010), silk (Patra et al. 2012) or fibrin (Godier-Furnémont et al. 2011), among others.

Interestingly in the last years several groups have reported the use of an engineered patch instead of a simply injection of cells into the damaged heart. In this regard the use of cell sheets and thermosensitive surfaces for cell culture provided interesting results within the last years (Miki et al. 2012; Haraguchi et al. 2013; Matsuura et al. 2011). These findings contrast with other studies reporting retention rates around 10% when injecting cells directly into the damaged cardiac tissue in experimental animals (Zhang et al. 2002; Dow et al. 2005; Nelson et al. 2009; Mauritz et al. 2011); or when intracoronary infusions have been executed in patients (Hofmann et al. 2005). Of note Okano's group (Yoshiki Sawa, Shigeru Miyagawa, Taichi Sakaguchi, Tomoyuki Fujita, Akifumi Matsuyama, Atsuhiro Saito, Tatsuya Shimizu 2012) have recently reported on the possibility to implant cell sheets into a patient with severe dilated cardiomyopathy.

3. STRATEGIES FOR GENE EDITING IN PSCs

Genome engineering is an encouraging discipline in biological science that opens new insights in Regenerative Medicine. Modification of basic cell function either by gene correction, deletion, addition, disruption or mutation permits to generate cell lines for drug discovery, cell tracking, and disease modelling or direct gene therapy. PSCs are an ideal source for genetic modification due to their fast and indefinitely proliferation in culture, as well as for their ability to differentiate into multiple cell types. However, gene transfer into PSCs has been a challenge in terms of identifying the ideal platform according to its final application. This choice is directly dependent on length and strength of expression required. There exist multiple methods which can be utilized to alter either ESCs or iPSCs and differ in parameters such as: stable or transient modification of the host cell, methods based on viral or non-viral vectors, integration or non-integration of the foreign DNA, site specific or random targeting in the host genome. All these issues are going to be extensively discussed below.

3.1. Gene Transduction

Gene transduction or insertion consists on introducing foreign DNA material in a host cell with the aim of modifying expression of certain gene of interest. Transfection of exogenous DNA into mammal cells can be done by multiple methods. Direct methods consist to introduce naked DNA either by electric pulse, electroporation or by microinjection, only possible in certain types of cells. Other methods are chemical-based, by using protein envelopes or transfection reagents to package DNA material and enter through cell membrane by diffusion. Most popular reagents are Lipofectamine®, Fugene® or StemFect®. When DNA of interest is difficult to introduce in cells, viral delivery systems are one of the best-proved alternatives. Combination of optimal vector design and cloning, containing proper signals for integration or selection and efficient gene delivery system are crucial to succeed modifying PSCs such hard to transfect.

Methodologies to integrate exogenous DNA information in a cell can be classified by multiple criteria. However, two major categories can clearly classify methods according to the destiny of foreign DNA, **Integrative** or **Non-integrative methods**.

3.1.1. Integrative Methods

Integrative methods are referred to those procedures that involve the insertion of the exogenous DNA material in the genome of the host cell. These methods enable users to create stable or long-term systems in PSCs. Integration of DNA can take place in a **Random** or in a **Directed** manner.

3.1.1.1. Random Integration methods

Random integration leads to variable copy number of the transgene of interest per cell; such approach leads to unpredictable transgene expression patterns depending on the locus where those fragments are integrated in. Sometimes, the locus of insertion can be partial or completely silenced in human PSCs due to culture pressure and selection leading to transgene silencing. Another major risk with random integration is the insertional mutagenesis resulting in genome instability and toxicity (Baum et al. 2006). Historically, random integration took advantage from viral vectors, since they can introduce their genetic material into the host cell taking advantage of the host replicative machinery. Commonly, lentiviral and retroviral vectors have been widely use in PSCs for different purposes. These methodologies are discussed below and are divided in: A) ***Viral*** and B) ***Non viral strategies*.**

A) Random integration methods with viral vectors

- Lentiviruses

Recombinant lentiviruses use common surface glycoproteins for cell membrane fusion; therefore they are able to infect a wide range of cell types including human PSCs.

Lentiviruses have been used as a genetic tool in human ESCs since Thomson's laboratory showed expression of GFP in ESCs with minimal silencing upon differentiation to hematopoietic precursors (Ma et al. 2003). Lentivirus transduction often requires a single infection and cells can be infected with minimal manipulation. Over-expression with lentivirus can provide a selection tool for pluripotency,

differentiation intermediates or markers of specific lineage promoters. Lentivirus have been used to over-express miRs into ESCs, and to identify and/or monitor repression and expression of key pluripotency genes (Xu et al. 2015). Unfortunately, their high transduction efficiencies are linked with a major risk of insertional mutagenesis and multiple proviral integrations, leading to alternative splicing and aberrant transcript expression (Moiani et al. 2012).

- Retroviruses

Retroviruses are enveloped viruses of single stranded RNA that replicate in a host cell through a process called reverse transcription. Once in the cytoplasm, RNA is retro-transcribed to a DNA intermediate able now to enter the nucleus and to incorporate into the host genome by an integrase enzyme. Those integrated fragments are called provirus and since that moment they are transcribed and translated as part of the cells genome. These infections persist indefinitevely, however they are replication competent virus, meaning they are able to infect only dividing cells. One of the first publications regarding the use of retroviruses for gene transfer came up in 1987, when Dr. Jack Price demonstrated that retroviruses encoding gene of β-galactosidase, the *lacZ* gene, could be successfully introduced into the rat retina to label cells to be detected histochemically upon transplantation (Price et al. 1987). Although retroviruses offer several interesting properties they lead to random insertional mutagenesis, as lentiviruses, due to their random integration in the genome host.

B) Random integration methods without viral vectors

- Nacked plasmids

The transfection of nacked plasmidic DNA by direct electroporation has been a wide used system for the expression of the gene of interest into the target cells. This method provides relatively simple integrating engineering platform since it requires no additional recombination or preparation *prior* transfection. In ESCs, plasmid insertion is limited when electroporation is used, since efficiency of transfection decreases when using large vectors (Moore et al. 2010). Commonly, DNA is previously linearized (by

means of enzymatic restriction) or encapsulated using commercial or in house transfection reagents (e.g; Fugene®, Poly-ethylenimine, etc).

- Transposons

Transposons or transposable elements (TEs) are DNA sequences capable to change their position within the genome, thus they are mobile genetic elements. These non-viral vectors offer efficient delivery and integration into the host genome and have been also widely used as mutagenesis tool.

The Sleeping Beauty (SB) transposon system has been used to generate modified human ESCs (Wilber et al. 2007). Subsequently SB- modified ESCs have been also used for further differentiation (Orbán et al. 2009). This system (Luft 2010) was awarded as the Molecule of the year in 2009, and it is currently investigated for use in human gene therapy. Other works have described the use of transposons PiggyBac or Tol2 for similar purposes (Ivics & Izsvák 2006; Wilson et al. 2007; Hackett et al. 2011).

3.1.1.2. Specific site integration methods

This is the ideal method to avoid variable expression patterns and copy number variation due to random integration triggered by the methods described above. By these technologies, researchers can select specific sites where silencing is minimized, in order to target the gene of interest and ensure the expression of integrated exogenous sequences. Selection is recommended when using these methods, since clones with low number of transgenic insertions can be selected. Methods following this strategy are described below.

- ***phiC31 integrase***

The phiC31 integrase is a bacteriophage sequence-specific recombinase that mediates recombination between two sequences termed attachment sites (*att*), one found in the phage and the other in the bacterial host (*attP*). phiC31 integrase has been show to function efficiently in many different cell types including mammalian cells. Human cells contain a number of pseudo *attP* sites, which allow for localized recombination. Moreover, there are hot spot *attP* sites, which are higher likelihood of recombination. In

the presence of phiC31 integrase, an *attB*- containing donor plasmid can be unidirectional integrated into a target genome through recombination at *pseudo-attP* sites. phiC31 integrase can integrate a plasmid of any size, as a single copy, and requires no co-factors. The integrated transgenes are stably expressed and heritable. One hot spot in hESCs is located on Chromosome 13, which is a known intronic region unaffected by chromatin remodeling during differentiation (Chalberg et al. 2006; Thyagarajan et al. 2008) and genes inserted into this site show sustained expression. This feature has been utilized to place R4 integrase target site to create a Target ESC line that can be rapidly re-targeted in order to generate modified ESCs expressing genes of interest (Thyagarajan et al. 2008; Liu et al. 2009; Xue et al. 2012).

- *Adeno-associated virus (AAV)*

Adeno-associated virus (AAV) vectors efficiently transduce various cell types in both, dividing and quiescent stages, and can produce long-term expression of transgenes *in vivo*. Although AAV vector genomes traditionally persist within cells as episomes, vector integration has been observed in various experimental settings. Vector integration can occur at non-homologous locations that are sites of DNA damage, or at specific sites via homologous recombination. Wild-type AAV can integrate in a site-specific manner in a region on the long arm of chromosome 19 (19q13-qter), termed the AAVS1 site (McCarty et al. 2004; Samulski et al. 1991; Kotin et al. 1990). However, it can also integrate at sites in the genome other than AAVS1, as demonstrated by the identification of a provirus on chromosome 1q31.1 in human tonsillar tissue and in multiple different chromosomes *in vitro* (Schnepp et al. 2005; Drew et al. 2007).

Synthetic AAV vectors have also been designed to integrate at the AAVS1 site, however it can only carry cassettes of maximum 1Kb size, an inconvenience and limiting factor when complex cassettes are needed. On the other side, AAV causes very mild immune response in human cells with apparent lack of pathogenicity. This feature has made AAV very attractive candidate for gene therapy and generation of isogenic human disease models. Nowadays, several promising clinical trials have been completed or are ongoing by this methodology. For example AAVs clinical trials have been designed for the treatment of age-related macular degeneration, cystic fibrosis or congestive heart failure (Kotterman & Schaffer 2014; Carter 2005).

3.1.2. Non-Integrative Methods

- Transient

Transient transfection is characterized by the limited period of time that the introduced nucleic acid sequence exists in the cell. Transiently transfected genetic material is not passed from generation to generation during cell division, and within few days most of the foreign DNA is degraded by nucleases, or diluted by cell division. Generally after one week the presence of the foreign DNA is no longer detected. However, the high copy number of the transfected genetic material leads to high levels of expressed protein within the period that it exists in the cell. The optimal time interval depends on the cell type, research goals, and specific expression characteristics of the introduced gene. Short interference RNAs (siRNAs), miRs, mRNAs, and even proteins can be also used for transient transfection. While transfected DNA is translocated into the nucleus for transcription, transfected RNA remains in the cytosol, where it is expressed within minutes after transfection (mRNA) or bound to mRNA to silence the expression of a target gene (siRNA and miRNA). The microRNAs for nuclear reprogramming of somatic cells are commonly used in many laboratories around the world in order to generate clinically grade iPSCs (Warren et al. 2010).

- Episomes

In eukaryotes, episomes are non-integrated extra-chromosomal circular DNA molecules that may replicate in the nucleus (Van Craenenbroeck et al. 2000; Colosimo et al. 2000). Episomes behave similarly to plasmids in prokaryotes, being stably maintained and conserved as replicative elements for a period of time in the host cell. Several cancer viruses such as Epstein-Barr Virus and Kaposi's sarcoma-associated herpesvirus (KSHV), have being commonly used in order to transiently express the sequences of interest in a wide range of cells. The Epstein Barr Nuclear Antigen 1 (EBNA) has been used for stable expression in mammalian for a variety of applications (Yates et al. 1984). In 2006, Ren and Zhao demonstrated that EBNA could be used for the expression of an enhanced green fluorescent protein (EGFP)/puromycin resistance gene cassette along with or without oriP in human ESCs (Ren et al. 2006). Later, in 2009, Thyagarajan and Scheyhing (Thyagarajan et al. 2009), achieved optimization and design of the vector oriP/EBNA1 and they generated stable GFP expressing human ESCs lines.

The vectors from Thyagarajan and Scheyhing, have two components of the Epstein-Barr virus, OriP and EBNA1. The EBNA1 sequence encodes a protein and expresses it from its viral promoter after transduction into human somatic cells. The EBNA1 protein in turns, recognizes the OriP sequence and induces plasmid amplification coincident with DNA amplification of the host cell. This system enables relatively high and long-term expression of the reprogramming factors. Interestingly, in 2013 Okita and colleagues successfully generate iPSCs from blood cells by the addition of EBNA for episomal amplification (Okita et al. 2013).

Episomal vectors are nowadays a well-described and even commercial system for producing transgene-free, virus-free iPSCs. Originally the findings were published by Okita K and collaborators in 2011 (Okita et al. 2011) by using a combination of 3 episomes with Oct4, Sox2, Lin28, Klf4, and c-Myc reprogramming factors.

- Minicircles

Minicircles are DNA plasmid-based vectors that do not contain bacterial elements such as OriP or antibiotic resistance, due to prior excision by recombination catalysed by PhiC31 integrase (Jia et al. 2010). Those mammalian expression minicircles have shown higher and longer expression. Moreover, these vectors provide an ideal non-integrating and foot print- free method (Chen ZY, He CY, Ehrhardt A 2003), thus this system has been used to generate human IPSCs (Jia et al. 2010) as well as to modify neural stem cells (Madeira et al. 2013).

- Baculovirus

As baculovirus does not retain ability to replicate and become infectious in mammalian cells, researchers have took advantage of this system to generate a transient over-expression tool by incorporating mammalian promoters: The Baculoviral Mammalian expression platform (BacMam). BacMam has become a standard gene transfer technique due to several advantages. From one side, its safety, easy transfection and apparent zero cytotoxic effects on human cells (Gao et al. 2002; Kost et al. 2005). On the other side, it has capacity for the transfer of large cassetes (>35kb) providing a flexible system for introduction of complex engineering fragments (Fornwald et al. 2007). BacMam has been used for example to directly drive bone differentiation by overexpression of key proteins in the process (Chuang et al. 2007).

3.1.2.1. Non-Integrative Methods with viral vectors

- Adenoviruses

Adenoviruses (AVs) carry a DNA molecule that is delivered directly into the nucleus of the host cell and is transcribed like any other gene in the host genome. However, AVs extra-genes are not replicated when the cell is about to undergo cell division, so descendants will not have the extra gene. This characteristic confers a drawback when using AVs as a method to generate genetically modified cells, as the transgene over-expression won´t last indefinitely. Interestingly, this disadvantage is tremendously useful in transient expression requirements, such as in reprogramming and cell conversion protocols, or furthermore, in cell and gene therapy treatments. Even though AVs were originally believed to be safe in the clinical setting, concerns were raised after the death of Jesse Gelsinger while participating in a gene therapy trial in 1999. Since then, work using AVs has focused on genetically crippled versions of the virus. Indeed, there are already approved treatments based on AVs such as p53-based adenoviral treatment for cancer [e.g: gendicine, approved by Chinese food and drug regulators in 2003; Advexin, turned down by the US Food and Drug Administration (FDA) in 2008].

3.2. Gene Targeting and Editing

Technologies that allow to routinely and efficiently edit the genome, by directing mutations in a targeted fashion, truly enhance understanding of basic biology and potentially leads to novel ways of treating human disease. Targeted gene editing is the method of choice to modify human ESCs and iPSCs in order to generate disease model phenotypes, corrected disease cell lines, and to engineer *knock out* and *knock in* cell lines.

Overall, these methods rely on naturally existing DNA-repair mechanisms found in most cells upon DNA damage. When double strand break occurs in the genome, the cell has the endogenous capacity to repair it either via Homologous Recombination (HR) or non-homologous end joining (NHEJ) mechanism. Gene targeting and editing

technologies take advantage of these endogenous mechanisms to incorporate, delete or change genes as desired.

3.2.1. Recombineering Methods for genome editing.

Recombineering is an *in vivo* method of genetic engineering based on HR. Naturally HR occurring in cells upon damage consists on the use of the second copy of a chromosome as a template to repair the damage and restore gene sequence.

Primarily used in *E.Coli,* (Sawitzke et al. 2007), recombineering allows insertion, deletion or alteration of any sequence precisely and is not dependent on the location of restriction sites. Exogenous linear DNAs, either double-stranded, provide the homologous substrates to create genetic changes. Recombineering is catalyzed by bacteriophage-encoded homologous recombination functions, such as the coliphage l Red system and the RecET system from the Rac prophage. While Mario Capecchi and colleagues were the first to suggest mammalian cells had the machinery capable for homologous recombination with exogenous DNA (Folger et al. 1982), Smithies and colleagues were the first ones to demonstrate that targeted gene insertion was possible in the β-globin gene (Smithies O, Gregg RG, Boggs SS, Koralewski MA 1985).

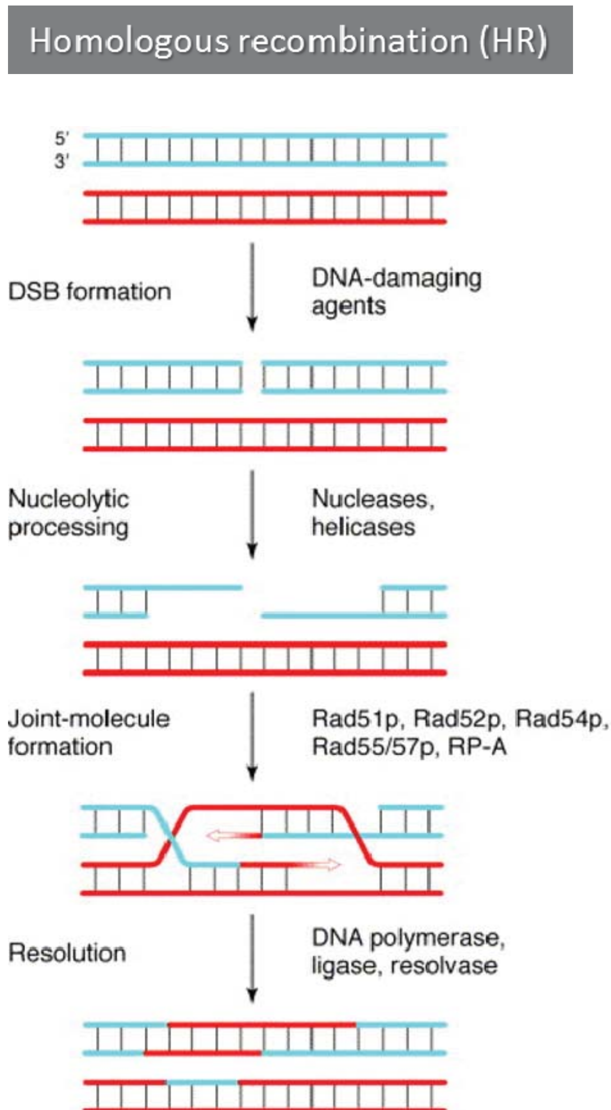


Figure 7: **Simplified scheme of double-strand break (DSB) repair by homologous recombination.** Since a double-strand break is involved in most initiation events, this model, it's the most general form, likely can apply to mechanisms of homologous recombination in all organisms. The break produced by endo- or exogenous factors is processed by nucleases and helicases to produce 3' single-strand tails. Recombination factors such as Rad proteins, nucleate onto the single-strand tails to form an active presynaptic filament. This complex invades the homologous DNA duplex to form a D-loop structure. Subsequent steps include DNA synthesis, resolution of DNA intermediates, and ligation to generate mature recombinants.

Ultimately, the advances of Evans in the culture of ESCs cells (Evans MJ 1981), together with the findings of Smithies and Capecchi on HR, were recognized with the Nobel Prize in Physiology or Medicine in 2007. The field of HR has allowed for many scientific discoveries, primarily through the creation of *knock out* animals and cell lines (T. Zwaka & Thomson 2003; Urbach et al. 2004; Irion et al. 2007; Di Domenico et al. 2008). In the field of ESCs, HR has been used to *knock in* genes of interest in specific locus aiming to generate reporter ESCs lines (T. P. Zwaka & Thomson 2003; Ng et al. 2008; Xue et al. 2015; Elliott et al. 2011a; Goulburn et al. 2011). Although this methodology has opened new venues for the generation of genetic modified organisms,

still the method shows low efficiencies and needs laborious and tedious manipulations due to the requirement of targeting constructs with long homology arms subjected to time consuming drug-selection processes.

3.2.1.1. Engineered DNA nucleases

Engineered DNA nucleases are enzymes that have been specifically developed to induce double strand breaks (DSB) at a unique and defined nucleotide in the cell genome. The reason of promoting DSB is to increase the rate of DNA repair by HR by 10^3 to 10^4 fold (Porteus & Carroll 2005). In general, engineered DNA nucleases, are formed by a nuclease domain plus a DNA binding domain whose sequence can be modified as desired.

3.2.1.1.1. Meganucleases

Meganucleases, also called homing endonucleases, are found in microbial species, with unique property of having very long recognition sequences (>14bp) thus making them naturally very specific (de Souza 2011; Smith et al. 2006). Meganucleases can be exploited to make site-specific DSB to promote HR. They can be artificially generated to recognize unique sequences (Chevalier et al. 2015; Smith et al. 2006). Use of these "molecular scissors" cause less toxicity in cells because of more stringent DNA sequence recognition. However, the construction of each specific enzyme is costly and time consuming, thus, they are not extensively used as a first choice method for HR.

3.2.1.1.2. Zinc Finger Nucleases (ZFN)

ZFNs are artificial nucleases generated as a product of fusion between a zinc finger DNA-binding domain and a DNA-cleavage domain. The DNA-binding domain is linked to the nuclease domain of the restriction enzyme FokI, naturally found in *Flavobacterium okeanokoites*. These ZFN dimers can be designed to bind to a genomic sequence of 18-36 nucleotides long (Porteus & Carroll 2005; Carroll 2011). ZFNs have become useful reagents for manipulating the genome of a wide range of species including human cells.

Lombardo et al. in 2007 (Lombardo et al. 2007), transferred ZFNs with specific donor DNA into hESCs and human lymphoblastoid cells achieving to integrate 800-bp cDNA into the IL2RG locus with a 5% of targeting efficiency. Thereafter, ZFNs were widely used to create or correct specific mutations in hPSCs (Hockemeyer et al. 2009; Asuri et al. 2012; DeKelver et al. 2010; Lombardo et al. 2011; Mussolino et al. 2011; Sebastiano et al. 2011; Soldner et al. 2011; Yusa et al. 2011; Zou, Sweeney, et al. 2011; Zou, Mali, et al. 2011). Results in these studies showed permanent and heritable gene modifications at specific endogenous loci.

One of the major concerns of ZFNs is the off-target effect. Some studies claim possible chromosomal instability and genotoxicity (Gabriel et al. 2011; Pattanayak et al. 2011). Even the on-target effect of ZFNs can cause genotoxicity, as repair of ZFN cleavage sites leads to frequent mutations (Lombardo et al. 2007; Joung et al. 2010) or abnormal integrations of donor DNA (Hockemeyer et al. 2009).

3.2.1.2. Transcription activator-like effectors (TALE) nucleases (TALEN)

TALE were first identified in the *Xanthomonas sp* bacteria. Natural structure of TALE proteins consist on a DNA binding domain composed of 33-34 aminoacids strongly conserved excepting amino acids in 12th and 13th position, which are highly variable and are called repeated variable diresidues (RVDs), those RVDs are responsible for nucleotide recognition (Boch et al. 2009; Moscou & Bogdanove 2009).

The intrinsic properties of TALEs have allowed for the engineering of specific DNA binding domains by selecting a combination of repeated segments containing appropriated RVDs (Scholze & Boch 2011). Engineered TALE DNA binding domains can be fused to different active domains generating a wide range of gene editing tools with custom DNA specificity. There are reported fusions to transcriptional activators (F. Zhang et al. 2011b), repressors (Cong et al. 2012), chromatin remodeling enzymes (Konermann et al. 2013; Mendenhall et al. 2013; Maeder et al. 2013), and recombinases (Mercer et al. 2012). Nowadays TALE DNA is commonly bound to nuclease domains such as FokI, I-TevI, PvuII and I-AniI (Christian et al. 2010; Beurdeley et al. 2013; Yanik et al. 2013; Boissel et al. 2014) enabling the generation of TALE nucleases

(TALEN). TALEN cleave DNA as dimers (Li et al. 2011) promoting DSB into the targeted sequence determined by the DNA binding domain.

TALENs have been tested in mammalian cells, and more recently, in both hESCs and hiPSCs for gene integration in the *PPP1R12C* (the *AAVS1* locus), *OCT4* and *PITX3* loci (Hockemeyer et al. 2012), at precisely the same positions as targeted earlier by the same group with ZFNs (Hockemeyer et al. 2009). Gene correction has been also successfully performed in different genes (*COL7A*, *HBB*, *DMD* and *XPC*) involved in recessive dystrophic epidermolysis bullosa (Osborn et al. 2013), in genes determinant for sickle cell anemia (SCD) (Ma et al. 2013), in Duchenne dystrophy (Li et al. 2015) and *Xeroderma pigmentosum* syndrome (Dupuy et al. 2013).

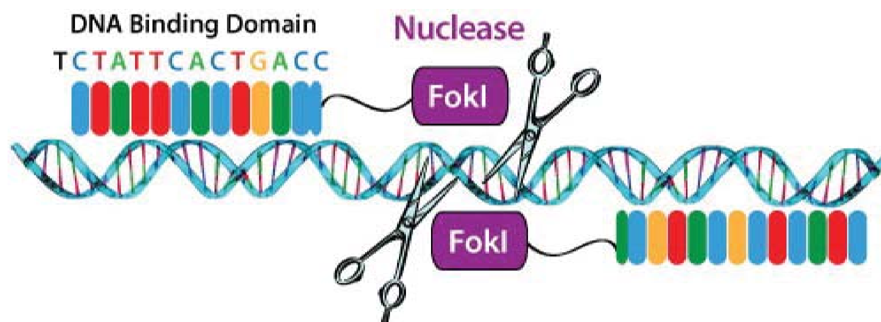


Figure 8: TALENs can be used to generate site-specific double-strand breaks to facilitate genome editing through non-homologous repair or homology directed repair. Two TALENs target a pair of binding sites flanking a 16-bp spacer. The left and right TALENs recognize the top and bottom strands of the target sites, respectively. Each TALE DNA-binding domain is fused to the catalytic domain FokI endonuclease; when FokI dimerizes, it cuts the DNA in the region between the left and the right TALEN-binding sites. Image modified from *http://www.biocat.com/products/CS810TN-1-SBI.*

3.2.1.3. Clustered regularly interspaced short palindromic repeats (CRISPR)/CAS9

The CRISPR-associated (CAS) system is an adaptive immune defence mechanism naturally present in bacteria and *archaea* against invasion of foreign nucleic acids from exogenous plasmids or bacteriophages (Barrangou et al. 2007; Marraffini & Sontheimer 2008; Bhaya et al. 2011; Garneau et al. 2010; Hale et al. 2015). The system use short RNAs to direct degradation of foreign nucleic acids. The CAS system is composed by a

short non-coding guide RNA (gRNA) with two molecular components, a target-specific CRISPR RNA (crRNA) and an auxiliary *trans*-activating crRNA (tracrRNA) and a nuclease associated to the CRISPR locus. The gRNA is the one in charge to guide nuclease protein CAS to the specific genomic locus. Once there, following Watson-Crick base pairing, the crRNA binds to the target sequence. The tracrRNA then is recruited and leads the CAS nuclease binding to the crRNA/tracrRNA/DNA complex. CAS protein then induces a double-stranded break at the specific target sequence (Sashital et al. 2012; Wiedenheft et al. 2012).

Different strategies have evolved in the prokaryotic kingdom but type II CRISPR/CAS9 system is the most frequently used tool for genome editing (Makarova et al. 2011). Components of this system have been further adapted for use in eukaryotic species by means of codon optimization (Mali et al. 2013), addition of nuclear localization signals to the open reading frame of the nuclease (Friedland et al. 2013), or even mitochondrial signal (Reddy et al. 2015), or fusion of crRNA and tracrRNA to generate a single guide RNA (sgRNA) (Jinek et al. 2012). Moreover, to overcome off-target cleavage reported effects mainly due to the short homology sequence in the sgRNA (just 20bp), different works have modified CAS9 in order to introduce nicks in only one strand (Mali et al. 2013; Cho et al. 2014). By combination of two sgRNAs simultaneously for sequences close to each other in the locus of interest the two generated single offset nicks are able to generate DSBs, a design that resembles analogous to TALENs that work as dimers.

The CAS9 system is revolutionizing the field of genome editing. To date, CAS9 has been broadly used to achieve genome editing in a variety of species and cell types (see Sander and Joung 2014 for a detailed list) (Sander & Joung 2014). DSBs induced by CAS9 have been used to introduce NHEJ-mediated Insertion-Deletion (Indel) mutations as well as to stimulate HR in the presence of DNA donor vectors double or single stranded. The capability to introduce DSBs at multiple sites in parallel using the CAS9 system is a unique advantage of this platform relative to meganucleases, ZFNs or TALENs. Multiple gRNAs have been used with CAS9 to induce small and large deletions or inversions at the DSB site (Cong et al. 2013; Xiao et al. 2013; Upadhyay et al. 2013; Horii et al. 2013). Also to simultaneously introduce mutations in multiple genes in rat cells (Li et al. 2013), mESCs (H. Wang et al. 2013) and somatic cells from zebrafish (Jao et al. 2013). CAS9 has been also used for disruption of DNA methyltransferases (DNMTs) in hESCs (J. Liao et al. 2015).

CAS9 system has been proposed as a new therapeutic strategy against viral infections. Probably one of the breaking studies of last months is the one carried by Liao HK and colleagues, whose taking HIV-1 infection as a model, have demonstrated that CRISPR/CAS9 system disrupts latently integrated viral genome and provides long-term adaptive defense against new viral infection, expression and replication in human cells (H.-K. Liao et al. 2015).

CRISPR/CAS9 has been also used to correct disease-causing mutations in iPSCs. That is the case of the study leaded by Ma and colleagues (2013), where they were able to correct and model Beta-thalassemia (also called Cooley's anemia) (Ma et al. 2013). In the same line Sun and Zhao (2014) applied CRISPR/CAS9 system to correct Sickle Cell Anemia (SCA) derived patient-specific iPSCs by targeting human β-globin gene (*HBB*) (Sun & Zhao 2014).

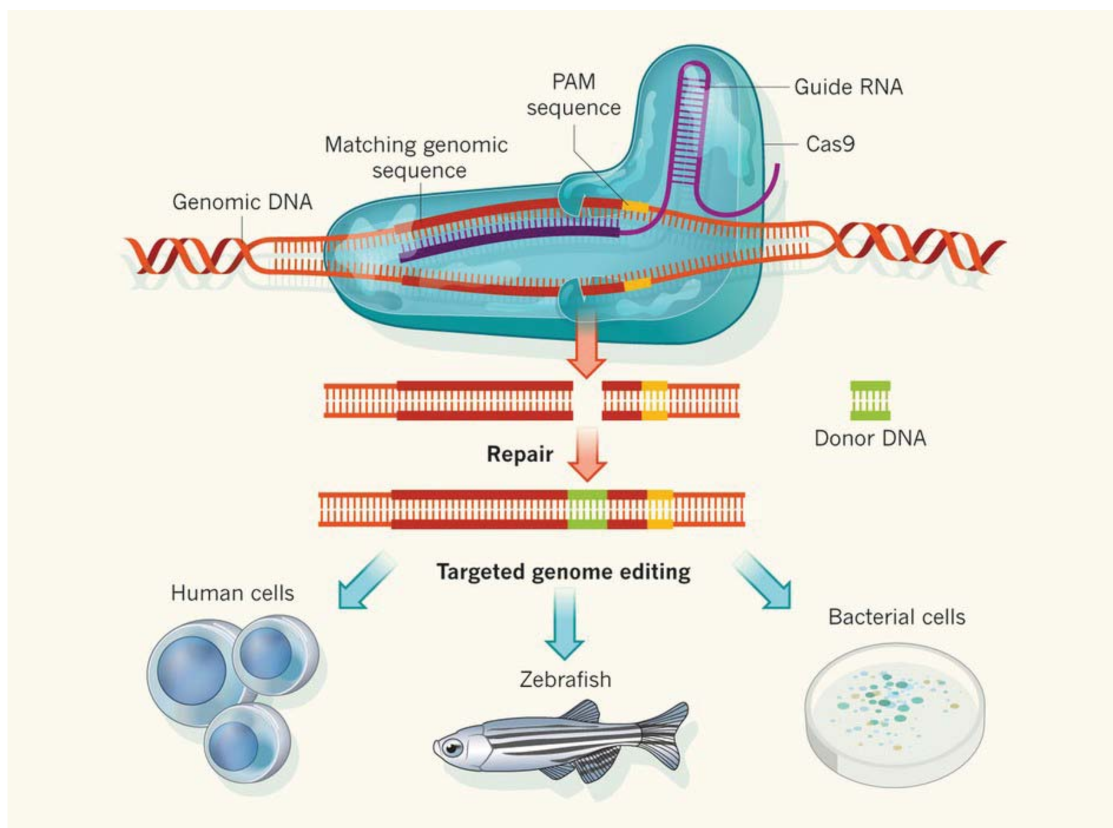


Figure 9: Targeted genome editing with RNA-guided Cas9. The Cas9 nuclease, found in bacteria, functions as part of a defence system against invading DNA molecules, such as viruses. Cas9 has two active sites that each cleave one strand of a double-stranded DNA molecule. The enzyme is guided to the target DNA by an RNA molecule that contains a sequence that matches the sequence to be cleaved, which

is demarcated by PAM sequences. RNA-guided Cas9 activity creates site-specific double-stranded DNA breaks, which are then repaired by either non-homologous end joining or homologous recombination. During homologous recombination, the addition of donor DNA enables new sequence information to be inserted at the break site. RNA-guided Cas9 systems can be used to engineer the genomes of human and mouse cell lines bacteria and zebrafish. Original image from Charpentier & Doudna Review, 2013 (Charpentier & Doudna 2013).

3.3. Reporter Cell lines

A reporter gene is a known gene that can be attached to a regulatory sequence from a gene of interest, thus conferring characteristics to the bacteria, cell or organism expressing it. In this particular setting reporter genes become easily traceable and quantifiable. Generally reporter genes are chosen as differential or selection markers in order to follow a biological process.

Most common used reporter genes in molecular biology that confer visually identifiable characteristics are related to fluorescent or luminescent proteins. The Jellyfish green fluorescent protein (GFP) is detected as a green protein under blue light. In the same line, red fluorescent protein (dsRed) can emit red fluorescence under green light. Luciferase is an enzyme capable to produce light in the presence of luciferin. All these proteins have been widely used for constructing reporter genes in the last decades. Other common reporter genes extensively used in bacteria are for example, the β-galactosidase (*lacZ*) gene from *Escherichia Coli*, responsible of encoding the β-galactosidase hydrolase enzyme, which confers cells expressing it to become blue when growing in a media containing a substrate analog (X-gal), or resistance to antibiotics like chloranfenicol, encoded by the Chloranfenicol acetyltransferase gene (CAT).

Reporter genes can be used for a variety of purposes. A popular use is to perform assays for the activity of promoters in a cell or organism. In this case, the reporter must be placed under the control of the target promoter; thus, the reporter gene product's activity can be quantitatively measured and correlated to the promoter's activity. Another common use is to monitor the expression of a gene of interest that may have a specific and known effect in the cell type or organism of interest. For these studies, the reporter is attached just after the gene of interest as a gene fusion, thus the two genes are under the same promoter. When the gene or promoter is turned on, the reporter gene is synthetized too, making possible to detect and localize the expression of genes or activation of promoter regions at different stages of development.

To artificially express an exogenous gene into an organism, bacteria or cell, it is necessary to introduce it by any of the gene editing techniques previously described. It is possible to make reporter cell lines by directly transducing the reporter gene of interest in a plasmid by electroporation, or by viral vectors transduction or, even in a

more sophisticated maner, by direct genome engineering technologies. Independently of the reporter generation method, it is important to introduce a selectable marker gene that allows selecting those cells in where the reporter gene has been successfully incorporated. Selectable marker genes encode easily detectable traits making reporter cells different from not reporter cells. Neomycin phosphotransferase gene is a common selectable marker that confers geneticin resistance (G418) in those cells successfully targeted.

Different types of reporters have been successfully used in ESCs research to identify gene regulation pathways, gene repressors, activators and promoters (Meyer et al. 2000; T. Zwaka & Thomson 2003; Hadjantonakis et al. 2002; Fischer et al. 2010)

3.3.1. Cardiac Reporter Cell lines

As cardiac disease is of world over interest, developing tools that can lead to understand heart development, heart disease, as well as to discover new possible regenerative therapies is one of the hot topics of research nowadays.

So far different approaches have been used to generate cardiac reporter cell lines. In this regard, direct transduction by electroporation of a plasmid containing a reporter gene under the transcriptional control of the promoter of interest has been the most extended approach. This is the case of a pioneering work of Dr. Metzeger and colleagues in 1996, who engineered a D3 ES cell clone, which expresses the *lacZ* gene under the transcriptional control of alpha-actin promoter (Metzger et al. 1996); also Dr. Kolossov and colleagues in 1998 established a similar D3 ES clone but with GFP as reporter gene (Kolossov et al. 1998). Alpha actin gene is a very useful reporter because it is expressed early during cardiac differentiation program, however different reports have demonstrated that is not exclusively targeting cells from cardiac tissues, not even from heart ventricles (Alonso et al. 1990; GE & , Buckingham ME 1991).

Chamber specification occurs early during embryonic heart development. Atrial and ventricular cells arise from divergent lineages that are determined in the primary myocardium before looping of the heart occurs (O'Brien et al. 1993; Miller-Hance et al. 1993). Thus, other cardiac specific markers have been chosen for effective targeting.

That was the case of ES reporter cell line generated by Meyer and colleagues already in 2000, which expresses the enhanced cyan variant of *Aequorea victoria* green fluorescent protein (ECFP) under the transcriptional control of the ventricular myosin light chain 2 (*MLC2v)* promoter. Alpha myosin heavy chain (*α-MHC* or *MYH6*), expressed both at early and late stages during cardiac differentiation, and has been used several times as reporter gene to generate transgenic mice. The simplest version probably is the mouse used in the work of Song and colleagues, in which the GFP expression was driven by a short form from *α-MHC* promoter sequence (Song et al. 2012). Then several complex and accurate reporter mice have been created, as the *α-MHC*-GFP/IRES-Puromycin mice (Ieda et al. 2010), or the *α-MHC*-CFP/Fsp1-Cre/tdTomato mice; the later used for direct cardiac reprogramming of fibroblasts into induced cardiomyocytes by means of micro RNAs overexpression (Jayawardena et al. 2012).

Very few cardiac reporter cell lines have been successfully constructed in the human setting. Probably the most used human reporter cell lines nowadays, due to their effectiveness and simplicity, are the ones created in Mercola's laboratory (Kita-Matsuo et al. 2009). Kita–Matsuo and colleagues (2009) generated a batch of several lentiviral vectors combining either *Brachyury* or *α-MHC* promoter sequences with mCherry or GFP fluorescent proteins and antibiotic resistance under the promoter of *Rex1,* Reduced Expression Protein 1 (a pluripotency-related gene). In that system authors avoided the contamination of ESCs in the final differentiated cardiac populations. Following a similar approach, Ritner and colleagues generated *α-MHC*-GFP human hESCs reporter cell line (Ritner et al. 2011).

Another reporter cell line that has successfully been used for the study of cardiac differentiation is the *NKX2*-5eGFP/w line by Elliott and colleagues (2012) (Elliott et al. 2011a). *NKX2.5* gene is expressed in the heart throughout life. In that particular study authors targeted EGFP coding sequences to the *NKX2.5* locus of human embryonic stem cells (hESCs) by HR. Moreover, the generation of *NKX2.5*eGFP/w hESCs line enabled the quantification of cardiac differentiation, the purification of hESC-derived committed cardiac progenitor cells (hESC-CPCs) and cardiomyocytes (hESC-CMs), and the standardization of a cardiac differentiation protocol. Furthermore, the group used NKX2.5 eGFP positive cells (eGFP+ cells) to identify VCAM1 and SIRPA as novel cell-surface markers expressed in cardiac lineages. A similar reporter cell line generated by Bacterial Artificial Chromosomes (BACs) recombineering was generated earlier by

Hsiao and colleagues (2008) (Hsiao et al. 2008). The hESCs reporter cell line *NKX2.5*_2AemGFP preserved all regulatory sequences from *NKX2.5* gene due to the targeting at the ATG start site of the gene.

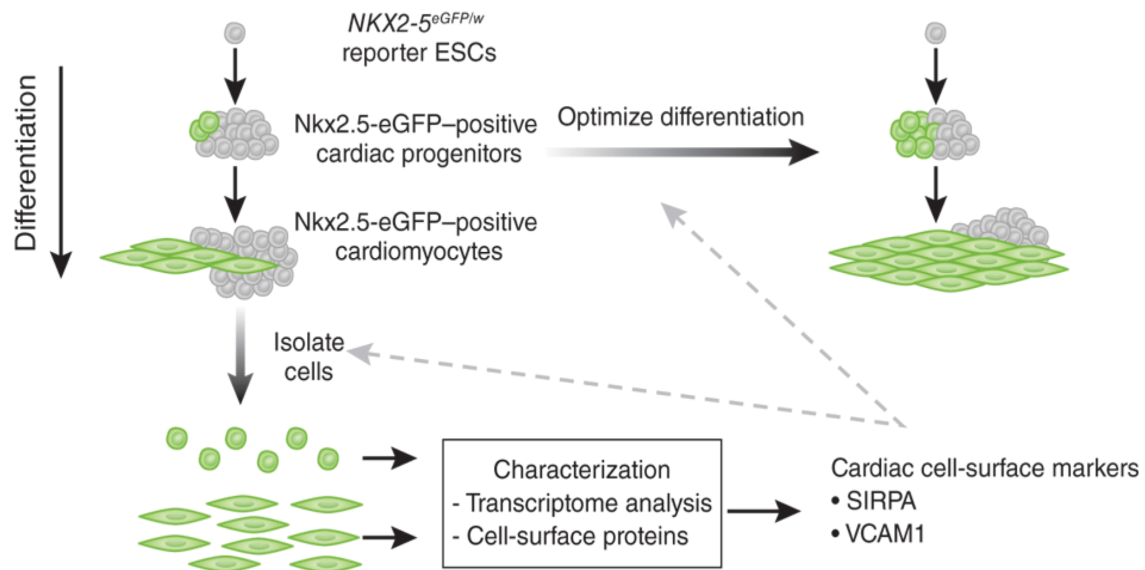


Figure 10: Applications of the *NKX2-5*$^{eGFP/w}$ reporter cell line. Picture from Kamp, T. et al 2011 (Kamp 2011) referring to *NKX2-5*$^{eGFP/w}$ reporter cell line generated by Elliot et al 2001 (Elliott et al. 2011b). Remarks the importance of a cardiac-specific reporter genetically engineered into human embryonic stem cells, which allows the optimization of differentiation protocols and the identification of cell-surface markers and cardiac features. It is a very useful tool to isolate and define cardiac cell lineages.

Overall reporter cell lines have demonstrated that by this approach final homogenous populations can be generated. Also that is possible to separate different specific sub-populations, and even to define new markers for the populations of interest through differentiation processes. Besides, reporter cell lines have been shown to explain different molecular processes *in vivo* (Kammili et al. 2010) or even to monitor cell transplantation evolution in animal models (Vandsburger et al. 2013).

In the context of the cardiac field common efforts have been guided towards the understanding of those molecular and cellular cues responsible of cardiac fate. The possibility to monitor cardiac differentiation from hESCs/hiPSCs opens a new scenario

where we could easily analyze those molecular and cellular events during early and late stages of cardiac differentiation. This information becomes essential when modelling cardiac disease with hiPSCs, and even for the generation of robust protocols for the generation of high yields for cardiac cells.

II. SPECIFIC AIMS

II.SPECIFIC AIMS

1. To generate induced pluripotent stem cells (iPSCs) from umbilical cord (uc) derived mesenchymal stem cells (ucMSCs) for the screening of novel reprogramming factors and cardiac differentiation protocols.

2. To generate a protocol for cardiac conversion from post-natal dermal fibroblasts.

3. To generate a cardiac reporter human embryonic stem cell (hESC) line for the study of cardiac differentiation.

III. MATERIALS AND METHODS

III A. MATERIALS

1. Bacterial strains

Name	Genotype	Use	Description
DH5α	*F-φ 80lac ZΔ M15 Δ (lac ZYA-arg F) U169 rec A1 end A1 hsd R17(rK-, mK+) pho A sup E44 λ- thi -1 gyr A96 rel A1.*	Amplification of plasmids and general cloning.	Chemical competent. Derived from DHB101 *E.Coli* strain.
Stbl3	*F-mcrB mrrhsdS20(rB-, mB-) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20(StrR) xyl-5 λ-leumtl-1*	In-Fusion® transformation	Electrocompetent
TOP10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG*	Amplification of plasmids and general cloning.	Electrocompetent cells. Similar to the DH10B *E.Coli* strain.

2. Cell lines

Name	Description
ES[4]	Human ESC cell line derived at the Stem Cell Bank of the CMR[B] from a cryopreserved donated human embryo at day +2 of development.
HEK293T (ATCC® CRL-3216™)	Stable cell line derived from human embryonic kidney expressing the SV40 large T antigen. Highly transfectable, contains the SV40 T-antigen. Commercial available.
HFFs	Primary cell line derived from human fibroblasts isolated from juvenile foreskin of a donor.
HFFs (ATCC® SCRC-1041™)	Newborn human fibroblasts derived from foreskin. Commercial available.
iCMS	Induced Cardiomyocytes from Pluripotent Stem Cells.
MEFs	Primary cell line of Mouse Embryonic Fibroblasts established from dissociated C57BL/6 mouse embryos at 13.5 day of gestation.
PA6	Mouse bone marrow–derived stromal cell line. Commercially available.
Phoenix Amphotropic Cells (ATCC® CRL-3213™)	Stable cell line from human embryonic kidney expressing the SV40 and Adeno viral sequences. Second-generation retrovirus producer cell line for the generation of helper-free ecotropic and amphotropic retroviruses. Commercially available.
uciPSCs 2F	Umbilical Cord induced pluripotent stem cells. Reprogrammed with two transcription factors.
uciPSCs 4F	Umbilical Cord induced pluripotent stem cells. Reprogrammed with four transcription factors.

ucMSCs	Umbilical Cord Mesenchymal stem cells derived from human cord blood.

3. Plasmid and Viral Vectors

Name	Description
GATA4 (OHS5894)	GATA4 cDNA without stop codon. From Thermo Scientific Orfeome library.
MYH6 BAC (RP11-929J10)	Bacterial Artificial Chromosome for MYH6 gene. Used to copy homology arms for donor vector plasmid cloning (BACPAC C.H.O.R.I.).
NI, HD,NG, NN (kit#100000019)	TALE monomer templates. From TALE Toolbox kit in Addgene. (Sanjana et al. 2012; Z. Zhang et al. 2011). Used for TALEN construction for the MYH6 gene locus targeting in ES[4]. Templates maps in ***Appendix* I Supplementary Figure 1A.**
pCAG_HAmCherry_Poly	Plasmid from laboratory of Polleux, F. at the Scripps Institute (La Jolla, CA) (Courchet et al. 2013). Used to obtain HA_mCherry fragment insert for cloning Donor Vector and gRNA of the CRISPR/CAS9 system. Plasmid map in ***Appendix* I Supplementary Figure 2.**
pCAS9_GFP (Addgene #44719)	Plasmid acquired from Addgene (#44719), Kiran Musunuru laboratory (Peters et al. n.d.). Plasmid optimized for expression in human pluripotent stem cells. Co-expression of human codon-optimized Cas9 nuclease and GFP. Used to target ES[4] at the MYH6 gene locus. Plasmid map in ***Appendix* I Supplementary Figure 3.**
pCMV_NLS (NI,HD,NG, NN)_FokI (kit#100000019)	TALE backbones for cloning monomers from TALE Toolbox kit in Addgene. (Sanjana et al. 2012; Z. Zhang et al. 2011). With CMV promoter, Nuclear signal and *FokI* nuclease. Used for TALEN construction for the MYH6 gene locus targeting in ES[4]. Backbones maps in ***Appendix* I Supplementary Figure 1B.**
pCR_Blunt II-TOPO (Addgene #41824)	Empty gRNA expression vector, used to create a gRNA to a specific sequence from George Church lab (Mali et al. 2013). Plasmid map in ***Appendix* I Supplementary Figure 4.**
pCRII_VP16_AgePTV_EBFP2	Plasmid derived in the Laboratory as intermediate step for final cloning of the retroviral vector pMSCV_HA_GATA4_VP16_ppGFP for cell conversion approach.. Contains VP16 transactivator domain and *AgeI* restriction site. Plasmid map in ***Appendix* I Supplementary Figure 5.**
pMax_ppGFP	Plasmid from Nucleofection Kit of Lonza (cat no. H3VPH-5012). Used as positive control for electroporation or nucleofection of ES[4] and transfection assays of HFFs and 293T cells. Transgene ppGFP was amplified by PCR to clone in the retroviral vector pMSCV_HA_GATA4_VP16_ppGFP for the cell conversion approach. Plasmid map in ***Appendix* I Supplementary Figure 6.**

pMSCV_Flag-tagged (OSKM) (OCT4 #20072, SOX2 #20073, KLF4 #20074 and c-MYC#20075)	Retroviral vectors kindly provided by Juan Carlos Izpisúa. Available in Addgene. Four retroviral vectors encoding for the four pluripotency factors (OSKM). Used for Reprogramming ucMSCs. Plasmid map in ***Appendix* I Supplementary Figure 7.**
pMSCV_puro (#634401)	Retroviral plasmid backbone commercially available from Clonetech (#634401). Retroviral vector optimized for expression of a gene in hematopoietic, embryonic stem, or embryonic carcinoma cells. This backbone was used to clone GATA4 TF with ppGFP reporter transgene to generate the final retroviral vector pMSCV_HA_GATA4_VP16_ppGFP for the cell conversion approach. Plasmid map in ***Appendix* I Supplementary Figure 8.**
pMXS_OVPS_Orange	Derived in the laboratory by Federico González. (Montserrat et al. 2012).Retroviral plasmid for the expression of OCT4 and SOX2 pluripotency transcription factors enhanced by VP16 transactivator domain and reported by mOrange fluorescent protein. Used to induce "plastic stage" in the cell-conversion approach. Plasmid map in ***Appendix* I Supplementary Figure 9.**
pZero_FRT_Neo	Plasmid derived at the Salk Institute by GH Liu (Liu et al. 2011). Used as backbone to clone the MYH6 donor vector needed for CRISPR/CAS9 and TALEN systems. Plasmid map in ***Appendix* I Supplementary Figure 10.**
MYH6 d-vector	Donor Vector plasmid with homologous arms to human *MYH6* locus flanking the reporter cassette. Co-trasnfected with TALEN and CRISPR/Cas9 nucleases to induce HR after DSB. Constructed in the laboratory. ***Appendix* I Supplementary Figure 11.**

4. Antibodies

Table I Immunoshistochemistry Primary Antibodies

Name (anti)	Isotype	Distributor	Ref	Dilution
GATA4	Rabbit IgG	Santa Cruz	sc-9053	1:25
GFAP	Rabbit IgG	Dako	Z0334	1:1000
HA	Mouse IgG1	Sigma	H9658	1:200
Heavy chain cardiac Myosin	Mouse IgG2	Abcam	ab15	1:200
MEF2	Rabbit IgG	Santa Cruz	sc-313	1:25
NANOG	Goat IgG	R&D Systems	AF1997	1:25
NKX2.5	Goat IgG	Santa Cruz	sc-8697	1:25
OCT4	Mouse IgG2b	Santa Cruz	sc-5279	1:25
RFP biotinilated	Rabbit IgG	AbCam	ab34771	1:400
Smooth muscle actin (SMA)	Mouse IgG	Sigma-Aldrich	A5228	1:400
SOX2	Rabbit IgG	ABR	PA1-16968	1:100
SSEA-3	Rat IgM	Iowa	MC-631	1:3
SSEA-4	Mouse IgG3	Iowa	MC-813-70	1:3
SSEA-1	Mouse IgM	Hybridoma Bank	MC-480	1:3
Tra-1-60	Mouse IgM	Chemicon	MAB4360	1:200

Tra-1-81	Mouse IgM	Chemicon	MAB4381	1:200
Tropomyosin	Mouse IgG1	Sigma	T 2780	1:400
Troponin I	Rabbit IgG	Santa Cruz	sc-15368	1:25
α-1-Fetoprotein	Rabbit IgG	Dako	A0008	1:400
α-Actinin sarcomeric (AAS)	Mouse IgG	Sigma	A7811	1:100
α-Sarcomeric actin (ASA)	Mouse IgM	Sigma	A2172	1:400
β-III-Tubulin Tuj1	Mouse IgG	Covance	MMS-435P	1:500

Table II Immunoshistochemistry Secondary Antibodies

Isotype	Conjugated	Source	Dis	Ref	Dilution
Goat IgG	DyLight649	Donkey	Jackson	705-605-147	1:200
Goat IgG	Cy3	Donkey	Jackson	705-165-147	1:200
Goat IgG	CY2	Donkey	Jackson	705-225-147	1:200
Mouse IgG	DyLight649	Donkey	Jackson	715-605-151	1:200
Mouse IgG	DyLight488	Donkey	Jackson	715-485-151	1:200
Mouse IgG	Cy3	Donkey	Jackson	715-165-151	1:200
Mouse IgM Fcmu spec	CY2	Donkey	Jackson	715-225-140	1:200
Mouse IgM Fcmu spec	Cy3	Donkey	Jackson	715-165-140	1:200
Rabbit IgG	DyLight649	Donkey	Jackson	711-606-152	1:200
Rabbit IgG	DyLight488	Donkey	Jackson	711-545-152	1:200
Rabbit IgG	Cy3	Donkey	Jackson	711-165-152	1:200
Rat IgM Fcmu spec	CY2	Goat	Jackson	112-225-020	1:200
Rat IgM Fcmu spec	Cy3	Goat	Jackson	112-165-020	1:200

Table III Fluorescent Activated Analysis and Cell Sorting Antibodies

Name (anti)	Isotype	Distributor	Ref	Conjugated
CD106	Mouse IgG1,k	BD	551147	APC
CD105	Mouse IgG1,k	eBioscience	17-1057-42	APC
CD34	Mouse IgG2a	Miltenyi Biotec	130-081-002	PE
CD45	Mouse IgG1,k	BD	555485	APC
CD73	Mouse IgG1,k	BD	550257	PE
CD90 (Thy1)	Mouse IgG1,k	BD	555596	PE

Table IV FACS Isotype Control Antibodies

Clone	Isotype	Distributor	Reference	Conjugated
MOPC-21	Mouse IgG1	BD	557702	AF488
MOPC-21	Mouse IgG1	BD	550854	APC

Mouse IgG1	Miltenyi	130-092-212	PE
Mouse IgG2a	Miltenyi	130-091-835	PE
Mouse IgM	Miltenyi	130-093-177	PE

5. Oligonucleotides

Table I Primers Real Time qPCR

gene	Forward primer 5'-3'	Reverse primer 5'-3'
Alfa1- Integrin (CD49a)	GCTGGCTCCTCACTGTTGTT	CTCCATTTGGGTTGGTGACT
BAF60C	GAAGAGGCCCATGAAGCAAA	CCGTCGGAATCCTCAGCAT
Beta1-Integrin	CAAAGGAACAGCAGAGAAGC	ATTGAGTAAGACAGGTCCATAAGG
BMP4	GGGAGGAGGAGGAAGAGCAG	GTCCCTGGGATGTTCTCCAGAT
BRACHYURY	CCCAGCCCCTATGCTCATC	CCAATTGTCATGGGATTGCA
CACNA1a	CGGGAAACCGTGTGATAAGAA	AGCATCAGAAACGAGCAGAGGAA
CD109	GTGGGGAGTCCTAAAGCGAAG	GCTGCCAGGAGTCAGAAAGTTT
CD34	CCCACAGGAGAAAGGCTGG	TGAGCCCCTCGGTTCACA
CDKN2C	GGGGACCTAGAGCAACTTACT	GGCAATCTCGGGATTTCCAAG
CRIPTO	CGGAACTGTGAGCACGATGT	GGGCAGCCAGGTGTCATG
DESMIN	GAGGAGAGCCGGATCAATCTC	GGACCTCAGAACCCCTTTGCT
DNMT3B	CCGCCATGGTGGTGTCTT	ACCGCATGCCAGACATAGC
FOXF2	CTACTTGCACCAGAACGCTC	CGCAGGGCTTAATATCCTGACA
GAPDH	CCTGCACCACCAACTGCTTAG	TGGCATGGACTGTGGTCATG
GATA2	GGCCACAGCCACCCCTCTCT	GCGGCCGACAGTCTTCGCTT
GATA4	GGGACGGGTCACTATCTGTG	GGTGGTGGTCTGGCAGTT
GATA6	CTCAGTTCCTACGCTTCGCAT	GTCGAGGTCAGTGAACAGCA
HAND2	GAAGAAGGAGCTGAACGAAATCTT	CGTCCGGCCTTTGGTTTT
ITGA4	CCCCAGGATCATCTTACTGGA	TATGCTGGCTCCGAAAATGAC
KDR	GGTCAGGCAGCTCACAGTCCTA	ACTTCGATGCTTTCCCCAATAC
KIT	GACGAGTTGGCCCTAGACTTAGAA	CTGGCTGCCAAGTCTCTGTGA
KLF4	GATGAACTGACCAGGCACTA	GTGGGTCATATCCACTGTCT
MEF2C	GCGCTCTTCACCTTGGTTCA	TGCAAGCTCCCAACTGACTGA
MESP1	CAGGCGATGGAGCCAAG	TCCTCAGGCAGCCACTC
MYH6	GACTGTTGTGGCCCTGTACC	GGAAGGATGAGCCCTTTTTC
MYL2	CGCCAACTCCAACGTGTTCT	CCATCCCTGTTCTGGTCCAT
MYL7	CCCATCAACTTCACCGTCTTCCT	AGAGAACTTGTCTGCCTGGGTCA
MYOZ2	CCAGGCTATTTAAGATGCGTCA	CCTTCCAAGTTACTTCCATCCAC

NANOG	ACAGAAATACCTCAGCCTCCAGC	CCATTGCTATTCTTCGGCCAGTT
NKX2.5	AAGTGTGCGTCTGCCTTT	GTTGTCCGCCTCTGTCTTC
OCT4	TGGAGGAAGCTGACAACAATGA	TCTCGATACTGGTTCGCTTTCTC
REX1	CCTGCAGGCGGAAATAGAAC	GCACACATAGCCATCACATAAGG
SIRPA	CGAGGACGTTCACTCTCAAGTCA	GAACTCGGATGGTCTCAGACAAG
SMYD1	AAGATGCCTACGCCCACGG	ACATTCTTGCAGTACTGGCCGC
SOX2	CCCAGCAGACTTCACATGT	CCTCCCATTTCCCTCGTTTT
TNNI3	CCAACTACCGCGCTTATGC	CTCGCTCCAGCTCTTGCTTT
TNNT2	CAAAGCCCAGGTCGTTCATG	CCGGTGGATGTCATCAAAGTC
VEGFC	GTCTCCAGTGTAGATGAACTC	ATCTGTAGACGGACACACATG
WNT3A	CCCGTGCTGGACAAAGCTA	CATGAGCGTGTCACTGCAAA

Table II Primers sequencing, PCR and cloning

Name	Sequence 5'-3'
pMSCV_gag F	CTACATCGTGACCTGGGAAG
P2A R	GTCTCCTGCTTGCTTTAACAG
PGK R	CTTCCATTTGTCACGTCCTG
Puro F	GCAACCTCCCTTCTACGAGC
Neo F	CGTTGGCTACCCGTGATATT
MYH6_seqF	GAAGGGATAACCAGGTGAGAAC
MYH6_seqF	GAAGAGGGATACAAGTGCAAGA
In-Fusion MYH6 5' F	TACCGAGCTCGGATCCGGTCAGTTGGAGGCCTAAGG
In-Fusion MYH6 5' R	GGATACATCATCTTGGTGCTTCCCCTGG
In-Fusion MYH6_HA_mCherry F	CCAAGATGATGTATCCGTATGATGTGCCGGATTATG
In-Fusion MYH6_HA_mCherry R	ACTTCGAATTCCATATGTGATAGACACAAAAAATTCCA ACACACTATTGC
In-Fusion MYH6 3' F	GCGAATTCTGCAGATATCAAGGAGCGTCTAGAGGCCC
In-Fusion MYH6 3' R	TAGATGCATGCTCGAGAGTTCACCCATCTACTCCACACT C
mCherry R	CTTGGTCACCTTCAGCTTGGC
mCherry F	AGGACGGCGAGTTCATCTACAAG

Table III Primer sequences for TALE construction

Name	Sequence 5'-3'
Ex-F1	TGCGTCcgtctcCGAACCTTAAACCGGCCAACATACCggtctcCTGACCCCAG AGCAGGTCGTG
Ex-F2	TGCGTCcgtctcCGAACCTTAAACCGGCCAACATACCggtctcGACTTACACC CGAACAAGTCGTGGCA ATTGCGAGC
Ex-F3	TGCGTCcgtctcCGAACCTTAAACCGGCCAACATACCggtctcGCGGCCTCAC

	CCCAGAGCAGGTCG
Ex-F4	TGCGTCcgtctcCGAACCTTAAACCGGCCAACATACCggtctcGTGGGCTCACCCCAGAGCAGGTCG
Ex-R1	GCTGACcgtctcCGTTCAGTCTGTCTTTCCCCTTTCCggtctcTAAGTCCGTGCGCTTGGCAC
Ex-R2	GCTGACcgtctcCGTTCAGTCTGTCTTTCCCCTTTCCggtctcAGCCGTGCGCTTGGCACAG
Ex-R3	GCTGACcgtctcCGTTCAGTCTGTCTTTCCCCTTTCCggtctcTCCCATGGGCCTGACATAACACAGGCAGCAACCTCTG
Ex-R4	GCTGACcgtctcCGTTCAGTCTGTCTTTCCCCTTTCCggtctcTGAGTCCGTGCGCTTGGCAC
In-F2	CTTGTTATGGACGAGTTGCCcgtctcGTACGCCAGAGCAGGTCGTGGC
In-F3	CCAAAGATTCAACCGTCCTGcgtctcGAACCCCAGAGCAGGTCGTG
In-F4	TATTCATGCTTGGACGGACTcgtctcGGTTGACCCCAGAGCAGGTCGTG
In-F5	GTCCTAGTGAGGAATACCGGcgtctcGCCTGACCCCAGAGCAGGTCGTG
In-F6	TTCCTTGATACCGTAGCTCGcgtctcGGACACCAGAGCAGGTCGTGGC
In-R1	TCTTATCGGTGCTTCGTTCTcgtctcCCGTAAGTCCGTGCGCTTGGCAC
In-R2	CGTTTCTTTCCGGTCGTTAGcgtctcTGGTTAGTCCGTGCGCTTGGCAC
In-R3	TGAGCCTTATGATTTCCCGTcgtctcTCAACCCGTGCGCTTGGCACAG
In-R4	AGTCTGTCTTTCCCCTTTCCcgtctcTCAGGCCGTGCGCTTGGCACAG
In-R5	CCGAAGAATCGCAGATCCTAcgtctcTTGTCAGTCCGTGCGCTTGGCAC
Hex-F	CTTAAACCGGCCAACATACC
Hex-R	AGTCTGTCTTTCCCCTTTCC
TALE-Seq- F1	CCAGTTGCTGAAGATCGCGAAGC
TALE-Seq- F2	ACTTACACCCGAACAAGTCG
TALE-Seq- R1	TGCCACTCGATGTGATGTCCTC
TALE-Seq- R2	CCCATGGGCCTGACATAA

Table IV. Other Primers

Name	Sequence 5’-3’
Southern MYH6 F	TGCCTCTGTTGTTCTTCACTCTCC
Southern MYH6 R	GGTCAGAGACAGGAGGGCTAT
Southern mCherry F	AAGGGCGAGGAGGATAACATGG
Southern mCherry R	GGTGTAGTCCTCGTTGTGGGA
Surveyor MYH6 F	CACTCAGCGCCAACCCTTAGCATACTCCAG
Surveyor MYH6 R	CCAGGGGTGATTCTCTTGGCTGGTGTGAG

6. Reagents

Table I Molecular Biology

Product	Provider
Ampicilin	Sigma-Aldrich Quimica cat no. A0166
Antartic Phosphatase	New England Biolabs M0289S
anti-digoxigenin	Roche cat no.11333089001
AP Staining Kit	Sigma-Aldrich cat no.AB0300
BigDye® v3.1	Life Technologies cat no.4337454
Blue Membrane Substrate solution kit	Sigma-Aldrich cat no.AB0300
CDP-Star® Chemiluminiscent Substrate solution	Sigma-Aldrich cat no.C0712
Cloned AMV First-Strand Synthesis kit	Invitrogen, Life Technologies cat no.12328-032
DNA ladder 1kb	Invitrogen, Life Technologies cat no.10787-018
DNeasy Blood & Tissue kit	QUIAGEN cat no.69504
EDTA	Sigma-Aldrich Quimica cat no.E5134
Ethanol, 100% vol/vol	Panreac cat no.361086
Ethidium Bromide (EtBr)	Life Technologies cat no.15585011
Glycerol	Merck cat no.1.04093.1000
Glycine	Sigma-Aldrich Quimica cat no.G7126
Herculase II high fidelity polymerase	Agilent Technologies cat no.600679
High Fidelity polymerase Q5 Hot Start Polymerase	New England Biolabs cat no.M0493S
In-Fusion® HD	Clontech cat no.011614
Kanamycin	Sigma-Aldrich Quimica cat no.K1377
Klenow enzyme	Roche Life Science cat no.11008404001
LA Taq DNA Polymerase	TAKARA Clontech cat no.RR002A
NaCl	Sigma-Aldrich Quimica cat no.S7653
NaN3	Merck cat no.1.06688.0100
NaOH	Panreac cat no.131687_1210
NucleoBond Xtra	TAKARA Clontech cat no.740410.50
Nylon membrane Hybond-N	RPN203N, GE Healthcare Europe GmbH
PCR DIG Probe Synthesis Kit	Roche Life Science cat no.11636090910
Phusion® Hot Start II DNA polymerase	Thermo Scientific cat no.F-549L
PlasmidSafe DNAse	Epicentre cat no.E3101K
Platinum Syber Green Super Mix	Invitrogen, Life Technologies cat no.11733-038
Polybrene	Millipore cat no. TR-1003-G
PrimeSTAR® GXL Polymerase	TAKARA Clontech cat no.R050Q
Propyl gallate	Sigma-Aldrich Quimica cat no.P3130
Proteinase K	Sigma-Aldrich cat no.P2308
QIAprep Spin Miniprep Kit	QUIAGEN cat no.27104
QIAquick Gel Extraction kit	QIAGEN cat no.28704
QIAquick PCR Purification Kit	QIAGEN cat no.28104
Restriction Enzymes	New England Biolabs
RNase A	Sigma-Aldrich cat no.R6513
SYBER® Green	Life Technologies cat no.4309155
SYBER® Safe DNA Gel Stain	Life Technologies cat no.S33102
T4 DNA Ligase	Roche Life Science cat no.11 635 379 001
Trizma base	Sigma-Aldrich Quimica cat no.T6791
Trizma-HCl	Sigma-Aldrich Quimica cat no.T6666
TRIzol® Reagent	Invitrogen, Life Technologies cat no.15596-026
TURBO DNAse	Ambion cat no.AM1907
Tween-20	Sigma-Aldrich Quimica cat no.P5927

Table II Cell Culture

Product	Provider
0.05% trypsin-EDTA	Invitrogen, Life Technologies cat no.25300 -054
0.25% trypsin-EDTA	Invitrogen, Life Technologies cat no.25200 -056
2-mercaptoethanol	Gibco®, Life Technologies cat no.31350-010
3-Isobutyl-methylxanthine (IBMX)	Sigma-Aldrich Quimica cat no.I5879
5-azactydine	Sigma-Aldrich Quimica cat no.A2385
Accumax	Stemcell Technologies cat no.07921
Alizarin Red S	Sigma-Aldrich Quimica cat no.A5533
Ascorbic acid (ascorbate-2-P)	Sigma-Aldrich cat no.A4544-25g
B-27® Supplement	Life Technologies cat no.0080085SA
B-27® Supplement Minus Insulin	Life Technologies cat no.0050129SA
bFGF	Peprotech cat no.100-18B
Blue Membrane Substrate solution kit	Sigma-Aldrich Quimica cat no.AB0300
BMP4	Peprotech cat no.120-05ET
BMP6	R&D Systems cat no.P22004
CHIR99021	Selleck catalog cat no. S2924
DAPI solution	Life Technologies D21490
Dexamethasone	Sigma-Aldrich Quimica cat no.D2915
Dispase	Stem Cell Technologies cat no.07923
Donkey Serum	Jackson 017-000-121
Dulbecco's modified Eagle's medium (DMEM)	Gibco®, Life Technologies cat no.01-055-1A
Dulbecco's modified Eagle's medium (DMEM) -high glucose	Gibco®, Life Technologies cat no.21969-035
Dulbecco's modified Eagle's medium (DMEM) /F12	Gibco®, Life Technologies cat no.21331-020
Fetal Bovine Serum (FBS)	Gibco®, Life Technologies cat no.10270-106
Fetal Calf Serum (FCS)	Gibco®, Life Technologies
Fugene6® reagent	Roche Life Science cat no.1814443001
GlutaMAX	Gibco®, Life Technologies cat no.35050-038
Human Stem Cell Nucleofector Kit 1	Lonza cat no.H3VPH-5012
Indomethacin (TLC)	Sigma-Aldrich Quimica cat no.I7378
Iscoves's Modified Dulbecco's Medium (IMDM)	Gibco®, Life Technologies cat no.21980-032
ITS	BD Biosciences cat no.354351
IWP4	Stemgent Catalog cat no.04-0036
Knockout Serum Replacement (KSR)	Gibco®, Life Technologies cat no.10828-028
Knockout-Dulbecco's modified Eagle's medium (DMEM)	Gibco®, Life Technologies cat no.10829-018
Lipofectamine 2000	Life Technologies cat no.11668-019
Matrigel™	BD Biosciences cat no.354277
mTeSR ™	Stem Cell Technologies cat no.05850
N-2 supplement	Invitrogen, Life Technologies cat no.17502-048
Non-essential amino acids (NEAA)	Cambrex, Lonza cat no.BE13-114E
Oil Red O lipid stain	Abcam cat no. ab150678
Opti-MEM	Gibco®, Life Technologies cat no.31985-062
Paraformaldehyde, 4% (PFA)	Sigma-Aldrich, cat. no. P6148-500G
PBS	Gibco®, Life Technologies cat no. 10010
Penicillin-streptomycin	Gibco®, Life Technologies cat no.15140-122
Polybrene	Millipore cat no.TR-1003-G
Proline	Sigma-Aldrich Quimica cat no.P3350000
ROCK inhibitor Y-27632	Sigma-Aldrich Quimica cat no.Y0503
RPMI 1640 medium	Life Technologies cat no.11875093

SURVEYOR® mutation detection Kit	Transgenomic cat no.706025
TGF-β3	R&D Systems cat no.243-B3-002
Toluidin Blue O	Sigma-Aldrich Quimica cat no. T3260
Triton X-100	Sigma-Aldrich Quimica cat no. 93443
Trypan blue solution	Sigma-Aldrich Quimica cat no. T8154
TrypLE Express	Invitrogen, Life Technologies cat no.NP0001
Ultra Pure 0.5M EDTA	Life Technologies cat no. 15575-020
α-MEM with Ribonucleosides/Deoxyribonucleosides	Gibco®, Life Technologies cat no.32571
β-mercaptoethanol	Gibco®, Life Technologies cat no.31350-010

III. B. METHODS

1. GENERAL PROTOCOLS

1.1. Molecular Biology and Biochemistry techniques.

1.1.1. Plasmid Cloning

1.1.1.1. Traditional cloning

General cloning design was performed with help of ApE-A Plasmid Editor free software, SnapGene and MacVector software.

- Digestion with restriction enzymes

Enzymatic digestion was the method to generate DNA fragments and open DNA vectors for subsequent cloning. Standard protocol was used to ensure the total digestion of the DNA substrate without generating unspecific cuts. DNA concentration at the final solution was between 20 and 200 ng/µl. Glycerol percentage never exceeded 10 % of the total volume. Digestions of 1 to 5 µg of DNA were performed in a final volume of 50 to 100 µl. One unit (U) of restriction enzyme per 1µg of DNA was the recommended ratio. Buffer type, temperature and time of the reaction were specific for each enzyme used, as well as temperature and time of incubation for subsequent inactivation. Restriction enzymes were supplied by New England Biolabs.

- Vector Dephosphorylation

To dephosphorylate 1 µg of DNA containing vector, 1U of Antarctic Phosphatase (New England Biolabs) was used. The final mix was incubated during 1 hour at 37°C. Next, Phosphatase was inactivated at 65°C during 10 minutes. DNA was purified using the PCR Purification Kit (Qiagen) following manufacturers' protocol. Final elution of DNA fragments was performed in DNAse free water (Ambion).

- Ligation

Once the purified vector and the insert were respectively obtained (for generation of inserts see *Inserts preparation* section below), ligation was performed in a general molar ratio vector: insert of 1:3 (insert: vector) for sticky ends, or 1:10 (insert: vector)

for blunt ends. The reaction was usually performed in a final volume of 10 μl in the presence of 1 U of T4 DNA Ligase (Roche) overnight (ON) at 16°C or during 1-2 hours at room temperature (RT).

1.1.1.2. In-Fusion® cloning method

In-Fusion® HD Cloning Kit (Clonetech) permits directional cloning of one or more fragments of DNA into any location of any vector. DNA fragments are fused efficiently and precisely by recognizing 15 base pair (bp) overlap at their ends. This 15 bp overlap can be engineered by designing primers for amplification of the desired sequences.

-Vector linearization

Generally 5 μg of the vector of interest was linearized by ON digestion with 50U of two different and compatible restriction enzymes in order to avoid re-ligation of the insert of interest. A final volume of 100 μl was used. Next day, digestion was inactivated for 20 minutes at 65ºC (standard inactivation protocol) and afterwards, de-phosphorylation was performed by Antartic Phosphatase (New England Biolabs) for 1 hour at 37ºC followed by 10 minutes of inactivation at 65ºC. Linearization was checked by standard electrophoresis in 1 % agarose gel*. 1 μl of the linearized vector was separated during 30 minutes at 90 Volts (V). Electrophoresis buffer was Tris-Acetate-EDTA* (TAE1X). DNA bands were visualized* using Bio-Rad Gel Documentation System (Bio-Rad).

**agarose gel preparation: appropriated amount of agarose (UltraPure Low Melting Point Agarose, Lonza) is weighted [1 gram (g) per 100 ml of 1XTAE* buffer for general applications; when resolving plasmids (>5 kb) 0.7-0.8% is the general standard. For small fragments (<0.5 kb) 2.5% gels are needed and dissolved by boiling in the microwave. Next agarose solution is placed in the appropriated cuvette for further applications (BioRad)].*

**1X TAE buffer: 40 mM Tris [pH 7.6], 20 mM acetic acid, 1 mM EDTA.*

**DNA visualization: Visualization of DNA fragments in the agarose gels was performed by SYBER® Safe DNA Gel Stain (Life Technologies) or Ethidium Bromide (EtBr). In order to label the gel both markers are added after agarose boiling in the microwave.*

To estimate the size of the DNA fragments weight markers from Life Technologies were used. Images were captured by Bio-Rad Gel Documentation System (Bio-Rad).

- Inserts preparation

Inserts were amplified with High Fidelity polymerase Q5 Hot Start Polymerase (New England Biolabs) with 1 µg of plasmid template in 50 µl of final volume mixtures following manufacturers' specifications. Primer specific design for In-Fusion® cloning was required. Primers had 15 base pair (bp) extensions complementary to the end of the vector of choice or to the adjoining insert. Insert products generated by Polymerase Chain Reaction (PCR) following the next conditions: 3 minutes at 94ºC; 30 cycles of 30 seconds at 94ºC/ 30 seconds at 60ºC/ 2 minutes at 68ºC; 5 minutes at 68ºC. Insert size and purity was checked by electrophoresis in 1 % agarose gel. 1 µl of the linearized vector was separated during 30 minutes at 90 Volts (V).

- In-Fusion® Cloning Reaction

The linearized vectors of choice and PCR products of interest were separated during 30 minutes at 90 Volts (V) in 0.8 % agarose gels in the absence of DNA ladder (in order to avoid DNA contamination). Bands were subsequently isolated and further purified with QIAquick PCR Purification Kit (Qiagen).

In-Fusion® cloning reaction was done in a final volume of 5µl in the presence of 1 µl of linearized vector (at a final concentration of 25ng/ µl), plus 1 µl of each insert (at a final concentration of 25ng/ µl), plus 1 µl of In-Fusion® HD Enzyme premix (Clonetech). All the reagents were mixed in a final volume of 5 ul of distilled water (H_2O, Clonetech). After 15 minutes of incubation at 50ºC, the reaction was placed on ice for further transformation *(see below*).

1.1.2. Transformation of DNA plasmids using Heat (A) or Electro (B) shock methods

A. Heat Shock transformation

Heat shock transformation uses a calcium rich environment provided by calcium

chloride to counteract the electrostatic repulsion between the plasmid DNA and bacterial cellular membrane. A sudden increase in temperature creates pores in the plasma membrane of the bacteria and allows for plasmid DNA to enter the bacterial cell. For heat shock transformation generally *Escherichia Coli* (*E.coli*) DH5alpha cells are used.

- Preparation of heat shock competent cells (DH5alpha)

One single colony of *Escherichia Coli* (*E.coli*) DH5alpha from a Luria-Bertani Broth (LB)-agar plate* was inoculated into 5 ml of LB media and grown at 37°C for 8 hours. Bacterial culture was diluted 1:50 with Super Optimal Broth (SOB) media and grown at 18°C to optical density OD_{600nm} up to 0.6. Next, bacterial culture was placed on ice for 10 minutes and centrifuged at 2.500 g for 10 min at 4°C. Supernatant was discarded and the pellet was resuspended in 80 ml ice-cold Terrific Broth (TB) media. Again, bacterial suspension was centrifuged at 2.500 g for 10 min at 4°C and the pellet was resuspended in 20 ml ice-cold TB media. Dimethylsulfoxide (DMSO) was added to a final concentration of 7% and placed on ice for 10 minutes. Several aliquots were prepared and frozen and stored immediately at -80°C.

**500ml Luria-Bertani broth (LB)-agar composition: 5g NaCl, 5g Tryptone, 2,5g Yeast Extract, 7,5g Agar and H_2O up to 500ml.*

**LB-agar plate preparation: ~20mL of LB agar are poured per 10cm polystyrene Petri dish (Nunc). Plates are left to cool for 30-60 minutes (until solidified. Then plates are inverted, and sit for several more hours or overnight. Generally LB-agar plates without antibiotic addition can be kept at 4ºC up to 3 weeks.*

**Super Optimal Broth (SOB) composition: 2% tryptone, 0, 5% yeast extract, 10 mM NaCl, 2, 5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.*

**1L Terrific Broth (TB) composition: 11, 8g Peptone 140, 23, 6g Yeast Extract, 9, 4g dipotassium hydrogen phosphate and 2, 2g potassium dihydrogen phosphate.*

- Transformation using the heat shock method

A suitable volume of cloning products after In-Fusion® reaction was added into 100µl of DH5alpha competent bacteria and mixed gently. Incubation of DNA-bacteria mixture

was performed for 30 min on ice. Next, the mixture was incubated for 45 seconds at 42°C, followed by 2 minutes on ice. LB media was added and incubated at 37°C shaking for 1 hour. The mixture was spread into LB-agar plates containing the corresponding antibiotic (100 µg/mL Ampicillin, 50 µg/mL Kanamycin), and incubated for 16-24 hours at 37°C.

B. Electro shock transformation

Electro-transformation is the process of subjecting living cells to a rapidly changing, high-strength electric field which results in producing transient pores in their outer membranes (Szostkova and Horakova, 1998). Consequently, diffusion and exchange of intracellular and extracellular components can take place during the lifespan of the membrane pore. The possibility to manipulate membrane pore stability offers the possibility to transfer biological cargo into living cells (e.g: DNA into living bacteria).

- Preparation of electrocompetent cells

Electrocompetent cells were made by streaking out a glycerol stock from bacterial strands One Shot® TOP10 E. coli (Life Technologies) or Stbl3 (Life Technologies) onto a LB-agar plate with no antibiotics. After overnight growing, 10 ml of TB media was inoculated with one single colony and cultured ON by shaking at 220 rpm at 37ºC. Next day, 1L of TB media was inoculated with 10 ml of the starter culture and it was grown at 37ºC shaking until OD_{600nm} was around 0,4. Then, immediately cells were transferred on ice and from that moment all the reagents were maintained in ice cold. Three washes were performed afterwards by pre-chilled ice cold H_2O with 10% glycerol and centrifugations at 1.000 relative centrifugal force (rcf) of 20 minutes at 4ºC. Final resuspension was performed in 4 ml ice cold H_2O 10% glycerol (final OD_{600nm} should be 200-250). Finally, 100 µl of electrocompetent bacteria was aliquoted in 1,5 ml tubes and frozen at -80ºC.

- Transformation of bacterial strains using the electro shock method

After thawing 100 µl of bacteria on ice, DNA was added and mixed gently avoiding pipetting. The DNA-bacteria mixtures were transferred to an electroporation cuvette and placed on ice for 10 minutes. Then, electric shock was performed by electroporation (Gene Pulser Xcell™ system from Biorad) with a pre-set protocol for bacteria: *E.coli* 2mm and 2,5kV. Then, 500 µl of SOB media were added immediately to recover cells from the cuvette and mixtures were transferred to a new tube with 400 µl SOC. Mixture were incubated at 37ºC shaking for 1 hour. Afterwards cells were centrifuged and spread into LB plates containing the corresponding antibiotic of choice* and incubated for 16-24 hours at 37°C.

**SOC media composition: SOB with 20mM glucose.*

** Antibiotic selection in LB-agar plates: when antibiotic selection is needed the appropriated amount of the desired antibiotic is added and mixed into LB-solution before plate preparation (500µL from 1,000X antibiotic stocks).*

- Bacterial Clone screening

For general clone screening, enzymatic digestions and subsequent electrophoresis analyses comparing non-cloned DNA and final cloning products was the general method of choice.

** Colony PCR for clone screening*

Single colonies were touched with a sterile tip and streaked onto a single square on a new gridded LB plate to save the colony. The tip was next swirled in 100 µl of distilled H_2O to dissolve the colony. Dissolved colonies are used as template for subsequent PCR reactions. 1 µl of the final 100-µl colony suspension were used in each PCR mix. PCR mix were as follows: 1 mM dNTPS, Taq-B polymerase Buffer 1X (New England Biolabs), 100nM of forward and reverse primers (Life Technologies), Taq-B polymerase 0. 02U/µl (New England Biolabs). The standard cycling conditions were as follows: 33 minutes at 94ºC; 30 cycles of 30 seconds at 94ºC/ 30 seconds at 60ºC/ 2 minutes at 68ºC; 5 minutes at 68ºC. PCR products were separated in 1% agarose gels during 45 minutes at 80 V.

1.1.3. Extraction of Nucleic Acids (DNA and RNA) from biological samples

- DNA fragments gel extraction and purification

The analysis, identification and isolation of DNA fragments were performed through agarose gel electrophoresis using non-denaturing conditions. The percentage of agarose used was variable, between 0.8 % and 2 %, depending on the size of the DNA fragments to analyze. DNA fragments were isolated from agarose gels and subsequently purified using QIAquick Gel Extraction Kit (Qiagen).

- Plasmid purification

The extraction of plasmidic DNA from *E.coli* cells was performed using the commercial QIAprep Spin Miniprep Kit (Qiagen). The procedure consists on the lysis of the cells, the precipitation of the insoluble cell debris together with bacterial genomic DNA and the binding of the plasmidic DNA to a positively charged membrane that retains the DNA while proteins and other components are washed away. The purified DNA was eluted into 25-50 µl of TE (Tris-EDTA) buffer or H_2O (both supplied by the Qiagen kit).

- Genomic DNA extraction

Genomic DNA (gDNA) extraction from cells was performed using DNeasy Blood & Tissue kit (Qiagen). Following manufacturers' indications for Animal Blood or Cells (Spin-Column Protocol, Qiagen). Briefly, pellets of approximately $2x10^6$ cells stored at -80ºC were taken and resuspended in Phosphate-buffer saline (PBS-Life Technologies) with 2mg/ml proteinase K and 2µg/µl of RNase (both supplied by the Qiagen kit). Then 200µl of AL buffer were added for lysis at 56ºC for 10 minutes. Before pipetting the mixture into the DNeasy Mini spin column (Qiagen) 200 µl of ethanol 98-100% were added and the sample was mixed by vortexing. Subsequent washing steps of the membrane were performed with AW1 and AW2 buffers by centrifugations of the spin column and finally, two elution steps were performed for maximum DNA yield in buffer AE (Qiagen kit).

- mRNA extraction

Total RNA extraction was done from frozen cell pellets stored at -80ºC following phenol-chloroform based method. For cell pellet generation, culture dishes content was scrapped mechanically with ice cold PBS (Life Technologies). Cell suspension was centrifuged and supernatants subsequently aspirated. Cell pellets were frozen at -80ºC until further use. After thawing cell pellet, 1ml of TRIzol® Reagent (Life Technologies), was added and cell disaggregation was performed with a syringe and a 21G needle help. Then, 250 µl chloroform and mixtures were incubated after intense vortex for 15 minutes. Next mixes were separated at 13.000 rpm at 4ºC. Organic and inorganic phases were further separated by pippeting. Aqueous phase (upper) was recovered and ice-cold 500 µl isopropanol was added to precipitate mRNA overnight at -80ºC (this incubation facilitates the isolation of higher yields of mRNA). Next day, samples were centrifuged for 20 minutes at 13.000rpm at 4ºC. After vacuum aspiration, 1 ml of 70% ethanol was added in order to wash final RNA containing pellets. After ethanol aspiration, RNA pellets were left for 5 minutes to air dry residual volumes of ethanol. Finally, RNA pellets were resuspended in 20 µl of MiliQ H_2O (MiliQ®). Finally, RNA solutions were treated with TURBO DNAse following manufacturers' instructions (Ambion).

1.1.4. Polymerase Chain Reaction (PCR)

- Standard PCR conditions

PCR was performed with multiple Polymerase types depending on the experiment of interest following manufacturers' conditions. Generally PrimeSTAR® GXL Polymerase was the enzyme of choice (TAKARA). PCR conditions used the following reaction mixture: 1X PrimeSTAR® GXL Buffer (TAKARA), 200 µM of each dNTP (Promega), 0, 25 µM each primer (Life Technologies), 1, 25 U/50µl of PrimeSTAR® GXL Polymerase (TAKARA), 50 µl of template (100 ug of gDNA, and 1 ng for plasmidic DNA-λDNA). PCR conditions were as follows: 30 cycles of 10 seconds at 98ºC for denaturation, 15 seconds at 60ºC for primer annealing and 1minute/kb at 68ºC for extension.

- Real Time quantitative PCR (qPCR)

To quantify mRNA expression levels of the genes of interest, total mRNA was isolated from frozen cell pellets using TRIzol® method described above (*mRNA extraction* section). Then RNA solutions were retrotranscribed using Cloned AMV First-Strand Synthesis kit (Life Technologies). 20ng of complementary DNA (cDNA) were used to quantify gene expression with Platinum Syber Green Super Mix (Life Technologies) in an ABI 7900 Fast Sequence Detection Instrument (Applied Biosystems). Primers listed in "Oligonucleotides Table I" were designed and validated with Primer 3 Software. Gene expression was normalized to the mean of housekeeping Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Ribosomal protein S18 (S18) genes. Fold change determined using the comparative Ct method.

1.1.5. Sanger Sequencing

DNA sequencing from plasmid vectors or gDNA were done by PCR amplification using GXL Polymerase (TAKARA) and primers listed in "Oligonucleotides Table II". Final PCR products were separated by electrophoresis and subsequently recovered and purified following QIAquick Gel Extraction Kit (Qiagen) recommendations. The purity and amount of DNA was quantified using Nanodrop (Thermo Scientific NanoDrop 2000c). Generally 10 ng of gDNA were used as template for subsequent sequencing reactions. Mixtures were as follows: with BigDye v3.1. PCR conditions were as follows: 3 minutes at 94ºC; 40 cycles of: 10 seconds 96ºC/5 seconds 50ºC/4minutes 60ºC; and 4ºC. Sanger sequencing was performed in a 3130XL DNA analyser (Applied Biosystems®). Sequencing results were analysed and compared by using ApE-A software (http://www.apesoftware.com/).

1.2. Biohistochemistry and Imaging

1.2.1. General Immunostaining and image captation

Cells were grown on plastic cover slide chambers (170920, Nunc) and fixed in 4% paraformaldehyde (PFA) (Sigma) during 15 minutes at RT. When mesodermal markers

were analyzed 2% PFA was used. Fixation was performed during 20 minutes at RT. Slides were washed three times with Tris-Buffered Saline* (TBS) and blocked with TBS+ (0.5% Triton X-100 in the presence of 6% donkey serum-Life Technologies) during 30 minutes at RT. Slides were incubated overnight with the primary antibody listed in “Antibodies Table I” in TBS++ (0.1% Triton X-100 and 6% donkey serum) at 4°C. The next day, slides were washed with TBS++ and incubated with CY-conjugated or DyLight-conjugated secondary anti-rabbit/mouse/goat listed in “Antibodies Table II”, during two hours at 37°C. Finally, slides were washed with TBS++ for 5 minutes at RT and counterstain with 4',6-diamidino-2-phenylindole (DAPI) at 1:10,000 diluted in H_2O (MiliQ®) from DAPI stock solution (Life Technologies) for 10 minutes at RT in the dark. Slides were covered with mounting medium* together with 24 x 40 mm Knittel Glass coverslip. Images were taken using Leica SP5 confocal microscope or Leica SPE. Captures were analyzed by Leica AF6000 software. Image processing was performed with Image J/FIJI software.

**Tris-Buffered Saline (TBS) preparation: 50 mM Tris-Cl and 150 mM NaCl dissolved in H_2O [pH 7.5]. TBS can be kept up to 1 month at 4ºC.*

**Mounting Medium, Recipe for 1L: 4,2g glycine, 0,21g NaOH, 5, 1 NaCl, 0,3g NaN_3, 50 g propyl gallate, 700ml glycerol and H_2O up to 1L.*

1.3. General Cell Culture and maintaining

All cell lines were incubated at 37 °C and 5% CO2.

- 293A, 293T, Phoenix Amphotropic Cells

293A, 293T, HEK293, Phoenix Amphotropic cells were purchased in ATTC. Cell lines were maintained and expanded in complete Dulbeccos’ Modified Eagle Media (Complete DMEM). When cells reached around 80% confluence, every 3-4 days, subcultures were performed enzymatic disaggregation* with Trypsin-EDTA 0.05%. Media was changed every two days.

**Complete DMEM composition: DMEM, 10% Fetal Bovine Serum (FBS) + Non-Essential Aminoacids (NEAA), 1% Glutamax (Glx), 1% Penicillin- Streptomycin (P/S) (100 U/ml).*

** Enzymatic disaggregation: Medium is aspirated and cultures are washed with PBS. Trypsin-EDTA 0.05% is added to cell cultures to completely cover the monolayer of cells and place in 37 °C incubator for approximately 2 minutes. Trypsin or trypsin-EDTA solution is removed by aspiration and Complete DMEM is added into the cell suspensions that are further centrifugued at 1200 revolutions per minute (rpm) during 5 minutes at RT. Cells are resuspended and subsequently cultured for further purposes.*

- Human Embryonic Stem Cells (hESCs)

ES[4] cell line was originally derived at the Center of Regenerative Medicine of Barcelona. ES[4] was cultured on Matrigel™ (BD Biosciences) coated plates in the presence of mTeSR ™ medium (Stem Cell Technologies) supplemented with 100 U/ml penicillin- streptomycin (P/S). Undifferentiated colonies were passaged periodically (every 5-7 days) with 0.5mM UltraPure EDTA (Life Technologies). Media was changed every two days.

- Human Foreskin Fibroblasts (HFFs)

HFFs from ATTC (HFF1, ATTC) were cultured in the presence of Iscoves's Modified Dulbecco's Medium (IMDM) media. Media was replaced every 2-3 days. When cultures reached 80-90% of confluence, cells were passaged by standard trypsinization with trypsin-EDTA 0, 25% (Life Technologies) and seeded at a density of 70.000 cells/ cm^2.

For irradiation, cells were grown until 100% of confluence, trypsinized, resuspended in IMDM media and irradiated at 45Gy in RS 2000 Biological Research Irradiator (Rad source technologies). IrHFF were frozen in freezing media* ($4x10^6$ irHFF per vial). IrHFF were plated on gelatin (Sigma) coated plates at a density of $3x10^6$ irHFF in a 10cm plate if freshly irradiated or at a density of $4x10^6$ irHFF in 10cm plate if thawed from a frozen vial.

**IMDM composition: IMDM (Gibco), 10% FBS, 1% P/S.*

**Freezing media preparation: complete DMEM plus 5-10% (v/v) DMSO. Generally freezing media is freshly used and not preserved for further use.*

- Mouse Embryonic Fibroblasts (MEFS)

MEFs were derived from E13,5-E14,5 day old CD1 strain embryos. Irradiation was performed as described above for irHFFs. IrMEFs were plated on gelatin (Chemicon) coated plates at a density of $3x10^6$ cells/ 10cm plate when freshly irradiated cells were used [when frozen stocks were used $4x10^6$ IrMEFs were seeded in 10cm plates (Nunc)]. irMEFs were cultured 1 day in the presence of DMEM 10% FBS. Next HES media* was added on confluent monolayers of IrMEFs in order to generated HES-conditioned media, suitable for the culture of hiPSCs. HES-conditioned media was generated by changing HES media every day during 10 consecutive days. Every day HES-conditioned media was filtered with 0,22um filters and supplemented with 5ng/ml bFGF (Prepotech). Media kept at -20ºC was preserved in the absence of bFGF after further use.

**HES media composition: Knock out (KO)-DMEM (Gibco), 20% Knockout Serum Replacement (KSR) (Gibco), 1% NEAA, 1% Glx, 0.1% β-Mercaptoethanol (Gibco), 0.5% P/S and 10ng/ml bFGF (Peprotech).*

- Human induced Pluripotent Stem Cells (hiPSCs)

hiPSCs were cultured on top of irradiated Human foreskin Fibroblasts (HFF, ATTC) feeder layers in the presence of HES media. Cells were passaged mechanically every 7-10 days. In order to obtain hiPSC without feeder contamination, hiPSC were seed on Matrigel™ (BD Biosciences) coated plates with $6x10^5$ irHFF in the presence of HES-conditioned media.

- Umbilical Cord Mesenchymal Stem Cells (ucMSCs)

ucMSCS were cultured in complete *Alpha-* Minimum Essential Medium. Media was changed every 2 days. Cells were subcultured by trypsinization every 5 or 6 days.

**Complete alpha-MEM composition: alpha-MEM (Life Technologies) with*

Ribonucleosides and Deoxyribonucleosides (Gibco), 10% FBS, 1% P/S, 1% Glx.

1.4. Fluorescent Activated Cell Analysis and Sorting (FACS)

Generally FACS was performed as follows: first lifting the cells with Trypsin–EDTA 0.05% (Life Technologies) or Accumax (Stem Cell Technologies) in case of either hESCs or hiPSCs. After trypsin inactivation cell suspensions were adjusted to $1x10^6$ cells/ml.

For direct-labeling, primary antibodies conjugated with FITC, PE or APC fluorescent proteins were used listed in "*Antibodies Table III*" Staining was performed following manufacturers' recommendations. Concentrations with the selected antibodies were performed for 30 minutes in the dark. Next, cells were washed twice with FACS buffer* and filtered with a nylon mesh (Falcon ®) before sorting. Negative controls were performed by incubating cell suspensions in the presence of antibodies isotypes listed in *"Antibodies table IV"*.

Direct-labeling was performed for characterization of ucMSCs. When cell suspensions were analyzed for the expression of mOrange or GFP expression after viral transduction for fibroblasts cell-conversion to cardiac-like cells direct labeling was not required.

FACS and FA-analysis were performed in a MoFlo^{TM} and a Gallios^{TM} (Beckman and Coulter) cytometer respectively. Dot blots were analysed by KALUZA and FlowJo analysis software.

**FACS buffer composition: ice-cold PBS, 5% FBS or BSA (without sodium azide addition).*

2. REPROGRAMMING OF HUMAN UCMSCs

2.1. ucMSCs derivation and characterization

Umbilical Cord Blood (UCB) samples were obtained from the *Banc de Sang i Teixits, Hospital Duran i Reynals, Barcelona.*

2.1.1. Isolation of ucMSCs

ucMSCs were isolated from UCB as previously described (Kern et al. 2006a). Briefly, the mononuclear cell (MNC) fraction was isolated by density gradient centrifugation (**Figure 1**) and seeded at a density of 1 x 10^6 MNC/ cm^2. Non-adherent cells were removed 12–18 hours after initial plating. Adherent ucMSCs appeared as fibroblastoid colony-forming units and were harvested at sub-confluence with trypsin-EDTA 0,25% and expanded for their subsequent characterization and further experiments. ucMSCs were cultured in complete DMEM.

Figure 1: Centrifufgation method for isolation of cells from cord blood. a) An average of 75 ml of umbilical cord blood is collected into a plastic blood bag by venipucture. **b**) Before centrifugation, two distinct layers can be clearly distinguished: the diluted blood (B) over the Lympholyte-H (F). **c**) After centrifugation, there should be a well-defined white layer (W) at the interface, red blood cells on the bottom below the Lympholyte (R) and plasma with platelets on the top (P). Adapted from (Giorgetti et al. 2010).

2.1.2. Differentiation potential in ucMSCs

Differentiation towards bone, cartilage and adipose cells was performed to further assess ucMSCs functionality.

2.1.2.1. Differentiation towards Osteogenic fate

In order to evaluate the differentiation capacity to osteogenic fate, ucMSCs were plated at 10.000 cells/cm^2 density in osteogenic media for 21 days.

The analysis of cell differentiation was performed by Alizarin Red S histochemical staining.

**Osteogenic media:* a*lpha-MEM with 10% FCS, 1% P/S, 10 mM β-glicerolphosphate (Sigma), 0.2 mM ascorbate-2-phosphate (Sigma) and 100 nM dexamethasone (Sigma).*

2.1.2.2. Differentiation towards Chondrogenic fate

Chondrogenic differentiation was performed by pelleting 200.000 ucMSCs cells/well in a 96 well plate with conical bottom and cultured in suspension in chondrogenic induction media for up to 21 days.

Analysis of cell differentiation was performed by histochemical staining by Toluidin blue.

**Chondrogenic media*: *DMEM-high glucose (Gibco) with 1% P/S, 10 ng/ml TGF- β 3 (R&D Systems), 50 mg/ml ITS + Premix (BD), 50 mg/ml of proline (Sigma), 500 ng/ml of BMP6 (R&D Systems), 50 µg/ml of ascorbate-2-P and 100 nM of dexamethasone*.

2.1.2.3. Differentiation towards Adipogenic fate

For adipogenic differentiation, ucMSCs were cultured to 95–100% confluence. After expansion complete DMEM was replaced with adipogenic media for 21 days.

Analysis of cell differentiation was performed by histochemical staining with Oil red O (Sigma).

**Adipogenic media: media alpha-MEM with 10% FCS, 1% P/S, 50 μM indomethacin (Sigma), 0.5 mM IBMX (Sigma) and 1μM dexamethasone.*

2.2. Somatic cell reprogramming

2.2.1. Generation of retroviral supernatants

Phoenix Amphotrophic cells 293 (ATTC) at passage 6 were plated in polystyrene plates (Nunc) and further expanded in complete DMEM medium. When cells reached 80% confluence, they were disaggregated in the presence of Trypsin–EDTA 0.05% (Life Technologies). Next $10x10^6$ cells were plated in 150 mm polystyrene culture dishes (Nunc). Next day, Phoenix Amphotropic 293 cells were transfected with FuGENE 6–DNA complex according to the manufacturers' instructions (Promega). For FuGENE 6_DNA complex formation, 27 uL FuGENE 6 was added to 1 ml of Opti-MEM (Life Technologies) in a 50ml tube (Falcon ®) and further incubated for 5 minutes at RT.

Next, tricistronic pMXs retroviral DNA plasmids encoding for reprogramming factors (Oct4, Sox2, Klf4 and c-Myc) described in (Montserrat et al. 2012) were added carefully to the solution, avoiding direct contact of the plastic tip to the walls of the falcon tube. After 5 minutes 20 ug from each pMXs retroviral based plasmid was added to the final mixtures and incubated at RT during 20 minutes. Next, the solution was added drop wise onto Phoenix Amphotropic cell culture dishes. Transfected cells were maintained overnight in the incubator at 37ºC. Next day, *complete DMEM* was replaced with new media and from that point, cells were incubated at 32ºC (in order to induce the generation of retroviral particles). Monitoring transfection efficiency was possible at this point because of the fluorescent reporters: mOrange (monitoring the expression of Oct4 and Sox2 transgenes) or GFP (monitoring the expression of Klf4 and c-Myc). 48 hours after transfection, first viral supernatant was collected and filtered through a 0.45-μm PVDF low binding protein filter (Falcon ®) to remove any residual cells. Two more collections were performed every 12 hours during two consecutive days. Viral supernatants were directly used after collection or further stored at -80ºC.

2.2.2. Retroviruses transduction to ucMSCs

The day before transduction, 80.000 ucMSCs were plated in each well of a 6 well plate. Fresh retroviral supernatants were supplemented with Polybrene (Sigma) at final concentration of 10ng/ml. Then, 1ml of each retroviral preparation was in each well and plates were further centrifuged for 45 minutes at 750 rcf at 32ºC. ucMSCS were infected three times at 12 hours intervals. One to three days after the last infection, infected cells were trypsinized with Trypsin-EDTA 0,05% (Life Technologies) and plated onto irMEFs in the presence of HES media.

2.2.3. Generation and culture of hiPSCs from ucMSCs

Infected ucMSCs were maintained on irMEFs in the presence of HES media for up to 10 days. 15 days after infection, small iPSCs appeared and by day 20 individual colonies were mechanically picked and subcultured on irHFFs in HES media. After couple of passages, iPSC were seed on Matrigel™ (BD Biosciences) coated plates in the presence of HES conditioned media. After 1-2 passages, iPSCs were seed on pure Matrigel™ (BD Biosciences) coated plates and further expanded by trypsinization with Trypsin-EDTA 0.05% (Life Technologies).

2.3. Molecular characterization of iPSCs derived from ucMSCs

2.3.1 qPCR analysis

Real Time qPCR was performed in order to analyse transgenes silencing and the endogenous expression of pluripotent related factors. General conditions are detailed above in *1.1.4. Polymerase Chain Reaction (PCR)-Real Time quantitative PCR (qPCR)* section. Primers used are listed in "*Oligonucleotides Table I*".

2.3.2 Immunophenotyping analysis by Fluorescent Activated Cell sorting (FACS)

Direct-labeling was performed as described above in "*1.4. Fluorescent Activated Cell Analysis and Sorting (FACS)"* section for characterization of ucMSCs with antibodies against CD34, CD45, CD73, and CD90, CD105, CD106 listed in *"Antibodies Table III"*.

2.4. *In vitro* differentiation to the three germ layers of the embryo

2.4.1. iPSCs *in vitro* differentiation into endoderm

Monolayers of cultured iPSCs were collected with a handmade glass instrument and left in suspension in low attachment plastic plates for 3. During this time iPSCs aggregates were induced to form Embryoid Bodies (EBs). Next EBs were transferred on ultra-low attachment cell culture plates (Nunc) in the presence of EB media* for 3 days more days in suspension. In order to proceed with subsequent experiments 6 to 10 EBs were plated per slide flask (Nunc) pre-coated with gelatin 0.1% (Sigma). After EBs formed, they were seeded on 0,1 % gelatin (Sigma) coated slide flasks and exposed to culture conditions for the generation of mesoderm, endoderm and neural derivatives (detailed below). Two to three weeks after EB formation, slide flasks were processed for immunohistochemistry.

**EB media composition: Knock out (KO)-DMEM (Gibco), 10% Fetal Bovine Serum (FBS) (Hyclone), 1% NEAA, 1% Glx, 0.1% β-Mercaptoethanol (Gibco), 1% P/S.*

2.4.2. iPSCs *in vitro* differentiation into mesoderm

iPSC were detached and cultured as EBs on ultra-low attachment cell culture plates in EB media for 3 days in suspension. 6-10 EBs were plated per slide flasks pre-coated with gelatin (Sigma) 0.1% and fed with EB media supplemented with 0.5mM ascorbic acid (AA) every 2-3 days until beating focuses appeared around day 20. Then, samples

were fixed with PFA 2% and immunostained against mesodermal markers as Smooth muscle actin (SMA) and alpha-sarcomeric actin (ASA). (Details for primary and secondary antibodies specificities and working dilution conditions are detailed in "*Antibodies Table I* and *Table II* respectively").

2.4.3. iPSCs *in vitro* differentiation into ectoderm

iPSCs were detached and cultured as EBs on ultra-low attachment cell culture plates in 1% N2 (Gibco) and 0,25% B27 (Gibco) media for 4 days in suspension. Next 6-10 EBs were plated on Matrigel™ (BD Biosciences) in neural basal media supplemented with N2/B27 media (Gibco) during 14-16 days, media was changed every to the other day. Samples were then fixed with PFA 4% and immunostained against ectodermal markers Neuron-specific class III beta-tubulin (Tuj1) and Glial fibrillary acidic protein (GFAP) were the markers of choice. (Details for primary and secondary antibodies specificities and working dilution conditions are detailed in "*Antibodies Table I* and *Table II* respectively").

3. GENERATION OF CARDIOMYOCYTE-LIKE CELLS *in vitro*

3.1. Induction of cardiac differentiation by the Embryoid Body (EB) method

Derivation of cardiomyocyte-like cells from ES[4] was performed following similar procedure described in section *2.4. In vitro differentiation to the three germ layers of the embryo (Mesoderm).* Since ES[4] cell line was maintained in fully defined culture conditions (growing on Matrigel® coated surface in mTeSR® media-both from Stemcell Technologies) EB generation method was slithly different. Cells were disaggregated up to single cell by Accumax (Stemcell Technologies). Then, about 100.000 cells were seeded on low attachment V-shaped bottom 96 well plates that were centrifuged for 10 minutes at 1.000 rpm. Plates containing cell aggregates were transferred to the incubator for 3-4 days until spheric EBs were formed. Afterwards, we proceeded exactly as described in the above mentioned section.

3.2. Chemical induction of cardiac differentiation from ES[4] and reporter ES[4] targeted in the *MYH6* locus

Derivation of cardiomyocyte-like cells from ES[4] and ES[4] reporter cell lines was performed by modifying previously described protocols (Lian et al. 2012a; Gu et al. 2014) For reporter cell lines generation see "*Gene targeting and Knock-in by TALEN and CRISPR/CAS9*" section below.

Single cell suspension of ES[4] and ES[4] reporter cell lines performed by Accumax (Stemcell Technologies) disaggregation, were seeded onto Matrigel™ (BD Biosciences) pre-coated cell culture dishes at a density of 125,000 cells per cm^2 in mTeSR ™ (Stem Cell Technologies) in the presence of ROCK inhibitor Y-27632 (Sigma) for 24 hours. Cells were then cultured in mTeSR ™ medium for another day and differentiation was initiated by treatment with 12µM CHIR99021 (Selleck) in complete RPMI/B27-insulin (all from Life Technologies) for 24 h (*day 0 to day 1*). Medium was then changed to complete RPMI/B27-insulin (all from Life Technologies). On *day 3,* 5µM of IWP4 (Stemgent) was added and cells were cultured without medium change for 48 h.

At *day 5*, cells were washed once to eliminate IWP4 inhibitor (Stemgent) and maintained in complete RPMI/B27/Insulin media (Life Technologies). Medium changes were performed every 2 to 3 days. Beating cells appeared around *day 7-12*.

On *day 15*, contracting cardiomyocyte-like sheets or cardiomyocyte-like clusters were collected by intensive mechanic washing or manually picking, re-plated onto fresh Matrigel™ (BD Biosciences) pre-coated dishes or human decellularized heart matrices and maintained in RPMI/B27 (Life Technologies). To avoid other cell contamination and proliferation, clusters were collected and plated in a non-coated plate ON. Next day, fibroblasts-like cells were attached in polystyrene plates (Nunc) and cardiomyocyte-like cells were sub-cultured for further analysis either in Matrigel™ (BD Biosciences) pre-coated dishes or human decellularized heart matrices.

**Complete RPMI and B27+/-insulin composition: RPMI 1640, 0,5% P/S, 1% Glx, 1% NEAA, 0,1% β-mercaptoethanol and 1X B27 +/- insulin supplement (Gibco).*

3.3. Direct conversion of human fibroblasts (HFFs) towards cardiac-like Cells

3.3.1. Retroviral based plasmid construction

Retroviral vector containing OCT4 fused to VP16 transactivator domain and SOX2 (pMXS_OVPS_Orange) (*Materials* section *3.plasmids and Viral Vectors*) was previously derived by Federico González. This plasmid was used either to generate viral particles for the cell-conversion protocol as well as basal vector for cloning the retroviral vector for GATA4 trancription factor. For GATA4 retrovirus, human cDNA without stop codon (OHS5894) was obtained from ORFeome library (Thermo Scientific). The reporter for the transgene ppGFP was amplified from pMax_GFP (Lonza) and cloned in a pCRII vector (Addgene) containing VP16. By classical cloning techniques, both proteins were cloned in a modified retrovirus vector HA tagged pMSCV retroviral vector (Addgene). The final construct was pMSCV_HA_GATA4_VP16_ppGFP. Plasmid map and detailed description in *Results* section *2.2. Figure 6*.

3.3.2 Two-step cell conversion protocol from HFFs towards cardiac fate.

Pools of $9x10^5$ HFFs were transduced with retroviral supernatants from Oct4, Sox2 transcription factors generated as previously described in "*2.2.1. Generation of retroviral supernatants"* section. Transduced cells were maintained on HES media and by *day 8* cells were splited with Trypsin-EDTA 0,05% (Life Technologies), counted and re-plated in Matrigel™ (BD Biosciences) coated plates at a cell density of 100.000 cells per well in 6 well plates (Nunc). Next day (day 9), serial retrovirus infection with GATA4 transcription factor were performed following same infection protocol as described in section *2.2.1* and *2.2.2*. On *day 10*, 12 hours after the third GATA4 infection, media was changed to EB media supplemented with 0.5mM of ascorbic acid (Sigma). From that day cell media was changed every to the other day.

4. GENE TARGETING AND *knock-in* BY TRANSCRIPTION ACTIVATOR-LIKE EFFECTORS NUCLEASES (TALEN) AND CLUSTERED REGULARLY INTERESPACED SHORT PALINDROMIC REPEATS (CRISPR)/CAS9

4.1. Design and Construction of TALENs

Because there exist yet-unknown sequence dependencies for efficient binding or site-specific constraints responsible for differences in functional activity, it is recommended (if possible) to construct two or three TALEN pairs for targeting the same locus. Thus, two different TALEN for *MYH6* locus were designed. For detailed sequence information go to *Appendix I*.

4.1.1. TALEN monomer library construction

To generate TALEN in the lab, first is necessary to construct a library of monomers for constructing custom 18-mer TALE DNA-binding domains that can be stored at -20ºC and are useful for multiple TALEN designs.

Designer TALEs with customized DNA binding domains were constructed using hierarchical ligation as described Sanjana NE 2012 protocol. Briefly, one 96-well plate was used to carry out 72 reactions, using 18 different primers combinations for each monomer template. For PCR reactions, Herculase II high fidelity polymerase (Agilent Technologies) was used following manufacturers' recommendations in a final volume of 100 µl, with primers detailed in "*Oligonucleotides Table III*". DNA templates are listed in "*Plasmids table I*". After reactions were completed, 18 reactions of each monomer amplification were verified using a 1% agarose gel. Then, PCR products were cleaned using NucleoBond Xtra column (Clonetech). For subsequent digestion and ligation reactions monomers were readjusted to equimolar concentrations. In order to normalize monomer concentration the initial concentration was estimated making use of electrophoresis reaction running the samples together with 1 µl of DNA ladder (Life

Technologies) with known mass (5, 10, 20, 40 and 80 ng). Quantitative imaging was performed using Image J software. Concentrations were adjusted by adding Elution Buffer (EB-buffer from Qiagen) to each monomer. Final concentrations of monomers 1, 6, 7, 12, 13 and 18 were adjusted to 15 ng/µl, the other monomers were at a final concentration of 18 ng/ µl.

4.1.2. TALEN target site selection

The left and right TALENs target sites were designed on opposite strands of DNA flanking the region of interest. Each TALEN targets 20-bp sequences. Left and right TALEN were chosen with spacing of 14-20 bases to facilitate *FoKI* nuclease dimerization. Therefore, for a pair of TALENs, each targeting 20-bp sequences, the complete target site should have the form 5'-TN^{19} N^{14-20} N^{19}A-3', where the left TALEN targets 5-TN^{19} -3' and the right TALEN targets the anti- sense strand of 5'-N^{19} A-3' (N = A, G, T or C).

TALEN targeting sites were identified using TAL effector Nucleotide Targeter program (Doyle et al. 2012).

4.1.3. Construction of custom 20-bp targeting TALENs

Best TALEN candidates suggested by Nucleotide Targeter program, were constructed using Golden Gate Assembly method with TALE Toolbox kit from Addgene (http://www.addgene.org/TALE_ Toolbox) (F. Zhang et al. 2011a; Sanjana et al. 2012). Briefly, this process consists on different phases. First, a Golden-Gate cut-ligation step to generate circularized hexamers with *BsmBI* and T7 ligase, and an exonuclease treatment to remove non-circularized hexamers with 0,66U/µl PlasmidSafe DNAse (Epicentre). Then an amplification step, using Herculase II high fidelity polymerase and gel purification and normalization of concentration. Finally, a second Golden-Gate round of cut-ligation to generate final TALEN constructs overnight at 16ºC, by *BsaI-HF* and T7 ligase (Promega) and TALE backbones detailed in "Plasmids Table I".

4.1.4. Verification of the correct TALEN repeats assembly

Transformation of TALEN constructs were done using Stabl3 competent cells following transformation protocol described in "*1.1.2. Transformation of DNA plasmids*" section. For the detection of TALEN clones were screened by PCR as detailed in "*1.1.2. Transformation of DNA plasmids-Colony PCR for clone screening*" section above. The correct sequence was verified by Sanger sequentiation as detailed in "*1.1.5. Sanger Sequencing"* section.

4.2. CRISPR/Cas9 design and construction: guiding RNAs (gRNAs)

For CRISPR/CAS9 experiments, guide RNA cloning plasmid was purchased from Addgene (#41824) (Plasmid Map in *Appendix I* **Supplementary Figure 4**). gRNA expression plasmid was constructed by modifying the gRNA cloning plasmid with HA_mCherry_PolyA cassette (Courchet et al. 2013) and the gRNA specific for MYH6. Best candidate guiding RNA sequence was selected following the protocol described in here: http://www.addgene.org/static/data/93/40/adf4a4fe-5e77-11e2-9c30-003048dd6500.pdf. PAM sequence (CCA) was targeting 5 nucleotides upstream the ATG site in the *MYH6* locus. In addition, last nucleotide of the gRNA core sequence was mutated from G to C in order to increase targeting efficiency**.** For the specific cut of the targeting site we made use of pCAS9_GFP expression plasmid from Addgene (#44719) (Derek T Peters, Chad A Cowan 2013) (Plasmid Map in *Appendix I* **Supplementary Figure 3**)

4.3. MYH6 Donor Vector (d-vector) design and construction

MYH6 donor vector (MYH6 d-vector) was generated by In-Fusion® cloning method (Clonetech) as described in "*1.1. Molecular Biology and Biochemistry techniques*" section. MYH6 5' homology arm PCR from the BAC (RP11-929J10) was purchased in BACPAC Resources Center (BPRC). At the same time, HA_mCherry_Poly cassette was amplified from pCAG_HA_mCherry_Poly based vector from The Scripps Institute

(Courchet et al. 2013). In-Fusion® reaction was done with pZero_FRT_Neo (Liu et al. 2011) double digested with *BamHI* and *NdeI* reestriction enzymes (New England Biolabs). Then MYH6 3' homology arm was amplified as 5' homology arm and subsequently cloned by In-Fusion® using pZero_5'arm_HAmCherry_FRT_Neo vector digested with *EcoRV* and *XhoI* restriction enzymes (all from New England Biolabs). PCRs were performed with primers listed in "*Oligonucleotides Table II*". PCR conditions are detailed in "*1.1. Molecular Biology and Biochemistry techniques*" section.

4.4. Functional Assay: Surveyor Assay

For Surveyor Assay, 293A cells were plated in 6 well plates 24 hours prior lipofection. Next day, cells were lipofected with Lipofectamine 2000 (Life Technologies) following manufacturer´s instructions. For this purpose cells at 70% of confluence were used. 8μg total DNA were lipofected in each reaction. The reactions were as follows:

-4 μg of each MYH6_gRNAs_mCherry plus 4 μg of pCAS9_GFP

-4 μg of MYH6_TALEN for 5' plus MYH6_TALEN for 3'

-4 μg of nuclease plus 4 μg carrier mock DNA as control.

Between day 3 and 4 transfection, cells were analysed for GFP and mCherry expression as detailed in "*2.3.2 Fluorescent Activated Cell Analysis and Sorting (FACS)*" section. Cells were mechanically lifted and stored as a cell pellet at -80ºC as described in *"1.1.3 Extraction of Nucleic Acids (DNA and RNA) from biological samples*" section. Next, gDNA extraction was performed following Qiagen DNeasy kit for Blood and Tissue manufacturers' instructions. The fragment of interest was amplified by PCR using TAKARA LA Taq pol Hot Start as detailed in "*1.1.4. Polymerase Chain Reaction (PCR)*" section above. Briefly; 50ng of genomic DNA were used as template with primers detailed in "*Oligonucleotides Table IV*". PCR conditions were: 94ºCx1minute; 35 cycles of 94ºCx20seconds, 68ºCx1minute; and 68ºCx5minutes; 4ºC. Then, quantification of the DNA product was performed in 1% agarose gel. 1μl of PCR reaction was loaded for each sample. 1kb DNA ladder (Invitrogen) at concentrations: 1000ng, 500ng, 250ng, 125ng, 62.5ng was used in order to perform standard curve of

DNA for further quantifications. Absolute quantification with ladder curve was performed by Transiluminator (BioRad). When each PCR product was adjusted to 40ng/µl, 20µl were taken to perform the heteroduplex annealing following transgenomic Surveyor kit mutation detection kit indications (Life Technologies). After 30 minutes, 8µl of PCR reactions were kept on ice to perform the Suveyor assay reaction (Life Technologies) by mixing PCR products with 1µl $MgCl_2$, 0,5µl nuclease S and 0,5µl enhancer S (Life Technologies). After 1hour of incubation at 42ºC, the reaction was stopped with stop reaction solution from the kit. Then, 10µl of each reaction were analysed by electrophoresis in a 1X Tris-borate-EDTA* (TBE) gel with 2% agarose without Etidium Bromide (EtBr). EtBr dying was performed afterwards by 30 minutes incubation in 1X TBE. Analysis of DNA fragments was done with transiluminator resulting images.

* *Tris-borate-EDTA (TBE) for 5X solution composition: 1.1M Tris, 900mM Borate, 25mM EDTA, pH8.3.*

4.5. ES[4] cell transfection with (TALEN) and (CRISPR)/CAS9

4.5.1. Electroporation

To Electroporate, ES[4] cells were incubated for 1-3hours with 10µM Rho-inhibitor (ROCK: Y-27632;Sigma). Then, single cell suspension was performed by Accumax exposure in PBS washed ES[4] monolayers for 4 or 6 minutes and inactivated by complete mTeSR™ media. After 4 minutes cell suspensions were centrifuged at 1.000 rpm. This process was done twice. Final cells suspensions were prepared at a final concentration of 10^6 ES[4] cells per 800 µl. Then, 800 µl ES[4] cells were mixed with 15 µg of each pair of TALEN constructs (30 µg total), plus 30 µg of donor vector when TALEN System was used. When CRISP/CAS9 technology was used 15µg of pCAS9_GFP were mixed together with 15µg of gRNA plasmid and 30µg of donor vector, and transferred to an electroporation cuvette (Biorad). Eletroporation conditions were fixed at 500µF; 200Ω; 250V in Gene Pulser Xcell™ system as described in "*1.1.2. Transformation of DNA plasmids using Heat (A) or Electro (B) shock methods*" section above.

After electric shock, cells were immediately transferred to Matrigel™ (BD Biosciences) coated plates with pre-warmed recovering medium consisting on (3:1) mTeSR®:HES conditioned medium, plus 10µM Rho-inhibitor (ROCK: Y-27632;Sigma). Each electroporation reaction was transferred into two wells. Next day, media was changed to mTeSR ®HES conditioned medium without Rho-inhibitor (ROCK: Y-27632;Sigma).

4.5.2. Nucleofection

For nucleofection, single cell suspension of ES[4] cells were performed exactly as mentioned for electroporation protocol. After centrifugation, ES[4] cells were suspended following manufacturers' conditions described in Human Stem Cell Nucleofector Kit (Lonza). After nucleofection ES[4] cells were carefully transferred to 1 well of a 6w plate (Nunc) at same conditions as the one described for electroporated ES[4] cells.

4.6. Determination of MYH6 targeting and copy number integration in transfected ES[4] cells

4.6.1. Selection of targeted ES[4] by antibiotic treatment

Three days after nucleofection, media was changed to normal mTeSR® (Stem Cell Technologies) plus 50 µg/ml of Neomycin (G418, GIBCO). Electroporated cells with the CRISPR/CAS9 system were separated by FACS based on GFP and mCherry expression. G418 was added to the media 48 hours after sorting. Resistant colonies were manually picked after neomycin selection. Half colony was plated in 1 well of 96 well plates for expansion and other half colony was transferred to 1.5 ml tube (Eppendorf®) for gDNA extraction and subsequent molecular assays to identify targeted clones. 7 days after, positive clones identified were sub-cultured in 1 well of a 12 well plate. Cells were maintained as normal ES[4] from this passage.

4.6.2 PCR clone selection: identification of ES[4] clones targeted in *MYH6* locus by TALEN and CRISPR/CAS9 technologies

- Genomic DNA Extraction

After washing cell pellets with PBS, home-made Lysis Buffer* containing 100 µg /ml Proteinase K (Clonetech) was added to cells and incubation at 50ºC for 2-3 hours was performed. After that, Phenol: Chloroform was added and after centrifugation, supernatant recovered was again mixed with Chloroform Isoamyl Alcohol. Again, after centrifugation, recovered supernatant was mixed with Isopropanol (Merck) with Blue DYE (Qiagen) for ON precipitation. Next day gDNA pellets were washed with ethanol 100% and ethanol 70%. Finally gDNA was air dried for 5 minutes and resuspended in TE buffer.

**Lysis buffer composition: 100mMTri-HCl [pH 8.5]; 200mM NaCl, 5mM EDTA and 0,2% SDS.*

- PCR for targeted clone screening

gDNA extraction was performed from half of a colony from each clone picked and transferred to 96well plates. PCR with GXL polymerase (TAKARA) was done to detect targeted clones as described in *"1.1.4. Polymerase Chain Reaction (PCR)"* section above. First by a forward primer homologous to a common region outside the targeting area, upstream the MYH6 starting site in combination with a reverse primer designed to recognize mCherry sequence. Similar short PCRs were performed by combination of outer primers homologous to MYH6 area and inner primers for PGK promoter, mCherry or Neomycin/Kanamycin sequence. Once targeted clones were further identified, long PCRs were performed with outer primers to detect all the integration cassette size.

Primers used are listed in "*Oligonucleotides Table II*"

4.6.3 Southern Blot Analysis: determination of reporter integration and location in ES[4] lines engineered by TALEN and CRISPR/Cas9 technologies

gDNA from each cell line was isolated using All Prep DNA/RNA columns (Qiagen), following manufacturer's guidelines. Each lane of the Southern blot corresponds to 5 ug of genomic DNA digested with 40 U of *BclII* restriction enzyme (New England Biolabs), electrophoresis on a 1% agarose gel, transferred to a neutral nylon membranes (Hybond-N, Amersham) and hybridized with DIG-dUTP labeled probes generated by PCR using the PCR DIG Probe Synthesis Kit (Roche Diagnostics). Probes were detected by an AP-conjugated DIG-Antibody (Roche Diagnostics) using CDP-Star (Sigma-Aldrich) as a substrate for chemiluminescence. Conditions were as per the instructions of the manufacturer. The probes were generated using MYH6, Neomycin and PGK cDNAs as templates with primers listed in "*Oligonucleotides Table IV*".

IV. RESULTS | CHAPTER 1

1. GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS WITH OCT4 AND SOX2.

Human mesenchymal stem cells (MSCs) derived from umbilical cord blood (ucMSCs) represent an ideal source in terms of their accessibility, painless procedures to the donors, few ethical issues and their abundance in the tissue source (Chase et al. 2011a). In the last years, common efforts have been done in order to identify clinically grade sources for the generation of human iPSCs. Due to the capability of ucMSCs to be transformed into other cell types in the presence of chemical compounds (i.e: ucMSCs have been previously transformed into cardiomyocyte-like cells under 5´azacytidine exposure: (Q. Qian et al. 2012) we hypothesized that ucMSCs plasticity could be screened for the generation of iPSCs by means of the overexpression of OCT4, SOX2, KLF4 and c-MYC. In order to test our hypothesis ucMSCs were first derived from umbilical cord (UC) units and subsequently exposed to retroviral supernatants for OCT4, SOX2, KLF4 and c-MYC transcription factors. Our protocol showed that in only 16 days, without the addition of any chemical compound, ucMSCs could efficiently be transformed into ucMSCs derived iPSCS (ucMSCs-iPS). More importantly, our protocol efficiently converted ucMSCs into iPSCS in the presence of only two transcriptional factors: OCT4 and SOX2 avoiding the use of KLF4 and c-MYC. In order to do so, OCT4 was fused with the VP16 transactivation domain, which has been described to increase dramatically the reprogramming efficiency in human fibroblasts (Wang et al. 2011) and human renal proximal tubular cells (Montserrat et al. 2012).

So far, few cell types have been reprogrammed with OCT4 and SOX2 (Giorgetti et al. 2009; Montserrat et al. 2012). In this regard cord blood-derived-prominin (CD133) positive cells (CD133+ cells) have been shown to be reprogrammed with such combination of factors, without the need of any additional chemical compound (Giorgetti et al. 2009). However, cord blood derived CD133+ cells are difficult to expand and maintain *in vitro,* since they need to be cultured in suspension and in the presence of specific cytokines and growth factors. Importantly, although the cytokines sustaining CD133+ cells have been successfully identified (Giorgetti et al. 2009), still these cells have been shown to spontaneously differentiate towards other blood related cell types (i.e: CD34+ cells) after short time periods *in vitro* (4 days). Thus, CD133+

represent a limited source of reprogrammable cells in terms of initial numbers and intrinsic properties. On the contrary, ucMSCs are easily expandable as adherent cells in the presence of chemically defined cell culture media. Moreover, ucMSCs have been demonstrated to retain their related features for more than 11 passages *in vitro* (Can & Balci 2011), representing an ideal cell source for reprogramming.

Overall we believe that the possibility to reprogram ucMSCs towards iPSCs with only *OCT4* and *SOX2* transcriptional factors offers an unprecedented platform for the high-throughput screening and discovery of small molecules able to induce pluripotency and differentiation in somatic cells.

1.1. Phenotype and purity of ucMSCs

Umbilical cord (UC) blood samples were obtained from the "Banc de Sang i Teixits, Hospital Duran i Reynals", Barcelona. UC derived MSCS (ucMSCs) were isolated from UC blood units as previously described by others (Kern et al. 2006a). Briefly, the mononuclear cell (MNC) fraction was isolated by density gradient centrifugation and seeded at a density of 1 x 10^6 MNC/cm^2. Non-adherent cells were removed 12–18 hours after initial plating. Adherent ucMSCs appeared as fibroblastoid colony-forming units (CFU-F). The generated aggregates were cultured in MSC complete medium for a couple of days and then seeded onto 0.1% gelatin-coated plates supplemented with MSC culture medium (*detailed in Material and Methods* section). Sub-confluent ucMSC were obtained within a week and passed at 5000 cells/cm2. At passage (p) 2 ucMSCs were then extensively characterized by Fluorescent Activated Cell Sorting (FACS) analysis for the expression of surface markers related to MSCs phenotype such as CD73, CD90, CD106, and CD106, and surface markers related to blood field such as CD34 and CD45. As expected, ucMSCs expressed CD73, CD90, CD106, and CD106 surface markers, and were negative for the expression of CD34 and CD45 (**Figure 1 A**). In our hands the ratio of success following this approach was 100% efficient. Specifically, we have been able to isolate 3 different clones of ucMSCs from 3 different UC blood initial samples. In order to generate iPSCs from ucMSCs we selected one clone taking in consideration the following features: morphology, clonogenic capacity (data not shown), expression of surface markers related to MSCs phenotype, and

differentiation potential (*detailed below*).

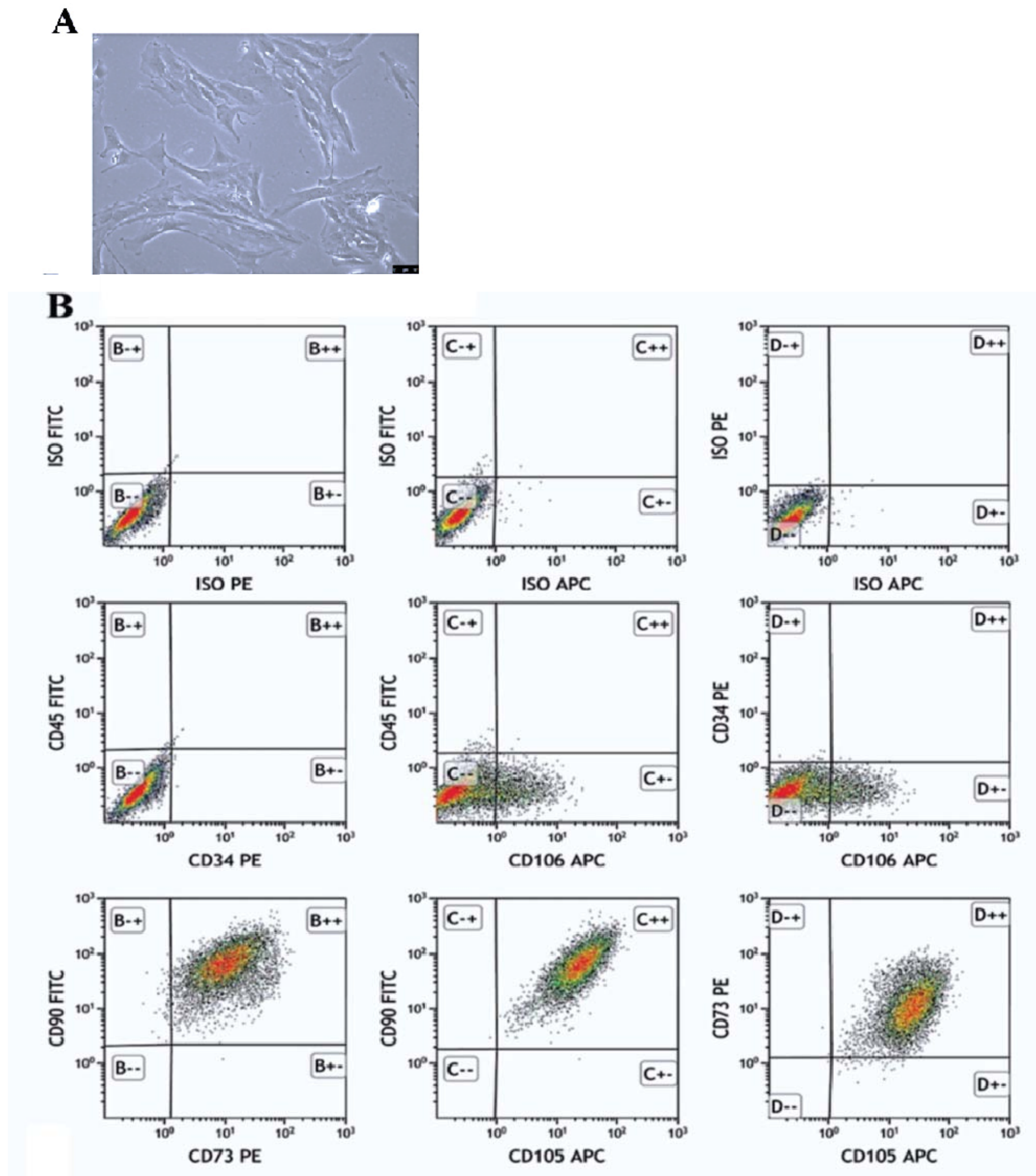


Figure 1. Characterization of ucMSCs. A) Representative image from early passage ucMSCs cultured on gelatin coated surface in the presence of specific media sustaining ucMSCs propierties. **B**) To ascertain the mesenchymal stem cell status of the generated cells expression of surface markers associated with MSCs development and/or progenitor cells such as CD106, CD73, CD90 and CD105 was assessed by FACS analysis. At the same time ucMSCS were stained against CD34 and CD45 surface markers, typically expressed in blood-related cells. Results demonstrate that ucMSCs expressed surface markers related with MSCs biology and are negative for CD34 and CD45 blood related markers.

1.2. ucMSCs can be differentiated towards bone, adipose and cartilage fate

MSCs are currently termed as “mesenchymal stem cells” based on their property of differentiating into a variety of mesodermal tissues including bone, cartilage and adipose (Kern et al. 2006b). In order to evaluate the differentiation capacity to osteogenic fate, cells were plated at 10,000 cells/cm2 in osteogenic media. For adipogenic differentiation, once cells reached upon 95–100% confluence, ucMSC expansion media was replaced with adipogenic, chondrogenic or either adipogenic media. (All differentiation protocols are described in the *Material and Methods* section *2.1.2. Differentiation potential in ucMSCs)*. MSCs were maintained approximately for 21 days in the different conditions sustaining osteogenic, adipogenic or chondrogenic differentiation. Analysis of MSCs differentiation was performed by different histochemical stainings following manufacturer’s protocols **(Figure 2).** Positive adipogenic induction was demonstrated by morphological changes towards larger cells with marked lipid droplet deposition stained with Oil Red O **(Figure 2.A).** Osteogenic induction after 21 days showed marked morphological changes and extra cellular calcium deposition as demonstrated by positive Von Kossa staining **(Figure 2.B).** Chondrogenic differentiation was assessed in cell aggregates by toluidine staining for glycosaminoglycans (**Figure 2.C**). As shown in **Figure 2**, ucMSCs show the capacity to be differentiated towards the three mesenchymal related lineages.

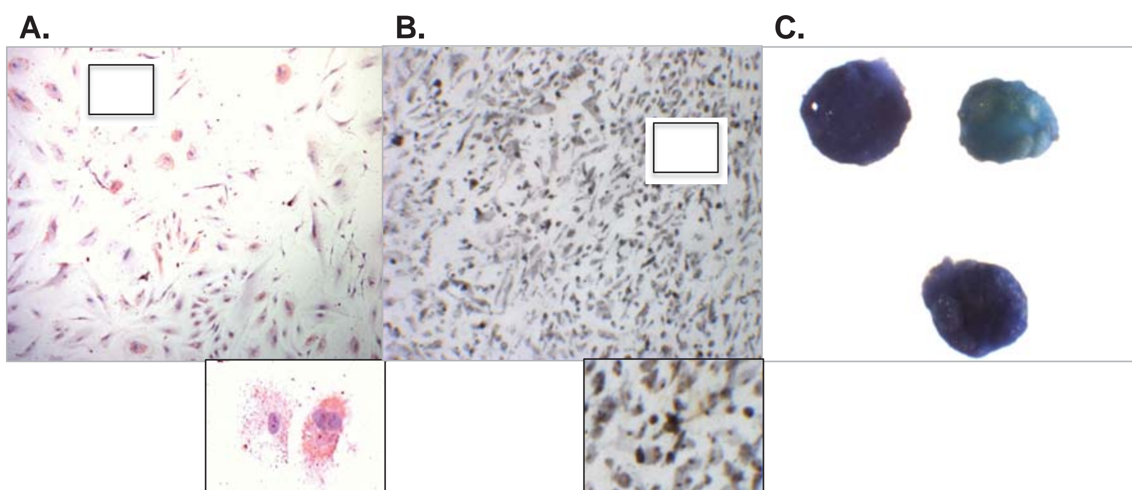


Figure 2. Analysis of the differentiation potential of ucMSCs. Representative images of ucMSCs differentiated towards adipose, bone and cartilage-like cells. Briefly, ucMSCs at passage 2 were exposed to specific protocols for the interrogation of the differentiation potential of ucMSCs. Results show that

under the specific cell culture conditions ucMSCs are capable to differentiate into the main cell types related with MSC potency. **A)** Positive adipogenic induction demonstrated by red lipid depositions stained with Oil Red O. **B)** Osteogenic induction demonstrated by calcium depositions stained by Von Kossa method. **C)** Chondrogenic induction demonstrated by toluidine blue staining, whereby cartilaginous extracellular matrix stain purple (metachromasia) while undifferentiated or fibrous tissue stain blue (top right aggregate corresponds to control aggregate).

1.3. Reprogramming of ucMSC and characterization of pluripotency signatures in ucMSC-iPSC lines

In order to obtain iPSC colonies, pools of 80,000 ucMSCs were transduced during two consecutive days with OCT4, SOX2, KLF4 and c-MYC (4F) or OCT4VP16-SOX2-Orange (2F) combinations. One day after the last viral infection, cells were transferred onto irradiated mouse embryonic fibroblasts (iMEFs) in the presence of human embryonic stem cell medium (hESm). After 9 days, little compact ESC-like colonies began to appear and approximately 13 days after transferring infected ucMSCs on ESC culture conditions, large and compact iPSC colonies with defined borders and ESC-like morphology were mechanically transferred onto a confluent monolayer of iHFFs in the presence of hESm (**Figure, 3A**). In the same manner ucMSCs derived iPSC lines (ucMSC-iPSC) generated with either 4F or 2F (ucMSC-iPS-4F and ucMSC-iPS-2F, respectively) were extensively characterized for the expression of pluripotency-related markers by means of immunofluorescence. We observed that ucMSC-iPS-4F and ucMSC-iPS-2F expressed nuclear pluripotency-related markers such OCT4, NANOG and SOX2; as well of surface markers typically related to hES biology like SSEA-3, SSEA-4, TRA-1-81 and TRA-1-60 (**Figure 3B**). In the same immunofluorescence analysis we observed that ucMSC-iPS-4F and ucMSC-iPS-2F cell lines had silenced transgene expression (**Figure 3B, 3C)** by anti-dsRED for the detection of Orange expression in colonies generated by retrovirus with pMXS_OVPS_Orange based plasmids (Montserrat et al., 2012) and anti-FLAG for the detection of FLAG expression in those iPS clones generated with pMSCV based plasmids (Aasen et al., 2008).

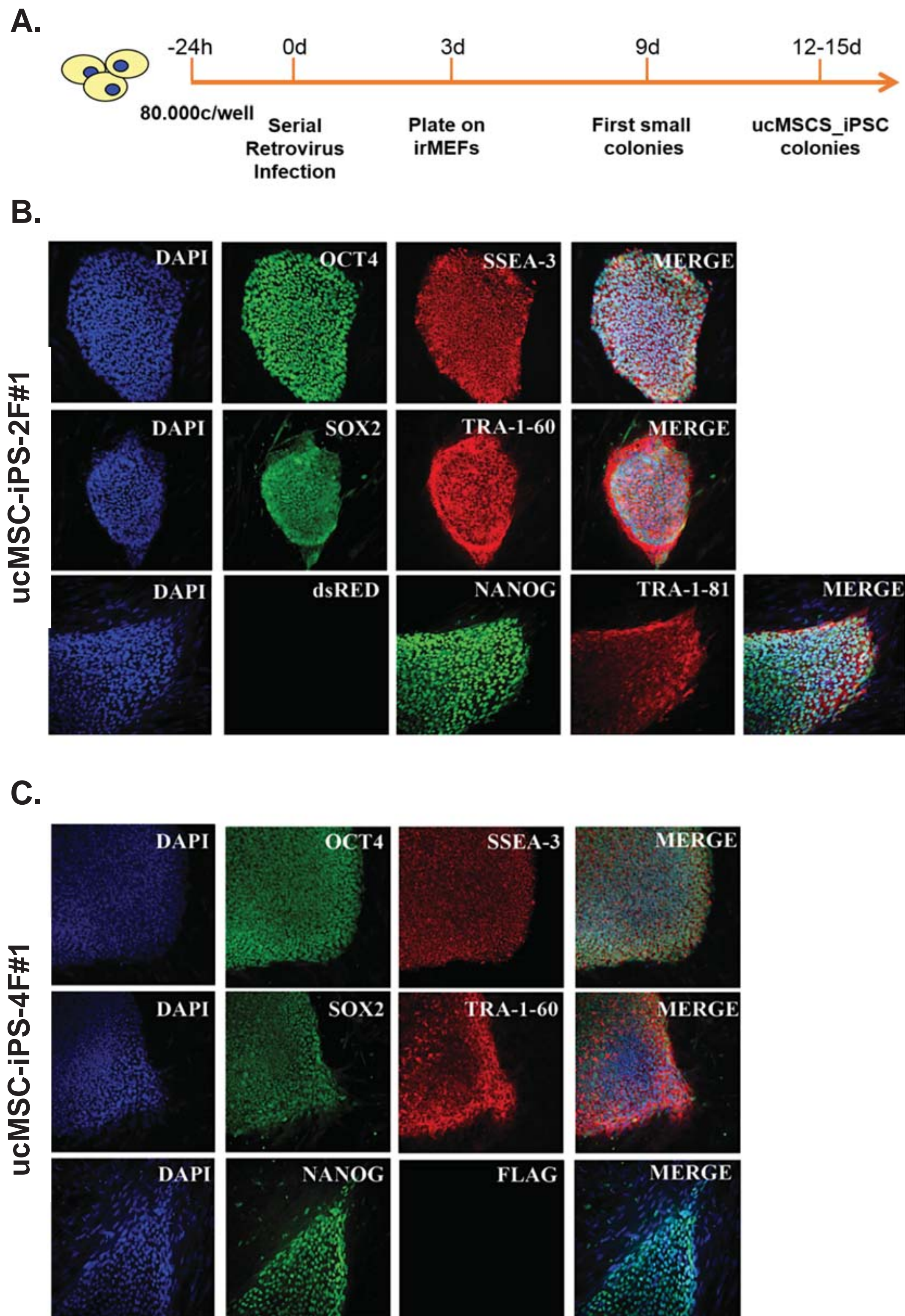


Figure 3. Generation and characterization of ucMSC-iPS-4F and ucMSC-iPS-2F. A) Reprogramming time-line. ucMSCs were transduced with either retroviral particles for OCT4, SOX2, KLF4, and c-MYC transcription factors or OCT4, SOX2 pluripotent related factors. Transduced cells were then transferred on top of iMEFs in the presence of HES media. For both conditions, and only 10 days after viral transduction, small tight colonies appeared. As soon as *day 15* after transduction putative

ucMSC-iPS colonies were manually dissected on top of iHFFs in presence of hES media. After one passage in culture ucMSC-iPS-4F and ucMSC-iPS-2F were manually transferred on top of iHFFs in presence of HES conditioned media from iMEFs. At passage 3 ucMSC-iPS-4F and ucMSC-iPS-2F were cultured in Matrigel® coated plates in presence of mTSER media or HES conditioned media from iMEFs for subsequent assays. **B**) ucMSC-iPS-2F#1 and **C**) ucMSC-iPS-4F#1 clones were analysed by immunofluorescence for the acquisition of markers related with pluripotent stem cells (PSCs) biology. Representative images show that ucMSC-iPS-2F#1 expresses nuclear transcription factors as OCT4, SOX2 and NANOG together with surface markers as Tra-1-60, Tra-1-81 and SSEA-3. Representative images show that ucMSC-iPS-4F#1 expresses nuclear transcription factors as OCT4, SOX2 and NANOG together with surface markers as Tra-1-60 and SSEA-3. Negative transgene detection was assessed by dsRED and FLAG. DNA was stained with DAPI. Images at 20X.

When we analysed gene expression at the mRNA level by RT-qPCR, we detected endogenous reactivation of the four pluripotency factors including a set of other pluripotency markers: DPPA4, REX1, DNMT3B and SALL4 (**Figure, 4).** The levels of expression of the selected markers were comparable to those found in hESC lines ES[2] and ES[4], revealing that the obtained ucMSC-iPSC lines had pluripotency properties similar to hESCs.

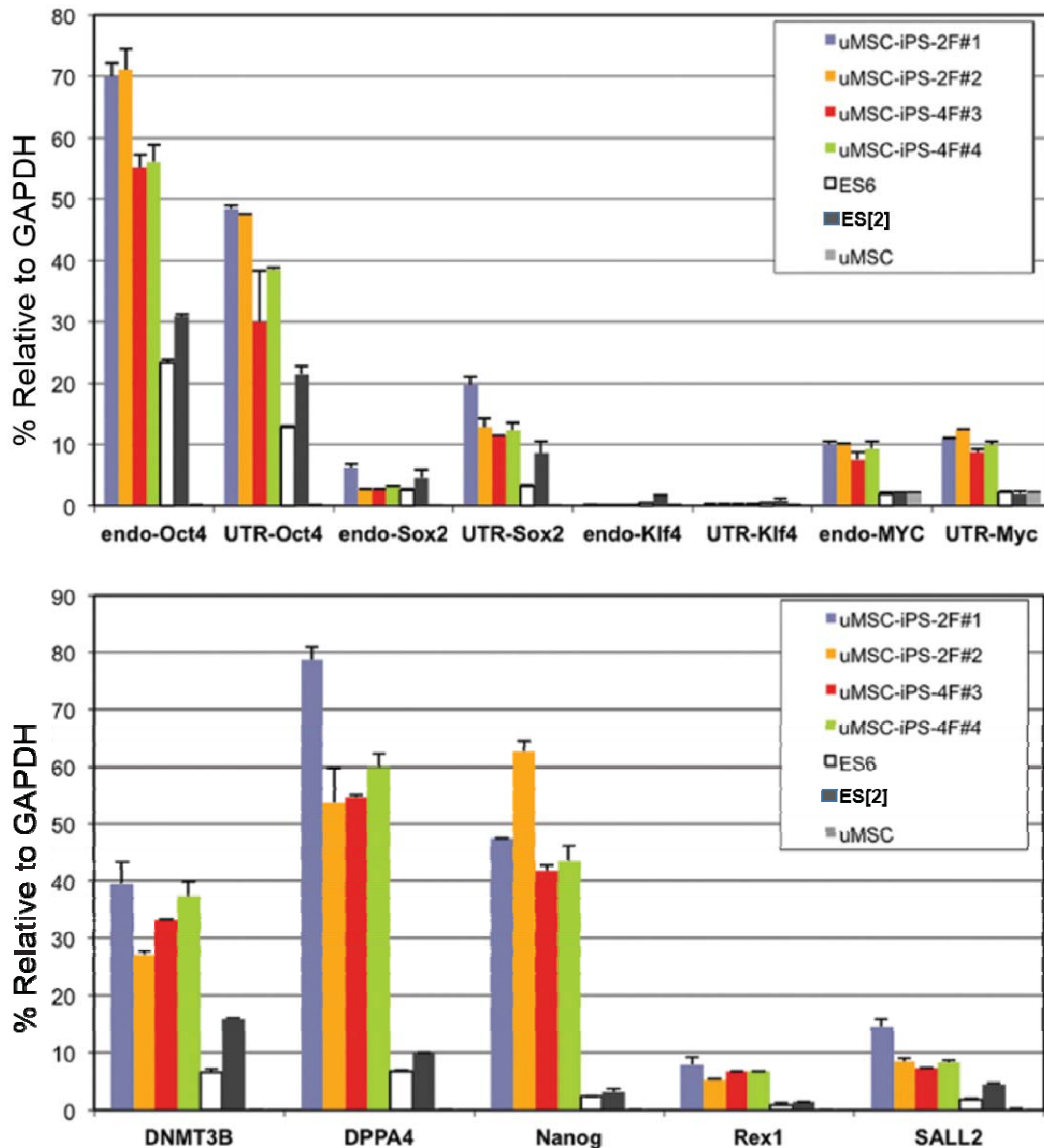


Figure 4. Expression of pluripotency-related markers by quantitative real time (qPCR). ucMSC-iPS-4F and ucMSC-iPS-2F were analyzed for the expression of pluripotent related markers by qPCR. ucMSC-iPS expressed endogenously pluripotency-related genes such as DPPA4, REX1, DNMT3B and SALL4. At the same time the analyzed lines were analyzed for the endogenous expression of the reprogramming factors, demonstrating that OCT4, SOX2, KLF4 and c-MYC expression was induced and sustained under our experimental conditions. ES[4] and ES [2] human embryonic stem cells were used as controls.

1.4. Reprogramming of ucMSCs: *in vitro* differentiation potential of ucMSC-iPS-2F cell lines

We also tested the *in vitro* differentiation potential of ucMSC-iPS-2F cell lines by generating EBs and differentiating them into the three germ layers of the embryo: ectoderm, endoderm and mesoderm (**Figure 5**). ucMSC-iPS-2F derived EBs grown in the presence of 5 μM retinoic acid expressed, Neuron-specific class III beta-tubulin (TUJ-1) and Glial fibrillary acidic protein (GFAP) neuronal markers after 21 days. When ucMSC- iPS-2F derived EBs were grown in the presence of EB media, cells positively stained for forkhead box a2 (FOXA2) were found after 21 days of differentiation. In the same manner, when ucMSC-iPS-2F derived EBs were grown in the presence of 100 μM ascorbic acid we were able to detect focus of beating cells that were positively stained for alpha sarcomeric actin (ASA) and alpha smooth muscle actin (ASMA) markers. Our results demonstrated that ucMSC-iPS-2F cell lines can be properly differentiated *in vitro* towards the three germ layers of the embryo, proving their pluripotency-related potential. Representative images for ectoderm, endoderm and mesoderm markers are shown in **Figure 5**.

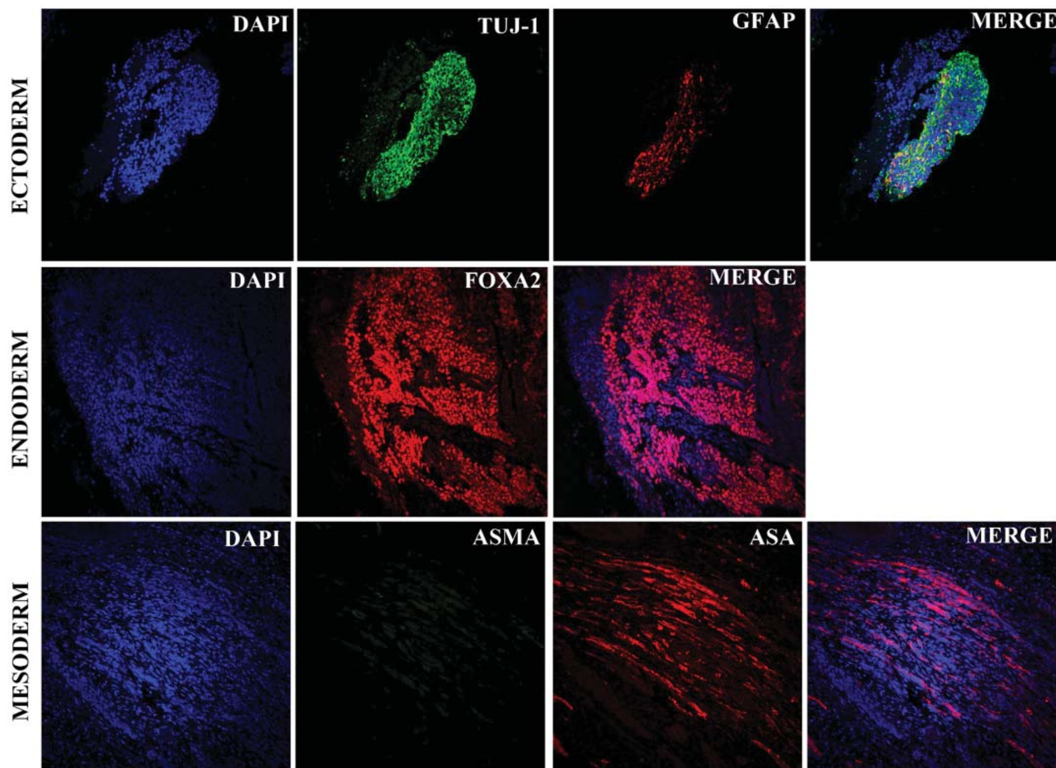


Figure 5. *In vitro* differentiation of ucMSC-iPS-2F#1. The iPSC line was differentiated *in vitro* through the formation of embryoid bodies (EBs). After three days in suspension, EBs were specifically directed to differentiate into ectoderm; for this purpose cells were cultured in Matrigel® in the presence of retinoic acid (RA) a potent inductor of neural lineage at early stages during embryo development. The image shows the merge of nuclear DAPI, Neuron-specific class III beta-tubulin (green) and GFAP (red). Same line was differentiated towards endoderm; for this purpose groups of 10 EBs were exposed to EB media and subsequently stained for the expression of forkhead box A2 (FOXA2-red). In an effort to differentiate ucMSC-iPS-2F#1 towards cardiac-like cells, groups of 8 to 10 EBs were exposed to EB media in the presence of ascorbic acid and subsequently stained for ASMA and ASA markers. Nuclei were stained with DAPI. 10X objective was used to take images.

Our study shows that ucMSCs can be converted towards iPSCs by the ectopic over-expression of OCT4 and SOX2 transcriptional factors. This is the first time, to our knowledge that ucMSCs are reprogrammed towards iPSCs with this factor combination and in the absence of chemical compounds boosting the reprogramming process. We believe that this finding will serve for the screening of chemical compounds and transcription factors replacing OCT4 and/or SOX2 during somatic reprogramming.

IV.RESULTS │ CHAPTER 2

2. CONVERSION OF DERMAL POST-NATAL FIBROBLASTS INTO CARDIAC-LIKE CELLS

The possibility to convert somatic cells into functional specific cell types has opened the door to the development of protocols for cell conversion. In this regard, the discovery that MyoD sufficed the transformation of dermal fibroblasts to skeletal contracting muscle cells represented the starting point for a new field in cell biology (Lassar et al. 1989). Generally the conversion of somatic cells to different cell types has been referred as transdifferentiation, where a master gene defining the final cell type of interest (i.e: MyoD) has been used for guiding cell conversion. The discovery made by Davis and colleagues almost thirty years ago changed our understanding about cell identity and cell plasticity. Considering the fact that only pluripotent stem cells derived from embryos at the blastocyst stage were believed to be derived into any cell type of our organism, the possibility to promote cell conversion between cells of equal or different embryonic origin represented an unprecedented opportunity for the generation of functional cell types avoiding the use of embryonic stem cells. On the other hand the discovery that almost any somatic cell of our body can be converted to induced pluripotent stem cells (iPSCs) also revealed that under certain conditions differentiated cells can be de-differentiated towards a more permissive cell type, as described in capable-regenerating organisms. In the last years different authors have induced the so called "permissive state", also referred as "plastic phase", by means of the ectopic over-expression of reprogramming factors. Here we present a simple protocol for the generation of cardiac-like cells by the exposure of dermal post-natal fibroblast to reprogramming factors (OCT4 and SOX2) and to a specific cardiac transcription factor (GATA 4).

In this work we are also making use of a specific culture media described before to promote cardiac differentiation from pluripotent stem cells. Although our results described the possibility to generate cardiac-like cells based in the acquisition of the expression of different cardiac-related markers at protein and mRNA levels, we are still far from the generation of functional cardiomyocytes. Other authors have also reported similar results when converting human fibroblasts different from cardiac fibroblasts, thus pointing out the need to develop further methodologies for cardiac conversion from

amenable clinical sources (like dermal fibroblasts). We believe that the possibility to convert dermal fibroblasts towards cardiac functional cells will represent a suitable approach for regenerative purposes.

2.1. Differentiation of hESCs towards cardiac like cells with ascorbic acid promotes the expression of cardiac-related markers.

Initial studies in murine ESCs already showed that the exposure of embryoid bodies (EBs) to ascorbic acid promotes cardiac differentiation in a process that takes less than 10 days (Czyz J 2001). Remarkably previous findings in our laboratory already proved the suitability of this protocol for generating cardiac-like cells from iPSCs (**Results 1**) and encouraged us to study which were the cardiac-related markers that were induced during the onset of differentiation. For this purpose we make use of a control hESC cell line, named ES[4], obtained from "Banco Nacional de Líneas Celulares" (Documento_Deposito_Lineas_v32_ES4_def.pdf) ES[4] cells grown in monolayer were induced to form EBs that were kept in suspension for a period of 3 days. Next, groups of 10 to 15 EBs were sub-cultured in gelatin-coated plates in the presence of EB culture media in the presence of ascorbic acid (*media composition is detailed in Material and Methods* section). Under these conditions we were able to produce beating foci in a process that last between *20* and *30 days* (***Appendix* I Video 1**) with efficiencies around 50%. Next we studied the expression of cardiac related markers at *day 20* during differentiation by immunofluorescence. As shown in **Figure 1** differentiated cells expressed a panel of markers related to cardiac fate. Interestingly GATA binding protein 4 (GATA4) expression was induced already at early stages during the differentiation process (**Figure 2**).

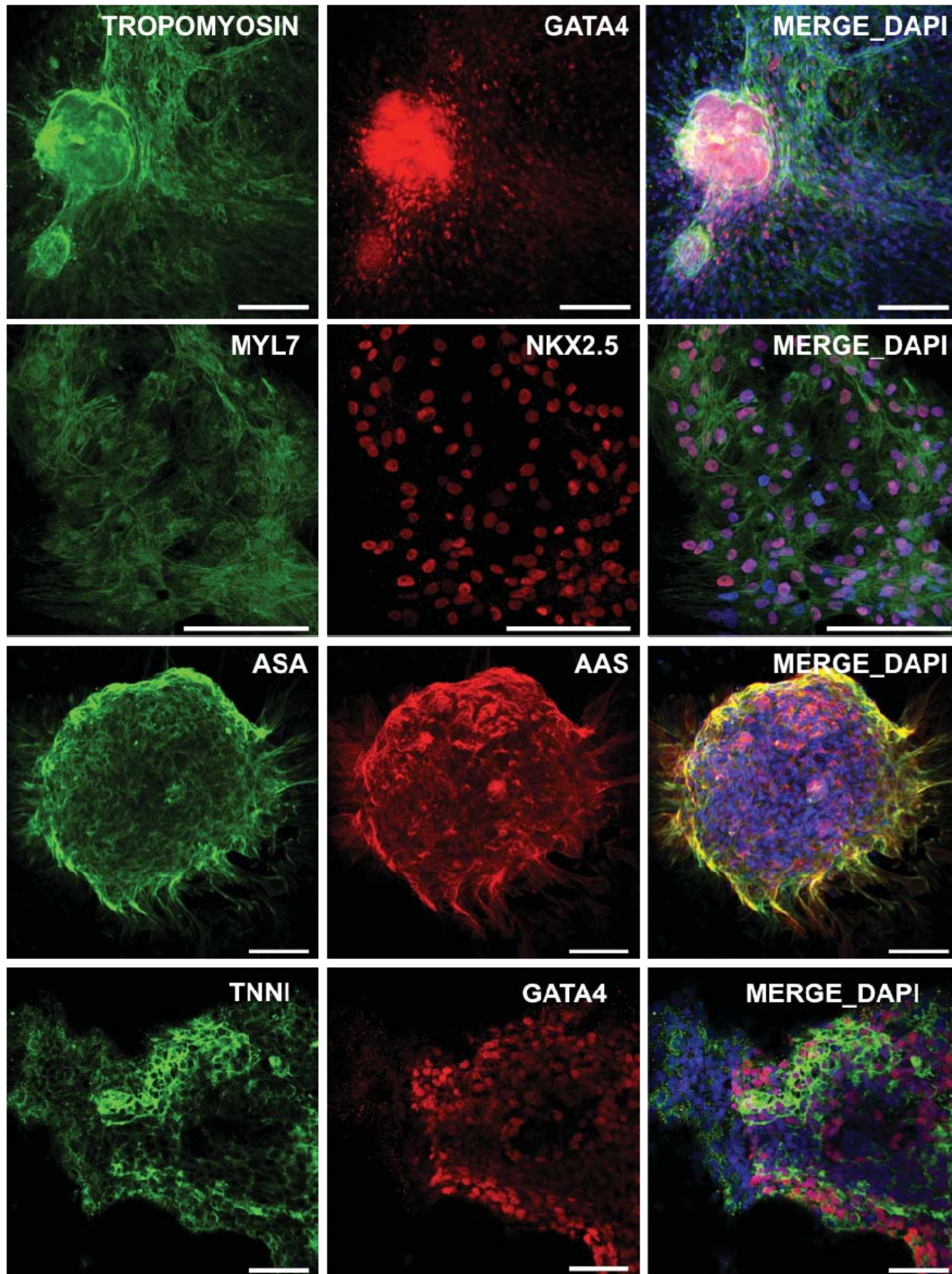


Figure 1. Immunofluorescence analysis by confocal microscopy of ES[4] at *day 20* of cardiac differentiation. Embryoid Bodies (EBs) were exposed to EB medium supplemented with 0.5mM of ascorbic acid. Beating EBs exhibit typical cardiac specific markers, including motor proteins as: Tropomyosin, TroponinI (TNNI), α-Sarcomeric Actin (ASA), α-Actinin Aarcomeric (AAS), Myosin light

chain 7 (MYL7) related to atrial myocytes and cardiac nuclear transcription factors (TFs) GATA4 and NKX2.5. Scale bars: 250µm on top, and 100 µm.

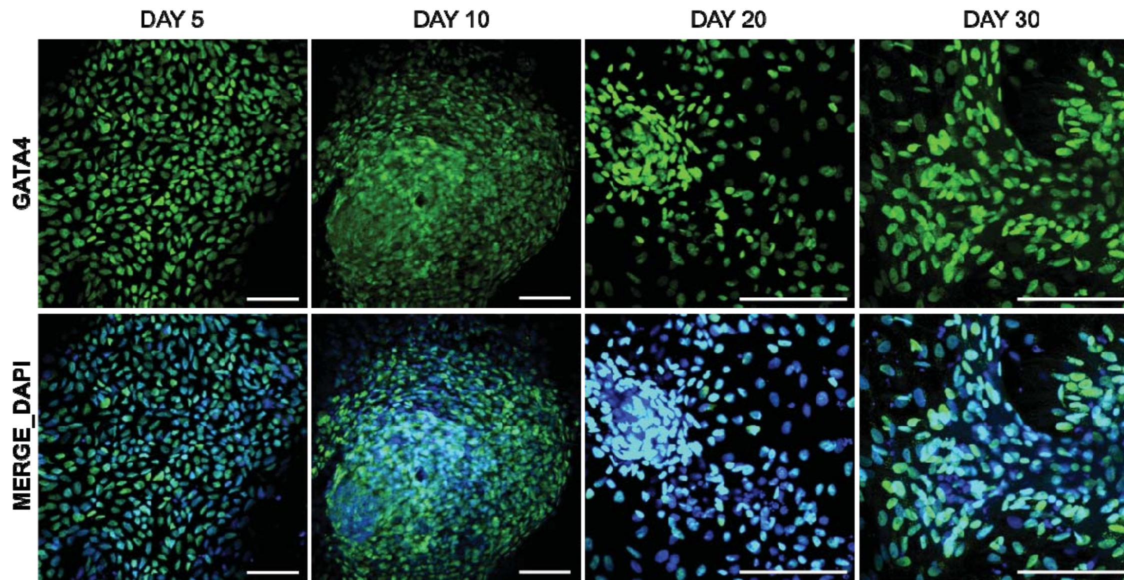


Figure 2. GATA 4 is expressed from early stages of cardiac differentiation in hESCs. Immunofluorescence pictures demonstrating GATA4 protein expression during the differentiation *in vitro* of ES[4] cell line to induced cardiomyocytes (iCMs) at different time points: *day 5*, d*ay 10*, *day 20* and *day 30*. . Scale bars: 100 µm.

2.2. Exposure of post-natal dermal fibroblasts to OCT4 and SOX2 promotes the expression of mesodermal and cardiac related genes.

Recently two independent groups have reported the reprogramming of mouse fibroblasts into cardiomyocyte-like cells by the over-expression of cardiac-related factors (Ieda et al. 2010; Efe et al. 2011). One group relayed in a first phase of exposure of dermal fibroblasts to Yamanaka factors (Ieda et al. 2010), and the other in the over-expression of cardiac-related factors in murine cardiac fibroblasts (Efe et al. 2011). Other studies in the human setting have demonstrated that is possible to convert cardiac fibroblasts into induced cardiomyocytes (Wada et al. 2013). This last report, however, did not rely in the induction of de-differentiation by means of Yamanaka factors, and

made use of a cocktail of five factors related with cardiac fate (GATA4, MEF2C, TBX5, ESRRG, MESP1, HAND2 and Myocardin). For this reason, and also considering that members of our team reported at that time the possibility to de-differentiate human fibroblasts and mesenchymal stem cells by the ectopic over-expression of Yamanaka factors (Kurian, Sancho-Martinez, Nivet, Aguirre, Moon, Pendaries, Volle-Challier, Bono, Herbert, Pulecio, Xia, Li, Montserrat, Ruiz, Dubova, Rodriguez, Denli, Boscolo, Thiagarajan, Gage, Loring, Laurent & Belmonte 2013), we decided to make use of OCT4 fused with VP16 trans-activator domain and SOX 2 together with KLF4 and c-MYC in order to induce fibroblast de-differentiation. For this purpose we did use of a retroviral plasmid vector encoding for OCT4VP16 and SOX2 together with Orange fluorescent protein. This tri-cistronic vector had been already used for the generation of iPSCs from proximal tubular cells (Montserrat et al. 2012) and ucMSCs (de Oñate et al., *in preparation*). We also performed retroviral particles from a retroviral plasmid vector encoding for OCT4 and SOX2 together with Orange fluorescent (OS-Or). In this manner we could compare the effect on fibroblast de-differentiation due to VP16 fusion with OCT4. We then proceed to perform viral particles for OvPS-Or (**Figure 3**), OS-Or and KLF4 and c-MYC (KM) in order to transduce dermal post-natal fibroblasts.

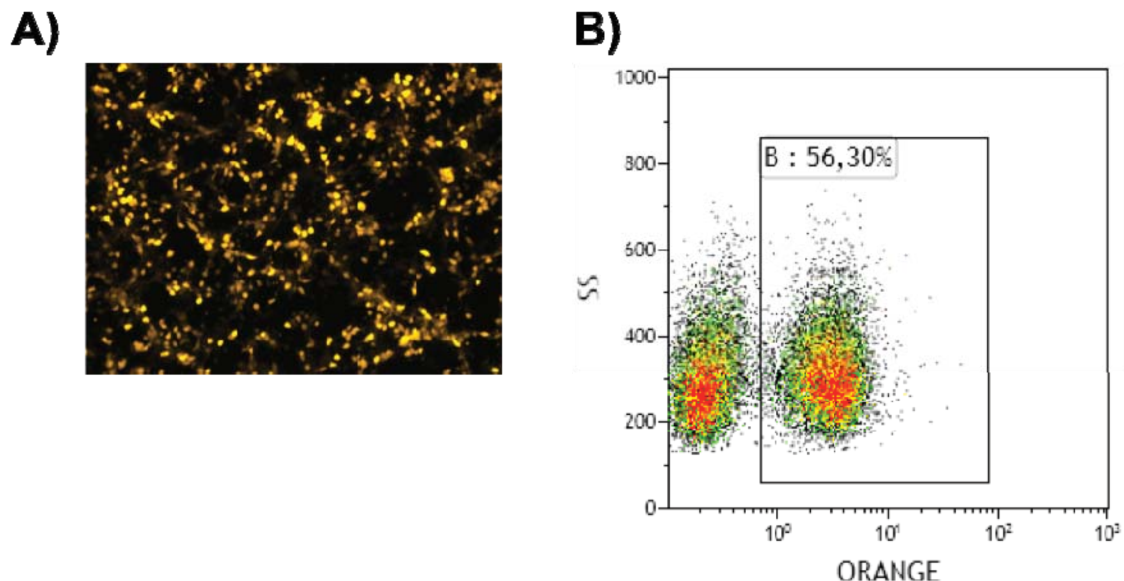


Figure 3. Generation of viral particles for the expression of Yamanaka factors in post-natal dermal fibroblasts. A) Phoenix Amphotropic 293 cells were transfected with Fugene HD® following manufacturer´s indications for the expression of pMXs OCT4VP16-SOX2-Orange plasmid. Image at 10X. After 24 hours cells were analyzed by optical microcopy. **B)** Post-natal dermal fibroblasts were

transduced with viral supernatants during two consecutive days at intervals of 12 hours. Cells were analyzed and separated by FACS for the expression of Orange fluorescent protein. Generally 24 hours after the last transduction 40% of the cells expressed Orange fluorescent protein.

In order to analyze if OCT4 and SOX2 were able to promote fibroblast de-differentiation pools of 80,000 fibroblasts were transduced with either OS-Or + KM or OVPS-Or + KM viral supernatants. Next samples were analyzed by real time qPCR for the expression of markers related with pluripotency. We observed that already at *day 4* after viral transduction NANOG and REX 1 mRNAs expression were upregulated at the different time points analyzed in fibroblasts transduced with OVPS-Or + KM when compared with OS-Or + KM. The rest of the analyzed markers were upregulated from *day 7* up to *day 14*. Our results indicated that the ectopic expression of OVPS-Or and KM induced the expression of several markers related with pluripotency, prompting us to test OVPS-Or as inducer of fibroblasts de-differentiation in the first part of our protocol of fibroblast cardiac conversion (**Figure 4**).

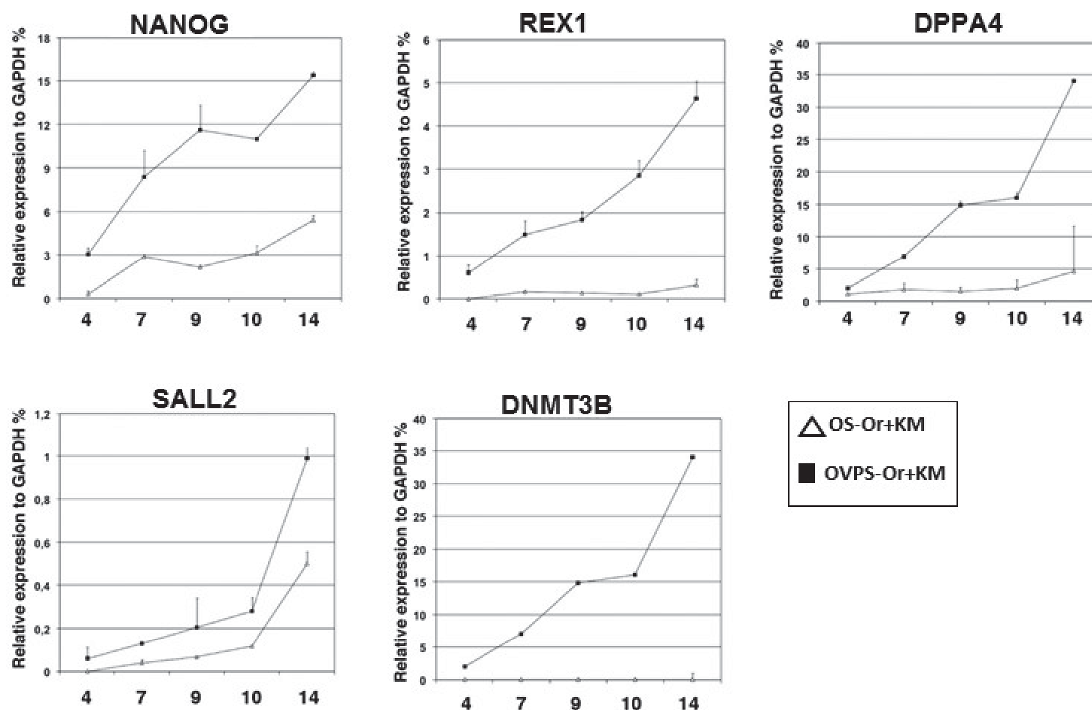


Figure 4. VP16 fused with OCT4 promotes fibroblast de-differentiation. Pools of fibroblasts were infected with different combinations of Yamanaka factors in order to analyze the expression of pluripotency-related markers by qPCR. From *day 4* in culture NANOG and REX 1 expression were upregulated and sustained 14 days after OVPS-Or infection. DPPA4, SALL2 and DNMT3B mRNAs were also up-regulated *7 days* after viral transduction. These results indicated that VP16 fusion with

OCT4 promotes the expression of the indicated markers when compared with OCT4 alone and indicate that under our specific conditions OVPS-Or particles could be used for the induction of de-differentiation in dermal post-natal fibroblasts.

In the same manner mesodermal and cardiac fate markers (early and late stages) were analyzed by qPCR at *day 4* and *day 6* in fibroblasts infected with OVPS-Or viral particles. Our results show that already at *day 4* there was a clear induction in the expression of the different analyzed mRNAs. Interestingly CD34 mRNA was over-expressed four days after viral transduction, as reported by other authors who transdifferentiated dermal fibroblast towards CD34+ cells by OCT4 over-expression (Szabo et al. 2010) (**Figure 5**).

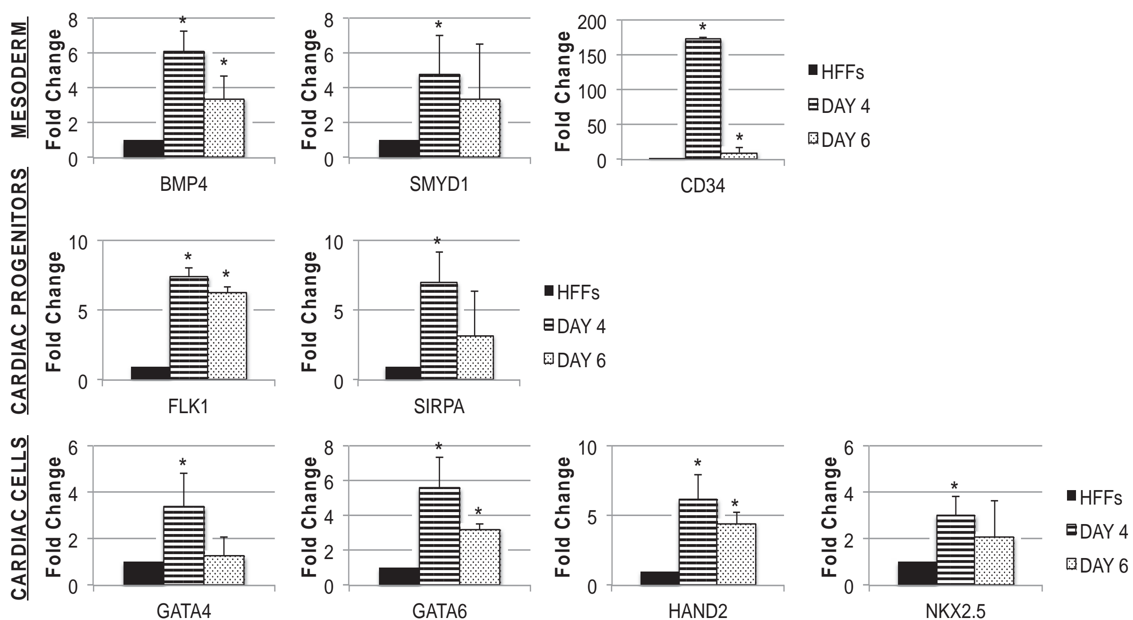


Figure 5. Real Time qPCR analysis time-course for post-natal dermal human fibroblasts (HFFs) at *day 0*, *day4* and *day 6* after the infection with OVPS_mOrange particles. Already at *day 4* the majority of the analyzed mRNAs were up-regulated when compared with HFFs in basal conditions (not infected). Genes for either mesoderm, cardiac progenitors or cardiac cells were analyzed. ΔΔCt analysis. Data are represented as mean +/- s.d. n=3, * $p<0,05$.

Considering the possibility to couple specific conditions promoting cardiac conversion in our first phase of the protocol, we decided to make use of GATA 4 transcription factor in order to boost cardiac cell conversion from de-differentiated fibroblasts. In our hands GATA4 expression was sustained during the process of differentiation from hESCs towards cardiac-like cells (**Figure 2**), and other authors already have proved its

role in cardiac conversion when over-expressed together with other cardiac transcription factors (Ieda et al. 2010; L. Qian et al. 2012; Hirai et al. 2013; Wada et al. 2013; Nam et al. 2013; Takeuchi & Bruneau 2009). For this purpose GATA 4 cDNA was sub-cloned in pMSCV_HA_puro retroviral based vector (for cloning information see *Methods* section *3.2.1. Retroviral based plasmid construction*) (**Figure 6 A**). Viral particles were subsequently produced in Phoenix Amphotropic 293 cells for the subsequent infection of post-natal dermal fibroblasts previously transduced with retroviral particles for OCT4 VP16-SOX2_Orange. (**Figure 6 B**).

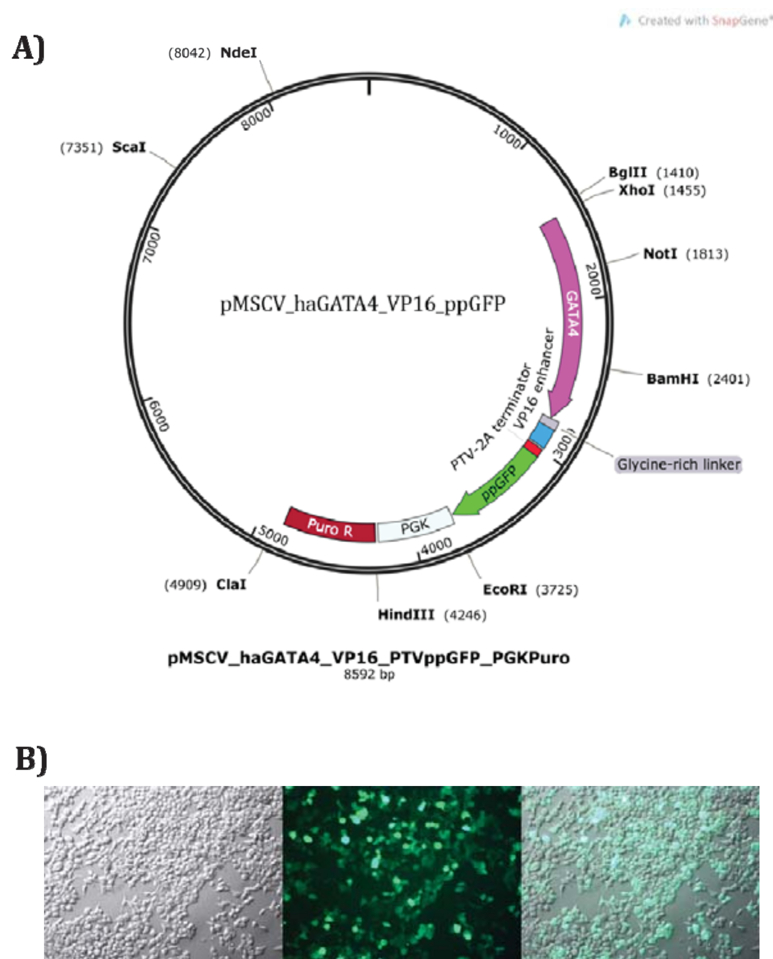


Figure 6. Construction of retroviral vector for GATA4. A) Plasmid map of retroviral vector pMSCV_haGATA4_VP16_ppGFP containing the MSCV (Murine Stem Cell Virus), a Retroviral Expression System vector optimized for introducing and expressing target genes in pluripotent cell lines, including murine or human hematopoietic, embryonic stem (ES), followed by GATA4 TF cDNA tagged in 5' with HA protein, a glycine-rich linker as spacer for VP16 transactivator domain, and a PTV-2A terminator sequence. In frame, ppEGFP and PGK constitutive promoter driving Puromycin Resistance. pMSCV_HA_GATA4_VP16_ppGFP was used to infect HFFs at day 8 and day 9 of the cell-conversion approach. **B)** Bright Field (BF) and fluorescent microscopy images of Phoenix Amphotropic 293 cells transfected with pMSCV_HA_GATA4_VP16_ppGFP at 72 hours. Images 10 X.

2.3. Coupling first phase of mesodermal induction by OCT4 and SOX2 with GATA4, promotes cardiac features in human post-natal dermal fibroblasts.

Once we generated the different tools for the conversion of post-natal dermal fibroblasts towards cardiac-like cells, we designed a two-step protocol based in a first phase inducing cell de-differentiation and mesoderm commitment by over-expressing OCT4 and SOX2 transcription factors. After *8 days* GATA4 was over-expressed in order to direct cardiac conversion. For this purpose cells were exposed to EBm supplemented with ascorbic acid, since we previously observed that under those conditions ES[4] line efficiently differentiated towards cardiac-like cells expressing GATA4 at the protein level. Our protocol is detailed in **Figure 7 A**.

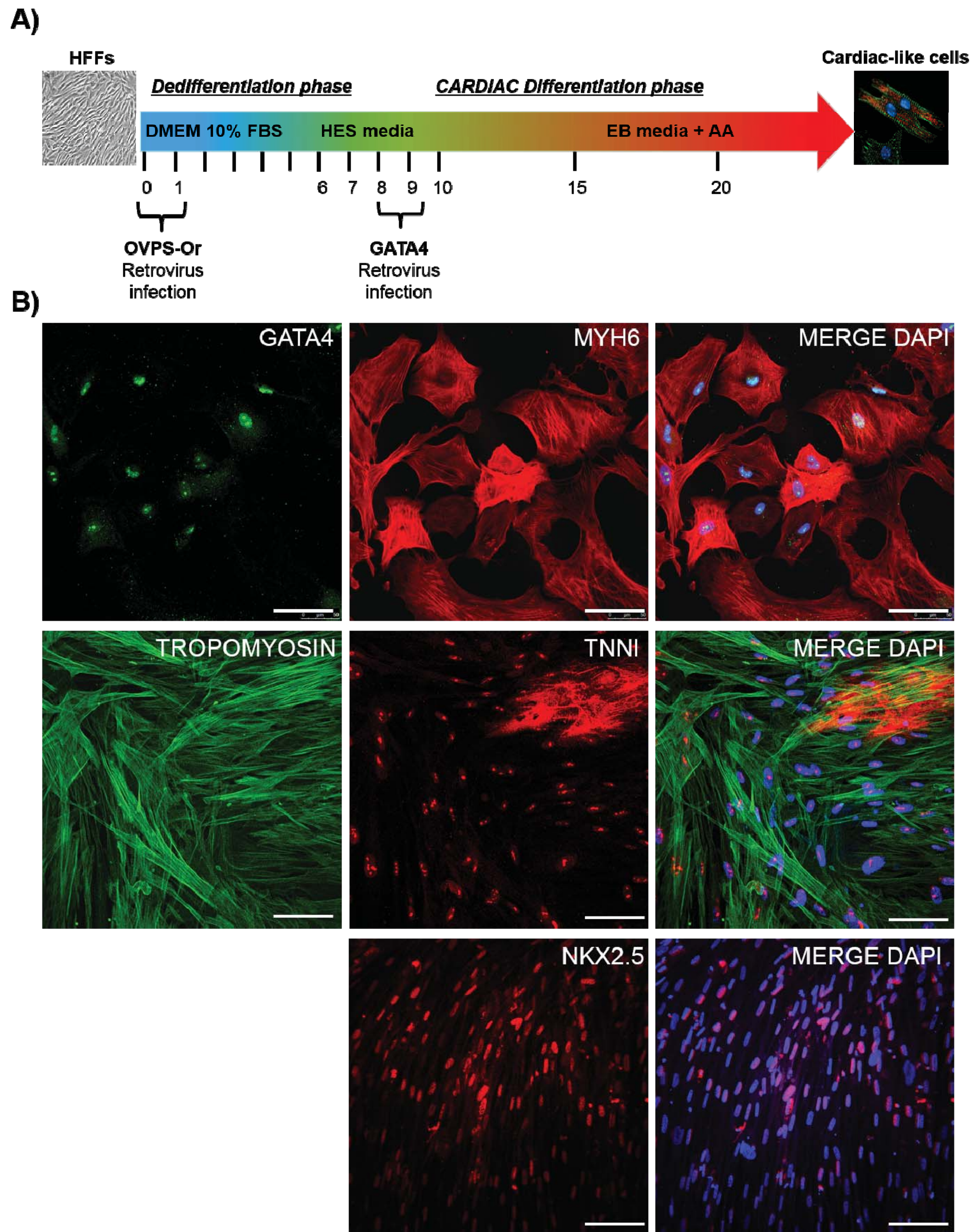


Figure 7. Protocol of cardiac conversion from dermal post-natal fibroblasts. A) Time-line of our protocol indicating culture conditions and transcription factors used during our two-step protocol. **B**) At *day 20* cells were analyzed by immunofluorescence for markers related with cardiac cells. Representative images performed by confocal microscopy show that cells exhibit typical cardiac specific markers, including motor proteins as: Tropomyosin, Troponin I (TNNI), α-myosin heavy chain (MYH6) and nuclear transcription factors and as GATA4 and NKX2.5. Scale bars: 50µm.

In order to exclude the possibility that GATA4 expression was due to transgene over-expression during the process of cardiac conversion, we analyzed the levels of HA expression by immunofluorescence in cardiac-like cells at *day 20*. Results indicated that GATA4 transgene silencing is not complete, since still some cells expressed HA together with endogenous GATA4 protein (**Figure 8**). Further analysis performed at *day 35* showed that GATA4 silencing was complete (*data not shown*), pointing out that culture conditions are crucial for the activation of endogenous programs sustaining cardiac fate.

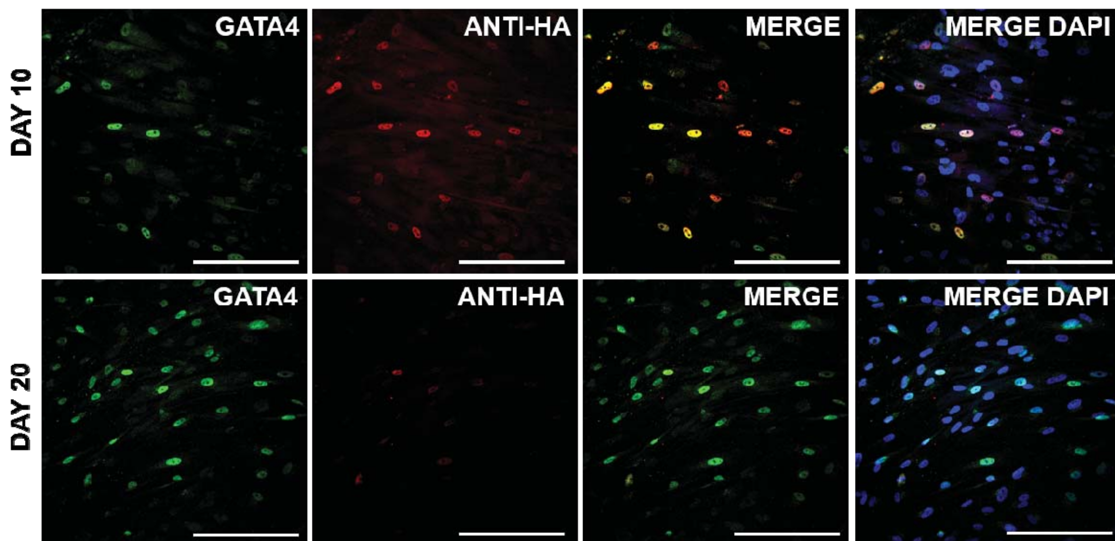


Figure 8. Monitoring GATA 4 transgene expression during the process of cardiac conversion. Immunofluorescence analysis by confocal microscopy on HFFs at *day 10* and *day 20* of the cell-conversion approach. In green, GATA4 protein expression, in red HA protein expression detecting GATA4 transgene. Merge corresponds to the overlay of anti-GATA4 and anti-HA images. On the right, DAPI staining for nucli merged with GATA4 and HA immunodetection. . Scale bars: 100 µm.

The possibility to control cell fate by overexpressing specific transcription factors represents an attracting approach for generating cell-specific types for clinical purposes. In this work we have set the basis for the description of a new protocol for the generation of cardiac-like cells from human post-natal dermal fibroblasts. Although our results indicated that final populations express cardiac markers only at the protein level, we are confident that further experiments will determine the final conditions for cardiomyocytes generation. In this regard, and comparing with other protocols recently published, we have observed that the over-expression of OCT4 and SOX2 could induce

a de-differentiated state in the original cells which is also characterized by the acquisition of the expression of genes related with cardiac fate (both at early and final stages). We believe that the possibility to screen for other cell culture conditions defined in the *Results III* section of the present thesis will results in the generation of beating foci out of primary fibroblasts.

IV.RESULTS │ CHAPTER 3

3. GENERATION OF HUMAN REPORTER CELL LINES FOR ALPHA MYOSIN HEAVY CHAIN (α–MHC) GENE: DEVELOPING TOOLS FOR CARDIAC DIFFERENTIATION

Although different groups, including our laboratory, have participated in the generation of efficient protocols for the derivation of cardiomyocyte-like cells from hESCs (Gu et al. 2014), there are several drawbacks that are still preventing the use of hESC/hiPSCs technology for Regenerative Medicine purposes. Generally, the protocols relaying on hESCs/hiPSCs lead to the derivation of final heterogeneous populations containing different cardiac cell types (i.e: cardiac-like cells of atrial and ventricle origin, cardiac precursors, etc), and even undifferentiated PSCs that may confer tumorigenic properties to the final cellular products to be used in the clinical setting. Other important issue is related to the production of low yields of cardiac-like cells by means of these approaches, a major limiting factor when translating this methodology for cardiac transplantation. In the last years our work has been devoted to the generation of specific tools in order to bypass all these important issues, and more importantly, to bring new knowledge in the field of cardiac development and regeneration. The generation of reporter cell lines for cardiac genes by genome engineering technologies could lead us to monitor the activation of cardiac specific genes, and more importantly, to purify and characterize hPSCs-derived cardiomyocytes. By a simple approach we will be able to identify the developmental cues triggering cardiac differentiation from hPSCs in order to further develop efficient protocols for cardiac differentiation.

3.1. α–MHC expression during cardiac differentiation from hESCs

In order to identify potential candidates for the generation of reporter hESCs lines for cardiac differentiation we decided to study the expression of MYH6 (α-MHC) gene at both mRNA and protein levels during our protocol of differentiation. MYH6 is an essential protein required for cardiomyocyte contraction and functionality and it is one of the best genes enabling to monitor cardiac differentiation, and even cardiomyocyte cell conversion from murine cardiac fibroblasts (Efe et al. 2011). MYH6 has been

shown to be expressed both, early and late during cardiac differentiation in humans, so we first checked if this same event takes place under our protocol of cardiac differentiation from hESC. For that purpose, we first differentiated our control hESC cell line ES[4] obtained from the "Banco Nacional de Líneas Celulares" (Documento_Deposito_Lineas_v32_ES4_def.pdf) to cardiomyocyte-like beating cells (**Figure 1**). In particular Embryoid Bodies (EBs) derived from ES[4] line were exposed to ascorbic acid every two days during 30 days. RNA from differentiating EBs was extracted at different time points for subsequent analysis.

Analysis of MYH6 mRNA expression by quantitative PCR (qPCR) showed that MYH6 mRNA is induced early during ES[4] differentiation towards cardiac-like cells, as soon as day 5 (**Figure 1A**). Moreover MYH6 mRNA expression is gradually increased through the differentiation process, being also preserved in later stages (up to day 30). In the same manner, immunostaining against MYH6 was performed at days 5, 10 and 20 during the differentiation process. We found that MYH6 detection was already evident at day 5 during our differentiation protocol. Increases in MYH6 expression and sarcomeric distribution were coincident with increases in time exposure to cardiac differentiation media, suggesting that our chemically defined media positively induces cardiac differentiation from human PSCs (**Figure 1B**). Our results coincided with those previously reported (Kwon et al. 2007b; Burridge et al. 2012; Lian et al. 2012a).

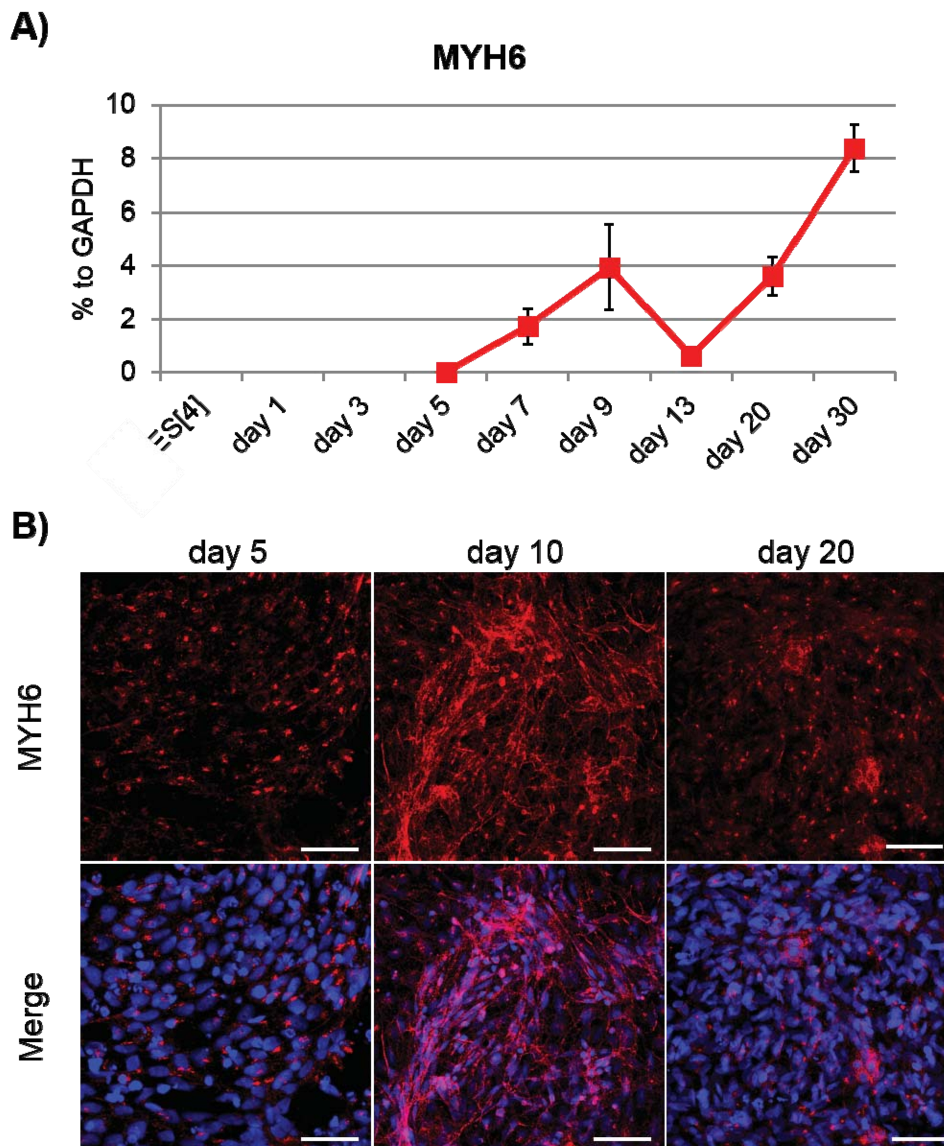


Figure 1. Cardiac differentiation in ES[4] line. **A**) Cardiac differentiation in ES[4] line induces MYH6 mRNA expression from day 5. Data are represented as mean +/- s.d., n=3. **B**) MYH6 expression was analysed by immunofluorescence during the time course of cardiac differentiation of ES[4] line. MYH6 cells (in red) appear as soon as day 5 during differentiation. Scale bars: 50µm.

3.2. *Knock in* on the *α-MHC* locus in hESCs by TALEN and CRISPR/Cas9 technologies

To generate our reporter cell line we chose two different genome-editing technologies allowing to *knock in* hESCs in a site-specific manner. The methods of choice were CRISPR/Cas9 and TALEN technologies. Based in our preliminary results (**Figure 1A**, **B**) we decided to target MYH6 after the starting codon (ATG) (positioned at

chromosome14: 23407220) (**Figure 2**). This strategy could help us to conserve intact all possible regulatory sequences upstream the transcription starting site, including the whole described promoter sequence (Ghosh et al. 2009).

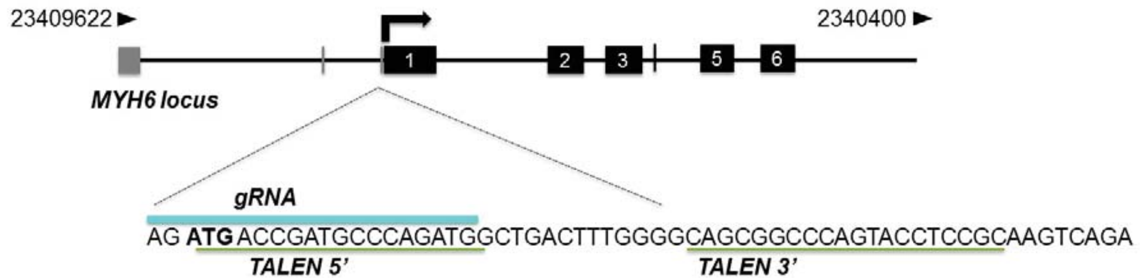


Figure 2. Schematic overview depicting the targeting strategy for MYH6 human locus making use of TALEN and CRISPR/Cas9. Targeting was designed into the 5´untranslated region of the MYH6 gene upstream of the MYH6 start codon (ATG), resulting in a mCherry-tagged MYH6 allele. gRNA (blue) and TALEN 3´and 5´sequences (green) are indicated in MYH6 sequence (Detailed information in *Appendix I*).

3.2.1. TALEN and CRISPR/Cas9 design, construction and functional test

Common donor vector (**Figure 3**) for both methodologies was designed (MYH6 d-vector) and cloned successfully (plasmid map in *Appendix I supplementary Figure 11*, cloning described in *Materials and Methods* section *4.1.5. MYH6 Donor Vector (d-vector) design and construction*). Plasmid clone screening was performed by enzymatic digestion (described i*n Materials and Methods* section *1.1.1.1. Traditional cloning*) and Sanger sequencing (described i*n Materials and Methods* section *1.1.5. Sanger Sequencing)* (data not shown). Design and synthesis of TALEN was performed as described in detail in *Materials and Methods s*ection *4.1. Construction of TALENs*, resulting in two correct TALEN pairs for functional testing (sequences detailed in *Appendix I supplementary Figure 12*). For the CRISPR/Cas9 method, three possible guiding RNAs were designed as described in *Materials and Methods s*ection *4.2. CRISPR/CAS9 design and construction: guiding RNAs (gRNAs)* (sequences detailed in *Appendix I supplementary Figure 13)*

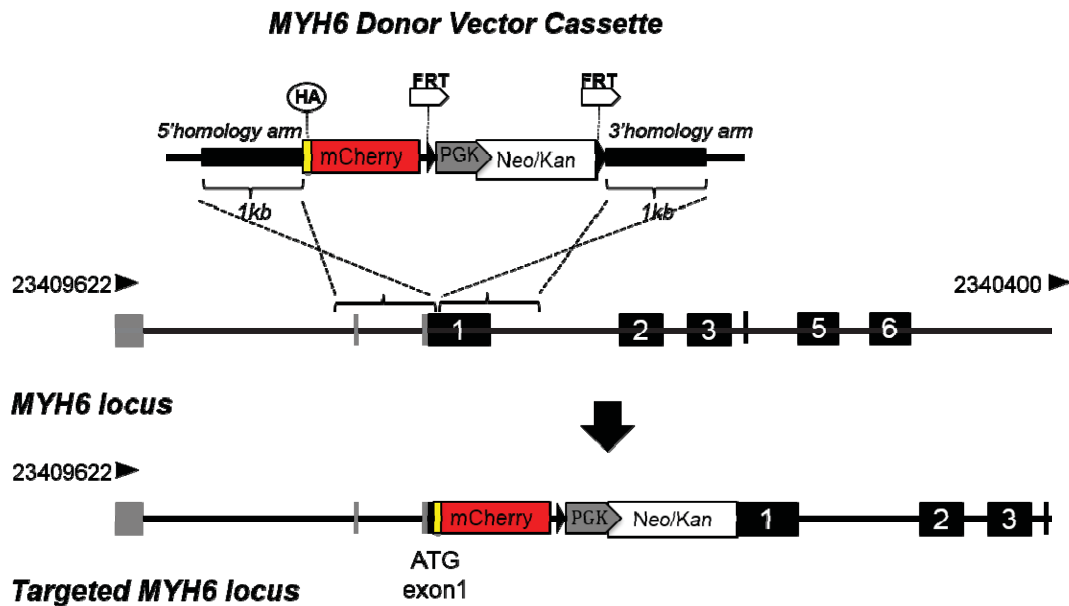


Figure 3. Schematic representation of MYH6 targeting. The MYH6 donor-vector (d-vector) includes a mCherry cassette HA-tagged (HA), a PGK promoter and a neomycin-kanamycin resistant cassette (neo/kan). Both PGK and Neo/Kan cassetes are flanked by FRT sites for further FLPo recognition. Dashed lines indicate the sites of homologous recombination (HR) in the *MYH6* locus. HR results in *knock in* of the complete cassette into the ATG start site of *MYH6* coding sequence, thus generating a mCherry-tagged *MYH6* allele.

Surveyor assay was performed in HEK293A cells in order to evaluate the targeting efficiency and specificity of the designed tools (**Figure 4**). Our results demonstrated that both techniques could efficiently cut and target *MYH6* locus in the site of choice. As shown in **Figure 4**, PCR on gDNA from HEK293A cells transfected with either mock (N.Control), a single TALEN (TALEN 5' control) and TALEN pairs (TALEN pairs 1, TALEN pair 2) (**Figure 4B**) and mock (N.Control), Control-Cas9 and gRNAs (gRNA1, gRNA2 and gRNA3) (**Figure 4C**) resulted in a product of 979 bp, corresponding to a fragment from MYH6 starting coding region gene (Primer sequences and PCR conditions are listed in *Materials and Methods* section *Oligonucleotides Table IV* and *Materials and Methods* section *4.2.1. Surveyor Assay* respectively) gDNA amplified sequence is detailed in *Appendix I supplementary Figure 14*). PCR products were subsequently digested by Surveyor enzyme and two different bands of ≅705 bp

and ≅274 bp were generated (**Figure 4**). Both TALEN pairs (TALEN pair 1 and TALEN pair 2) were selected for further targeting purposes, whereas only Cas9 gRNA1 was selected for *MYH6* targeting using CRISPR/Cas9 system.

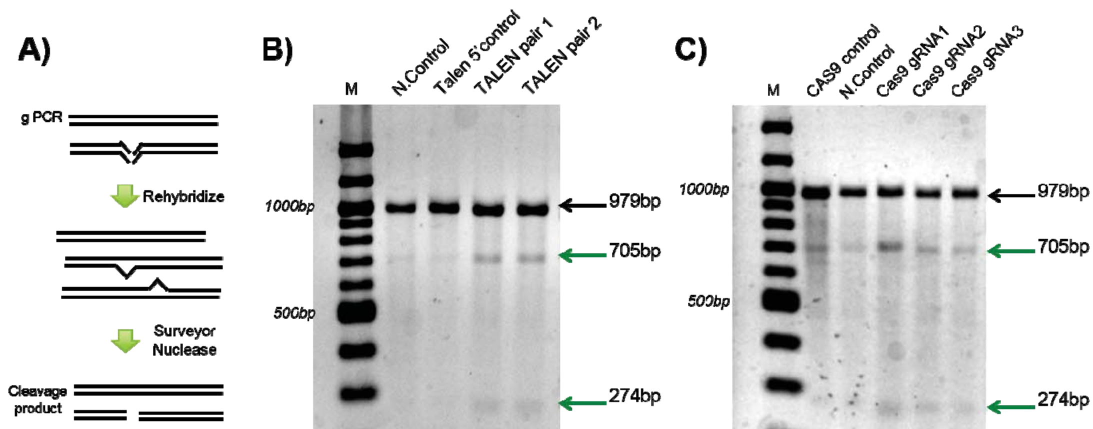


Figure 4. Agarose gel electrophoresis for Surveyor Nuclease digestion in HEK293 cells. A) Schematic of the Surveyor nuclease assay used to determine TALEN and Cas9 cleavage efficiency. First, genomic PCR (gPCR) is used to amplify the TALEN target region from a heterogeneous population of TALEN- or Cas9-modified and TALEN- or Cas9-unmodified cells, and the gPCR products are reannealed slowly to generate heteroduplexes. The reannealed heteroduplexes are cleaved by Surveyor nuclease, whereas homoduplexes are left intact. TALEN cleavage efficiency is calculated based on the density of the fraction of cleaved DNA. **B**) HEK293 transfected with either mock (N.Control) or 5´TALEN did not produced a cleavage product, thus the complete 979 bp band amplified appeared. When MYH6 TALEN pairs (TALEN pair 1 and TALEN pair 2 lanes, respectively) were transfected in HEK293, a part from the non-cleavaged band (979 bp), two more bands of ~705bp and ~274bp were generated showing that TALE-Nuclease mediated the cutting at MYH6 defined genomic position. **C**) HEK293 transfected with Cas9_GFP alone or mock as control, did not produced the expected cleavaged products. When Cas9 gRNA candidates (Cas9 gRNA1, Cas9 gRNA2, Cas9 gRNA3, respectively) were transfected together with CAS_GFP nuclease in HEK293, again, two bands of ~705bp and ~274bp were generated, indicating that Cas9-mediated cleavage occurred in the expected site. M=DNA Ladder 1 kilobase (kb).

3.2.2. Targeting of *MYH6* locus in ES[4] line

ES[4] line was transfected by electroporation and/or nucleofection with either: a) Selected TALEN pairs together with the MYH6 d-vector; b) Cas9_GFP and gRNA1-

mCherry together with MYH6 d-vector (details described in *Materials and Methods* section *4.3. ES[4] cell transfection with Transcription activator-like effectors nucleases (TALEN) and Clustered regularly interspaced short palindromic repeats [(CRISPR)/CAS9])*. After 24hours, only in the CRISPR/Cas9 system method, we analyzed the transduction efficiency evaluating GFP and mCherry expression by optical microscopy (**Figure 5A)**. In the same manner double positive GFP+/mCherry + ES[4] cells were sorted by fluorescent activated cell sorting (FACS) analysis and cultured in feeder-free conditions for further purposes. In our hands the recovery of double positive GFP+/mCherry+ ES[4] was higher when ES[4] cells were nucleofected compared with electroporation. Estimation of efficiency was calculated by the number of double positive GFP+/mCherry+ correlated to the number of total cells. Our results showed that after nucleofection ≅ 25% cells were expressing GFP and mCherry (**Figure 5B**). Similar results were obtained when ES[4] line was electroporated (≅ 22%). However, the total number of cells positively transfected with gRNA_mCherry (≅78,2% mCherry+ after nucleofection and ≅61,72% mCherry+ after electroporation) was much higher than those expressing Cas9_GFP (≅ 25,5% GFP+ after nucleofection and ≅23,56% GFP+ after electroporation) suggesting the importance of plasmid size for transduction efficiency (gRNA plasmid size ≅ 3,6 kilobases (kb) and Cas9_GFP size ≅ 9,2kb) (**Figure 5B**).

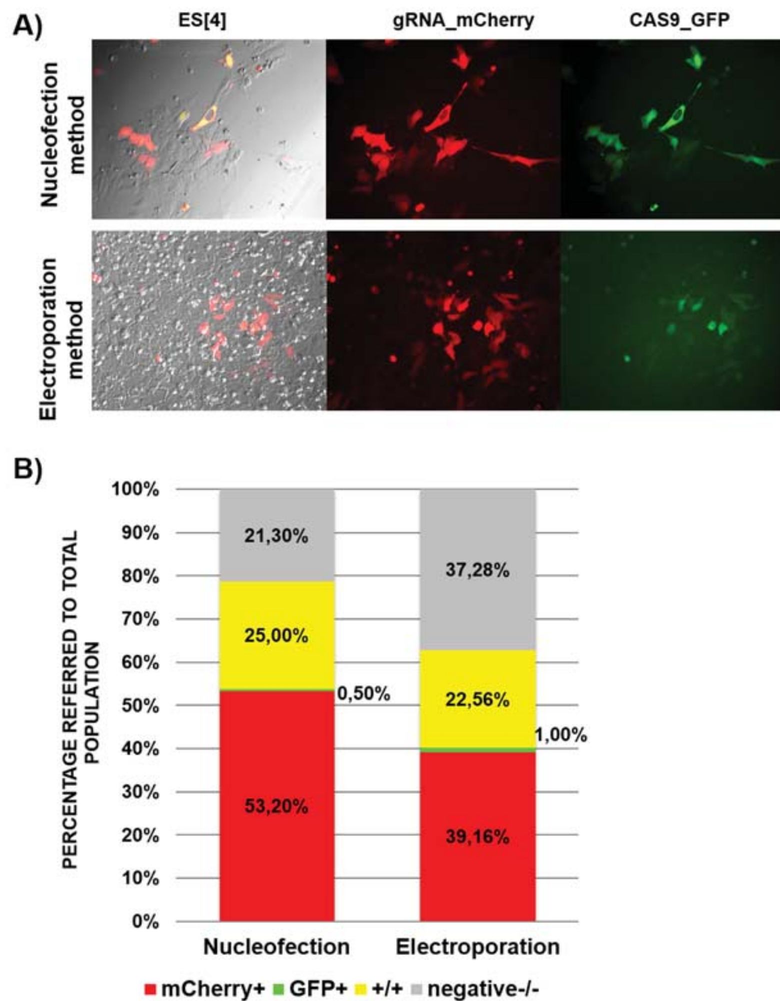


Figure 5. Monitoring CRISPR/Cas9 transfection on ES[4] cells. A) Fluorescence microscopy of ES[4] cells expressing gRNA_mCherry and Cas9_GFP confirmed that cells were positively transfected with either nucleofection or electroporation methods. **B**) Quantification of CRISPR/Cas9 transfection on ES[4] cells by FACS analysis shows that both methodologies gave rise to similar efficiencies of transfection (~22-25% of cells are expressing both plasmids).

3.2.3. Clone selection

To further select ES[4] expressing MYH6 transgenic elements we performed Neomycin (Geneticin G418) selection in order to isolate those ES[4] cells in where homologous recombination (HR) of the donor vector in the site of cut was achieved. After three days in culture with recovering media (3:1 mTeSR/CM), as described i*n Materials and Methods* section *4.4. Determination of MYH6 targeting and copy number integration in transfected ES[4] cells*, we proceeded to add Neomycin (G418) during 48 hours. After

72 hours of Neomycin treatment small groups of ES[4] cells appeared in culture. The Neomycin resistant clones were further expanded for future analysis described below.

3.2.4. Assessment of reporter transgene integration by PCR screening

One week after further expansion of ES[4] under Neomycin selection around 24 colonies from either TALEN or CRISPR/Cas9 methods were isolated for subsequent screening by PCR analysis. Genomic DNA (gDNA) from the different clones was analyzed for the amplification of the mCherry and Neomycin transgene cassette. In order to determine those clones that positively integrated the reporter transgenes, we performed different PCR reactions. First we performed a fast screening for the amplification of short fragments by PCR; primers flanking PGK and Neomycin cassette were used (S1:S2 primers) as shown in **Figure 6 A.** Then, we performed a second round of PCR on those clones showing the expected bands in the first screening. Primers flanking the reporter transgene cassette, homologous upstream and downstream the *MYH6* targeting site were used (L1:L2) (**Figure 6A**). (For detailed information go to *Materials and Methods* section *4.4.2 PCR clone selection: identification of ES[4] clones targeted in MYH6 locus by TALEN and CRISPR/CAS9 technologies).* PCR screening show putative positive clones generated with either TALEN or CRISPR/Cas9 methods (**Figure 6B).** In our hands, the total number of positive clones identified by PCR screening was higher when using TALEN genome engineering technology.

For further analysis we picked four clones generated from each technology which demonstrated positive results in both short and long PCRs. Those clones were further sub-cultured for next analysis of the reporter cassette mCherry_Neo integration at the desired targeting site in the genomic locus *MYH6*, as detailed below.

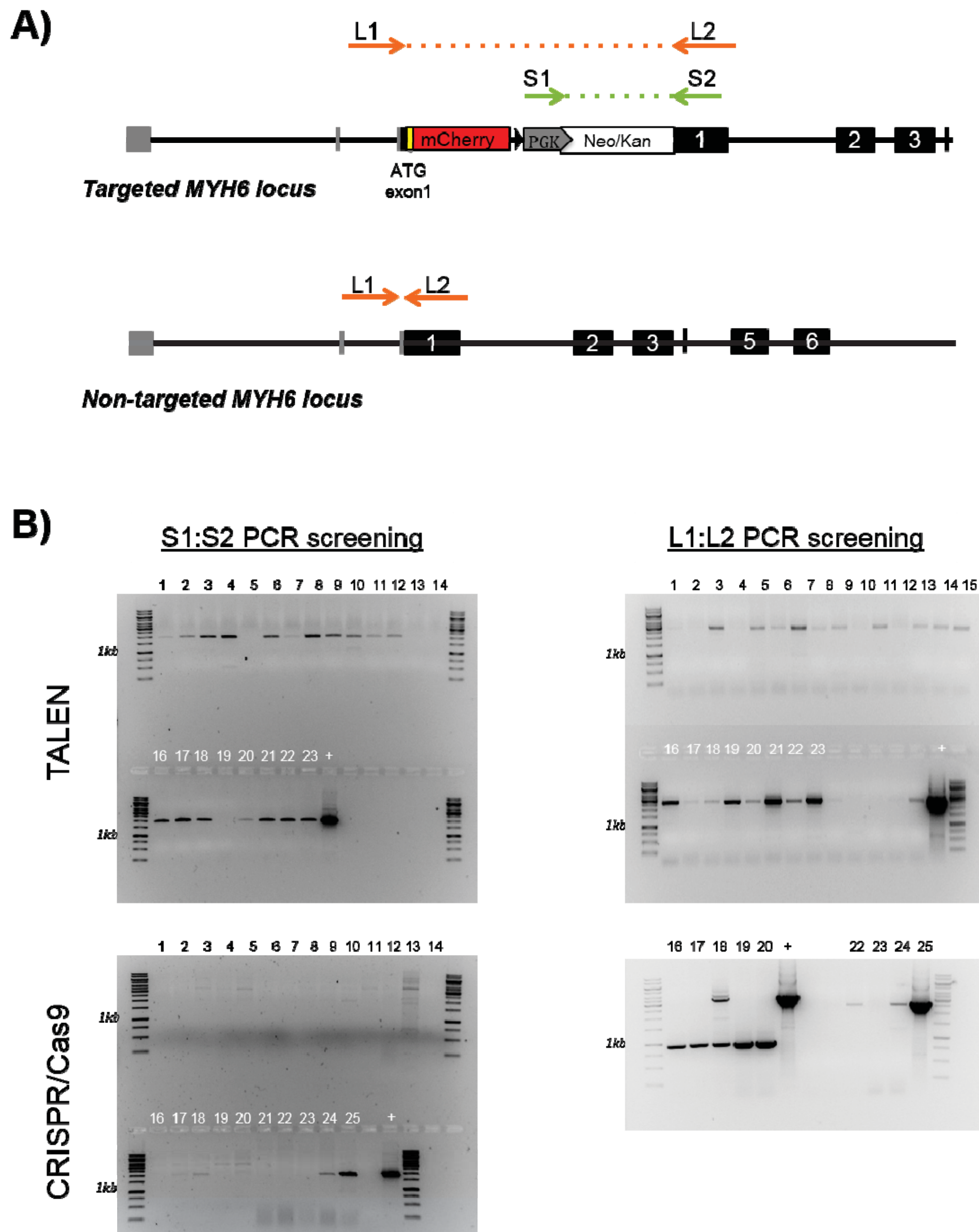


Figure 6. PCR screening for the reporter cassette integration. **A)** Schematic representation of PCR primer binding on the targeted and non-targeted gDNA of ES[4]-transfected clones. **B)** Representative images of electrophoresis analysis for two different PCR screenings for TALEN (top) and CRISPR/Cas9 (bottom) isolated clones. On the left, short PCR. On the right, long PCR of some of the clones. ES[4]-targeted clones show amplification of different fragments [Short (S) and Long (L) fragments]. S-primer pair (PGK5´: MYH6 3´; 1768 bp) and L-primer pair (MYH6 5´:MYH6 3´; 3369 bp) (Primer sequences are detailed in *Materials and Methods* Section *Oligonucleotides Table II*). M=DNA ladder 1kb. MYH6d-vector was used as PCR positive control.

3.2.5 Assessment of reporter transgene copy number integration and location by Southern Blot

In order to select those reporter ES[4] lines that positively integrated the reporter transgene, we performed Southern blot analysis. In this manner we could verify the existence of reporter transgene copy numbers in the different putative ES[4] targeted lines. Analysis on gDNA of 4 clones from either TALEN or CRISPR/Cas9 methodologies that were positively targeted by PCR analysis was ran in denaturing conditions for further hybridization against digoxigenin-labelled probes for different regions of MYH6. Probes were designed to recognize either mCherry, Neomycin or MYH6 (**Figure 7A**). When using the MYH6 probe, we confirmed that all the analyzed clones contained the expected 10kb band corresponding to the MYH6 wild type allele. This results were confirmed by the detection of the same band in gDNA isolated from non-targeted ES[4] (lane 1). Among the four clones analyzed three clones generated with TALEN technology showed a band of 2,5kb size, corresponding to the transgene **(Figure 7B)**. In the same line, only one clone out of the four generated by CRISPR/Cas9 technology was positive for the detection of the transgene band **(Figure 7B)**. This new band appears from the generation of a new restriction site acquired after the HR of the MYH6 donor vector in the specific region of interest, the ATG starting site. Moreover, the precise size of the band and re-hybridation of the same Blot membrane with mCherry and Neomycin probes (data not shown) confirmed the *knock in* in the expected site and the heterozygosity after targeting. Thus, we generated 3 TALEN- and 1 CRISPR/Cas9 clone in which mCherry was targeted after ATG starting codon in *MYH6* human locus, offering the possibility to monitor cardiac differentiation. We next proceeded to deeply characterize one TALEN ES[4] clone.

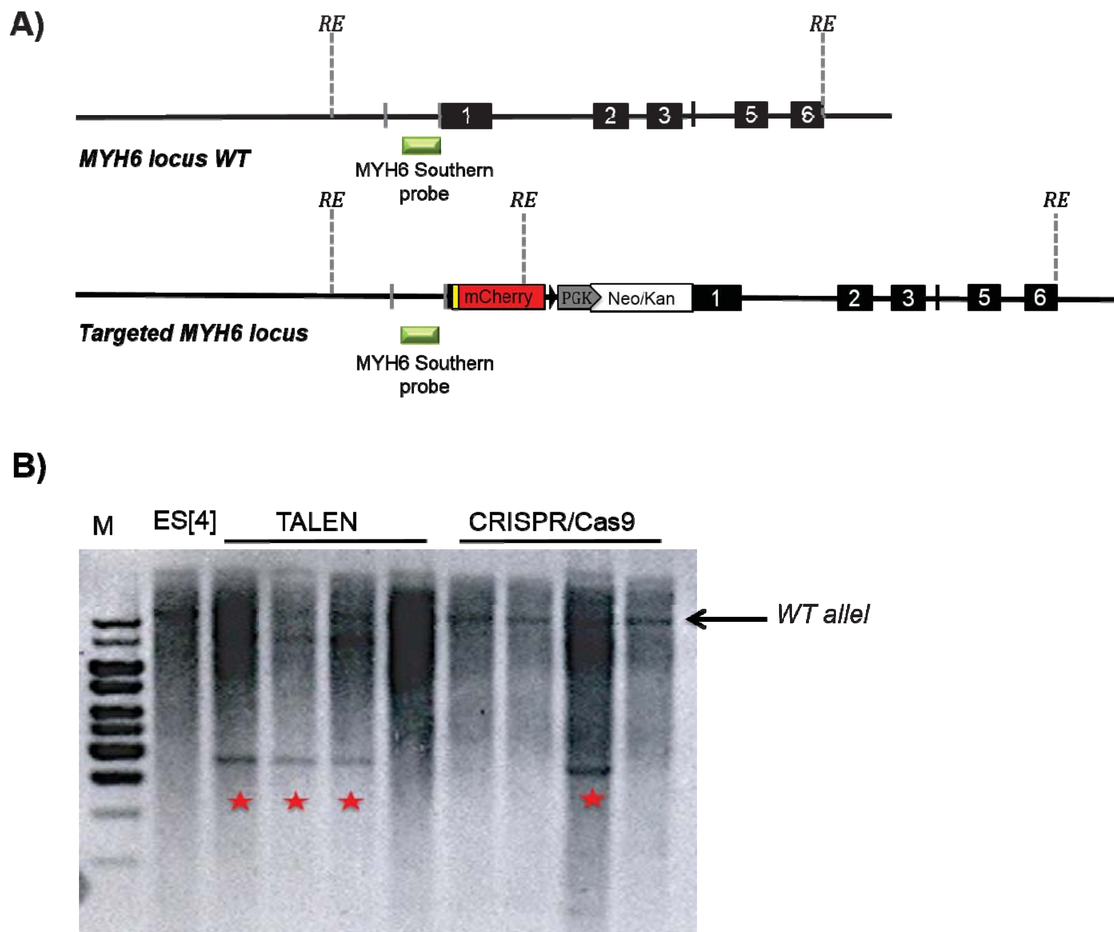


Figure 7. Southern blot analysis after MYH6 targeting. A) Schematic for MYH6 probe recognition site in wild type (WT) and targeted *MYH6* locus and possible generated fragments after restriction enzyme (RE) digestion for Southern blot analysis. **B)** Southern blot for MYH6. Copy number and allel integration of the reporter cassette in genomic DNA was evaluated by Southern blot in four clones TALEN and CRISPR/Cas9, respectively. ES[4] lane shows only the presence of wild type MYH6. Red stars indicate those clones that integrated the reporter transgene. The approximate molecular weights (kb) corresponding to the bands are 10kb for the wild type and 2,5kb for the transgene.

3.3. Characterization of TALEN ES[4] MYH6_mCherry cell line

It has been widely described that human pluripotent stem cells are sensitive to culture pressure and growth expansion (Laurent et al. 2011). Based on those previous findings, and due to the fact that ES[4] manipulation during our targeting experiments involved the selection of the clones in stressing conditions (feeder-free culture conditions) together with small compounds activating specific pathways related with cell survival, we proceed to deeply characterize TALEN ES[4] MYH6_mCherry cell line for the

expression of pluripotency related markers, the presence of chromosomal aberrations, and the capacity to differentiate i*n vitro* towards the three germ layers of the embryo.

3.3.1. Undifferentiated TALEN ES[4] MYH6_mCherry cell line shows stable karyotype

Culture pressure during the processes of single cell clone selection after nucleofection, and subsequent exposure of nucleofected cells to feeder-free conditions is known to induce genetic instability (Laurent et al. 2011). In order to make sure that the methodology described here does not account for major chromosomal aberrations in ES[4] parental line we performed a karyotype assay that revealed no major genetic abnormalities (e.g: deletions, translocations and/or insertions) in TALEN ES[4] MYH6_mCherry cell line. Our results demonstrated that *MYH6* targeting did not have any impact in the genomic stability of TALEN ES[4] MYH6_mCherry cell line (**Figure 8**).

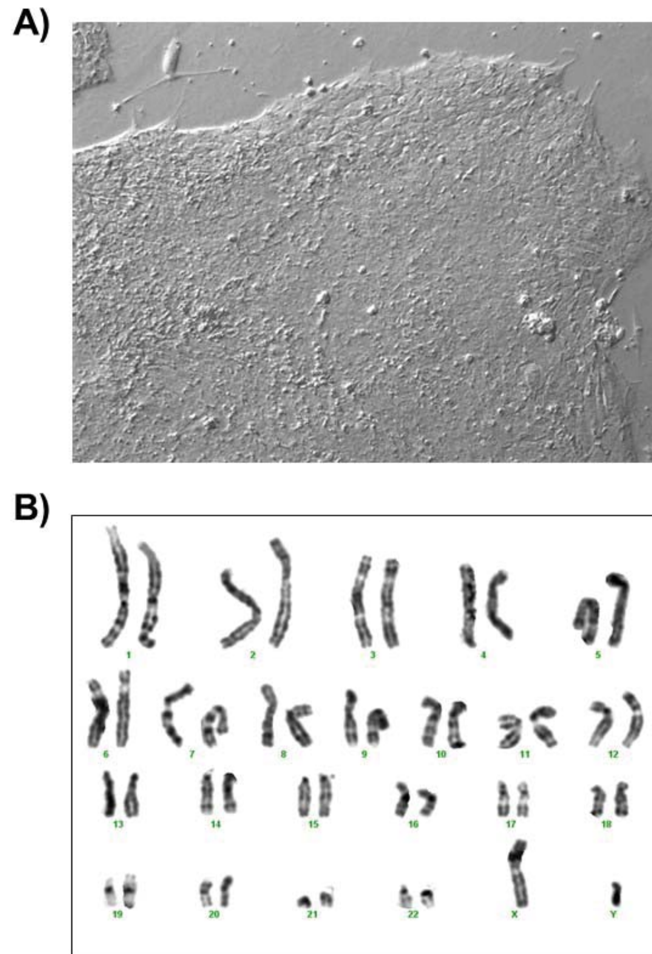


Figure 8. TALEN ES[4] MYH6_mCherry shows hESC-like morphology and stable karyotype. A) One week after Neomycin selection TALEN ES[4] MYH6_mCherry line grew as compact and tight adherent colonies. Undifferentiated TALEN ES[4] MYH6_mCherry cells were negative for mCherry expression. **B**) High-resolution, G-banded karyotype indicating a normal diploid male chromosomal content in the TALEN ES[4] MYH6_mCherry cell line.

3.3.2. Undifferentiated TALEN ES[4] MYH6_mCherry cell line expresses pluripotency-related genes

As expected TALEN ES[4] MYH6_mCherry cell line presented a high ratio of nucleus to cytoplasm and prominent nucleoli as well as compact flat colony structure **(Figure 8).** We next analyzed the mRNA expression levels of pluripotency related genes DPPA4, NANOG, OCT4, REX1, SOX2, CRIPTO1, DNMT3B, SALL2 and UTF1 by RT-qPCR (conditions and primers used are detailed in *Materials* section *5. Oligonucleotides* and *Methods* section *1.1.4. Polymerase Chain Reaction (PCR)*

respectively). Undifferentiated TALEN ES[4] MYH6_mCherry cell line expressed all pluripotency related genes analysed at similar levels as the control cell line ES[4]. Those genes were downregulated after 24 days of differentiation to cardiac fate in both cell lines (**Figure 9**).

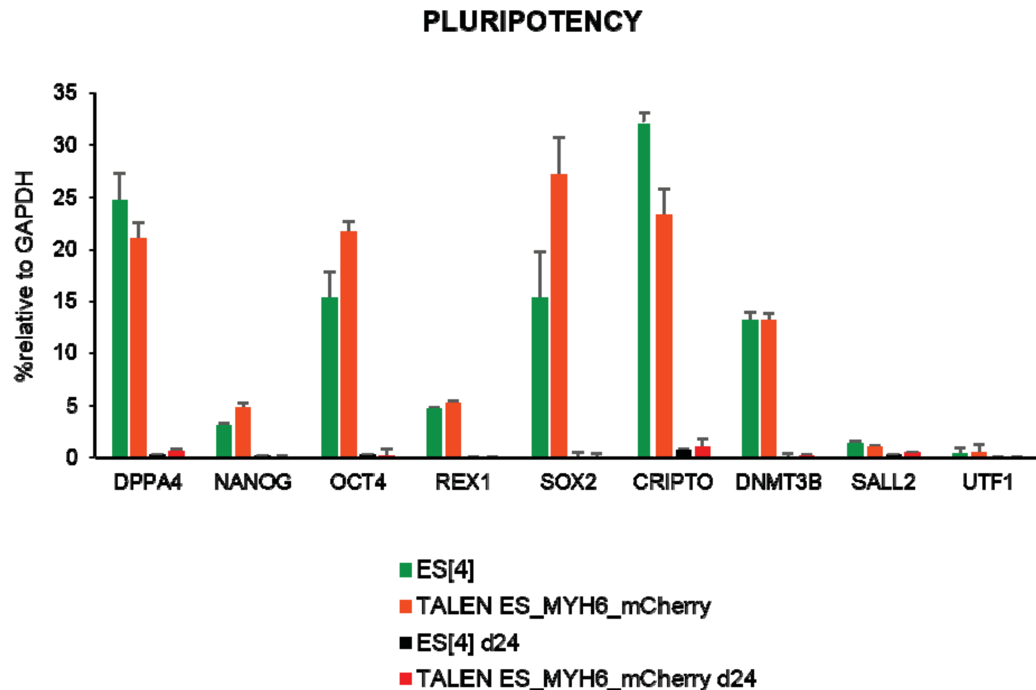


Figure 9. Gene targeting at MYH6 locus does not alter the expression of pluripotent-related markers. RT-qPCR for the indicated markers in ES[4] and TALEN ES[4]_MHY6_ mCherry line before and after cardiac differentiated at the indicated time points.

Moreover, when nuclear transcription factors as OCT4, NANOG and surface markers as TRA-1-81 were analysed by immunofluorescence (conditions for immunofluorescence and image capture are detailed in *Materials and Methods* section *1.2.1. General Immunostaining and image captation*), TALEN ES[4] MYH6_mCherry cell line positively expressed all the analyzed pluripotency-related markers **(Figure 10).** Our results also showed that undifferentiated TALEN ES[4] MYH6_mCherry cell line was negative for mCherry expression. Together these results demonstrated that MYH6 targeting did not have any impact in pluripotency-related features of TALEN ES[4] MYH6_mCherry cell line.

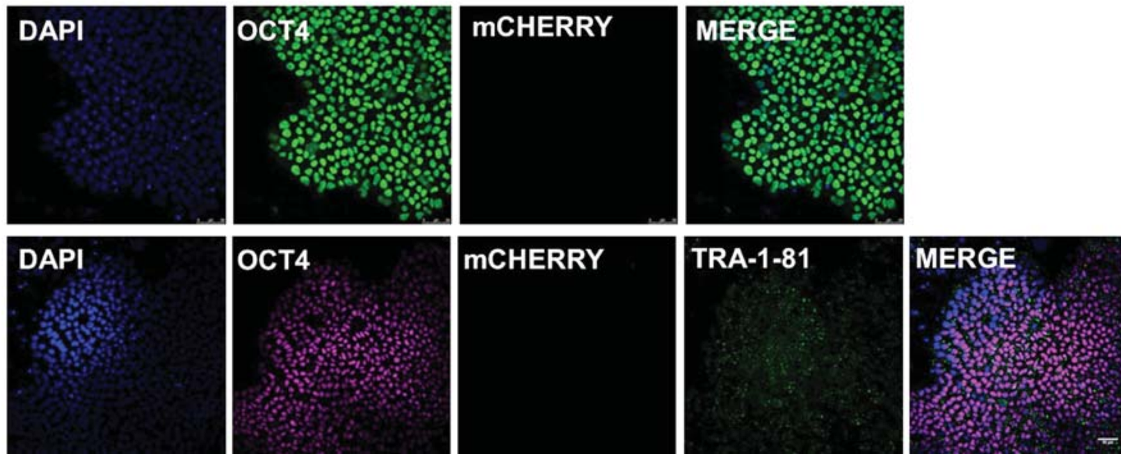


Figure 10. ES[4] MYH6_mCherry cell line preserves major signatures related to pluripotency. Immunofluorescence analysis of TALEN ES[4] MYH6_mCherry cell line for pluripotency markers. Colonies exhibit typical pluripotency markers including nuclear OCT4 and the surface marker TRA-1–81. Of note, there is no mCherry signal when the ES[4] MYH6_mCherry cell line remains undifferentiated. Image capture at 40x on top and 20x bottom.

3.3.3 TALEN ES[4] MYH6_mCherry cell line differentiate *in vitro* towards the three germ layers of the embryo

We further evaluated if TALEN ES[4] MYH6_mCherry cell line could be properly differentiated towards the three germ layers of the embryo. For this purpose colonies of TALEN ES[4] MYH6_mCherry cell line were disaggregated, and groups of 100.000 cells were centrifugated in order to force spheric re-aggregation and Embryoid Bodies (EBs) formation in suspension for 3 to 5 days. During this time, media was gradually changed to EB media. Once we had growth EBs, those were seeded on gelatin-coated slide flasks and exposed to culture conditions for the generation of mesoderm, endoderm and neural derivatives following the same protocol for ucMSCs_iPSC differentiation potential assessment described previously and detailed in *Materials and Methods* section *2.1.2. Differentiation potential in ucMSCs*. Two to three weeks after EB formation, slide flasks were processed for immunohistochemistry and TALEN ES[4] MYH6_mCherry cell line was analyzed for the expression of endodermal markers [Alpha 1 fetoprotein (AFP), Forkhead Box Protein A2 (FOXA2)], neural markers [Paired Box 6 (PAX6); Microtubule-Associated Protein 2 (MAP2)] and

mesodermal markers [Alpha Actinin Sarcomeric (AAS) and NK2 Homeobox 5 (NKX2.5)]. Our results demonstrated that MYH6 targeting did not have any impact in the differentiation of TALEN ES[4] MYH6_mCherry cell line differentiation *in vitro* (conditions for immunofluorescence and image capture are detailed in *Materials and Methods* section *1.2.1. General Immunostaining and image captation*). Results are shown in **Figure 11.**

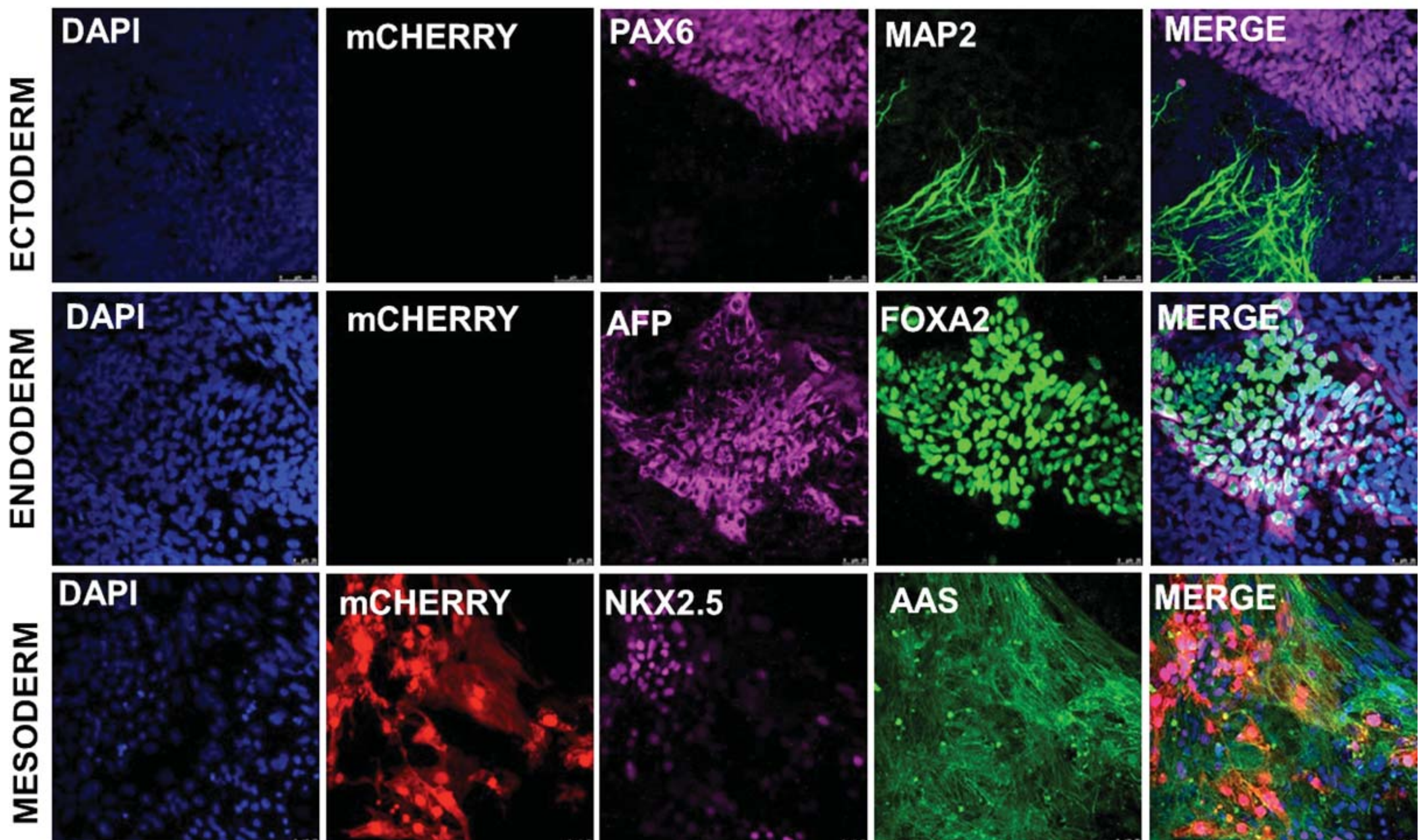


Figure 11. ALEN ES[4] MYH6_mCherry cell line differentiation potential. Representative images of in vitro differentiation of the TALEN ES[4] MYH6_mCherry cell line into the three primary germ cell layers [ectoderm-neuron-specific class III β-tubulin (TUJ-1) and Paired Box 6 (PAX 6); endoderm-α-fetoprotein (AFP), Forkhead box protein A2 (FOXA2); mesoderm-NKX 2.5 and α-sarcomeric actin (ASA) for mesoderm]. Endoderm image capture at 40X. Ectoderm and mesoderm image capture at 20X.

3.4. TALEN ES[4] MYH6_mCherry cell line as a tool for guiding cardiac differentiation

One of the major impacts from the development of reporter cell lines from PSCs is the possibility to develop protocols monitoring phenotypic and molecular changes during differentiation. In the cardiac context Elliot and colleagues have already demonstrated that eGFP targeting in *NKX2.5* locus enables the definition of new protocols for cardiac differentiation, and also the identification of new surface markers for cardiomyocyte detection (Elliott et al. 2011a). In this same line, other authors have generated a double reporter cell line for MESP1 and NKX2.5 loci, demonstrating that is possible to separate and enrich different cardiac populations during the onset of differentiation (Den Hartogh et al. 2015).

In order to test the suitability of our newly generated reporter cell line we first sought to define a more robust cardiac differentiation protocol from human PSCs than the one based on EBs formation and ascorbic acid supplementation. We wanted to ensure the robustness of our procedure as well as its standardization, and for this reason we proceeded to differentiate TALEN ES[4] MYH6_mCherry cell line into cardiomyocyte-like cells starting from cultures grown as monolayers instead generating EBs. Previously, other studies have demonstrated that this approach could lead to the generation of beating foci in less than 10 days (Lian et al. 2012a; Willems et al. 2011; Ren et al. 2011; Tran et al. 2009). Indeed, Lian and colleagues (2012) (Lian et al. 2012a) demonstrated that the exposure of hESCs to consecutive treatments of WNT effectors and inhibitors lead to the generation of pure populations of cardiac-like cells. Later, Gu and colleagues (2013) (Gu et al. 2014) used a similar protocol for the definition of the transcriptomic events driving cardiac differentiation from hESCs.

Based in all these observations we decided to disaggregate TALEN ES[4] MYH6_mCherry previously treated with ROCK inhibitor making use of three different starting cell densities. We used 75.000 cells per cm^2 (0,75X), 100.000 cells per cm^2 (1X as described by Lian et al., (2012)(Lian et al. 2012a)) and 125.000 cells per cm^2 (1.25X). Our first attempts demonstrated that when starting density was 1.25X, under the conditions described below, cardiac beating monolayers appeared already at *day 7* (**Video 1**). One day after ROCK inhibitor treatment TALEN ES[4] MYH6_mCherry

was cultured with mTeSR® for another 24 additional hours. If monolayer nets were homogenously formed we added GSK-3β inhibitor (CHIR9902-CHIR) in the presence of chemically defined media (RPMI/B27 without insulin) (**Figure 12A, 12B**). The conditions defining our differentiation protocol are detailed in **Figure 12A**. Of interest, already at *day 5-7* mCherry expression was detected by optical microscopy (**Figure 12B**). In our hands starting cell densities were a major limiting factor for the generation of high yields of mCherry beating cells, since CHIR exposure induced a lot of mortality if cell densities were not properly adjusted.

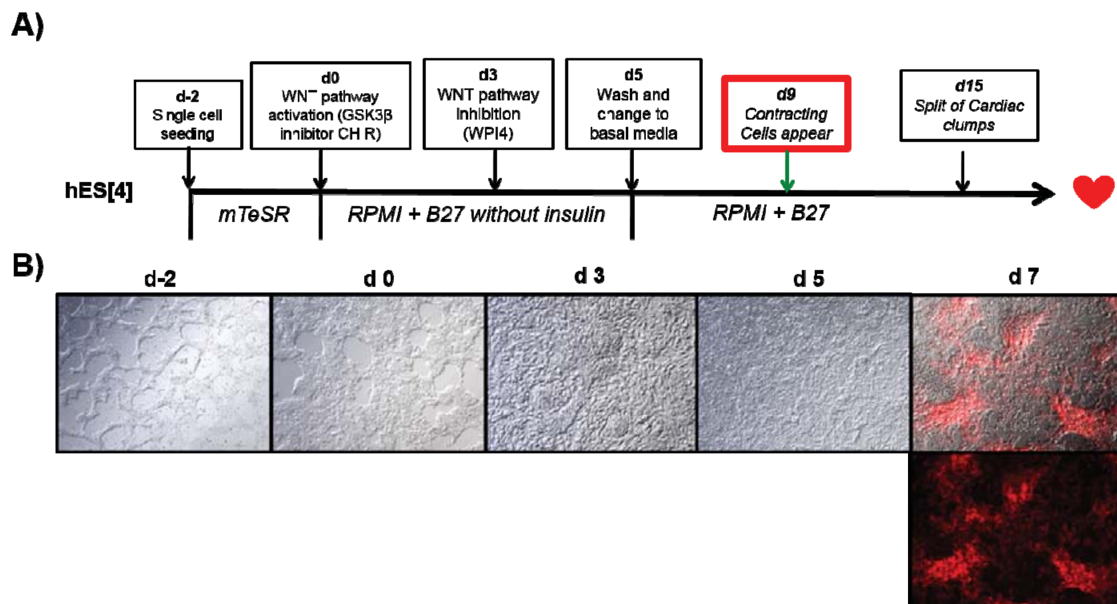


Figure 12. TALEN ES[4] MYH6_mCherry differentiation towards cardiac-like cells. A) Time-line for the derivation of cardiac-like cells by WNT signaling pathway modulation. **B)** Morphological changes during the process of differentiation. Notice that at day 7 mCherry expression is already detected by optical microscopy. Image capture at 10X and 20X.

After one day of CHIR treatment (*day 0* to *day 1*) we performed qPCR analysis in order to monitor the major changes in mRNA expression during the process of cardiac differentiation. Of interest, already at *day 1* the mRNA levels of Brachyury (T) and KDR, both markers of early phases of cardiac specification, were already induced (**Figure 13**). By *day 2* and *day 3* the mRNA levels of the pluripotency-related markers OCT4, NANOG and SOX2 were downregulated (**Figure 13**). Later, by *day 5*, after 48 hours of WNT inhibitor (IWP4), we detected an increase in the levels of mRNA

expression of BMP4 (mesodermal related gene) together with KDR, ISL1, c-KIT, HAND2 and BAF60C (cardiac precursor related genes) (**Figure 13**). Other genes related with cardiac program were also analyzed. Overall in our hands, the protocol developed here induced the expression of different genes related to early and late cardiac program. Remarkably, expression of MYH6 mRNA was detected at *day 5* of the differentiation protocol, and its expression was sustained through all the process of differentiation, thus confirming our previous findings when using ascorbic acid (AA) supplementation. Importantly these results reveal the suitability of MYH6 for monitoring cardiac differentiation from PSCs.

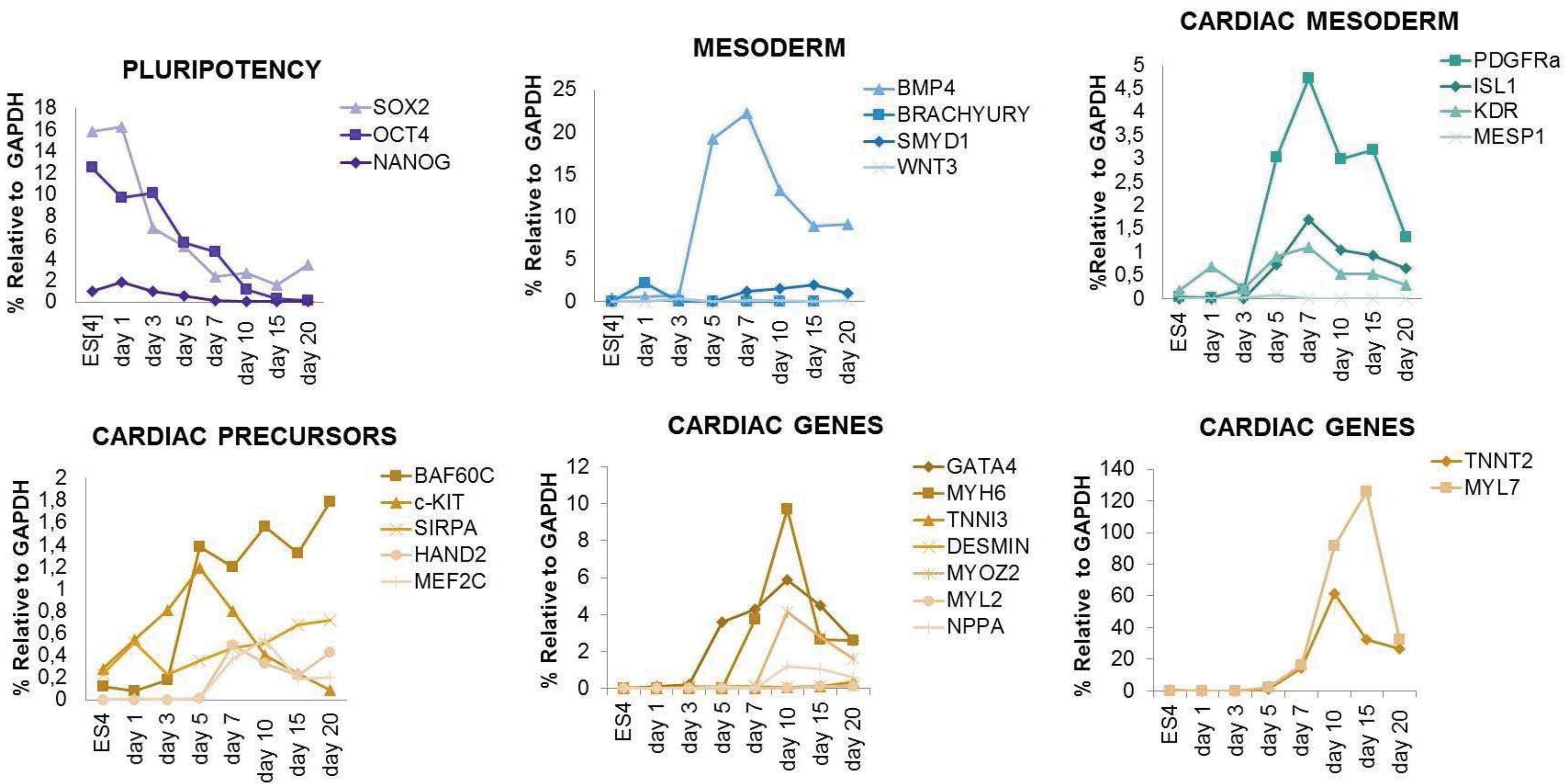


Figure 13. Differentiation of TALEN ES[4] MYH6_mCherry towards cardiac-like cells. Quantitative PCR (qPCR) analysis at the different time points indicated for the expression of markers related to pluripotency, mesoderm, cardiac mesoderm, cardiac precursors and cardiac fate. The activation of WNT pathway promotes the silencing of mRNAs related to pluripotency. When WNT pathway is inhibited the levels of BMP4 mRNA are induced. After WNT pathway activation and inhibition the defined culture conditions seem to promote cardiac program.

In an effort to generate an optimal tool for the study of the developmental cues driving cardiac differentiation from PSCs we next wondered if TALEN ES[4] MYH6_mCherry cell line could help us to monitor cardiac differentiation by means of the expression of mCherry protein as soon as endogenous MYH6 positive cells appeared under our defined culture conditions. In our hands, beating focuses appeared between day 7 and 10 of differentiation (**Video 1**). Positive signal from mCherry fluorescent protein was visible by optical microscopy at *day 7* during differentiation (**Figure 12**)**,** before first beating focuses appeared, confirming that our reporter cell line is a precise tool for monitoring and tracing cardiac differentiation.

3.5. Our new protocol for cardiac derivation sustains TALEN ES[4] MYH6_mCherry - and ES[4]- cell lines differentiation toward cardiac-like cells

In order to test the effectiveness of our protocol in hESCs we performed side by side the same experiment described above using both TALEN ES[4] MYH6_mCherry and ES[4] cell lines. For this reason we performed qPCR analysis during the time course of differentiation (*day 0*-undifferentiated cells and *day 20*-differented cardiac-like cells) (**Figure 14**) in order to monitor the expression of cardiac related mRNAs during cardiac differentiation in both non-targeted ES[4] and TALEN ES[4] MYH6_mCherry cell lines. We first assessed the levels of expression of the pluripotent related mRNAs OCT4, SOX2 and NANOG, demonstrating that as expected, cardiac differentiation silenced their expression. Also we evaluated the mRNA expression of cardiac genes relevant for early stages of cardiac development. Overall ES[4] and TALEN ES[4] MYH6_mCherry cell lines showed similar levels of expression for the different analyzed markers. Similarly, when ES[4] and TALEN ES[4] MYH6_mCherry cell lines were analyzed for the levels of expression of mRNAs related with later stages of cardiac program similar results were observed. In resume, our results confirmed that MYH6 targeting did not have any impact in cardiac differentiation efficiency of TALEN ES[4] MYH6_mCherry cell line compared with non-targeted ES[4] line.

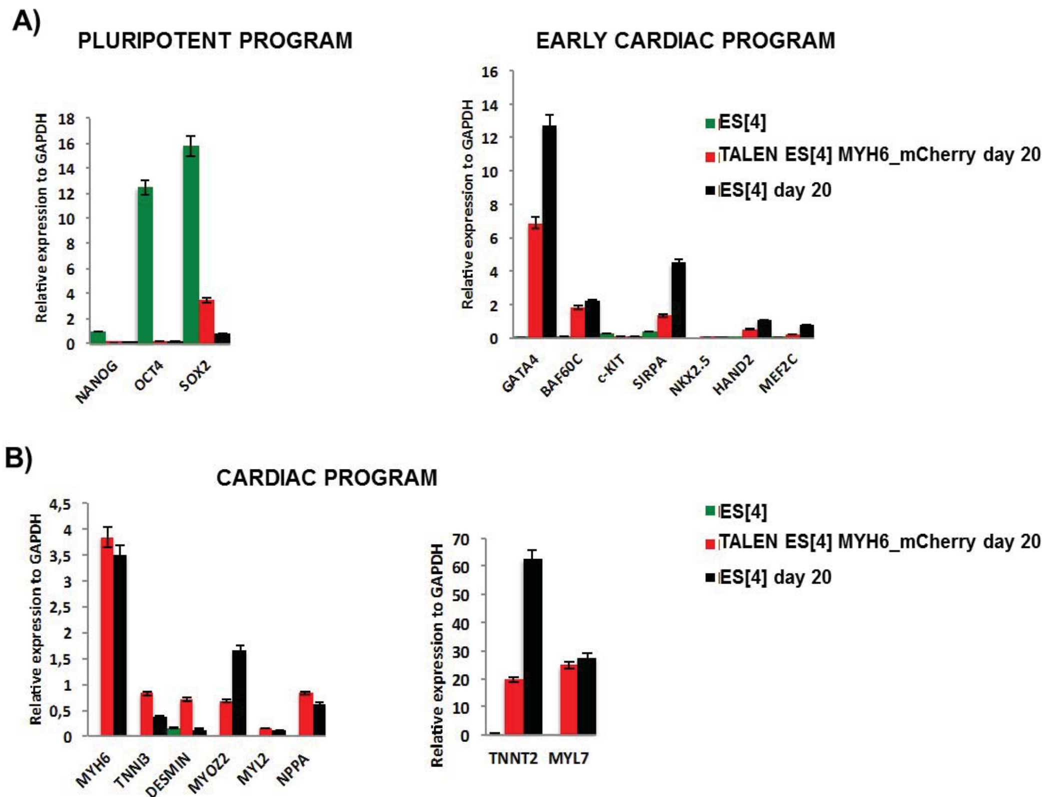


Figure 14. Targeting of MYH6_mCherry by TALEN does not affect cardiac differentiation efficiency in TALEN ES[4] MYH6_mCherry cell line. Evaluation of mRNA expression of genes from pluripotent stem cells and cardiac program by qPCR in TALEN ES[4] MYH6_mCherry and ES[4] cell lines at day 20 during differentiation. ES[4] cell line was used as a control of undifferentiated stages. Our results show that differentiation potential is not compromised after gene targeting when compared with ES[4] cell line. Of note, levels of mRNA expression of MYH6 in either control line and TALEN ES[4] MYH6_mCherry are very similar, thus suggesting that although the targeting, there is not a negative effect on the MYH6 regulation. Data are represented as mean +/- s.d.

When different time points were analyzed for mCherry expression by optical microscopy, we could detect mCherry expression before beating at *day 5-7*. Then, red signal was brighter in those beating areas appearing as soon as 7 days after differentiation. mCherry expression was also monitored at *day 12* and *day 20* during cardiac differentiation (**Figure 15**) with positive signal in every time point analyzed and even later (>day 30 not shown) confirming its maintaining on time. The analysis evinced that mCherry signal was restricted to beating areas (*Appendix I* **Video 2**). Overall our data confirms that mCherry expression is a positive cardiac read out in our experimental setting.

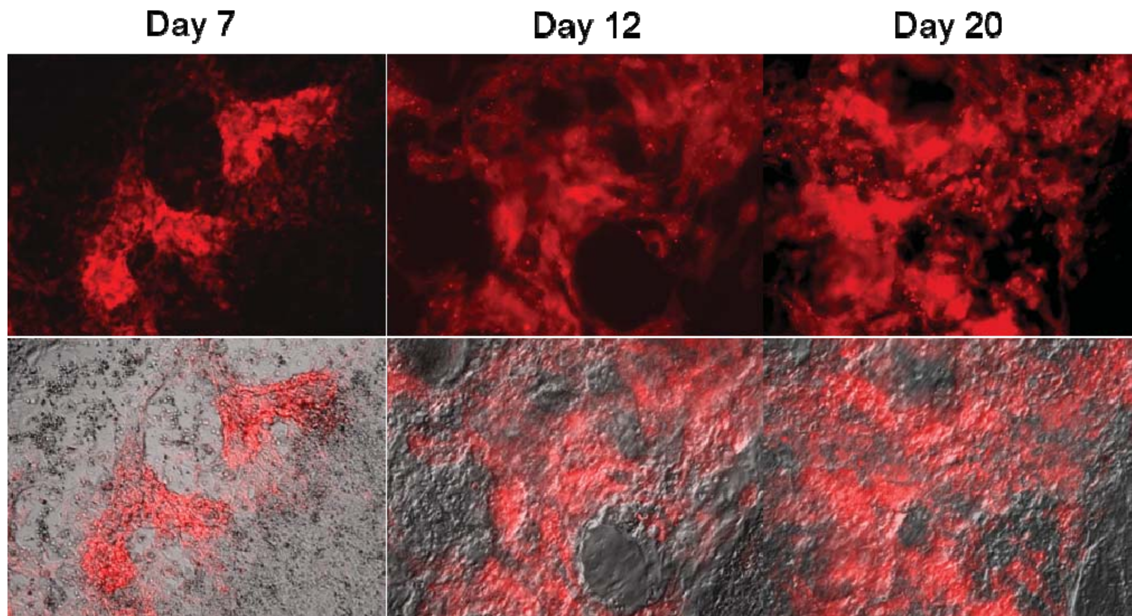


Figure 15. mCherry expression monitoring upon cardiac differentiation of TALEN ES[4] MYH6_mCherry cell line. Optical microscopy images showing red fluorescence emission at different time points (day 7, 12 and 20 of differentiation protocol) confirming mCherry reporter functionality. Top lane corresponds to red channel; bottom lane corresponds to merge with bright field (BF). Of note, mCherry brighter signal was restricted t beating areas. Day 7 images at 10X. Day 12 and 20 at 20X.

3.6. TALEN ES[4] MYH6_mCherry derived cardiomyocytes expressed cardiac related markers by immunofluorescence.

We next proceed to analyze a bigger panel of cardiac related markers by immunofluorescence in TALEN ES[4] MYH6_mCherry line during the onset of differentiation. When immunostaining assay was performed at *day 9* during cardiac differentiation we were able to localize GATA Binding Protein 4 (GATA4), Alpha Sarcomeric Actin (ASA), Troponin I (TNNI), NK2 Homeobox 5 (NKX2.5) and Myocyte Enhancer Factor 2C (MEF2) (**Figure 16**). Interestingly cells expressing the analyzed cardiac markers were frequently localized in beating focuses.

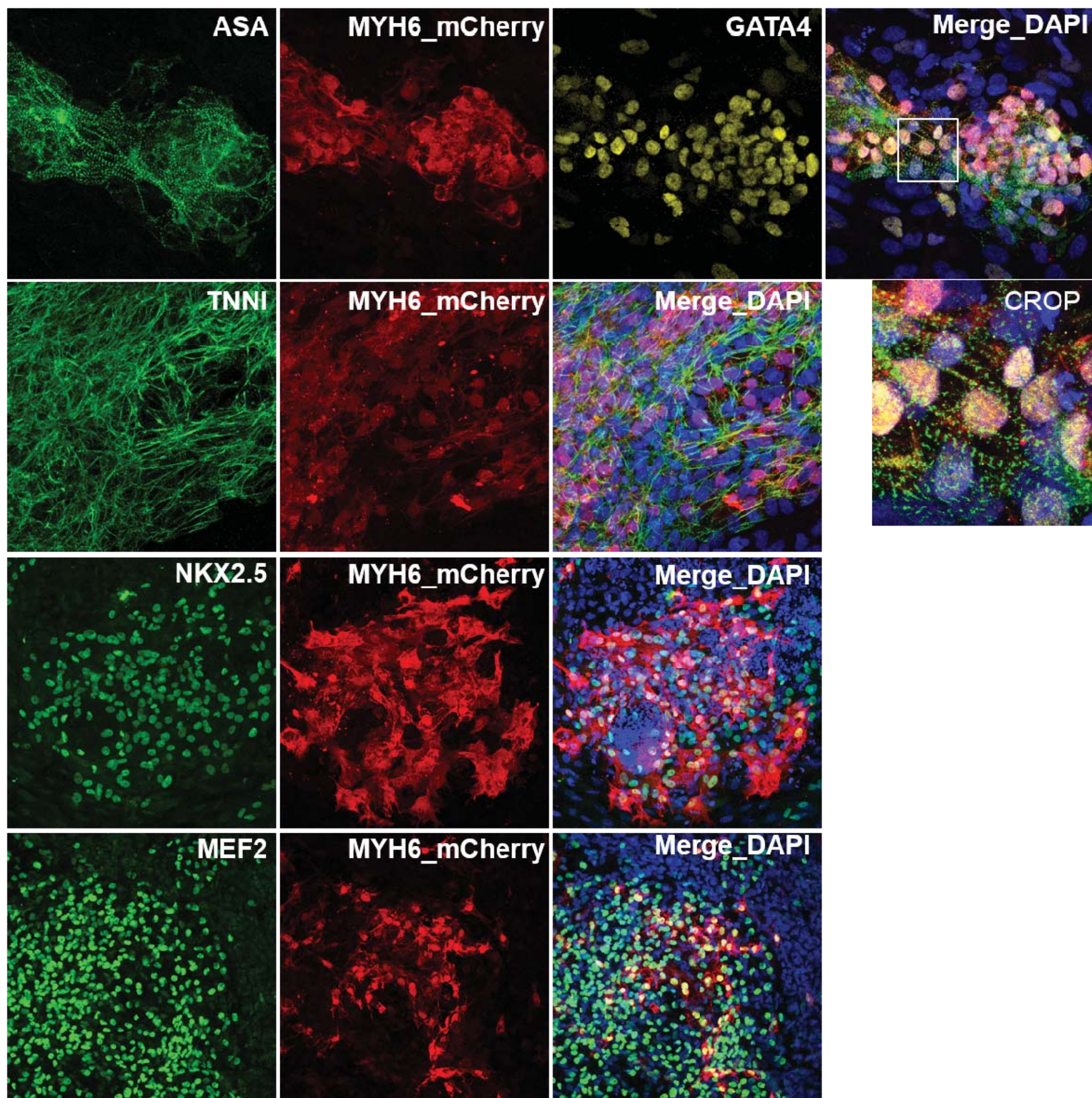


Figure 16. Cardiac differentiation in TALEN ES[4] MYH6_mCherry cell line. Representative images of i*n vitro* differentiation of the TALEN ES[4] MYH6_mCherry cell line into cardiomyocyte-like cells shows that GATA Binding Protein 4 (GATA4), Alpha Sarcomeric Actin (ASA), Troponin I (TNNI), NK2 Homeobox 5 (NKX2.5) and Myocyte Enhancer Factor 2 (MEF2), NKX 2.5 and MYH6 (mCherry) are already expressed at *day 9* during cardiac differentiation. Crop image shows typical stripped pattern of cardiomyocytes sarcomeres. Image capture by 40X objective.

3.7. TALEN ES[4] MYH6_mCherry derived cardiomyocytes recapitulate cardiac features when co-cultured in human cardiac matrices

Due that a major need in cardiac tissue engineering is to restore left ventricular function we decide to test the possibility to mature TALEN ES[4] MYH6_mCherry derived cardiomyocytes on top of 3-D cardiac extracellular matrix (dECM), generated in the "Hospital General Universitario Gregorio Marañón". For this purpose our collaborators sub-cultured cardiomyocyte-like derived cells from TALEN ES[4] MYH6_mCherry o in the presence of cardiac maturation media (RPMIN/B27 media) on 3-D human cadaveric 3-D dECM ventricles. Based on our preliminary data TALEN ES[4] MYH6_mCherry derived cardiomyocytes at *day 16* during the differentiation process were splitted on top of 3-D dECM ventricles and after four days matrices were analyzed by confocal microscopy for the expression of NKX2.5 and mCherry markers (**Figure 17**). Overall our results indicated that TALEN ES[4] MYH6_mCherry represents an innovative approach for the generation of human cardiac-like cells that can be easily monitored based on the expression of mCherry reporter on human cadaveric 3-D dECM ventricles.

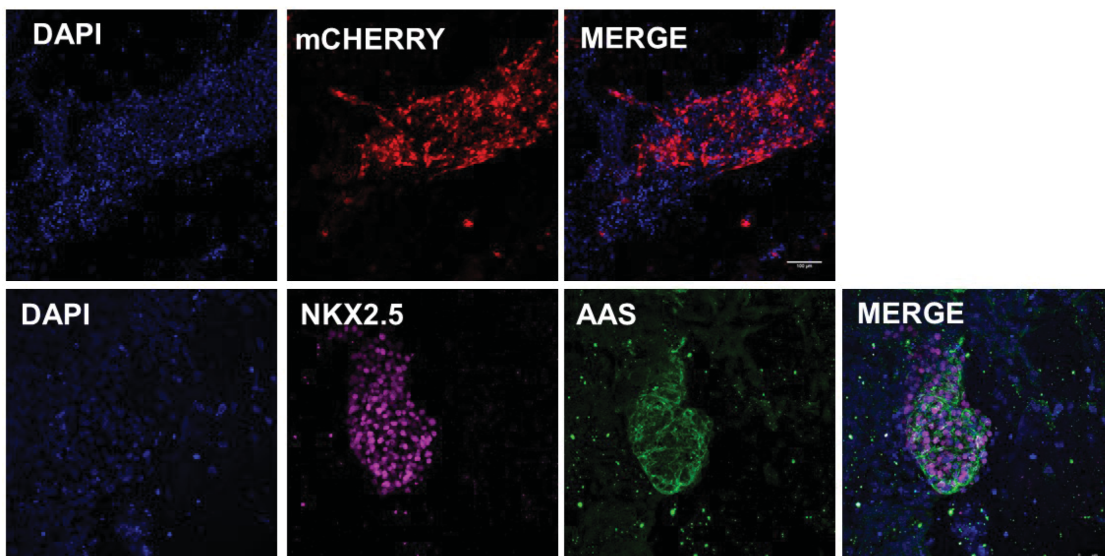


Figure 17. Culture of TALEN ES[4] MYH6_mCherry derived cardiomyocytes on top of 3-D cardiac extracellular matrix (dECM). TALEN ES[4] MYH6_mCherry derived cardiomyocytes were co-cultured on top of 3-D dECM from day 20 to day 24 in the presence of maturation media. Representative images of are already expressed at day 20 during cardiac differentiation. Scale bars, 100 µm

Here we described the generation of a reporter cell line for the study of cardiac differentiation in hESCs. Specifically two different approaches have been followed for the targeting of mCherry in the ATG starting codon of *MYH6* locus: TALEN and CRISPR/Cas9. Although we were able to generate reporter cell lines with both approaches, in our hands, TALEN technology has demonstrated to be more efficient than CRISPR/Cas9 as summarized in the box below (**Table 1**).

	Targeting efficiency			HR efficiency				mCherry expression	
Method	**n G418R colonies**	**positive SHORT PCR**	**%**	**positive LONG PCR**	**%**	**+Southern**	**%**	**Differentiated**	**%**
TALEN pair1	>100	18/24	75	14/18	77,7	2/2	100	2/2	100
TALEN pair2	>100	14/24	58,3	11/14	78,5	1/2	50	1/1	100
CAS9 + gRNA1	<100	12/48	25	9/12	75	1/4	25	1/1	100

Table 1 Gene-targeting and transgene *knock in* efficiencies at the *MYH6* locus with TALEN and CRISPR/CAS9 nuclease. The table indicates de ratio of success after G418 selection and Short PCR screening (Targeting efficiency), and the ratio of success for long PCR screening and the detection of transgene allele by Shouthern Blot (Homologous Recombination-HR efficiency). We also indicate the percentage of success when experiments of cardiac differentiation were evaluated for the acquisition of mCherry expression.

In our hand TALEN ES[4] MYH6_mCherry line perserves the all the analyzed characteristics related to pluripotency (expression of endogenous pluripotent related markers and capability to give rise to cells from the three germ layers of the embryo) proving the feasibility of this approach for genome editing in hESCs. On the other hand we have been able to develop a robust protocol for the differentiation of this reporter cell line towards cardiac-like cells aiming to underscore in the near future the epigenetic and cellular mechanisms responsible of different fate decisions during cardiac differentiation.

V. DISCUSSION

V.DISCUSSION

The isolation and derivation of hESCs by Thompson and colleagues in 1998 attracted significant attention in the Regenerative Medicine field (Thomson 1998). Indeed, regenerative cell transplantation therapies have been expected to treat incurable diseases, such as spinal cord injury (Nakamura & Okano 2013), neurodegenerative disease (CA & Akimov SS 2014), heart failure (Fujita et al. 2012; Hsiao et al. 2012), diabetes (Holditch et al. 2014), and retinal disease (Ramsden et al. 2013), among others.

Nowadays, clinical application of hESCs still shows many concerns regarding the use of human embryos, tissue rejection after transplantation, and tumor formation. However, hESCs possess the dual ability to proliferate indefinitely without phenotypic alterations, and more importantly, to differentiate, theoretically, into all cell types in the human body. These qualities suggest extensive utility of hESCs in applications varying from the definition of differentiation protocols, to the generation of drug screening platforms for disease treatment. Thus, hESCs represent an ideal source for understanding cardiac development and disease.

The generation of induced pluripotent stem cells (iPSCs), especially the generation of patient-derived pluripotent stem cells (PSCs) suitable for disease modelling *in vitro*, opens the door for the potential translation of stem-cell related studies into the clinic. Successful replacement, or augmentation, of the function of damaged cells by patient-derived differentiated stem cells would provide a novel cell-based therapy for cardiac and other related diseases. Since iPSCs resemble human embryonic stem cells (hESCs) in their ability to generate cells of the three germ layers (ectoderm, mesoderm and endoderm), patient-specific iPSCs offer definitive solutions for the ethical and histo-incompatibility issues related to hESCs. Indeed human iPSC (hiPSC)-based autologous transplantation is heralded as the future of Regenerative Medicine. Interestingly, during the last years intense research has been published on disease-specific hiPSCs derivation and differentiation into relevant tissues/organs providing a unique scenario for modelling disease progression and to screen patient-specific drugs.

Somatic reprogramming was first described using mouse embryonic fibroblasts (MEFs) (Takahashi & Yamanaka 2006). Since that moment, different research groups have shown that iPSC technology can be applied to reprogram a huge variety of human

somatic cells, independently of their embryonic origin (Takahashi et al. 2007; Aasen et al. 2008; Giorgetti et al. 2009; González et al. 2011; Montserrat et al. 2012). Interestingly, during the last years the generation of protocols avoiding the use of lentiviral or retroviral vectors for the expression of Yamanaka factors has involved the definition of novel strategies for hiPSCs generation, including the use of recombinant proteins (D. Kim et al. 2009; Zhou et al. 2009), episomal vectors (Okita et al. 2011), or mRNAs (Anokye-Danso et al. 2011; Warren et al. 2010), among others (González et al. 2011).

A part from issues related to the development of safe strategies for somatic reprogramming, many efforts have been done in the quest to the proper cell to be converted into iPSCs. In this regard, first reports relayed in the use of amenable reprogrammable cell sources, betting for those requiring minimal invasive and quick procedures. So far, iPSC lines have been generated from many cell types including dermal fibroblasts (Takahashi et al. 2007), keratinocytes (Aasen et al. 2008), mesenchymal stem cells (MSCs) from adipose tissue (Sun, Panetta, et al. 2009), CD34+ fraction of blood samples (Meng et al. 2012), neural stem cells (J. B. Kim et al. 2009), CD133+ cells from cord blood samples (Giorgetti et al. 2009), and renal tubular epithelial cells (Montserrat et al. 2012), among others. Overall, it has been widely demonstrated that the efficiency, speed and number of transcription factors depends on the cell source of origin. Thus, cells that express endogenously high levels of certain reprogramming factors are more amenable to be reprogrammed. In this regard, Kim et al, showed that neural stem cells, which express endogenously high levels of SOX2, could be reprogrammed to pluripotency with only OCT4 (J. B. Kim et al. 2009). In the same manner, Giorgetti et al. demonstrated that cord blood cells expressing CD133 (CD133+), which express high levels of KLF4 and c-MYC could be reprogrammed with only OCT4 and SOX2 (Giorgetti et al. 2009).

MSCs derived from umbilical cord blood (ucMSCs) represent an ideal source in terms of their accessibility, painless procedures to the donors, few ethical issues and their abundance in the tissue source (Chase et al. 2011b). In the present thesis we describe the generation of iPSC lines form ucMSCs by the overexpression of OCT4, SOX2, KLF4 and c-MYC in only 16 days, without the addition of any chemical compound previously reported to enhance or increase reprogramming efficiency (Huangfu et al. 2008;

Lyssiotis et al. 2009). Since ucMSCs are derived from cord blood cells (CD34+ population), we hypothesized that the use of only OCT4 and SOX2 could convert ucMSCs into iPSCs. In order to achieve our objective OCT4 was fused with the VP16 transactivation domain (OCT4VP16), which was previously described to efficiently increase the reprogramming efficiency when using human fibroblasts (Wang et al. 2011) and to enable the reprogramming of human renal proximal tubular cells when over-expressed with SOX2 (Montserrat et al. 2012). Thus, we proceeded to transduce ucMSCs with OCT4VP16 together with SOX2 viral particles under our specific cell culture conditions obtaining in a process of 12-16 days iPS-like colonies.

Although CD133+ cells have been shown to be efficiently reprogrammed towards iPSCs, their *in vitro* expansion potential and maintenance represent two major obstacles in the context of iPSC generation. On the contrary, ucMSCs can be harvested without any risk for the donors and easily expanded *ex-vivo* for clinical applications (Fan et al. 2011). On the other hand, when compared with adipose tissue (AT) or bone marrow (BM) derived MSCs, ucMSCs show no differences regarding morphology and immune phenotype, and more importantly, can be cultured during longer time periods showing the highest proliferation potential among the three MSCs types (Kern et al. 2006b). It has been previously shown that BM-MSCs, AT-MSCs and ucMSCs can be reprogrammed to iPSCs efficiently (Jinglei Cai et al. 2010; Kunisato et al. 2010; Sun, Lee, et al. 2009; Y. Wang et al. 2013) indicating that MSCs are promising candidates for the generation of iPSC lines in a clinical setting. However, this is the first time that ucMSCs are reprogrammed with only OCT4 and SOX2 transcription factors opening the door to new studies for the screening of chemical compounds and transcription factors replacing OCT4 and SOX2 during the reprogramming process in the human setting.

In order to define new conditions for the generation of cardiac-like cells from ucMSCs derived iPSCs (ucMSCs_iPS) we screened several conditions previously reported to convert iPSCs towards cardiac-like cells. In particular, we employed embryoid body media (EBm) supplemented with ascorbic acid in both monolayer and EBs derived from ccMSCs_iPS generated with either 4 or 2 factors. As expected our results demonstrated that ucMSCs_iPS could be efficiently converted towards cardiac-like cells in a process that last only 15 days.

Within the last two decades intense research in the area of direct cell conversion has been done (Sancho-Martinez et al. 2012). The possibility to restore lost parts of a functional organ (i.e: tissue, or specific cell types, etc) opens the door to the development of autologous sources avoiding the use of PSCs (i.e: hESCs nor patient-specific iPSCs). In this regard adult MSCs show promise for repair and regeneration of cardiac tissues. The ability of MSCs to differentiate into mesoderm- and non-mesoderm-derived tissues, their immunomodulatory effects, their quick availability, their easy culture expansion, and their key role in maintaining and replenishing endogenous stem cell niches have rendered them one of the most deeply investigated and clinically tested type of stem cell (Si et al. 2011; Hoogduijn et al. 2011; Jung et al. 2012). From a translational point of view up to day accumulating data from preclinical and early phase clinical trials document their safety when delivered as either autologous or allogeneic forms in a range of cardiovascular diseases, but also importantly, define parameters of clinical efficacy that justify further investigation in larger clinical trials.

Interestingly MSCs have been also investigated in terms of their capacity to give rise to cardiac-like cells *in vitro*. In this regard seminal studies have already demonstrated that murine BM-MSCs could be converted into cardiac-like cells after exposure into 5´-azacytidine (5-aza) (Makino et al. 1999). Although the efficiency of the process was reported to be low, and 5-aza is highly toxic, those first results encouraged the scientific community to screen for new strategies aiming to generate cardiac-like cells from cell sources different from PSCs. From that moment, other demethylating agents have been invested for their potential action on cardiomyocyte conversion, such as zebularine (Naeem et al. 2013). Thus in the last years several methods have been explored to promote cardiac differentiation from adult stem cells into cardiomyocytes, including the co-culture of stem cells (Wang et al. 2006), treatment with cardiac tissue extracts (Liu et al. 2008), angiotensin II exposure (Xing et al. 2012), and the use of nitric oxide donors (Kanno et al. 2004; Rebelatto et al. 2009). Although challenging, these methods are still inefficient, and mostly reported using mouse or rat cells.

Based on our expertise in the fields of somatic reprogramming and cardiac differentiation we explored the possibility to direct convert ucMSCs and other mesodermal-like somatic cells, as dermal fibroblast cells, towards cardiac-like cells. In order to achieve our objective we decided to design a two-step protocol for the

generation of cardiac-like cells from these and other cell sources. At that time previous findings described that the exposure of human fibroblasts to OCT4, SOX2, KLF4 and c-MYC induced an initial conversion phase defined as “plastic state”, in where “primed” fibroblast exposed to a specific culture media able to differentiate hESCs into angioblast-like cells, were efficiently converted into that specific population (Kurian et al 2013). Based on this and other observations (OCT4 alone was reported to induce blood generation from human fibroblasts; (Szabo et al. 2010)), and to the fact that in our hands OCT4 fused with VP16 trans-activator domain (OCT4VP16) together with SOX2 sufficed for the reprogramming of ucMSCs into iPSCs, we decided to transduce dermal fibroblasts with these same factors in order to induce the so-called “plastic phase” with no need of KLF4 and c-MYC. Our first screening showed that the exposure of dermal fibroblasts to OCT4VP16 and SOX2 in the presence of fibroblast media, already induces the expression of markers related with mesodermal lineage. In this regard, this was the first time, to our knowledge, that OCT4 and SOX2 in the absence of KLF4 and c-MYC were shown to promote mesodermal fate under these conditions. In order to promote cardiac differentiation we then exposed “primed” fibroblasts to EB media supplemented with ascorbic acid, since we previously observed that the same treatment efficiently converted ucMSC derived iPSC into cardiac like-cells (**Objective 1**). Interestingly, by this approach we could convert fibroblasts into cardiac cells, although the efficiency was very low.

These preliminary results encouraged us to explore for additional transcription factors promoting cardiomyocyte conversion from dermal post-natal fibroblasts. For this reason we monitored the expression of different proteins previously related to cardiac developmental program in ES[4] line aiming to identify novel conditions for fibroblast cardiac conversion. Interestingly, we found that GATA4 was expressed as soon as 5 days during our differentiation protocol, so we decided to select this transcription factor as a major regulator of fibroblast cardiac conversion. Interestingly at that time also several authors reported that GATA4 together with other transcription factors efficiently converted mouse cardiac fibroblasts into cardiac-like cells (Efe et al. 2011). Thus, we decided to transduce primed fibroblasts (previously infected with OCT4VP16 and SOX2) with retroviral particles for GATA4 fused with VP16 trans-activator domain (GATA4VP16). Our results demonstrated that our protocol allowed the generation of

cardiac-like cells (based in the acquisition of cardiac markers at the protein level) in a process that last 20 days.

During the last years other groups have demonstrated the possibility to generate cardiac-like cells out of cardiac (Wada et al. 2013; Nam et al. 2013), dermal (Wada et al. 2013; Fu et al. 2013; Nam et al. 2013), and neonatal human fibroblast (Nam et al. 2013). Those protocols made use of different combinations of cardiac transcription factors and/or cardiac related factors for the generation of induced cardiomyocytes (iCMs). Specifically the different works made use of either GATA4, HAND2, Myocardin, TBX5, miR1 and miR133 (Nam et al. 2013); GATA4, MEF2C, TBX5 MESP1 and Myocardin (Wada et al. 2013) ; GATA4, MEF2C, TBX5, ESGRRG, ZFPM2, Myocardin, MESP1 and Myocardin (Fu et al. 2013). Although those first reports pointed out the feasibility of this approach, it seems that only human cardiac fibroblasts could be converted into beating cardiomyocytes (Wada et al. 2013). Thus, there is a need to generate further protocols for iCMs production from clinically amenable somatic cells (e.g: dermal fibroblasts).

Overall, our protocol shows that making use of only one cardiac transcription factor (GATA4) we are able to generate cardiac-like cells based in the acquisition of the expression of cardiac markers at the protein level. Indeed our results demonstrate that we are able to generate cells expressing cardiac markers such as Troponin I, GATA4, MYH6, Tropomyosin and NKX2.5, similarly to previous findings in the human setting (Nam et al. 2013; Fu et al. 2013). Due to the fact that our protocol relays in the use of a single cardiac factor together with a very simple culture media (EB media with ascorbic acid), we are convinced that further experiments, conducted nowadays in the laboratory, will provide novel information about the final cell culture conditions promoting the generation of functional iCMs from post-natal dermal fibroblasts.

In the quest for the definition of robust protocols for the derivation of cardiac-like cells from either PSCs or somatic cell sources we decided to explore the possibility to generate a cardiac reporter cell line. Due to the fact that PSCs are a suitable platform for the generation of expandable undifferentiated cells, we decided to design two forefront approaches (TALEN and CRISPR/CAS9 techniques) for the derivation of a cardiac reporter cell line in hESCs. In this manner we were going to be able to monitor the

process of cardiac differentiation, and more importantly, to generate a tool for further studies in cardiac tissue engineering.

In particular we decided to target Alpha Myosin heavy chain (α-*MHC* or *MYH6*) locus based on our own preliminary data, that showed that during the onset of cardiac differentiation MYH6 expression is induced at early stages of the process, being sustained until day 30. Moreover, other authors already pointed out the suitability of this factor when establishing reporter cell lines in either murine or human hESCs. Indeed Song et al., (2012) already picked this same gene in order to monitor GFP expression under the control of a short form of MYH6 promoter in mice (Song et al. 2012). Then other groups reported on the generation of transgenic mice as MYH6-GFP/IRES-Puromycin mice (Ieda et al. 2010), or the MYH6-CFP/Fsp1-Cre/tdTomato mice; the later used for direct cardiac reprogramming of fibroblasts into iCMS by micro RNAs overexpression (Jayawardena et al. 2012).

In the human setting still few data for the definition of robust protocols for cardiac differentiation arises from the use of cardiac reporter cell lines. In this regard most of the examples make use of integrative approaches, as the one described by Mercola and colleagues (2009), who demonstrated the feasibility of lentiviral vectors for the expression and further selection of cardiac reporter genes in hESCs [329].They reported on the generation of hESCs reporter cell lines for either Brachyury (T) or *MYH6* promoter sequences with mCherry or GFP fluorescent proteins and antibiotic resistance under the promoter of Rex1 (a pluripotency-related gene), avoiding the presence of undifferentiated hESCs at the end of the differentiation protocol. Following this same approach Ritner and colleagues (2011) generated MYH6-GFP human hESCs reporter cell line (Ritner et al. 2011).

By the time we decided to generate our cardiac reporter cell line, only one laboratory have described the generation of a cardiac reporter cell line in hESCs by gene targeting. Interestingly Elliot and colleagues (Elliott et al. 2011a) targeted eGFP sequences to the *NKX2.5* locus by means of homologous recombination (HR) mediated by DNA meganucleases. In that particular work authors demonstrated that by means of this technique NKX2-5eGFP/w hESCs facilitated the quantification of cardiac differentiation, purification of hESC-derived committed cardiac progenitor cells (hESC-CPCs) and cardiomyocytes (hESC-CMs). In a very elegant manner authors also

identified that VCAM1 and SIRPA cell-surface markers could be used for the identification of cardiac specific cell subtypes during differentiation (Dubois et al. 2011). Interestingly this year (2015) other authors have published the generation of a double reporter cell line taking advantage of the one published before by Elliott. In the same manner, authors have targeted the early cardiac progenitor marker MESP1 by homologous recombination and, successfully generated a *knock in* cell line by mCherry /Neomycin reporter integration at *MESP1* genome locus (Den Hartogh et al. 2015).

Since TALEN and CRISPR/CAS9 technologies were described as powerful tools for gene targeting in PSCs, we decided to make use of these forefront methodologies in order to generate a MYH6-mCherry/w hESCs. Our design preserved all regulatory sequences from MYH6 gene due to the targeting at the ATG start site of the gene. In this manner, and using the same donor vector, we were able to generate 3 MYH6-mCherry/w hESCs with TALEN technology and one with CRISPR/CAS9. We decided to characterize one out of the three MYH6-mCherry/w hESCs generated with TALEN in an effort to provide a tool for the screening of the best conditions for the generation of cardiac-like cells. In particular TALEN_MYH6-mCherry/w hESCs preserved the different morphological and phenotypic properties related to hESCs biology (i.e: expression of endogenous pluripotent-related markers, capacity to give rise to cells from the three germ layers of the embryo *in vitro*, stable karyotype). Moreover, when exposed to different protocols of cardiac differentiation (detailed below) cells expressed mCherry fluorescent protein together with other markers related to cardiac fate, pointing out the suitability of this approach for the generation of new protocols for cardiac differentiation.

Next and in an effort to define a robust protocol for cardiac differentiation we exposed TALEN_MYH6-mCherry/w hESCs into conditions reported to either stimulate or inhibit WNT signaling pathway, both key events during cardiac development when using human PSCs (Lian et al. 2012b; Gu et al. 2014). For that reason we activated WNT signaling pathway by means of CHIR99021 in the presence of specific cell culture conditions previously defined to induce early stages of cardiac differentiation (Lian et al. 2012b; Gu et al. 2014). Our analysis demonstrated that already after 3 days WNT signaling pathway activation was coincident with silencing of pluripotent-related genes. When WNT pathway was inhibited other markers related with cardiac program

(i.e: cardiac precursor genes) were upregulated and sustained up to 20 days under our specific cell culture conditions. Overall, and by means of the modification of several parameters defined before (i.e: confluency status of undifferentiated cells) we were able to set up a protocol of cardiac differentiation giving rise to MYH6 positive cells as soon as day 7 after differentiation. When wild type hESCs were exposed to our new conditions similar results were observed (i.e: expression of cardiac markers at protein and mRNA levels were similar), proving the suitability of our TALEN_MYH6-mCherry/w hESCs line for the definition of new protocols for cardiac differentiation. Also, when TALEN_MYH6-mCherry/w hESCs at *day 20* during the differentiation protocol were co-cultured with cadaveric matrices from human donors we observed that under our defined conditions TALEN_MYH6-mCherry/w hESC derived cardiomyocytes behave as functional cells, since electrophysiological parameters are improved when comparing TALEN_MYH6-mCherry/w hESCs derived cardiomyocytes cultured in ventricular decellularized matrices than in Matrigel® (data not shown generated in collaboration with Dr. Fernández-Avilés)

In resume we believe that the generation of TALEN_MYH6-mCherry/w hESC reporter cell line will serve for the establishment of new conditions for the generation of cardiac-like cells from PSCs. Indeed, preliminary data generated in the last months of this thesis (*data not shown*) points out that the use of our differentiation protocol in fibroblasts infected with OCT4VP16, SOX2 and GATA4VP16 promotes the generation of cardiac-like cells in a process that last 20 days. Although the data is still very preliminary we are confident that the use of this protocol will boost cardiac conversion from dermal fibroblasts. Also, the possibility to select mCherry fluorescent cardiac-expressing cells will allow for the interrogation of epigenetic mechanisms on-going during cardiac differentiation from PSCs, leading the possibility to identify new molecular drivers at different stages of the process.

Overall the thesis presented here illustrates different approaches for the generation of cardiac-like cells. First, and in an effort to bring new knowledge in the field of somatic reprogramming, we have demonstrated that ucMSCs-iPS lines generated with two factors can be efficiently differentiated towards cardiac-like cells. Second, we have developed a protocol for cardiac conversion from post-natal dermal fibroblasts applying two methodologies related with PSCs biology (overexpression of pluripotent

transcription factors coupled with cardiac media promoting cardiac differentiation from PSCs). Third, we have generated cardiac reporter cell lines in order to define new conditions for the generation of cardiac-like cells from both PSCs and somatic cell sources. We believe that the different approaches developed here provide new information for the generation of cardiac-like cells for Regenerative Medicine purposes.

In summary the different approaches aiming to generate cardiomyocytes with an impact in Regenerative Medicine are detailed in **Figure 1**. Overall the data generated in the context of this thesis set the bases for the definition of new differentiation protocols and forefront approaches for cardiovascular disease treatment.

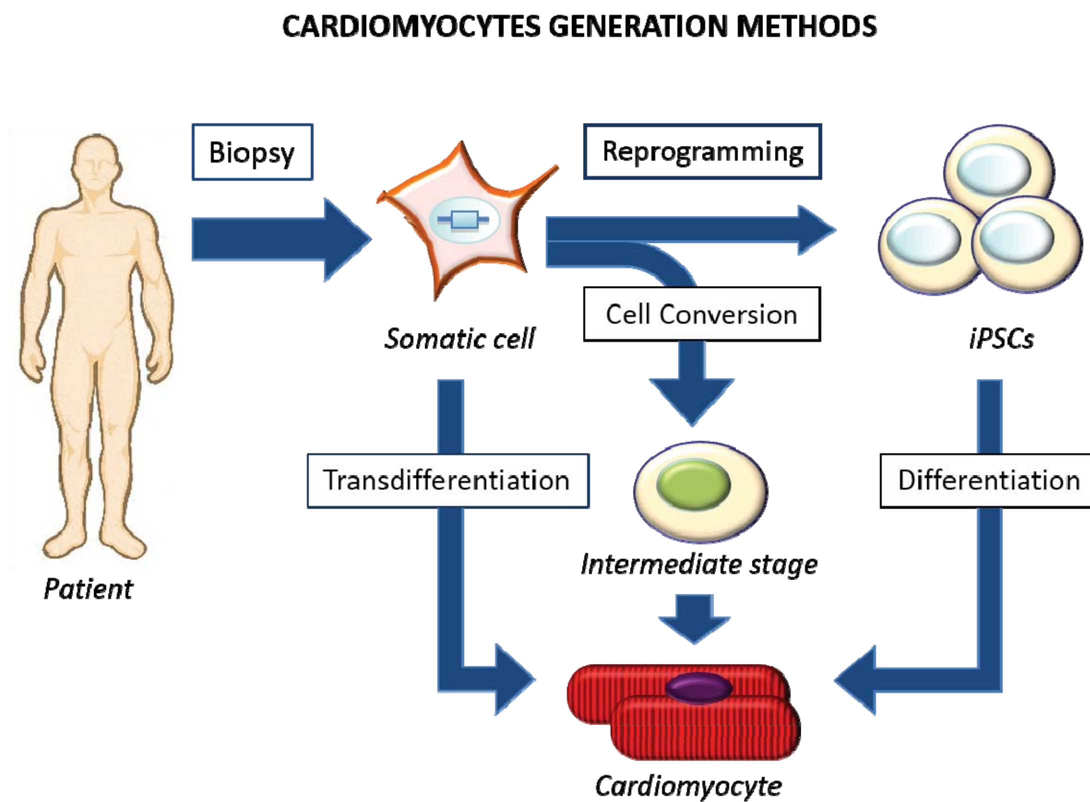


Figure 1 Research on cardiac differentiation from human Pluripotent Stem Cells: how to get beating cells in a dish. Overview of different approaches described in this thesis aiming to generate cardiac-like cells from different cell sources. First, we have reported on the possibility to generate iPSCs for the generation of cardiac cells from ucMSCs . Second, and in an effort to develop new protocols for cardiac direct conversion we have explored the possibility to de-differentiate post-natal dermal fibroblast with reprogramming factors and to subsequent express cardiac-related transcription factors together with specific cuture conditions promoting cardiac differentiation from iPSCs. Third we have generated a cardiac reporter line from human embryonic stem cells aiming to define robust protocols for cardiac differentiation. Overall these approaches demonstrate the possibility to study cardiac differentiation and specification by means of forefront methodologies applicable to the human setting

VI. CONCLUSIONS

VI.CONCLUSIONS

1. Umbilical cord derived mesenchymal stem cells (ucMSCs) can be efficiently reprogrammed to induced Pluripotent Stem Cells (ucMSC-iPS) with four transcriptional factors: OCT4, SOX2, KLF4 and c-MYC (ucMSC-iPS-4F).

2. ucMSC-iPS-4F exhibit characteristics related to human embryonic stem cells (hESCs), such as expression of pluripotent-related markers and capability to be differentiated *in vitro* towards the three germ layers of the embryo.

3. Umbilical cord derived mesenchymal stem cells (ucMSCs) can be efficiently reprogrammed to Induced Pluripotent Stem Cells (ucMSC-iPS) with two transcriptional factors: OCT4 and SOX2, in the absence of chemical compounds (ucMSC-iPS-2F).

4. ucMSC-iPS-2F exhibit characteristics related to human embryonic stem cells (hESCs), such as expression of pluripotent-related markers and capability to be differentiated *in vitro* towards the three germ layers of the embryo.

5. The forced expression of OCT4 and SOX2 in dermal post-natal fibroblasts induces the expression of pluripotent and mesodermal related genes.

6. When de-differentiated dermal post-natal fibroblasts are transduced with GATA 4 cardiac-related transcription factor in the presence of embryoid body media supplemented with ascorbic acid, cells expressing proteins related to cardiac fate are detected by immunofluorescence after 10 days.

7. TALEN and CRISPR/CAS9 can efficiently target mCherry fluorescent protein after the ATG starting codon in the alpha myosin heavy chain (*MYH6*) locus in a human ESC line, namely ES [4] line, without altering pluripotent-related properties, neither chromosomal stability.

8. ES[4] MYH6-mCherry reporter cell line generated with TALEN technology (TALEN ES[4] MYH6_mCherry line) can be efficiently differentiated towards cardiac-like cells in a period of 7 days by means of a chemically defined protocol.

9. The expression of mCherry fluorescent protein in TALEN ES[4] MYH6_mCherry derived cardiomyocytes is preserved after the co-culture with tri-dimensional cardiac extracellular matrices from human cadaveric hearts, indicating the feasibility of this approach for human heart engineering.

VII. BIBLIOGRAPHY

VII. BIBLIOGRAPHY

Aasen, T. et al., 2008. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nature biotechnology*, 26, pp.1276–1284.

Ahmed, T.A.E. & Hincke, M.T., 2014. Mesenchymal stem cell - Based tissue engineering strategies for repair of articular cartilage. *Histology and Histopathology*, 29(6), pp.669–689.

Ai, D. et al., 2007. Canonical Wnt signaling functions in second heart field to promote right ventricular growth. *Proceedings of the National Academy of Sciences of the United States of America*, 104(22), pp.9319–9324.

Alonso, S. et al., 1990. Genetic analysis of the interaction between cardiac and skeletal actin gene expression in striated muscle of the mouse. *Journal of Molecular Biology*, 211(4), pp.727–738. Available at: http://www.sciencedirect.com/science/article/pii/002228369090073U.

Alsan, B.H. & Schultheiss, T.M., 2002. Regulation of avian cardiogenesis by Fgf8 signaling. *Development (Cambridge, England)*, 129(8), pp.1935–1943.

ALTMAN, J. & DAS, G.D., 1965. Post-Natal Origin of Microneurones in the Rat Brain. *Nature*, 207(5000), pp.953–956. Available at: http://dx.doi.org/10.1038/207953a0.

Anokye-Danso, F. et al., 2011. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell*, 8, pp.376–388.

Aoi, T. et al., 2008. Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science (New York, N.Y.)*, 321(5889), pp.699–702.

Appelbaum, F.R. et al., 2015. Blood and Marrow Transplant Clinical Trials Network State of the Science Symposium 2014. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation*, 21(2), pp.202–224. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4426907/.

Asuri, P. et al., 2012. Directed Evolution of Adeno-associated Virus for Enhanced Gene Delivery and Gene Targeting in Human Pluripotent Stem Cells. *Mol Ther*, 20(2), pp.329–338. Available at: http://dx.doi.org/10.1038/mt.2011.255.

Aviss, K.J., Gough, J.E. & Downes, S., 2010. Aligned electrospun polymer fibres for skeletal muscle regeneration. *European Cells and Materials*, 19, pp.193–204.

Barrangou, R. et al., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science (New York, N.Y.)*, 315(5819), pp.1709–1712.

Baum, C. et al., 2006. Retrovirus Vectors: Toward the Plentivirus? *Mol Ther*, 13(6), pp.1050–1063. Available at: http://dx.doi.org/10.1016/j.ymthe.2006.03.007.

Becker, A. J., McCulloch, E. A. & Till, J.E., 1963. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*, 197, pp.452–454.

Belay, E. et al., 2010. Novel hyperactive transposons for genetic modification of induced pluripotent and adult stem cells: A nonviral paradigm for coaxed differentiation. *Stem Cells*, 28(10), pp.1760–1771.

Beqqali, A. et al., 2006. Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. *Stem cells*, 24(8), pp.1956–1967.

Bethesda, MD: National Institutes of Health, U.S.D. of H. and H.S., 2009. Human Embryonic Stem Cell Policy Under Former President Bush (Aug. 9, 2001–Mar. 9, 2009). *Stem Cell Information*. Available at: http://stemcells.nih.gov/policy/pages/2001policy.aspx.

Beurdeley, M. et al., 2013. Compact designer TALENs for efficient genome engineering. *Nature Communications*, 4, p.1762. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3644105/.

Bhaya, D., Davison, M. & Barrangou, R., 2011. CRISPR-Cas Systems in Bacteria and Archaea: Versatile Small RNAs for Adaptive Defense and Regulation. *Annual Review of Genetics*, 45(1), pp.273–297.

Bjorklund, L.M. et al., 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proceedings of the National Academy of Sciences of the United States of America*, 99(4), pp.2344–2349.

Blau, H.M., Chiu, C.P. & Webster, C., 1983. Cytoplasmic activation of human nuclear genes in stable heterokaryons. *Cell*, 32, pp.1171–1180.

Boch, J. et al., 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science (New York, N.Y.)*, 326(5959), pp.1509–1512.

Bogert, P.T. & LaRusso, N.F., 2007. Cholangiocyte biology. *Current opinion in gastroenterology*, 23(3), pp.299–305.

Boissel, S. et al., 2014. megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering. *Nucleic Acids Research*, 42(4), pp.2591–2601. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3936731/.

Brade, T., Männer, J. & Kühl, M., 2006. The role of Wnt signalling in cardiac development and tissue remodelling in the mature heart. *Cardiovascular Research*, 72(2), pp.198–209. Available at: http://cardiovascres.oxfordjournals.org/content/72/2/198.abstract.

Brolén, G. et al., 2010. Hepatocyte-like cells derived from human embryonic stem cells specifically via definitive endoderm and a progenitor stage. *Journal of Biotechnology*, 145(3), pp.284–294.

Bu, L. et al., 2009. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature*, 460(7251), pp.113–117.

Buckingham, M., Meilhac, S. & Zaffran, S., 2005. Building the mammalian heart from two sources of myocardial cells. *Nature reviews. Genetics*, 6(11), pp.826–835.

Burridge, P.W. et al., 2011. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS ONE*, 6(4).

Burridge, P.W. et al., 2007. Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. *Stem cells*, 25(4), pp.929–938.

Burridge, P.W. et al., 2012. Production of de novo cardiomyocytes: Human pluripotent stem cell differentiation and direct reprogramming. *Cell Stem Cell*, 10(1), pp.16–28. Available at: http://dx.doi.org/10.1016/j.stem.2011.12.013.

CA, R. & Akimov SS, 2014. Human-induced pluripotent stem cells: potential for neurodegenerative diseases. *Human Molecular Genetics*, 15(23), pp.17–26.

Cai, C.-L. et al., 2008. A myocardial lineage derives from Tbx18 epicardial cells. *Nature*, 454(7200), pp.104–108.

Cai, J. et al., 2010. Generation of human induced pluripotent stem cells from umbilical cord matrix and amniotic membrane mesenchymal cells. *The Journal of biological chemistry*, 285, pp.11227–11234.

Cai, J. et al., 2010. Modeling co-expression across species for complex traits: insights to the difference of human and mouse embryonic stem cells. *PLoS Comput Biol*, 6(3), p.e1000707. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20300647.

Callis, T.E. & Wang, D.Z., 2008. Taking microRNAs to heart. *Trends in Molecular Medicine*, 14(6), pp.254–260.

Cameron, H.A. & McKay, R., 1998. Discussion point. Stem cells and neurogenesis in the adult brain. *Current Opinion in Neurobiology*, 8(5), pp.677–680.

Can, A. & Balci, D., 2011. Isolation, culture, and characterization of human umbilical cord stroma-derived mesenchymal stem cells. *Methods Mol Biol*, 698, pp.51–62. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21431510.

Cao, N. et al., 2011. Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells promoting the proliferation of cardiac progenitor cells. *Cell Res*, 22(1), pp.219–236. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22143566.

Cao, N. et al., 2012. Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells through promoting the proliferation of cardiac progenitor cells. *Cell Res*, 22(1), pp.219–236. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22143566 [Accessed January 9, 2015].

Carroll, D., 2011. Genome engineering with zinc-finger nucleases. *Genetics*, 188(4), pp.773–782.

Carter, D.B.J., 2005. Adeno-Associated Virus Vectors in Clinical Trials. *Human Gene Therapy*, 16(5), pp.541–550.

Chalberg, T.W. et al., 2006. Integration specificity of phage φC31 integrase in the human genome. *Journal of Molecular Biology*, 357(1), pp.28–48.

Chang, C.W. et al., 2009. Polycistronic lentiviral vector for “hit and run” reprogramming of adult skin fibroblasts to induced pluripotent stem cells. *Stem Cells*, 27(5), pp.1042–1049.

Charpentier, E. & Doudna, J.A., 2013. Biotechnology: Rewriting a genome. *Nature*, 495(7439), pp.50–51. Available at: http://dx.doi.org/10.1038/495050a.

Chase, L.C.G., Rao, M.S.G. & Vemuri, M.S., 2011a. *Mesenchymal Stem Cell Assays and Applications Methods in Molecular Biology*,

Chase, L.C.G., Rao, M.S.G. & Vemuri, M.S., 2011b. *Mesenchymal Stem Cell Assays and Applications Methods in Molecular Biology*, Available at: http://www.springer.com/life+sciences/cell+biology/book/978-1-60761-998-7.

Chen, J.-F. et al., 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet*, 38(2), pp.228–233. Available at: http://dx.doi.org/10.1038/ng1725.

Chen ZY, He CY, Ehrhardt A, K.M., 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Molecular TherapyTher*, 8(3), pp.495–500.

Chevalier, B.S. et al., 2015. Design, Activity, and Structure of a Highly Specific Artificial Endonuclease. *Molecular Cell*, 10(4), pp.895–905. Available at: http://dx.doi.org/10.1016/S1097-2765(02)00690-1.

Chiba, S. et al., 2004. Anatomical and functional recovery by embryonic stem cell-derived neural tissue of a mouse model of brain damage. *J Neurol Sci*, 219(1-2), pp.107–117. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15050446.

Chimenti, I. et al., 2011. Human cardiosphere-seeded gelatin and collagen scaffolds as cardiogenic engineered bioconstructs. *Biomaterials*, 32(35), pp.9271–9281.

Cho, S.W. et al., 2014. Analysis of off-target effects of CRISPR / Cas-derived RNA-guided endonucleases and nickases. , pp.132–141.

Choi, J. et al., 1990. MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *Proceedings of the National Academy of Sciences of the United States of America*, 87(20), pp.7988–7992.

Chong, J.J.H. et al., 2014. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature*, 510(7504), pp.273–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24776797.

Christian, M. et al., 2010. Targeting DNA Double-Strand Breaks with TAL Effector Nucleases. *Genetics*, 186(2), pp.757–761. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2942870/.

Chuang, C.-K. et al., 2007. Baculovirus as a new gene delivery vector for stem cell engineering and bone tissue engineering. *Gene therapy*, 14(19), pp.1417–1424.

Cohen, E.D. et al., 2007. Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *The Journal of clinical investigation*, 117(7), pp.1794–1804.

Colosimo, A. et al., 2000. Transfer and expression of foreign genes in mammalian cells. *BioTechniques*, 29(2), pp.314–331.

Cong, L. et al., 2012. Comprehensive interrogation of natural TALE DNA-binding modules and transcriptional repressor domains. *Nat Commun*, 3, p.968. Available at: http://dx.doi.org/10.1038/ncomms1962.

Cong, L. et al., 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, N.Y.)*, 339(6121), pp.819–23. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3795411&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Courchet, J. et al., 2013. XTerminal axon branching is regulated by the LKB1-NUAK1 kinase pathway via presynaptic mitochondrial capture. *Cell*, 153(7).

Van Craenenbroeck, K., Vanhoenacker, P. & Haegeman, G., 2000. Episomal vectors for gene expression in mammalian cells. *European Journal of Biochemistry*, 267(18), pp.5665–5678. Available at: http://dx.doi.org/10.1046/j.1432-1327.2000.01645.x.

Czyz J, W.A., 2001. Embryonic stem cell differentiation: the role of extracellular factors. *Differentiation*, 68(4-5), pp.167–74.

Daar, A.S. & Greenwood, H.L., 2007. A proposed definition of regenerative medicine. *Journal of Tissue Engineering and Regenerative Medicine*, 1(3), pp.179–184.

Davis, R.L., Weintraub, H. & Lassar, A.B., 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*, 51(6), pp.987–1000.

Davis, R.P. et al., 2012. Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease. *Circulation*, 125(25), pp.3079–3091.

Dekel, I. et al., 1992. Conditional conversion of ES cells to skeletal muscle by an exogenous MyoD1 gene. *The New biologist*, 4, pp.217–224.

DeKelver, R.C. et al., 2010. Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Research*, 20(8), pp.1133–1142.

Derek T Peters, Chad A Cowan, and K.M., 2013. Genome editing in human pluripotent stem cells. In *Stem Book*.

Di Domenico, A.I. et al., 2008. Sequential genetic modification of the hprt locus in human ESCs combining gene targeting and recombinase-mediated cassette exchange. *Cloning and stem cells*, 10(2), pp.217–230.

Dow, J. et al., 2005. Washout of transplanted cells from the heart: A potential new hurdle for cell transplantation therapy. *Cardiovascular Research*, 67(2), pp.301–307.

Doyle, E.L. et al., 2012. TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: Tools for TAL effector design and target prediction. *Nucleic Acids Research*, 40(W1).

Drew, H.R., Lockett, L.J. & Both, G.W., 2007. Increased complexity of wild-type adeno-associated virus-chromosomal junctions as determined by analysis of unselected cellular genomes. *Journal of General Virology*, 88(6), pp.1722–1732.

Dubois, N.C. et al., 2011. SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nature Biotechnology*, 29(11), pp.1011–1018. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22020386 [Accessed January 2, 2015].

Dupuy, A. et al., 2013. Targeted Gene Therapy of Xeroderma Pigmentosum Cells Using Meganuclease and TALEN^{TM} S. Xu, ed. *PLoS ONE*, 8(11), p.e78678. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3827243/.

Dyer, L.A. & Kirby, M.L., 2009. Sonic hedgehog maintains proliferation in secondary heart field progenitors and is required for normal arterial pole formation. *Developmental Biology*, 330(2), pp.305–317.

Dzierzak, E. & Speck, N.A., 2008. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature immunology*, 9(2), pp.129–136.

E., H., 1966. Constancy, variation and type of determination and differentiation in cells from male genitalia rudiments of Drosophila melanogaster in permanent culture in vivo. *Developmental biology*, 13(3), pp.424–509.

Eaves, C.J., 2015. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood*, 125(17), pp.2605–2613. Available at: http://www.bloodjournal.org/content/125/17/2605.abstract.

Efe, J. a et al., 2011. Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nature cell biology*, 13(3), pp.215–222. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21278734 [Accessed July 10, 2014].

Elkabetz, Y. et al., 2008. Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes and Development*, 22(2), pp.152–165.

Elliott, D. a et al., 2011a. NKX2-5eGFP/w hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nature Methods*, 8(12), pp.1037–1040. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22020065 [Accessed January 5, 2015].

Elliott, D. a et al., 2011b. NKX2-5eGFP/w hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nature Methods*, 8(12), pp.1037–1040. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22020065 [Accessed January 5, 2015].

Evans MJ, K.M., 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 292(5819), pp.154–6.

Fan, C.-G., Zhang, Q. & Zhou, J., 2011. Therapeutic potentials of mesenchymal stem cells derived from human umbilical cord. *Stem cell reviews*, 7, pp.195–207.

Feng, R. et al., 2008. PU.1 and C/EBPα/β convert fibroblasts into macrophage-like cells. *Proceedings of the National Academy of Sciences* , 105 (16), pp.6057–6062. Available at: http://www.pnas.org/content/105/16/6057.abstract.

Fischer, Y. et al., 2010. NANOG reporter cell lines generated by gene targeting in human embryonic stem cells. *PLoS.One.*, 5(1932-6203 (Electronic)), pp.1–11. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2932718&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Folger, K.R. et al., 1982. Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. *Molecular and cellular biology*, 2(11), pp.1372–1387.

Fornwald, J.A. et al., 2007. Gene expression in mammalian cells using BacMam, a modified baculovirus system. *Methods in molecular biology (Clifton, N.J.)*, 388, pp.95–114.

Freund, C. et al., 2008. Insulin redirects differentiation from cardiogenic mesoderm and endoderm to neuroectoderm in differentiating human embryonic stem cells. *Stem cells*, 26(3), pp.724–733.

Friedland, A.E. et al., 2013. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. *Nature methods*, 10(8), p.10.1038/nmeth.2532. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3822328/.

Fu, J. et al., 2013. Stem Cell Reports. , 1.

Fu, J.-D. & Srivastava, D., 2015. Direct Reprogramming of Fibroblasts into Cardiomyocytes for Cardiac Regenerative Medicine. *Circulation Journal*, 79(2), pp.245–254. Available at: https://www.jstage.jst.go.jp/article/circj/79/2/79_CJ-14-1372/_article.

Fujita, J. et al., 2012. Myocardial cell sheet therapy and cardiac function. *American journal of physiology. Heart and circulatory physiology*, 303, pp.H1169–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23001836.

Fusaki, N. et al., 2009. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proceedings of the Japan Academy. Series B, Physical and biological sciences*, 85, pp.348–362.

Gabriel, R. et al., 2011. An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat Biotech*, 29(9), pp.816–823. Available at: http://dx.doi.org/10.1038/nbt.1948.

Gage, F.H., 2000. Mammalian Neural Stem Cells. *Science*, 287(5457), pp.1433–1438.

Gao, R. et al., 2002. High efficiency gene transfer into cultured primary rat and human hepatic stellate cells using baculovirus vectors. *Liver*, 22(1), pp.15–22.

Garneau, J.E. et al., 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, 468(7320), pp.67–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21048762 [Accessed July 11, 2014].

Gaur, M. et al., 2010. Timed inhibition of p38MAPK directs accelerated differentiation of human embryonic stem cells into cardiomyocytes. *Cytotherapy*, 12(6), pp.807–817.

GE, L. & , Buckingham ME, M.H., 1991. alpha-Actin proteins and gene transcripts are colocalized in embryonic mouse muscle. *Development*, 111(2), pp.451–4.

Gehring, W., 1967. Clonal analysis of determination dynamics in cultures of imaginal disks in Drosophila melanogaster. *Developmental Biology*, 16(5), pp.438–456. Available at: http://www.sciencedirect.com/science/article/pii/0012160667900589.

Ghosh, T.K. et al., 2009. Physical interaction between TBX5 and MEF2C is required for early heart development. *Molecular and cellular biology*, 29(8), pp.2205–2218.

Giorgetti, a, 2009. Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. *Cell Stem Cell*, 5, pp.353–357. Available at: http://dx.doi.org/10.1016/j.stem.2009.09.008.

Giorgetti, A. et al., 2009. Generation of Induced Pluripotent Stem Cells from Human Cord Blood Using OCT4 and SOX2. *Cell Stem Cell*, 5, pp.353–357.

Giorgetti, A. et al., 2010. NIH Public Access. , 5(4), pp.353–357.

Godier-Furnémont, A.F.G. et al., 2011. Composite scaffold provides a cell delivery platform for cardiovascular repair. *Proceedings of the National Academy of Sciences* , 108 (19), pp.7974–7979. Available at: http://www.pnas.org/content/108/19/7974.abstract.

González, F., Boué, S. & Belmonte, J.C.I., 2011. Methods for making induced pluripotent stem cells: reprogramming à la carte. *Nature Reviews Genetics*, 12(4), pp.231–242. Available at: http://www.nature.com/doifinder/10.1038/nrg2937.

Goulburn, A.L. et al., 2011. A Targeted NKX2.1 Human Embryonic Stem Cell Reporter Line Enables Identification of Human Basal Forebrain Derivatives. *STEM CELLS*, 29(3), pp.462–473. Available at: http://dx.doi.org/10.1002/stem.587.

Graf, T. & Enver, T., 2009. Forcing cells to change lineages . *Nature*, 462(7273), pp.587–594. Available at: http://dx.doi.org/10.1038/nature08533.

Greenhough, S., Medine, C.N. & Hay, D.C., 2010. Pluripotent stem cell derived hepatocyte like cells and their potential in toxicity screening. *Toxicology*, 278(3), pp.250–255.

Griffith, L.M. et al., 2014. Primary Immune Deficiency Treatment Consortium (PIDTC) Report. *The Journal of allergy and clinical immunology*, 133(2), pp.335–347.e11. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3960312/.

Grigoriadis, a. E., Heersche, J.N.M. & Aubin, J.E., 1988. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: Effect of dexamethasone. *Journal of Cell Biology*, 106(6), pp.2139–2151.

Gu, Y. et al., 2014. Global DNA methylation and transcriptional analyses of human ESC-derived cardiomyocytes. *Protein and Cell*, 5(1), pp.59–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23982742 [Accessed January 9, 2015].

Gurdon, J.B., 2013. The egg and the nucleus: A battle for supremacy (nobel lecture). *Angewandte Chemie - International Edition*, 52(52), pp.13890–13899.

Gurdon, J.B. & Byrne, J. a., 2005. The first half-century of nuclear transplantation. *Bioscience Reports*, 24(4-5), pp.547–557.

Gurdon, J.B. & Melton, D.A., 2008. Nuclear reprogramming in cells. *Science (New York, N.Y.)*, 322(5909), pp.1811–1815.

GURDON JB, ELSDALE TR, F.M., 1958. Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. *Nature*, 182(4627), pp.64–5.

Hackett, P.B. et al., 2011. Efficacy and Safety of Sleeping Beauty Transposon-Mediated Gene Transfer in Preclinical Animal Studies. *Current Gene Therapy*, 11(5), pp.341–349.

Hadjantonakis, A.-K., Macmaster, S. & Nagy, A., 2002. Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal. *BMC biotechnology*, 2, p.11.

Hale, C.R. et al., 2015. RNA-Guided RNA Cleavage by a CRISPR RNA-Cas Protein Complex. *Cell*, 139(5), pp.945–956. Available at: http://dx.doi.org/10.1016/j.cell.2009.07.040.

Hanna, J. et al., 2008. Direct Reprogramming of Terminally Differentiated Mature B Lymphocytes to Pluripotency. *Cell*, 133(2), pp.250–264.

Haraguchi, Y. et al., 2013. Simple suspension culture system of human iPS cells maintaining their pluripotency for cardiac cell sheet engineering. *Journal of Tissue Engineering and Regenerative Medicine*, p.n/a–n/a. Available at: http://dx.doi.org/10.1002/term.1761.

Den Hartogh, S.C. et al., 2015. Dual Reporter MESP1 mCherry/w -NKX2-5 eGFP/w hESCs Enable Studying Early Human Cardiac Differentiation. *Stem Cells*, 33(1), pp.56–67. Available at: http://doi.wiley.com/10.1002/stem.1842.

Hayes, M. & Zavazava, N., 2013. Strategies to Generate Induced Pluripotent Stem Cells. In N. Zavazava, ed. *Embryonic Stem Cell Immunobiology SE - 6*. Methods in Molecular Biology. Humana Press, pp. 77–92. Available at: http://dx.doi.org/10.1007/978-1-62703-478-4_6.

He, J.-Q. et al., 2003. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circulation research*, 93(1), pp.32–39.

Hirai, H. et al., 2013. Accelerated direct reprogramming of fibroblasts into cardiomyocyte-like cells with the MyoD transactivation domain. *Cardiovascular Research*, 100(1), pp.105–113.

Hochedlinger, K. & Jaenisch, R., 2002. Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature*, 415(6875), pp.1035–1038. Available at: http://dx.doi.org/10.1038/nature718.

Hockemeyer, D. et al., 2009. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nature biotechnology*, 27(9), pp.851–857.

Hockemeyer, D. et al., 2012. Genetic engineering of human ES and iPS cells using TALE nucleases. *Nat Biotechnol*, 29(8), pp.731–734.

Hofmann, M. et al., 2005. Monitoring of bone marrow cell homing into the infarcted human myocardium. *Circulation*, 111(17), pp.2198–2202.

Holditch, S.J., Terzic, A. & Ikeda, Y., 2014. Concise Review: Pluripotent Stem Cell-Based Regenerative Applications for Failing β-Cell Function. *Stem cells translational medicine*, 3, pp.653–61. Available at: http://stemcellstm.alphamedpress.org/content/early/2014/03/18/sctm.2013-0184.abstract.

Hoogduijn, M.J. et al., 2011. Immunological aspects of allogeneic and autologous mesenchymal stem cell therapies. *Human gene therapy*, 22(12), pp.1587–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21732766 [Accessed January 9, 2015].

Hopwood, N.D., Pluck, A. & Gurdon, J.B., 1989. MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. *The EMBO journal*, 8(11), pp.3409–3417.

Horii, T. et al., 2013. Generation of an ICF Syndrome Model by Efficient Genome Editing of Human Induced Pluripotent Stem Cells Using the CRISPR System. *International Journal of Molecular Sciences*, 14(10), pp.19774–19781. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3821585/.

Hou, P. et al., 2013. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. *Science (New York, N.Y.)*, 341(6146), pp.651–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23868920.

Hsiao, E.C. et al., 2008. Marking embryonic stem cells with a 2A self-cleaving peptide: A NKX2-5 emerald GFP BAC reporter. *PLoS ONE*, 3(7), p.e2532. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2430532&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Hsiao, L.-C. et al., 2012. Review Article: Stem Cell-based Therapy for Ischemic Heart Disease. *Cell transplantation*, 22, pp.663–675. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23044395.

Huang, N.F. et al., 2006. Myotube assembly on nanofibrous and micropatterned polymers. *Nano Letters*, 6, pp.537–542.

Huangfu, D. et al., 2008. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nature biotechnology*, 26, pp.1269–1275.

Hudson, J. et al., 2011. Primitive Cardiac Cells from Human Embryonic Stem Cells. *Stem cells and development*, 00(00). Available at: http://www.ncbi.nlm.nih.gov/pubmed/21933026.

Hwang, Y. et al., 2014. WNT3A promotes myogenesis of human embryonic stem cells and enhances in vivo engraftment. *Scientific reports*, 4, p.5916. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25084050.

Ieda, M. et al., 2010. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*, 142(3), pp.375–386.

Irion, S. et al., 2007. Identification and targeting of the ROSA26 locus in human embryonic stem cells. *Nat Biotech*, 25(12), pp.1477–1482. Available at: http://dx.doi.org/10.1038/nbt1362.

Ivics, Z. & Izsvák, Z., 2006. Transposons for gene therapy! *Current gene therapy*, 6(5), pp.593–607.

Jao, L.-E., Wente, S.R. & Chen, W., 2013. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences of the United States of America*, 110(34), pp.13904–13909. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3752207/.

Jayawardena, T.M. et al., 2012. MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. *Circulation Research*, 110(11), pp.1465–1473.

Jeevanantham, V. et al., 2013. Clinical Trials of Cardiac Repair with Adult Bone Marrow- Derived Cells. *Methods in molecular biology (Clifton, N.J.)*, 1036, pp.179–205. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4270749/.

Jia, F. et al., 2010. A nonviral minicircle vector for deriving human iPS cells. *Nature methods*, 7(3), pp.197–199.

Jinek, M. et al., 2012. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, 337(6096), pp.816–821. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22745249 [Accessed July 9, 2014].

Jopling, C. et al., 2010. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature*, 464(7288), pp.606–609.

Joung, J.K., Voytas, D.F. & Cathomen, T., 2010. Reply to "Successful genome editing with modularly assembled zinc finger nucleases." *Nature methods*, 7(1), pp.91–92. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2987589/.

Jung, Y., Bauer, G. & Nolta, J.A., 2012. Concise review: Induced pluripotent stem cell-derived mesenchymal stem cells: Progress toward safe clinical products. *Stem Cells*, 30, pp.42–47.

Kaji, K. et al., 2009. Virus free induction of pluripotency and subsequent excision of reprogramming factors. *Nature*, 458(7239), pp.771–775. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2667910/.

Kammili, R.K. et al., 2010. Generation of Novel Reporter Stem Cells and Their Application for Molecular Imaging of Cardiac-Differentiated Stem Cells In Vivo. *Stem Cells and Development*, 19(9), pp.1437–1448. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3121874/.

Kamp, T.J., 2011. Recognizing heart cells in a crowd. *Nat Meth*, 8(12), pp.1013–1016. Available at: http://dx.doi.org/10.1038/nmeth.1780.

Kanno, S. et al., 2004. Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101(33), pp.12277–12281. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC514469/.

Kattman, S.J. et al., 2011. Stage-Specific Optimization of Activin/Nodal and BMP Signaling Promotes Cardiac Differentiation of Mouse and Human Pluripotent Stem Cell Lines. *Cell Stem Cell*, 8, pp.228–240. Available at: <Go to ISI>://000287633400015.

Keating, A. et al., 2013. Autologous blood cell transplantation versus HLA-identical sibling transplantation for acute myeloid leukemia in first complete remission: a registry study from the Center for International Blood and Marrow Transplantation Research. *Haematologica*, 98(2), pp.185–192. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3561424/.

Kehat, I. et al., 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest*, 108(3), pp.407–414.

Kern, S. et al., 2006a. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem cells (Dayton, Ohio)*, 24, pp.1294–301.

Kern, S. et al., 2006b. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem cells (Dayton, Ohio)*, 24, pp.1294–301. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16410387.

Kim, D. et al., 2009. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell stem cell*, 4, pp.472–476.

Kim, D. et al., 2003. In vivo functioning and transplantable mature pancreatic islet-like cell clusters differentiated from embryonic stem cell. *Pancreas*, 27(2), pp.e34–e41.

Kim, J.B. et al., 2009. Direct reprogramming of human neural stem cells by OCT4. *Nature*, 461, pp.649–643.

Kim, K. et al., 2011. Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. *Nat Biotech*, 29(12), pp.1117–1119. Available at: http://dx.doi.org/10.1038/nbt.2052.

Kita-Matsuo, H. et al., 2009. Lentiviral vectors and protocols for creation of stable hESC lines for fluorescent tracking and drug resistance selection of cardiomyocytes. *PLoS ONE*, 4(4), p.e5046. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2662416&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Kocaefe, Ç. et al., 2010. Reprogramming of Human Umbilical Cord Stromal Mesenchymal Stem Cells for Myogenic Differentiation and Muscle Repair. *Stem Cell Reviews and Reports*, 6(4), pp.512–522.

Kolossov, E. et al., 1998. Functional Characteristics of ES Cell–derived Cardiac Precursor Cells Identified by Tissue-specific Expression of the Green Fluorescent Protein. *The Journal of Cell Biology* , 143 (7), pp.2045–2056. Available at: http://jcb.rupress.org/content/143/7/2045.abstract.

Konermann, S. et al., 2013. Optical control of mammalian endogenous transcription and epigenetic states. *Nature*, 500(7463), pp.472–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3856241&tool=pmcentrez&rendertype=abstract.

Kost, T.A., Condreay, J.P. & Jarvis, D.L., 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nature biotechnology*, 23(5), pp.567–575.

Kotin, R.M. et al., 1990. Site-specific integration by adeno-associated virus. *Proceedings of the National Academy of Sciences of the United States of America*, 87(6), pp.2211–2215. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC53656/.

Kotterman, M.A. & Schaffer, D. V, 2014. Engineering adeno-associated viruses for clinical gene therapy. *Nat Rev Genet*, 15(7), pp.445–451. Available at: http://dx.doi.org/10.1038/nrg3742.

Krishnan, J. et al., 2008. Essential role of developmentally activated hypoxia-inducible factor la for cardiac morphogenesis and function. *Circulation Research*, 103(10), pp.1139–1146.

Kristjánsson, B. & Honsawek, S., 2014. Current Perspectives in Mesenchymal Stem Cell Therapies for Osteoarthritis. , 2014.

Kuhn, H.G. & Svendsen, C.N., 1999. Origins, functions, and potential of adult neural stem cells. *BioEssays*, 21(8), pp.625–630. Available at: http://dx.doi.org/10.1002/(SICI)1521-1878(199908)21:8<625::AID-BIES1>3.0.CO.

Kunisato, A. et al., 2010. Generation of induced pluripotent stem cells by efficient reprogramming of adult bone marrow cells. *Stem cells and development*, 19, pp.229–238.

Kurian, L., Sancho-Martinez, I., Nivet, E., Aguirre, A., Moon, K., Pendaries, C., Volle-Challier, C., Bono, F., Herbert, J.-M., Pulecio, J., Xia, Y., Li, M., Montserrat, N., Ruiz, S., Dubova, I., Rodriguez, C., Denli, A.M., Boscolo, F.S., Thiagarajan, R.D., Gage, F.H., Loring, J.F., Laurent, L.C. & Belmonte, J.C.I., 2013. Conversion of human fibroblasts into angioblast-like multipotent progenitor cells. *Nature methods*, 10(1), pp.77–83. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3531579/.

Kurian, L., Sancho-Martinez, I., Nivet, E., Aguirre, A., Moon, K., Pendaries, C., Volle-Challier, C., Bono, F., Herbert, J.-M., Pulecio, J., Xia, Y., Li, M., Montserrat, N., Ruiz, S., Dubova, I., Rodriguez, C., Denli, A.M., Boscolo, F.S., Thiagarajan, R.D.,

Gage, F.H., Loring, J.F., Laurent, L.C. & Izpisua Belmonte, J.C., 2013. Conversion of human fibroblasts to angioblast-like progenitor cells. *Nature methods*, 10(1), pp.77–83. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3531579&tool=pmcentrez&rendertype=abstract [Accessed January 4, 2015].

Kwon, C. et al., 2007a. Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proceedings of the National Academy of Sciences of the United States of America*, 104(26), pp.10894–10899.

Kwon, C. et al., 2007b. Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proceedings of the National Academy of Sciences of the United States of America*, 104(26), pp.10894–10899.

Laflamme, M.A. et al., 2007. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature biotechnology*, 25(9), pp.1015–1024.

Lassar, A.B. et al., 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell*, 58(5), pp.823–831.

Lassar, A.B., Paterson, B.M. & Weintraub, H., 1986. Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell*, 47(5), pp.649–656.

Laurent, L.C. et al., 2011. Dynamic Changes in the Copy Number of Pluripotency and Cell Proliferation Genes in Human ES and iPS Cells during Reprogramming and Time in Culture. *Cell stem cell*, 8(1), pp.106–118. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3043464/.

Lee, D.H. et al., 2004. Enhancement of re-closure capacity by the intra-amniotic injection of human embryonic stem cells in surgically induced spinal open neural tube defects in chick embryos. *Neuroscience Letters*, 364(2), pp.98–100.

Lemaigre, F. & Zaret, K.S., 2004. Liver development update: New embryo models, cell lineage control, and morphogenesis. *Current Opinion in Genetics and Development*, 14(5), pp.582–590.

Levenberg, S. et al., 2007. Endothelial potential of human embryonic stem cells. *Blood*, 110(3), pp.806–814.

Li, H.L. et al., 2015. Precise Correction of the Dystrophin Gene in Duchenne Muscular Dystrophy Patient Induced Pluripotent Stem Cells by TALEN and CRISPR-Cas9. *Stem Cell Reports*, 4(1), pp.143–154. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4297888/.

Li, T. et al., 2011. TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. *Nucleic Acids Research*, 39(1), pp.359–372. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3017587/.

Li, W. et al., 2013. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotech*, 31(8), pp.684–686. Available at: http://dx.doi.org/10.1038/nbt.2652.

Lian, X. et al., 2012a. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proceedings of the …*, 109(27), pp.E1848–57. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3390875&tool=pmcentrez&rendertype=abstract\nhttp://www.pnas.org/content/109/27/E1848.short [Accessed July 11, 2014].

Lian, X. et al., 2012b. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 109(27), pp.E1848–57. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3390875&tool=pmcentrez&rendertype=abstract.

Liao, H. & Zhou, G.-Q., 2009. Development and progress of engineering of skeletal muscle tissue. *Tissue engineering. Part B, Reviews*, 15, pp.319–331.

Liao, H.-K. et al., 2015. Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. *Nat Commun*, 6. Available at: http://dx.doi.org/10.1038/ncomms7413.

Liao, J. et al., 2015. Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. *Nat Genet*, 47(5), pp.469–478. Available at: http://dx.doi.org/10.1038/ng.3258.

Le Lièvre, C.S. & Le Douarin, N.M., 1975. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *Journal of embryology and experimental morphology*, 34(1), pp.125–154.

Lin, L. et al., 2007. Beta-catenin directly regulates Islet1 expression in cardiovascular progenitors and is required for multiple aspects of cardiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 104(22), pp.9313–9318.

Liu, G.H. et al., 2011. Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs. *Cell Stem Cell*, 8, pp.688–694.

Liu, H. et al., 2010. Generation of Endoderm derived Human iPS cells from Primary Hepatocytes. *Hepatology (Baltimore, Md.)*, 51(5), pp.1810–1819. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2925460/.

Liu, X.N. et al., 2008. Tissue extracts from infarcted myocardium of rats in promoting the differentiation of bone marrow stromal cells into cardiomyocyte-like cells. *Biomedical and Environmental Sciences*, 21(2), pp.110–117.

Liu, Y. et al., 2009. Generation of platform human embryonic stem cell lines that allow efficient targeting at a predetermined genomic location. *Stem cells and development*, 18(10), pp.1459–1472.

Lombardo, A. et al., 2007. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nature biotechnology*, 25(11), pp.1298–1306.

Lombardo, A. et al., 2011. Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nature Methods*, 8(10), pp.861–869.

Lopez-Sanchez, C. et al., 2002. Induction of cardiogenesis by Hensen's node and fibroblast growth factors. *Cell and Tissue Research*, 309(2), pp.237–249.

Lough, J.W. & Sugi, Y., 2000. Endoderm and heart development. *Developmental Dynamics*, 217(4), pp.327–342.

LR., K., 1992. The future of multihospital systems. *Topics in Health Care Financing*, 18(4), pp.32–45.

Lu, S.-J. et al., 2011. Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice. *Cell Research*, 21(3), pp.530–545. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3193430/.

Lu, Z. et al., 2015. Synergistic effect of nanomaterials and BMP-2 signalling in inducing osteogenic differentiation of adipose tissue-derived mesenchymal stem cells. *Nanomedicine: Nanotechnology, Biology and Medicine*, 11(1), pp.219–228. Available at: http://dx.doi.org/10.1016/j.nano.2014.09.008.

Luft, F., 2010. Sleeping Beauty jumps to new heights. *Journal of Molecular Medicine*, 88(7), pp.641–643. Available at: http://dx.doi.org/10.1007/s00109-010-0626-1.

Lyssiotis, C.A. et al., 2009. Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4. *Proceedings of the National Academy of Sciences* , 106 (22), pp.8912–8917. Available at: http://www.pnas.org/content/106/22/8912.abstract.

Ma, N. et al., 2013. Transcription Activator-like Effector Nuclease (TALEN)-mediated Gene Correction in Integration-free β-Thalassemia Induced Pluripotent Stem Cells. *The Journal of Biological Chemistry*, 288(48), pp.34671–34679. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3843079/.

Ma, Y. et al., 2003. High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. *Stem Cells*, 21(1), pp.111–117.

Madeira, C. et al., 2013. Nonviral gene delivery to neural stem cells with minicircles by microporation. *Biomacromolecules*, 14(5), pp.1379–1387.

Maeder, M.L. et al., 2013. Targeted DNA Demethylation and Endogenous Gene Activation Using Programmable TALE-TET1 Fusions. *Nature biotechnology*, 31(12), pp.1137–1142. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3858462/.

Makarova, K.S. et al., 2011. Evolution and classification of the CRISPR-Cas systems. *Nature reviews. Microbiology*, 9(6), pp.467–477.

Makino, S. et al., 1999. Cardiomyocytes can be generated from marrow stromal cells in vitro. *Journal of Clinical Investigation*, 103(5), pp.697–705. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC408125/.

Mali, P. et al., 2013. RNA-guided human genome engineering via Cas9. *Science (New York, N.Y.)*, 339(6121), pp.823–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3712628&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Mandel, E.M. et al., 2010. The BMP pathway acts to directly regulate Tbx20 in the developing heart. *Development (Cambridge, England)*, 137(11), pp.1919–1929.

Maria C. N. Marchetto, Gene W. Yeo, Osamu Kainohana, Martin Marsala, Fred H. Gage, A.R.M., 2009. Transcriptional Signature and Memory Retention of Human-Induced Pluripotent Stem Cells. *PLoS ONE*, 4(9), p.e7076.

Marraffini, L.A. & Sontheimer, E.J., 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science (New York, N.Y.)*, 322(5909), pp.1843–1845.

Matsuura, K. et al., 2011. Creation of mouse embryonic stem cell-derived cardiac cell sheets. *Biomaterials*, 32(30), pp.7355–7362.

Mauritz, C. et al., 2011. Induced pluripotent stem cell (iPSC)-derived Flk-1 progenitor cells engraft, differentiate, and improve heart function in a mouse model of acute myocardial infarction. *European Heart Journal*, 32(21), pp.2634–2641.

McCarty, D.M., Young, S.M. & Samulski, R.J., 2004. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annual review of genetics*, 38, pp.819–845.

McDevitt, T.C., Laflamme, M.A. & Murry, C.E., 2005. Proliferation of cardiomyocytes derived from human embryonic stem cells is mediated via the IGF/PI 3-kinase/Akt signaling pathway. *Journal of Molecular and Cellular Cardiology*, 39(6), pp.865–873.

Mendenhall, E.M. et al., 2013. Locus-specific editing of histone modifications at endogenous enhancers using programmable TALE-LSD1 fusions. *Nature biotechnology*, 31(12), pp.1133–1136. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3858395/.

Meng, X. et al., 2012. Efficient reprogramming of human cord blood CD34+ cells into induced pluripotent stem cells with OCT4 and SOX2 alone. *Molecular therapy : the journal of the American Society of Gene Therapy*, 20, pp.408–16. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3277237&tool=pmcentrez&rendertype=abstract.

Mercer, A.C. et al., 2012. Chimeric TALE recombinases with programmable DNA sequence specificity. *Nucleic Acids Research*, 40(21), pp.11163–11172. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3510496/.

Mertens, J.P. et al., 2014. Engineering muscle constructs for the creation of functional engineered musculoskeletal tissue. *Regenerative medicine*, 9, pp.89–100. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24351009.

Metzger, J.M., Lin, W.-I. & Samuelson, L.C., 1996. Vital Staining of Cardiac Myocytes During Embryonic Stem Cell Cardiogenesis In Vitro. *Circulation Research* , 78 (4), pp.547–552. Available at: http://circres.ahajournals.org/content/78/4/547.abstract.

Meyer, N. et al., 2000. A fluorescent reporter gene as a marker for ventricular specification in ES-derived cardiac cells. *FEBS Letters*, 478, pp.151–158.

Michalek, A.J. et al., 2015. Autologous adipose tissue-derived stromal vascular fraction cells application in patients with osteoarthritis. , pp.1–36.

Miki, K. et al., 2012. Bioengineered myocardium derived from induced pluripotent stem cells improves cardiac function and attenuates cardiac remodeling following chronic myocardial infarction in rats. *Stem cells translational medicine*, 1(5), pp.430–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3659710&tool=pmcentrez&rendertype=abstract.

Miller-Hance, W.C. et al., 1993. In vitro chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation. *Journal of Biological Chemistry*, 268(33), pp.25244–25252.

Min, J.-Y. et al., 2002. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *Journal of Applied Physiology*, 92(1), pp.288–296. Available at: http://jap.physiology.org/content/92/1/288.abstract.

Miyoshi, N. et al., 2015. Reprogramming of Mouse and Human Cells to Pluripotency Using Mature MicroRNAs. *Cell Stem Cell*, 8(6), pp.633–638. Available at: http://dx.doi.org/10.1016/j.stem.2011.05.001.

Moiani, A. et al., 2012. Lentiviral vector integration in the human genome induces alternative splicing and generates aberrant transcripts. *Journal of Clinical Investigation*, 122(5), pp.1653–1666.

Montserrat, N. et al., 2012. Generation of induced pluripotent stem cells from human renal proximal tubular cells with only two transcription factors, OCT4 and SOX2. *The Journal of biological chemistry*, 287, pp.24131–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3397840&tool=pmcentrez&rendertype=abstract.

Moore, J.C. et al., 2010. Efficient, high-throughput transfection of human embryonic stem cells. *Stem Cell Research & Therapy*, 1(3), p.23. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2941115/.

Morris, S.A. et al., 2015. Dissecting Engineered Cell Types and Enhancing Cell Fate Conversion via CellNet. *Cell*, 158(4), pp.889–902. Available at: http://dx.doi.org/10.1016/j.cell.2014.07.021.

Moscou, M.J. & Bogdanove, A.J., 2009. A simple cipher governs DNA recognition by TAL effectors. *Science (New York, N.Y.)*, 326(5959), p.1501.

Mummery, C. et al., 2003. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation*, 107(21), pp.2733–2740.

Mummery, C.L. et al., 2012. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: A methods overview. *Circulation Research*, 111(3), pp.344–358.

Mussolino, C. et al., 2011. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Research*, 39(21), pp.9283–9293.

Naeem, N. et al., 2013. DNA methylation inhibitors, 5-azacytidine and zebularine potentiate the transdifferentiation of rat bone marrow mesenchymal stem cells into cardiomyocytes. *Cardiovascular Therapeutics*, 31(4), pp.201–209. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22954287 [Accessed January 9, 2015].

Nakamura, M. & Okano, H., 2013. Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells. *Cell research*, 23, pp.70–80. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3541652&tool=pmcentrez&rendertype=abstract.

Nakano, T. et al., 2012. Self-Formation of Optic Cups and Storable Stratified Neural Retina from Human ESCs. *Cell Stem Cell*, 10(6), pp.771–785. Available at: http://dx.doi.org/10.1016/j.stem.2012.05.009.

Nam, Y.-J. et al., 2013. Reprogramming of human fibroblasts toward a cardiac fate. *Proceedings of the National Academy of Sciences of the United States of America*, 110(14), pp.5588–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3619357&tool=pmcentrez&rendertype=abstract.

Di Nardo, P., Minieri, M. & Ahluwalia, A., 2011. Engineering the Stem Cell Niche and the Differentiative Micro- and Macroenvironment: Technologies and Tools for Applying Biochemical, Physical and Structural Stimuli and Their Effects on Stem Cells. In G. M. Artmann, S. Minger, & J. Hescheler, eds. *Stem Cell Engineering SE - 2*. Springer Berlin Heidelberg, pp. 41–59. Available at: http://dx.doi.org/10.1007/978-3-642-11865-4_2.

Nashun, B., Hill, P.W.S. & Hajkova, P., 2015. Reprogramming of cell fate: epigenetic memory and the erasure of memories past. *The EMBO Journal*, 34(10), pp.1296–1308. Available at: http://emboj.embopress.org/content/34/10/1296.abstract.

Nelson, T.J. et al., 2009. Repair of acute myocardial infarction with iPS induced by human stemness factors. *Circulation*, 120(5), pp.408–416. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2768575/.

Ng, E.S. et al., 2008. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. *Nat. Protocols*, 3(5), pp.768–776. Available at: http://dx.doi.org/10.1038/nprot.2008.42.

Ng, E.S. et al., 2005. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. *Blood*, 106(5), pp.1601–1603.

Niederreither, K. et al., 1997. Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mechanisms of Development*, 62(1), pp.67–78.

Niibe, K. et al., 2011. Purified mesenchymal stem cells are an efficient source for iPS cell induction. *PLoS ONE*, 6(3).

Nishimura, F. et al., 2003. Potential Use of Embryonic Stem Cells for the Treatment of Mouse Parkinsonian Models: Improved Behavior by Transplantation of In Vitro Differentiated Dopaminergic Neurons from Embryonic Stem Cells. *STEM CELLS*, 21(2), pp.171–180. Available at: http://dx.doi.org/10.1634/stemcells.21-2-171.

Niu, Z. et al., 2007. Serum response factor micromanaging cardiogenesis. *Current Opinion in Cell Biology*, 19(6), pp.618–627.

Noguchi, H., 2010. Pancreatic stem/progenitor cells for the treatment of diabetes. *The review of diabetic studies : RDS*, 7(2), pp.105–111.

O'Brien, T.X., Lee, K.J. & Chien, K.R., 1993. Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proceedings of the National Academy of Sciences of the United States of America*, 90(11), pp.5157–5161. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC46674/.

Oberwallner, B. et al., 2014. Human cardiac extracellular matrix supports myocardial lineage commitment of pluripotent stem cells. *European journal of cardio-thoracic*

surgery : official journal of the European Association for Cardio-thoracic Surgery, p.ezu163–. Available at: http://ejcts.oxfordjournals.org/content/early/2014/04/25/ejcts.ezu163.long.

Okita, K. et al., 2011. A more efficient method to generate integration-free human iPS cells. *Nature methods*, 8(5), pp.409–412. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21460823 [Accessed July 9, 2014].

Okita, K. et al., 2013. An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. *Stem Cells*, 31(3), pp.458–466. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23193063 [Accessed January 8, 2015].

Okita, K. et al., 2008. Generation of mouse induced pluripotent stem cells without viral vectors. *Science (New York, N.Y.)*, 322(5903), pp.949–953.

Orbán, T.I. et al., 2009. Applying a “double-feature” promoter to identify cardiomyocytes differentiated from human embryonic stem cells following transposon-based gene delivery. *Stem Cells*, 27(5), pp.1077–1087.

Osafune, K. et al., 2008. Marked differences in differentiation propensity among human embryonic stem cell lines. *Nature biotechnology*, 26(3), pp.313–315.

Osakada, F. et al., 2009. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. *Journal of cell science*, 122(Pt 17), pp.3169–3179.

Osborn, M.J. et al., 2013. TALEN-based Gene Correction for Epidermolysis Bullosa. *Molecular Therapy*, 21(6), pp.1151–1159. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3677309/.

Paige, S.L. et al., 2010. Endogenous Wnt/beta-catenin signaling is required for cardiac differentiation in human embryonic stem cells. *PloS one*, 5(6), p.e11134.

Pan, Y. et al., 2015. Engraftment of Embryonic Stem Cells and Differentiated Progeny by Host Conditioning with Total Lymphoid Irradiation and Regulatory T Cells. *Cell Reports*, 10(11), pp.1793–1802. Available at: http://linkinghub.elsevier.com/retrieve/pii/S2211124715002089.

Pankratz, M.T. et al., 2007. Directed Neural Differentiation of Human Embryonic Stem Cells via an Obligated Primitive Anterior Stage. *STEM CELLS*, 25(6), pp.1511–1520. Available at: http://dx.doi.org/10.1634/stemcells.2006-0707.

Park, I.-H. et al., 2008. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*, 451, pp.141–146.

Passier, R. et al., 2005. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem cells (Dayton, Ohio)*, 23(6), pp.772–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15917473.

Patra, C. et al., 2012. Silk protein fibroin from Antheraea mylitta for cardiac tissue engineering. *Biomaterials*, 33(9), pp.2673–2680. Available at: http://www.sciencedirect.com/science/article/pii/S0142961211015067.

Pattanayak, V. et al., 2011. Revealing Off-Target Cleavage Specificities of Zinc Finger Nucleases by In Vitro Selection. *Nature Methods*, 8(9), pp.765–770. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3164905/.

Pearl, J.I. et al., 2012. Pluripotent Stem Cells: Immune to the Immune system? *Science translational medicine*, 4(164), pp.164ps25–164ps25. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3638038/.

Peerani, R. & Zandstra, P.W., 2010. Enabling stem cell therapies through synthetic stem cell-niche engineering. *Journal of Clinical Investigation*, 120(1), pp.60–70.

Peters, D.T., Cowan, C.A. & Musunuru, K., Genome editing in human pluripotent stem cells. , (617), pp.1–14.

Phillips, H.M. et al., 2005. Vangl2 acts via RhoA signaling to regulate polarized cell movements during development of the proximal outflow tract. *Circulation Research*, 96(3), pp.292–299.

Pick, M., 2013. Generation of Megakaryocytes and Platelets from Human Pluripotent Stem Cells. In Methods in Molecular Biology. Humana Press, pp. 1–8. Available at: http://dx.doi.org/10.1007/7651_2013_58.

Polo, J.M. et al., 2010. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotech*, 28(8), pp.848–855. Available at: http://dx.doi.org/10.1038/nbt.1667.

Porteus, M.H. & Carroll, D., 2005. Gene targeting using zinc finger nucleases. *Nature biotechnology*, 23(8), pp.967–973.

Poss, K.D., Keating, M.T. & Nechiporuk, A., 2003. Tales of regeneration in zebrafish. *Developmental Dynamics*, 226(2), pp.202–210. Available at: http://dx.doi.org/10.1002/dvdy.10220.

Poulsom, R. et al., 2002. Adult stem cell plasticity. *The Journal of Pathology*, 197(4), pp.441–456. Available at: http://dx.doi.org/10.1002/path.1176.

Price, J., Turner, D. & Cepko, C., 1987. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*, 84(1), pp.156–160. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC304161/.

Qian, L. et al., 2012. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature*, 485(7400), pp.593–598.

Qian, Q. et al., 2012. 5-Azacytidine Induces Cardiac Differentiation of Human Umbilical Cord-Derived Mesenchymal Stem Cells by Activating Extracellular

Regulated Kinase. *Stem Cells and Development*, 21(1), pp.67–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3245671&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Ramsden, C.M. et al., 2013. Stem cells in retinal regeneration: past, present and future. *Development (Cambridge, England)*, 140, pp.2576–85. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3666384&tool=pmcentrez&rendertype=abstract.

Rangappa, S., Makkar, R. & Forrester, J., 2010. Current Status of Myocardial Regeneration: New Cell Sources and New Strategies. *Journal of cardiovascular pharmacology and therapeutics*, 15(4), pp.338–343.

Rebelatto, C.K. et al., 2009. Expression of cardiac function genes in adult stem cells is increased by treatment with nitric oxide agents. *Biochemical and Biophysical Research Communications*, 378(3), pp.456–461.

Reddy, P. et al., 2015. Selective Elimination of Mitochondrial Mutations in the Germline by Genome Editing. *Cell*, 161(3), pp.459–469. Available at: http://dx.doi.org/10.1016/j.cell.2015.03.051.

Ren, C. et al., 2006. Establishment and Applications of Epstein-Barr Virus-Based Episomal Vectors in Human Embryonic Stem Cells. *Stem Cells*, 24(5), pp.1338–1347. Available at: http://dx.doi.org/10.1634/stemcells.2005-0338.

Ren, Y. et al., 2011. Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. *Journal of Molecular and Cellular Cardiology*, 51(3), pp.280–287.

Ritner, C. et al., 2011. An engineered cardiac reporter cell line identifies human embryonic stem cell-derived myocardial precursors carissa ritner. *PLoS ONE*, 6(1), p.e16004. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3014940&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Roncalli, J. et al., 2011. 058 The ongoing MESAMI translational research program. *Archives of Cardiovascular Diseases Supplements*, 3(1), p.19. Available at: http://www.sciencedirect.com/science/article/pii/S187864801170060X.

Van Rooij, E., Marshall, W.S. & Olson, E.N., 2008. Toward microRNA-based therapeutics for heart disease: The sense in antisense. *Circulation Research*, 103(9), pp.919–928.

Rosellini, E. et al., 2009. Preparation and characterization of alginate/gelatin blend films for cardiac tissue engineering. *Journal of Biomedical Materials Research - Part A*, 91(2), pp.447–453.

Saga, Y., Kitajima, S. & Miyagawa-Tomita, S., 2000. Mesp1 expression is the earliest sign of cardiovascular development. *Trends in Cardiovascular Medicine*, 10(8), pp.345–352.

Samulski, R.J. et al., 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *The EMBO Journal*, 10(12), pp.3941–3950. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC453134/.

Sánchez, P.L. et al., 2015. Acellular human heart matrix: A critical step toward whole heart grafts. *Biomaterials*, 61(0), pp.279–289. Available at: http://www.sciencedirect.com/science/article/pii/S0142961215004433.

Sancho-Martinez, I., Baek, S.H. & Izpisua Belmonte, J.C., 2012. Lineage conversion methodologies meet the reprogramming toolbox. *Nature Cell Biology*, 14(9), pp.892–899. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22945254 [Accessed January 8, 2015].

Sander, J.D. & Joung, J.K., 2014. CRISPR-Cas systems for genome editing, regulation and targeting. *Nature Biotechnology*, 32(4), pp.347–355.

Sanjana, N.E. et al., 2012. A transcription activator-like effector toolbox for genome engineering. *Nature protocols*, 7(1), pp.171–92. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3684555&tool=pmcentrez&rendertype=abstract [Accessed July 11, 2014].

Sashital, D.G., Wiedenheft, B. & Doudna, J.A., 2012. Mechanism of Foreign DNA Selection in a Bacterial Adaptive Immune System. *Molecular Cell*, 46(5), pp.606–615.

Sawitzke, J. a. et al., 2007. Recombineering: In Vivo Genetic Engineering in E. coli, S. enterica, and Beyond. *Methods in Enzymology*, 421(06), pp.171–199. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17352923 [Accessed January 9, 2015].

Scarritt, M.E., Pashos, N.C. & Bunnell, B.A., 2015. A Review of Cellularization Strategies for Tissue Engineering of Whole Organs. *Frontiers in Bioengineering and Biotechnology*, 3, p.43. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378188/.

Schmitz, M. et al., 2010. Dendritic cell-based immunotherapy for prostate cancer. *Clinical and Developmental Immunology*, 2010.

Schnepp, B.C. et al., 2005. Characterization of Adeno-Associated Virus Genomes Isolated from Human Tissues. *Journal of Virology*, 79(23), pp.14793–14803. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1287572/.

Schoenebeck, J.J., Keegan, B.R. & Yelon, D., 2007. Vessel and Blood Specification Override Cardiac Potential in Anterior Mesoderm. *Developmental Cell*, 13(2), pp.254–267.

Scholze, H. & Boch, J., 2011. TAL effectors are remote controls for gene activation. *Current Opinion in Microbiology*, 14(1), pp.47–53.

Schulz, L.C. et al., 2008. Human Embryonic Stem Cells as Models for Trophoblast Differentiation. *Placenta*, 29(SUPPL.), pp.10–16.

Schwartz, S.D., Hubschman, J.-P., et al., 2015. Embryonic stem cell trials for macular degeneration: a preliminary report. *The Lancet*, 379(9817), pp.713–720. Available at: http://dx.doi.org/10.1016/S0140-6736(12)60028-2.

Schwartz, S.D., Regillo, C.D., et al., 2015. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *The Lancet*, 385(9967), pp.509–516. Available at: http://dx.doi.org/10.1016/S0140-6736(14)61376-3.

Sebastiano, V. et al., 2011. In Situ Genetic Correction of the Sickle Cell Anemia Mutation in Human Induced Pluripotent Stem Cells Using Engineered Zinc Finger Nucleases. *Stem Cells (Dayton, Ohio)*, 29(11), pp.1717–1726. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3285772/.

Seo, S. & Na, K., 2011. Mesenchymal stem cell-based tissue engineering for chondrogenesis. *Journal of biomedicine & biotechnology*, 2011, p.806891. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3191858&tool=pmcentrez&rendertype=abstract.

Shi, Y. et al., 2000. BMP signaling is required for heart formation in vertebrates. *Developmental biology*, 224(2), pp.226–237.

Shimoji, K. et al., 2015. G-CSF Promotes the Proliferation of Developing Cardiomyocytes In Vivo and in Derivation from ESCs and iPSCs. *Cell Stem Cell*, 6(3), pp.227–237. Available at: http://dx.doi.org/10.1016/j.stem.2010.01.002.

Si, Y.L. et al., 2011. MSCs: Biological characteristics, clinical applications and their outstanding concerns. *Ageing Research Reviews*, 10(1), pp.93–103. Available at: http://dx.doi.org/10.1016/j.arr.2010.08.005.

Si-Tayeb, K. et al., 2010. Highly efficient generation of human hepatocyte–like cells from induced pluripotent stem cells. *Hepatology*, 51(1), pp.297–305. Available at: http://onlinelibrary.wiley.com/doi/10.1002/hep.23354/full.

SJ, K., 1977. Studies of the developmental potential of 4- and 8-cell stage mouse blastomeres. *J Exp Zool.*, 200(3), pp.365–76.

Smith, A.W. et al., 2013. Direct reprogramming of mouse fibroblasts to cardiomyocyte-like cells using Yamanaka factors on engineered poly(ethylene glycol) (PEG) hydrogels. *Biomaterials*, 34, pp.6559–6571.

Smith, J. et al., 2006. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. *Nucleic Acids Research*, 34(22), pp.e149–e149. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1702487/.

Smithies O, Gregg RG, Boggs SS, Koralewski MA, K.R., 1985. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature*, 317, pp.230–234.

Soldner, F. et al., 2011. Generation of isogenic pluripotent stem cells differing exclusively at two early onset parkinson point mutations. *Cell*, 146(2), pp.318–331.

Song, K. et al., 2012. Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature*, 485(7400), pp.599–604.

De Souza, N., 2011. Primer: genome editing with engineered nucleases. *Nature Methods*, 9(1), pp.27–27.

Srivastava, D., 2006a. Making or Breaking the Heart: From Lineage Determination to Morphogenesis. *Cell*, 126(6), pp.1037–1048.

Srivastava, D., 2006b. Making or Breaking the Heart: From Lineage Determination to Morphogenesis. *Cell*, 126(6), pp.1037–1048. Available at: http://www.sciencedirect.com/science/article/pii/S0092867406011500.

Stadtfeld, M., Brennand, K. & Hochedlinger, K., 2008. Reprogramming of Pancreatic β Cells into Induced Pluripotent Stem Cells. *Current Biology*, 18(12), pp.890–894. Available at: http://dx.doi.org/10.1016/j.cub.2008.05.010.

Sun, N., Panetta, N.J., et al., 2009. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 106, pp.15720–15725.

Sun, N., Lee, A. & Wu, J.C., 2009. Long term non-invasive imaging of embryonic stem cells using reporter genes. *Nature protocols*, 4, pp.1192–1201.

Sun, N. & Zhao, H., 2014. Seamless correction of the sickle cell disease mutation of the HBB gene in human induced pluripotent stem cells using TALENs. *Biotechnology and Bioengineering*, 111(5), pp.1048–1053. Available at: http://dx.doi.org/10.1002/bit.25018.

Swijnenburg, R.-J. et al., 2008. In Vivo Imaging of Embryonic Stem Cells Reveals Patterns of Survival and Immune Rejection Following Transplantation. *Stem Cells and Development*, 17(6), pp.1023–1029. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2657199/.

Szabo, E. et al., 2010. Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature*, 468(7323), pp.521–526. Available at: http://dx.doi.org/10.1038/nature09591.

Tabar, V. et al., 2005. Migration and differentiation of neural precursors derived from human embryonic stem cells in the rat brain. *Nature biotechnology*, 23(5), pp.601–606.

Takahashi, K. et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131, pp.861–872. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18035408.

Takahashi, K. & Yamanaka, S., 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*, 126, pp.663–676.

Takayama, N. et al., 2010. Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. *The Journal of experimental medicine*, 207(13), pp.2817–2830.

Takei, S. et al., 2009. Bone morphogenetic protein-4 promotes induction of cardiomyocytes from human embryonic stem cells in serum-based embryoid body development. *American journal of physiology. Heart and circulatory physiology*, 296(6), pp.H1793–H1803.

Takeuchi, J.K. & Bruneau, B.G., 2009. Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature*, 459(7247), pp.708–711.

Tamayol, A. et al., 2013. Fiber-based tissue engineering: Progress, challenges, and opportunities. *Biotechnology Advances*, 31, pp.669–687.

Tapscott, S.J. et al., 1988. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science (New York, N.Y.)*, 242(4877), pp.405–411.

Tatarkiewicz, K. et al., 2001. Porcine neonatal pancreatic cell clusters in tissue culture: benefits of serum and immobilization in alginate hydrogel. *Transplantation*, 71(11), pp.1518–1526.

Temple, S. & Alvarez-Buylla, A., 1999. Stem cells in the adult mammalian central nervous system. *Current opinion in neurobiology*, 9(1), pp.135–141.

Thomson, J.A., 1998. Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science*, 282, pp.1145–1147.

Thyagarajan, B. et al., 2009. A single EBV-based vector for stable episomal maintenance and expression of GFP in human embryonic stem cells. *Regenerative medicine*, 4(2), pp.239–250.

Thyagarajan, B. et al., 2008. Creation of engineered human embryonic stem cell lines using phiC31 integrase. *Stem cells (Dayton, Ohio)*, 26(1), pp.119–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17962703.

Till, J. E., & McCulloch, E.A., 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Res*, 14, p.213—222.

Till, J. E., McCulloch, E. A. & Siminovitch, L., 1964. A stochastic model of stem cell proliferation based on the growth of spleen colony-forming cells. *Proc. Natl Acad. Sci.USA*, 51, p.29—36.

Touboul, T., Vallier, L. & Weber, A., 2010. Robust differentiation of fetal hepatocytes from human embryonic stem cells and iPS. *Medecine sciences : M/S*, 26(12), pp.1061–1066.

Tran, T.H. et al., 2009. Wnt3a-induced mesoderm formation and cardiomyogenesis in human embryonic stem cells. *Stem Cells*, 27(8), pp.1869–1878.

Twal, W., Roze, L. & Zile, M.H., 1995. Anti-retinoic acid monoclonal antibody localizes all-trans-retinoic acid in target cells and blocks normal development in early quail embryo. *Developmental biology*, 168(2), pp.225–234.

Ueno, S. et al., 2007. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 104(23), pp.9685–9690.

Von Unge, M., Dirckx, J.J.J. & Olivius, N.P., 2003. Embryonic stem cells enhance the healing of tympanic membrane perforations. *International journal of pediatric otorhinolaryngology*, 67(3), pp.215–219.

Uosaki, H. et al., 2011. Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. *PLoS ONE*, 6(8).

Upadhyay, S.K. et al., 2013. RNA-Guided Genome Editing for Target Gene Mutations in Wheat. *G3: Genes|Genomes|Genetics*, 3(12), pp.2233–2238. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3852385/.

Urbach, A., Schuldiner, M. & Benvenisty, N., 2004. Modeling for Lesch-Nyhan Disease by Gene Targeting in Human Embryonic Stem Cells. *STEM CELLS*, 22(4), pp.635–641. Available at: http://dx.doi.org/10.1634/stemcells.22-4-635.

Vandsburger, M.H. et al., 2013. MRI Reporter Genes: Application to Imaging of Cell Survival, Proliferation, Migration, and Differentiation. *NMR in biomedicine*, 26(7), pp.872–884. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3713407/.

Wachowska, M. et al., 2014. 5-Aza-2′-deoxycytidine potentiates antitumour immune response induced by photodynamic therapy. *European Journal of Cancer*, 50(7), pp.1370–1381. Available at: http://www.sciencedirect.com/science/article/pii/S0959804914000902.

Wada, R. et al., 2013. Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. *Proceedings of the National Academy of Sciences of the United States of America*, 110(31), pp.12667–72. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3732928&tool=pmcentrez&rendertype=abstract [Accessed November 25, 2014].

Waddington, C.H., 1957. *The Strategy of the Genes* G. A. & Unwin, ed., London.

Wagner, J.E. et al., 2007. Unrelated donor bone marrow transplantation for the treatment of Fanconi anemia. *Blood*, 109(5), pp.2256–2262. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1801062/.

Walters, B.D. & Stegemann, J.P., 2014. Strategies for directing the structure and function of three-dimensional collagen biomaterials across length scales. *Acta Biomaterialia*, 10, pp.1488–1501.

Walters, M.J., Wayman, G.A. & Christian, J.L., 2001. Bone morphogenetic protein function is required for terminal differentiation of the heart but not for early expression of cardiac marker genes. *Mechanisms of Development*, 100(2), pp.263–273.

Wang, H. et al., 2010. Improved myocardial performance in infarcted rat heart by co-injection of basic fibroblast growth factor with temperature-responsive Chitosan hydrogel. *Journal of Heart and Lung Transplantation*, 29(8), pp.881–887.

Wang, H. et al., 2013. One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering. *Cell*, 153(4), pp.910–918. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969854/.

Wang, T. et al., 2006. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. *International Journal of Cardiology*, 109(1), pp.74–81.

Wang, Y. et al., 2013. Induced Pluripotent Stem Cells from Human Hair Follicle Mesenchymal Stem Cells. *Stem Cell Reviews*, 9(4), pp.451–460. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3742959/.

Wang, Y. et al., 2011. Reprogramming of mouse and human somatic cells by high-performance engineered factors. *EMBO reports*, 12, pp.373–378.

Wang, Z.Z. et al., 2007. Endothelial cells derived from human embryonic stem cells form durable blood vessels in vivo. *Nat Biotech*, 25(3), pp.317–318. Available at: http://dx.doi.org/10.1038/nbt1287.

Warren, L. et al., 2010. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell*, 7, pp.618–630.

Weintraub, H. et al., 1990. MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. *Proceedings of the National Academy of Sciences of the United States of America*, 87(15), pp.5623–5627.

Weismann, A., 1893. *The Germ-plasm: a theory of heredity*, New York: Scribner. Available at: http://dx.doi.org/10.5962/bhl.title.25196.

Wiedenheft, B., Sternberg, S.H. & Doudna, J.A., 2012. RNA-guided genetic silencing systems in bacteria and archaea. *Nature*, 482(7385), pp.331–338.

Wilber, A. et al., 2007. Messenger RNA as a Source of Transposase for Sleeping Beauty Transposon-mediated Correction of Hereditary Tyrosinemia Type I. *Mol Ther*, 15(7), pp.1280–1287. Available at: http://dx.doi.org/10.1038/sj.mt.6300160.

Willems, E. et al., 2011. Small-molecule inhibitors of the Wnt pathway potently promote cardiomyocytes from human embryonic stem cell-derived mesoderm. *Circulation Research*, 109(4), pp.360–364.

Wilmut, I. et al., 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385(6619), pp.810–813. Available at: http://dx.doi.org/10.1038/385810a0.

Wilson, M.H., Coates, C.J. & George, A.L., 2007. PiggyBac transposon-mediated gene transfer in human cells. *Molecular therapy : the journal of the American Society of Gene Therapy*, 15(1), pp.139–145.

Woltjen, K. et al., 2009. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*, 458(7239), pp.766–770. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758996/.

Xiao, A. et al., 2013. Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Research*, 41(14), pp.e141–e141. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3737551/.

Xing, Y. et al., 2012. The combination of angiotensin II and 5-azacytidine promotes cardiomyocyte differentiation of rat bone marrow mesenchymal stem cells. *Molecular and Cellular Biochemistry*, 360(1-2), pp.279–287.

Xu, C., 2002. Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells. *Circulation Research*, 91(6), pp.501–508.

Xu, N. et al., 2015. MicroRNA-145 Regulates OCT4, SOX2, and KLF4 and Represses Pluripotency in Human Embryonic Stem Cells. *Cell*, 137(4), pp.647–658. Available at: http://dx.doi.org/10.1016/j.cell.2009.02.038.

Xu, R.-H. et al., 2002. *BMP4 initiates human embryonic stem cell differentiation to trophoblast.*,

Xu, X.Q. et al., 2008. Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. *Differentiation; research in biological diversity*, 76(9), pp.958–970.

Xue, Y. et al., 2012. Deleterious- and disease-allele prevalence in healthy individuals: Insights from current predictions, mutation databases, and population-scale resequencing. *American Journal of Human Genetics*, 91(6), pp.1022–1032.

Xue, Y. et al., 2015. Genome-wide Analysis of PTB-RNA Interactions Reveals a Strategy Used by the General Splicing Repressor to Modulate Exon Inclusion or

Skipping. *Molecular Cell*, 36(6), pp.996–1006. Available at: http://dx.doi.org/10.1016/j.molcel.2009.12.003.

Yang, L. et al., 2008. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature*, 453(7194), pp.524–528.

Yang, L. et al., 2006. Isl1Cre reveals a common Bmp pathway in heart and limb development. *Development (Cambridge, England)*, 133(8), pp.1575–1585.

Yanik, M. et al., 2013. TALE-PvuII Fusion Proteins – Novel Tools for Gene Targeting S. Xu, ed. *PLoS ONE*, 8(12), p.e82539. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3857828/.

Yates, J. et al., 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. *Proceedings of the National Academy of Sciences of the United States of America*, 81(12), pp.3806–3810.

Yildirimman, R. et al., 2011. Human embryonic stem cell derived hepatocyte-like cells as a tool for In Vitro hazard assessment of chemical carcinogenicity. *Toxicological Sciences*, 124(2), pp.278–290.

Ying, Q.-L. et al., 2015. BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3. *Cell*, 115(3), pp.281–292. Available at: http://dx.doi.org/10.1016/S0092-8674(03)00847-X.

Yoshida, N. et al., 2014. First-line treatment for severe aplastic anemia in children: bone marrow transplantation from a matched family donor versus immunosuppressive therapy. *Haematologica*, 99(12), pp.1784–1791. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4258757/.

Yoshiki Sawa, Shigeru Miyagawa, Taichi Sakaguchi, Tomoyuki Fujita, Akifumi Matsuyama, Atsuhiro Saito, Tatsuya Shimizu, T.O., 2012. Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. *surgery today*, 42(2), pp.181–184.

Yusa, K. et al., 2011. Targeted gene correction of α(1)-antitrypsin deficiency in induced pluripotent stem cells. *Nature*, 478(7369), pp.391–394. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3198846/.

Zaffran, S. & Frasch, M., 2002. Early signals in cardiac development. *Circulation Research*, 91(6), pp.457–469.

Zhang, F. et al., 2011a. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nature biotechnology*, 29(2), pp.149–153.

Zhang, F. et al., 2011b. LETTErs Efficient construction of sequence-specific for mTAL effectorsodulating mammalian transcription. *Online*, 29(2), pp.149–154.

Zhang, S.C. et al., 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nature biotechnology*, 19(12), pp.1129–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11731781.

Zhang, Y.M. et al., 2002. Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. *Circulation*, 106(10), pp.1294–1299.

Zhang, Z. et al., 2011. Efficient generation of fully reprogrammed human iPS cells via polycistronic retroviral vector and a new cocktail of chemical compounds. *PLoS ONE*, 6(10), pp.1–9.

Zhao, Y., Samal, E. & Srivastava, D., 2005. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature*, 436(7048), pp.214–220.

Zhou, B. et al., 2011. Administering human adipose-derived mesenchymal stem cells to prevent and treat experimental arthritis. *Clinical Immunology*, 141(3), pp.328–337. Available at: http://www.sciencedirect.com/science/article/pii/S1521661611002488.

Zhou, H. et al., 2009. Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins. *Cell Stem Cell*, 4, pp.381–384.

Zhou, W. et al., 2007. Modulation of morphogenesis by noncanonical Wnt signaling requires ATF/CREB family-mediated transcriptional activation of TGFbeta2. *Nature genetics*, 39(10), pp.1225–1234.

Zhou, W. & Freed, C.R., 2009. Adenoviral Gene Delivery Can Reprogram Human Fibroblasts to Induced Pluripotent Stem Cells. *STEM CELLS*, 27(11), pp.2667–2674. Available at: http://dx.doi.org/10.1002/stem.201.

Zou, J., Sweeney, C.L., et al., 2011. Oxidase-deficient neutrophils from X-linked chronic granulomatous disease iPS cells: Functional correction by zinc finger nuclease-mediated safe harbor targeting. *Blood*, 117(21), pp.5561–5572.

Zou, J., Mali, P., et al., 2011. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. *Blood*, 118(17), pp.4599–4608.

Zwaka, T. & Thomson, J., 2003. Homologous recombination in human embryonic stem cells. *Nature biotechnology*, 21(3), pp.319–321. Available at: http://dx.doi.org/10.1038/nbt788 [Accessed December 11, 2014].

Zwaka, T.P. & Thomson, J. a, 2003. Homologous recombination in human embryonic stem cells. *Nature biotechnology*, 21(March), pp.319–321.

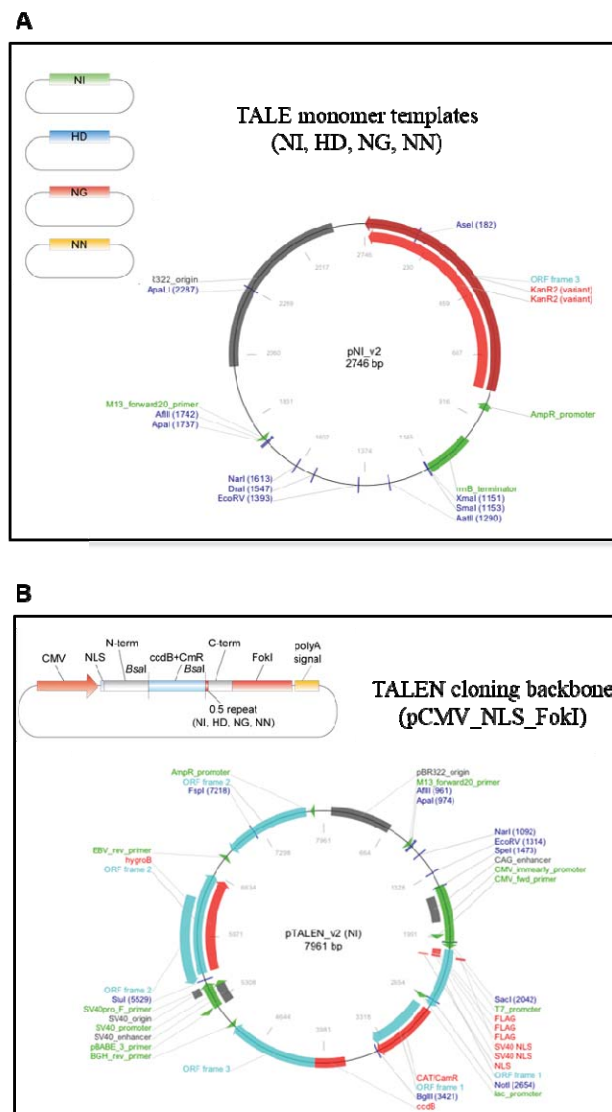
Zwi, L. et al., 2009. Cardiomyocyte differentiation of human induced pluripotent stem cells. *Circulation*, 120(15), pp.1513–1523.

VIII. APPENDIX

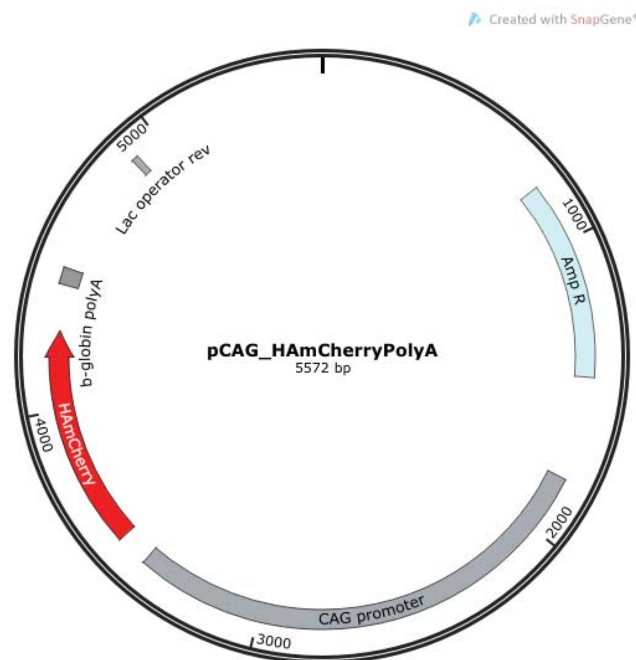
VIII. APPENDIX I

SUPLEMENTARY FIGURES

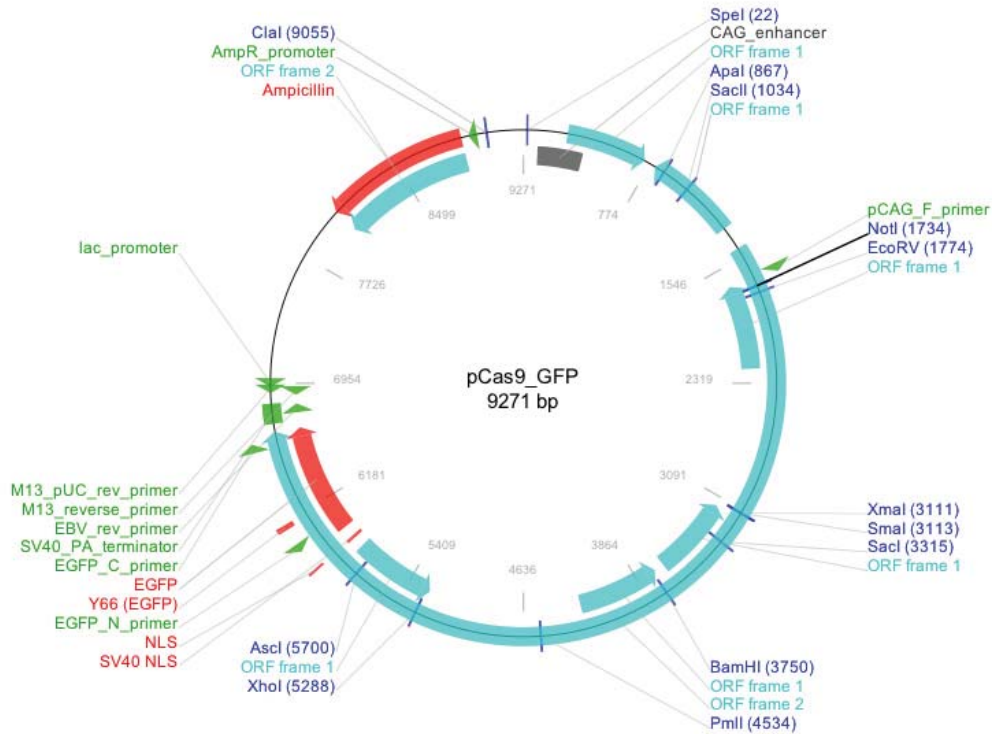
Supplementary Figure 1: Backbones and templates for building TALENs. A) NI, HD,NG, NN or TALE monomer templates supplied in TALE toolbox kit (Addgene #100000019) for building TALENs. Used for monomer library construction. **B) pCMV_NLS (NI,HD,NG, NN)_FokI** or TALEN cloning backbone supplied in TALE toolbox kit (Addgene #100000019) for building TALENs by adding library monomers. Contains Nuclear Localization Signal (NLS), CMV constitutive promoter and FokI nuclease.



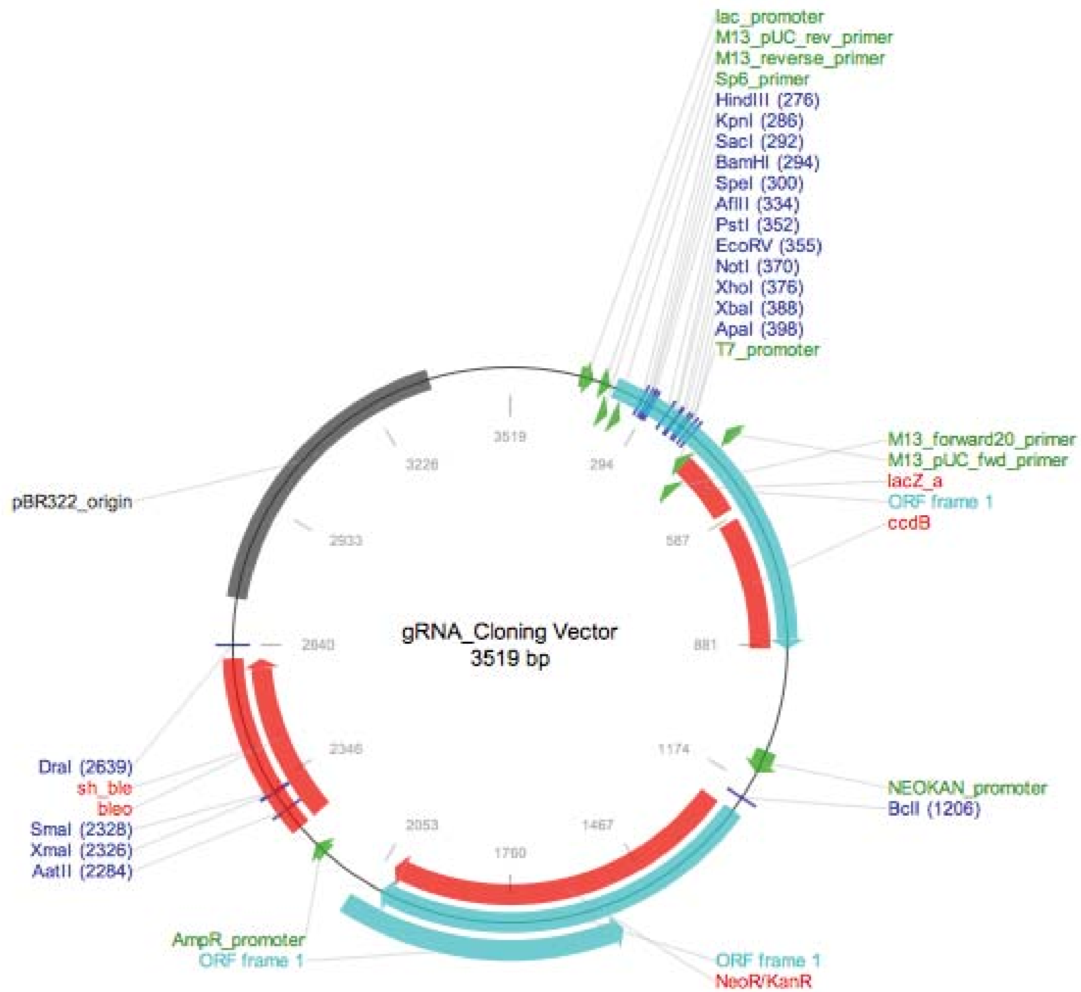
Supplementary Figure 2: pCAG_HAmCherryPolyA. Plasmid from laboratory of Polleux, F. at the Scripps Institute (La Jolla, CA). Plasmid with CAG constitutive promoter driving expression of mCherry fluorescent protein with a β-globin polyA tale. Contains HA protein tag C-terminal on mCherry insert. Encodes for Ampicillin resistance in bacteria.



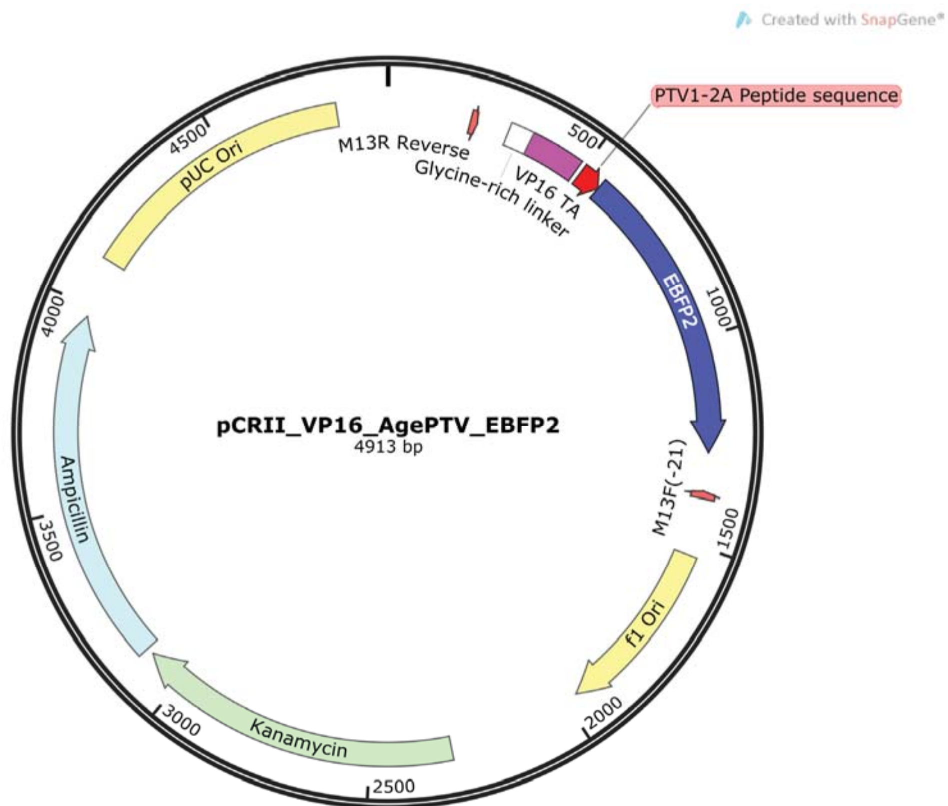
Supplementary Figure 3: pCAS9_GFP. Plasmid optimized for expression in human pluripotent stem cells acquired from Addgene (#44719). Contains CAG constitutive promoter driving the expression of codon-optimized CAS9 nuclease and GFP transgene. Contains nuclear localization signal (NLS) and ampicillin resistance for bacteria.



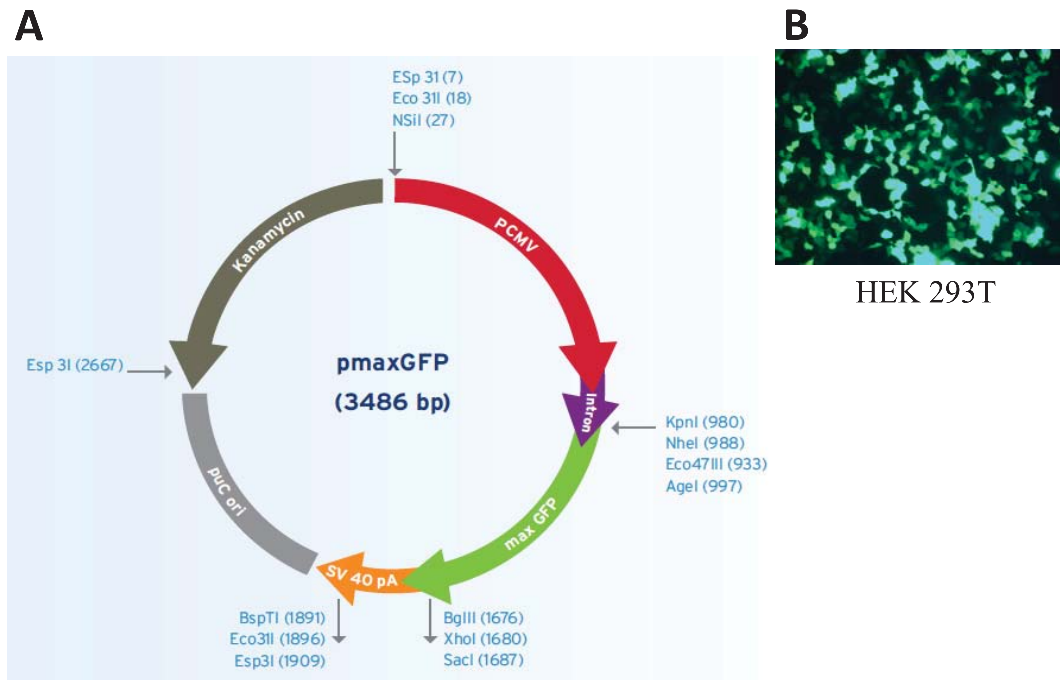
Supplementary Figure 4: pCR_Blunt II-TOPO. Empty mammalian expression vector from Addgene (#41824) for gRNA cloning for CRISPR/CAS9 system. Contains *AflII* site for Gibson Assembly of gRNA sequence. For bacteria selection contains LacZ reporter and kanamycin resistance.



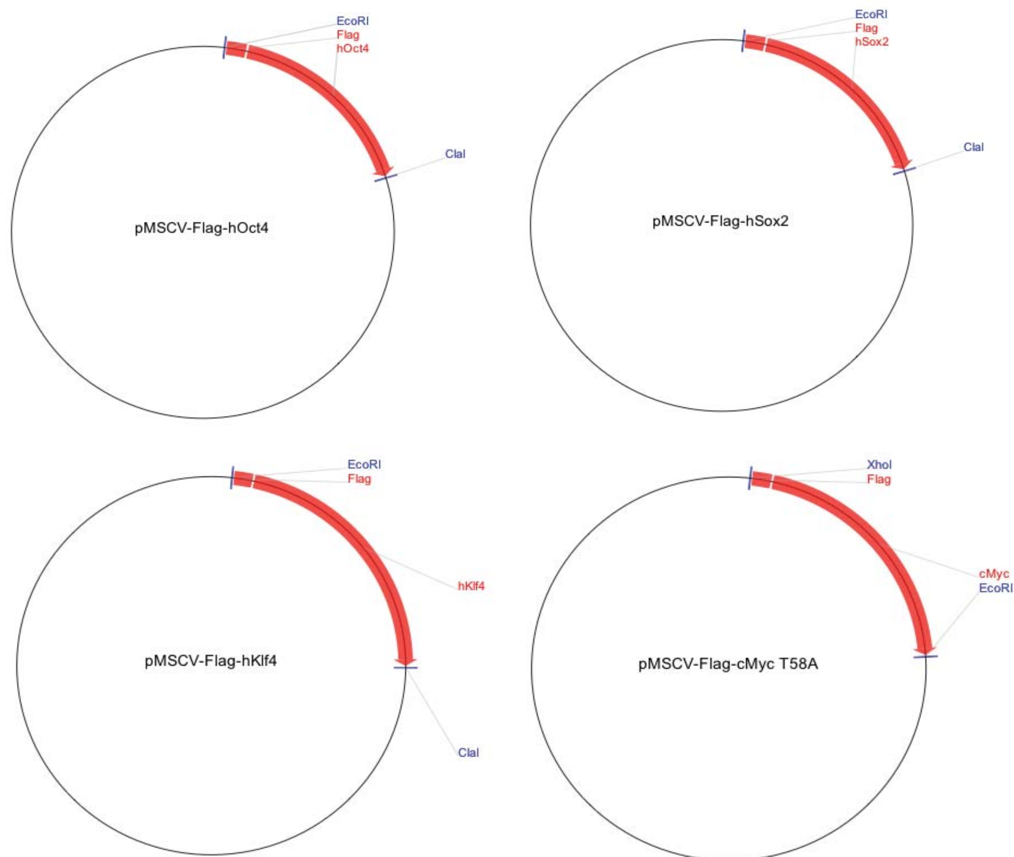
Supplementary Figure 5: pCRII_VP16_AgePTV_EBFP2. Plasmid derived in the Laboratory. Mammalian expression vector pCRII. Contains VP16 transactivator domain followed by an *AgeI* restriction site generated by PCR to clone inside other transgenes. Then, a PTV1-2A fusion protein sequence (C terminal on insert) followed by a EBFP2 (blue) reporter gene. This plasmid encodes for bacterial resistance to Kanamycin and Ampicillin.



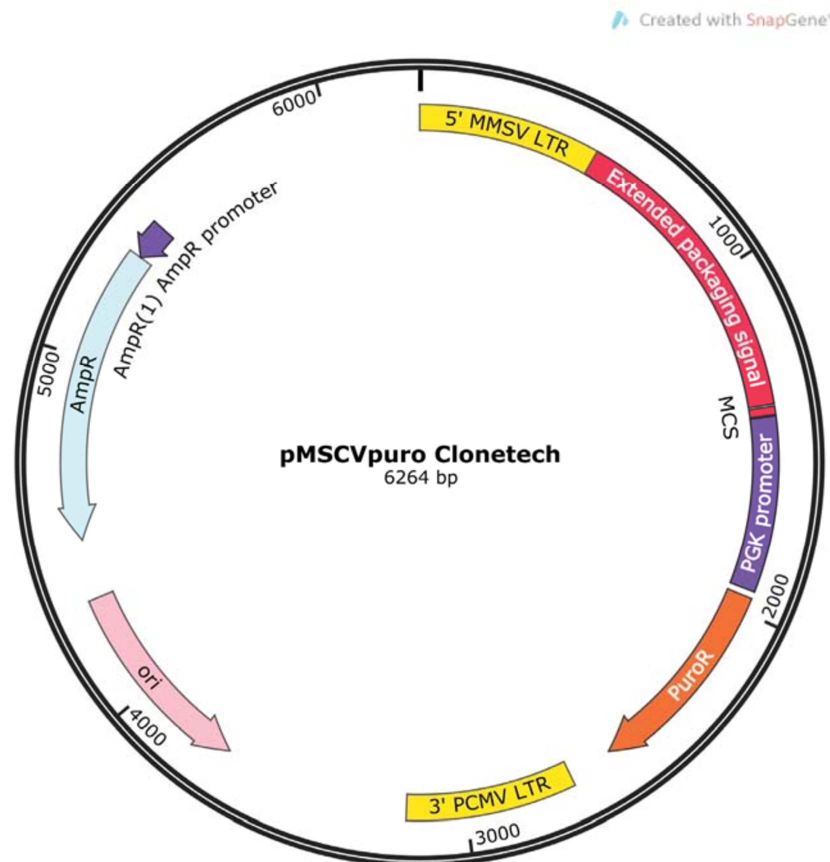
Supplementary Figure 6: pMax_ppGFP. A) pMax_ppGFP from Nucleofection Kit of Lonza (cat no.H3VPH-5012). Mammalian expression vector. Contains citomegalovirus (CMV) promoter upstream of a multicloning site (MSC). Express ppGFP transcript ending in a poly(A) tail (using the SV40 poly(A) signal). Encodes for bacterial resistance to Kanamycin. **B**) HEK 293T cells 48h post-transfection with pMAX_ppGFP in one of the control experiments.



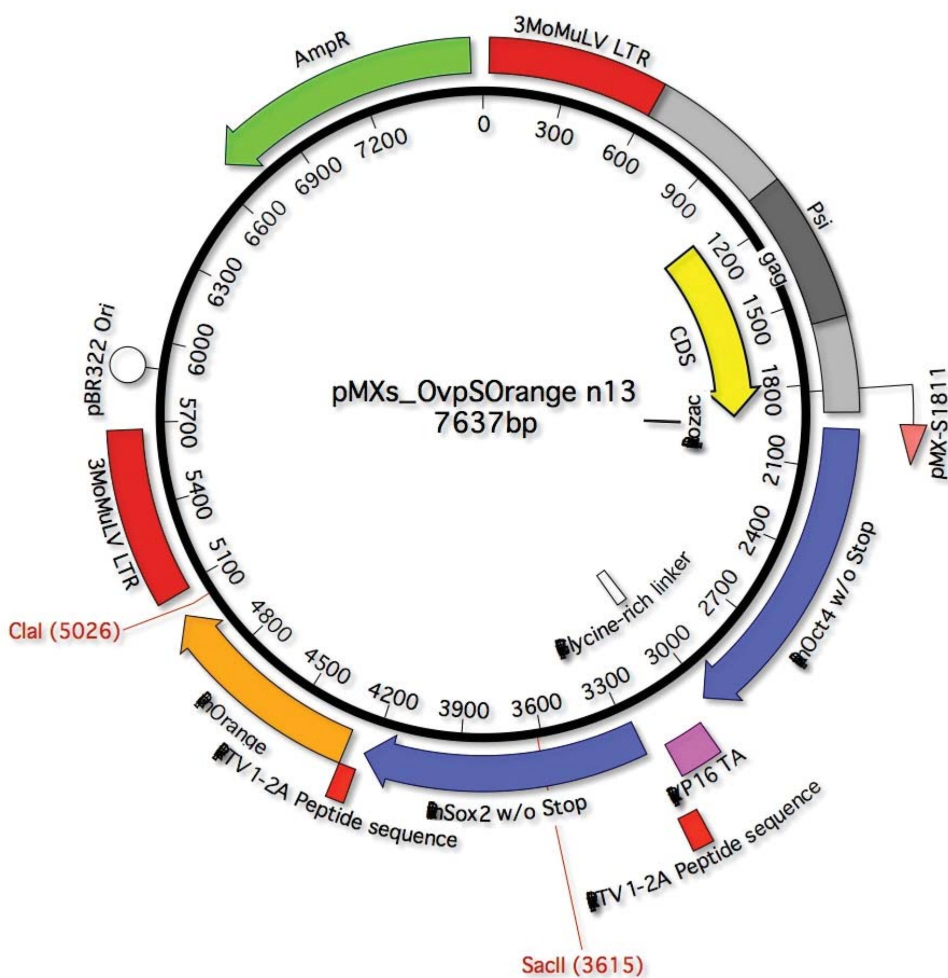
Supplementary Figure 7: pMSCV_Flag-tagged (OSKM) retroviral vectors. Retroviral plasmids cloned in Juan Carlos Izpisúas Laboratory and commercially available in Addgene. Plasmids optimized for expression of a gene in hematopoietic, embryonic stem, or embryonic carcinoma cells. Contains retroviral LTR promoter sequences flanking each of the four reprogramming factors (OCT4 #20072, SOX2 #20073, KLF4 #20074 and c-MYC#20075). These plasmids encode for Ampicillin resistance in bacteria.



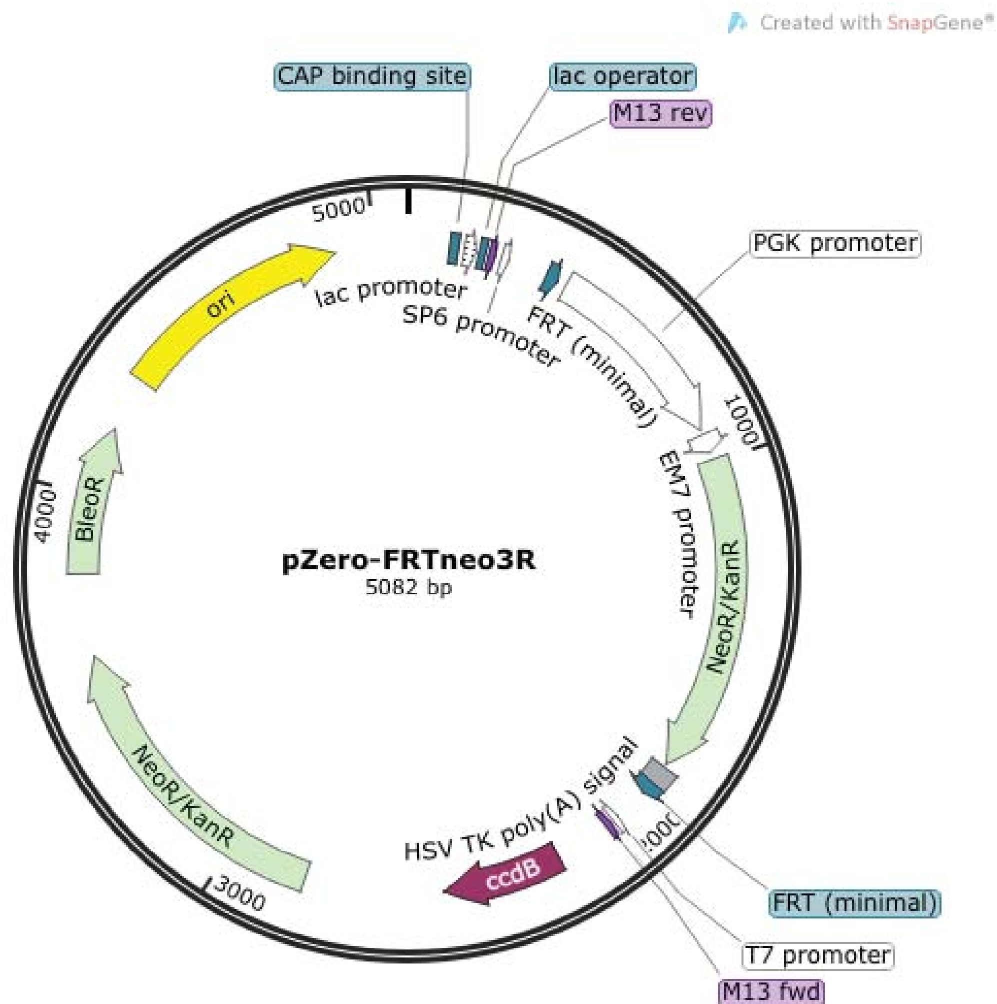
Supplementary Figure 8: pMSCV_Puro. Retroviral plasmid commercially available from Clonetech (#634401). Empty backbone optimized for expression of a gene in hematopoietic, embryonic stem, or embryonic carcinoma cells. Contains retroviral LTR promoter sequences flanking a MCS followed by a PGK constitutive promoter for mammalian cells that drives expression of Puromycin resistance gene. This plasmid encodes for Ampicillin resistance in bacteria.



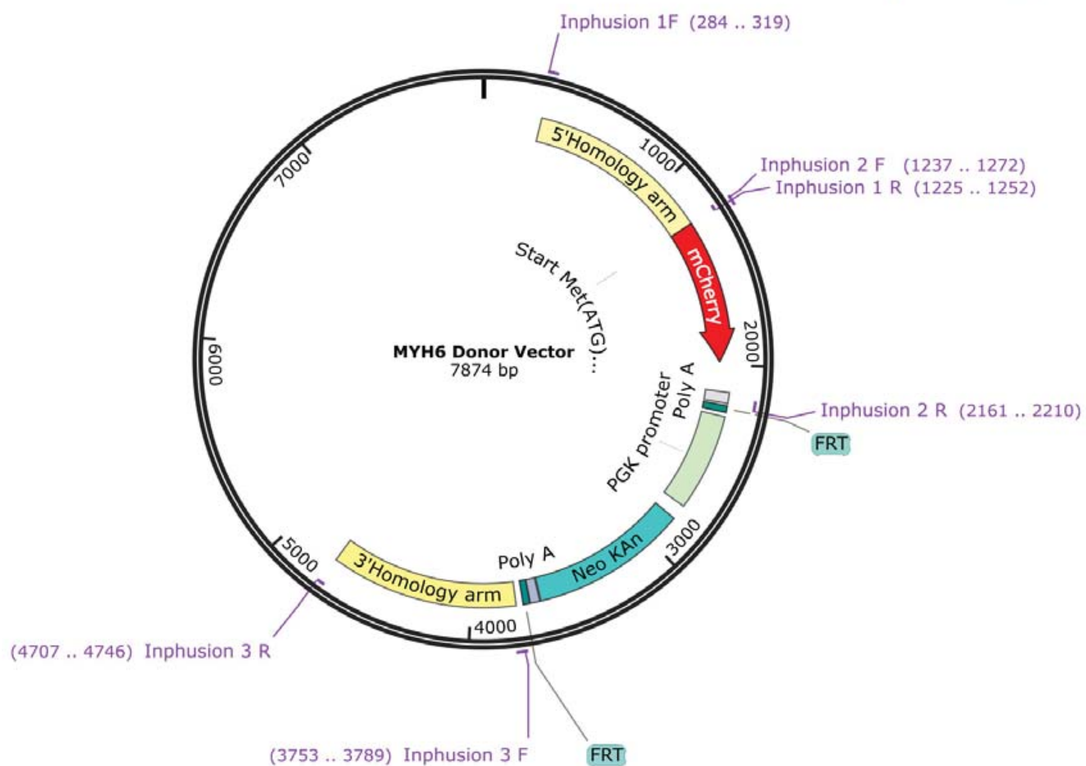
Supplementary Figure 9: pMXs_OVPS_Orange Plasmid map of retroviral vector pMXS_OVPS_mOrange cloned in the laboratory and used for dedifferentiation and plastic stage induction in HFFs for the cell-conversion approach. Contains retroviral LTR promoter sequences flanking OCT4 coding sequence followed by a glycine rich linker, and the VP16 transactivator domain and a PTV-2A peptide to separate protein product from SOX2. In the same manner, SOX2 has a PTV-2A (N-terminal) to separate the protein product from Orange fluorescent protein afterwards. This plasmid encodes for Ampicillin resistance in bacteria.



Supplementary Figure 10: pZero_FRT_Neo. Plasmid kindly provided by GH Liu at the Salk Institute. Contains FRT sites flanking Neomycin (G418) /Kanamycin resistance transgenes under the PGK/EM7 dual promoter (Mammalian and Bacteria) FRT sites allow to excise antibiotic resistance when desired. pZero backbone allows for high-efficiency cloning of DNA inserts with sticky or blunt ends.



Supplementary Figure 11: MYH6 d-vector. Donor Vector plasmid for homologous recombination (HR) in the human *MYH6* locus when co-transfected with either TALEN or CRISPR/Cas9. Generated by Infusion cloning (primer binding sites detailed in the map) Contains 1kb 5' homology upstream the ATG starting site of the human *MYH6* gene followed by an extra Kozac sequence to enhance mCherry expression, followed by mCherry fluorescent protein coding sequence HA tagged and PolyA tail. Then, a PGK dual promoter for Neomycin resistance expression in eukariotic cells. This Neo cassette is flanked by FRT sites for subsequent excision if desired by FLpo. Finally, a second homology arm in 3' corresponding to the downstream region after the starting ATG site of *MYH6* locus. Original backbone is pZero_FRT_Neo. Encodes for Ampicillin resistance in bacteria. Designed and cloned in the laboratory.



Supplementary Figure 12: TALEN specific sequences for human *MYH6* locus targeting.

(TALEN 1) MYH6_1 Target site

T CTGACCCAGGGGAAGCACC aagatgaccgatgccc AGATGGCTGACTTTGGGGC A

MYH6_1 **-5' TALEN**:

HD NG NH NI HD HD HD NI NH NH NH NH NI NI NH HD NI HD

+pTALEN (HD)

1C 2T 3G 4A 5C 6C 7C 8A 9G 10G 11G 12G 13A 14A 15G 16C 17A 18C

GC 68,4%

MYH6_1 **-3' TALEN**:

NH HD HD HD HD NI NI NI NH NG HD NI NH HD HD NI NG HD

+pTALEN (NG)

1G 2C 3C 4C 5C 6A 7A 8A 9G 10T 11C 12A 13G 14C 15C 16A 17T 18C

GC 57,89%

(TALEN2) MYH6_2 Target site

T GACCGATGCCCAGATGGCT gactttggggcagcggcc CAGTACCTCCGCAAGTCAG A

MYH6_2 **-5' TALEN**:

NH NI HD HD NH NI NG NH HD HD HD NI NH NI NG NH NH HD

+pTALEN (NG)

1G 2A 3C 4C 5G 6A 7T 8G 9C 10C 11C 12A 13G 14A 15T 16G 17G 18C

GC 63,15%

***MYH6_2* -3' TALEN**:

HD NG NH NI HD NG NG NH HD NH NH NI NH NH NG NI HD NG

+pTALEN (NH)

1C 2T 3G 4A 5C 6T 7T 8G 9C 10G 11G 12A 13G 14G 15T 16A 17C 18T

GC 57,89%

Supplementary Figure 13: human *MYH6* locus WT (sequence region of interest). Detailed sequence, TALEN and CRISPR/Cas9 guiding RNA (gRNA) specific recognition regions and binding sites for primers used in several assays reported in the present work regarding the targeting procedure.

```
>gi|568815584|ref|NC_000014.9| Homo sapiens chromosome
14, GRCh38.p2 Primary Assembly
```

AAGAAATCTGCAAGCTTGAGTGGGTAAGTGTGAAAAATCTGCATGTGTGGCT
GAAGATGGGCACAGACACGGTCAAGTCTGTATGTGAGAGTGCTGAACTGGGG
TTCTGTGTGAAAATCTGCCTGAGGCGGCAGGGAGAATCACTGCCATTGCGTG
AGCAGGTTGGATGTTGGCCACTCTATCAGGAGCATTAGGGAAGGGGTGGGGA
CTCCAGACGTGTCCCCAAACCAGGGTGGCCTCAAGACCTTGGGAGAACACTT
GTCTGAAGACTTGGGGAACAGAAGGAGACCAGGCATGGCACTTATGCAGACT
GAGGCCAGGACAGAATTTCCTGACAAAAGAAAACTGAGCCATGGAGATGGAC
AACAGATCCCTTCCCTGGGCACCATACTGCAGCTTTTAGTCCCTAGCACTGGG
GGCTCCAGTACTAACAGCAGGAAGATGCTCCCAGCCTGGGACTGTGTGAGGG
AGGTCAGAATGGGAAGGAGAGGCTGGGGAACAGGGGAGGAAAGCCCATGGT
TGGGAGGCGGAGGACAGGCATTTGGCCTGCAGGAGAAGGTGACCCTCACCCA
TGTTTTCAGTTCACCCTTCGGGTTAAAAATAACTGAGGTAAGGGCCATGGCAG
GGTGGGAGAGGCGGTGTGAGAAGGTCCTGTCTTCCCACTATCTGCTCATCAG
CCCTTTGAAGGGGAGGAATGTGCCCAAGGACTAAAAAAAGGCCGTGGAGCCA
GAGAGGCTGGGGCAGCAGACCTTTCTTGGGCAAATCAGGGGGCCCTGCTGTC
CTCCTGTCACCTCCAGAGCCAAAGGATCAAAGGAGGAGGAGCCAGGAGGGGA
GAGAGGTGGGAGGGAGGGTCCCTCCGGAAGGACTCCAAATTTAGACAGAGG
GTGGGGGAAACGGGATATAAAGGAACTGGAGCTTTGAGGACAGATAGAGAGA
CTCCTGCGGCCCAGGTAAGAGGAGGTTTGGGGTGGGATGCCCTGCAGCCCGT
CCACAGAGCCCCCACCGTGAGGGACCTCCTTCACCAGGAGTGGGGTGCAGG
TCAGTTGGAGGCCTAAGGGCTCTATTAAAACTGCCTATCTCCAGGCCCAGG
GAAGTTCCCCCTGACACAAGGAGGTTCCACAGGAAACCCAGAAACCTCTTT
TCTCCTTCTCTGACTCTCCATTTCTTTCTCTGCATCATTCTGAGTCTCCTACAT
GTTGTCTCCATCTTTCCATCTTCCTTCCTCCTTTGGATGGCTTCCTTCCCTTGA
TCCTGGTTTTATCTTGCCTCTTGGTCTTCATCGACACTTGTCACAATCATGCT
TCTTTGTCTCTCTCCCTTGTCCTTCCTTCTTGGCACGTGTTCTCACCTCCCTGC
CTCTCTGCTTCTAACCCTGTTTCCACACCCCGTCCCTCGCACTCATATTGACT
CGGTGCCCTTTCTTTTCTGCCTCTGCGTCTTTCCCTTTCTGACTCCCTGGTCTG
TCCTGCCTGTCTGCGCTCTGGGGCTGCCTCCATCCCCGGGTGGCCTGCCTCT
GTTGTTCTTCACTCTCCTCATCTGTTCTTCTCTCTGCCCGGCTCTACCTCTGTT
GTTCCTTGCTCCACCCACGGTCCAGATTCTTCAGGATTCTCCGTGAAGGGAT
AACCAGGTGAGAACTGCCCCCATTTTCTCTGCAGAGACTGGGGCATGCTTCT
CCTGGGAGCCGGATTGCTGGACCAGGGGTCTGCTGTCCCAAGCACTCAGCG
CCAACCCTTAGCATACTCCAGCCAATGCCACCCCAGGGAAACCCCTTACAG
AGATTGTCCTTCAGCATCACCTCAGAGGGCAGGAGAAGCAGAGCCCTGAGT
AGGGGAGGGTGCAACAGCAGGTGCCTCTCCCAGGGTGGAGGAGAGGAGCG
GGGGTAGGGAGGGGGGCTGCAGAGGACAAAGCCACTCGCTGGAGCCTGGG

CTCCCTCAGGAGTAACATAGCCCTCCTGTC***TCTGACCCAGGGGAAGCACCAA***
GATGACCGATGCCCAGATGGCTGACTTTGGGGCAGCGGCCCAGTACCTCCG
CAAGTCAGAGAAGGAGCGTCTAGAGGCCCAGACCCGGCCCTTTGACATTCG
CACTGAGTGCTTCGTGCCCGATGACAAGGAAGAGTTTGTCAAAGCCAAGAT
TTTGTCCCGGGAGGGAGGCAAGGTCATTGCTGAAACCGAGAATGGGAAGGT
GAGTAGGGCATGGCGCCGGGGCAGAAGGGAAGGAGGTCTGGGAAAGAAGA
TGCAGAGGTGGAGCCACTTGCAGGGGGAGCTGAGAGGGCTGGAGAAAAGC
CAAGGCCAGTGGGGATGCCAGGACATGCTCCTTTGAGGAGCCCAGAATCTG
ATCCCTCTCAAATTGACCTGAGCTGGTGCAACAGGTGCCACCCAGGGCCAT
GTTCCCCCTGCCAGAGAGGATGCTGAGGAAGAAGAACCTCAGTGTTCGCCT
AAGAGGGGTCTTGTAGATAAAGAGGGCACAGACACAGCATTAAATGATGCC
CCCTTCTTGCACTTGTATCCCTCTTCCCTGTGCCTCAGTTTCCTCCATGAGTC
CACTTTCTCAAATTCCGTTCACCCAAATCAAGAGTAATTCTTAGACCCAGAT
GAACACAAAGATCAGAAACTTTTGAGCTGAGCACTCTCCTTGACTGGCACT
CAGAAGCTCTGGTCCCTGGTTTGCTCACACCAGCCAAGAGAATCACCCCTG
GTTACCAGCTGCGGCTCAGGGCTGTGTGCCTCATGAACTCGTTGACTGAATG
TTACAACCCATTGAAGTGTAGAATAACAGGCCACAATCCCCTGGGGCTTTTG
ACTCTGATCCCAGCTCTGCCACCCGCTAGTCACTGTGCAGGCAAATCATTTA
GTCATTTAGAGCCACGGATTTCTCCACTATAAAAACACTGGAATACCTACT
GGCAGGATCTAATGACATCAGGGCATGGCAAACTGACTGCTGCCAATCAAA
CCACACCAACAGTGATGGATGGGGAGTGTGGAGTAGATGGGTGAACTACTT
TTCCAGCAGGGGTGAAGGTTTGCCCTGAGCAACAGATACCCTAAAGCGCTG
CCCGCGGGAGACAGCCTCGGGGTCAGCATAAGGTGTGCACAGATCTGAGAG
CTGCCAATCTCCAGGTCTGCCCCAAGACCCTTGGAACATAGGGACTGAAGA
GTGATGGTCATGGGCACAGGGTGTCCCCAGGATGGTCTGGGGATCTGGCAA
GAGAAAGGTACCCTAGGACAGTCTCTAGGATGGGAGATACAATGGGAAGG
GAAATTACCTGGGGAAAGTGTCCCAGGGGACATCGGGGTAGGGGCCGGGG
CACTGGTCAGAGCAAGGGGAGCAAGGCCAAGTCCCTGTGTCCTGGGAGGAG
GTCAGTGGGCAGTGCTGGCAAGGGTCCCGGAGGGATTGTGGTCACTCATCC
TCCTGCTTATGCGCCCCCTCCAGACGGTGACTGTGAAGGAGGACCAGGTGTT
GCAGCAGAACCCACCCAAGTTCGACAAGATTGAGGACATGGCCATGCTGAC
CTTCCTGCACGAGCCCGCGGTGCTTTTCAACCTCAAGGAGCGCTACGCGGCC
TGGATGATATAT

First MET (starting Site) **ATG**

MYH6_seq F: 5’-GAAGGGATAACCAGGTGAGAAC-3’

MYH6_seq R: 5’-GAAGAGGGATACAAGTGCAAGA-3’

Southern MYH6 F: 5’-TGCCTCTGTTGTTCTTCACTCTCC-3’

Southern MYH6 R: 5’-GGTCAGAGACAGGAGGGCTAT-3’

gRNA1 : AGATGACCGATGCCCAGAT

TALEN 1:

TCTGACCCAGGGGAAGCACCAAGATGACCGATGCCCAGATGGCTGACTTTGG GGCA

TALEN2 :

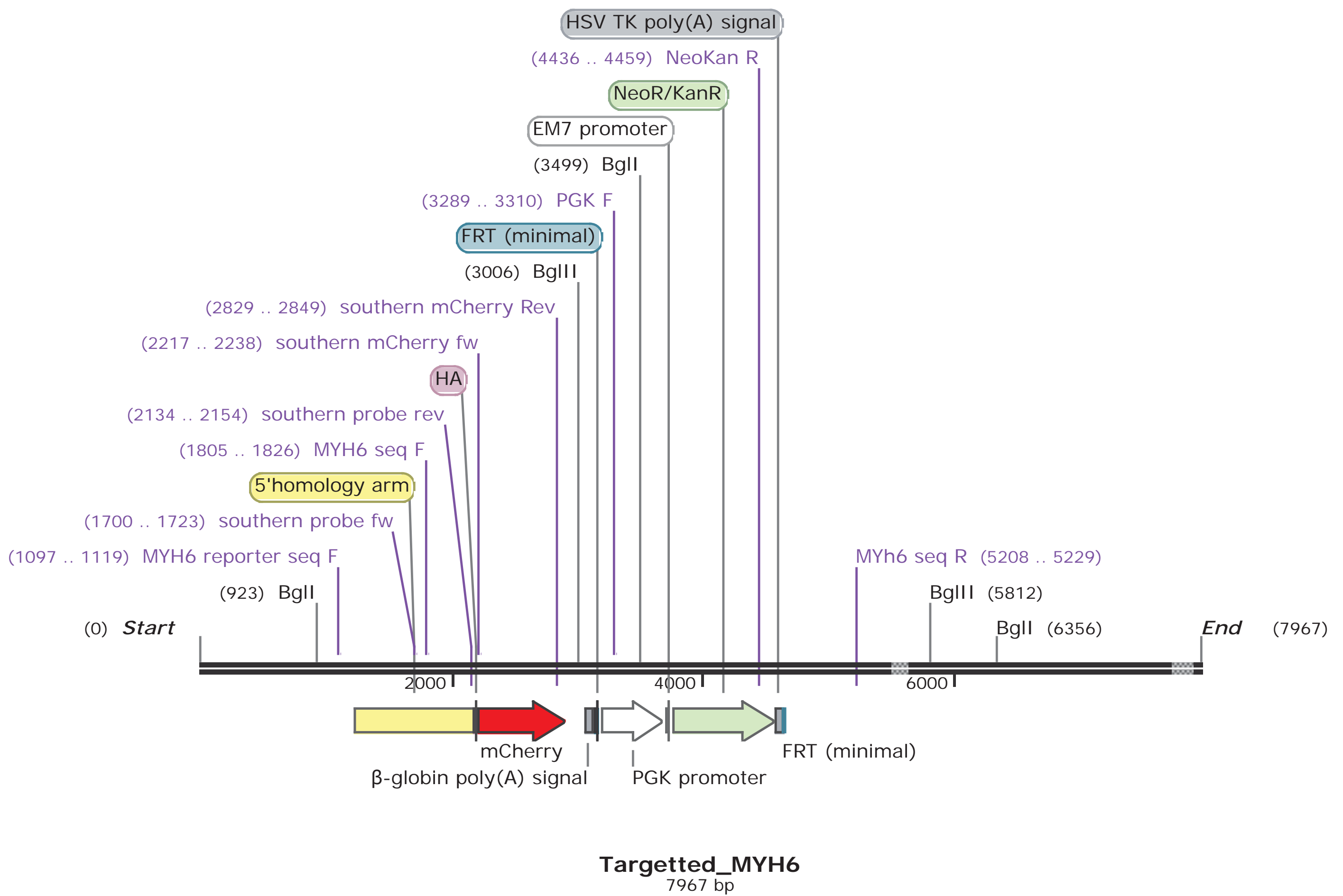
ACCGATGCCCAGATGGCTGACTTTGGGGCAGCGGCCCAGTACCTCCGCAAGT CAGA

MYH6_Promoter : AAGAAATCTGCAAGCTTG….

Suplementary Figure 14: Human *MYH6* genomic locus targeted final sequence. Detailed sequence of MYH6 locus with the successful integration of the reporter cassette mCherry/Neo.

Sequence: Targetted_MYH6.dna (Linear / 7967 bp)
Enzymes: < Unsaved Enzyme Set > (2 of 653 total)
Features: 10 visible, 10 total
Primers: 9 visible, 9 total

Unique Cutters **Bold**



Targetted_MYH6
7967 bp

Start (0)

```
5′ ctggcagcccggtgaggcagaaggggacagcccctcacccacccccctcctctctcaggc  60
3′ gaccgtcgggccactccgtcttcccctgtcggggagtgggtggggggaggagagagtccg

   cctaggattagcaccatcttcttctgcccaccccaccccaggagcagagggtagcagggg  120
   ggatcctaatcgtggtagaagaagacgggtggggtggggtcctcgtctcccatcgtcccc

   agggcaaaagcctatgtgtgcaaatgcggatgtgcaaatgtgtgcatccatgtacacaaa  180
   tcccgttttcggatacacacgtttacgcctacacgtttacacacgtaggtacatgtgttt

   agaaagtagaaaagaaatctgcaagcttgagtgggtaagtgtgaaaaatctgcatgtgtg  240
   tctttcatcttttctttagacgttcgaactcacccattcacactttttagacgtacacac

   gctgaagatgggcacagacacggtcaagtctgtatgtgagagtgctgaactggggttctg  300
   cgacttctacccgtgtctgtgccagttcagacatacactctcacgacttgaccccaagac

   tgtgaaaatctgcctgaggcggcagggagaatcactgccattgcgtgagcaggttggatg  360
   acacttttagacggactccgccgtccctcttagtgacggtaacgcactcgtccaacctac

   ttggccactctatcaggagcattagggaaggggtggggactccagacgtgtccccaaacc  420
   aaccggtgagatagtcctcgtaatcccttccccacccctgaggtctgcacaggggtttgg

   agggtggcctcaagaccttgggagaacacttgtctgaagacttggggaacagaaggagac  480
   tcccaccggagttctggaaccctcttgtgaacagacttctgaaccccttgtcttcctctg

   caggcatggcacttatgcagactgaggccaggacagaatttcctgacaaaagaaaactga  540
   gtccgtaccgtgaatacgtctgactccggtcctgtcttaaaggactgttttcttttgact

   gccatggagatggacaacagatcccttccctgggcaccatactgcagcttttagtcccta  600
   cggtacctctacctgttgtctagggaagggacccgtggtatgacgtcgaaaatcagggat

   gcactgggggctccagtactaacagcaggaagatgctcccagcctgggactgtgtgaggg  660
   cgtgacccccgaggtcatgattgtcgtccttctacgagggtcggaccctgacacactccc

   aggtcagaatgggaaggagaggctggggaacaggggaggaaagcccatggttgggaggcg  720
   tccagtcttacccttcctctccgaccccttgtcccctcctttcgggtaccaaccctccgc

   gaggacaggcatttggcctgcaggagaaggtgaccctcacccatgttttcagttcaccct  780
   ctcctgtccgtaaaccggacgtcctcttccactgggagtgggtacaaaagtcaagtggga
```

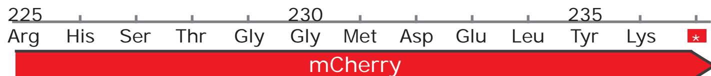
```
tcgggttaaaaataactgaggtaagggccatggcagggtgggagaggcggtgtgagaagg
agcccaatttttattgactccattcccggtaccgtcccaccctctccgccacactcttcc    840

tcctgtcttcccactatctgctcatcagccctttgaaggggaggaatgtgcccaaggact
aggacagaagggtgatagacgagtagtcgggaaacttcccctccttacacgggttcctga    900

                                     BglI
aaaaaaaggccgtggagccagagaggctggggcagcagacctttcttgggcaaatcaggg
tttttttccggcacctcggtctctccgaccccgtcgtctggaaagaacccgtttagtccc    960

ggccctgctgtcctcctgtcacctccagagccaaaggatcaaaggaggaggagccaggag
ccgggacgacaggaggacagtggaggtctcggtttcctagtttcctcctcctcggtcctc    1020

gggagagaggtgggagggagggtccctccggaaggactccaaatttagacagagggtggg
ccctctctccaccctccctcccagggaggccttcctgaggtttaaatctgtctcccaccc    1080

                  MYH6 reporter seq F
                  ggaactggagctttgaggacAGA
ggaaacgggatataaaggaactggagctttgaggacAGATAGAGAGACTCCTGCGGCCCA
cctttgccctatatttccttgacctcgaaactcctgTCTATCTCTCTGAGGACGCCGGGT    1140

Ggtaagaggaggtttggggtgggatgccctgcagcccgtccacagagcccccaccgtgag
Ccattctcctccaaaccccaccctacgggacgtcgggcaggtgtctcgggggtggcactc    1200

ggacctccttcaccaggagtggggtgcaggtcagttggaggcctaagggctctattaaaa
cctggaggaagtggtcctcaccccacgtccagtcaacctccggattcccgagataatttt    1260
                                    5'homology arm

ctgcctatctccaggcccagggaagttccccctgacacaaggaggttccacaggaaaccc
gacggataggggtccgggtcccttcaagggggactgtgttcctccaaggtgtcctttggg    1320
5'homology arm

agaaacctcttttctccttctctgactctccatttctttctctgcatcattctgagtctc
tctttggagaaaagaggaagagactgagaggtaaagaaagagacgtagtaagactcagag    1380
5'homology arm

ctacatgttgtctccatctttccatcttccttcctcctttggatggcttccttcccttga
gatgtacaacagaggtagaaaggtagaaggaaggaggaaacctaccgaaggaagggaact    1440
5'homology arm
```

```
tcctggtttatcttgcctcttggtcttcatcgacacttgtcacaatcatgcttctttgt      1500
aggaccaaaatagaacggagaaccagaagtagctgtgaacagtgttagtacgaagaaaca
5'homology arm

ctctctcccttgtccttccttcttggcacgtgttctcacctccctgcctctctgcttcta      1560
gagagagggaacaggaaggaagaaccgtgcacaagagtggagggacggagagacgaagat
5'homology arm

accctgtttccacaccccgtccctcgcactcatattgactcggtgccctttcttttctgc      1620
tgggacaaaggtgtggggcagggagcgtgagtataactgagccacgggaaagaaaagacg
5'homology arm

ctctgcgtctttccctttctgactccctggtctgtcctgcctgtctgcgctctggggctg      1680
gagacgcagaaagggaaagactgagggaccagacaggacggacagacgcgagaccccgac
5'homology arm

                          southern probe fw
                  tgcctctgttgttcttcactctcc
cctccatccccgggtggcctgcctctgttgttcttcactctcctcatctgttcttctctc      1740
ggaggtaggggcccaccggacggagacaacaagaagtgagaggagtagacaagaagagag
5'homology arm

tgcccggctctacctctgttgttccttgctccacccacggtccagATTCTTCAGGATTCT      1800
acgggccgagatggagacaacaaggaacgaggtgggtgccaggtcTAAGAAGTCCTAAGA
5'homology arm

          MYH6 seq F
   GAAGGGATAACCAGgtgagaac
CCGTGAAGGGATAACCAGgtgagaactgccccatttctctgcagagactggggcatgc      1860
GGCACTTCCCTATTGGTCcactcttgacggggtaaaagagacgtctctgaccccgtacg
5'homology arm

ttctcctgggagccggattgctggaccaggggtctgctgtcccaagcactcagcgccaac      1920
aagaggaccctcggcctaacgacctggtccccagacgacagggttcgtgagtcgcggttg
5'homology arm

ccttagcatactccagccaatgccaccccagggaaaccccttacagagattgtccttcag      1980
ggaatcgtatgaggtcggttacggtggggtccctttggggaatgtctctaacaggaagtc
5'homology arm
```

```
catcacctcagagggcaggagaagcagagccctgagtaggggagggtgcaacagcaggtg   2040
gtagtggagtctcccgtcctcttcgtctcgggactcatcccctcccacgttgtcgtccac
                      5'homology arm

cctctcccagggtggaggagaggagcgggggtagggagggggctgcagaggacaaagcc   2100
ggagagggtcccacctcctctcctcgcccccatccctcccccgacgtctcctgtttcgg
                      5'homology arm

actcgctggagcctgggctccctcaggagtaacatagccctcctgtctctgacccagGGG   2160
tgagcgacctcggacccgagggagtcctcattgtatcgggaggacagagactgggtcCCC
                      5'homology arm
                                  tatcgggaggacagagactgg
                                    southern probe rev

                                              southern mCherry fw
                                                             aagg
AAGCACCAAGATGACCGATGatgtatccgtatgatgtgccggattatgcggtgagcaagg   2220
TTCGTGGTTCTACTGGCTACtacataggcatactacacggcctaatacgccactcgttcc
                        1           5
                       Tyr Pro Tyr Asp Val Pro Asp Tyr Ala  Val Ser Lys
5'homology arm                         HA                     mCherry

  southern mCherry fw
gcgaggaggataacatgg
gcgaggaggataacatggccatcatcaaggagttcatgcgcttcaaggtgcacatggagg   2280
cgctcctcctattgtaccggtagtagttcctcaagtacgcgaagttccacgtgtacctcc
5             10            15            20
Gly Glu Glu Asp Asn Met Ala Ile Ile Lys Glu Phe Met Arg Phe Lys Val His Met Glu
                            mCherry

gctccgtgaacggccacgagttcgagatcgagggcgagggcgagggccgcccctacgagg   2340
cgaggcacttgccggtgctcaagctctagctcccgctcccgctcccggcggggatgctcc
25            30            35            40
Gly Ser Val Asn Gly His Glu Phe Glu Ile Glu Gly Glu Gly Glu Gly Arg Pro Tyr Glu
                            mCherry

gcacccagaccgccaagctgaaggtgaccaagggtggccccctgcccttcgcctgggaca   2400
cgtgggtctggcggttcgacttccactggttcccaccggggggacgggaagcggaccctgt
45            50            55            60
Gly Thr Gln Thr Ala Lys Leu Lys Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Trp Asp
                            mCherry
```

```
tcctgtcccctcagttcatgtacggctccaaggcctacgtgaagcaccccgccgacatcc   2460
aggacaggggagtcaagtacatgccgaggttccggatgcacttcgtggggcggctgtagg
65              70              75              80
Ile Leu Ser Pro Gln Phe Met Tyr Gly Ser Lys Ala Tyr Val Lys His Pro Ala Asp Ile
mCherry

ccgactacttgaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcg   2520
ggctgatgaacttcgacaggaaggggctcccgaagttcaccctcgcgcactacttgaagc
85              90              95              100
Pro Asp Tyr Leu Lys Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val Met Asn Phe
mCherry

aggacggcggcgtggtgaccgtgacccaggactcctccctgcaggacggcgagttcatct   2580
tcctgccgccgcaccactggcactgggtcctgaggagggacgtcctgccgctcaagtaga
105             110             115             120
Glu Asp Gly Gly Val Val Thr Val Thr Gln Asp Ser Ser Leu Gln Asp Gly Glu Phe Ile
mCherry

acaaggtgaagctgcgcggcaccaacttccccctccgacggccccgtaatgcagaagaaga   2640
tgttccacttcgacgcgccgtggttgaagggggaggctgccggggcattacgtcttcttct
125             130             135             140
Tyr Lys Val Lys Leu Arg Gly Thr Asn Phe Pro Ser Asp Gly Pro Val Met Gln Lys Lys
mCherry

ccatgggctgggaggcctcctccgagcggatgtaccccgaggacggcgccctgaagggcg   2700
ggtacccgaccctccggaggaggctcgcctacatggggctcctgccgcgggacttcccgc
145             150             155             160
Thr Met Gly Trp Glu Ala Ser Ser Glu Arg Met Tyr Pro Glu Asp Gly Ala Leu Lys Gly
mCherry

agatcaagcagaggctgaagctgaaggacggcggccactacgacgctgaggtcaagacca   2760
tctagttcgtctccgacttcgacttcctgccgccggtgatgctgcgactccagttctggt
165             170             175             180
Glu Ile Lys Gln Arg Leu Lys Leu Lys Asp Gly Gly His Tyr Asp Ala Glu Val Lys Thr
mCherry

cctacaaggccaagaagcccgtgcagctgcccggcgcctacaacgtcaacatcaagttgg   2820
ggatgttccggttcttcgggcacgtcgacgggccgcggatgttgcagttgtagttcaacc
185             190             195             200
Thr Tyr Lys Ala Lys Lys Pro Val Gln Leu Pro Gly Ala Tyr Asn Val Asn Ile Lys Leu
mCherry

acatcacctcccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggcc   2880
tgtagtggagggtgttgctcctgatgtggtagcaccttgtcatgcttgcgcggctcccgg
205             210             215             220
Asp Ile Thr Ser His Asn Glu Asp Tyr Thr Ile Val Glu Gln Tyr Glu Arg Ala Glu Gly
mCherry
        agggtgttgctcctgatgtgg
        southern mCherry Rev
```

```
gccactccaccggcggcatggacgagctgtacaagtaagcggccgcaattcactcctcag   2940
cggtgaggtggccgccgtacctgctcgacatgttcattcgccggcgttaagtgaggagtc
225            230            235
Arg His Ser Thr Gly Gly Met Asp Glu Leu Tyr Lys *
```




```
gtgcaggctgcctatcagaaggtggtggctggtgtggccaatgccctggctcacaaatac   3000
cacgtccgacggatagtcttccaccaccgaccacaccggttacgggaccgagtgtttatg

BglII
cactgagatcttttccctctgccaaaaattatggggacatcatgaagccccttgagcat   3060
gtgactctagaaaagggagacggtttttaataccccctgtagtacttcggggaactcgta

ctgacttctggctaataaaggaaatttatttcattgcaatagtgtgttggaattttttg   3120
gactgaagaccgattatttcctttaaataaaagtaacgttatcacacaaccttaaaaaac
```

```
tgtctatcaCATATGGAATTCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGGCCC   3180
acagatagtGTATACCTTAAGCTTCAAGGATAAGAGATCTTTCATATCCTTGAAGCCGGG
```

```
CTGCAGGTCAATTCTAccgggtaggggaggcgcttttcccaaggcagtctggagcatgcg   3240
GACGTCCAGTTAAGATggcccatcccctccgcgaaaagggttccgtcagacctcgtacgc
```

```
                                                PGK F
                                                ctcgcacacatt
ctttagcagccccgctgggcacttggcgctacacaagtggcctctggcctcgcacacatt   3300
gaaatcgtcggggcgacccgtgaaccgcgatgtgttcaccggagaccggagcgtgtgtaa
```

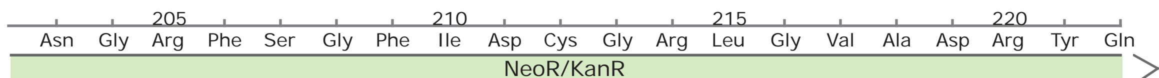
```
PGK F
ccacatccac
ccacatccaccggtaggcgccaaccggctccgttctttggtggccccttcgcgccacctt   3360
ggtgtaggtggccatccgcggttggccgaggcaagaaaccaccggggaagcgcggtggaa
```

```
ctactcctccccctagtcaggaagttcccccccgccccgcagctcgcgtcgtgcaggacgt   3420
gatgaggaggggatcagtccttcaagggggggcggggcgtcgagcgcagcacgtcctgca
```

```
gacaaatggaagtagcacgtctcactagtctcgtgcagatggacagcaccgctgagcaat   3480
ctgtttaccttcatcgtgcagagtgatcagagcacgtctacctgtcgtggcgactcgtta
PGK promoter

                    BglI
ggaagcgggtaggcctttggggcagcggccaatagcagctttgctccttcgctttctggg   3540
ccttcgcccatccggaaaccccgtcgccggttatcgtcgaaacgaggaagcgaaagaccc
PGK promoter

ctcagaggctgggaaggggtgggtccgggggcgggctcaggggcgggctcaggggcgggg   3600
gagtctccgacccttccccacccaggcccccgcccgagtccccgcccgagtccccgcccc
PGK promoter

cgggcgcccgaaggtcctccggAGGCCCGGCATTCTGCACGCTTCAAAAGCGCACGTCTG   3660
gcccgcgggcttccaggaggccTCCGGGCCGTAAGACGTGCGAAGTTTTCGCGTGCAGAC
PGK promoter

CCGCGCTGTTCTCCTCTTCCTCATCTCCGGGCCTTTCGACCTGCAGCCTGTTGACAATTA   3720
GGCGCGACAAGAGGAGAAGGAGTAGAGGCCCGGAAAGCTGGACGTCGGACAACTGTTAAT
PGK promoter                                  EM7 promoter

ATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGGAACTAAACCATGGG   3780
TAGTAGCCGTATCATATAGCCGTATCATATTATGCTGTTCCACTCCTTGATTTGGTACCC
                                                       1
                                                       Met Gly
EM7 promoter                                           NeoR/KanR

ATCGGCCATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCT   3840
TAGCCGGTAACTTGTTCTACCTAACGTGCGTCCAAGAGGCCGGCGAACCCACCTCTCCGA
    5              10             15             20
Ser Ala Ile Glu Gln Asp Gly Leu His Ala Gly Ser Pro Ala Ala Trp Val Glu Arg Leu
NeoR/KanR

ATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCT   3900
TAAGCCGATACTGACCCGTGTTGTCTGTTAGCCGACGAGACTACGGCGGCACAAGGCCGA
    25             30             35             40
Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser Asp Ala Ala Val Phe Arg Leu
NeoR/KanR

GTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGA   3960
CAGTCGCGTCCCCGCGGGCCAAGAAAAACAGTTCTGGCTGGACAGGCCACGGGACTTACT
    45             50             55             60
Ser Ala Gln Gly Arg Pro Val Leu Phe Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu
NeoR/KanR
```

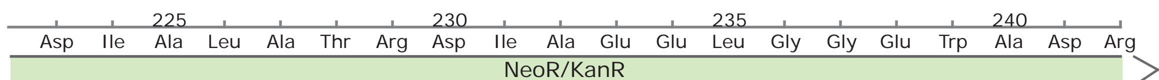
```
ACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGC   4020
TGACGTCCTGCTCCGTCGCGCCGATAGCACCGACCGGTGCTGCCCGCAAGGAACGCGTCG
        65                  70                  75                  80
 Leu  Gln  Asp  Glu  Ala  Ala  Arg  Leu  Ser  Trp  Leu  Ala  Thr  Thr  Gly  Val  Pro  Cys  Ala  Ala
NeoR/KanR

TGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGG   4080
ACACGAGCTGCAACAGTGACTTCGCCCTTCCCTGACCGACGATAACCCGCTTCACGGCCC
        85                  90                  95                  100
 Val  Leu  Asp  Val  Val  Thr  Glu  Ala  Gly  Arg  Asp  Trp  Leu  Leu  Leu  Gly  Glu  Val  Pro  Gly
NeoR/KanR

GCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGC   4140
CGTCCTAGAGGACAGTAGAGTGGAACGAGGACGGCTCTTTCATAGGTAGTACCGACTACG
        105                 110                 115                 120
 Gln  Asp  Leu  Leu  Ser  Ser  His  Leu  Ala  Pro  Ala  Glu  Lys  Val  Ser  Ile  Met  Ala  Asp  Ala
NeoR/KanR

AATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACA   4200
TTACGCCGCCGACGTATGCGAACTAGGCCGATGGACGGGTAAGCTGGTGGTTCGCTTTGT
        125                 130                 135                 140
 Met  Arg  Arg  Leu  His  Thr  Leu  Asp  Pro  Ala  Thr  Cys  Pro  Phe  Asp  His  Gln  Ala  Lys  His
NeoR/KanR

TCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGA   4260
AGCGTAGCTCGCTCGTGCATGAGCCTACCTTCGGCCAGAACAGCTAGTCCTACTAGACCT
        145                 150                 155                 160
 Arg  Ile  Glu  Arg  Ala  Arg  Thr  Arg  Met  Glu  Ala  Gly  Leu  Val  Asp  Gln  Asp  Asp  Leu  Asp
NeoR/KanR

CGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCC   4320
GCTTCTCGTAGTCCCCGAGCGCGGTCGGCTTGACAAGCGGTCCGAGTTCCGCGCGTACGG
        165                 170                 175                 180
 Glu  Glu  His  Gln  Gly  Leu  Ala  Pro  Ala  Glu  Leu  Phe  Ala  Arg  Leu  Lys  Ala  Arg  Met  Pro
NeoR/KanR

CGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGA   4380
GCTGCCGCTCCTAGAGCAGCACTGGGTACCGCTACGGACGAACGGCTTATAGTACCACCT
        185                 190                 195                 200
 Asp  Gly  Glu  Asp  Leu  Val  Val  Thr  His  Gly  Asp  Ala  Cys  Leu  Pro  Asn  Ile  Met  Val  Glu
NeoR/KanR
```

AAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCA
4440
TTTACCGGCGAAAAGACCTAAGTAGCTGACACCGGCCGACCCACACCGCCTGGCGATAGT



NeoKan R

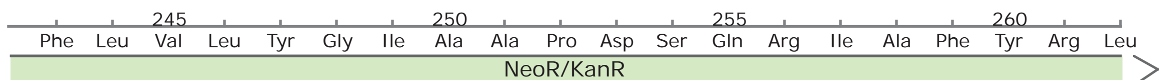
GGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCG
4500
CCTGTATCGCAACCGATGGGCACTATAACGACTTCTCGAACCGCCGCTTACCCGACTGGC



CCTGTATCGCAACCGATGG

NeoKan R

CTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCT
4560
GAAGGAGCACGAAATGCCATAGCGGCGAGGGCTAAGCGTCGCGTAGCGGAAGATAGCGGA



TCTTGACGAGTTCTTCTGAggggatcggcaataaaaagacagaataaaacgcacgggTGT
4620
AGAACTGCTCAAGAAGACTcccctagccgttatttttctgtcttattttgcgtgcccACA

TGGGTCGTTTGTTCGGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGAATTCTTAA
4680
ACCCAGCAAACAAGCCCTTCAAGGATAAGAGATCTTTCATATCCTTGAAGCTTAAGAATT

TTAAAAGGGCgaattctgcagatatcCCCAGATGGCTGACTTTGGGGCAGCGGCCCAGTA
4740
AATTTTCCCGcttaagacgtctatagGGGTCTACCGACTGAAACCCCGTCGCCGGGTCAT

CCTCCGCAAGTCAGAGAAGGAGCGTCTAGAGGCCCAGACCCGGCCCTTTGACATTCGCAC
4800
GGAGGCGTTCAGTCTCTTCCTCGCAGATCTCCGGGTCTGGGCCGGGAAACTGTAAGCGTG

TGAGTGCTTCGTGCCCGATGACAAGGAAGAGTTTGTCAAAGCCAAGATTTTGTCCCGGGA
4860
ACTCACGAAGCACGGGCTACTGTTCCTTCTCAAACAGTTTCGGTTCTAAAACAGGGCCCT

GGGAGGCAAGGTCATTGCTGAAACCGAGAATGGGAAGgtgagtagggcatggcgccgggg
4920
CCCTCCGTTCCAGTAACGACTTTGGCTCTTACCCTTCcactcatcccgtaccgcggcccc

cagaagggaaggaggtctgggaaagaagatgcagaggtggagccacttgcagggggagct 4980
gtcttcccttcctccagaccctttcttctacgtctccacctcggtgaacgtccccctcga

gagagggctggagaaaagccaaggccagtggggatgccaggacatgctcctttgaggagc 5040
ctctcccgacctcttttcggttccggtcacccctacggtcctgtacgaggaaactcctcg

ccagaatctgatccctctcaaattgacctgagctggtgcaacaggtgccacccagggcca 5100
ggtcttagactagggagagtttaactggactcgaccacgttgtccacggtgggtcccggt

tgttcccctgccagagaggatgctgaggaagaagaacctcagtgttcgcctaagagggg 5160
acaaggggacggtctctcctacgactccttcttcttggagtcacaagcggattctcccc

tcttgtagataaagagggcacagacacagcattaaatgatgccccttcttgcacttgta 5220
agaacatctatttctcccgtgtctgtgtcgtaatttactacggggaagaacgtgaacat

agaacgtgaacat
MYh6 seq R

tccctcttccctgtgcctcagtttcctccatgagtccactttctcaaattccgttcaccc 5280
agggagaagggacacggagtcaaaggaggtactcaggtgaaagagtttaaggcaagtggg

agggagaag
MYh6 seq R

aaatcaagagtaattcttagacccagatgaacacaaagatcagaaacttttgagctgagc 5340
tttagttctcattaagaatctgggtctacttgtgtttctagtctttgaaaactcgactcg

actctccttgactggcactcagaagctctggtccctggtttgctcacaccagccaagaga 5400
tgagaggaactgaccgtgagtcttcgagaccagggaccaaacgagtgtggtcggttctct

atcacccctggttaccagctgcggctcagggctgtgtgcctcatgaactcgttgactgaa 5460
tagtggggaccaatggtcgacgccgagtcccgacacacggagtacttgagcaactgactt

tgttacaacccattgaagtgtagaataacaggccacaatcccctggggcttttnnnnnnn 5520
acaatgttgggtaacttcacatcttattgtccggtgttaggggaccccgaaaannnnnnn

nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn 5580
nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn

nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnctaatgacatcagg 5640
nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnngattactgtagtcc

```
gcatggcaaactgactgctgccaatcaaaccacaccaacagtgatggatggggagtgtgg
cgtaccgtttgactgacgacggttagtttggtgtggttgtcactacctacccctcacacc    5700

agtagatgggtgaactacttttccagcaggggtgaaggtttgccctgagcaacagatacc
tcatctacccacttgatgaaaaggtcgtccccacttccaaacgggactcgttgtctatgg    5760

                                                   BglII
ctaaagcgctgcccgcgggagacagcctcggggtcagcataaggtgtgcacagatctgag
gatttcgcgacgggcgccctctgtcggagccccagtcgtattccacacgtgtctagactc    5820

agctgccaatctccaggtctgccccaagacccttggaacatagggactgaagagtgatgg
tcgacggttagaggtccagacggggttctgggaaccttgtatccctgacttctcactacc    5880

tcatgggcacagggtgtccccaggatggtctggggatctggcaagagaaaggtaccctag
agtacccgtgtcccacaggggtcctaccagacccctagaccgttctctttccatgggatc    5940

gacagtctctaggatgggagatacaatgggaagggaaattacctggggaaagtgtcccag
ctgtcagagatcctaccctctatgttacccttccctttaatggacccctttcacagggtc    6000

gggacatcggggtaggggccggggcactggtcagagcaaggggagcaaggccaagtccct
ccctgtagccccatccccggccccgtgaccagtctcgttcccctcgttccggttcaggga    6060

gtgtcctgggaggaggtcagtgggcagtgctggcaagggtcccggagggattgtggtcac
cacaggaccctcctccagtcacccgtcacgaccgttcccagggcctccctaacaccagtg    6120

tcatcctcctgcttatgcgcccctccagACGGTGACTGTGAAGGAGGACCAGGTGTTGC
agtaggaggacgaatacgcgggggaggtcTGCCACTGACACTTCCTCCTGGTCCACAACG    6180

AGCAGAACCCACCCAAGTTCGACAAGATTGAGGACATGGCCATGCTGACCTTCCTGCACG
TCGTCTTGGGTGGGTTCAAGCTGTTCTAACTCCTGTACCGGTACGACTGGAAGGACGTGC    6240

AGCCCGCGGTGCTTTTCAACCTCAAGGAGCGCTACGCGGCCTGGATGATATATgtgagtg
TCGGGCGCCACGAAAAGTTGGAGTTCCTCGCGATGCGCCGGACCTACTATATAcactcac    6300

                                                       BglI
gctcctgcacactgcagaggcttcctgtgctgcgtggaggcctaaataagccaggggggc
cgaggacgtgtgacgtctccgaaggacacgacgcacctccggatttattcggtcccccg    6360

tctcccaagaagagggggagagactcccaagggacccaagtccccttcccccctccaccc
agagggttcttctccccctctctgagggttccctgggttcaggggaaggggggaggtggg    6420
```

```
ctagtcagctgcaggaggagtagagccagctggagtgaacagggacatgcctggctgcca    6480
gatcagtcgacgtcctcctcatctcggtcgacctcacttgtccctgtacggaccgacggt

ccactgcctgtcccaggctctccccaccaacctcatgcccagccttgtctcctgctccag    6540
ggtgacggacagggtccgagaggggtggttggagtacgggtcggaacagaggacgaggtc

ACCTACTCGGGCCTCTTCTGTGTCACTGTCAACCCCTACAAGTGGCTGCCGGTGTACAAT    6600
TGGATGAGCCCGGAGAAGACACAGTGACAGTTGGGGATGTTCACCGACGGCCACATGTTA

GCCGAGGTGGTGGCCGCCTACCGGGGCAAGAAGAGGAGTGAGGCCCCGCCCCACATCTTC    6660
CGGCTCCACCACCGGCGGATGGCCCCGTTCTTCTCCTCACTCCGGGGCGGGGTGTAGAAG

TCCATCTCCGACAACGCCTATCAGTACATGCTGACAGgtgagcctggtggcccctggtct    6720
AGGTAGAGGCTGTTGCGGATAGTCATGTACGACTGTCcactcggaccaccggggaccaga

ctgctcctcctcccagacacccacccagatcctcagccctgaccccattgcttctcctct    6780
gacgaggaggagggtctgtgggtgggtctaggagtcgggactggggtaacgaagaggaga

tttttcttccagATCGGGAGAACCAGTCCATCCTCATCACgtgagcgagtgccatcctcc    6840
aaaaagaaggtcTAGCCCTCTTGGTCAGGTAGGAGTAGTGcactcgctcacggtaggagg

cacagaagggactgggctgggggcatacacgctgatgcctagggtgtagttgggaggaga    6900
gtgtcttccctgacccgacccccgtatgtgcgactacggatcccacatcaaccctcctct

ggtttaaggctgggattgcagggagcatggggcactgagctctcattagaggggtgccag    6960
ccaaattccgaccctaacgtccctcgtaccccgtgactcgagagtaatctccccacggtc

agcaatgaaccatgtcaggcaaatcctgctgggtgctgggctctgatggccaggctggga    7020
tcgttacttggtacagtccgtttaggacgacccacgacccgagactaccggtccgaccct

aggggagcatgtgatgctgagccctgtatggagaacagtaggaatcctgagttttgattg    7080
tcccctcgtacactacgactcgggacatacctcttgtcatccttaggactcaaaactaac

gtctctgtggcccccagGGGAGAATCCGGGGCGGGGAAGACTGTGAACACCAAGCGTGTC    7140
cagagacaccgggggtcCCCTCTTAGGCCCCGCCCCTTCTGACACTTGTGGTTCGCACAG

ATCCAGTACTTTGCCAGCATTGCAGCCATAGGTGACCGTGGCAAGAAGGACAATGCCAAT    7200
TAGGTCATGAAACGGTCGTAACGTCGGTATCCACTGGCACCGTTCTTCCTGTTACGGTTA

GCGAACAAGgtgccatgggggacacaggctcggcagaacaggggttggggggcaggctga    7260
CGCTTGTTCcacggtaccccctgtgtccgagccgtcttgtccccaaccccccgtccgact
```

VIII. APPENDIX II

LIST OF ABBREVIATIONS

aa	amino acids
AAS	Alpha actinin sarcomeric
AAV	Adeno-associated virus
AFP	Alpha Fetoprotein
aMSC(s)	Adipose Tissue Mesenchymal Stem Cells
AP	Alkaline Phosphatase
ASA	Alpha-Sarcomeric Actin
ASC(s)	Adult stem cells
AVs	Adenoviruses
bFGF	Basic Fibroblasts Growth Factor
BM	Bone Marrow
BMMSC(s)	Bone Marrow Mesenchymal Stem Cells
BMP4	Bone morphogenetic protein 4
bp	base pair
C/EBP	CCAAT/enhancer-binding protein
CAT	Committee For Advanced Therapies
CAT	Chloranfenicol acetyltransferase gene
CB	Coord Blood
cDNA	Complementary DNA
CM(s)	Cardiomyocytes
c-MYC	V-Myc Avian Myelocytomatosis Viral Oncogene
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNA	deoxyribonucleic acid
DNMT(s)	of DNA methyltransferases
DSB	double Strand Breaks
E(n)	Embryonic day (n)
EB(s)	Embryoid body or bodies
EBNA	Epstein Barr Nuclear Antigen 1
ECFP	Enhanced Cyan fluorescent protein from *Aequorea Victoria*
ECM	Cardiac Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EMT	Epithelial to Mesenchymal Ttransition
EpiSCs	Epiblast-derived Stem Cells
ES[4]	Embryonic Stem Cell Line no 4 derived from human Blastocyst
ESC(s)	Embryonic Stem Cells

FACS Fluorescence-Activated Cell Sorting
FBS Fetal Bovine Serum
FDA Food and Drug Administration
FGF Fibroblast Growth Factor
FGFR1 FGF receptor 1
FHF first heart field
G418 Neomycin
GFP Green Fluorescent Protein
GMP Good manufacturing Practice
gRNA Guiding RNA
GSK3 Glycogen synthase kinase-3
hbFGF Human basic Fibroblast Growth factor
hESC(s) Human Embryonic Stem Cells
HFF(s) Human Foreskin Fibroblasts
hiPSC(s) Human Induced Pluripotent Stem Cells
HIV-1 Human Immunodeficiency Virus
hPSC(s) Human Pluripotent Stem Cells
HR Homologous Recombination
HSC(s) Hematopoietic Stem Cells
ICM Inner Cell Mass
IGF Insulin growth factor
IMDM Iscove's Modified Dulbecco's Medium
iPSC(s) Induced Pluripotent Stem Cells
irHFF(s) Irradiated Human Foreskin Fibroblasts
irMEF(s) Irradiated Mouse Embryonic Fibroblasts
IWP4 Inhibitor Wnt signalling pathway 4
Kan Kanamycin
KLF4 Kruppel-Like Factor 4
KO Knock Out
KO-SR Knock Out Serum Replacement
lacZ β-galactosidase
LIF Leukemia inhibitory factor
MEF(s) Mouse embryonic fibroblasts
mESC(s) Mouse embryonic StemCells
miPSC(s) Mouse Induced pluripotent Stem Cells
miR MicroRNA
miRNA(s) MicroRNAs
MLC2v ventricular myosin light chain 2
mRNA Messenger RNA
MSC(s) Mesenchymal Stem Cells
MYH6 alpha myosin heavy chain
NEAA Non-Essential Aminoacids
Neo Neomycin
NHEJ Non-homologous end joining
OCT4 or OCT3/4 octamer-binding transcription factor 4 (also known as POU5F1, POU domain class 5 transcription factor 1)
ORF Open Reading Frame

OS OCT3/4, SOX2
OSK Oct4, Sox2, Klf4
OSKM Oct4, Sox2, Klf4 and c-Myc
P/S Penicilin-Streptomycin
PB-MNC peripheral blood mononuclear cells
PBS Phosphate buffered saline
PEI Poly-ethylenimine
PGC(s) primordial germ Cells
PSC(s) Pluripotent Stem Cells
RA Retinoic Acid
RNA Ribonucleic Acid
RVDs repeated variable diresidues
SB Sleeping Beauty trasnposon system
SCA Sickle Cell Anemia
SCNT Somatic cell nuclear transfer
SCs Stem Cells
SDS Sodium dodecyl sulfate
sgRNA single guide RNA
SHF Second heart field
SHH Sonic hedgehog
siRNA(s) Small Interfiring RNA
SMA Smooth Muscle Actin
SOX2 SRY (sex determining region Y)-box 2
SRF Serum Response factor
SSEA Stage specific Embryonic antigen
TAE Tris-acetate-EDTA
TAL Transcription Activator-like
TALE Transcription Activator-like Effectors
TALEN Transcription Activator-like Effectors nucleases
TBS Tris-buffered saline
TE(s) Transposable Elements
TF Transcription Factor
TIL tumor-infiltrating lymphocytes
tracrRNA *trans*-activating crRNA
ucMSC(s) Umbilical Cord Mesenchymal Stem Cells
VPA valproic acid
Yamanaka factors Oct4, Sox2, Klf4 and c-Myc
ZFN Zinc Finger Nucleases
MHC alpha myosin heavy chain

VIII. APPENDIX III

LIST OF PUBLICATIONS

1. Published:

Research on skeletal muscle diseases using pluripotent stem cells.
de Oñate L, Garreta E, Tarantino C, Martínez E, Capilla E, Navarro I, Gutiérrez J, Samitier J, Campistol J, Muñoz P, Montserrat N.
Intech 2015-Book Chapter.

Generation of feeder-free pig induced pluripotent stem cells without Pou5f1.
Montserrat N, de Oñate L, Garreta E, González F, Adamo A, Eguizábal C, Häfner S, Vassena R, Izpisúa Belmonte JC.
Cell Transplant. 2012;21(5):815-25

Accumulation of instability in serial differentiation and reprogramming of parthenogenetic human cells.
Vassena R, Montserrat N, Carrasco Canal B, Aran B, de Oñate L, Veiga A, Izpisúa Belmonte JC..
Hum Mol Genet. 2012 Aug 1;21(15):3366-73

2. Peer-reviewed accepted:

Characterization of the epigenetic changes during human gonadal primordial germ cells reprogramming.
Eguizábal C, de Oñate L, Montserrat N, Hajkova P, Izpisúa Belmonte JC.
EMBOReports-2015-40645V1

3. In preparation:

Alpha myosin heavy chain (MYH6) reporter cell line generated by direct targeting human embryonic stem cells
de Oñate L, Garreta E, Suzuki K., Montserrat N.*, Izpisúa Belmonte J.C.*

Generation of induced pluripotent stem cells from umbilical cord derived mesenchymal stem cells with OCT4 and SOX2.
de Oñate L, Cortina C, González F, Garreta E, Montserrat N.*, Izpisúa Belmonte J.C*.

de Oñate L, Garreta E, Tarantino C, Martínez E, Capilla E, Navarro I, Gutiérrez J, Samitier J, Campistol JM, Muñoz-Cánovas P, Montserrat N. Research on skeletal muscle diseases using pluripotent stem cells. Dins: Muscle cell and tissue. Sakuma K (editor). Intech, 2015. Chapter 12. doi:10.5772/60092

Montserrat N, de Oñate L, Garreta E, González F, Adamo A, Eguizábal C, Häfner S, Vassena R, Izpisua Belmonte JC. Generation of feeder-free pig induced pluripotent stem cells without Pou5f1. Cell Transplant. 2012;21(5):815-25. doi: 10.3727/096368911X601019

Vassena R, Montserrat N, Carrasco Canal B, Aran B, de Oñate L, Veiga A, Izpisua Belmonte JC. Accumulation of instability in serial differentiation and reprogramming of parthenogenetic human cells. Hum Mol Genet. 2012 Aug 1;21(15):3366-73. doi: 10.1093/hmg/dds168

Eguizabal C, Herrera L, De Oñate L, Montserrat N, Hajkova P, Izpisua Belmonte JC. Characterization of the Epigenetic Changes During Human Gonadal Primordial Germ Cells Reprogramming. Stem Cells. 2016 Sep;34(9):2418-28. doi: 10.1002/stem.2422