Universitat Internacional de Catalunya
Regenerative Medicine Research Institute

Osteogenic differentiation strategies of Dental Pulp Pluripotent-like Stem Cells (DPPSC) for their potential application in Bone Tissue Engineering

PhD Thesis

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"Leave this world a little better than you found it"

Robert Baden-Powell

(Founder of the World Scouting Movement)

Agraïments / Acknowledgements

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Abstract

Bone defects due to trauma or disease affect millions of people worldwide, placing an even larger demand on the healthcare system to replace and restore bone loss. Recently, bone tissue engineering (BTE), combining biomaterials, cells and growth factors, has emerged as a promising therapeutic approach to treat large bone defects. For this purpose, pluripotent stem cells (PSC) are an attractive option. These cells can proliferate indefinitely *in vitro*, have self-renewal, high replicative capacity and are capable of differentiating into most cell types of the body. In addition, PSC have a higher value when testing the differentiation capacity of biomaterials; since these undifferentiated cells need to be guided merely by the biomaterial to their final fate. However, there is still a need to find a cell type with genetic stability, stemness characteristics and no ethical problems to be used in BTE approaches.

In previous studies, our group described a pluripotent-like population of dental pulp stem cells derived from the third molars (DPPSC) that show genetic stability and share some pluripotent characteristics with embryonic stem cells. Until now, it has been studied the differentiation capacity of DPPSC into cells of different tissues from the three embryonic layers. In fact, it has been demonstrated they capacity to differentiate into bone-like tissue, even more than other dental pulp stem cells. This doctoral thesis introduces the use of DPPSC as a good alternative model for BTE approaches, either to direct bone regeneration therapy or to evaluate biomaterials before being applied. For this purpose, the thesis is divided in three main chapters.

As a first step, the osteogenic differentiation process in DPPSC and their ability to grow, attach and differentiate were evaluated with different types of biomaterials commonly used in BTE studies, such as metals or natural scaffolds. Results reveal high osteogenic and adhesion potential of DPPSC on both biomaterials without acquiring genetic alterations. Thus, proposing the use of DPPSC as a good model to evaluate the biocompatibility and the osteogenic capacity of different biomaterials.

Afterwards, different strategies were used to improve the osteogenesis of DPPSC for their potential application in BTE approaches:

Firstly, a novel polymeric nanoparticle system was used as a non-viral gene delivery method to improve the osteogenic differentiation of DPPSC by silencing the expression of pluripotency genes (OCT3/4 and NANOG) and enhancing the expression of the osteogenic gene RUNX2. Thus, the combination of poly(β-amino ester)s (pBAEs) with natural oligopeptides was used in order to prove their biocompatibility with DPPSC and to simultaneously deliver anti-OCT3/4 siRNA, anti-NANOG siRNA, and RUNX2 plasmid in DPPSC. Results show that DPPSC can be transfected with these vectors whereas they maintain their viability and genetic stability. Furthermore, the delivery of siOCT3/4 in combination with pRUNX2 robustly accelerates the osteogenic differentiation of DPPSC.

Secondly, different strategies were tested in order to induce the vascularization of the BTE constructs using DPPSC. This vascularization consequently, should improve the viability of the construct and the osteogenic differentiation process. Previous results showed that DPPSC also have a high endothelial potential. Thus, we suggested the combination of bone-like DPPSC and endothelial-like DPPSC to induce vascularized bone formation from the same stem cell population. Hence, different co-culture systems were analysed. Furthermore, in order to take this approach towards clinical assays, the osteogenic and endothelial medium compositions were tested using human serum (HS) to supplement the medium, instead of the commonly used animal-derived fetal bovine serum (FBS). In addition, the effect of bioactive glass (BaG) ions, characterized for their high osteogenic properties, was examined in DPPSC co-cultures and monocultures. Results demonstrate that endothelial medium with BaG extracts can provide an effective way to enhance both, osteogenic and endothelial processes, supporting the formation of vascular-like structures in DPPSC co-cultures. Therefore, the co-culture of osteogenic and endothelial pre-differentiated DPPSC in combination with BaG and xeno-free medium conditions provides a new promising system for the *in vitro* vascularization of the BTE constructs. Taken together, the findings described in this doctoral thesis propose DPPSC as a good alternative stem cell population for different BTE approaches.

Resum

Actualment, els defectes ossis, ja sigui deguts a traumes o a malalties, afecten a milions de persones de tot el món suposant una demanda constant al sistema sanitari per al reemplaçament o la restitució d'os. En els últims anys, les tècniques de medicina regenerativa i d'enginyeria tissular d'ós que combinen l'ús de biomaterials, cèl·lules i factors de creixement, han esdevingut un recurs terapèutic molt prometedor per al tractament de defectes ossis. Les cèl·lules mare pluripotents poden ser de gran valor en enginyeria tissular degut al seu potencial d'auto-renovació, la seva alta capacitat de proliferació i el seu potencial de diferenciació a la majoria de llinatges cel·lulars del cos. A més, poden servir com a model per a testar l'eficiència dels nous biomaterials ja que necessiten ser guiades pròpiament pel material per a diferenciar-se. Tot i així, encara no s'ha identificat un tipus cel·lular amb característiques pluripotents, controlat genèticament i sense problemes ètics per a ser utilitzat en tècniques d'enginyeria tissular d'os. En estudis previs, el nostre grup ha descrit una nova població de cèl·lules mare amb característiques pluripotents de la polpa dental del tercer molar: les DPPSC (de l'anglès, Dental Pulp Pluripotent-like Stem Cells). Aquestes cèl·lules semblen estar presents fins a edat avançada, tenen una bona estabilitat genètica i mostren característiques pluripotents típiques de cèl·lules mare embrionàries. Fins ara, s'ha demostrat la seva capacitat de diferenciar-se a cèl·lules de teixits de les tres capes embrionàries. A més a més, semblen tenir una major capacitat de diferenciar-se a cèl·lules del teixit ossi que altres poblacions de cèl·lules mare de la polpa dental.

Aquesta tesis doctoral pretén doncs introduir l'ús de les DPPSC com a model cel·lular alternatiu per a tècniques de medicina regenerativa i d'enginyeria tissular d'os. La tesis s'ha dividit en tres parts principals:

Com a primer pas, el procés de diferenciació osteogènica de les DPPSC i les seves habilitats per créixer, adherir-se i diferenciar-se han estat avaluades a partir de diferents tipus de biomaterials tradicionalment utilitzats en estudis de regeneració òssia (metalls o materials d'origen natural). Els resultats mostren un alt potencial de diferenciació osteogènica i d'adhesió de les DPPSC als materials estudiats, així com una alta estabilitat genètica. Proposant en

conseqüència l'ús de les DPPSC com a bon model cel·lular per avaluar la biocompatibilitat i la capacitat osteogènica de diferents tipus de biomaterials.

A continuació, diferents estratègies s'han utilitzat per millorar el procés d'osteogènesis a partir de les DPPSC per a la seva potencial aplicació en teràpies d'enginyeria tissular d'os.

Una nova família de polímers ha estat utilitzada com a teràpia gènica no viral per a la millora de la diferenciació osteogènica en DPPSC a partir del silenciament de gens de pluripotència i la sobre-expressió de gens de osteogènesis. Concretament, s'ha utilitzat com a vector la combinació de poli(β-amino ester)s (pBAEs) amb oligopèptids d'origen natural per tal de millorar la seva biocompatibilitat amb les DPPSC i alliberar-ne simultàniament anti-OCT3/4 siRNA, anti-NANOG siRNA i plàsmid RUNX2. Els resultats mostren que les DPPSC poden ser perfectament transfectades amb aquests polímers mantenint la viabilitat cel·lular i l'estabilitat genètica. A més a més, la combinació de silenciar OCT3/4 i potenciar RUNX2 n'accelera la diferenciació osteogènica.

Seguidament, diferents estratègies han estat provades per tal d'induir vascularització en els constructes d'enginyeria tissular fets a partir de les DPPSC, fet important per a garantir la supervivència dels constructes un cop implantats *in vivo* i que a l'hora permet millorar la osteogènesis. Resultats previs mostren que les DPPSC tenen un alt potencial endotelial. Per tant, en aquest estudi, s'ha suggerit la combinació de DPPSC pre-diferenciades a teixit ossi amb DPPSC pre-diferenciades a teixit endotelial com a una bona estratègia per induir os vascularitzat a partir d'una única població de cèl·lules mare. Així, diferents sistemes de co-cultius amb DPPSC han estat analitzats. A més a més, per tal d'apropar els resultats a la teràpia clínica, tant les diferenciacions en monocultiu com en co-cultiu de les DPPSC han estat testades en medis lliures de components animals, reemplaçant el sèrum fetal boví per sèrum humà. Per altra banda, l'efecte de vidres bioactius (BaG, de l'anglès Bioactive Glasses), caracteritzats per les seves propietats osteogèniques, també ha estat investigat en monocultius i co-cultius de DPPSC. Els resultats mostren que el medi endotelial condicionat amb extractes de BaG pot promoure tant el potencial osteogènic com l'endotelial de les DPPSC, promovent la formació d'estructures vasculars en els sistemes de co-cultiu. Així, el co-cultiu de DPPSC

prediferenciades a teixit ossi i endotelial en combinació amb BaG i en medis lliures d'origen animal proporciona un nou sistema per a la vascularització *in vitro* de constructes ossis d'enginyeria tissular.

En conclusió, els resultats dels estudis realitzats en aquesta tesis doctoral proposen l'ús de les DPPSC per diferents tècniques d'enginyeria tissular d'os i medicina regenerativa.

Original Publications

I. Chatakun P, **Núñez-Toldrà R**, Díaz López EJ, Gil-Recio C, Martínez-Sarrà E, Hernández-Alfaro F, Ferrés-Padró E, Giner-Tarrida L, Atari M. The effect of five proteins on stem cells used for osteoblast differentiation and proliferation: a current review of the literature. *Cellular and Molecular Life Sciences*. 2014;71:113-42.

II. **Núñez-Toldrà R**, Martínez-Sarrà E, Gil-Recio C, Carrasco MA, Al Madhoun A, Montori S, Atari M. Dental pulp pluripotent-like stem cells (DPPSC), a new stem cell population with chromosomal stability and osteogenic capacity for biomaterials evaluation. *BMC Cell Biology*. 2017;18:21.

III. **Núñez-Toldrà R**, Dosta P, Montori S, Ramos V, Atari M, Borrós S. Improvement of osteogenesis in dental pulp pluripotent-like stem cells by oligopeptide-modified poly(beta-amino ester)s. *Acta Biomaterialia*. 2017;53:152-64.

Congress Participation

I. **Núñez-Toldrà R**, Martínez-Sarrà E, Gil-Recio C,Hategan I, Carrió N, Ferrés-Amat E, Giner L, Montori S, Atari M. Dental pulp pluripotent-like stem cells (DPPSC) as a new source of adult Stem Cells with chromosomal stability. *World Conference on Regenerative Medicine*. Leipzig, November 2013. (Poster Presentation).

II. **Núñez-Toldrà R**, Martínez-Sarrà E, Gil-Recio C, Piñera M, Giner-Tarrida L, Montori S, Atari M. DPPSC as a new cell type for osteogenic differentiation and biomaterials evaluation. *International Bone & Mineral Society - Herbert Fleisch Workshop*. Bruges, April 2014. (Poster Presentation).

III. **Núñez-Toldrà R**, Martínez-Sarrà E, Gil-Recio C, Giner L, Montori S, Atari M. Dental pulp pluripotent-like stem cells (DPPSC) as new stem cells with chromosomal stability for osteogenic differentiation and biomaterials evaluation. *Gene Regulation, Stem Cells & Cancer, 13th CRG Symposium.* Barcelona, November 2014. (Poster Presentation).

IV. **Núñez-Toldrà R**, Martínez-Sarrà E, Gil-Recio C, Giner-Tarrida L, Montori S, Atari M. Dental pulp pluripotent-like stem cells (DPPSC) as a new cell population with genetic stability for biomaterials evaluation in bone regeneration studies. *International Bone Tissue Engineering Congress.* Stuttgart, October 2015. (Oral Communication).

Table of Contents

Abbreviations

AcONa: Sodium Acetate

ADSC: Adipose Derived Stem Cells

ALP: Alkaline Phosphatase

ASC: Adult Stem Cells

BaG: Bioactive Glass

BM-MSC: Bone Marrow Mesenchymal Stem Cells

B.DPPSC: Bone-like DPPSC 15 days differentiated

BM: Basal Medium

BMP-4: Bone Morphogenic Protein 4

BSP: Bone Sialoprotein

BTE: Bone Tissue Engineering

CCC: Collagen I-based Cell Carrier

CD31: Cluster of differentiation 31

C/EBPβ: CCAAT/enhancer-binding protein β

COL1: Collagen type I

COL2: Collagen type II

COL IV: Collagen type IV

DLX5: Distal-less homeobox 5

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic Acid

DPMSC: Dental Pulp Mesenchymal Stem Cells

DPSC: Dental Pulp Stem Cells

DPPSC: Dental Pulp Pluripotent-like Stem Cells

EC: Endothelial Cells

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

EGM-2: Endothelial Growth Medium 2

EM: Endothelial Medium

EM-BaG: Endothelial Medium conditioned with BaG ions dissolution

EM-FBS: Endothelial Medium supplemented with FBS

EM-HS: endothelial medium supplemented with HS

ESC: Embryonic Stem Cells

FACS: Fluorescent Activated Cell Sorter

FBS: Foetal Bovine Serum

FGF: Fibroblast Growth Factor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GA: Gentamicin / Amphotericin-B

GAM: Gene Activated Matrix

GAGs: Glycosaminoglycans

GFP: Green Fluorescence Protein

GMP: Good manufacturing practices

HCA: Hydroxyl Carbonate Apatite

HLA-DR: Human Leukocyte Antigen - antigen D Related

HS: Human Serum

HUVEC: Human Umbilical Endothelial Cells

IF: Immunofluorescence

IHC: Immunohistochemistry

iPSC: Induced Pluripotent Stem Cells

ITGα3: integrin α3

ITGαV: integrin αV

KLF4: Kruppel-like factor 4

LA-BSA: Linoleic Acid Bovine Serum Albumin

LIF: Leukemia Inhibitory Factor

MAPC: Multipotent Adult Progenitor Cells

MIAMI: Marrow Isolated Adult Multilineage Inducible

miRNA: micro ribonucleic acid

MPC: Mesodermal Progenitor Cells

MSC: Mesenchymal Stem Cells

NANOG: Nanog homeobox

OCT4/POU5F1: Octamer-binding Transcription Factor 4

OC: Osteocalcin

OM: Osteogenic Medium

OM-BaG: Osteogenic Medium conditioned with BaG ions dissolution

OM-FBS: Osteogenic Medium supplemented with FBS

OM-HS: Osteogenic Medium supplemented with HS

OM/EM: mixture of Osteogenic and Endothelial Medium

OPN: Osteopontin

OSX: Osterix

pBAEs: poly (β -Amino Ester) polymers

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

PDGF: Platelet-derived growth factor

PDLSC: Periodontal Ligament Stem Cells

PPARγ : Peroxisome Proliferator-Activated Receptor γ

pRUNX2: RUNX2 Plasmid

PSC: Pluripotent Stem Cell

P/S: Penicillin / Streptomycin

qALP: Quantitative Alkaline Phosphatase Activity

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

RISC: RNA-Induced Silencing Complex

RNA: Ribonucleic Acid

RPLP0: Large Ribosomal Protein P0

RT-PCR: Retro-Transcriptase Polymerase chain reaction

RUNX2: Runt-related transcription factor 2

SAOS-2: Human osteosarcoma cell line

SCAP: Stem Cells from Apical Papilla

SEM: Scanning electron microscopy

SHED: Stem Cells from Human Exfoliated Deciduous Teeth

Short-CGH: Short-Comparative Genomic Hybridization

siOCT3/4: anti-OCT3/4 siRNA

siNANOG: anti-NANOG siRNA

siRNA: Small Interference Ribonucleic Acid

SOX2: Sex determining region Y-box2

SSEA: Stage Specific Embryonic Antigen

TGF-β: Transforming growth factor beta

Ta: Talantum

Ti: Titanium

TI DPPSC: DPPSC on Ti alloy disks.

TI B.DPPSC: B.DPPSC differentiating on TI alloy disks.

TI SAOS-2: SAOS-2 differentiating on Ti alloy disks.

VEGF: vascular endothelial growth factor

VEGFR2/FLK1/KDR/: Vascular Endothelial Growth Factor Receptor 2

VLA4: Integrin α4 β1

VSEL: Very Small Embryonic like

vWF: von Willebrand Factor

WNT: portmanteau of Wingless and integration 1

CHAPTER 1: INTRODUCTION

1.1 BON TISSUE

1.1.1 Bone Structure

Bones are vascularized and innervated organs that are composed of bone tissue, bone marrow and periosteum, an adjacent connective tissue. They assist a number of body functions such as: locomotion, load-bearing capacity of the skeleton, protective casing for the internal organs of the body and sound transmission. Moreover, bones are involved in homeostasis through its storage of calcium and phosphate ions and by regulating the concentration of key electrolytes in the blood. In total, the adult human skeleton is composed of 213 bones (excluding the sesamoid bones); ranging from the long bones of our limbs, short bones in the wrists and ankles, flat bones in the sternum and skull to the irregular bones such as the pelvis and vertebrae [1-3].

Bone tissue is the rigid calcified portion of bone in a continual chemical exchange and structural remodelling due to both internal mediators and external mechanical demands. Bone tissue can be classified in cortical (compact) bone or trabecular (spongy) bone. 80% of cortical bone and 20% of trabecular bone composed the adult human skeleton. However different bones have different ratios of cortical to trabecular bone [5].

Cortical bone is a dense and solid tissue found on bone peripheral regions. It is highly mineralized and is important for mechanical and structural functions. On the other hand, trabecular bone is involved in calcium homeostasis and acid/base regulation. It is found in the interior of bones adjacent to the marrow cavity. It is porous (80%) and exhibits higher surface area than cortical bone [2, 4].

Both cortical and trabecular bone are composed of osteons formed in concentric lamellae where the osteocytes are entrapped between them. Trabecular osteons are called packets and cortical osteons are called Haversian systems. The outer surface of the cortical bone is called the periosteum and the inner surface is called the endosteum. The remodelling activity of the bone is focussed on these surfaces. In the periosteum the bone formation typically exceeds bone resorption, which is important for fracture repair and appositional growth, hence bones normally increase in diameter with aging. In

contrast, the reverse is characteristically in the endosteum, where the bone marrow space increases with aging (Figure 1.1) [3].

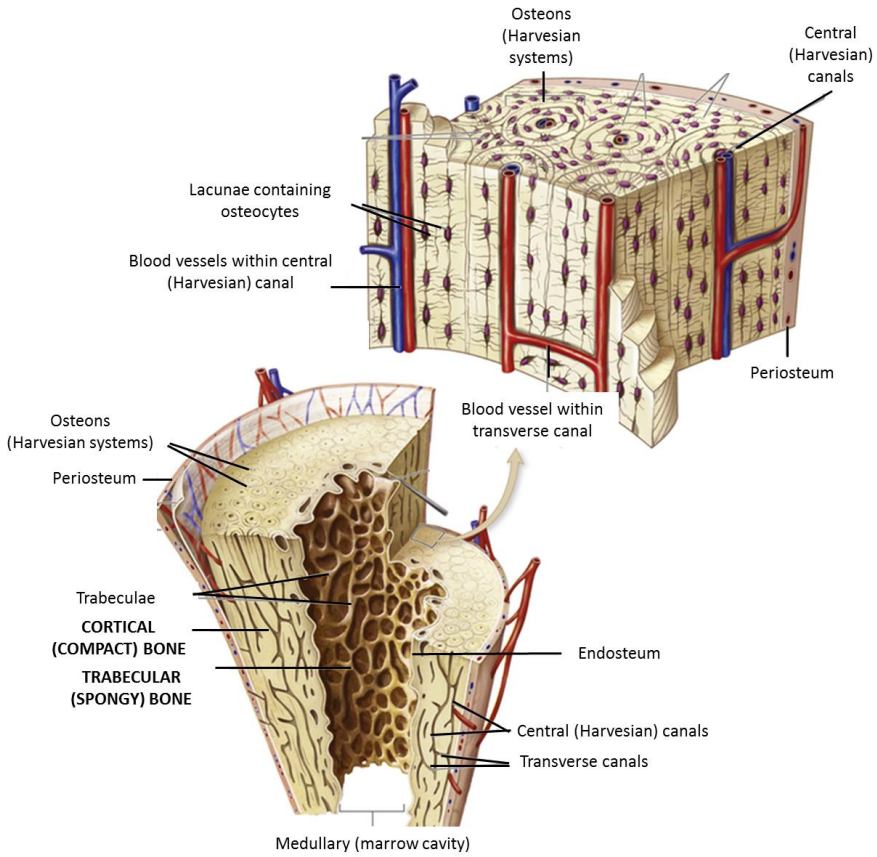


Figure 1.1. Structure of the long bone. Modified from Gunson, Gropp & Varela (2013) [5].

1.1.2 Bone Development

Bone formation occurs by two distinct modes of ossification, intramembranous ossification and endochondral ossification. In each case, first of all, mesenchymal cellular condensation occurs and serves as a template for subsequent bone formation (Figure 1.2) [2, 4, 6].

Intramembranous ossification involves mesenchymal progenitor cells that differentiate directly into osteoblasts without a pre-existing cartilage model. It takes place in the formation of flat and irregularly shaped bones, such as the cranial bones [4, 7].

In contrast, endochondral ossification occurs in several steps. The process begins with mesenchymal condensations followed by the differentiation to cartilage producing cells, chondrocytes, and subsequent growth generates a cartilage template for future bone tissue. Then, the cartilage begins to be calcified. As the cartilage template calcifies, the chondrocytes become hypertrophic and attract blood vessels through the production of angiogenic factors. Moreover, they direct adjacent perichondral cells to become osteoblasts, and thereafter undergo apoptotic cell death, creating bone marrow cavity. Endochondral ossification is characteristic in the formation of bones on the trunk and extremities [4, 7].

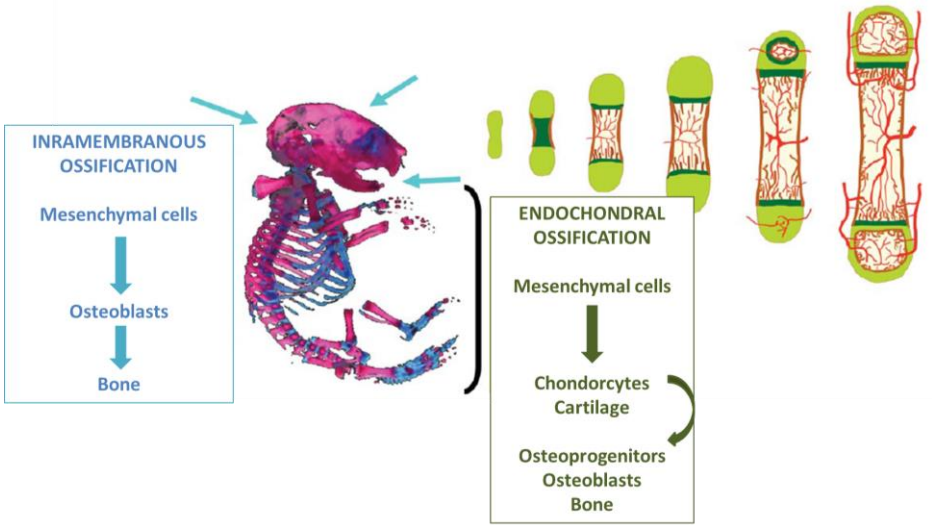


Figure 1.2. Mechanisms of bone development. Different skeletal elements develop through intramembranous or endochondral ossification, as indicated on a skeletal preparation stained with Alizarin red (bone) and Alcian blue (cartilage). Extracted from Dirckx *et al.* (2013) [7].

1.1.3 Bone Regeneration

Each bone constantly undergoes remodelling during life to adapt to changing biomechanical forces, as well as to remove old or microdamaged bone and replace it with a new, mechanically stronger bone [3]. Bone formation and bone remodelling are controlled by three main cell types: osteoblasts, osteocytes, and osteoclasts.

Osteoblasts are the bone matrix-forming cells that also regulate mineralisation. They have extensive cell-cell and cell-matrix contacts. Osteoblasts deposit osteoid, the unmineralized extracellular matrix (ECM), which successively becomes calcified. Through this process, a proportion of osteoblasts become trapped in their own calcified matrix, changes their phenotype and, finally, become osteocytes. Osteocytes remain connected creating an extensive network with other similar cells and quiescent bone-lining cells. This is the lacunar-canalicular network, used for nutrient and waste transfer, as well as for communication between osteocytes via gap junctions. The other proportion of osteoblasts becomes bone-lining cells, which are flat cells lining the surface of bone. On the other hand, osteoblasts also influence the differentiation of osteoclasts, multinucleated bone resorbing cells of the family of monocyte/macrophage lineage derived from hematopoietic stem cells [4, 8]. Hence, the remodelling bone cycle can be divided in four consecutive phases (Figure 1.3) [3]:

- **Activation:** hormonal or physical stimuli recruit mononuclear pre-osteoclasts from the circulation to the bone remodelling site. Following attachment to the bone surface, cells fuse creating multinucleated osteoclasts.

- **Resorption:** osteoclasts initiate resorption of organic and mineral bone components. Then, osteoclasts originate Howship's lacunae in trabecular bone and a cutting cone in cortical bone. After these cavities reach a certain size, apoptosis of osteoclasts terminates bone resorption.

- **Reversal:** the resorbed bone surface is smoothed by mononuclear macrophage-like cells and prepared for matrix deposition.

- **Formation:** osteoblasts lay down new bone by secreting a collagen matrix and controlling its mineralization. During this process, some osteoblasts become trapped within the matrix and differentiate to osteocytes which reside in the fully mineralized lacunar-canalicular system. After 4–6 months, this phase is completed and the other osteoblasts turn into bone-lining cells or enter apoptosis.

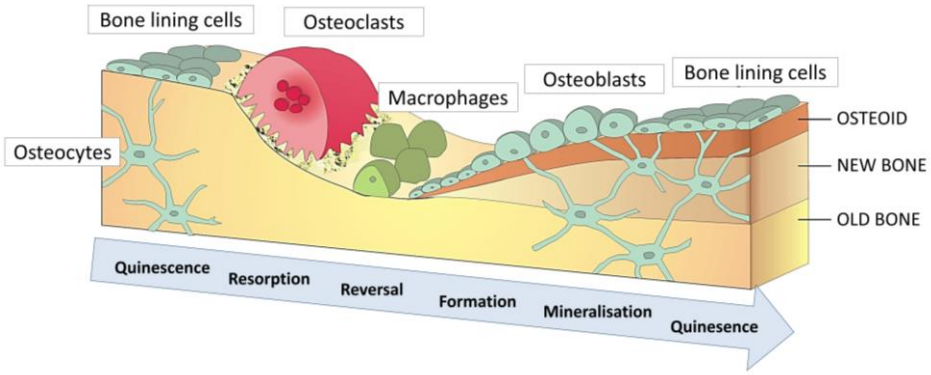


Figure 1.3. Diagram of the bone remodelling process. Bone is continuously remodelled at discrete sites in the skeleton in order to maintain the integrity of the tissue. During this process, old bone is resorbed by osteoclasts and replaced with new osteoid, secreted by osteoblasts. First osteoclasts are activated, and the resorption phase takes approximately 10 days. Following resorption, unclassified macrophage-like cells are found at the remodelling site in the intermediate, or reversal phase. Osteoblast precursors are then recruited and started to proliferate and differentiate into mature osteoblasts before secreting new bone matrix. Afterwards, the matrix then mineralises to generate new bone and this completes the remodelling process. Extracted from © *Biomedical Tissue Research, University of York*.

Upon a fracture, bone is repaired by a process that recapitulates many of the events of both intramembraneous and endochondral ossification. Firstly, hematoma formation occurs accompanied by an inflammatory response and the recruitment of signalling molecules involved in the regulation of new bone

formation (i.e., ILs, TNF-α, FGFs, BMPs, PDGF, VEGF). This series of events allow subsequent steps by initializing angiogenesis, chemotaxis, and mesenchymal stem cell differentiation [9, 10]. At the cortex and periosteum, intramembranous bone formation immediately occurs. In contrast, in the external soft tissues the fracture is stabilized by the formation of a callus, which subsequently undergoes chondrogenesis, and then a process highly similar to endochondral ossification. Briefly, after the callus forms, chondrocyte proliferation decreases as the tissues begin to mature and calcify the matrix. Then, in-growing blood vessels transport chondroclasts, responsible for resorbing the calcified cartilage, and osteoblastic progenitors, which initiate new bone formation. The mechanical continuity of the cortex is achieved by subsequent remodelling of the newly formed bone [1, 7].

1.1.4 Osteoblasts differentiation process

Osteoblast lineage cells, including osteoprogenitors, osteoblasts, and osteocytes, derive from mesenchymal stem cells (MSC). MSC are adult stem cells from bone marrow, periosteum and other tissue sources that are capable of differentiating towards the osteoblastic, chondrogenic, adipogenic, and myogenic cell lineage.

Recruitment, proliferation and differentiation of MSC within bone tissue are regulated by the expression of different osteogenic genes. Hence, the osteoblastic development can be divided in different developmental stages with characteristic changes in gene expression: cell proliferation, cell differentiation, ECM synthesis, development and maturation and, finally, mineralization [7, 11] (Figure 1.5). During the first osteoblastic stages, the pre-osteoblastic cells predominantly express genes which support proliferation and ECM synthesis. Then, the first transition producing the initiation of osteoblastic gene expression occurs after the active proliferation period, where osteoprogenitors express two essential transcription factors: Runt-related transcription factor 2 (RUNX2) and osterix (OSX).

Various cytokines, such as BMP2, TGFβ and portmanteau of Wingless and integration1 (Wnt) ligands, can enhance the expression of RUNX2 and OSX

through different pathways. After the pathway is activated, RUNX2 and OSX play different particular roles in different stages of osteoblastic lineage. RUNX2 is considered to be the "master gene" of osteogenesis because it regulates the differentiation of progenitor cells to pre-osteoblasts and is required for the expression of non-collagenous proteins such as bone sialoprotein (BSP) and osteocalcin (OC) [12]. OSX is determined to act downstream RUNX2 and it is also essential for osteoblast differentiation and function. Both, RUNX2 and OSX support the downstream effects of multiple osteogenic factors and regulate gene expression of major bone matrix proteins during osteoblast differentiation [12, 13]. However, RUNX2 can also inhibit the differentiation of pre-osteoblasts into active osteoblasts (Figure 1.4).

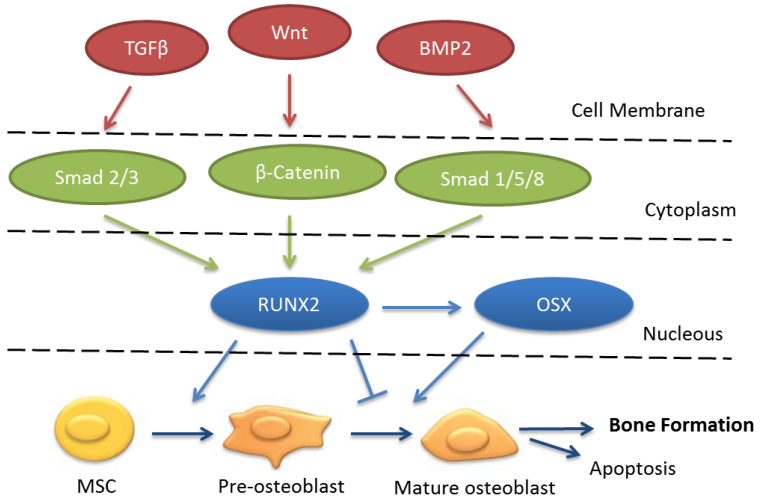


Figure 1.4. Schematic diagram with the signalling pathway for RUNX2 and OSX during the osteoblast differentiation process.

As they mature, pre-osteoblasts produce abundant matrix proteins, such as collagen type I (COL1) which they deposit as osteoid, the non-mineralized bone matrix [14]. On the other hand, pre-osteoblasts begin to express alkaline phosphatase (ALP), which regulates phosphate metabolism by hydrolysing phosphate esters and functioning as a plasma membrane transporter for

inorganic phosphates contributing to the mineralization of the ECM [15]. When pre-osteoblasts transform to fully differentiated osteoblasts, high expression of OC and BSP occurs with the beginning of mineralization. BSP is non-collagenous protein that binds to COL1 and nucleates the hydroxyapatite crystal formation, indicating the initiation of the mineralization stage. OC, the most abundant osteoblast-specific non-collagenous protein is involved in the binding of calcium and hydroxyapatite [7]. Osteopontin (OPN) is also up-regulated during the mineralization process [16]. Therefore, the osteoid becomes mineralized by the formation of hydroxyapatite. Finally, an osteoblast can become surrounded by its own matrix and terminally differentiates into an osteocyte playing an important role in bone cell communication. Otherwise, a mature osteoblast becomes a lining cell or undergoes apoptosis [7] (Figure 1.5). However, although the temporal expression of various osteogenic markers has been described, the progress of differentiation is a more heterogeneous process in practice, because the osteogenic population contains cells of various differentiation stages [17].

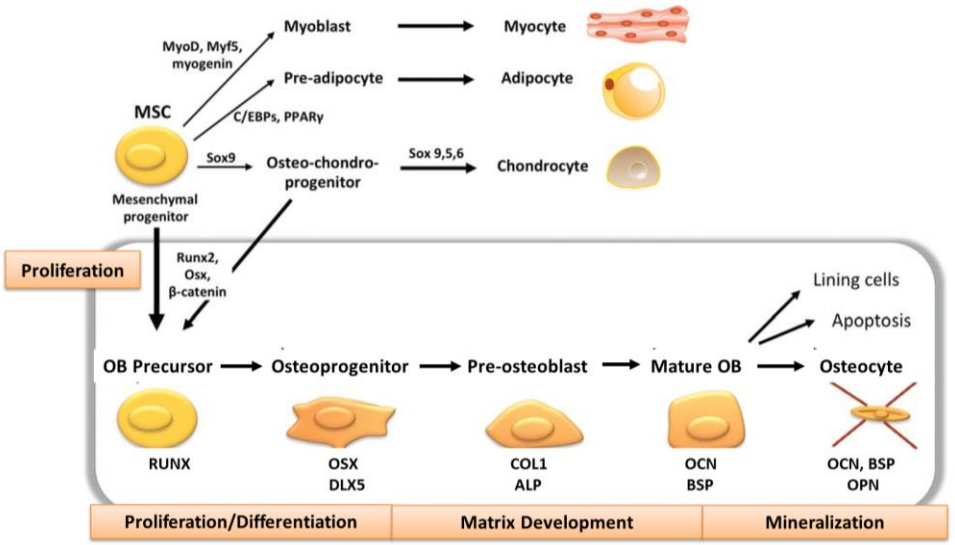


Figure 1.5. Osteogenic differentiation process. Stepwise representation of osteoblast (OB) differentiation from mesenchymal stem cells with indication of typical

differentiation stages and their dependent markers. Modified from Dirckx *et al.* (2013) [7].

1.1.5 Clinical need for bone regeneration

The worldwide incidence of bone disorders is continuously increasing and is expected to double by 2020, particularly in populations where aging is coupled with increased obesity and poor physical activity [18]. Furthermore, elderly people are the fastest growing population in the world and, as people age, bone mass declines and the risk of fractures increases. An estimated 2.2 million bone graft procedures are performed every year to promote fracture healing, fill defects, or repair spinal lesions [19].

In addition, one of the main public health problems throughout the world and that currently affects approximately 200 million women is osteoporosis. Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture. Although the possibility of developing osteoporosis currently is highest in North America and Europe, it will increase in developing countries as population longevity continues to increase [20].

Even though most of the fractures will heal well without the need for major intervention due the high regenerative capacity of bone, some large bone defects, as observed after bone tumour resections and severe non-union fractures, usually require surgical interventions. Extensive studies have reported the considerable limitations of current clinical treatments for bone regeneration; these include autologous and allogeneic transplantations using autografts and allografts [18, 21, 22].

Currently, the gold standard treatment is the use of autografts (autologous bone transplants) because they are histocompatible and non-immunogenic, and they offer all of the properties required of a bone graft material [1, 23]. Nevertheless, it is a very expensive procedure, and it may result in significant donor site injury, morbidity, deformity and scarring. Moreover, autografts are associated with surgical risks as well: bleeding, inflammation, infection and chronic pain [24-26]. Furthermore, when the defect site requires larger bone sizes it can be a null treatment option.

On the other hand, allografts, the second most common bone-grafting treatment; involve transplanting donor bone tissue, often from a cadaver. Allogeneic bone is also likely histocompatible, and is available in various forms depending on the host-site requirements. However, there are immunoreactions and transmission of infections risks associated with these types of transplants. Moreover, they present reduced osteoinductive properties and they have no cellular component, because donor grafts are devitalized [27, 28]. Furthermore, the bone grafting market is experiencing a great demand and a shortage in allograft bone material [26]. Other commonly used bone repair techniques may involve distraction osteogenesis, bone cement fillers and bone morphogenic proteins.

Although the previously mentioned clinical interventions have been shown to improve bone repair, none possess all of the ideal characteristics: high osteoinductive and angiogenic potentials, biological safety, low patient morbidity, no size restrictions, ready access to surgeons, long shelf life and reasonable cost [1]. Therefore, the search for new bone regeneration strategies is a key international priority due to the debilitating pain associated with bone damage, and the increasing medical and socioeconomic challenge of the aging population.

1.2 BONE TISSUE ENGINEERING

The field of bone tissue engineering (BTE), initiated nearly three decades ago, has been considered as a potential alternative to the current clinical treatments, that will ideally eliminate the previously described issues [29, 30].

BTE aims to induce new functional bone regeneration via the synergistic combination of biomaterials, cells, and cell-inductive stimuli, such as growth factors or physical stimuli. Therefore, the classic BTE highlights four important factors:

1. A biocompatible biomaterial or scaffold that closely mimics the natural bone extracellular matrix niche,
2. Osteogenic cells to establish the bone tissue matrix,

3. Morphogenic signals or factors to direct the cells towards the osteogenic differentiation,
4. Adequate vascularization to allow nutrient supply.

After the implantation, the construct may influence the host by releasing osteogenic and vasculogenic growth factors in order to accelerate cell homing, vascularization, and bone regeneration of the defect site. Although much progress has been made, BTE practices have not progressed to usual clinical practice due to some challenges not yet resolved [1]:

- The use of pluri- or multipotent stem cells.
- The identification of key genes, growth factors and signal transduction cascades that mediate bone formation.
- The physical process of bone formation.
- The progressive remodelling and restructuring of pre-existing tissue structures.
- The importance of the tissue microenvironment's physical properties.
- The angiogenesis and the neo-vascularization of the newly formed bone tissue.

1.2.1 Biomaterials used for Bone Tissue Engineering

Biomaterials are used to develop scaffolds, which provide a template for cells to organize and restore structure and function of damaged or dysfunctional tissues. Guidance can be achieved through biophysical and biochemical cues that direct cell behaviour, morphology, adhesion and motility [31]. Biomaterials can allocate drugs, bioactive factors and genetic material that help directing cells into specific differentiation processes or organ repair. There is an extensive spectrum of materials that are clinically relevant to help the restoring of the damaged tissue or organ [32, 33].

In the past, materials for implantation were designed to be "bio-inert", that refers to any material that once placed in the human body has minimal interaction with its surrounding tissue. However, in recent years, there has been increasing emphasis in the design of "bioactive" materials that integrate

with biological molecules or cells and regenerate tissues. These materials are derived from both natural and synthetic sources, and possess a broad spectrum of structural and functional properties that make them suitable for many clinical applications. In the case of bone, materials should be *osteoinductive* (capable of promoting the differentiation of progenitor cells to the osteoblast lineage), *osteoconductive* (support bone growth and encourage the ingrowth of surrounding bone), *osseointegrative* (integrate into surrounding bone) and *resorbable* [4, 34, 35].

There are several strategies that can tailor the described properties by tuning their physical, chemical and biological parameters to create appropriate regenerative host microenvironments. In general, we can classify the biomaterials used for bone regeneration in terms of composition or morphology [36].

1.2.1.1 Biomaterials compositions

- **Ceramics**: Ceramic-based biomaterials are widely used as bone graft substitutes. These common biomaterials are made primarily from calcium phosphates, calcium sulphate or Bioactive Glasses (BaG) [33, 37]. Calcium phosphate bone graft substitutes are usually either β-tricalcium phosphate or hydroxyapatite, which is the primary mineral in bone. BaG are surface reactive amorphous biomaterials, which are able to bond with bone due to the formation of a surface apatite layer. Moreover, BaG release soluble ions, such as silica and calcium, which can recruit and stimulate osteoprogenitor cells [37]. In general, ceramics exhibit good bone integration, osteoconductive properties and display a high compressive strength. The main inconvenient of all the hydroxyapatite-based ceramic bone graft substitutes is that they are slowly resorbed by the body and are very brittle [4].

- **Metal-Based Materials:** Metals have high compressive strengths and excellent fatigue resistance. Hence, porous metallic scaffolds have been investigated for bone related applications due to their excellent physical

properties and their ability to promote tissue ingrowth [38]. The most commonly used metal-based materials are titanium (Ti) and tantalum (Ta). Ti shows high biocompatibility and mechanical strength with a good corrosion resistance. Ti structure displays mechanical properties closer to bone, and enhances as well osteoblast adhesion, proliferation and differentiation [39]. Moreover, Ti has often been incorporated into alloys. Different studies showed that modification of Ti surfaces has been shown to increase osteoconductivity. A rat model of distal femur defect suggested that higher porosity of Ti6Al4V structures increased mineralization and bone tissue formation [40].

- **Polymers**: Polymeric scaffolds have unique characteristics such as high surface-to-volume ratio, high porosity with very small pore size, biodegradation and mechanical properties. Moreover, they offer different advantages of biocompatibility, versatility of chemistry and the biological properties which are significant in the application of tissue engineering and organ substitution. According their origin, we can differentiate:

  - Natural polymers: A wide range of natural-origin polymers, including proteins and polysaccharides, are used as carriers for cells and bioactive molecules [41]. Natural polymers are advantageous due to their biocompatibility and their inherent biological domains. They also have reached clinical use with minimal adverse immunological reports and low toxicity. Protein-based natural polymers include collagen, gelatin, silk fibroin, fibrin, elastin and soybean [42]. Collagen is a major component of the extracellular matrix, it is the natural cell scaffold that interacts with cells in all tissues providing essential signals for the regulation of cell adhesion, migration, proliferation, differentiation and survival [43]. As a result, collagen has been studied for different approaches of tissue engineering: artificial skin (collagen IV), bone (collagen I) and cartilage (collagen II) [32]. Despite their inherent excellent biological properties, they lack reproducibility due to the batch to batch variations.

- **Synthetic polymers**: Synthetic polymers are generally composed of monomers that react to form chains of diverse lengths. Depending on the types of monomers used and the extent of reaction, a broad control of the physical and mechanical properties can be achieved, which allows to tune the properties of the polymers according to the requirements. Synthetic polymers are often cheaper than biologic scaffolds because these can be produced in large uniform quantities and they have a long shelf time [4, 36]. Moreover, they exhibit predictable and reproducible mechanical and physicochemical properties such as tensile strength, elastic modulus and degradation rate comparable to those of biological tissues [44]. Synthetic polymers represent the largest group of biodegradable polymers.

- **Composites:** Composites are the combination of materials that have different properties, such as the combination of polymers with ceramics [36]. The composites allow to combine the advantages of two distinct domains of materials and thus, to overcome the innate weak properties of individual materials. For instance, these combinations can improve the mechanical properties of materials, such as in the case of ceramics, which are known to be very brittle. So, this combination can reduce their brittleness by combining them with polymeric materials. On the other hand, some biomaterials, such as synthetic polymers, have limited biological behaviour and can be combined with natural polymers to achieve optimized biological [1].

1.2.1.2 Biomaterials morphology

- **Porous/Fibrous Scaffolds:** Porous/fibrous is the most widely used form of biomaterials for tissue regeneration purposes, especially for the growth of host tissue, bone regrowth or tissue vascularization. Their porous network simulates the ECM architecture, allowing a good interaction between cells and their environment. Scaffolds are designed to present high porosities with interconnected and large-sized pores (tens to hundreds of micrometres) to enable nutrient supply and cellular ingrowth [45, 46].

- **Hydrogels:** Hydrogels are polymer networks full of aqueous medium. They are soft and provide a cell-friendly 3D matrix condition similar to that found in native tissues [47]. These materials can typically support cell adhesion, cell migration and angiogenesis. Moreover, they facilitate the incorporation of biologically active molecules which are released in a controlled location at specific time points, which can act directly to support the development and differentiation of cells [48]. Hydrogels can be moulded into various forms and are currently used for different applications, such as cartilage wound healing, bone regeneration, wound dress or as carriers for drug delivery [48, 49].

- **Microparticles/Nanoparticles:** Microparticles are considered micro-scaffolds that are commonly prepared with spherical sizes of 100-400 µm to allowing the incorporation and release of molecules [44]. Furthermore, their micron sized morphology allows the culture of cells on the microspheres to use them as microcarriers. On the other hand, nanoparticles scaffolds, from 20 to 200 nm, are increasingly used as drug delivery systems and in advanced tissue engineering applications, such as gene therapy or antibiotic treatment of infected bone [50]. The influence of nanotechnology on scaffold design and the possibility of release growth factors via microspheres are showing promising development in tissue engineering and gene therapies.

1.2.2 Cell models in Bone Tissue Engineering

Cells are the building blocks of tissues, and play a critical role in promoting tissue healing and regeneration. Within tissue engineering strategies, cells may be a component of the *in vitro* construct or may be recruited *in vivo* with the aid of immobilized or soluble signals [32]. Cell types utilized for tissue engineering are selected from a variety of sources, which include autologous cells from the patient, allogeneic cells from other human and xenogeneic cells from different species. Common cell types used in BTE include , differentiated mature cells (osteoblasts and osteocytes cell models) [51, 52], immortalized and osteosarcoma cell lines [53] and stem cells (capable of self-renewal and

differentiate into multiple lineages) [54]. Stem cells include embryonic stem cells (ESC), adult stem cells (ASC) and induced pluripotent stem cells (iPSC).

1.2.2.1 Differentiated mature cells

- **Primary Osteoblasts:** Primary osteoblasts can be isolated from human bones and cultivated relatively easily [51]. Moreover they are a good candidate for clinical research since outcomes are not influenced by interspecies differences. However, primary human osteoblasts are a heterogeneous population and their behaviour depends on several factors, including isolation and donor age [55]. All of these factors can be better controlled by isolating primary cells from animals, including rats and mice [56, 57]. The potential of differentiated adult cells is often limited due to their low proliferation capability, loss of phenotype, and dedifferentiation in culture.

- **Primary Osteocytes:** Although osteocytes are the most abundant cell type in bone, their isolation and culture is very challenging due to their location within the bone matrix. Primary osteocytes have been successfully isolated from chicken calvariae, mice and most recently from humans. However, primary osteocyte culture still has many obstacles. For example, the output of osteocytes after isolation is usually low and since they are terminally differentiated cells they also lack the capability to proliferate in culture [58].

1.2.2.2 Immortalized Cell Lines

- **Immortalized osteoblast cell lines:** Cell lines can provide more homogeneous cell populations and an unlimited number of cells. They are either generated from immortalized primary cells, such as MC3T3-E1 (from newborn mouse calvaria), human osteoblast-like cells, and human fetal osteoblast-like cells or they are derived from osteosarcomas.

- **Osteosarcoma cell lines:** Osteosarcoma cell lines, such as MG-63 and SAOS-2 [59] can be a useful tool for investigating specific aspects of cell function in bone such as cell adhesion. However, in certain aspects they behave very different to normal bone cells, specifically their growth characteristics and ALP activities differ considerably from primary osteoblasts [60]. Moreover, these cell lines, due to their cancer origin, usually possess phenotype changes between passages, aberrations in mitotic processes and lack of growth inhibition, which limits their use in long-term investigations.

1.2.2.3 Stem Cells

Stem cells are found in almost all multicellular organisms and are capable of renewing themselves through cell division. Moreover, under certain stimuli, they can differentiate into tissue-specific cells [61, 62]. Therefore, stem cells serve as a reservoir and repair system capable of replacing differentiated cells lost either naturally through apoptosis or as a result of trauma or disease. Therefore, the potential of stem cells to renew and differentiate makes them attractive candidates for regenerative medicine [63]. These basic stem cell properties differ among various sources of stem cells, and they can be classified based on their origin and/or differentiation potential [62].

- Potency of Stem Cells

The capacity to differentiate into specialized cell types and be able to originate a mature cell type is referred to as potency. Stem cells can be classified depending on their differentiation potential in toti- (omni-), pluri-, multi- and unipotent (Figure 1.6) [64].

- **Totipotent stem cells** can differentiate into embryonic and extraembryonic cell types. Such cells can construct a complete, viable organism. The only totipotent cells are the fertilized egg and the cells produced by the first few divisions of this fertilized egg.

- **Pluripotent stem cells (PSC)** are the descendants of totipotent cells and they can self-renew and differentiate into any of the three germ layers,

ectoderm, endoderm and mesoderm, from which all tissues and organs develop.

- **Multipotent stem cells** can self-renew and differentiate only in a closely related family of cells from the same germ layer tissues.

- **Unipotent progenitor cells** can produce only one cell type, their own, but have the property of self-renewal, which differentiates them from non-stem cells. One example could be the epithelial tissue, that is in constant self-renew through adult life [64].

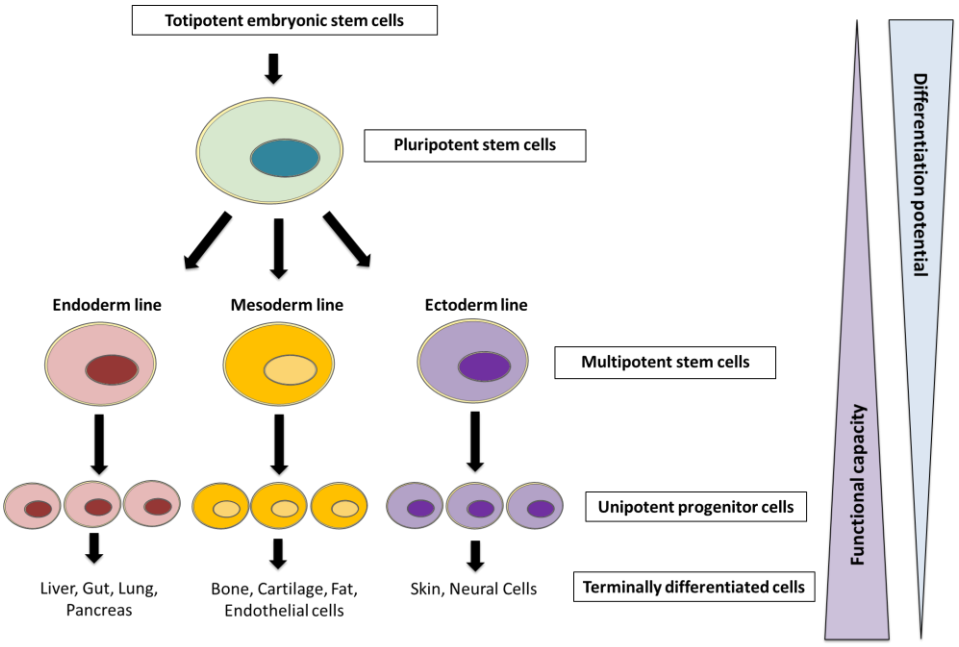


Figure 1.6. Stem cell hierarchy. Classification of stem cells depending on their differentiation potential and functional capacity.

- *Types of Stem Cells*

- **Embryonic Stem Cells (ESC):** Embryonic stem cells are cells derived from blastocyst, 5–6 days after fertilization. The outer layer of blastocyst is

formed by the trophoblast cells and inside the blastocyst there is the inner cell mass (ICM) or embryoblast. While the trophoblast will ultimately form the outer chorionic sac and the foetal component of the placenta, the ICM will give rise to all embryonic tissues and to some of the extraembryonic membranes. Therefore, ESC are the cells from the ICM. They are PSC that originate all cell types of the tissue layers (ectoderm, mesoderm, and endoderm) that will make up the embryo [61]. ESC have the ability to keep dividing (self-renewing) over many generations [65]. Because ESC are pluripotent cells, with high proliferative activity, they can potentially be used as a single source for the derivation of multiple lineages present in adult bone, including osteogenic cells, vascular cells, osteoclasts and nerve cells for bone regeneration [66]. However, ethical, religious and political issues are relating to human ESC and there are still a number of obstacles to be resolved before these cells can be widely used for cell-based therapy.

- **Adult Stem Cells (ASC):** ASC refer to any cell found in a developed organism that has two properties: the ability to divide and create another cell like itself or even to create a cell more differentiated than itself. They are found both in children, as well as in adults. ASC have been isolated from several tissue sources, including the central nervous system, bone marrow, adipose tissue, dental pulp and skeletal muscle [46]. Most ASC are lineage-restricted; they have multipotent potential and are generally referred to by their tissue origin. However, a small amount of pluripotent ASC can be found in a number of adult tissues, including umbilical cord blood or dental pulp, although they are rare. ASC treatments have been successfully used for many years to treat leukaemia and related bone/blood cancers through bone marrow transplants. Moreover, the use of ASC isolated from patients could solve immunological problems associated to cell transplant [50]. Mesenchymal Stem Cells (MSC), which are multipotent ASC that can be isolated from different tissues, such as bone marrow, adipose tissue, dental pulp, umbilical cord and blood. MSC can be used as osteoblast cell model since they originate the osteoblasts precursors. They are also a good model for gaining a better understanding of the mechanisms guiding MSC differentiation to mesodermal tissues [67]. Nevertheless, it has been

demonstrated that long-term MSC cultures cause morphological, genetic and immune-phenotypical changes which lead to cell senescence and alternations in their differentiation potential [68].

- **Induced Pluripotent Stem Cells (iPSC):** iPSC are reprogrammed somatic cells with pluripotent capabilities. In 2006, Takahashi et al. [69, 70] showed that the introduction of four specific genes encoding transcription factors (OCT4, SOX2, c-MYC and KLF4) could convert mouse adult cells (fibroblasts) into PSC known as iPSC. They have the ability to propagate indefinitely and to give rise to every cell from the three germ layers. They are similar to ESC in morphology, proliferation and teratoma capacity formation. Moreover, iPSC are useful in the production of new disease models and in drug development [71]. However, genomic integration of the transcriptor factors might result in risk of oncogene activation or mutagenesis in iPSC.

	ESC	ASC	iPSC
ORIGIN	- Blastocyst of embryo	- Bone morrow, circulation or resident tissues	- Reprogramming of somatic cells
ATTRIBUTES	- Pluripotent (3 germ layers) - Self-renewal and high replicative capacity	- Autologous - Clinical safety and efficacy data - Multipotent: Typically lineage committed	- Pluripotent (3 germ layers) - Autologous (HLA histocompatibility) - Large reservoir of cells - Easy to obtain - No ethical issues
LIMITATIONS	- Immunological concerns - Difficult obtaining - Limited: blastocysts days 5-14 - Subject to ethical debate - Risk of teratomas.	- Limited number - Limited replicative capacity - Lineage restricted	- Oncogen activation risk - Risk of mutagenesis - Risk of teratoma - Additional cost

Table 1.1. Types of Stem Cells. Origin, attributes and limitations of Embryonic Stem Cells (ESC), Adult Stem Cells (ASC) and induced Pluripotent Stem Cells (iPSC).

1.2.3 Cell Constructs and *in vitro* models in Bone Tissue Engineering

Tissue engineering uses a set of tools at the interface of the biomedical and engineering sciences to support the growth of living cells or to attract endogenous cells to induce tissue formation or regeneration. Approaches in BTE can be classified into two main categories: (A) *In situ* regeneration using a combination of a scaffold and growth factors as a guiding template to induce host cell regeneration of the tissue *in vivo.* (B) Transplantation of a tissue grown *in vitro* consisting of an artificial scaffold with cells and growth factors (Figure 1.7).

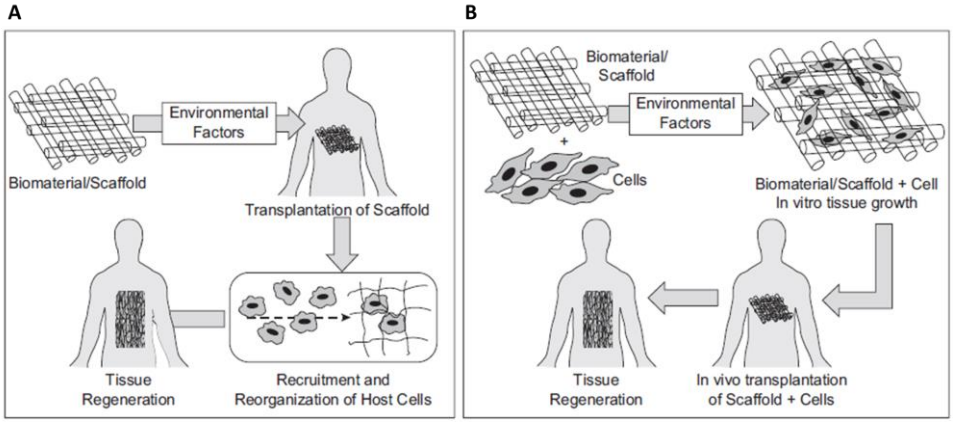


Figure 1.7. Tissue engineering approaches. **A)** *In vivo* tissue engineering by transplantation of scaffold, recruitment and reorganization of host cells. **B)** *In vitro* tissue engineering followed by transplantation; Modified from D. Sarkar *et al.* (2013) [32].

In the most frequent paradigm, cells are seeded on a scaffold, a tissue is matured *in vitro*, and the construct is implanted as a prosthesis [72]. Therefore, the first phase is the *in vitro* formation of a cell-tissue construct, by placing the cells in a scaffold with a supportive environment of growth media and the appropriate signalling factors, in which the cells proliferate, differentiate and elaborate the extracellular matrix. Then, in the second phase, the construct is implanted in the appropriate anatomic location, where remodelling and

potentially growth of the tissue *in vivo* are intended to recapitulate the normal functional architecture [73] (Figure 1.8).

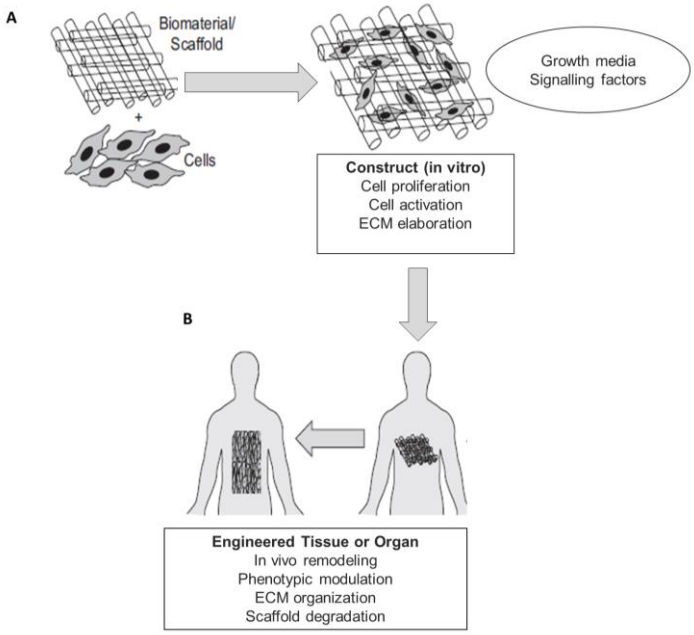


Figure 1.8. Tissue engineering paradigm. A) First step of the typical tissue engineering approach, differentiated or undifferentiated cells are seeded on a scaffold and then the construct matured *in vitro*. The cells proliferate and elaborate extracellular matrix (ECM) to form a new tissue. **B)** Second step, the construct is implanted in the appropriate anatomical position, where remodelling *in viv*o is intended to recapitulate the normal tissue/organ structure and function. Modified from D. Sarkar *et al.* (2013) [32].

In both tissue engineering approaches it is very important to assess the biocompatibility of novel biomaterials as well as their ability to conduct cells to differentiate into specific lineages before they can be transplanted *in vivo*. Hence, many established cell lines and models have emerged to address the surge in research in this field [74]. However, the use of differentiated cells, e.g. osteoblast cells, limits the relevance of the biomaterial since it is already expected that the biomaterial will allow expression of osteogenic markers. For

this purpose, the use of pluripotent cells that can potentially differentiate into any lineage can be properly guided by the biomaterial and hence demonstrating the efficiency of the biomaterial [75]. While this is the main purpose of PSC, as previously mentioned, ESC and iPSC have limited applications for biomaterials testing due to ethical reasons or low efficient transfections [75]. Therefore, there is still a need to find a cell type with genetic stability, stemness characteristics and no ethical problems to be used in BTE approaches, either to direct bone regeneration therapy or to evaluate biomaterials before cell therapy applications.

1.3 DENTAL PULP PLURIPOTENT-LIKE STEM CELLS (DPPSC)

Several types of adult stem cells have been isolated from teeth, including stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), dental follicle precursor cells (DFPC), stem cells from apical papilla (SCAP) and dental pulp stem cells (DPSC). These post-natal populations have MSC-like qualities such as the capacity for self-renewal and the potential to differentiate into multiple tissues including adipose, bone, endothelial and neural-like tissue [76-79].

The dental pulp is a soft connective tissue within the dental crown housing neural crest-derived stem cells. This niche contains several populations of multipotent stem/progenitor cells as a group designated dental pulp stem cells (DPSC or DPMSC) since their first isolation in 2000 by Gronthos *et al.* [80]. DPSC are isolated by enzymatic digestion of pulp tissue after separating the crown from the roots. DPSC can proliferate extensively, can be safely cryopreserved, possess immunosuppressive properties and express markers such as CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146, and STRO-1, but do not express CD14, CD24, CD34, CD45, CD19 and HLA-DR (Human Leukocyte Antigen-antigen D Related). They have the ability to differentiate into odontoblasts, osteoblasts, adipocytes, neural cells, cardiomyocytes, myocytes, and chondrocytes *in vitro*. DPSC represent less than 1% of the total cell population present in the dental pulp [81, 82]. In this way, the dental pulp tissue contains other types of

progenitor cells, which differ in regards to the rate of proliferation, renewal ability and differentiation potential [82, 83].

In previous studies, our group has described a new population from the dental pulp of the third molars named Dental Pulp Pluripotent-like Stem Cells (DPPSC) [84, 85]. The late development of the third molar could allow the presence of these progenitor cells with interesting characteristics. Thus, these cells express pluripotency markers such as OCT4, NANOG and SOX2, and show embryonic-like behaviour. Until now, our group has demonstrated the differentiation of DPPSC into cells of different tissues from the three embryonic layers: endothelium, neurons, skeletal muscle, smooth muscle, dental tissue, bone and hepatic tissue (Figure 1.9). Moreover, although the percentage of DPPSC decreases with age, it has been demonstrated that a population of these cells is always present [84].

DPSC/DPMSC was the first characterized population characterized from the dental pulp, with multi-potential capability. DPPSC and DPMSC share the same isolation protocol as well as some characteristics. The phenotypical analysis of DPPSC showed high expression levels of CD29 and CD105 markers, and low expression levels of CD45, indicating that DPPSC share several similarities with DPMSC. However, they differ in a higher expression level of embryonic markers (OCT3/4, NANOG, SOX2 and SSEA4) as well as a lower expression level of the membrane protein CD73 in DPPSC versus DPMSC. Moreover, DPPSC, instead of DPMSC from the same donor, maintain the pluripotency and the genetic stability in advanced culture passages [86, 87] The culture conditions are also different; DPPSC need low density and a specific medium that contains growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and leukemia inhibitory factor (LIF) to allow the maintenance of the pluripotent state [84].

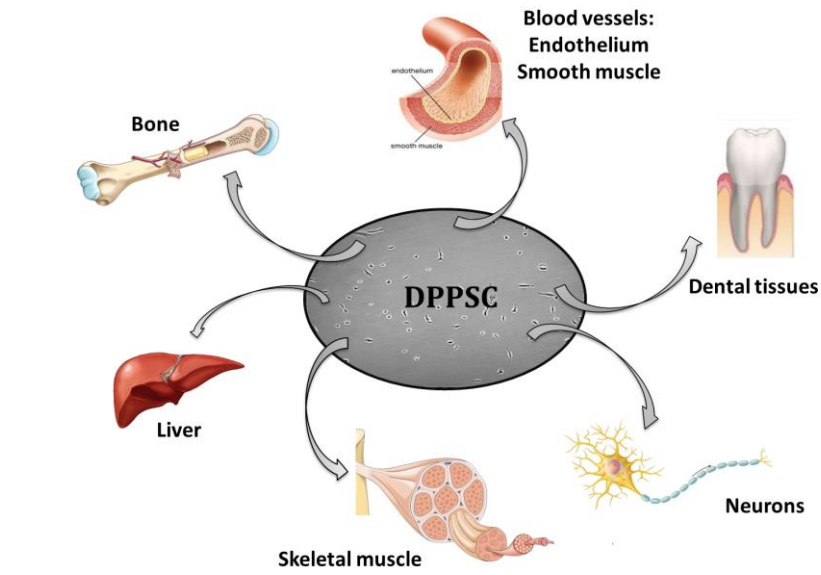


Figure 1.9. Differentiation potential of DPPSC.

Although the unique characteristics of these cells are still under investigation, it has been demonstrated that DPPSC show pluripotent-like properties that have not been found in cells of any other adult source: the ability to form EB-like and teratoma-like structures [84], which had been thought to be exclusive to ESC and iPSC [88].

DPPSC are not the first stem cell population isolated from adult tissues with pluripotency-like capacities. Indeed, several populations have been also identified: very small embryonic-like (VSEL) [89], multipotent adult progenitor cells (MAPC) [90], mesodermal progenitor cells (MPC) [91] and marrow-isolated adult multilineage inducible (MIAMI) cells [92].

1.3.1 DPPSC mesodermal differentiation potential

Previous studies of our group demonstrated the mesodermal differentiation capacity of DPPSC toward osteogenic, endothelial, smooth muscle and skeletal muscle tissues [84, 86, 93, 94].

It is well known the potential of DPMSC to differentiate into osteoblasts and the contribution of these cells to bone formation. The osteogenic potential of DPPSC and DPMSC isolated from the same donor and cultivated in the same osteogenic medium was compared by different methods in a previous study of our group [93]. Results showed significant differences between both cell types for the expression of bone markers, calcium deposition and ALP activity, being higher in DPPSC [93]. Furthermore, the thickness of collagen fibers was greater in differentiated DPPSC than that achieved with DPMSC. Lastly, when the architecture of bone-like tissue obtained from DPPSC was compared with human maxillary bone tissue, new bone-like tissue formed by DPPSC was in perfect continuity with the trabecular host bone structure (Figure 1.10).

Therefore, although the osteogenic differentiation potential of DPPSC is quite established, it is necessary to further investigate and optimize the osteogenic differentiation protocol of these cells and to evaluate their *in vitro* biocompatibility in different biomaterials and strategies for their use in the field of BTE.

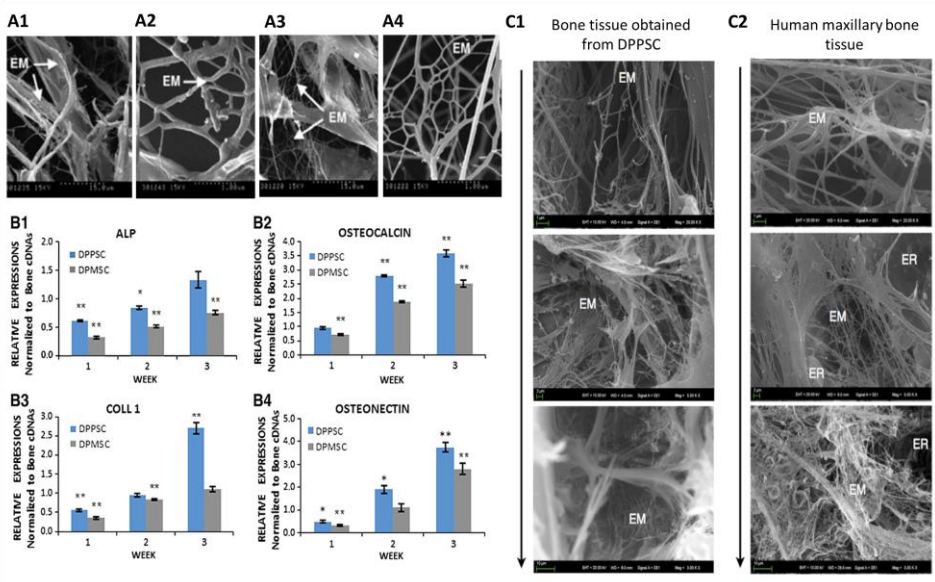


Figure 1.10. Osteogenic potential of DPPSC and DPMSC. A) SEM images of the extracellular matrix with collagen-like structures (EM) observed in **(A1-2)** DPPSC and **(A3-4)** DPMSC. Scale bars: 15 µm (A1, A3); 1 µm (A2, A4). **B)** Quantitative RT-PCR

analysis of the mRNA levels of bone-specific genes (ALP, Osteocalcin, COL1, Osteonectin) in DPPSC versus DPMSC. Data normalized to Bone cDNA. **C)** Comparison between SEM images of **(C1)** Bone tissue morphology obtained from DPPSC after 21 days of differentiation and **(C2)** Human maxillary bone tissue. Scale bars from top to down: 1 µm; 2 µm; 10 µm. Images extracted from Atari *et al.* (2012) [93].

1.4 REFERENCES

[1] Amini AR, Laurencin CT, Nukavarapu SP. Bone tissue engineering: recent advances and challenges. Critical reviews in biomedical engineering. 2012;40:363-408.

[2] Bilezikian JP, Raisz, L. G. and Rodan, G.A. Principles of bone biology. San Diego, CA: Academic Press; 2002.

[3] Clarke B. Normal bone anatomy and physiology. Clinical journal of the American Society of Nephrology : CJASN. 2008;3 Suppl 3:S131-9.

[4] Justin L. Brown SGKaCTL. Chapter II.6.7: Bone Tissue Engineering (3rd Edition). In: D. Ratner ASH, Frederick J. Schoen,Jack E. Lemons, editor. Biomaterials Science. Canada: Elsevier Inc.; 2013. p. 1194-214.

[5] Gunson D, Gropp, K.E. & Varela, A. Bone and Joints. In: W.M. Haschek CGRMAW, editor. Haschek and Rousseaux's Handbook of Toxicologic Pathology (Third Edition). Boston: Academic Press,; 2013. p. 2761-858.

[6] Franz-Odendaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. Developmental dynamics : an official publication of the American Association of Anatomists. 2006;235:176-90.

[7] Dirckx N, Van Hul M, Maes C. Osteoblast recruitment to sites of bone formation in skeletal development, homeostasis, and regeneration. Birth defects research Part C, Embryo today : reviews. 2013;99:170-91.

[8] William V. Giannobile HFR, Niklaus P. Lang. Chapter 4: Bone as a Tissue. In: Jan Lindhe NPL, Thorkild Karring, editor. Clinical periodontology and implant dentistry. Oxford: Ames, Iowa: Blackwell Munksgaar; 2008.

[9] Fazzalari NL. Bone fracture and bone fracture repair. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2011;22:2003-6.

[10] Shapiro F. Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. European cells & materials. 2008;15:53-76.

[11] Aubin JE. Regulation of osteoblast formation and function. Reviews in endocrine & metabolic disorders. 2001;2:81-94.

[12] Nakashima K, de Crombrugghe B. Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends in genetics : TIG. 2003;19:458-66.

[13] Miron RJ, Zhang YF. Osteoinduction: a review of old concepts with new standards. Journal of dental research. 2012;91:736-44.

[14] Tye CE, Hunter GK, Goldberg HA. Identification of the type I collagen-binding domain of bone sialoprotein and characterization of the mechanism of interaction. The Journal of biological chemistry. 2005;280:13487-92.

[15] Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, et al. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2000;15:1879-88.

[16] Rodriguez DE, Thula-Mata T, Toro EJ, Yeh YW, Holt C, Holliday LS, et al. Multifunctional role of osteopontin in directing intrafibrillar mineralization of collagen and activation of osteoclasts. Acta biomaterialia. 2014;10:494-507.

[17] Karner E, Backesjo CM, Cedervall J, Sugars RV, Ahrlund-Richter L, Wendel M. Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. Biochimica et biophysica acta. 2009;1790:110-8.

[18] Baroli B. From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. Journal of pharmaceutical sciences. 2009;98:1317-75.

[19] Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. Injury. 2005;36 Suppl 3:S20-7.

[20] Lane NE. Epidemiology, etiology, and diagnosis of osteoporosis. Am J Obstet Gynecol. 2006;194:S3-S11.

[21] Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC medicine. 2011;9:66.

[22] Finkemeier CG. Bone-grafting and bone-graft substitutes. The Journal of bone and joint surgery American volume. 2002;84-A:454-64.

[23] Bauer TW, Muschler GF. Bone graft materials. An overview of the basic science. Clinical orthopaedics and related research. 2000:10-27.

[24] Ebraheim NA, Elgafy H, Xu R. Bone-graft harvesting from iliac and fibular donor sites: techniques and complications. The Journal of the American Academy of Orthopaedic Surgeons. 2001;9:210-8.

[25] Silber JS, Anderson DG, Daffner SD, Brislin BT, Leland JM, Hilibrand AS, et al. Donor site morbidity after anterior iliac crest bone harvest for single-level anterior cervical discectomy and fusion. Spine. 2003;28:134-9.

[26] St John TA, Vaccaro AR, Sah AP, Schaefer M, Berta SC, Albert T, et al. Physical and monetary costs associated with autogenous bone graft harvesting. American journal of orthopedics. 2003;32:18-23.

[27] Delloye C, Cornu O, Druez V, Barbier O. Bone allografts: What they can offer and what they cannot. The Journal of bone and joint surgery British volume. 2007;89:574-9.

[28] Greenwald AS, Boden SD, Goldberg VM, Khan Y, Laurencin CT, Rosier RN, et al. Bone-graft substitutes: facts, fictions, and applications. The Journal of bone and joint surgery American volume. 2001;83-A Suppl 2 Pt 2:98-103.

[29] O'Keefe RJ, Mao J. Bone tissue engineering and regeneration: from discovery to the clinic--an overview. Tissue engineering Part B, Reviews. 2011;17:389-92.

[30] Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell and tissue kinetics. 1987;20:263-72.

[31] Langer R, Tirrell DA. Designing materials for biology and medicine. Nature. 2004;428:487-92.

[32] Debanjan Sarkar WZ, Sebastian Schaefer, James A. Ankrum, Grace S. L., Teo Maria Nunes Pereira, Lino Ferreira and Jeffrey M. Karp. Chapter II.6.2 Overview of tissue engineering concepts and applications. In: D. Ratner ASH, Frederick J. Schoen,Jack E. Lemons, editor. Biomaterials Science. Canada: Elsevier Inc.; 2013. p. 1122-37.

[33] Lee EJ, Kasper FK, Mikos AG. Biomaterials for tissue engineering. Annals of biomedical engineering. 2014;42:323-37.

[34] Stevens MM. Biomaterials for bone tissue engineering. Materials Today. 2008;11:18-25.

[35] Bose S, Roy M, Bandyopadhyay A. Recent advances in bone tissue engineering scaffolds. Trends in biotechnology. 2012;30:546-54.

[36] Perez RA, Jung CR, Kim HW. Biomaterials and Culture Technologies for Regenerative Therapy of Liver Tissue. Advanced healthcare materials. 2016.

[37] Rahaman MN, Day DE, Bal BS, Fu Q, Jung SB, Bonewald LF, et al. Bioactive glass in tissue engineering. Acta biomaterialia. 2011;7:2355-73.

[38] Simon M, Lagneau C, Moreno J, Lissac M, Dalard F, Grosgogeat B. Corrosion resistance and biocompatibility of a new porous surface for titanium implants. European journal of oral sciences. 2005;113:537-45.

[39] Takemoto M, Fujibayashi S, Neo M, Suzuki J, Kokubo T, Nakamura T. Mechanical properties and osteoconductivity of porous bioactive titanium. Biomaterials. 2005;26:6014-23.

[40] Bandyopadhyay A, Espana F, Balla VK, Bose S, Ohgami Y, Davies NM. Influence of porosity on mechanical properties and in vivo response of Ti6Al4V implants. Acta biomaterialia. 2010;6:1640-8.

[41] Lee SH, Shin H. Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. Advanced drug delivery reviews. 2007;59:339-59.

[42] Malafaya PB, Silva GA, Reis RL. Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Advanced drug delivery reviews. 2007;59:207-33.

[43] Yang C, Hillas PJ, Baez JA, Nokelainen M, Balan J, Tang J, et al. The application of recombinant human collagen in tissue engineering. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy. 2004;18:103-19.

[44] Dhandayuthapani B, Yoshida Y, Maekawa T, Kumar DS. Polymeric Scaffolds in Tissue Engineering Application: A Review. International Journal of Polymer Science. 2011.

[45] Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues--state of the art and future perspectives. Journal of biomaterials science Polymer edition. 2001;12:107-24.

[46] Perez RA, Mestres G. Role of pore size and morphology in musculo-skeletal tissue regeneration. Materials science & engineering C, Materials for biological applications. 2016;61:922-39.

[47] Ferreira LS, Gerecht S, Fuller J, Shieh HF, Vunjak-Novakovic G, Langer R. Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells. Biomaterials. 2007;28:2706-17.

[48] Lee KY, Mooney DJ. Hydrogels for tissue engineering. Chemical reviews. 2001;101:1869-79.

[49] Slaughter BV, Khurshid SS, Fisher OZ, Khademhosseini A, Peppas NA. Hydrogels in regenerative medicine. Advanced materials. 2009;21:3307-29.

[50] Perez RA, El-Fiqi A, Park JH, Kim TH, Kim JH, Kim HW. Therapeutic bioactive microcarriers: co-delivery of growth factors and stem cells for bone tissue engineering. Acta biomaterialia. 2014;10:520-30.

[51] Jonsson KB, Frost A, Nilsson O, Ljunghall S, Ljunggren O. Three isolation techniques for primary culture of human osteoblast-like cells: a comparison. Acta orthopaedica Scandinavica. 1999;70:365-73.

[52] Prideaux M, Schutz C, Wijenayaka AR, Findlay DM, Campbell DG, Solomon LB, et al. Isolation of osteocytes from human trabecular bone. Bone. 2016;88:64-72.

[53] Czekanska EM, Stoddart MJ, Richards RG, Hayes JS. In search of an osteoblast cell model for in vitro research. European cells & materials. 2012;24:1-17.

[54] Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. Journal of cellular biochemistry. 1997;64:295-312.

[55] Martinez ME, del Campo MT, Medina S, Sanchez M, Sanchez-Cabezudo MJ, Esbrit P, et al. Influence of skeletal site of origin and donor age on osteoblastic cell growth and differentiation. Calcified tissue international. 1999;64:280-6.

[56] Orriss IR, Taylor SE, Arnett TR. Rat osteoblast cultures. Methods in molecular biology. 2012;816:31-41.

[57] Bakker AD, Soejima K, Klein-Nulend J, Burger EH. The production of nitric oxide and prostaglandin E(2) by primary bone cells is shear stress dependent. Journal of biomechanics. 2001;34:671-7.

[58] Stern AR, Stern MM, Van Dyke ME, Jahn K, Prideaux M, Bonewald LF. Isolation and culture of primary osteocytes from the long bones of skeletally mature and aged mice. BioTechniques. 2012;52:361-73.

[59] Kartsogiannis V, Ng KW. Cell lines and primary cell cultures in the study of bone cell biology. Molecular and cellular endocrinology. 2004;228:79-102.

[60] Clover J, Gowen M. Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype? Bone. 1994;15:585-91.

[61] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-7.

[62] Kolios G, Moodley Y. Introduction to stem cells and regenerative medicine. Respiration; international review of thoracic diseases. 2013;85:3-10.

[63] Taupin P. Stem cells engineering for cell-based therapy. Journal of neural engineering. 2007;4:R59-63.

[64] Ilic D, Polak JM. Stem cells in regenerative medicine: introduction. British medical bulletin. 2011;98:117-26.

[65] Mimeault M, Batra SK. Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. Stem cells. 2006;24:2319-45.

[66] Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. Nature biotechnology. 2005;23:699-708.

[67] Kreke MR, Goldstein AS. Hydrodynamic shear stimulates osteocalcin expression but not proliferation of bone marrow stromal cells. Tissue engineering. 2004;10:780-8.

[68] Wagner W, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, et al. Aging and replicative senescence have related effects on human stem and progenitor cells. PloS one. 2009;4:e5846.

[69] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861-72.

[70] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663-76.

[71] Lahti AL, Kujala VJ, Chapman H, Koivisto AP, Pekkanen-Mattila M, Kerkela E, et al. Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. Disease models & mechanisms. 2012;5:220-30.

[72] Langer R, Vacanti JP. Tissue engineering. Science. 1993;260:920-6.

[73] Orlando G, Baptista P, Birchall M, De Coppi P, Farney A, Guimaraes-Souza NK, et al. Regenerative medicine as applied to solid organ transplantation: current status and future challenges. Transplant international : official journal of the European Society for Organ Transplantation. 2011;24:223-32.

[74] Czekanska EM, Stoddart MJ, Ralphs JR, Richards RG, Hayes JS. A phenotypic comparison of osteoblast cell lines versus human primary osteoblasts for biomaterials testing. Journal of biomedical materials research Part A. 2013.

[75] Perez RA, Choi S-J, Han C-M, Kim J-J, Shim H, Leong KW, et al. Biomaterials control of pluripotent stem cell fate for regenerative therapy. Progress in Materials Science. 2016;82:234-93.

[76] Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. Proceedings of the National Academy of Sciences of the United States of America. 2003;100:5807-12.

[77] Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, et al. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. Journal of endodontics. 2008;34:166-71.

[78] Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 2004;364:149-55.

[79] Vollner F, Driemel O, Reichert T, Morsczeck C. Isolation and characterization of dental follicle precursor cells (DFPCs). Journal of stem cells & regenerative medicine. 2007;2:130.

[80] Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2000;97:13625-30.

[81] Sloan AJ, Waddington RJ. Dental pulp stem cells: what, where, how? International journal of paediatric dentistry. 2009;19:61-70.

[82] Graziano A, d'Aquino R, Laino G, Papaccio G. Dental pulp stem cells: a promising tool for bone regeneration. Stem cell reviews. 2008;4:21-6.

[83] Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. Journal of dental research. 2002;81:531-5.

[84] Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, et al. Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science. 2012;125:3343-56.

[85] Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferres Padro E, et al. Isolation of pluripotent stem cells from human third molar dental pulp. Histology and histopathology. 2011;26:1057-70.

[86] Martínez-Sarrà E. Characterization of Dental Pulp Pluripotent-like Stem Cells (DPPSC) and their mesodermal differentiation potential. Barcelona: Universitat Internacional de Catalunya; 2017.

[87] Gil-Recio C. Obtaining hepatocyte-like cells from dental pulp pluripotent-like stem cells: Universitat Internacional de Catalunya; 2015.

[88] Benton G, George J, Kleinman HK, Arnaoutova IP. Advancing science and technology via 3D culture on basement membrane matrix. Journal of cellular physiology. 2009;221:18-25.

[89] Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, et al. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia. 2006;20:857-69.

[90] Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002;418:41-9.

[91] Petrini M, Pacini S, Trombi L, Fazzi R, Montali M, Ikehara S, et al. Identification and purification of mesodermal progenitor cells from human adult bone marrow. Stem cells and development. 2009;18:857-66.

[92] D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. Journal of cell science. 2004;117:2971-81.

[93] Atari M, Caballe-Serrano J, Gil-Recio C, Giner-Delgado C, Martinez-Sarra E, Garcia-Fernandez DA, et al. The enhancement of osteogenesis through the use of dental pulp pluripotent stem cells in 3D. Bone. 2012;50:930-41.

[94] Pulmonary risks; the latest on lung cancer. Smokers and nonsmokers both need to know that genetics play a role, food can be a preventative, and new tests can lead to earlier diagnosis. Health News. 2006;12:12-3.

CHAPTER 2:
OBJECTIVES & HYPOTHESES

2.1 OBJECTIVES

The overall aim of this thesis is to improve the osteogenic differentiation protocol of DPPSC for their potential combination with biomaterials for bone tissue engineering approaches.

The specific aims of the study are listed below:

1. To characterize and to understand the osteogenic differentiation of DPPSC.

2. To assess the biocompatibility and osteogenic capacity of DPPSC cultured on different types of biomaterials commonly used in bone regeneration studies.

3. To evaluate the genetic stability of DPPSC during the osteogenic differentiation process.

4. To improve the osteogenic differentiation of DPPSC by a novel polymeric nanoparticle non-viral gene delivery system.

5. To investigate the effect of the endothelial differentiated DPPSC on the osteogenic differentiation of DPPSC in xeno-free culture conditions.

6. To study the osteogenic and angiogenic potential of DPPSC by different monoculture and co-culture systems using Bioactive Glasses for vascularized bone tissue engineering constructs.

2.2 HYPOTHESES

H1. DPPSC are able to differentiate into osteogenic cells showing an expression pattern similar to that occurs during human osteoblasts development.

H2. DPPSC have the ability to proliferate and differentiate into osteogenic cells using different types of biomaterials.

H3. DPPSC maintain the genetic stability during the osteogenic differentiation process in combination with biomaterials.

H4.1. DPPSC can be transfected by oligopeptide-modified poly (β-amino ester)s maintaining cell viability and genetic stability.

H4.2. DPPSC improve the osteogenic differentiation process by silencing the expression of pluripotency genes (OCT3/4 and NANOG) and enhancing RUNX2 expression.

H5.1. The combination of endothelial differentiated DPPSC and osteogenic differentiated DPPSC can enhance osteogenesis and angiogenesis in both populations.

H5.2. DPPSC can differentiate into osteogenic and endothelial cells cultured in a xeno-free medium.

H6. BaG extracts can enhance the endothelial and osteogenic potential either in monocultures or co-cultures of DPPSC.

CHAPTER 3:
MATERIALS AND METHODS

3.1 Patient selection

The third molars of healthy patients were extracted for orthodontic and prophylactic reasons from 10 patients from both genders with ages comprised between 14 and 21 years old. Dental pulp tissues used for these experiments were obtained with informed consent from donors. All experiments were performed in accordance with the guidelines on human stem cell research issued by the Committee on Bioethics of the UIC Barcelona with the study code: BIO-ELB-2013-03 (appendix). Clinical information about the patients and the third molars can be found in the supplementary data (Table S1).

3.2 Cell Cultures

3.2.1 Isolation and Culture of DPPSC

The isolation and culture of DPPSC were performed as described in previous studies of our group [1, 2]. In short, after extraction, teeth were washed using gauze soaked in 70% ethanol. A second wash was performed with distilled water. The dental pulp was then extracted from the teeth using a sterile nerve-puller file 15 and forceps if the apexes were still open or, otherwise, fracturing the teeth and taking the dental pulp using forceps. The dental pulp was placed in falcon tubes containing sterile 1X phosphate-buffered saline (PBS) with 5% of 0.25% trypsin-EDTA (Life Technologies) and 1% Penicillin-Streptomycin (Life Technologies). The samples were then transferred to the laboratory. Dental pulps were disaggregated with collagenase type I (3 mg/ml; Sigma-Aldrich) for 60 min at 37°C in continuous shaking.

After washing twice with PBS (5 min, 1800 rpm), dental pulp tissue extracts were cultivated in DPPSC medium, which consisted of 60% Dulbecco's modified Eagle's medium (DMEM)-low glucose (Life Technologies) and 40% MCDB-201 (Sigma-Aldrich) supplemented with 1X selenium-insulin-transferrin-ethanolamine (SITE; Sigma-Aldrich), 1X linoleic acid-bovine serum albumin (LA-BSA; Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma-Aldrich), 100 units of penicillin/1000 units of streptomycin (Life Technologies), 2% foetal bovine serum (FBS; Sigma), 10 ng/ml hPDGF-BB (R&D Systems) and 10 ng/ml EGF (R&D Systems) in 650 ml flasks pre-coated with 100 ng/ml fibronectin and incubate

overnight at 37°C in a 5% CO_2 incubator. During the 2 weeks of primary culture, the medium was changed every 4-5 days. To propagate DPPSC, the cells were detached at 30% confluence by adding 0.25% trypsin-EDTA (Life Technologies) and re-pleated at 100 cells/cm^2 of density. Seeding DPPSC at the appropriate cell density with uniform distribution and correct timing of passage entails the most crucial part of DPPSC culture.

For xeno-free conditions, DPPSC were cultured in a medium supplemented with 1% of Human Serum (HS; Biowest) and that only contains xeno-free components. Moreover TrypLE™ Express Enzyme (Life Technologies) was used for cell detachment instead of 0.25% trypsin-EDTA (Life Technologies).

3.2.2 Isolation and Culture of DPMSC

Dental pulp mesenchymal stem cells (DPMSC) from the same donors as DPPSC were used for direct comparison of the differentiation potential. Therefore, adult human DPMSC were isolated from the same dental pulps as DPPSC. The isolation protocol was the same as for DPPSC, as described above. However, the culture medium in DPMSC consisted of DMEM 4.5 g/l glucose (Life Technologies) supplemented with 10% FBS (Biochrom) and 1X Penicillin/Streptomycin (Life Technologies). Cells were grown in 650 ml flasks at 37°C in a 5% CO_2 incubator. The medium was changed after 3 days and every 2 days thereafter. To propagate the DPMSC, the cells were detached at 80% confluence by the addition of PBS containing 0.25% trypsin-EDTA (Life Technologies) and replated at a density of $2x10^3$ cells/cm^2.

3.2.3 Culture of HUVEC

Human Umbilical Vein Endothelial Cells (HUVEC; Sigma-Aldrich) were maintained with EGM-2 BulletKit (Lonza), the medium was changed every 2 days and the cells were expanded when they reached 70-85% confluence using PBS containing 0.25% trypsin-EDTA (Life Technologies) and then replated at a density of $2.5x10^3$ cells/cm^2. HUVEC were used for co-cultures differentiation systems.

3.2.4 Culture of SAOS-2

The commercially available human osteosarcoma (SAOS-2) cell line (Sigma-Aldrich) were seeded at density of 10^3 cells/cm^2 in DMEM 4.5 g/l glucose (Life Technologies) supplemented with 10% FBS (Biochrom), 2 mM L-glutamine and 1% penicillin/streptomycin (Life Technologies), at 37°C in a 5% CO_2 incubator. The medium was changed every 3 days. After reaching 90% confluence, cells were detached by the addition of 0.25% trypsin-EDTA (Life Technologies).

3.3 Differentiation protocols

3.3.1 Osteogenic differentiation

DPPSC isolated from different donors between passage 6 and 8 were cultured in osteogenic medium (OM) for 15 or 21 days, depending on the experiment (refers to each chapter). The OM contained RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Biochrom), 10 mM β-glycerol phosphate (Sigma-Aldrich), 50 μM L-ascorbic acid (Sigma-Aldrich), 0.01 μM Dexamethasone (Sigma-Aldrich) and 1% penicillin/streptomycin (Life Technologies). Cells were cultured on 12, 24 or 48 well culture plates (depending on the experiment) at a cell density of 5×10^3 cells/cm^2. The cell line SAOS-2 was used as a control in biomaterials evaluation experiments (Chapter 5) and seeded at the same density and medium as DPPSC. The medium was changed every 2 days. For the comparison of DPPSC and DPMSC (Chapter 5), DPMSC from the same donors and culture passage (passage 6) as DPPSC were also differentiated in the same conditions as DPPSC.

In order to establish an osteogenic differentiation xeno-free medium for DPPSC more qualified for GMP conditions, we supplemented the medium with 5% of HS (Biowest) as a replacement of 10% FBS (Biochrom). Moreover TrypLE^{TM} Express Enzyme (Life Technologies) was used for cell detachment instead of 0.25% trypsin-EDTA (Life Technologies).

3.3.2 Endothelial differentiation

For the endothelial differentiation experiments, DPPSC were seeded in 12, 24 or 48 well culture plates at $2x10^4$ cells/cm^2 and cultured in EGM-2 bulletkit (Lonza) during 15 days. EGM-2 bulletkit contains the Endothelial Basal Medium-2 (EBM-2) and the following growth supplements: human Epidermal Growth Factor (hEGF); Vascular Endothelial Growth Factor (VEGF); R3- Insulin-like Growth Factor-1 (R3-IGF-1); Ascorbic Acid; Hydrocortisone; human Fibroblast Growth Factor-Beta (hFGF-β); Heparin; 0.1% of Gentamicin/Amphotericin-B (GA-1000) and 2% of Foetal Bovine Serum (FBS). The cell line HUVEC was used as a control in some experiments and seeded at the same density as DPPSC. The medium was changed every 2 days.

In order to establish an endothelial differentiation xeno-free medium more qualified for GMP conditions, the endothelial medium was supplemented with 2% of HS (Biowest) as a replacement of 2% FBS. Moreover TrypLE™ Express Enzyme (Life Technologies) was used for cell detachment instead of 0.25% trypsin-EDTA (Life Technologies).

3.3.3 Chondrogenic differentiation

In vitro chondrogenesis was performed in DPPSC and DPMSC from the same donors (Passage 6) during 21 days by a micro-mass culture method. Expanded DPPSC and DPMSC were trypsinized and aggregates of 2.5×10^5 cells were formed through centrifugation at 2000 rpm for 5 min in V-bottom 96-well plates (Greiner Cellstar). Chondrogenic differentiation was induced by treatment with DMEM 4.5 g/l glucose (Life Technologies) containing 100 nM dexamethasone (Sigma-Aldrich), 1 % 100 × ITS + 3 (insulin–transferrin–selenium) (ITS+3; Sigma-Aldrich), 200 µM l-ascorbic acid 2-phosphate (Sigma-Aldrich), 1 mM sodium pyruvate (Gibco Invitrogen), 10 ng/ml recombinant human transforming growth factor β1 (TGF β1; R&D Systems) and 1% FBS. Then, aggregates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and medium was changed three times a week.

The proteoglycan content after 21 days of differentiation was assessed by Alcian blue staining. Briefly, pellets were fixed in 4% paraformaldehyde (PFA)

for 2 h. Then, pellets were dehydrated, embedded in paraffin, and sectioned at 6-µm thickness. The sections were stained with Alcian blue (pH 1.0) to verify the presence of sulfated glycosaminoglycans (GAGs).

3.3.4 Adipogenic differentiation

For adipogenesis, DPPSC and DPMSC (passage 6) were seeded at $2.1x10^4$cells/cm^2, incubated in adipogenic induction medium consisting of 0.5 mM 3-isobatyl-1-methylxanthine (IBMX; Sigma-Aldrich), 10 µM insulin (Life Technologies), 1 µM dexamethasone, 200 µM idomethacin (Sigma-Aldrich) and 10% FBS (Biochrom) in DMEM 4.5 g/l glucose (Life Technologies). Cells were cultured 37 °C in a humidified atmosphere containing 5% CO_2 during 21 days, and medium was changed three times a week.

After 21 days of adipogenic induction culture, differentiation was assessed by qualitative Oil Red-O staining (Sigma-Aldrich), indicating the formation of intracellular lipid accumulation. In brief, the cells were washed with DPBS and fixed with 4% paraformaldehyde for 40 min followed by several washes with deionized water. Cells were pre-treated with 60% isopropanol 5 min at RT and stained with 0.5% Oil Red-O staining solution in 60% isopropanol (Sigma-Aldrich) for 15 min. Then, after several washes cells were stained with Hematoxylin solution (Fisher) for 1 min and washed again. DPBS was added and images were taken with an optical microscope (Olympus CKX41 microscope; Nikon DS-Fi1 camera).

3.4 Cell viability and Cell Proliferation

3.4.1 Live/Dead staining

Cell attachment and viability were evaluated qualitatively using Live/dead staining probes (Live Technologies). The viability was evaluated at 7- and 15-day time points. Briefly, DPPSC were incubated for 45 mins at room temperature with a mixture of 0.5 µM calcein acetoxymethyl ester (Calcein AM) and 0.25 µM ethidium homodimer-1 (Eth-1). Images of the viable cells (green fluorescence) and dead cells (red fluorescence) were obtained using an

Olympus IX51 phase contrast microscope with fluorescence optics and an Olympus DP30BW camera (Olympus Corporation, Tokyo, Japan).

3.4.2 CYQUANT Cell Proliferation Assay

The cell number was analysed by determining the amount of total DNA with a CyQUANT Cell Proliferation Assay Kit (CyQUANT; Life Technologies) according to the manufacturer's protocol. CyQUANT GR dye expresses fluorescence when bound to cellular nucleic acids. The cell number was analysed at days 7 and 15. On these days, the cells were washed with DPBS and lysed with 0.1% Triton-X 100 buffer (Sigma-Aldrich). The Triton cell lysates were frozen and stored at -70°C until analysis. After thawing, three parallel 20 µL samples of each cell lysate were pipetted onto a 96-well plate (Nunc) and mixed with 180 µL of working solution containing CyQUANT GR dye. Fluorescence was measured at 480/520 nm with a microplate reader (Victor 1420 Multilabel Counter).

3.4.3 MTT Cell Proliferation Assay

In chapter 5, polymer toxicity was measured using MTT Cell Proliferation Kit (Sigma-Aldrich) at 48 h after siRNAs transfection and 21 days after osteogenic differentiation according to the manufacturer's instructions. MTT assay measures the activity of living cells via mitochondrial dehydrogenase activity. Briefly, MTT stock solution (5 mg/ml) was added to each culture well, being assayed to equal 1/10 of the original culture volume and incubated for 3 h. Afterwards, DMSO was added in an amount equal to the original culture volume. Finally, the cell viability was determined by measuring the absorbance at 570 nm, and subtracting background absorbance at 690 nm.

3.5 Immunofluorescence analyses

For immunofluorescence (IF) analyses, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) supplemented with 0.2% Triton-X 100 (Sigma-Aldrich) (15 min at RT) and blocked with 1% bovine serum albumin (BSA) for 1 h at 4 ^{o}C. Then, after 3X PBS washes, cells were incubated with the

primary antibodies (Table 3.1) diluted in 1% BSA overnight at 4^{o}C in slight shaking. After 3 washes, the samples were treated with the corresponding secondary antibodies and dilutions (Table 3.1) for 1h at RT and washed 3 times again. The nuclei were stained with DAPI (dilution 1:2000) during the third wash after the secondary antibody treatment. Moreover, for 3D co-cultures in PuraMatrix (Corning), the cells were fixed with 4% paraformaldehyde, washed with PBS (Sigma-Aldrich) and the actin cytoskeleton was stained with phalloidin (dilution 1:1000; Abcam) which was incubated overnight. Afterwards, samples were incubated with DAPI (dilution 5:1000) for 1 h and then rinse two times with PBS. Finally the PBS was removed and images were performed with fluorescent microscope (Olympus, Tokyo, Japan) immediately after staining.

Primary Antibody against	Company	Cat. Number	Dilution	Secondary Antibody and dilution
OCT3/4	Santa Cruz Biotechnology	sc-5279	1:50	Alexa fluor 488 anti-mouse IgG (1:500)
NANOG	Abcam	ab109250	1:100	Alexa fluor 568 anti-rabbit IgG (1:500)
Osteocalcin	Abcam	ab13420	1:100	Alexa fluor 488 anti-mouse IgG (1:800)
Collagen IV	Abcam	ab6311	1:100	Alexa fluor 488 anti-mouse IgG (1:500)
Collagen I	Abcam	ab6308	1:2000	Alexa fluor 488 anti-mouse IgG (1:800)
Von Willebrand factor	Abcam	ab9378	1:500	Alexa fluor 488 anti-rabbit IgG (1:800)
CD31	Dako	M082329	1:40	Alexa fluor 488 anti-mouse IgG (1:800)

Table 3.1. List of antibodies used for protein detection in IF assays.

3.6 Immunohistochemistry analyses

In chapter 4, immunohistochemistry (IHC) assays were performing using specific anti-OC and anti-OPN antibodies in CCC sections. In chapter 6, the endothelial effects of the direct co-culture of bone-like DPPSC and HUVEC were analysed by IHC using anti-CD31 antibody. For this purpose, the co-cultures were performed in an 8 well chamber slide (Millicell® EZ SLIDES; Millipore) coated with fibronectin (Millipore).

Briefly, in both experiments, samples were fixed in 4% PFA for 2 h. Dehydrated, embedded in paraffin, and sectioned at 5-µm thickness for immunohistochemical staining. Then, paraffin slides were deparaffinised in xylene, rehydrated using graded percentages of ethanol followed by washed in distilled water and blocked against endogenous peroxidase in 0.3% hydrogen peroxide for 15 min and 10% normal goat serum in PBS for 1h at RT. The slides were then incubated with the primary antibodies against OC, OPN (dilution 1:200) or CD31 (dilution 1:200) at 4°C overnight. After washing with PBS, the specimens were incubated with biotinylated goat anti-mouse secondary antibody (Zymed) and streptavidin peroxidase (Zymed) at RT for 10 min each. Finally, the specimens were visualised using a diaminobenzidine reagent kit (Zymed). The immunostained sections were counterstained with Haematoxylin-Eosin staining. This procedure was performed by the Anatomopathology Department of *Hospital General de Catalunya*.

3.7 Flow cytometry

FACS analysis was performed to confirm the phenotype of the undifferentiated DPPSC (Chapter 4, Appendix). The following fluorochrome labelled monoclonal antibodies were used: CD105-FITC (R&D Systems), CD29-PE (R&D Systems), CD146-FITC (BD Pharmingen), CD45-PE (BD Pharmingen), NANOG-FITC and OCT3/4-FITC (R&D Systems). To analyse the control samples, different IgG isotypes coupled to PE and FITC fluorochromes (BD Pharmingen) were used. Briefly, cells were detached by adding PBS containing 0.25% trypsin-EDTA (Life Technologies), suspended in PBS with 2% FBS and incubated with the corresponding antibody for 45 min at 4°C in the absence of light. Subsequently,

cells were washed twice with 2% FBS-PBS and centrifuged (6 min at 1800 rpm), thereby removing any residual fluorochrome to avoid false positive results. The pellets were re-suspended in volumes between 600 to 1000 µl (depending on the number of cells) of PBS with 2% FBS.

In chapter 5, screening analysis of end-modified pBAEs was performed using GFP reporter plasmid and fluorescently-labelled siRNA. For plasmid screening, cells were incubated with nanoparticles containing pmaxGFP plasmid and GFP expression was analysed by FACS at 48 h post-transfection. For siRNA screening, cells were transfected using AlexaFluor 546-labelled siRNA-F during 2 h and fluorescence was analysed by FACS. In both cases, cells were washed twice in PBS and detached by adding PBS containing 0.25% trypsin-EDTA (Life Technologies). Afterwards, trypsin-EDTA was neutralized by the same amount of culture medium and cells were centrifuged and washed twice with PBS (6 min at 1800 rpm). Finally pellets were resuspended in 1 ml of PBS.

The flow cytometry measurements were made using a FACS cytometer (FACS Calibur, BD Biosciences) and analysed with WinMDI 2.8 software. To detect and exclude nonspecific unions and autofluorescence, at least $5x10^5$ cells were used for each sample.

3.8 Western blot analyses

Total protein was extracted from undifferentiated or differentiated DPPSC using Trizol Reagent (Life Technologies). Proteins are isolated from the phenol-ethanol supernatant layer left over after the DNA precipitation step and then precipitated according to the manufacturer's instructions. Afterwards, protein quantification was performed using Bradford Reagent (Sigma-Aldrich). Aliquots of cell lysates at a concentration of 20 µg/µl were loaded on SDS-PAGE using 12% polyacrylamide gels (BioRad) and transferred onto PVDF membranes (BioRad), previously activated with methanol. The membranes were then blocked with 1% (wt/vol) BSA in PBS containing 0.1% Tween-20. Next, membranes were incubated with the corresponding primary antibodies overnight, followed by washing and incubation with secondary antibodies (1h, RT). The Western blot membrane was finally developed using Luminata Forte

Western HRP substrate (Millipore) and quantified by Image J software. The antibodies used in Western blot assays are depicted in Table 3.2.

Primary Antibody against	Company	Cat. Number	Dilution	Secondary Antibody and dilution
OCT3/4	Santa Cruz Biotechnology	sc-5279	1:200	Anti-Mouse IgG H&L (1:5000)
OC	Millipore	AB10911	1:500	Anti-Rabbit HRP IgG H&L (1:5000)
OPN	Santa Cruz Biotechnology	sc-73631	1:200	Anti-Mouse IgG H&L (1:5000)
COL1	R&D Systems	AF6220	1:200	Anti-Sheep IgG H&L (1:5000)
GAPDH	Invitrogen	39-8600	1:500	Anti-Rabbit HRP IgG H&L (1:5000)

Table 3.2. List of antibodies used for protein detection in Western blot analyses.

3.9 RT-PCR analyses

Samples of total RNA were extracted from undifferentiated or differentiated cells using Trizol (Invitrogen). RNA was isolated following manufacturer's instructions. 2 µg of total RNA with a ratio 260/280 between 1.8 and 2 were treated with DNase I (Invitrogen) and afterwards reverse-transcribed (RT) using Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's instructions. PCR was performed using the primers listed on Table 3.3 for the amplification of the desired cDNA. TopTaq MasterMix kit (Qiagen) for regular PCR (RT-PCR) or FastStart Universal SYBR Green Master (Roche) for quantitative Real-Time PCR (qRT-PCR) were used. Finally, the amplifications were done in a T100 Thermal Cycler (Bio-Rad) for regular PCR and in a CFX96 Real-Time PCR Detection System (Bio-Rad) for Real-Time PCR.

GENE	Accession Number	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	USE
OCT 3/4	NM_002701	GTGGAGAGCAACTCCGATG	TGCAGAGCTTTGATGTCCTG	RT-PCR qRT-PCR
NANOG	NM_024865	CAGAAGGCCTCAGCACCTAC	ATTGTTCCAGGTCTGGTTGC	RT-PCR qRT-PCR
ALP	NM_000478	GGACATGCAGTACGAGCTGA	GTCAATTCTGCCTCCTTCCA	RT-PCR
ALP	NM_000478	CCGTGGCAACTCTATCTTTGG	GCCATACAGGATGGCAGTGA	qRT-PCR
COL1A1	NM_000088	ACTGGTGAGACCTGCGTGTA	CAGTCTGCTGGTCCATGTA	RT-PCR
COL1A1	NM_000088	CCCTGGAAAGAATGGAGATGAT	ACTGAAACCTCTGTGTCCCTTCA	qRT-PCR
OC	NM_199173	GTGCAGCCTTTGTGTCCAA	GCTCACACACCTCCCTCCT	RT-PCR
OC	NM_199173	AAGAGACCCAGGCGCTACCT	AAC TCGTCACAGTCCGGATTG	qRT-PCR
OSN	NM_001309444	ATCTTCCCTGTACACTGGCAGTTC	CTCGGTGTGGGAGAGGTACC	qRT-PCR
RUNX2	XM_017011396	TTACTGTCATGGCGGGTAAC	GGTTCCCGAGGTCCATCTA	RT-PCR
RUNX2	NM_001278478	AGCAAGGTTCAACGATCTGAGAT	TTTGTGAAGACGGTTATGGTCAA	qRT-PCR
OSX	AF477981	TGAGCTGGAGCGTCATGTG	TCGGGTAAAGCGCTTGGA	qRT-PCR
DLX5	NM_005221.5	ACCATCCGTCTCAGGAATCG	CCCCCGTAGGGCTGTAGTAGT	qRT-PCR
VLA4	NM_000885	CGAACCGATGGCTCCTAGTG	CACGTCTGGCCGGGATT	RT-PCR qRT-PCR
ITGα3	NM_002204	TCCGAGTCAATGTCCACAGA	GCTGGGCTACCCTATTCCTC	RT-PCR qRT-PCR
ITGαV	NM_002210	CCTTGCTGCTCTTGGAACTC	ATTCTGTGGCTGTCGGAGAT	RT-PCR qRT-PCR

SOX9	NM_000346	GTACCCGCACTTGCACAAC	GTAATCCGGGTGGTCCTTCT	qRT-PCR
COL2A1	NM_033150	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT	qRT-PCR
C/EBPβ	NM_005194	CACAGCGACGACTGCAAGATCC	CTTGAACAAGTTCCGCAGGGTG	qRT-PCR
PPARγ2	NM_015869	TGTCTCATAATGCCATCAGGTTTG	GATAACGAATGGTGATTTGTCTGTT	qRT-PCR
FLK1	NM_002253	TGGCATCGCGAAAGTGTATC	AAAGGGAGGCGAGCATCTC	qRT-PCR
vWf	NM_000552	GTCGAGCTGCACAGTGACAT	CCACGTAAGGAACAGAGA CCA	qRT-PCR
CD31	NM_000442	ACTGCACAGCCTTCAACAGA	TTTCTTCCATGGGGCAAG	qRT-PCR
GAPDH	NM_002046	CTGGTAAAGTGGATATTGTTGCCAT	TGGAATCATATTGGAACATGTAAACC	RT-PCR qRT-PCR
RPLP0	NM_001002	AATCTCCAGGGGCACCATT	CGCTGGCTCCCACTTTGT	qRT-PCR

Table 3.3. List of primers used for cDNA amplification in RT-PCR and qRT-PCR.

3.10 Short-comparative genomic hybridization

The genetic stability of undifferentiated DPPSC, differentiated DPPSC in 2D conditions (well plates), differentiated DPPSC on biomaterials (titanium disks and collagen carrier) and siOCT3/4-pRUNX2 transfected DPPSC were analysed by short-Comparative Genomic Hybridization (short-CGH)

Short-CGH is an aneuploidy screening that allows the detection of chromosome imbalances generated by aberrant segregation and structural differences for fragments larger than 10–20 Mb. The technique was performed as described in Rius M. *et al.* [3]. Briefly, 15-20 single cells from an homogeneous culture of each sample were collected and amplified using degenerate oligonucleotide-primed PCR (DOP-PCR). Then, whole genome amplification products were fluorescently labelled by nick translation. DNA from test samples was labelled in Red-dUTP, whereas control reference DNA (47, XXY) was labelled with Green-dUTP. After nick translation, reference and test DNA were mixed in equimolar proportions and ethanol precipitated.

Finally, hybridization was performed on normal male (46, XY) metaphase spread. The capture of metaphases was performed with an epifluorescence microscope and an average of 12 metaphases per sample was captured and evaluated using Isis CGH software (Meta Systems). The ratio between red and green fluorescence is 1:1 when there is the same proportion of reference and test DNA. The thresholds used to diagnose losses and gains were 0.8 and 1.2, respectively. This procedure was performed by an external service (Universitat Autònoma de Barcelona).

3.11 Alkaline phosphatase activity

3.11.1 Alkaline phosphatase activity staining

Alkaline phosphatase (ALP) activity was assessed qualitatively in undifferentiated DPPSC (day 0) and during the osteogenic differentiation of DPPSC in different culture conditions by ALP staining Kit (CosmoBio) following the manufacturer's instructions. Briefly, cells were fixed with 10% Formalin Neutral Buffer Solution, for 20 min at RT. After 3 washes with 1 ml of deionized water, 200 µl of Chromogenic Substrate were added to each well and incubated for 20 min at 37^{o}C. Finally, to stop the reaction, the staining were washed again with 1ml of deionized water and observed the obtained blue staining under an optical microscope. In chapter 5, the biomineralization of ALP staining was quantified by Image J software.

3.11.2 Alkaline phosphatase activity quantification

ALP was determined quantitatively in undifferentiated DPPSC (day 0) and during the osteogenic differentiation of DPPSC (day 7, day 15) in different culture conditions. In short, at each time point cells were lysed with 0.1% Triton-X 100 (Sigma-Aldrich) buffer. Then 20 µl of each lysate was pipetted in three parallel samples into the wells of a MicroAmp™ Optical 96-well plate (Applied Biosystems, Life Technologies). In order to initiate the ALP enzyme reaction, 90 µl of working solution containing 1:1 stock substrate solution (p-nitrophenol phosphate) (Sigma-Aldrich) and 1.5 M alkaline buffer solution (2-amino-2-methyl propanol) (Sigma-Aldrich) were added to each well with a

multichannel pipette. After 15 at 37ºC, 50 µl of 1 M NaOH (Sigma-Aldrich) were added to the wells to stop the reaction. Finally, the absorbance was measured at 405 nm.

3.12 Mineralization assays

3.12.1 Von Kossa staining

In chapter 4, mineralization was assessed qualitatively in undifferentiated DPPSC (day 0) and during the osteogenic differentiation of DPPSC at different time points by Von Kossa Method for Calcium staining (Polysciences), following the manufacturer's protocol.

Briefly, cells were fixed with 10% Formalin Neutral Buffer Solution, for 20 min at RT and stained with 0.5 ml of 3% Silver Nitrate Solution under UV light for 45 min. After 3 washes with 0.5 ml of deionized water, 0.5 ml of 5% sodium thiosulfate were added to each well for 2 min at RT, and washed again with 1 ml of deionized water 3 times. Finally, the samples were counterstained in Nuclear Fast Red for 5 min at RT and photographed with an optic microscope (Olympus CKX41 microscope; Nikon DS-Fi1 camera).

3.12.2 Alizarin Red S staining and quantification

In chapters 4, 5 and 6, mineralization of differentiated DPPSC was determined by Alizarin red S staining, which stains the calcium minerals in red. In short, cells were fixed with 70% ethanol for 1 h (-20ºC) and stained with 2% Alizarin red S solution (pH 4.1–4.3; Sigma-Aldrich) for 10 min at RT. The excess of colon was washed away with three consecutive water washes and one wash more with 70% ethanol, after which the samples were photographed. Finally, in order to quantify the results, the dye was extracted with 100 mM cetylpyridinium chloride (Sigma-Aldrich). After 3 h of extraction, the absorbance was measured at 544 nm. In chapter 5, the biomineralization of Alizarin Red S staining was quantified by Image J software.

3.13 Osteogenic differentiation on biomaterials

In chapter 4, in order to evaluate if DPPSC are appropriate to test the osteogenic capacity of well-known biomaterials, DPPSC from the same donors used also in 2D differentiations, were differentiated on biomaterials. The chosen biomaterials based on the extensive previous research using different types of cells, were collagen I based cell carriers (CCC) and titanium Ti6Al4V disks.

3.13.1 Collagen Cell Carriers

The collagen cell carrier (CCC) sheets (Viscofan Bio Engineering) were prepared as described in T. Schmidt *et al.* [4]. In short, CCC sheets were equilibrated 30 min at RT in distilled water (200 µL per sheet) at 37°C. The disks were transferred into 48-well plates preloaded with distilled water. After the removal of residual water, the culture plates containing the CCC sheets were dried overnight at RT under sterile conditions in a Laminar Air-Flow Cabinet. Before cell seeding, the dried CCC sheets were equilibrated with culture medium for 10 min at 37°C. Due to the drying process, the collagen sheets firmly attached to the plastic well without the entrapment of air. Thus, cells could only adhere to the upper surface of the CCC. DPPSC were seeded at a density of 5×10^3 cells/cm^2 with OM for 21 days. SAOS-2 cells were seeded and differentiated under the same conditions as a control.

3.13.2 Ti6Al4V disks

Ti6Al4V disks were obtained by cutting commercially available titanium alloy Ti6Al4V into 2.0-mm-thick disks with a 14 mm diameter and, subsequently, the surface was alumina-blasted and acid-etched treated to induce roughness, thus increasing the surface area (provided by *MIS Implants Technologies Ltd.*). The osteogenic differentiation was performed on Ti6Al4V disks in 24-well plates at a density of 5×10^3 cells/cm^2 using OM for 15 days. For bone differentiation on titanium disks two DPPSC models were analysed. The first one, coded TI DPPSC, consist on undifferentiated DPPSC seeded directly on the disk surfaces for 15

days. In the second model, coded as TI B.DPPSC, before being seeded on disks, DPPSC were differentiated for 15 days under 2D conditions, obtaining bone-like DPPSC (B.DPPSC). These osteogenic-like cells were then cultured on titanium disks and maintained during 15 days more in OM.

3.13.3 Scanning electron microscopy

Scanning electron microscopy (SEM) analysis of cells cultured on biomaterials (titanium disks and collagen carrier) was performed after DPPSC and SAOS-2 cells were differentiated on the biomaterials. In short, the samples were fixed with 2.5% glutaraldehyde (Ted Pella Inc.) in 0.1 M Na-cacodylate buffer (EMS, Electron Microscopy Sciences, Hatfield, PA, USA) (pH 7.2) for 1 h on ice. After fixation, the samples were treated with 1% osmium tetroxide (OsO4) for 1 h and dehydrated in serial solutions of acetone (30–100%) with the scaffolds mounted on aluminium stubs. Finally, samples were examined with a Zeiss 940 DSM scanning electron microscope.

3.14 Transfection of DPPSC by oligopeptide-modified poly (β-amino ester)s

3.14.1 Materials for DPPSC polymer transfections

Reagents and solvents used for polymer synthesis were purchased from Sigma-Aldrich and Panreac. Oligopeptide moieties used on the polymer modification (H-Cys-Arg-Arg-Arg-NH2, H-Cys-Lys-Lys-Lys-NH2, H-Cys-His-His-His-NH2 and H-Cys-Asp-Asp-Asp-NH2) were obtained from GL Biochem (Shanghai) Ltd with a purity of at least 98%. Polyplus Interferin and Lipofectamine 2000 transfection reagents were purchased from VWR and used according to manufacturer instructions. Knockdown of OCT3/4 was performed using ON-TARGETplus Human POU5F1siRNA SMART Pool (L-019591-00), knockdown of NANOG using ON-TARGETplus Human NANOGsiRNA SMART Pool (L-014489-00) and scramble siRNA control using ON-TARGETplus Non-targeting Control Pool (D-001810-10), all of them obtained from Thermo GE Dharmacon. Labelled siRNA (All Stars Neg. siRNA AF546) for uptake experiments was purchased from Quiagen. For

plasmid transfection, RUNX2 gene overexpression was obtained using RUNX2 plasmid ((Myc-DDK-tagged)-Human RUNX2) (OriGene) and pmaxGPF (Amaxa) was used as a scramble control.

3.14.2 Synthesis of oligopeptide end-modified poly (*β*-amino ester)s

Poly(*β*-amino ester)s (pBAEs) were synthesized following a two-step procedure as previously described [5, 6]. Briefly, acrylate-terminated C32 intermediate polymer was obtained by conjugate addition of 5-amino-1-pentanol to 1,4-butanediol diacrylate during 24 h at 90ºC. Then, oligopeptide-modified pBAEs were obtained by end-capping modification of the resulting acrylate-terminated polymer with thiol-bearing oligopeptides at 1:2,5 molar ratios in dimethyl sulfoxide (DMSO) during 12 h at 200 rpm at RT (Figure 3.1). These experiments were carried out by the *Grup d'Enginyeria de Materials* (*Institut Químic de Sarrià*, Barcelona).

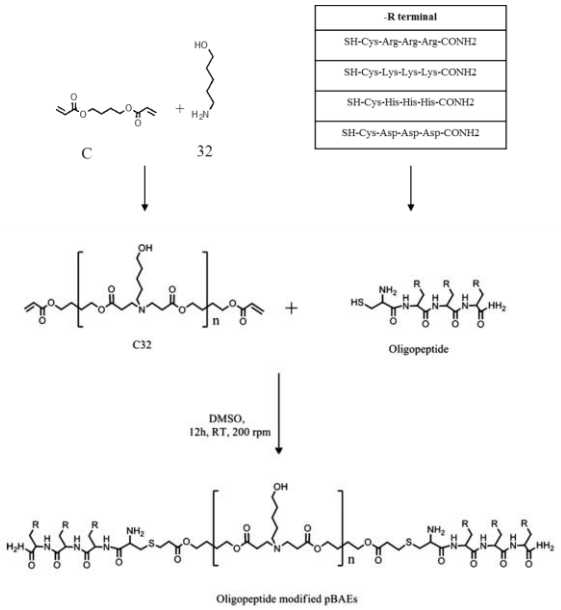


Figure 3.1. Structure and synthetic scheme of oligopeptide-modified poly (β-amino ester) polymers formation. R terminal can be arginine-, lysine-, histidine- and aspartic acid- oligopeptide.

3.14.3 Transfection of DPPSC using siNANOG, siOCT3/4 and pRUNX2

In chapter 5, siRNAs anti-NANOG, siRNAs anti-OCT3/4 and RUNX2 plasmid were delivered in DPPSC by a non-viral gene delivery system using the C32-CR3/CD3 polymer formulation. Briefly, siRNA polyplexes were performed at 200:1 polymer/siRNA ratio and plasmid polyplexes were performed at 50:1 polymer/plasmid ratio using AcONa buffer (25mM, pH 5.5). Cells were washed with PBS and polyplexes were added. siRNA transfection was performed working at a final concentration of 50 nM, plasmid transfection was carried out at 0.95 μg/mL and the co-transfection of siRNA-plasmid was performed at 25 nM and 0.48 μg/mL, respectively. At 3 h post-transfection, the remaining complexes were removed and replaced with OM. Scramble siRNA and scramble plasmid were used as a negative control in all experimental conditions following the same procedure of transfection.

3.15 Bioactive glass ions in DPPSC differentiations

3.15.1 Bioactive glass granules manufacture

Bioactive glasses (BaG) S53P4 granules were kindly supplied by *Adult Stem Cells Group* (*Biomeditech, University of Tampere*) and performed M. Ojansivu *et al.* as described in [7]. Briefly, BaG S53P4 were prepared from batches of analytical grade reagents Na_2CO_3, K_2CO_3, $CaCO_3$, MgO, $CaHPO_4 \cdot 2H_2O$, and Belgian quartz sand (Sigma-Aldrich, USA). The batches giving 300 g glass were melted in a platinum crucible for 3 h at 1360^{o}C, cast, annealed, crushed and remelted to ensure homogeneity. The oxide composition of the S53P4 is described in Table 6.1. Annealed glass blocks were crushed and sieved to give a 500–1000 μm size range fraction. The crushing was done according to the ISO 719 procedure without milling. After crushing, the granules were washed with acetone in an ultrasound batch at least five times to minimize the fine grained particles attached on their surface. Finally, the acetone was evaporated and the particles were dried at 120^{o}C.

3.15.2 Bioactive glass extracts preparation

The S53P4 BaG granules (500–1000 μm) to be used in the extract preparations were first disinfected with ethanol (two times with 70% ethanol for 10 min), after which they were let dry at room temperature for 2 h. In order to dissolute ions from the BaG granules, 87.5 mg/ml granules were incubated for 24 h at 37^{o}C in cell culture dishes (diameter 10 cm) with the extraction medium.

The extraction medium for the osteogenic induction contained RPMI 1640 Medium GlutaMAX™ (Life Technologies) supplemented with 1% antibiotics (penicillin/streptomycin; Life Technologies). The extraction medium for the endothelial induction contained Endothelial Basal Medium-2 (EBM-2) supplemented with 0.1% antibiotics (Gentamicin/Amphotericin-B; GA; from EGM-2 Bulletkit, Lonza).

After incubation, the extracts were sterile filtered (0.2 μm) and HS (Biowest) was added to the RPMI (5% of HS) or EBM-2 (2% of HS) extract medium. The medium composition of RPMI extract medium and HS is referred to as basic medium extract (BM extract). In order to obtain osteogenic medium extracts (OM extracts), RPMI extracts with HS were supplemented with osteogenic factors (10 mM β-glycerol phosphate, 50 μM L-ascorbic acid, 0.01 μM Dexamethasone). On the other hand, to obtain endothelial medium extracts (EM extracts), the EBM-2 extracts with HS were supplemented with the endothelial factors of the EGM-2 Bulletkit (hEGF, VEGF, R3-IGF-1, ascorbic acid, hydrocortisone, hFGF-β and heparin to the manufacturer's concentrations) (Figure 3.2). The maximum storage time of the BaG extracts media was 14 days at 4^{o}C. No visible precipitate was formed during this time.

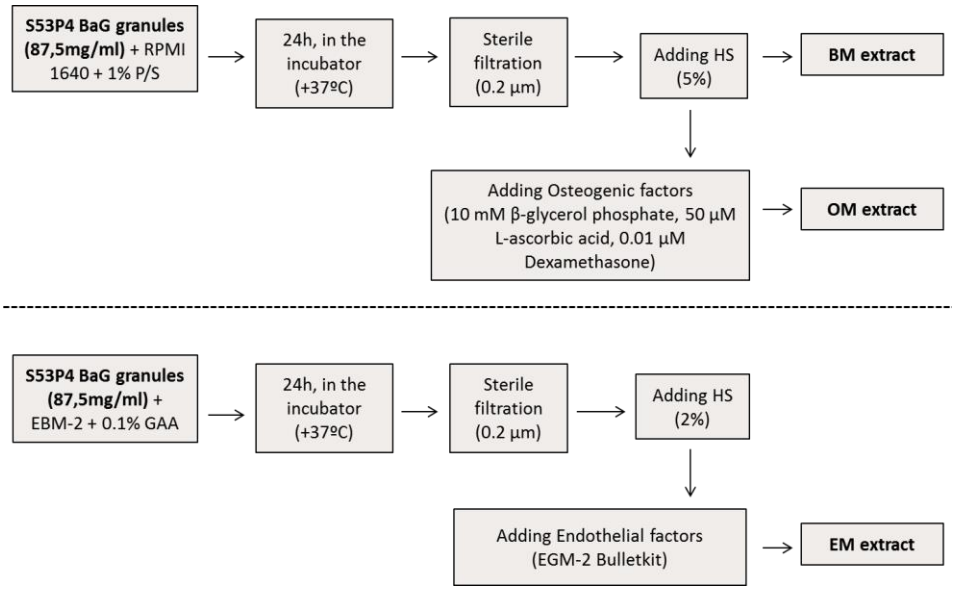


Figure 3.2. Schematic representation of the BaG extract preparation. BaG: Bioactive glass; BM: basal culture medium; OM: osteogenic medium; EM: endothelial medium.

3.16 DPPSC co-cultures

Different types of direct and indirect co-culture models including DPPSC were studied in chapter 6. A diagram of the experimental design of DPPSC co-cultures is provided in chapter 6 (Figure 6.10).

3.16.1 Indirect co-cultures

- **Bone-like DPPSC and HUVEC**

A preliminary study evaluating the effect of the indirect co-culture of DPPSC with HUVEC cells was performed. Firstly, DPPSC at passage 5 were cultivated in OM in 12-well culture plates at $5x10^3$ cells/cm^2 for 2 weeks to induce bone-like DPPSC before starting co-culture experiments. Then, for the indirect co-cultures of HUVEC and bone-like DPPSC, porous cell chambers were used to physically separate the cells (ThinCert™ Cell Culture Inserts; Greiner Bio-One). HUVEC were cultured above (in the chamber) at $2x10^4$ cells/cm^2 and bone-like DPPSC

below (on the plastic surface) at $5x10^3$ cells/cm^2. Co-cultures were incubated during 2 weeks in 4 different culture media: osteogenic medium (OM), endothelial medium (EM), a mixture of Osteogenic and Endothelial Medium (OM/EM) and Basal Medium (BM). At the same time bone-like DPPSC were cultured in a monoculture with OM ($5x10^3$ cells/cm^2) for 14 days as a control condition. During all the experiments fresh media was given to the cells three times a week.

- **Bone-like DPPSC and Endothelial-like DPPSC**

For the indirect co-cultures of bone-like DPPSC and endothelial-like DPPSC, DPPSC at passage 5 were previously cultivated either in OM ($5x10^3$ cells/cm^2) or in EM ($2x10^4$ cells/cm^2) in 12 -well culture plates during 2 weeks in order to induce bone-like DPPSC and endothelial-like DPPSC for the co-culture experiments. The indirect DPPSC co-cultures were performed as HUVEC-DPPSC indirect co-cultures and ThinCert™ Cell Culture Inserts (Greiner Bio-One) were used to physically separate the cells. In this case, endothelial-like DPPSC were seeded above at $2x10^4$ cells/cm^2 and bone-like DPPSC below at $5x10^3$ cells/cm^2.

Then, co-cultures were also cultivated during 2 weeks in the 4 different media (OM, EM, OM/EM, BM). Bone-like DPPSC were cultured as a monoculture in OM ($5x10^3$ cells/cm^2) during 14 days as a control condition. During all the experiments fresh media was given to the cells three times a week.

3.16.2 Direct co-cultures

- **Bone-like DPPSC and HUVEC**

Firstly, as in indirect co-cultures, DPPSC at passage 5 were cultivated in OM at $5x10^3$ cells/cm^2 for 2 weeks to induce bone-like DPPSC for co-culture experiments. Then, direct co-cultures were performed with a cell ratio of 3:2 (bone-like: HUVEC). Briefly, bone-like DPPSC were seeded on 24 -well culture plates coated with fibronectin (Millipore) at a density of $3x10^5$ cells/well and cultivated in OM. After 24 h, HUVEC were added at a density of $2x10^5$

cells/well. Co-cultures were cultured in the 4 different culture media (OM, EM, OM/EM, BM) for 14 days. Bone-like DPPSC were also cultured as monoculture at the same density corresponding to the co-culture seeding ratio ($3x10^5$ cells/well). During all the experiments fresh media was given to the cells three times a week.

- **Bone-like DPPSC and Endohelial-like DPPSC**

Firstly, as in indirect co-cultures with DPPSC, DPPSC at passage 5 were cultivated in OM ($5x10^3$ cells/cm^2) or in EM ($2x10^4$ cells/cm^2) during 2 weeks in order to induce bone-like DPPSC and endothelial-like DPPSC for the co-culture experiments. Direct co-cultures of DPPSC, were also performed at the cell ratio of 3:2 (bone-like:endothelial-like DPPSC). Bone-like DPPSC were seeded on 24 - well culture plates coated with fibronectin (Millipore) at a density of $3x10^5$ cells/well in OM. After 24 h, endothelial-like DPPSC were added at a density of $2x10^5$ cells/well using OM, EM, OM/EM or BM xeno-free culture media supplemented with or without S53P4 BaG extracts (87.5 mg/ml; 0.08% w/v) as previously described. Bone-like DPPSC and endothelial-like DPPSC were also cultivated as monocultures at the same density of co-cultures. During all the experiments fresh media was given to the cells three times a week.

3.16.3 3D co-cultures

DPPSC co-cultures were studied in a 3D peptide-hydrogel system during 15 days of culture in xeno-free EM with and without BaG extracts (87.5 mg/ml; 0.08% w/v).

DPPSC monocultures and co-cultures (3 bone-like:2 endothelial-like DPPSC) were encapsulated in 0.5% of PuraMatrix (Corning). Briefly, the viscosity of PuraMatrix stock solution (1% w/v) was reduced by vortexing, and air bubbles were removed by centrifuging. The cells were detached by TrypLE™ Express Enzyme (Life Technologies) and centrifuged at $1x10^6$ cells/ml. The supernatant medium was removed from the cell pellet, and the cells were resuspended at the desired density in sterile 10% sucrose. Then, the cells were centrifuged again to remove any remaining salts and resuspended in 10% sucrose at twice

the final desired cell concentration. Equal volumes of PuraMatrix and 2x cell/sucrose mixture were mixed and then carefully added to the center of the well to avoid the air bubbles.

Gelation of the PuraMatrix was initiated by running culture media down the side of the well on top of the hydrogel. The medium was changed twice over the next hour to equilibrate the pH of the hydrogel.

3.16.4 Vascular endothelial growth factor quantification

The supernatants of co-cultures and endothelial monocultures were collected at various time points and stored at -20^{o}C. The vascular endothelial growth factor (VEGF) was quantified by an enzyme-linked immunosorbent assay using the human VEGF Elisa Kit (Sigma-Aldrich) according to the manufacturer's protocol. The culture medium was used as blank. Briefly, serially diluted standards were prepared prior to start the assay. Then, 100 µL of each standard and sample were added into appropriate wells. The wells were covered and incubated over night at 4°C with gentle shaking. Afterwards, the solution was discarded and wells were washed 4X with 1X Wash Solution (300 µL) using a multi-channel pipette. After the last wash, 100 µL of 1X Biotinylated VEGF Detection Antibody were added to each well and incubated for 1 h at RT with gentle shaking. Then, the samples were washed again 4 times and 100 µL of 1X HRP-Streptavidin solution were added to each well and incubated for 45 min at RT with gentle shaking. After washing 4 times, 100 µL of TMB One-Step Substrate Reagent were added to each well for 30 min at RT in the dark with gentle shaking. Finally, 50 µL of Stop Solution were added to each well and the absorbance of each sample was measured at 450 nm immediately.

3.17 Statistical analysis

Statistical analyses were performed with SPSS Statistics version 21.0 (IBM). The results were analysed by applying the two-way analysis of variance (ANOVA) test for multiple factors. Confidence intervals were fixed at 95% ($P<0.05$). GraphPad Prism was used to graph all the quantitative data presented as mean

and standard deviation (SD). See figure legends for specific information regarding the number of independent experiments or biological replicates.

3.18 REFERENCES

[1] Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, et al. Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science. 2012;125:3343-56.

[2] Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferres Padro E, et al. Isolation of pluripotent stem cells from human third molar dental pulp. Histology and histopathology. 2011;26:1057-70.

[3] Rius M, Obradors A, Daina G, Cuzzi J, Marques L, Calderon G, et al. Reliability of short comparative genomic hybridization in fibroblasts and blastomeres for a comprehensive aneuploidy screening: first clinical application. Human reproduction. 2010;25:1824-35.

[4] Schmidt T, Stachon S, Mack A, Rohde M, Just L. Evaluation of a thin and mechanically stable collagen cell carrier. Tissue engineering Part C, Methods. 2011;17:1161-70.

[5] Segovia N, Dosta P, Cascante A, Ramos V, Borros S. Oligopeptide-terminated poly(beta-amino ester)s for highly efficient gene delivery and intracellular localization. Acta biomaterialia. 2014;10:2147-58.

[6] Dosta P, Segovia N, Cascante A, Ramos V, Borros S. Surface charge tunability as a powerful strategy to control electrostatic interaction for high efficiency silencing, using tailored oligopeptide-modified poly(beta-amino ester)s (PBAEs). Acta biomaterialia. 2015;20:82-93.

[7] Ojansivu M, Vanhatupa S, Bjorkvik L, Hakkanen H, Kellomaki M, Autio R, et al. Bioactive glass ions as strong enhancers of osteogenic differentiation in human adipose stem cells. Acta biomaterialia. 2015;21:190-203.

CHAPTER 4:
DPPSC FOR BONE TISSUE REGENERATION AND BIOMATERIALS EVALUATION

The contents of this chapter have been previously published [1].

4.1 INTRODUCTION

Recently, a great deal of efforts has focused in the field of BTE, and, particularly, in the area of stem cell biology and how to modulate their behaviour through environmental cues [2].

Biomaterials are widely used to regenerate or substitute bone tissue, because they allow the guidance of stem cells *in vitro* as well as *in vivo*. Therefore, in order to assess their biocompatibility and their ability to differentiate cells into specific lineages, they need to be tested in an *in vitro* cell culture model.

In this chapter, we propose the use of DPPSC, a new pluripotent-like subpopulation of stem cells from the dental pulp, as a good alternative model to evaluate different types of biomaterials commonly used for bone regeneration studies. Hence, we have characterized the osteogenic process in DPPSC and their ability to grow, attach and differentiate using different types of biomaterials, such as metals or natural scaffolds.

4.1.1 *In vitro* models for biomaterials evaluation

Several established cell lines and models have emerged in order to evaluate the potential use of biomaterials for clinical applications [3]. For instance, for bone regeneration, most studies have examined the osteogenic potential of biomaterials using immature osteoblasts, immortalized cell lines or mesenchymal stem cells among others. Primary cells, such as lineage-specific osteoblasts, can be isolated and cultivated relatively easily; however, they have a limited lifespan [4]. Immortalized cell lines, such as the human sarcoma osteogenic cell line (SAOS-2) have been frequently used in applied biology since they are from human origin while providing unlimited number of cells [3, 5]. Nevertheless, these cell lines, due to their cancer origin, usually possess phenotype changes between passages, aberrations in mitotic processes and

lack of growth inhibition, which limits their use in long-term investigations [6]. On the other hand, mesenchymal stem cells (MSC), which can be isolated from several adult tissues, are an attractive cell source for tissue engineering. These cells possess self-renewable and high proliferative capacity and multi-lineage differentiation potential [7]. However, long-term culture conditions, for maintenance and expansion, cause morphological and immune-phenotypical changes which lead to cell senescence and alternations in their differentiation potential [8]. Therefore, there is a need to find a cell type with genetic stability and stemness characteristics to be used to evaluate biomaterials in cell therapy applications.

4.1.2 DPPSC for biomaterials evaluation

As it was mentioned previously, the use of differentiated cells, e.g. osteoblast cells, limits the relevance of the biomaterial since it is already expected that the biomaterial will allow expression of osteogenic markers. For this reason, the use of pluripotent cells that can potentially differentiate into any lineage can be properly guided by the biomaterial and therefore demonstrating its efficiency [9]. While this is the main purpose of pluripotent stem cells, up to date, embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) have limited applications for biomaterials testing due to ethical reasons or low efficient transfections [9]. As it has been described in chapter 1, a new adult stem cell population from the human third molar dental pulp has been isolated by our group. These cells, called DPPSC, under particular culture conditions, express pluripotency markers until late passages and are able to differentiate into cells from the three germ layers [10, 11], thus, resembling the pluripotent characteristics of ESC and iPSC. We, therefore, consider that DPPSC are a promising alternative cell population for evaluating the biological properties of biomaterials, overcoming the current limitations of specific cell lineages or other PSC.

4.1.3 Biomaterials for BTE

Scaffolds are manufactured from several biomaterials including metals, ceramics, synthetic polymers or natural polymers.

4.1.3.1 Collagen Cell Carrier

Currently, the components of the extracellular matrix play an important role as natural substrates for cells cultured *in vitro* [12]. In this sense, collagen is regarded as an ideal scaffold for tissue engineering, because it is the major component of the extracellular matrix in mammals and it provides support to connective tissues [13-15]. Additionally, it is a highly conserved protein that is ubiquitously expressed among mammalian species. Therfore purified porcine or bovine collagen I also represents appropriate biocompatible sources for degradable scaffolds in human applications. Hence, Collagen type I based materials are extensively used for basic cell culture applications, as well as in the fields of bioreactor technology and tissue engineering [16-18]. Particularly, in this chapter, we evaluated the biocompatibility and the osteogenic capacity of DPPSC in a cell carrier based on fibrillar bovine collagen I in form of thin planar sheets (purchased from Viscofan).

4.1.3.2 Titanium alloy disks

Titanium (Ti) has been widely used in medical practice, showing excellent biocompatibility and safety [19]. Moreover, Ti and Ti alloys are primarily used in bone implants. Hence, tissue compatibility, osseointegration and functional maintenance of functions are fundamental criteria for the long-term success of the endosseous implants [20]. In addition, some studies report that Ti stimulates the adhesion, proliferation and the secretion of specific proteins to the ECM of the osteoblasts [21]. Here, we cultured DPPSC on a commercially available Ti alloy Ti6Al4V cut into 2.0-mm-thick disks with a 14 mm diameter and treated with alumina-blasted and acid-etched to induce roughness and increase the surface area (provided by *MIS Implants Technologies Ltd.*).

4.2 OBJECTIVES

Therefore, the main purposes of this chapter were to characterize the osteogenic differentiation process in DPPSC, and to assess their biocompatibility and their osteogenic capacity in the presence of different types of biomaterials commonly used in bone regeneration studies, such as metals (Ti6Al4V disks, Ti disks) or natural scaffolds (Collagen Cell Carrier scaffolds, CCC).

4.3 RESULTS

For the following experiments, DPPSC from 6 different donors were previously isolated, expanded and characterized as previously described [11]. The results with their pluripotent-like characteristics are shown in Appendix (Figure S1).

4.3.1 DPPSC and DPMSC mesodermal differentiation potential

Firstly, in order to better characterize the mesodermal potential of DPPSC, the multilineage differentiation capacity between DPPSC and DPMSC were compared. We differentiated both populations from the same dental pulp donors into osteogenic, adipogenic and chondrogenic lineage over a period of 21 days. Results were evaluated by specific stainings and qRT-PCRs of the specific lineage genes (Figures 4.1 and 4.2).

Based on chondrogenic differentiation, both DPPSC and DPMSC populations were able to initiate similarly the differentiation based on the Alcian blue staining of proteoglycans (Figure 4.1A). However, although DPMSC showed higher levels of the early chondrogenic marker SOX9, only DPPSC showed expression of the more advanced marker COL2A1 after 21 days of culture (Figure 4.2A).

In the adipogenic induction cultures, the rounded and swollen morphology of the cells indicated that the differentiation of DPPSC and DPMSC towards adipogenic cells was initiated. However, it did not progress efficiently which was shown by fewer oil droplets in Oil Red-O staining (Figure 4.1B). Moreover,

DPPSC showed lower expression of the adipogenic markers (C/EBPβ, PPARγ) by qRT-PCR than DPMSC, which expressed certain levels of the early adipogenic marker C/EBPβ since their undifferentiated stage (Day 0) (Figure 4.2B).

Finally, both dental pulp cell populations showed osteogenic differentiation capacity. However, ALP and von Kossa stainings indicated a slightly enhanced capacity to undergo osteogenic differentiation in DPPSC compared with DPMSC cultures, which was shown by higher ALP activity (in blue) and mineralization (in brown) (Figure 4.1C-D). In addition, although there were no differences in OC expression levels, COL1A1 expression was higher in DPPSC than in DPMSC (Figure 4.2C).

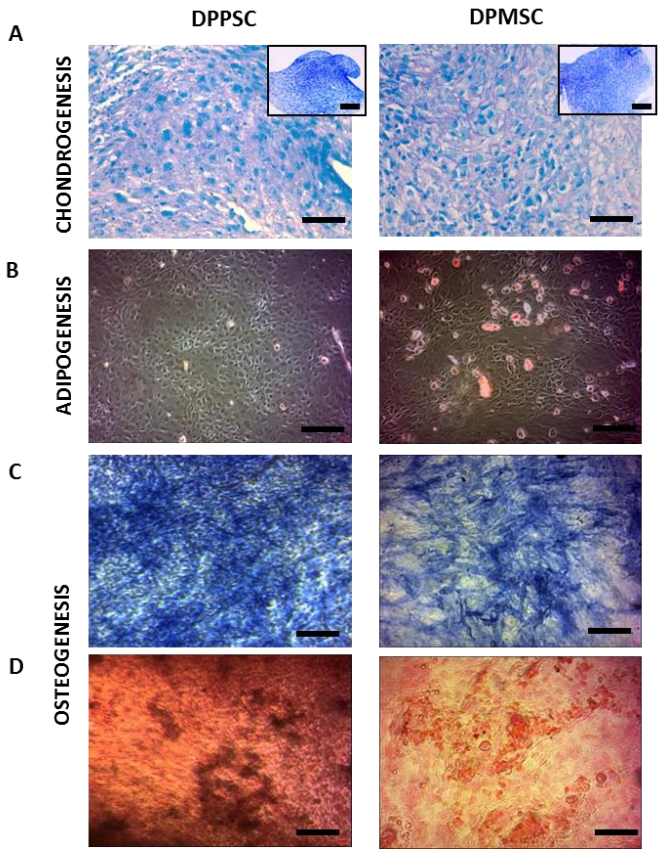


Figure 4.1. Multilineage differentiation potential of DPPSC and DPMSC from the same donors. A) Alcian blue staining after chondrogenic differentiation. Scale bars: 100 µm.

B) Oil Red-O staining after adipogenic differentiation. Scale bars: 200 µm. **C)** ALP staining after osteogenic differentiation (in blue). **D)** Von Kossa staining showing mineralization (in brown) after osteogenic differentiation. Scale bars: 200 µm. Representative images from 1 of 3N.

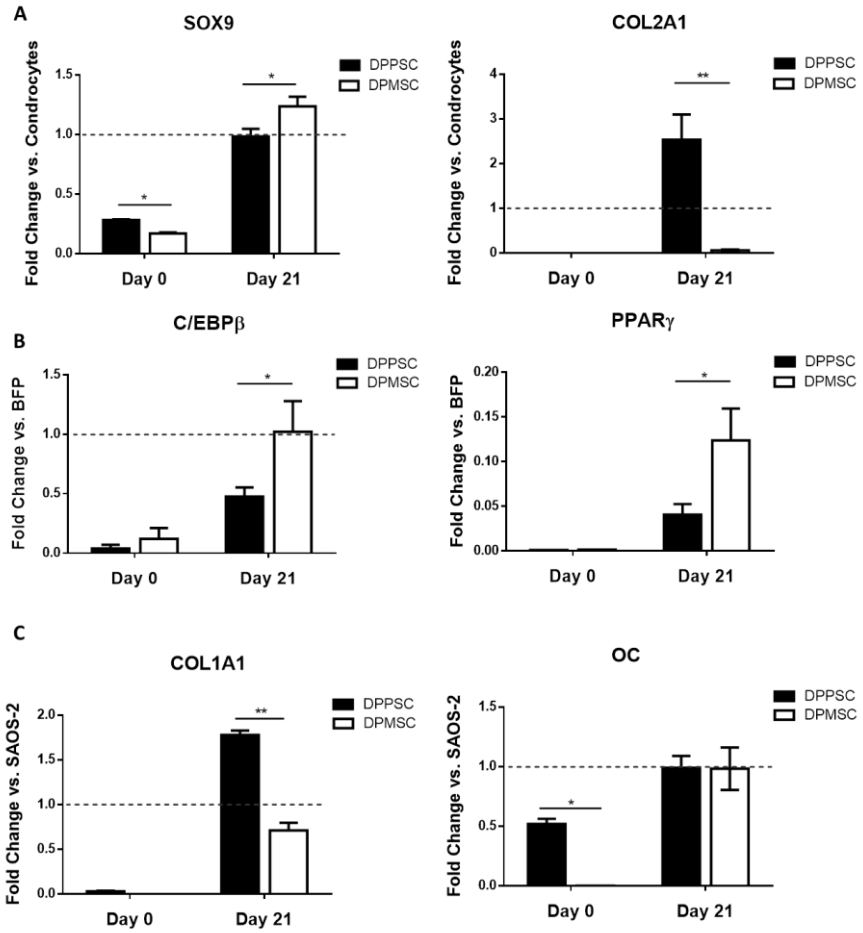


Figure 4.2. Multilineage differentiation potential of DPPSC and DPMSC from the same donors. A) Relative gene expression of chondrogenic markers (SOX9, COL2) compared to chondrocytes as a positive control. **B)** Relative gene expression of adipogenic markers (C/EBPβ, PPARγ) compared to differentiated buccal fat pad cells (BFP) as a positive control. **C)** Relative gene expression of osteogenic markers (COL1A1, OC) compared to SAOS-2 cells as a positive control. All data were normalized to the expression of the housekeeping gene GAPDH. **P < 0.05*; ***P < 0.01.* N=3.

4.3.2 DPPSC osteogenic differentiation potential

After cell characterization, DPPSC at different culture passages (P1, P5 and P10) were cultured in Osteogenic Medium (OM) for 21 days in order to characterize and understand better the osteogenic differentiation process of these pluripotent-like stem cells.

4.3.2.1 Osteogenic induction medium in DPPSC cultures

Firstly, we demonstrated the effect of the OM in DPPSC cultures over 15 days in terms of viability, proliferation and osteogenic differentiation. Results were compared with DPPSC cultured in Basal Medium (BM), without osteogenic induction components.

Thus, the survival and the proliferation of DPPSC were evaluated by Live/Dead staining and CyQUANT Cell Proliferation Assay after 7 and 15 days of culture in both medium conditions. Based on Live/Dead staining, no dead cells were detected in any of the conditions at neither of the time points studied (Figure 4.3A). At 7 days of culture in OM, the cell amount increased and cells adopted a more elongated shape. Moreover, cell proliferation assay also indicated a significant increase in cell number in OM compared to BM (Figure 4.3B).

Furthermore, the protein expression of the osteogenic markers Collagen Type I (COL1) and Osteocalcin (OC) was evaluated by IF analyses (Figure 4.3C-D). Results showed that after 7 days of culture, COL1, the major organic component of bone ECM, was still predominantly located intracellularly around the nucleus in both culture conditions. However, the amount of COL1 was greater in OM than in BM conditions. In addition, at day 15, COL1 was secreted to the ECM only in the OM cultures (Figure 1C). On the other hand, IF analyses also revealed that DPPSC in BM could not induce OC production after 15 days of culture. In contrast, OM samples showed an intracellular OC expression present at day 7 of differentiation and large amounts of OC majority located in

the ECM at day 15, indicating that at this point, DPPSC have secreted this protein to be part of the mineralized matrix.

Based on functional analyses, Figure 4.3E showed the total ALP activity of the samples treated with OM and BM. Although during the first week of culture no significant differences existed between cells in OM or in BM, at day 15 the ALP activity was significantly increased by OM. Finally, Alizarin Red S staining was conducted after 15 days of culture to see whether the OM can induce mineralization of DPPSC. As seen in Figure 4.3D, DPPSC in OM showed more extensive mineral formation compared to cells cultured in BM with significant differences as seen after the staining quantification.

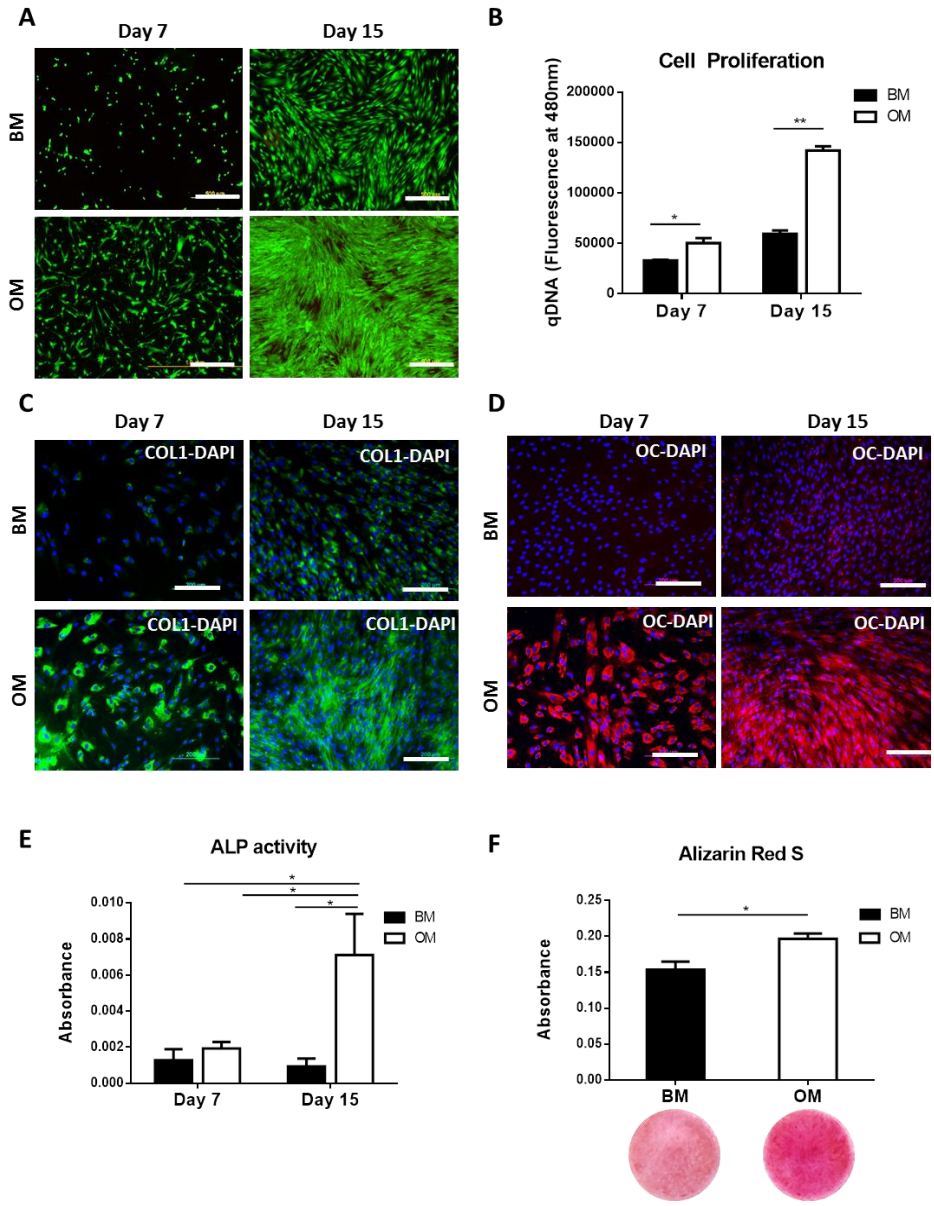


Figure 4.3. Effect of the OM in DPPSC cultures. A-B) Cell viability and proliferation of DPPSC cultured in BM and OM after 7 and 15 days of culture by **(A)** Live/Dead staining and **(B)** CyQUANT Cell Proliferation Assay kit. Scale bars: 500 µm. **C-D)** Protein expression by **(C)** COL1 and **(D)** OC immunostainings in DPPSC treated with BM or OM after 7 and 15 days of culture. COL1 is stained green, OC is stained red, and nuclei are

stained blue with DAPI. Scale bars: 200 µm. **E)** Quantitative ALP activity determined after 7 and 15 days of culture using a colorimetric assay. **F)** Mineralization of DPPSC by Alizarin Red S staining after 15 days of culture. Each image represents a whole well in 24-well plate (1.5 cm of diameter). Moreover, Alizarin Red S staining was quantified by extracting the color with cetylpyridinium chloride and measuring the absorbance at 544 nm. *$P<0.05$, **$P<0.01$. N=3.

4.3.2.2 Gene expression profile in DPPSC osteogenic differentiation

Under 2D conditions, the osteogenic differentiation of DPPSC was further investigated by examining the gene expression profile of pluripotent and osteogenic markers during 21 days of culture in OM (Figure 4.4).

First of all, qRT-PCR analysis revealed a gradual decrease in the expression of pluripotency markers OCT3/4 and NANOG during DPPSC differentiation (Figure 4.4A-B). On the other hand, the transcript expression of the early osteogenic marker RUNX2 was shown to increase and peaked at day 7, showing notably lower expression at the end of the differentiation. COL1A1 and ALP expression levels increased significantly along the differentiation and OC expression was also upregulated reaching the highest levels at 15 days of differentiation (Figure 4.4C-F).

Moreover, RT-PCR results at days 11 and 21 of the differentiation confirmed that the expression of the osteogenic markers ALP, COL1A1 and OC, was pronouncedly increasing until week 3, while RUNX2 expression was decreasing after first week of differentiation (Figure 4.5).

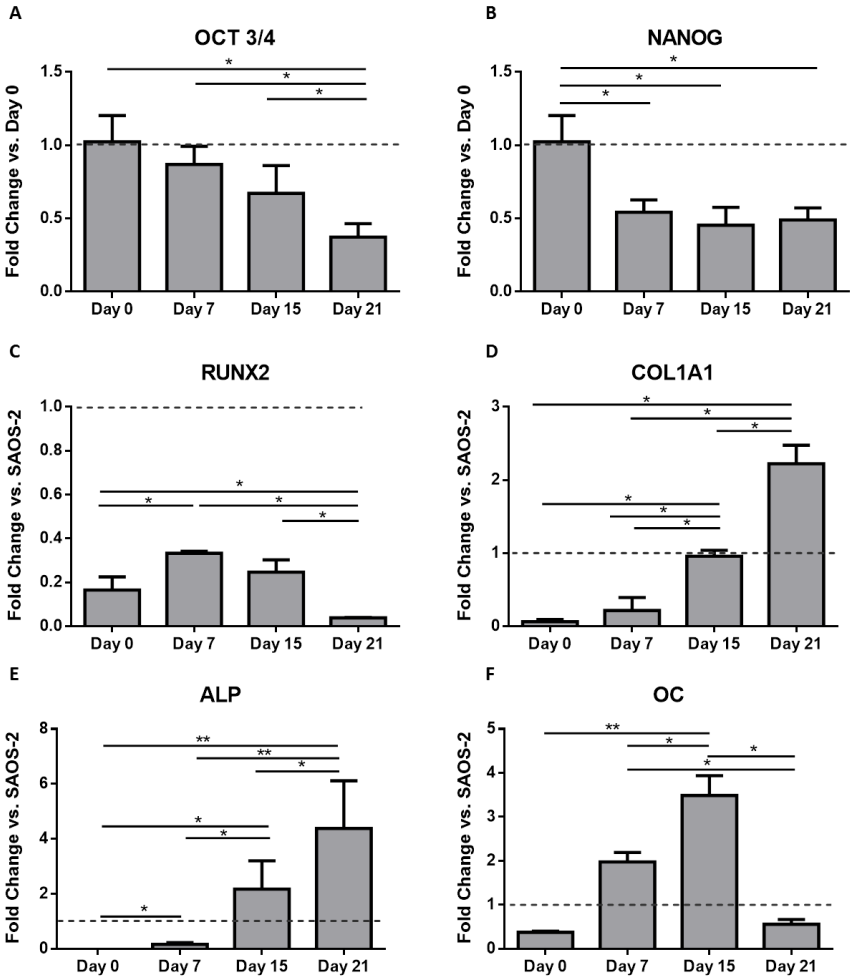


Figure 4.4. Gene expression during DPPSC osteogenic differentiation. A-B) Relative expression of pluripotency (OCT3/4, NANOG) markers versus DPPSC at Day 0 (dotted line). **C-F)** Relative expression of osteogenic markers (RUNX2, COL1A1, ALP, OC) versus SAOS-2 cells (dotted line). Data were normalized to the expression of housekeeping gene GAPDH. **P < 0.05; **P < 0.01*. N=4.

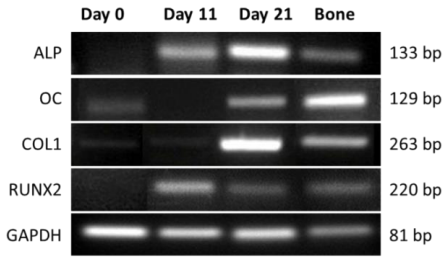


Figure 4.5. Gene expression during DPPSC osteogenic differentiation. RT-PCR of osteogenic markers (ALP, OC, COL1A1, RUNX2) at days 0, 11 and 21 of differentiation. Bone cDNA were used as a positive control and GAPDH as a housekeeping gene.

4.3.2.3 Cell morphology and Functional activity

DPPSC morphology was examined by optical microscopy along the osteogenic differentiation at different time points. At day 0, undifferentiated DPPSC are small-sized cells with rounded morphology, large nuclei and low cytoplasm content. By the second week of differentiation DPPSC adopted an elongated morphology and started to mineralize *in vitro* as observed by phase contrast microscopy at days 15 and 21 (Figure 4.6A).

In addition, functional osteogenic activity was qualitatively detected at different time points by ALP and von Kossa stainings. ALP activity enhancement, detected in blue, was clearly observed in a time dependent manner over the differentiation process (Figure 4.6B). Finally, as a mineralization assay, von Kossa staining showed in red regions rich in osteoid as well as in brown, calcium phosphate depositions (Figure 4.6C).

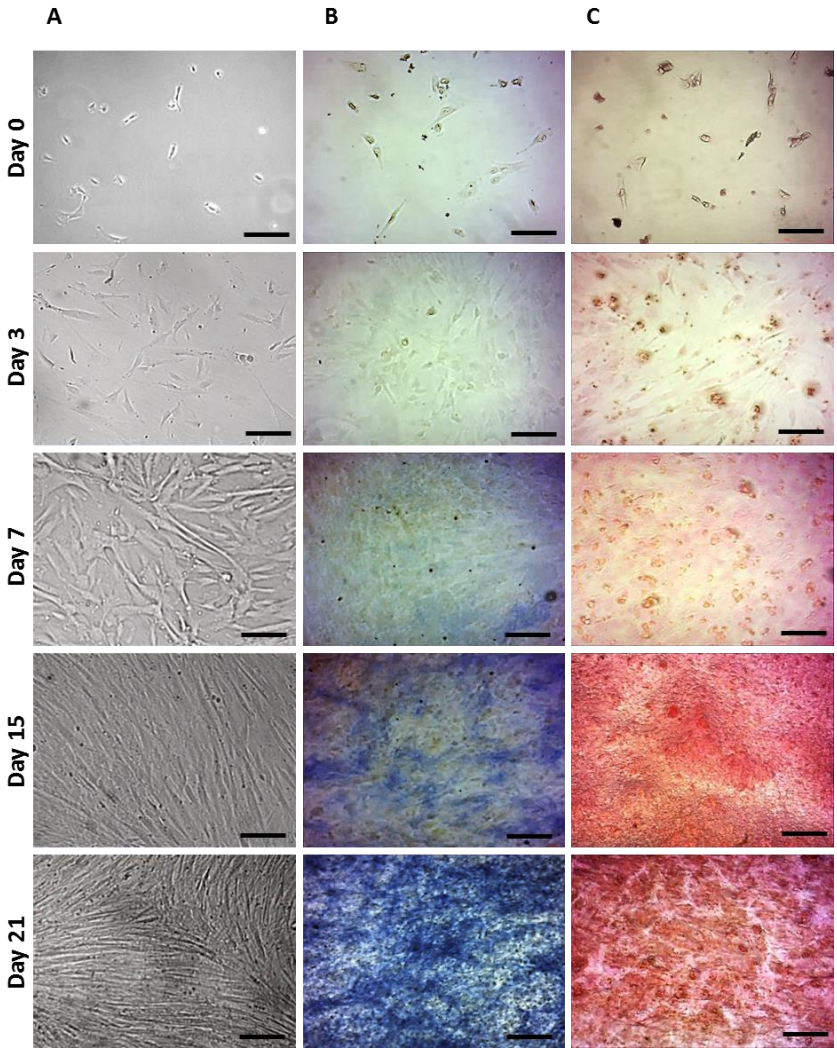


Figure 4.6. Cell morphology and functional activity during DPPSC osteogenic differentiation. A) Cell morphology of DPPSC observed with optic microscopy at days 0, 3, 7, 15 and 21 of osteogenic differentiation. **B)** ALP activity at days 0, 3, 7, 15 and 21 by an ALP staining (blue). **C)** Images of mineralization at days 0, 3, 7, 15 and 21 stained by von Kossa method showing mineralized bone (brown) and osteoid (red). Scale bars: 200 µm. Representative images from 1 of 4N.

4.3.2.4 Protein expression

In order to corroborate qRT-PCR results, the protein expression of different osteogenic markers was also analysed. IF analyses showed that COL-IV was secreted to the ECM acquiring a fibril-like structure while OC expression was detected in the cytoplasm (Figure 4.7A).

In addition, at day 21 of differentiation, high protein expressions of OC, OPN and COL1 were also detected by Western blot comparable to that observed in SAOS-2 cells (Figure 4.7B).

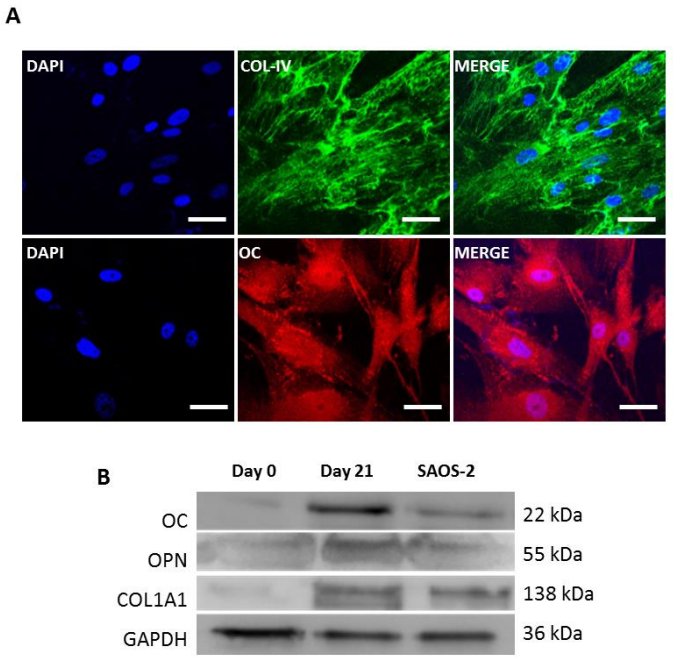


Figure 4.7. Protein expression after DPPSC osteogenic differentiation. A) COL-IV (in green) and OC (in red) immunostainings in 21 days differentiated DPPSC. Nuclei are stained DAPI (in blue) was used as a nucleus control. Scale bars: 50 µm. **B)** Western Blot analysis of osteogenic markers (OC, OPN, COL1A1) at day 21 of differentiation. GAPDH was used as a housekeeping control and SAOS-2 cells as a positive control.

4.3.2.5 Summary of the osteogenic differentiation in DPPSC

Finally, in order to recapitulate all the results and summarize the osteogenic differentiation process in DPPSC, a schematic representation of the different stages was performed (Figure 4.8). Shortly, in their undifferentiated stage, DPPSC express pluripotency markers such as NANOG and OCT3/4. Once in osteogenic medium, cultures reach confluency around day 7, when the expression of these pluripotency markers is down-regulated followed by an up-regulation of RUNX2. Then, RUNX2 supports the downstream effects of multiple osteogenic factors and regulates the gene expression of major bone matrix proteins. As they mature, pre-osteoblasts express COL1, which produce the non-mineralized bone matrix, and ALP, which transport inorganic phosphates contributing to the matrix mineralization. Then, when pre-osteoblasts transform to mature osteoblasts, OC is up-regulated and the matrix is mineralized by the formation of hydroxyapatite. Finally, after 21 days, the mature osteoblasts express also OPN and become surrounded by its own matrix and terminally differentiate into an osteocyte.

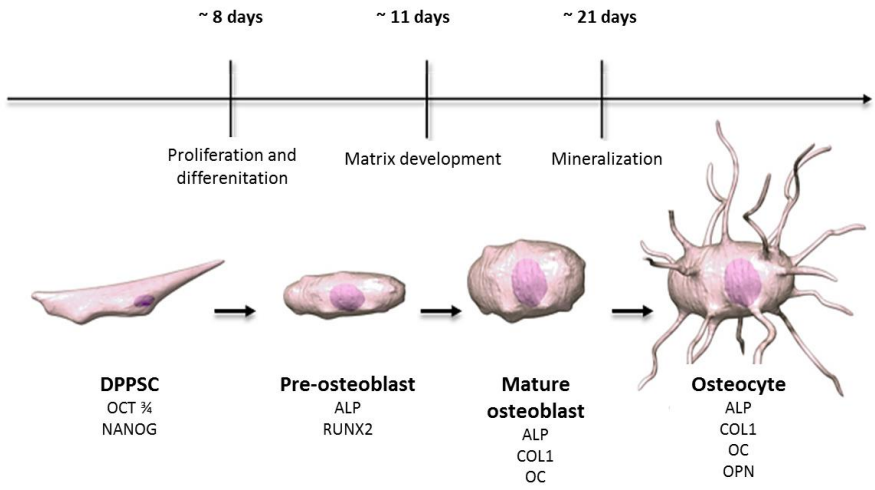


Figure 4.8. Stages of DPPSC osteoblastic differentiation during 21 days. Proposed scheme for osteogenic DPPSC culture with recognizable stages of differentiation.

4.3.3 DPPSC for biomaterials evaluation

In order to evaluate if DPPSC are appropriate to test the osteogenic capacity of well-known biomaterials, DPPSC from 3 of the same donors used also in previous 2D differentiations, were differentiated on biomaterials. Based on previous research, the chosen biomaterials were collagen I based cell carriers (CCC) and titanium Ti6Al4V disks. Moreover, the genetic stability was evaluated by short-CGH following each differentiation step. SAOS-2, an osteosarcoma cell line commonly used in BTE, was used as a control model in each differentiation. A diagram of the experimental design is provided in Figure 4.9.

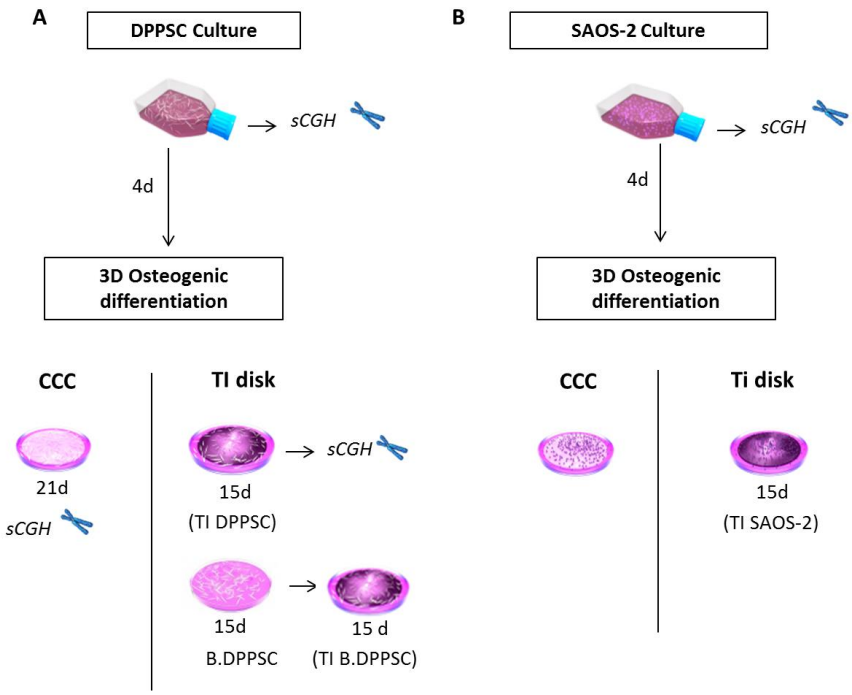


Figure 4.9. Schematic diagram of the experimental design. **A)** Osteogenic differentiation of DPPSC cultures. Undifferentiated DPPSC were cultivated and characterized until passage 15 (N=6). The genetic stability was checked in passages 1, 5, 10 and 15 by short-CGH. 3D osteogenic differentiation: DPPSC at passage 10, were differentiated on biomaterials (CCC and Ti disks) during 21 and 15 days (N=3). DPPSC after 15 days of 2D differentiation (B.DPPSC) were also seeded on Ti disks and maintained during 15 days more in osteogenic medium (TI B.DPPSC). Short-CGH was

performed in all DPPSC differentiations on biomaterials. **B)** SAOS-2 at passage 10 was used as a control cell line in all experiments. Genetic instability of SAOS-2 was evaluated before osteogenic induction experiments.

4.3.3.1 Osteogenic differentiation on Collagen I- based Cell Carrier (CCC)

DPPSC were cultured on CCC and the differentiation was induced for 21 days. SEM was utilized to visualize the scaffold/cells constructs and obtain a better understanding of the cell morphology (Figure 4.10). CCC scaffolds with the attached cells exhibited a dense microstructure. Figures 4.10A-C showed that cells were well dispersed and attached, covering the entire collagen surface and penetrating inside the scaffold.

Moreover, SEM images showed an ECM formed by collagen structures (Figure 4.10D-F) and some precipitates which resemble calcium phosphate depositions (Figure 4.10G-I), also confirmed by an atomic microanalysis with the presence of calcium (0.43 %) and phosphorus (3.16 %) ions (Figure 4.11).

In addition, RT-PCR analysis for RNA extracted after the osteogenic induction, revealed an enhancement of the osteogenic markers, ALP, OC, RUNX2 and COL1 as well as adhesion markers, VCAM1 and VLA4 (Figure 4.12A) at similar levels to that of 2D-differentiated DPPSC or SAOS-2 cells. Immunohistochemistry using specific OC and OPN antibodies showed the presence of these proteins implied in the ECM mineralization (Figure 4.12B-C).

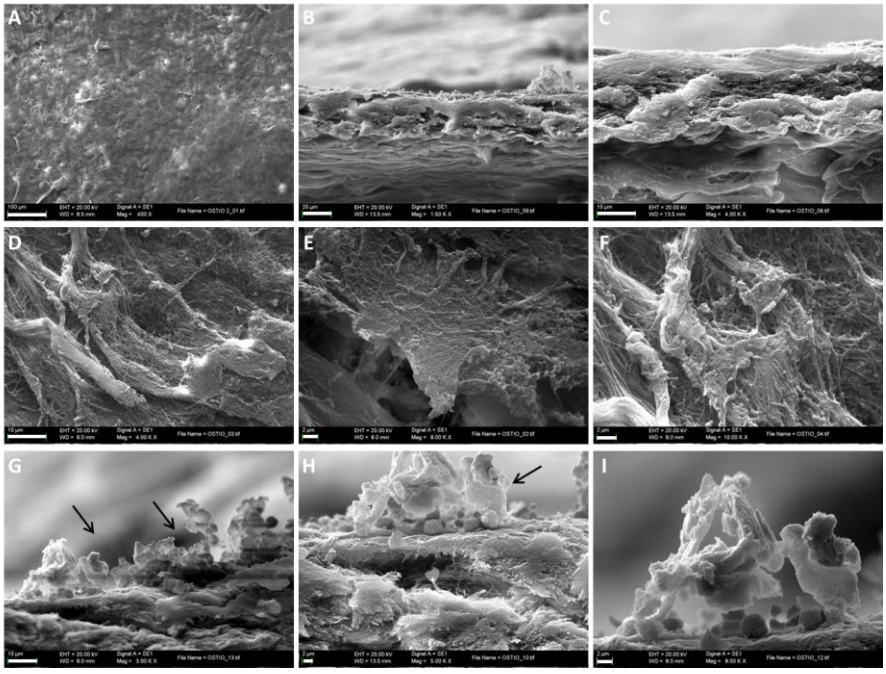


Figure 4.10. SEM images of the DPPSC osteogenic differentiation on CCC. A-C) Differentiated DPPSC adhered and penetrated on CCC (N=3). **D-F)** Collagen structures formed by differentiated DPPSC. **G-I)** Calcium phosphate depositions (black arrow) on the surface of CCC with differentiated DPPSC. Scale bars: 100 µm (A), 20 µm (B), 10 µm (C,D,G), 2 µm (E,F,H,I). Representative images from 1 of 3N.

ELEMENT	C	O	P	Ca	Fe	Total
ATOMIC %	73.79	22.51	3.16	0.43	0.11	100

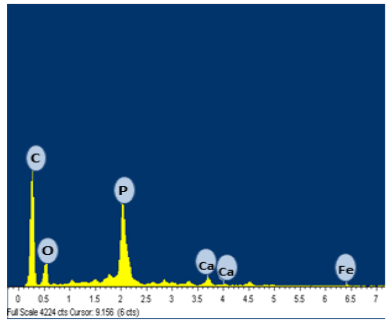


Figure 4.11. Atomic concentration microanalysis of the CCC surface with differentiated DPPSC.

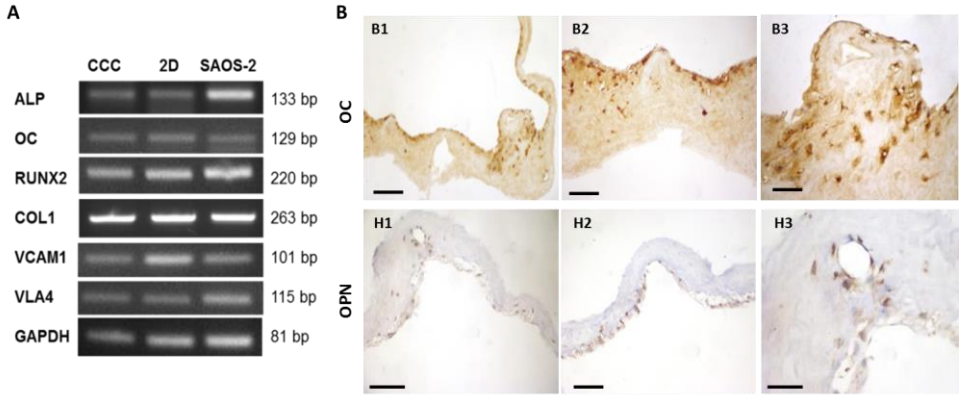


Figure 4.12. DPPSC osteogenic differentiation on Collagen I-based Cell Carrier (CCC). A) RT-PCR gene expression of osteogenic (OC, ALP, COL1A1) and adhesion markers (VCAM1, VLA4) in DPPSC differentiated in CCC, DPPSC differentiated in 2D and SAOS-2 cultured in CCC. GAPDH was used as a housekeeping gene. **B-C)** Immunohistochemistry of differentiation markers (OC, OPN) in DPPSC differentiated on CCC. Scale bars: 1000 µm (B1, C1), 400 µm (B2, C2); 200 µm (B3, C3). Representative images from 1 of 3N.

4.3.3.2 Osteogenic differentiation on Ti6Al4V disks

DPPSC (TI DPPSC), 15 day bone-like DPPSC (TI B.DPPSC) and SAOS-2 cells were cultivated and differentiated on titanium alloy disks with OM for 15 days.

SEM micrographs showed a high-density cell mass on the surface of the disk for all cell populations, indicating that the cells adhered and grew favourably (Figure 4.13), whereas TI B.DPPSC seemed to cover more surface than the other cell types (Figure 4.13B). Results of the ALP assay showed that the ALP activity increased significantly over time in TI DPPSC, TI B.DPPSC and TI SAOS-2, indicating that the cells acquired this functional activity during osteogenic differentiation (Figure 4.14). The behaviour of ALP activity in TI DPPSC and TI B.DPPSC was similar; differences between cell types were only statistically significant when comparing TI SAOS-2 cells with the DPPSC cell types.

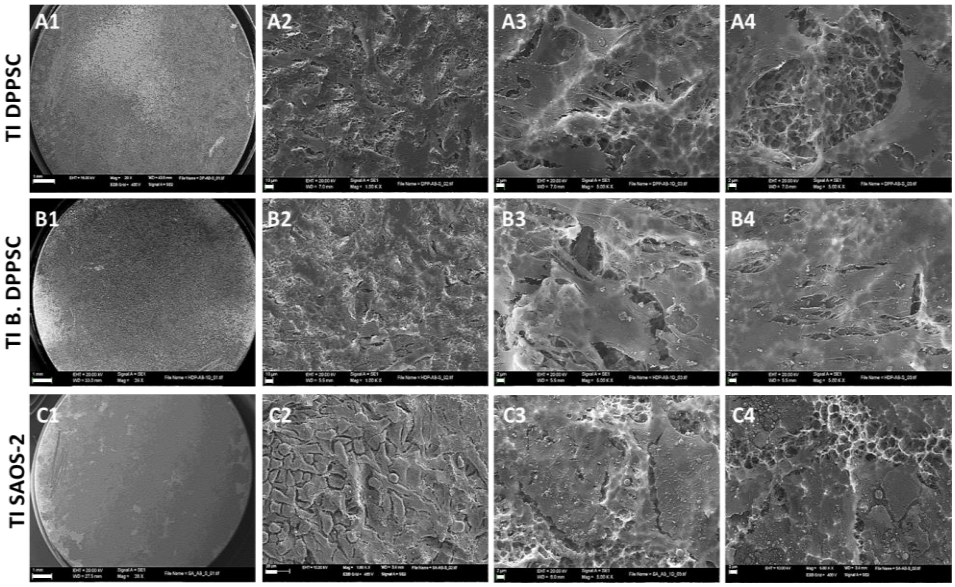


Figure 4.13. DPPSC osteogenic differentiation on Ti alloy disks. SEM images of **A)** DPPSC; **B)** Bone-like DPPSC (DPPSC 15 days pre-differentiated); **C)** SAOS-2 cells treated with OM during 15 days of culture on Ti alloy disks. Stars indicate the Ti surface without cells. Scale Bars: 1 mm (A1, B1, C1), 10 µm (A2, B2, C2), 2 µm (A3-4, B3-4, C3-4). Representative images from 1 of 3N.

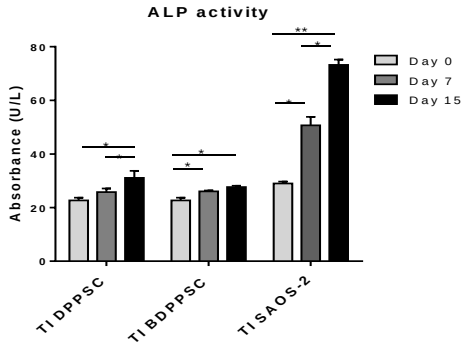


Figure 4.14. DPPSC osteogenic differentiation on Ti alloy disks. ALP activity of DPPSC (TI DPPSC); Bone-like DPPSC: DPPSC 15 days pre-differentiated (TI B.DPPSC) and SAOS-2 cells (TI SAOS-2) treated with OM during 15 days of culture on Ti alloy disks. **P < 0.05; **P < 0.01.* N=3.

To further characterize the differentiation status of DPPSC on titanium surfaces, we looked for the expression of several osteogenic and adhesion markers, at the end of the differentiation process using SAOS-2 cells for normalization (Figure 4.15). Compared to SAOS-2, TI DPPSC and TI B.DPPSC showed less expression of the early osteogenic marker RUNX2 and more expression of the advanced osteogenic markers COL1A1 and OC, with the highest expression in TI B.DPPSC (Figure 4.15A). Moreover, the expression of the adhesion genes showed that differentiated DPPSC have higher expression of integrin genes than SAOS-2 cells, confirming the high adhesion of these cells to the surface (Figure 4.15B-C).

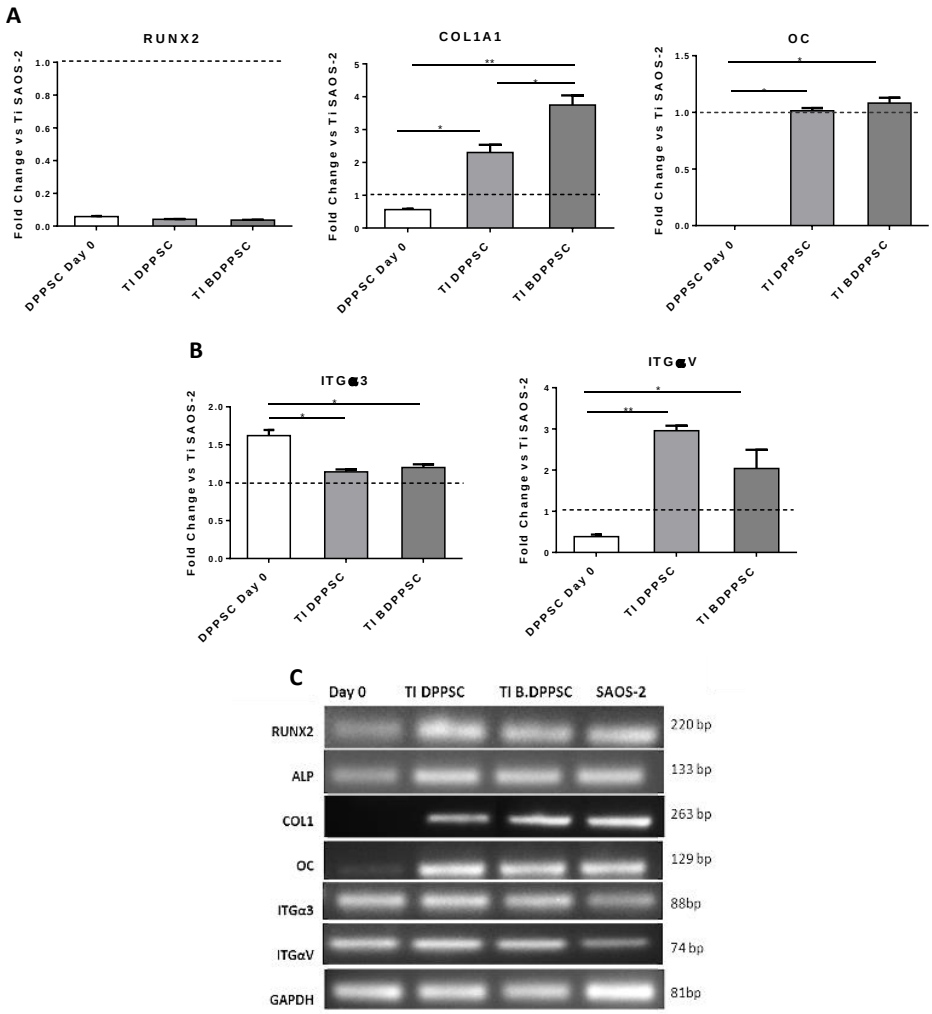


Figure 4.15. DPPSC osteogenic differentiation on Ti alloy disks. Gene expression of DPPSC (TI DPPSC); Bone-like DPPSC: DPPSC 15 days pre-differentiated (TI B.DPPSC) and SAOS-2 cells (TI SAOS-2) treated with OM during 15 days of culture on Ti alloy disks. **A-B)** Relative gene expression of **(A)** osteogenic markers (RUNX2, COL1A1, OC) and **(B)** adhesion markers (ITGα3, ITGαV) respect to TI SAOS-2. DPPSC at day 0 of differentiation were used as negative control and all data were normalized to the expression of the housekeeping gene GAPDH and TI SAOS-2 (dotted line). **P < 0.05; **P < 0.01*. N=3. **C)** RT-PCR of osteogenic (RUNX2, ALP, COL1A1, OC) and adhesion (ITGα3, ITGαV) markers. GAPDH was used as a housekeeping gene.

4.3.4 Genetic Stability

The development of potential therapeutic strategies using stem cells depends on their ability to undergo large scale *in vitro* amplification that could imply genetic instability. It has been reported that in some cell types used in BTE this instability appears, hence it was crucial to analyze the genetic stability in DPPSC.

We checked the genetic stability in undifferentiated DPPSC and during their osteogenic differentiation on culture plates and on biomaterials by short-CGH. Undifferentiated and differentiated DPPSC exhibited a normal karyotype with no presence of any aneuploidy or any chromosome structural alteration throughout several passages. Therefore, results showed that DPPSC maintained the stability before and after the differentiation process (Figure 4.16A-D).

Moreover, short-CGH confirmed a normal karyotype of DPPSC cultured on CCC and Ti6Al4V disks after 21 days of differentiation (Figure 4.16E-F)

We also evaluated the genetic stability of SAOS-2 cells. As we expected, SAOS-2 cells showed genetic mutations in numerous chromosomes by short-CGH, probably due to their carcinogenic origin (Figure 4.17).

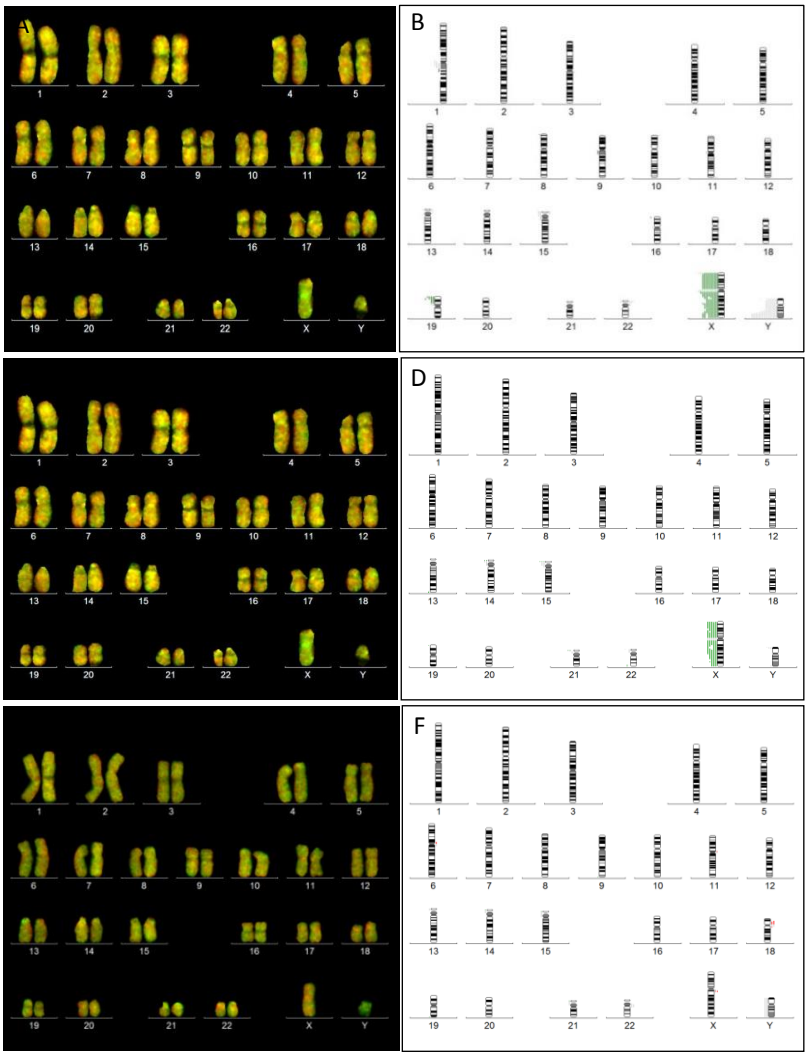


Figure 4.16. Genetic stability of DPPSC, before and after osteogenic differentiation by short-CGH analysis. A-B) short-CGH summary from undifferentiated DPPSC at passages 1, 5, 10 and 15 (N=6, n=12). **C-D)** Short-CGH summary from DPPSC at passages 1, 5 and 10 at day 21 of 2D osteogenic differentiation (N=6, n=12). **E-F)** Short-CGH summary from DPPSC after 21 days of osteogenic differentiation on biomaterials (CCC and Ti alloy disks) (N=3, n=12). In all analyses 47, XXY control samples (labelled in green) and DPPSC samples (labelled in red) were co-hybridized onto 46, XY metaphases (n=12). A gain of X or Y chromosome dosage was due to sex differences.

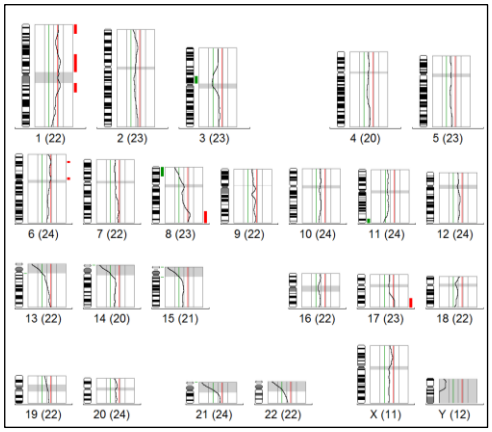


Figure 4.17. Genetic instability of SAOS-2 cells. Short-CGH summary from SAOS-2 cells at passage 10 (N=2, n=12). 47, XXY control samples (labelled in green) and SAOS-2 samples (labelled in red) were co-hybridized onto 46, XY metaphases.

4.4 DISCUSSION

The third molar represents a very accessible organ, which is often extracted for dental reasons, and due to its late development, allows the presence of progenitor cells. Previously, our group identified DPPSC as a new population of stem cells from the dental pulp cultivated in particular culture conditions to maintain their pluripotent-like state. In addition it has been demonstrated that DPPSC are able to differentiate into adult tissues from the three embryonic germ layers [11]. Moreover, DPPSC maintain the pluripotency ability and higher genetic stability in advanced culture passages.

We consider that PSC have higher value when testing the differentiation capacity of biomaterials, since these cells provide highly undifferentiated cells which need to be guided merely by the biomaterial. Hence, we consider that the use of osteoblastic-like cells to determine the osteogenic capacity of biomaterials is meaningless compared to the use of pluripotent-like cells. For all these reasons, in this study, we elected DPPSC to perform the osteogenic differentiations on biomaterials.

4.4.1 DPPSC and DPMSC are different dental pulp populations with distinct differentiation potential

First of all, in order to better characterize the mesodermal potential of DPPSC, the multilineage differentiation capacity of DPPSC and DPMSC from the same donors was investigated.

Although the osteogenic potential of DPPSC and DPMSC were previously studied [11, 23-25], here we have analysed for the first time the ability to form adipocyte-like and chondrocyte-like cells from DPPSC in comparison with DPMSC. Results showed that DPPSC were favourable to differentiate towards chondrogenic and osteogenic cells, whereas DPMSC showed more adipogenic potential.

Concretely, both DPPSC and DPMSC showed chondrogenic differentiation potential. However, DPMSC had higher expression of the initial marker SOX9 and only DPPSC showed expression of the advanced marker COL2A1, indicating that DPPSC were in a more mature stage at the end of the chondrogenic differentiation.

On the other hand, it is widely known that DPMSC are able to differentiate into adipocytes [26, 27]. Nevertheless, our results showed that, in this case, neither of both dental pulp populations were completely differentiated into adipocyte-like cells under the adipogenic medium conditions used. Hence, adipogenic differentiation of DPPSC and DPMSC was initiated but not progressed efficiently. However, qRT-PCR results indicated that DPMSC showed more pronounced adipogenic capacity in their undifferentiated stage than DPPSC.

Finally, based on the osteogenic differentiation, our results confirmed that DPPSC seem to present a higher osteogenic potential than DPMSC [10].

Therefore, these results also confirmed that, despite their common origin and isolation protocol, DPPSC and DPMSC are different dental pulp populations with distinct differentiation potential. In fact, previous reports showed that each cell population expressed a different profile of cell surface and intracellular markers [10, 11]. Although both dental pulp stem cell populations share several surface markers (CD29+, CD105+, CD45-), DPPSC show higher

expression of pluripotency markers such as OCT3/4, NANOG, SOX2 and SSEA4 [11, 28].

It was suggested that the distinction of DPPSC and DPMSC must depend on the culture medium and the density at which the cells are seeded. DPPSC, instead of DPMSC, are cultivated at low cell culture density in a medium containing low concentration of FBS (2%) and some factors to maintain their pluripotent state such as LIF, EGF and PDGF [11, 28].

Therefore, the pluripotency and osteogenic capabilities of DPPSC, could make them a good model for the evaluation of BTE biomaterials.

4.4.2 DPPSC are able to differentiate into bone-like tissue

To address the requirement to well-characterize the osteogenic differentiation of DPPSC, one of the first aims of this doctoral thesis was to analyse the osteogenesis process of this new stem cell population and to establish the expression profile of bone related genes during DPPSC differentiation.

Firstly, we demonstrated the effect of the OM in DPPSC cultures over 15 days. Results showed that DPPSC survived perfectly, being the cell increment higher in OM cultures than in BM cultures. Moreover, protein and functional analyses showed that DPPSC in OM were able to differentiate towards osteogenic lineage, while DPPSC in BM could not be differentiated. Therefore, the supplementation of the medium with ascorbic acid, β-glycerolphosphate and dexamethasone is required for the osteogenic differentiation of DPPSC, confirming that DPPSC are an undifferentiated stem cell population. In fact, previous studies have indicated that the initiation of mineral formation is highly dependent on the presence of β-glycerolphosphate and L-ascorbic acid 2-phosphate that serve as phosphate sources required for the mineralization [29].

Moreover, we analysed in more detail the DPPSC osteogenic differentiation process during 21 days. In general, our results in gene expression indicate that the osteogenic differentiation of DPPSC is in accordance with the human osteoblastic development, which can be divided into three chronological

stages: proliferation, matrix development and mineralization [30, 31]. Thus, RUNX2 is expressed during early osteoblast differentiation and is strictly required for the differentiation and appropriate functioning of osteoblasts [32]. Here, we found that RUNX2 was highly expressed during the first week of DPPSC differentiation, while the expression levels of pluripotent markers OCT3/4 and NANOG were reduced, indicating the end of the proliferation stage. At the second week, during the extracellular matrix maturation, RUNX2 expression levels were progressively down-regulated while differentiated cells began to express OC, in agreement with the reports that RUNX2 is an upstream gene of OC [31, 32]. Moreover, there was a remarkable peak of OC at day 15 that indicated an early mineralization of the differentiated DPPSC.

By comparing the gene expression of differentiated DPPSC with differentiated SAOS-2 cells, we can observe that SAOS-2 showed more expression of the initial osteogenic marker RUNX2 and similar levels of the advanced marker OC. It was probably due to the uncontrolled proliferation rate of SAOS-2, which constantly produces immature cells. SAOS-2 cells have been frequently used in applied biology since they are from human origin, they provide an unlimited number of cells and therefore, they are a fast and a cheap cell model to test osteogenesis in biomaterials. However, we consider that the use of pluripotent-like cells that can potentially differentiate into any lineage assesses better the role of the biomaterial in the osteogenic differentiation. Moreover, as DPPSC are in a previous stage of differentiation than SAOS-2 cells, this allows the analysis of the osteogenic differentiation since the beginning of the process.

Furthermore, RT-PCR analysis revealed that ALP was also expressed during the phase of matrix development, increasing from the first week until the end of the differentiation. This triggers the mineralization stage, commonly observed by the production of hydroxyapatite crystals [33]. At the same time, COL1A1 was detected during the matrix development until the mineralization stage, indicating that the maturation process of osteoblast-like cells produced abundant matrix proteins that were deposited as osteoid or non-mineralized bone matrix [34]. Moreover, results showed very similar osteogenic genes expression levels between DPPSC differentiation and the expression levels of

human bone cDNA, in accordance with a previous study on the osteogenic differentiation of DPPSC, where the bone-like tissue formed by DPPSC in 3D were similar in complexity to human bone tissue [10].

Finally, to evaluate the functional activity of bone-like DPPSC, Calcium and ALP stainings were performed through the differentiation. By the third week, both staining demonstrated an osteoid mineralization by the accumulation of calcium phosphate in the form of hydroxyapatite. In addition, protein analysis with high expression of late osteogenic markers, OC and OPN, confirmed the osteocyte-like phenotype of DPPSC. In summary, our results indicated that differentiated DPPSC show a behaviour pattern similar to human primary osteoblasts (HOb) [3, 35].

4.4.3 DPPSC show high osteogenic and adhesion potential in different types of biomaterials

It is known that natural materials, metals and synthetic polymers scaffolds organize stem cells into complex spatial groupings that mimics native tissue [36]. In this study, we evaluated the capacity of DPPSC to grow and differentiate in a natural collagen scaffold (CCC). Natural biological and mechanical properties of native collagen provide a bio-mimetic environment for stem cells as well as a mechanical support. Hence, collagen based biomaterials are commonly used as a support for cell culture and tissue engineering [37]. Here, the analysis of the scaffold surface and cell morphology by SEM emphasized the high affinity of DPPSC to grow in CCC, the homogenous cell distribution and the high level of calcification at the final stage of the differentiation. The expression of osteogenic and adhesion markers in differentiated DPPSC on CCC was comparable to the 2D differentiation on culture plates and SAOS-2 CCC culture. Moreover, an immunohistochemistry assay showed the expression of late osteogenic markers, corroborating the presence of osteocyte-like cells and the complete differentiation and biocompatibility of DPPSC over CCC *in vitro*.

Some studies report that titanium favours the osseointegration, stimulating the functions of osteoblasts on the surface, such as the adhesion, proliferation or secretion of specific proteins composing the ECM [38]. Currently, the most common cell sources used for the evaluation of titanium surfaces are immortalized cell lines and fibroblasts [39, 40]. However, these cell types have different characteristics from stem cells involved in bone regeneration *in vivo.* There are few studies regarding the osseointegration of titanium with stem cells [41]. Therefore, this study attempts to evaluate the osteogenic capacity of TI disks with an adult pluripotent-like stem cell population. Moreover, two populations of DPPSC were used: DPPSC and B.DPPSC. B.DPPSC were DPPSC pre-differentiated for 15 days, when they reached the beginning of the mineralization stage, corresponding with the peak of OC expression. This previous differentiation of the cells on plates before transferring to titanium surfaces can be used as another strategy for biomaterials evaluation, accelerating the process and reducing the costs (differentiated cells proliferate more rapidly and can be cultured at higher densities).

SEM images obtained on TI disks showed a complete coverage of the surface with all cell types, suggesting a major density, expansion and adhesion in TI B.DPPSC. On the other hand, the high ALP activity of SAOS-2 could be explained by some studies that revealed that this property of SAOS-2 can differ considerably from primary osteoblasts behaviour [3]. In addition, at the end of differentiation, TI SAOS-2 cells had higher expression of the initial markers ALP and RUNX2 and lower expression of advanced markers COL1 or OC than DPPSC. These results could indicate that this carcinogenic cell line has cells in a more immature stage than DPPSC lines, probably due to their constant and uncontrolled proliferation rate. Moreover, we found that the expression of COL1, the most important protein in the non-mineralized matrix, was higher in TI B.DPPSC. Finally, TI DPPSC and TI B.DPPSC had also a higher expression of the adhesion markers than SAOS-2, suggesting that DPPSC had a strong capacity to adhere on titanium surfaces.

4.4.4 DPPSC maintain genetic stability during culture expansion and differentiation on biomaterials

After analysing the expression profile of osteogenic genes during DPPSC differentiation, we analysed the genetic stability of this pluripotent-like stem cell population before and after their differentiation process on biomaterials.

There are few studies that used adult stem cells with genetic stability to assess the quality and the osteogenic capacity of biomaterials [10, 42]. Therefore, we analysed the genetic stability of DPPSC and SAOS-2 by short-CGH, a direct aneuploidy screening that allows the detection of chromosome imbalances generated by aberrant segregation and structural differences for fragments larger than 10–20 Mb [43]. Based on this analysis, our results showed a normal chromosomal dosage during DPPSC culture expansion until passage 15. This was also evident at the end of the differentiation process, both on culture plates and on biomaterials. Nevertheless, we confirmed some genetic instability in SAOS-2 cells that have been probably related to the progression and genesis of the osteosarcoma. This low stability of SAOS-2 could induce phenotype alterations, aberrations in mitotic processes or lack of growth inhibition affecting the results of biomaterial testing [6].

Some reports demonstrate a direct correlation between culture density and the occurrence of DNA damage and genomic alterations during the culture of stem cells *in vitro* [44]. These effects are largely caused by the accumulation of lactic acid in the culture medium and the associated medium acidification [44, 45]. Here, the particular culture conditions of DPPSC (medium composition, low serum levels, low cell confluence before passaging and low cell culture density) could facilitate the preservation of the genomic stability, making DPPSC an stable cell model for testing biomaterials in bone regeneration studies.

4.5 CONCLUSIONS

These results support the capacity of DPPSC, a new pluripotent-like population of dental pulp stem cells, to differentiate into bone-like tissue. Moreover, DPPSC show high osteogenic and adhesion potential in different types of

biomaterials, whilst seem to maintain genetic stability during their culture expansion and differentiation.

In conclusion, we propose the use of DPPSC as a good alternative model to evaluate the biocompatibility and the differentiation capacity of different types of biomaterials commonly used for bone regeneration studies.

4.6 REFERENCES

[1] Nunez-Toldra R, Martinez-Sarra E, Gil-Recio C, Carrasco MA, Al Madhoun A, Montori S, et al. Dental pulp pluripotent-like stem cells (DPPSC), a new stem cell population with chromosomal stability and osteogenic capacity for biomaterials evaluation. BMC cell biology. 2017;18:21.

[2] Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnic M, Vunjak-Novakovic G. Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. Current stem cell research & therapy. 2008;3:254-64.

[3] Czekanska EM, Stoddart MJ, Ralphs JR, Richards RG, Hayes JS. A phenotypic comparison of osteoblast cell lines versus human primary osteoblasts for biomaterials testing. Journal of biomedical materials research Part A. 2013.

[4] Amini AR, Laurencin CT, Nukavarapu SP. Bone tissue engineering: recent advances and challenges. Critical reviews in biomedical engineering. 2012;40:363-408.

[5] Rodan SB, Imai Y, Thiede MA, Wesolowski G, Thompson D, Bar-Shavit Z, et al. Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. Cancer research. 1987;47:4961-6.

[6] Hausser HJ, Brenner RE. Phenotypic instability of Saos-2 cells in long-term culture. Biochemical and biophysical research communications. 2005;333:216-22.

[7] Kassem M. Mesenchymal stem cells: biological characteristics and potential clinical applications. Cloning and stem cells. 2004;6:369-74.

[8] Wagner W, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, et al. Aging and replicative senescence have related effects on human stem and progenitor cells. PloS one. 2009;4:e5846.

[9] Perez RA, Choi S-J, Han C-M, Kim J-J, Shim H, Leong KW, et al. Biomaterials control of pluripotent stem cell fate for regenerative therapy. Progress in Materials Science. 2016;82:234-93.

[10] Atari M, Caballe-Serrano J, Gil-Recio C, Giner-Delgado C, Martinez-Sarra E, Garcia-Fernandez DA, et al. The enhancement of osteogenesis through the use of dental pulp pluripotent stem cells in 3D. Bone. 2012;50:930-41.

[11] Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, et al. Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science. 2012;125:3343-56.

[12] Malafaya PB, Silva GA, Reis RL. Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Advanced drug delivery reviews. 2007;59:207-33.

[13] Chevallay B, Herbage D. Collagen-based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy. Medical & biological engineering & computing. 2000;38:211-8.

[14] Lee CH, Singla A, Lee Y. Biomedical applications of collagen. International journal of pharmaceutics. 2001;221:1-22.

[15] Delgado LM, Bayon Y, Pandit A, Zeugolis DI. To cross-link or not to cross-link? Cross-linking associated foreign body response of collagen-based devices. Tissue engineering Part B, Reviews. 2015;21:298-313.

[16] Lee W, Debasitis JC, Lee VK, Lee JH, Fischer K, Edminster K, et al. Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. Biomaterials. 2009;30:1587-95.

[17] Micol LA, Ananta M, Engelhardt EM, Mudera VC, Brown RA, Hubbell JA, et al. High-density collagen gel tubes as a matrix for primary human bladder smooth muscle cells. Biomaterials. 2011;32:1543-8.

[18] Schmidt T, Stachon S, Mack A, Rohde M, Just L. Evaluation of a thin and mechanically stable collagen cell carrier. Tissue engineering Part C, Methods. 2011;17:1161-70.

[19] Kato H, Nakamura T, Nishiguchi S, Matsusue Y, Kobayashi M, Miyazaki T, et al. Bonding of alkali- and heat-treated tantalum implants to bone. Journal of biomedical materials research. 2000;53:28-35.

[20] Covani U, Giacomelli L, Krajewski A, Ravaglioli A, Spotorno L, Loria P, et al. Biomaterials for orthopedics: a roughness analysis by atomic force microscopy. Journal of biomedical materials research Part A. 2007;82:723-30.

[21] Branemark R, Branemark PI, Rydevik B, Myers RR. Osseointegration in skeletal reconstruction and rehabilitation: a review. Journal of rehabilitation research and development. 2001;38:175-81.

[22] Khanna-Jain R, Vuorinen A, Sandor GK, Suuronen R, Miettinen S. Vitamin D(3) metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells. The Journal of steroid biochemistry and molecular biology. 2010;122:133-41.

[23] Alge DL, Zhou D, Adams LL, Wyss BK, Shadday MD, Woods EJ, et al. Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. Journal of tissue engineering and regenerative medicine. 2010;4:73-81.

[24] Tomic S, Djokic J, Vasilijic S, Vucevic D, Todorovic V, Supic G, et al. Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. Stem cells and development. 2011;20:695-708.

[25] Harrington J, Sloan AJ, Waddington RJ. Quantification of clonal heterogeneity of mesenchymal progenitor cells in dental pulp and bone marrow. Connective tissue research. 2014;55 Suppl 1:62-7.

[26] Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2000;97:13625-30.

[27] Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue engineering. 2006;12:2813-23.

[28] Martínez-Sarrà E. Characterization of Dental Pulp Pluripotent-like Stem Cells (DPPSC) and their mesodermal differentiation potential. Barcelona: Universitat Internacional de Catalunya; 2017.

[29] Fratzl-Zelman N, Fratzl P, Horandner H, Grabner B, Varga F, Ellinger A, et al. Matrix mineralization in MC3T3-E1 cell cultures initiated by beta-glycerophosphate pulse. Bone. 1998;23:511-20.

[30] Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. Bone. 1995;17:77S-83S.

[31] Miron RJ, Zhang YF. Osteoinduction: a review of old concepts with new standards. Journal of dental research. 2012;91:736-44.

[32] Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell and tissue research. 2010;339:189-95.

[33] Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, et al. Functional characterization of osteoblasts and osteoclasts from alkaline

phosphatase knockout mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2000;15:1879-88.

[34] Dallas SL, Bonewald LF. Dynamics of the transition from osteoblast to osteocyte. Annals of the New York Academy of Sciences. 2010;1192:437-43.

[35] Karner E, Backesjo CM, Cedervall J, Sugars RV, Ahrlund-Richter L, Wendel M. Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. Biochimica et biophysica acta. 2009;1790:110-8.

[36] Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425:836-41.

[37] Glowacki J, Mizuno S. Collagen scaffolds for tissue engineering. Biopolymers. 2008;89:338-44.

[38] Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC. Review: ex vivo engineering of living tissues with adult stem cells. Tissue engineering. 2006;12:3007-19.

[39] Nothdurft FP, Fontana D, Ruppenthal S, May A, Aktas C, Mehraein Y, et al. Differential Behavior of Fibroblasts and Epithelial Cells on Structured Implant Abutment Materials: A Comparison of Materials and Surface Topographies. Clinical implant dentistry and related research. 2014.

[40] Vandrovcova M, Jirka I, Novotna K, Lisa V, Frank O, Kolska Z, et al. Interaction of human osteoblast-like Saos-2 and MG-63 cells with thermally oxidized surfaces of a titanium-niobium alloy. PloS one. 2014;9:e100475.

[41] Hou Y, Cai K, Li J, Chen X, Lai M, Hu Y, et al. Effects of titanium nanoparticles on adhesion, migration, proliferation, and differentiation of mesenchymal stem cells. International journal of nanomedicine. 2013;8:3619-30.

[42] Mendonca G, Mendonca DB, Simoes LG, Araujo AL, Leite ER, Duarte WR, et al. The effects of implant surface nanoscale features on osteoblast-specific gene expression. Biomaterials. 2009;30:4053-62.

[43] Griffin DK, Sanoudou D, Adamski E, McGiffert C, O'Brien P, Wienberg J, et al. Chromosome specific comparative genome hybridisation for determining the origin of intrachromosomal duplications. Journal of medical genetics. 1998;35:37-41.

[44] Jacobs K, Zambelli F, Mertzanidou A, Smolders I, Geens M, Nguyen HT, et al. Higher-Density Culture in Human Embryonic Stem Cells Results in DNA Damage and Genome Instability. Stem cell reports. 2016;6:330-41.

[45] Chen X, Chen A, Woo TL, Choo AB, Reuveny S, Oh SK. Investigations into the metabolism of two-dimensional colony and suspended microcarrier cultures of human embryonic stem cells in serum-free media. Stem cells and development. 2010;19:1781-92.

CHAPTER 5: IMPROVEMENT OF OSTEOGENESIS IN DPPSC BY OLIGOPEPTIDE-MODIFIED POLY(ß-AMINO ESTER)S

The following experiments have been performed in collaboration with the *Grup d'Enginyeria de Materials (Institut Químic de Sarrià, Universitat Ramón LLull,* Barcelona). The contents of this chapter have been previously published [1].

5.1 INTRODUCTION

One of the possibilities of BTE is the use of genetic modification techniques to stimulate bone formation [2-4]. Moreover, controlling pluripotent stem cell (PSC) differentiation via genetic manipulation is a promising procedure in the regenerative medicine field. However, the lack of safety and the low efficiency in delivering genetic vehicles limits its application [3, 5].

Non-viral vectors are an attractive option because they present lower immunogenicity than viral vectors and they are easy to manufacture [2]. Recently, a new family of poly (β-amino ester)s polymers (pBAEs) with oligopeptide-modified termini that shows high transfection efficiency of both siRNA and DNA plasmids, has been developed [6, 7]. In this chapter, oligopeptide-modified pBAEs were used to simultaneously deliver anti-OCT3/4 siRNA and RUNX2 plasmid into DPPSC in order to promote their osteogenic differentiation.

5.1.1 Gene therapy in Bone Tissue Regeneration

Gene therapy is an experimental technique that implies the introduction of functional genes into the cells in order to enhance or enforce their expression. Moreover, gene therapy can induce an inhibitory effect by small interfering ribonucleic acid (siRNA) or micro ribonucleic acid (miRNA) [2].

Use of gene therapies for bone tissue regeneration does not involve the replacement of a non-functional gene as in genetic disorders therapies, but comprises the delivery of transcription factors (RUNX2, SOX9, OSX) or growth factors (BMPs, IGF1, VEGF, TNFα) [8-10]. Many proteins are rapidly degraded, resulting in the delivery of up to milligram dosages to provide a sufficient

stimulus that lasts for few days in order to induce osteogenic differentiation and subsequent bone formation [2, 3].

Current strategies to deliver DNA can be divided in viral and non-viral transfections, that can be conducted *in vivo* and *ex vivo* [3]. Viral transfection is an effective way of gene transfer for many cell types that provides high transfection efficiency [11, 12]. However, viral vectors, such as adenovirus, retrovirus and lentivirus, shows safety concerns (e.g. mutagenesis or immunogenicity), limited nucleic acid packaging and difficulties in scale-up production, which limits their use in clinical trials [13, 14]. On the other hand, non-viral vectors, such as cationic liposomes and cationic polymers, have the potential to address many of these limitations, making them an attractive alternative [2]. These synthetic vectors present architecture and gene delivery system similar to viral vectors and allow complete definition and control of their design and properties. Furthermore, non-viral vectors present lower immunogenicity than viral vectors and are manufactured in an easier way. In addition, in recent years, transfection efficiency of synthetic vectors has been improved in order to become an important tool for human gene therapy.

In vivo and *ex vivo* BTE approaches have led to successful osteogenic differentiation and bone formation [3, 4]. During *in vivo* gene therapy, the vector is directly administered to the fracture gap and resident cells are expected to be transfected and, then, they will locally produce the osteogenic protein [15]. The administration can be via direct injection or combined with a biomaterial in a gene activated matrix (GAM) [16]. On the other hand, in the *ex vivo* approach, autologous cells will be harvested and transduced outside the body [17, 18]. The transduced cells are subsequently implanted in the fracture gap. Again, the main application method can be a direct injection or using a biomaterial as a carrier (Figure 5.1).

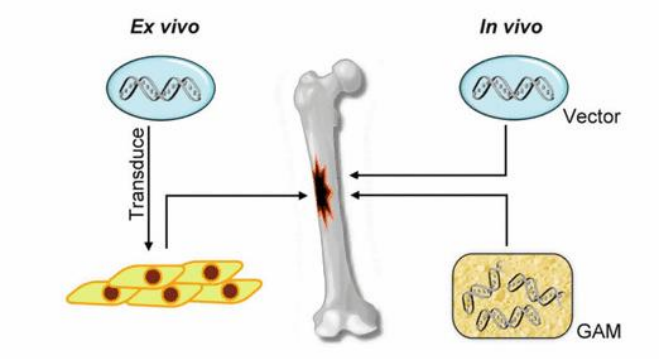


Figure 5.1. Different gene therapy methods for BTE. *Ex vivo* and *in vivo* approaches to induce osteogenic differentiation and bone formation. GAM: Gene activated matrix.

5.1.2 Oligopeptide-modified poly(β-amino ester)s

Poly(ß-amino ester) (pBAE) are non-viral vectors that have emerged in recent years. Currently, they are one of the most promising cationic polymers for gene delivery due to their transfection efficiency and their promising biophysical characteristics, like biodegradability and biocompatibility [19]. Hence, pBAEs polymer vectors are capable of condensing both DNA and RNA into discrete nanometric particles that protect nucleic acids from nuclease degradation [20]. Furthermore, their chemical structure allows further modifications of their termini, making easy their design and allowing the introduction of specific features for their final application [20, 21].

Different types of pBAEs have been used successfully in a large amount of therapeutic applications including vaccination, gene therapy for cancer, gene silencing and stem cell modification [22-26].

A powerful strategy to improve the biocompatibility of these non-viral vectors is the use of natural molecules, such as amino acids or lipids. These natural cationic and anionic amino acids reduce the toxicity of polymers. A new family of pBAEs polymers with natural oligopeptide-modified termini has been developed recently. These oligopeptide-modified poly(β-amino ester)s have demonstrated cell-type specificity and high transfection efficiency even in hard-to-transfect cell lines, such as stem cells and endothelial cells [6, 7]. Moreover,

the use of selected oligopeptides in the pBAEs formulation leads to preferential intracellular localization of the particles and makes them suitable polymers in terms of biodegradability and biocompatibility.

In general, oligopeptide-modified pBAEs are obtained by an end-modification of the acrylate-terminated polymer C32. Therefore, terminated pBAEs are modified with different cationic and anionic oligopeptides: arginine (CR3), lysine (CK3), histidine (CH3) as a cationic oligopeptides and aspartate acid (CD3) and glutamate acid (CE3) as anionic oligopeptides. Resulted polymers can show different behaviour according to their chemical structures [6, 7]. The cationic polymers play an important role in the nanoparticle formation due to their positive charge, which interacts with the negative charge of phosphate groups of nucleic acids. On the other hand, anionic polymers can provide several advantages when are combined with cationic polymers, such as improvement of the encapsulation efficiency by charge balance or destabilization of the nucleic acid-polymer complex inside the cells improving nucleic acids release [7].

5.1.3 Nanoparticles transfection mechanism

Non-viral vectors can be used to deliver DNA, mRNA and short double-stranded RNA, including small interfering RNA (siRNA) and microRNA (miRNA) [27, 28]. The transfection mechanism of nanoparticles can be divided in different stages (Figure 5.2):

1. **Nanoparticles formation:** pBAE polymers are able to condense siRNA or DNA plasmids by electrostatic interactions between positive charge of pBAEs and negative charge of phosphate groups of nucleic acids forming nanoparticles to transfect the cells. The carrier protects the nucleic acid from degradation and improves the cellular uptake of the complex.

2. **Endocytosis (cell entry):** the positive polyplex interacts with the negatively-charged phospholipid bilayer of the cell membrane and an endosomal compartment is formed to mediate cell entry.

3. **Endosome escape:** once the endosome is inside the cell, the protonation of polymers induce an inflow of water across the endosome membrane leading to an osmotic swelling. This phenomenon causes the rupture of the endosome and the release of the polyplexes.

4. **Polymer unpacking and degradation:** DNA/RNA is released from the particle and the polymer is degraded under physiological conditions and released to the ECM.

5. **Nucleic acid activation:** siRNA and miRNA mimics must be loaded into the RNA-induced silencing complex (RISC) to cleave the target mRNA strand complementary to the siRNA. mRNA must bind to the translational machinery. DNA has to be further transported to the nucleus to be transcripted and then, to the ribosomes to be translated into a functional protein.

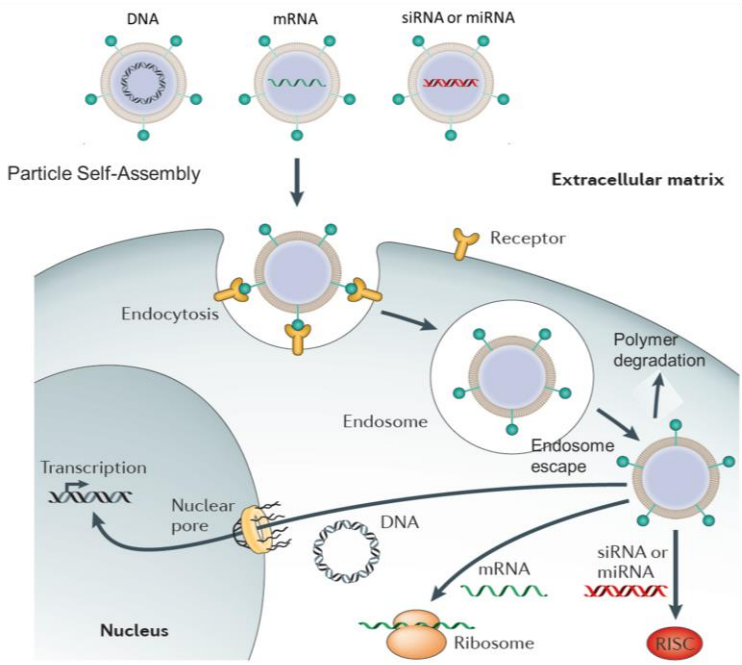


Figure 5.2. Nanoparticles transfection mechanism by non-viral gene therapy. Modified from Yin *et al*. (2014) [27].

5.1.4 Stem cells and gene therapy for Bone Tissue Engineering

Currently, most of the gene therapy strategies used in bone regeneration rely on the constitutive expression of certain transcription factors involved in the regulation of bone development that are rapidly degraded, such as RUNX2, SOX9 and OSX [8, 29-31]. RUNX2 is an essential regulator of bone development that controls the expression of a number of target genes during the osteogenic differentiation [8]. It has been shown that forced expression of RUNX2 in non-osteoblastic cells induces expression of multiple osteoblast-specific genes, upregulating the osteoblastic differentiation [32]. However, their positive function is restricted to the early differentiation stage of osteoblast development, because RUNX2 inhibits the late stage of the osteoblast maturation. In addition, it has been reported that continued overexpression of RUNX2 might generate adverse effects, such as severe osteopenia and bone fractures [10, 33].

On the other hand, it is known that after the osteogenic induction of stem cells, cultures retain an undifferentiated population of cells expressing pluripotency markers, such as OCT3/4 and NANOG, which maintain their stemness and their high proliferative potential [34, 35]. It has been demonstrated that the expression of these pluripotency factors is essential for the beginning of the osteogenic differentiation of stem cells [36]. However, their residual expression at the end of the process could impede their total differentiation into osteocytes [37, 38]. Therefore, this phenomenon can limit the clinical application of stem cells.

The most relevant advantages of gene therapy include the flexibility to express the protein locally and focally, or in a disseminated manner, as needed. Therefore, a possible strategy to improve bone regeneration with stem cells might be the control of the expression of some key genes simultaneously, such as the silence of pluripotency genes and the enhancement of the osteogenic genes expression during the osteogenic process. However, the development of safe and efficient gene delivery methods for stem cells gene therapy would be necessary.

5.2 OBJECTIVES

The main objective of this chapter was to describe a safer way to accelerate the osteogenic differentiation process of DPPSC using pBAE polymers as gene delivery method.

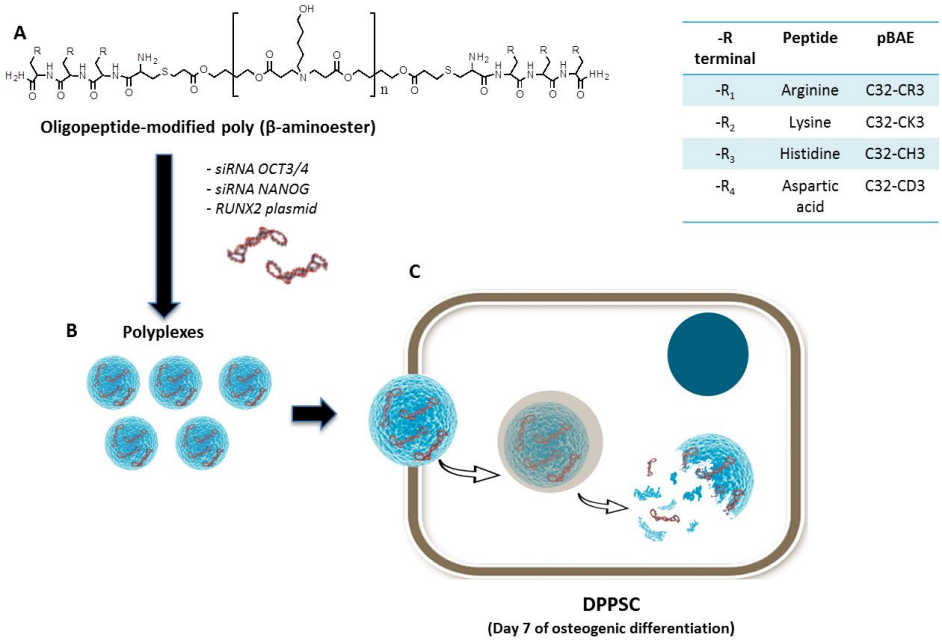
More specifically, we evaluated the effect of a double transfection with pBAE polymers containing plasmid DNA vectors to induce transient overexpression of RUNX2 and, simultaneously, siRNAs to silence the pluripotency genes OCT3/4 and NANOG.

5.3 RESULTS

Oligopeptide-modified pBAEs were used as a non-viral gene therapy to simultaneously deliver anti-OCT3/4 siRNA, anti-NANOG siRNA, and RUNX2 plasmid into DPPSC after 7 days of osteogenic differentiation (Figure 5.3).

End-modified pBAE synthesis by different oligopeptide moieties was performed as described in previous reports [6, 7]. Briefly, C32 polymer was further modified with different oligopeptide moieties via addition of arginine-, lysine-, histidine- or aspartic acid- oligopeptides in the R-terminal of C32 to co-deliver siRNA and plasmids in DPPSC (Figure 5.3A).

Hence, the first step of our experiments was focused on the identification of the better polymer formulation to achieve the optimal co-delivery of nucleic acids into DPPSC. Afterwards, we investigated the effect of silencing the pluripotency genes OCT3/4 and NANOG by siRNAs in DPPSC. Finally, we evaluated the effect of siOCT3/4 and RUNX2 plasmid co-delivery on the osteogenic differentiation of DPPSC. In addition, cell viability and genetic stability assays were performed at different time points along the differentiation to evaluate the safety of this co-delivery strategy in DPPSC.



-R terminal	Peptide	pBAE
$-R_1$	Arginine	C32-CR3
$-R_2$	Lysine	C32-CK3
$-R_3$	Histidine	C32-CH3
$-R_4$	Aspartic acid	C32-CD3

Figure 5.3. Oligopeptides modified pBAEs were used to co-deliver siRNA and plasmids in DPPSC. A) Chemical structure of synthetized oligopeptide end-modified poly(β-amino ester)s. -R terminal can be arginine-, lysine-, histidine- and aspartic acid-oligopeptide. **B)** Polyplexes formation by siRNA OCT3/4, siRNA NANOG or RUNX2 plasmid condensation. **C)** Polyplexes interaction with DPPSC membrane and cell entry by endocytosis.

5.3.1 Identification of the best-performing polymer formulation for DPPSC transfection

Firstly, in order to achieve the optimal co-delivery of nucleic acids into DPPSC, we focused on the identification of the top-performing polymer formulation. Therefore, the analysis of end-modified pBAEs entry was performed using a GFP reporter plasmid and a fluorescently-labelled siRNA by flow cytometry (Figure 5.4). Results demonstrated that pmaxGFP plasmid showed different GFP protein expression levels depending on the oligopeptide termini (Figure 5.4A-B). In general, mixtures of arginine (CR3) or lysine (CK3) oligopeptides with aspartic acid (CD3) or histidine (CH3) presented a greater GFP expression than

polyplexes prepared with either arginine (CR3) - or lysine (CK3) -pBAEs alone (Figure 5.4A). Results showed that the formulation composed by arginine- and aspartic acid-terminated polymer (CR3/CD3) was identified as the top-performing formulation under these conditions. CR3/CD3 was able to transfect the 60% of DPPSC, achieving a 2.3-fold increase in GFP expression compared to the positive control.

On the other hand, the siRNA screening using fluorescently-labelled siRNA in different oligopeptide-modified pBAEs showed that all the tested polymer formulations presented more than 4-fold increase in cellular uptake than the positive control. As for plasmid transfection, oligopeptide mixtures presented higher siRNA uptake than arginine- (CR3) or lysine- (CK3) pBAEs alone. Lysine-/histidine- (CK3/CH3) and arginine-/aspartic (CR3/CD3) modified pBAE were the best performing polymer formulations for DPPSC transfection, achieving 62.4% and 62.0% of transfection, respectively (Figure 5.4C). Taken together, C32-CR3/CD3 presents the highest cell-specificity and transfection efficiency for the delivery of both siRNAs and DNA plasmids in DPPSC.

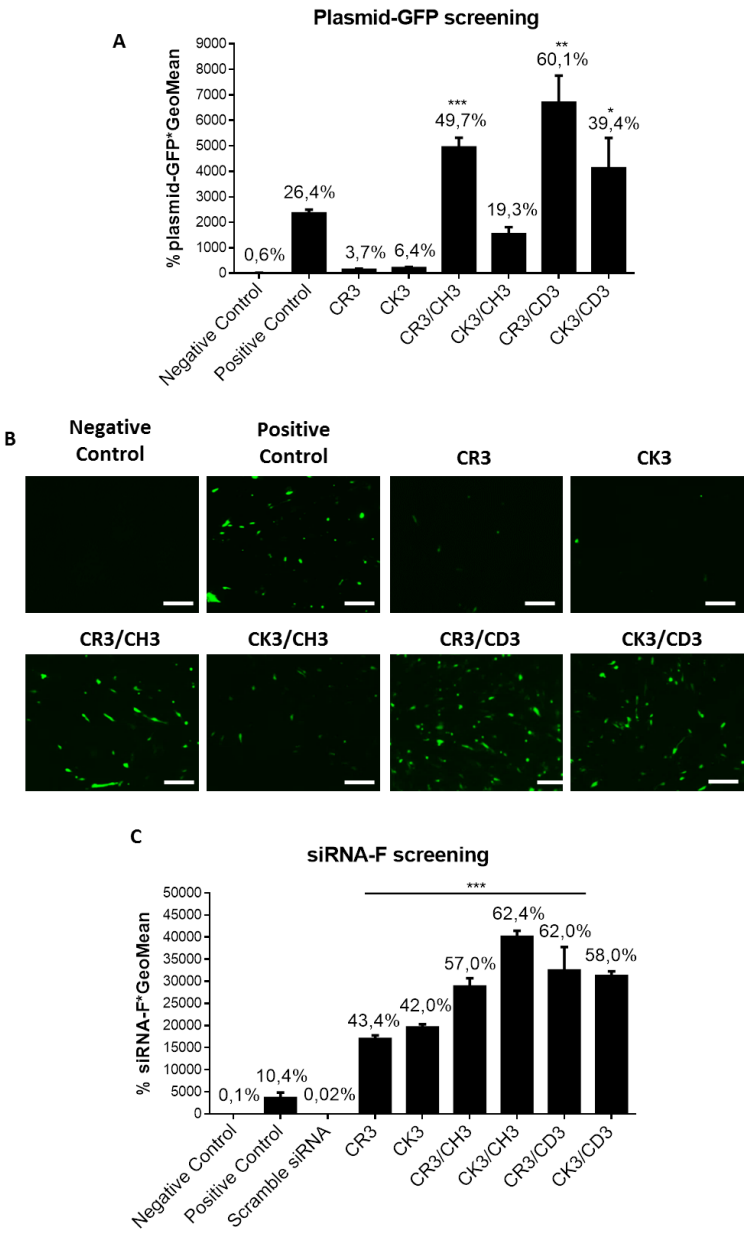


Figure 5.4. Screening of different- modified pBAEs for siRNAs and plasmids delivery into DPPSC. A) The GFP expression was evaluated in differentiated DPPSC using different oligopeptide-modified pBAE formulations and plasmid encoding GFP gene (pmaxGFP) by flow cytometry analysis. GFP expression was determined after 48 h by flow cytometry and plotted as a percentage of GFP-positive cells multiplied by the

GeoMean fluorescence of the positive population (N=3). **B)** Confocal laser scanning microscopic images of DPPSC cells using pmaxGFP plasmid and different oligopeptide end-modified pBAEs. Scale bars: 200 μm. **C)** Screening of labelled siRNA (AlexaFluor 546-labelled siRNA-F) was carried out in 7 days differentiated DPPSC using different oligopeptide end-modified poly(beta-amino ester)s. Fluorescence expression was determined 2 h post-transfection by flow cytometry and plotted as the percentage of positive cells multiplied by the GeoMean fluorescence. Statistical significance was determined versus positive control cells (transfected with the commercial reagent Lipofectamine 2000) (N=3). **P< 0.05, ** P < 0.01, *** P< 0.001*.

5.3.2 Pluripotency silencing in DPPSC

Previously, we have demonstrated that DPPSC showed expression of the pluripotency markers OCT3/4 and NANOG in their undifferentiated stage (Appendix, Figure S1).

In this study, to further characterize the change of the pluripotency markers expression during the osteogenic process, RNA was isolated during standard DPPSC osteogenic differentiation at days 0, 7, 15 and 21. By means of qRT-PCR it was demonstrated a progressive decrease of OCT3/4 during the differentiation process and a rapid downregulation of the NANOG expression during the first week of the differentiation, which was maintained throughout the differentiation process. However, at the end of the osteogenic induction, low levels of these pluripotency markers still remained (Figure 5.5A). Then, OCT3/4 and NANOG expression was silenced using C32-CR3/CD3 polymer formulation in both undifferentiated and initial differentiated DPPSC.

Therefore, first of all, in order to verify the silencing efficiency of these pluripotency genes, transfection with different combinations of siRNAs was performed to undifferentiated DPPSC. qRT-PCR results showed that OCT3/4 and NANOG expression decreased after 48h of transfection when compared with the scramble siRNA control. In addition, the simultaneous silencing of OCT3/4 and NANOG reached the lowest levels of both pluripotency markers (Figure 5.5B).

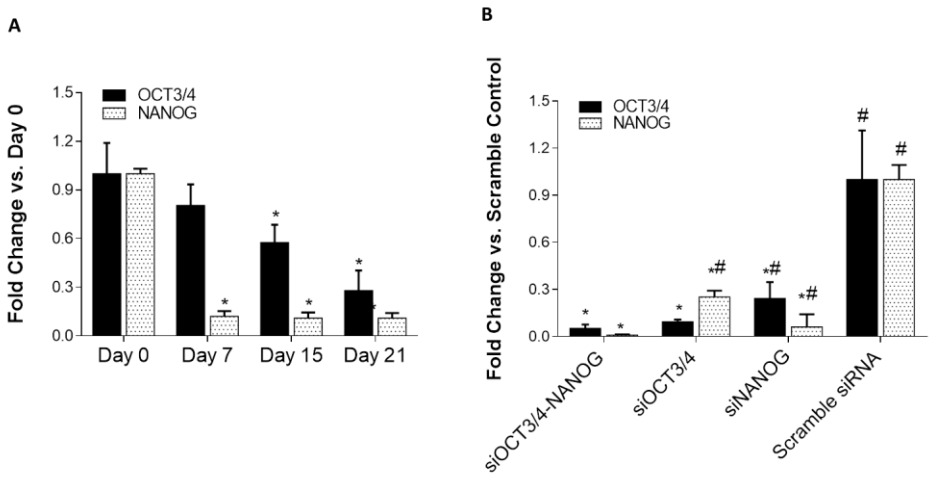


Figure 5.5. Silencing the pluripotency genes in undifferentiated DPPSC. A) Expression profile of pluripotent genes (OCT3/4, NANOG) during DPPSC osteogenic differentiation. NANOG and OCT3/4 expression were evaluated at days 7, 15, and 21 of osteoblast differentiation versus undifferentiated DPPSC (Day 0) (N=3). **P <0.05 versus* Day 0, N=3. **B)** NANOG and OCT3/4 knockdown using siRNA-C32-CR3/CD3 polyplexes in undifferentiated DPPSC (N=3). Results were evaluated by qRT-PCR at 48h post transfection *versus* scramble siRNA*. *P <0.05 versus* scramble siRNA*; #P <0.05 versus* siOCT3/4-NANOG*.*

Once we evaluated the pluripotency silencing in undifferentiated DPPSC, we transfected DPPSC at day 7 of the osteogenic differentiation with OCT3/4 and NANOG siRNAs to reduce the expression of these pluripotency genes and consequently, improve the osteogenic process (Figure 5.6). The silencing efficiency of OCT3/4 and NANOG genes was confirmed by RT-PCR at 48h post-transfection (Figure 5.6A). Results showed that OCT3/4 was reduced after siOCT3/4 delivery while NANOG was reduced after siNANOG delivery. However, there was high silencing of both NANOG and OCT3/4 using siOCT3/4 alone.

Afterwards, in order to evaluate the enhancement of the osteogenic differentiation after the silencing of pluripotency genes, RNA was isolated at day 21 of the osteogenic induction and key osteogenic markers were analysed by RT-PCR (Figure 5.6B). Results showed higher expression of the osteogenic genes in DPPSC transfected with anti-OCT3/4 or anti-NANOG siRNAs compared

to DPPSC transfected with the scramble siRNA control. Moreover, the levels of ALP and OCN were more upregulated after the transfection with anti-OCT3/4 siRNA.

In addition, qRT-PCR revealed that RUNX2 levels remained higher expressed in cells with pluripotency silencing than in control cells. This phenomenon was maintained until the end of the osteogenic induction. Furthermore, the siOCT3/4 transfected cells reached the highest levels of RUNX2, either at day 9 and day 21 of differentiation (Figure 5.6C).

Finally, cell viability after silencing of pluripotency genes was determined by MTT assay at 48h post-transfection. There were few differences between the different tested siRNAs, with viabilities between 80% and 100% compared to control cells (Figure 5.6D).

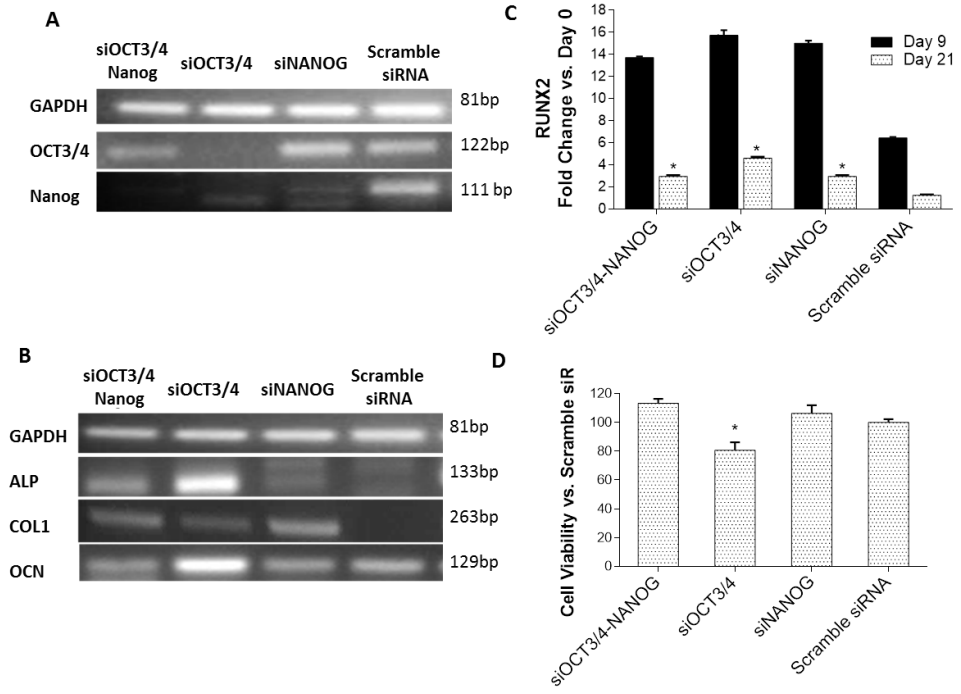


Figure 5.6. Pluripotency silencing in DPPSC osteogenic differentiation. A) OCT3/4 and NANOG gene expression were controlled by siOCT3/4 and/or siNANOG delivery at 7 days of osteogenic differentiation in DPPSC (N=3). Pluripotency markers were analysed at gene level at 48h post-transfection. Scramble siRNA was used as negative control. **B)** RT-PCR of osteogenic markers (ALP, COL1, OCN) at the end of the osteogenic differentiation (day 21) were analysed (N=3). **C)** RUNX2 expression was analysed at 48h post transfection (day 9) and at the end of the osteogenic differentiation (day 21) versus undifferentiated DPPSC (Day 0) by qRT-PCR. The relative value for undifferentiated DPPSC (Day 0) was considered 1 (N=3). **D)** Cell viability was determined at 48 h post siRNAs delivery by MTT assay respect Scramble siRNA (day 9 of osteogenic differentiation) (N=3). Scramble siRNA was used as a negative control. **P < 0.05 versus* scramble siRNA control at same day of differentiation.

5.3.3 siOCT3/4 and pRUNX2 co-delivery into DPPSC

Once the silencing of the pluripotency genes in DPPSC confirmed its effect in osteogenic differentiation, DPPSC were also transfected at day 7 of differentiation with a RUNX2 plasmid (pRUNX2) in order to further enhance and accelerate the differentiation process. Hence, DPPSC were transfected with

anti-OCT3/4 siRNA and pRUNX2 simultaneously. At the same time, control cells were transfected with both scramble siRNA and control plasmid (pmaxGFP plasmid).

qRT-PCR assays demonstrated that OCT3/4 expression levels decreased in all the transfected cells along the differentiation. However, at day 21, low levels of this pluripotency marker still remained in the cells transfected only with pRUNX2. In contrast, pRUNX2-siOCT3/4 transfected DPPSC showed the lowest OCT3/4 expression levels at each time point (Figure 5.7A).

On the other hand, RUNX2 expression was down-regulated in all transfections along the differentiation and was clearly over-expressed in the pRUNX2 and pRUNX2-siOCT3/4 transfected cells (Figure 5.7B).

In addition, DPPSC viability after the transfection with both pRUNX2 plasmid and anti-OCT3/4 siRNA was determined by MTT assay in order to evaluate the effect of the simultaneous transfection. MTT results demonstrated that, after 21 days of differentiation, DPPSC viability was greater than 70% in all the transfections. However, transfection with both nucleic acids, pRUNX2-siOCT3/4, resulted in lower cell viability than transfections with either pRUNX2 or siOCT3/4 alone (73%, 87% and 100% of viability respectively). Similar cell viability values were observed for control cells transfected with the scramble control, suggesting that the differences in cellular viability derived basically from the double or single transfection process (Figure 5.7C).

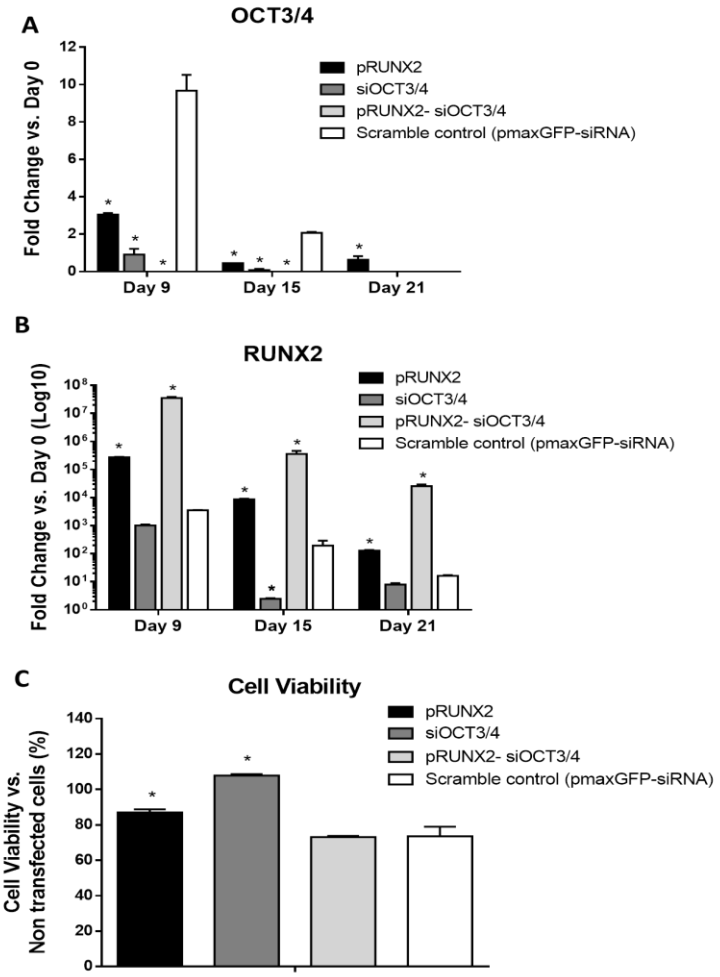


Figure 5.7. Efficiency and viability of single and double transfections in DPPSC during osteogenic differentiation. A-B) Relative expression of **(A)** OCT3/4 and **(B)** RUNX2 after pRunx2 and siOCT3/4 single and double transfections in DPPSSC at day 9 (48h post transfection), 15, and 21 of osteogenic differentiation *versus* undifferentiated DPPSC (Day 0) (N=3). The relative value for undifferentiated DPPSC (Day 0) was considered 1. **C)** Cell viability assay was performed at day 21 of the osteogenic process by MTT assay compared to non-transfected cells. Scramble control was used as negative control. **P<0.05 versus* scramble control.

Afterwards, the quality of the osteogenic induction after the transfection with pRUNX2-siOCT3/4 was evaluated by gene expression of osteogenic markers and functional assays.

Increasing levels of ALP were observed by qRT-PCR during the osteogenic differentiation in all transfected populations. However, pRUNX2-siOCT3/4-treated cells were the only with detectable ALP expression at 48 h post-transfection (day 9 of differentiation) (Figure 5.8A). COL1A1 and OSN levels were also increased significantly during the osteogenesis, showing the highest levels in cells co-transfected with pRUNX2-siOCT3/4 at each time point studied (Figure 5.8B-C). Furthermore, the quantification of the Western blot results, normalized with GAPDH and the scramble control, revealed that it was a higher COL1 expression in pRUNX2 and pRUNX2-siOCT3/4 transfected cells (Figure 5.9).

Finally, the functional osteogenic activity of the transfected cells was evaluated after 21 days of differentiation by ALP and Alizarin Red S stainings (Figure 5.10). Representative images from bone-like DPPSC showed that pRUNX2, siOCT3/4 and pRUNX2-siOCT3/4 transfected cells produced higher ALP activity and mineralization than scramble control cells. In addition, the differences were significant as seen after stainings quantification.

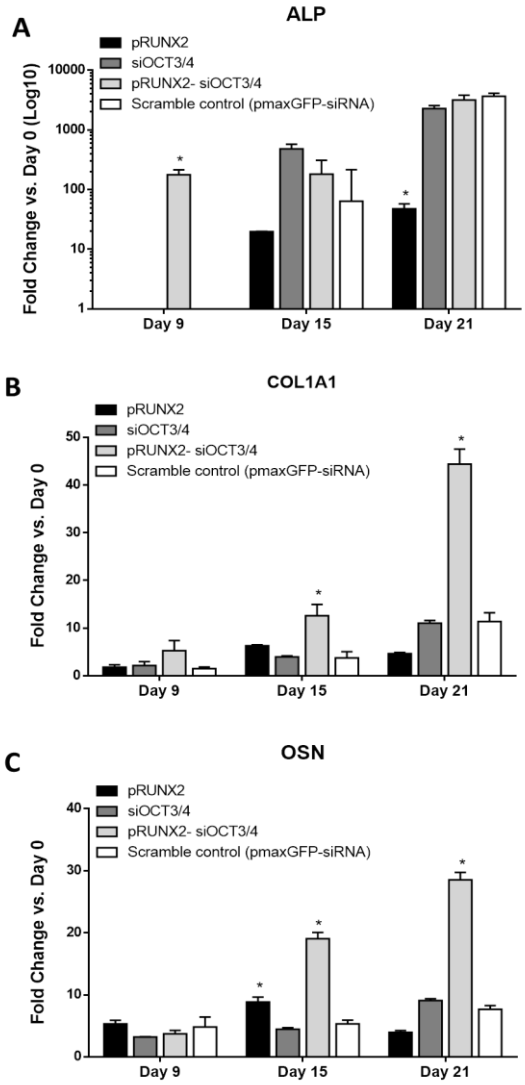


Figure 5.8. Effect of siOCT3/4 and pRUNX2 co-delivery during osteogenic differentiation of DPPSC, changes in gene expression. A-C) Changes in the expression of osteogenic markers ALP, COL1 and OSN after DPPSC transfections versus undifferentiated DPPSC (Day 0) were analysed at days 9, 15, and 21 of osteogenic differentiation (N=3). The relative value for undifferentiated DPPSC (Day 0) was considered 1 and scramble control was used as a negative control. **P<0.05 versus* scramble control.

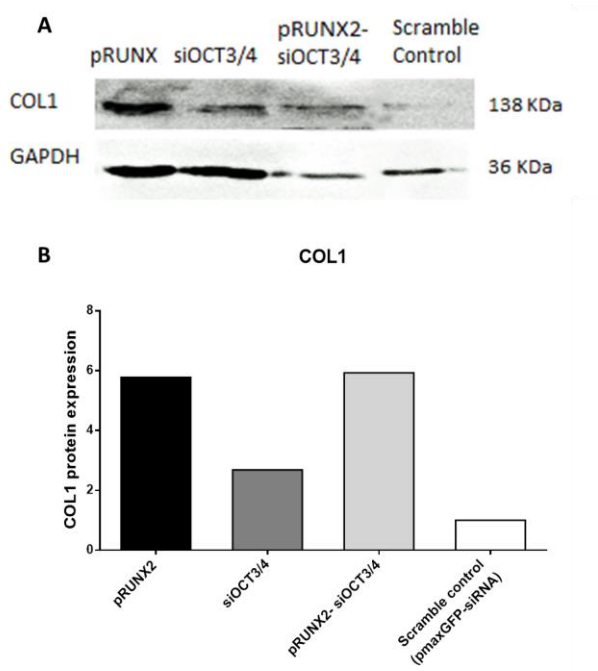


Figure 5.9. Effect of siOCT3/4 and pRUNX2 co-delivery during osteogenic differentiation of DPPSC, changes in Col1 protein expression. A) Western blot analysis of COL1 after 21 days of differentiation. GAPDH was used as a housekeeping control. **B)** COL1 protein expression quantification normalized to GAPDH and referred to the scramble control by Image J software.

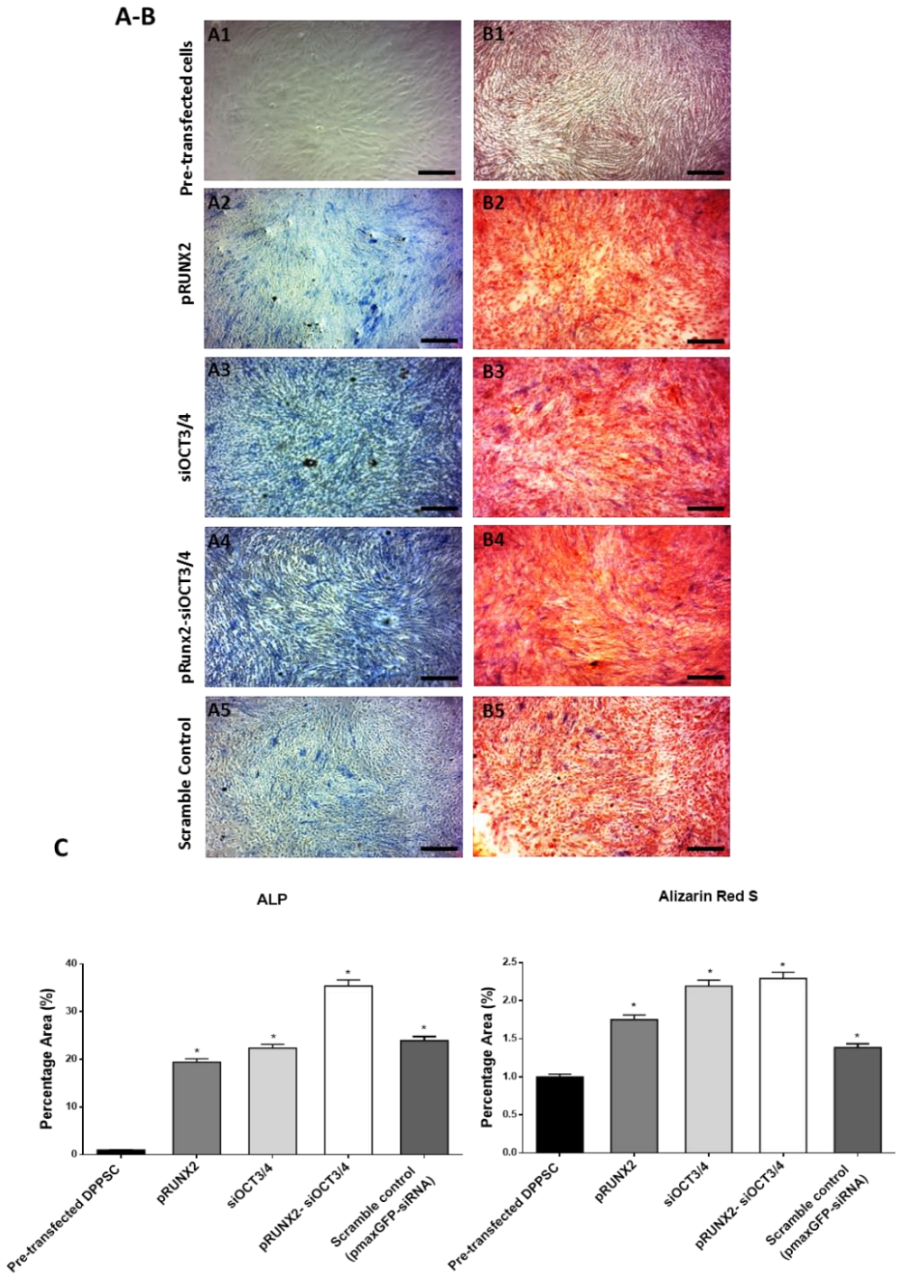


Figure 5.10. Functional activity in differentiated DPPSC after siOCT3/4 and pRUNX2 transfection. A) ALP staining in **(A1)** DPPSC before transfection (at day 7 of osteogenic induction) and **(A2-A5)** DPPSC after different transfection conditions, at 21 days of

differentiation. **B)** Images of mineralization in red by Alizarin Red S staining in DPPSC **(B1)** before transfection (at day 7 of osteogenic induction), and **(B2-B5)** after different transfection conditions, at day 21 of osteogenic differentiation. Scale bars: 200 µm. **C)** ALP and Alizarin Red S staining quantifications by Image J software. Results were normalized versus pre-transfected DPPSC. **P<0.05 versus* pre-transfected DPPSC. Representative images from 1 of 3N.

5.3.4 Genetic stability of pRUNX2-siOCT3/4 transfected DPPSC

The short-CGH allows the detection of all the aneuploidies. We evaluated the genetic stability of DPPSC before their osteogenic differentiation (Day 0), 48h post pRUNX2-siOCT3/4 transfection (Day 9) and during the osteogenic process of pRUNX2-siOCT3/4 transfected DPPSC (Day 15, Day 21). The results demonstrated that DPPSC in their undifferentiated stage showed no chromosome aneuploidies or imbalances in a summary of short-CGH with fixed limits (Figure 5.11A). Moreover, DPPSC maintained a normal chromosome dosage 48 h post transfection with pRUNX2-siOCT3/4 (Figure 5.10B) and also at the end of the osteogenic induction (Figure 5.11C-D). There were only losses in the Y chromosome dosage (labelled in green) due to the sex of the donors (females).

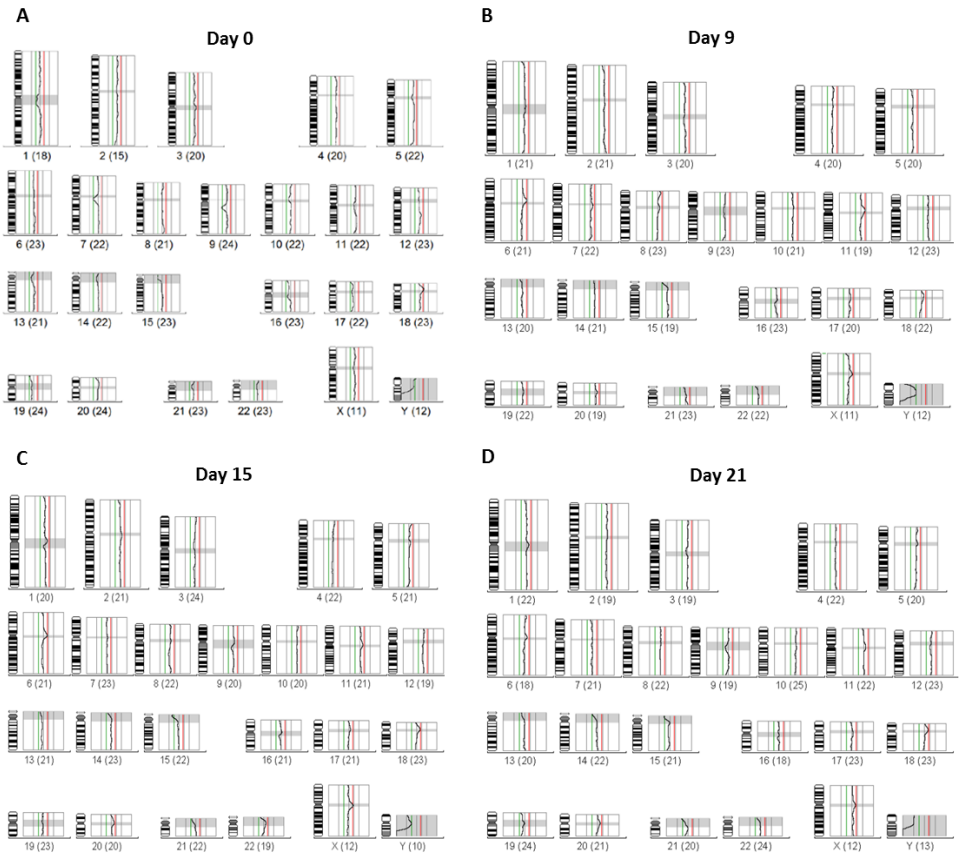


Figure 5.11. Genetic stability of DPPSC before and after pRUNX2-siOCT3/4 transfection with modified-pBAEs by short-CGH. Fixed limits summary of 47, XXY control samples (labelled in green) and pRUNX2-siOCT3/4 DPPSC samples (labelled in red) co-hybridization onto 12 normal metaphases (46, XY) (N=3). **A)** Short-CGH from undifferentiated DPPSC (day 0 of differentiation); **B)** Short-CGH from 48h post transfected DPPSC (day 9 of differentiation). **C)** Short-CGH from DPPSC at day 15 of differentiation. **D)** Short-CGH from DPPSC at day 21 of differentiation. Losses in the Y chromosome dosage were due to the sex of the donors (females).

5.4 DISCUSSION

Current research to stimulate bone regeneration is mainly focused on the use of conditioned media and/or scaffolds that mimic the natural bone

environment to promote osteogenic differentiation [39]. One alternative approach is focusing on the delivery of nucleic acids as a direct genetic reprogramming strategy. Recently, synthetic vectors have been used to efficiently differentiate MSC into osteoblasts [25, 40]. However, these carriers have shown to induce undesirable effects, such as cytotoxicity, mutagenesis, and genetic instability [41]. To address this concern, a new family of oligopeptide-modified polymers that combine different natural amino acids with pBAEs has been developed in order to improve the polymer biocompatibility. The oligopeptide composition allows the modulation of the size and surface charge of the resulting nanoparticles. Therefore, specific oligopeptide-terminated pBAEs formulation allows the development of cell- and tissue-specific nanoparticles [7, 42].

5.4.1 Arginine/aspartic polymer formulation (C32-CR3/CD3) shows the highest cell-specificity to DPPSC to delivery nucleic acids.

Polymers of varying amino acid compositions were evaluated based on their ability to transfect either pmaxGFP or fluorescently-labelled siRNA. The GFP expression was markedly increased in cells transfected with the polymer formulation C32-CR3/CD3 when compared to the positive control. Furthermore, siRNA uptake was also increased using this polymer formulation. Based on these results, it can be concluded that a mixture of positively- and negatively -charged oligopeptides promoted a preferential delivery of both siRNAs and plasmids to DPPSC. Specifically, the arginine/aspartic acid oligopeptide mixture (C32-CR3/CD3) was the most efficient. This result corroborates recent studies demonstrating that arginine peptides increase cellular uptake and nuclear trafficking [43, 44]. Moreover, the presence of negatively-charged oligopeptides (aspartic acid) decreases the overall charge of the polyplexes, hence decreasing their cytotoxicity [45].

5.4.2 Silencing of pluripotent genes improves the expression of osteogenic markers in DPPSC differentiation

Once the most efficient polymer formulation for DPPSC was identified, it was applied to improve the osteogenic differentiation of DPPSC, controlling key pluripotency and osteogenic genes. It has been shown that stem cells are stabilized in their undifferentiated stage by the pluripotency factors collaboratively inhibiting the differentiation of stem cells [46, 47]. In the present study, it has been demonstrated the expression of pluripotency markers OCT3/4 and NANOG in undifferentiated DPPSC and their decrease during the osteogenic process. However, a little expression of these markers still remains at the end of the differentiation process. In fact, other studies described that a potentially undifferentiated population of cells can remain during stem cells differentiation, maintaining their high proliferative potential but inhibiting a complete differentiation into osteocytes [37, 38].

It has been studied that different expression levels of the pluripotency factors lead stem cells into several differentiation fates [48, 49]. While adipogenic differentiation of MSC is further enhanced following longer ectopic expression of OCT3/4, osteogenic differentiation is not further improved after longer ectopic expression of OCT3/4 [50]. Hao *et al.* found that although both 4 and 10 days of overexpression of OCT3/4 increased the osteogenesis of MSC, prolonged overexpression of OCT3/4 in these cells did not further increase osteogenesis [50]. The present study examined, for the first time, the effects of the temporal silencing of OCT3/4 and NANOG genes in the first week of the osteogenic differentiation of DPPSC.

Initially, we evaluated the silencing in undifferentiated DPPSC. The results of siOCT3/4 and siNANOG transfections showed that both markers were downregulated in silenced cells. Interestingly, independently if the silencing was targeted either to one or both pluripotency genes, a decrease in both genes was observed. However, complete silencing of both pluripotent genes occurred only when the cells were simultaneously transfected with anti-OCT3/4 and anti-NANOG siRNA. Chambers *et al*. suggest that NANOG is expressed in OCT3/4-deficient embryos, and NANOG overexpression does not revert the differentiation program of ESC triggered by OCT3/4 downregulation [51]. They

conclude that NANOG is not just a downstream version of OCT3/4; indeed, NANOG and OCT3/4 work in concert to support stem cell potency and self-renewal [51, 52]. Our results also confirmed that there is a co-expression and a genetic interaction between these two pluripotency markers in DPPSC.

Once the potential silencing efficiency of this system was confirmed, it was evaluated in DPPSC differentiated during 7 days, which is approximately the time when pluripotency begins to decrease. Results showed a downregulation of OCT3/4 and NANOG genes after the siRNA transfections without affecting the cell viability. Furthermore, at the end of the osteogenic differentiation, there was an increased expression of the osteogenic markers in all the silencing conditions, being the silencing of OCT3/4 the condition that showed the highest improvement in osteogenic differentiation. High levels of RUNX2 expression at 48h post-transfection and at the end of the differentiation process after anti-OCT3/4 siRNA delivery confirmed the efficiency of this condition. For this reason, the silencing of OCT3/4 combined with the expression of RUNX2 was selected as the most promising strategy to improve the osteogenic differentiation of DPPSC.

5.4.3 Co-delivery of siOCT3/4 and pRUNX2 accelerates the osteogenic differentiation of DPPSC while maintaining cell viability

During the first week of the osteogenic induction, occur the proliferation and the first stages of stem cells differentiation. This process corresponds to an increase of some pluripotency markers that later, will be down-regulated, allowing the expression of the osteogenic genes.

A critical factor in osteogenic gene therapy is the timing that RUNX2 plasmid is delivered into the cells. The early stage of the osteogenesis is the most active period of bone formation and it occurs during the first week of differentiation [53, 54]. Some studies suggest that RUNX2 promotes the proliferation and differentiation of osteoblasts in the early stage of osteogenesis, but inhibits osteoblast maturity in the late stage, leading to osteopenia and fragility [10, 33]. Therefore, transient expression of both anti-

OCT3/4 siRNA and RUNX2 plasmid was conducted at day 7 of DPPSC differentiation, being the plasmid and the siRNA degraded along the next 72 h. There are only few studies where stem cells have been co-transfected at the same time with plasmids and siRNAs using synthetic vectors because it is not a trivial task [55]. Our analysis demonstrated satisfactory transfection and viability of the double transfection in DPPSC using C32-CR3/CD3 polymer formulation.

It is well known that RUNX2 upregulates osteoblastic gene expression [56]. In this study, it has been demonstrated that DPPSC transfected with pRUNX2 exhibited increased osteoblastic gene expression and produced significantly higher quantities of mineralized matrix compared to the control cells. However, RUNX2 transfection alone was not enough to promote and accelerate the osteogenic process. In this way, the co-transfection of pRUNX2 and siOCT3/4 induced a significantly higher expression of osteogenic markers (ALP, COL1 and OSN) and an earlier expression of some of these genes in co-transfected DPPSC. Additionally, doubly transfected cells resulted in a higher matrix mineralization and ALP activity at the end of the differentiation process. Therefore, the administration of pRUNX2 and siOCT3/4 at day 7 of the DPPSC osteogenic differentiation seems to allow the improvement and the acceleration of this process.

5.4.4 pRUNX2-siOCT3/4 transfection with modified-pBAEs does not induce cytotoxicity neither genetic instability of DPPSC

Observed cell viability was consistently greater than 75% in all tested conditions. In fact, cell viability of double transfection with pRUNX2-siOCT3/4 was not statistically different than the viability observed for cells transfected with the double scramble control. These results are in accordance with previous results using the same oligopeptide-modified pBAEs that showed cell viabilities higher than 80%, and even 90%, in different cells types [6, 7].

It is common that stem cells acquire genomic changes during cell culture or cell transfection. In fact, generation of iPSC using pBAE polymers has previously shown integration of exogenous genetic material [57-59]. Therefore, genomic

stability of stem cells before and after any treatment should be monitored carefully in basic research and even more in clinical trials [60]. ASC should satisfy all the safeness requirements for being used in human cell therapy. Hence, karyotype analysis of these cells is mandatory to confirm the absence of genetic instability and ensure their quality and safety [61]. Our results of DPPSC transfections using oligopeptide-modified pBAEs showed only a limited toxicity in the cell viability assays.

Finally, the short-CGH analysis of DPPSC did not show genetic alterations neither before nor after transfection. In addition, this genetic stability remained until the end of the osteogenic differentiation.

5.5 CONCLUSIONS

In this chapter we have identified a specific polymer formulation, of arginine/aspartic (C32-CR3/CD3) modified pBAE for highly efficient co-delivery of siRNAs and plasmids in DPPSC, as a non-viral gene therapy.

Furthermore, the delivery of siOCT3/4 in combination with pRUNX2 accelerates the expression of key osteogenic markers. Hence, the double transfection strategy produces higher matrix mineralization and ALP activity in the differentiated cells. Finally, we observed that the transfection with modified pBAEs did not induce chromosomal instability and did not reduce the viability of DPPSC. Therefore, this combination of DPPSC and biocompatible polymers to deliver/silencing specific genes may be a powerful BTE technique to improve and accelerate bone regeneration.

5.6 REFERENCES

[1] Nunez-Toldra R, Dosta P, Montori S, Ramos V, Atari M, Borros S. Improvement of osteogenesis in dental pulp pluripotent-like stem cells by oligopeptide-modified poly(beta-amino ester)s. Acta biomaterialia. 2017;53:152-64

[2] Wegman F, Oner FC, Dhert WJ, Alblas J. Non-viral gene therapy for bone tissue engineering. Biotechnology & genetic engineering reviews. 2013;29:206-20.

[3] Balmayor ER, van Griensven M. Gene therapy for bone engineering. Frontiers in bioengineering and biotechnology. 2015;3:9.

[4] Evans CH. Gene delivery to bone. Advanced drug delivery reviews. 2012;64:1331-40.

[5] Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nature reviews Genetics. 2003;4:346-58.

[6] Dosta P, Segovia N, Cascante A, Ramos V, Borros S. Surface charge tunability as a powerful strategy to control electrostatic interaction for high efficiency silencing, using tailored oligopeptide-modified poly(beta-amino ester)s (PBAEs). Acta Biomaterialia. 2015;20:82-93.

[7] Segovia N, Dosta P, Cascante A, Ramos V, Borros S. Oligopeptide-terminated poly(beta-amino ester)s for highly efficient gene delivery and intracellular localization. Acta Biomaterialia. 2014;10:2147-58.

[8] Lai QG, Yuan KF, Xu X, Li DR, Li GJ, Wei FL, et al. Transcription factor osterix modified bone marrow mesenchymal stem cells enhance callus formation during distraction osteogenesis. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2011;111:412-9.

[9] Kawai M, Maruyama H, Bessho K, Yamamoto H, Miyazaki J, Yamamoto T. Simple strategy for bone regeneration with a BMP-2/7 gene expression cassette vector. Biochemical and biophysical research communications. 2009;390:1012-7.

[10] Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, et al. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. The Journal of cell biology. 2001;155:157-66.

[11] Lim KI. Retroviral integration profiles: their determinants and implications for gene therapy. BMB reports. 2012;45:207-12.

[12] Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer cell. 2002;2:243-7.

[13] Devroe E, Silver PA. Therapeutic potential of retroviral RNAi vectors. Expert opinion on biological therapy. 2004;4:319-27.

[14] Tomanin R, Scarpa M. Why do we need new gene therapy viral vectors? Characteristics, limitations and future perspectives of viral vector transduction. Current gene therapy. 2004;4:357-72.

[15] van Griensven M, Lobenhoffer P, Barke A, Tschernig T, Lindenmaier W, Krettek C, et al. Adenoviral gene transfer in a rat fracture model. Laboratory animals. 2002;36:455-61.

[16] Fang J, Zhu YY, Smiley E, Bonadio J, Rouleau JP, Goldstein SA, et al. Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. Proc Natl Acad Sci U S A. 1996;93:5753-8.

[17] Li R, Stewart DJ, von Schroeder HP, Mackinnon ES, Schemitsch EH. Effect of cell-based VEGF gene therapy on healing of a segmental bone defect. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2009;27:8-14.

[18] Hsu WK, Sugiyama O, Park SH, Conduah A, Feeley BT, Liu NQ, et al. Lentiviral-mediated BMP-2 gene transfer enhances healing of segmental femoral defects in rats. Bone. 2007;40:931-8.

[19] Akinc A, Anderson DG, Lynn DM, Langer R. Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery. Bioconjugate chemistry. 2003;14:979-88.

[20] Zugates GT, Tedford NC, Zumbuehl A, Jhunjhunwala S, Kang CS, Griffith LG, et al. Gene delivery properties of end-modified poly(beta-amino ester)s. Bioconjug Chem. 2007;18:1887-96.

[21] Zugates GT, Peng W, Zumbuehl A, Jhunjhunwala S, Huang YH, Langer R, et al. Rapid Optimization of Gene Delivery by Parallel End-modification of Poly(beta-amino ester)s. Molecular therapy : the journal of the American Society of Gene Therapy. 2007;15:1306-12.

[22] Little SR, Lynn DM, Ge Q, Anderson DG, Puram SV, Chen J, et al. Poly-beta amino ester-containing microparticles enhance the activity of nonviral genetic vaccines. Proc Natl Acad Sci U S A. 2004;101:9534-9.

[23] Huang YH, Zugates GT, Peng W, Holtz D, Dunton C, Green JJ, et al. Nanoparticle-delivered suicide gene therapy effectively reduces ovarian tumor burden in mice. Cancer research. 2009;69:6184-91.

[24] Kamat CD, Shmueli RB, Connis N, Rudin CM, Green JJ, Hann CL. Poly(beta-amino ester) nanoparticle-delivery of p53 has activity against small cell lung cancer in vitro and in vivo. Molecular cancer therapeutics. 2013;12:405-15.

[25] Tzeng SY, Hung BP, Grayson WL, Green JJ. Cystamine-terminated poly(beta-amino ester)s for siRNA delivery to human mesenchymal stem cells and enhancement of osteogenic differentiation. Biomaterials. 2012;33:8142-51.

[26] Yang F, Cho SW, Son SM, Bogatyrev SR, Singh D, Green JJ, et al. Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles. Proceedings of the National Academy of Sciences of the United States of America. 2010;107:3317-22.

[27] Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. Nature reviews Genetics. 2014;15:541-55.

[28] Jin L, Zeng X, Liu M, Deng Y, He N. Current progress in gene delivery technology based on chemical methods and nano-carriers. Theranostics. 2014;4:240-55.

[29] Park SJ, Na K. The transfection efficiency of photosensitizer-induced gene delivery to human MSCs and internalization rates of EGFP and Runx2 genes. Biomaterials. 2012;33:6485-94.

[30] Yun YR, Jang JH, Jeon E, Kang W, Lee S, Won JE, et al. Administration of growth factors for bone regeneration. Regenerative medicine. 2012;7:369-85.

[31] Pan K, Sun Q, Zhang J, Ge S, Li S, Zhao Y, et al. Multilineage differentiation of dental follicle cells and the roles of Runx2 over-expression in enhancing osteoblast/cementoblast-related gene expression in dental follicle cells. Cell proliferation. 2010;43:219-28.

[32] Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell. 1997;89:747-54.

[33] Geoffroy V, Kneissel M, Fournier B, Boyde A, Matthias P. High Bone Resorption in Adult Aging Transgenic Mice Overexpressing Cbfa1/Runx2 in Cells of the Osteoblastic Lineage. Molecular and Cellular Biology. 2002;22:6222-33.

[34] Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, et al. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nature genetics. 2006;38:431-40.

[35] Liu L, Wu L, Wei X, Ling J. Induced overexpression of Oct4A in human dental pulp cells enhances pluripotency and multilineage differentiation capability. Stem cells and development. 2015;24:962-72.

[36] Yeo JC, Ng HH. The transcriptional regulation of pluripotency. Cell research. 2013;23:20-32.

[37] Darr H, Mayshar Y, Benvenisty N. Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features. Development. 2006;133:1193-201.

[38] Kochupurakkal BS, Sarig R, Fuchs O, Piestun D, Rechavi G, Givol D. Nanog inhibits the switch of myogenic cells towards the osteogenic lineage. Biochemical and biophysical research communications. 2008;365:846-50.

[39] Brammer KS, Frandsen CJ, Jin S. TiO2 nanotubes for bone regeneration. Trends in biotechnology. 2012;30:315-22.

[40] Kc RB, Kucharski C, Uludag H. Additive nanocomplexes of cationic lipopolymers for improved non-viral gene delivery to mesenchymal stem cells. Journal of Materials Chemistry B. 2015;3:3972-82.

[41] Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nature reviews Drug discovery. 2005;4:581-93.

[42] Alexander C. Convergence of synthetic and natural polymers: next generation nanomedicines? Nanomedicine (Lond). 2008;3:749-51.

[43] Vazquez E, Roldan M, Diez-Gil C, Unzueta U, Domingo-Espin J, Cedano J, et al. Protein nanodisk assembling and intracellular trafficking powered by an arginine-rich (R9) peptide. Nanomedicine (Lond). 2010;5:259-68.

[44] Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB. Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res. 2000;56:318-25.

[45] Putnam D, Gentry CA, Pack DW, Langer R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc Natl Acad Sci U S A. 2001;98:1200-5.

[46] Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature. 2008;455:1124-8.

[47] Young RA. Control of the embryonic stem cell state. Cell. 2011;144:940-54.

[48] Kopp JL, Ormsbee BD, Desler M, Rizzino A. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem cells. 2008;26:903-11.

[49] Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet. 2000;24:372-6.

[50] Hao Q, An JQ, Hao F, Yang C, Lu T, Qu TY, et al. Inducible Lentivirus-Mediated Expression of the Oct4 Gene Affects Multilineage Differentiation of Adult Human Bone Marrow-Derived Mesenchymal Stem Cells. Cell Reprogram. 2015;17:347-59.

[51] Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell. 2003;113:643-55.

[52] Mallon BS, Chenoweth JG, Johnson KR, Hamilton RS, Tesar PJ, Yavatkar AS, et al. StemCellDB: the human pluripotent stem cell database at the National Institutes of Health. Stem Cell Res. 2013;10:57-66.

[53] Karner E, Backesjo CM, Cedervall J, Sugars RV, Ahrlund-Richter L, Wendel M. Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. Biochimica et biophysica acta. 2009;1790:110-8.

[54] Miron RJ, Zhang YF. Osteoinduction: a review of old concepts with new standards. Journal of dental research. 2012;91:736-44.

[55] Barker GA, Diamond SL. RNA Interference Screen to Identify Pathways That Enhance or Reduce Nonviral Gene Transfer During Lipofection. Mol Ther. 2008;16:1602-8.

[56] Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell and tissue research. 2010;339:189-95.

[57] Medina-Kauwe LK, Xie J, Hamm-Alvarez S. Intracellular trafficking of nonviral vectors. Gene Ther. 2005;12:1734-51.

[58] Montserrat N, Garreta E, Gonzalez F, Gutierrez J, Eguizabal C, Ramos V, et al. Simple generation of human induced pluripotent stem cells using poly-beta-amino esters as the non-viral gene delivery system. The Journal of biological chemistry. 2011;286:12417-28.

[59] Shan Y, Luo T, Peng C, Sheng R, Cao A, Cao X, et al. Gene delivery using dendrimer-entrapped gold nanoparticles as nonviral vectors. Biomaterials. 2012;33:3025-35.

[60] Ben-David U, Benvenisty N. Analyzing the genomic integrity of stem cells. StemBook. Cambridge (MA). 2008.

[61] Torre ML, Lucarelli E, Guidi S, Ferrari M, Alessandri G, De Girolamo L, et al. Ex vivo expanded mesenchymal stromal cell minimal quality requirements for clinical application. Stem cells and development. 2015;24:677-85.

CHAPTER 6: VASCULARIZED BONE TISSUE ENGINEERING BY DPPSC CO-CULTURE SYSTEMS

The following experiments have been performed in collaboration with the *Adult Stem Cell Group (BioMediTech, University of Tampere,* Finland*).*

6.1 INTRODUCTION

Vascularization of large bone grafts is one of the main challenges that limit the clinical application of BTE approaches. Thick BTE grafts often suffer poor cellular survival due to inadequate exchange of nutrients and oxygen that produce an eventual failure of the graft. Thus, an important amount of research is focusing on the vascularization of the tissue engineered constructs. In this way, the co-culture of cells, which involve the cross-talk between vasculogenic cells and osteoprogenitor cells, have recently shown to be an effective pre-vascularization strategy.

Previous results of our group revealed that DPPSC have high osteogenic and endothelial potential [5, 13]. Hence, in this chapter, we propose the combination of bone-like DPPSC and endothelial-like DPPSC as a good strategy to induce vascularized bone from a unique stem cell population.

Moreover, we also studied the effect of bioactive glass (BaG) ions, biomaterials characterized for their high osteogenic and angiogenic properties, in DPPSC differentiations. In addition, we carried out all the experiments under xeno-free conditions, replacing Foetal Bovine Serum (FBS) with Human Serum (HS) from the used media, in order to enable the extrapolation of our results to the development of clinical-orientated BTE applications.

6.1.1 GMP in cell therapy

In order to provide cells with defined safe culture conditions for the patient, good manufacturing practice (GMP) needs to be employed. GMP quality, defined by both the European Medicines Agency and the Food and Drug Administration (USA), is a requirement for clinical-grade cells [1]. To reach the GMP goals, all parts of the process must be defined and controlled. These include facilities, cells isolation, culture methods, procedures, materials and

quality control. Quality control not only addresses microbiological safety and phenotyping of the cells, but also ensures their genetic stability [1-3].

Most of the current techniques for expansion and differentiation of ASC require the use of medium containing animal products such as FBS [4-6]. FBS contains a great quantity of growth factors to promote cell growth, cell attachment and cell differentiation. However, animal-derived reagents present safety issues in clinical therapy. The use of FBS is associated with possible allergic reactions caused by xenogenic FBS proteins internalized in the stem cells and risk of transmission of prion diseases and zoonosis [7, 8].

Recent clinical trials of stem cells have used media supplemented with FBS screened for prion and some viral contaminants. However, neither immunologic reaction against xenogenic serum antigens nor possible contamination with viruses or zoonosis can be totally excluded in these cultures. A safer solution could be the use of media supplemented with autologous or allogenic HS to expand the cells. Although the use of autologous serum could be preferably, in some case of patients, such as elderly, children or anaemic individuals may also be a scarcely or qualitatively affected source. The use of culture media supplemented with HS has been already studied with other stem cell populations such as DPMSC, ESC, ADSC or BM-MSC [9-12].

Therefore, an indispensable factor for DPPSC clinical application is to establish a GMP-approved protocol that allows their culture isolation, expansion and differentiation under xeno-free conditions. Previously, it has been already reported a DPPSC culture media GMP-approved that replaces FBS with HS (from 1 to 10%) and that only contains xeno-free components [13]. This medium allows the isolation of DPPSC and their subsequent culture maintaining their morphology, growth rate and genetic stability for at least 10 passages. Moreover, it was observed that in these conditions DPPSC increased the expression of the pluripotency markers OCT3/4 and NANOG [13].

6.1.2 Bioactive Glasses in Bone Tissue Engineering

Originally developed to fill and restore bone defects, bioactive glasses (BaG) are currently being investigated for BTE applications. BaG belong to a group of

surface reactive amorphous biomaterials, which are able to stimulate the osteogenic differentiation of stem and progenitor cells without adding any chemical supplement [14-16]. Moreover, these biomaterials display good biocompatibility and biodegradation properties [17].

BaG were originally described by Hench and coworkers, who developed the most widely known bioactive glass composition, 45S5 or Bioglass® as a commercial name [18]. This oldest BaG composition consists of a silicate network (45 wt % SiO_2) incorporating high amounts of Na_2O and CaO, as well as the relatively high CaO/P_2O_5 ratio, that make the glass surface highly reactive in physiological environments [16, 18]. The bioactivity of BaG is greatly dependent on the composition and the relative proportion of the main components.

Bioactive silicate glasses showed three important advantages for BTE applications. Firstly, chemical reactions on the material surface lead to a strong bond to bone produced by a hydroxyl carbonate apatite (HCA) layer [19]. Secondly, dissolution products and ion release from BaG such as silica and calcium, can up-regulate gene expression in osteoprogenitor cells that rapidly enhances bone regeneration [17, 20]. Thirdly, angiogenic effects of BaG have been also demonstrated such an increased secretion of vascular endothelial growth factor (VEGF) in fibroblasts, endothelial cells proliferation and endothelial tubules formation [21]. Moreover, possible antibacterial effects of BaG have also been investigated [17, 22]. A schematic overview of biological responses to ionic dissolution products of BaG is given in Figure 6.1.

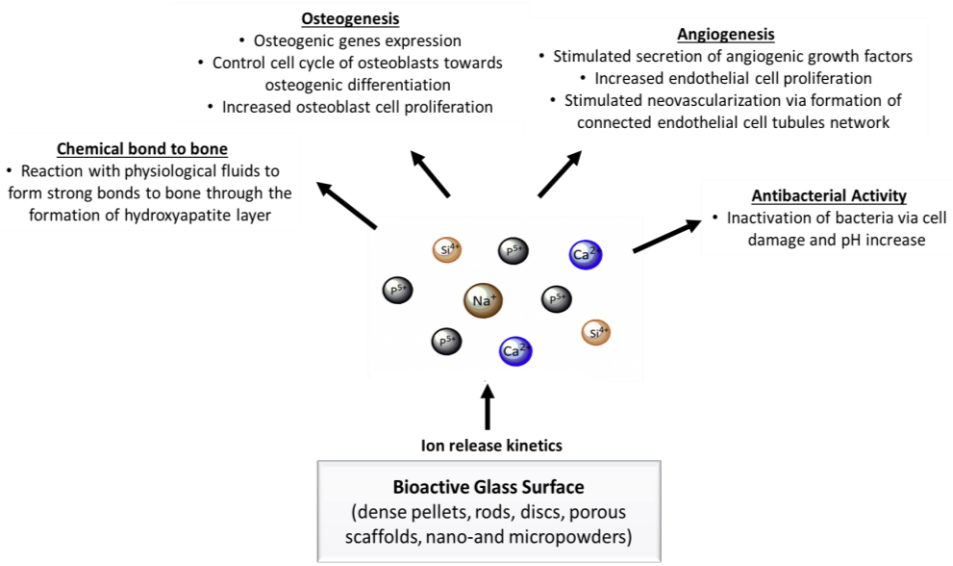


Figure 6.1. Overview of biological responses to ionic dissolution products of BaG. Modified from Hope et al. (2011) [17].

Recent efforts to stimulate angiogenesis have focused on the delivery of growth factors, such as VEGF and basic fibroblast growth factor (bFGF), gene therapy or cell-based therapy [23, 24]. However, growth factors are expensive, and the optimal delivery strategies are unclear. The ability of a BaG to induce angiogenesis could provide a robust alternative approach to the use of expensive growth factors for stimulating neovascularization of engineered tissues [24].

Since the discovery of BaG, several variations of the original 45S5 composition, as well as some completely novel compositions have been developed. S53P4 glass, commercially available as BonAlive®, is a variation composition of 45S5 glass. The oxide compositions of both BaG are depicted in Table 6.1. S53P4 is a bone substitute with proven antibacterial and bone bonding properties [22]. Moreover, it has also proven to perform well in clinical settings [25]. S53P4 BaG is known to induce osteogenic differentiation of human MSC from cultured in direct contact with the surface, as well as in indirect contact, implying that the ions from this BaG alone are capable of inducing osteogenic differentiation [26, 27]. Moreover, it has been

demonstrated that S53P4 BaG can also act as an angiogenic factor and induce increased vascularization when is incorporated in tissue-engineered scaffolds [21].

wt. %				
	Na_2O	CaO	P_2O_5	SiO_2
45S5	24.5	24.5	6.0	45.0
S53P4	23.0	20.0	4.0	53.0

Table 6.1. Compositions of BaG 45S5 (Bioglass®) and S53P4 (BonAlive®).

6.1.3 Vascularization in Bone Tissue Engineering

One of the critical problems in BTE is the development of a rapid vascularization after implantation to provide nutrients and oxygen to the osteoblast cells to grow and survive, as well as to remove the CO_2 and waste products. Moreover, in the case of bone, the vasculature also supplies the calcium and phosphate needed for the mineralization process [28, 29].

Hence, the success and survival of a biomaterial after implantation is usually dependent of the vascularization process. If the tissue-engineered construct does not contain or allow a rapid ingrowth of blood vessels, would not supply nutrients and oxygen and consequently, lead to hypoxia and death of the implanted cells. Moreover, it would avoid the ingrowth of bone cells from the host [29, 30].

It has been demonstrated that capillary-like structures created *in vitro* have a structure containing a lumen and tight junctions similar to capillaries *in vivo* [31-33] that will anastomose rapidly after implantation and supply blood to the biomaterial construct. As a consequence, in recent years several studies have examined a variety of human osteoblast and endothelial cell co-culture systems in order to distribute osteoblasts on all parts of the bone scaffold and at the same time offer conditions for the endothelial cells to migrate to form a network of capillary-like structures through the osteoblast-colonized scaffold. The movement and proliferation of endothelial cells to form capillary-like structures is known as angiogenesis and is dependent on a variety of pro-

angiogenic factors [28]. In addition, it has been demonstrated that angiogenesis is able to regulate the recruitment of stem cells and their orientation to the osteoblastic lineage [28, 34].

In vitro co-cultures can be categorized into two dimensional (2D) and three-dimensional (3D) systems (Figure 6.2). The 2D co-culture methods, including a direct contact culture and a noncontact (indirect) culture, offer detailed information of cellular events governing the differentiation of osteogenic cells that are in contact with endothelial cells or their conditioned media [35, 36]. On the other hand, 3D co-culture systems, including scaffold cultures and spheroid cultures (without scaffold), supply a preferable environment for cell survival, cell-to-cell interaction, cell alignment and a rapid formation of capillary structures [36, 37].

It is known that cell-to-cell communication in co-cultures can be achieved by different modes of crosstalk that arise depending on whether the cells were co-cultured in direct contact with each other or indirectly. Hence, cells can communicate through two different mechanisms: (i) via secretion of diffusible factors that can activate specific receptors on the target cells, and (ii) via direct cytoplasmic connections between adjacent cells by adherents, tight junctions or gap junctions.

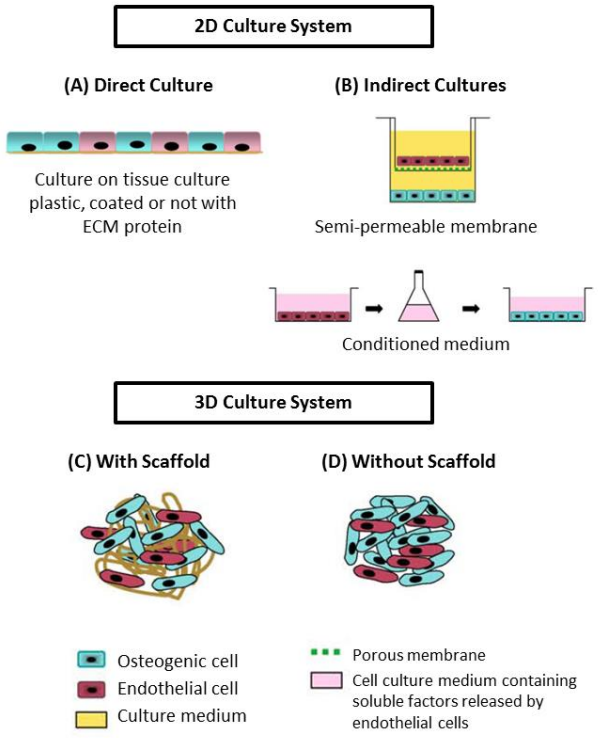


Figure 6.2. Common co-culture model systems used for pre-vascularized bone tissue. In the 2D system: **A)** Direct culture on tissue culture dish that can be coated with specific ECM proteins, such as collagen, laminin or fibronectin; **B)** Indirect culture through porous membranes or conditioned medium. In the 3D system: **C)** Direct culture with scaffold; **D)** Spheroid culture without scaffold. Modified from Grellier *et al.* (2009) [36].

The choice of the cell type is critical for the efficacy of these co-culture systems. Currently, there are different studies that combine osteogenic and endothelial cells from different sources. Primary osteoblasts isolated from bone tissue or human osteoblasts cell lines are commonly used as osteogenic cells [38]. On the other hand, human umbilical vein endothelial cells (HUVEC) or human dermal microvascular endothelial cells (HDMEC) are used as endothelial cells [39, 40]. Primary osteoblast and endothelial cells can be isolated and cultivated relatively easily. Nevertheless it is difficult to obtain sufficient numbers of cells before they begin to change, loose specific phenotypes and

undergo de-differentiation. In recent years, endothelial progenitor cells (EPC) have been isolated from blood using antibodies against specific surface markers, and they are commonly known as early and late outgrowth endothelial cells (OEC) [33]. Nevertheless, only late OEC have the proving ability to form microcapillary-like structures [40].

Furthermore, an optimal approach for clinical translation of co-culture constructs would be the obtainment of both endothelial and osteoblast cells from a single individual source in a minimally invasive extraction manner. Nonetheless, there are few reports with co-cultures of cells originated from a single stem cell population [41, 42]. Some studies have demonstrated that endothelial and osteogenic cells have also been generated from mesenchymal stem cells (MSC). Although they were easily differentiated into bone-like cells, microcapillary-like structures were not frequently observed in all of these reports [41, 42].

On the other hand, pluripotent stem cells (PSC), such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) present high differentiation potential into cells from the three germ layers. Hence, they can differentiate into endothelial-like cells, which can form microcapillary-like structures *in vitro* as well as neovascularization *in vivo*. Furthermore, osteoblast-like cells from ESC and iPSC demonstrate high osteogenic capacity after implantation [43, 44]. These PSC show unlimited passage potential, mature phenotypes and can be re-implanted into the same donor. However, as we detailed in Chapter 1, they commonly show ethical problems or safety risks and, up to date, co-cultures with only PSC targeting BTE approaches have not been yet reported.

Finally, another important concern of co-culture systems is the choice of the culture media that should be considered carefully for the maintenance of cellular viability of both co-cultured cell types as well as tissue development [45, 46]. In addition, cell culture medium itself might modulate the cell interaction between both cell types. Thus, cell culture conditions need to be optimized in each co-culture study.

6.2 OBJECTIVES

The main objective of this chapter was to investigate the effect of the endothelial differentiation of DPPSC on the osteogenic differentiation of DPPSC in xeno-free culture conditions. Moreover, the osteogenic and angiogenic potential of DPPSC was evaluated by different monoculture and co-culture systems using BaG for vascularized BTE constructs.

6.3 RESULTS

6.3.1 DPPSC differentiations under xeno-free culture conditions

In order to establish new differentiation culture mediums qualified for GMP conditions, the differentiation potential of DPPSC towards osteogenic and endothelial cells was evaluated in xeno-free culture conditions using HS supplemented medium. Results were compared with the common culture conditions using FBS.

Before differentiation, cells from the same donors were expanded during 5 culture passages in the corresponding culture condition. Thus, DPPSC isolated and expanded in HS medium were also differentiated in HS mediums while cells isolated and expanded in FBS medium were differentiated in FBS mediums.

6.3.1.1 Osteogenic differentiation under xeno-free conditions

First of all, cell viability and proliferation of DPPSC cultured in osteogenic medium supplemented with HS (OM-HS) or FBS (OM-FBS) were evaluated by Live/Dead staining and CyQUANT Cell Proliferation Assay after 7 and 15 days of culture. Based on Live/Dead staining, both culture conditions supported the viability of the cells as indicated in Figure 6.3A. Although at the end of the process the cell amount appeared similar in both culture mediums, at 7 days of differentiation in OM-HS the cell amount was higher than in OM-FBS. This phenomenon was also observed in the quantitative cell proliferation assay (Figure 6.3B).

Afterwards, the osteogenic differentiation potential in both culture conditions was evaluated by different functional and expression assays. ALP activity quantification showed higher activity in DPPSC cultured in OM-HS after 15 days of differentiation (Figure 6.3C). Moreover, Alizarin Red S staining and quantification demonstrated more mineral formation also in OM-HS versus OM-FBS cultures (Figure 6.3D).

The gene expression of typical osteogenic markers was also evaluated in both culture conditions by qRT-PCR (Figure 6.4A). At day 7 of differentiation, the initial osteogenic marker RUNX2, showed greater expression in OM-HS cultures and then, at day 15 decreased in HS but increased in FBS. DLX5 had similar expression levels in the two culture conditions and, at last, OC expression was also higher in OM-HS treated cells than in OM-FBS treated cells at both differentiation time points.

Finally, results of COL1 and OC IF analyses revealed that although at day 7 COL1 was predominantly located intracellularly in both culture conditions, the amount of COL1 was greater in DPPSC cultured in OM-HS. At day 15, collagen was secreted to the ECM in both culture conditions, but the amount was still higher in OM-HS than in OM-FBS. On the other hand, OC staining showed that OM-HS cultures began to secrete the protein earlier than OM-FBS cultures. Moreover, at day 15, larger amounts of OC in OM-HS cultures were observed (Figure 6.4B-C).

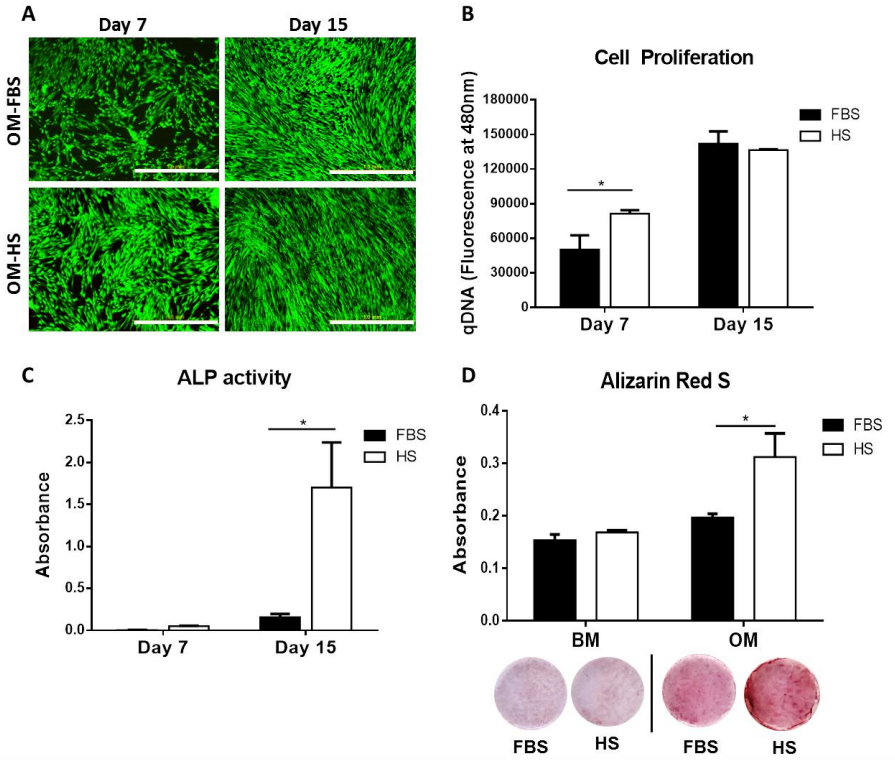


Figure 6.3. DPPSC osteogenic differentiation under xeno-free conditions. A-B) Viability and proliferation of DPPSC cultured with FBS or HS supplemented osteogenic medium after 7 and 15 days of culture (N=3) by **(A)** Live/Dead staining and **(B)** CyQUANT Cell Proliferation Assay Kit. Scale bars: 1 mm. **C)** Quantitative ALP activity determined after 7 and 15 days of culture. **D)** Mineralization assay by Alizarin Red S staining after 15 days of culture. Images of the whole wells in 24-well plate (1.5 cm of diameter) and quantification by cetylpyridinium chloride extracts absorbance (544nm). *$P<0.05$.

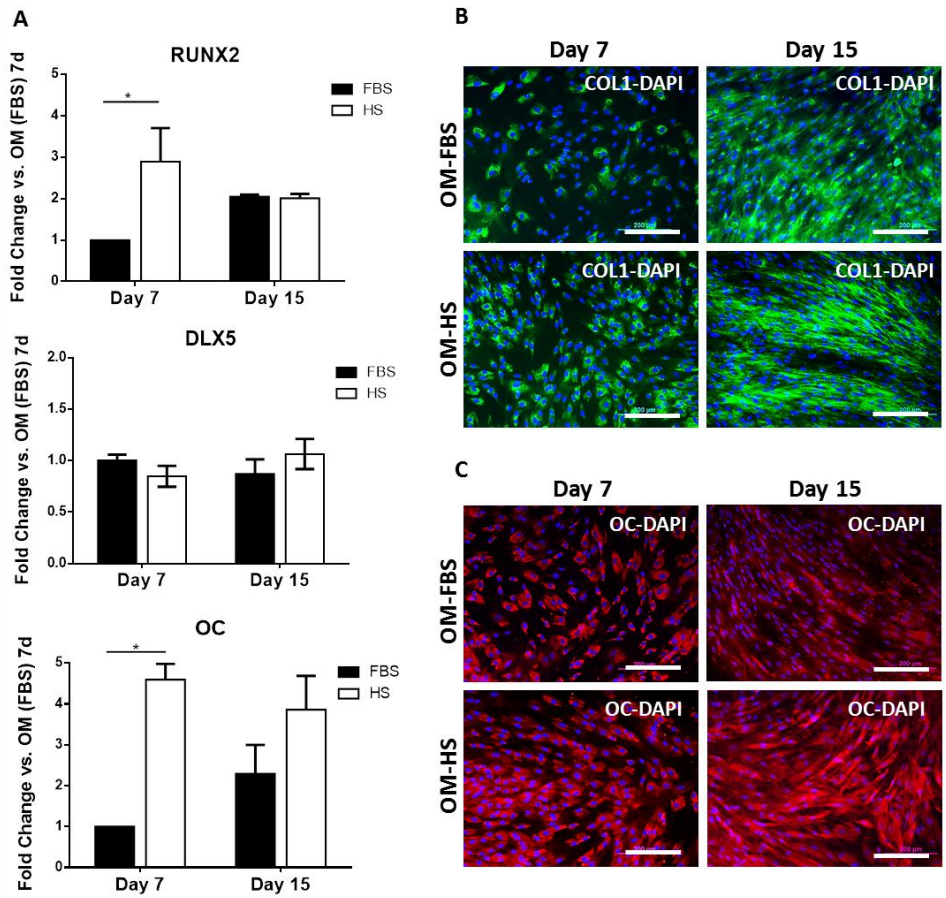


Figure 6.4. DPPSC osteogenic differentiation under xeno-free conditions. A) Gene expression after 7 and 15 days of culture by qRT-PCR of the osteogenic markers RUNX2, DLX5 and OC relative to cells cultured in OM-FBS at day 7 (N=3). Data were normalized to the expression of housekeeping gene RPLP0. **P<0.05.* **B-C)** Protein expression by COL1 and OC immunostaining in DPPSC treated with FBS or HS supplemented osteogenic medium after 7 and 15 days of culture (N=2). **(B)** COL1 is stained green, **(C)** OC is stained red and **(B-C)** nuclei are stained blue with DAPI. Scale bars: 200 µm.

6.3.1.2 Endothelial differentiation under xeno-free conditions

The effects of HS medium in the endothelial differentiation of DPPSC (EM-HS) were analysed after 15 days of culture and compared to the effects of FBS medium (EM-FBS). Live/Dead staining and CyQUANT Cell Proliferation Assay

demonstrated that the cells differentiated in EM-HS survived and proliferated as well as cells in EM-FBS, with no significant differences (Figure 6.5A-B).

Furthermore, the endothelial differentiation potential in both culture conditions was evaluated after 15 days of culture. qRT-PCR and IF analyses of CD31 showed that cells cultured in EM-HS upregulated CD31 expression levels when compared with cells cultured in EM-FBS (Figure 6.5C-D). Moreover, vWF IF showed more positive expression for this endothelial marker in cells cultured in EM-HS (Figure 6.5D).

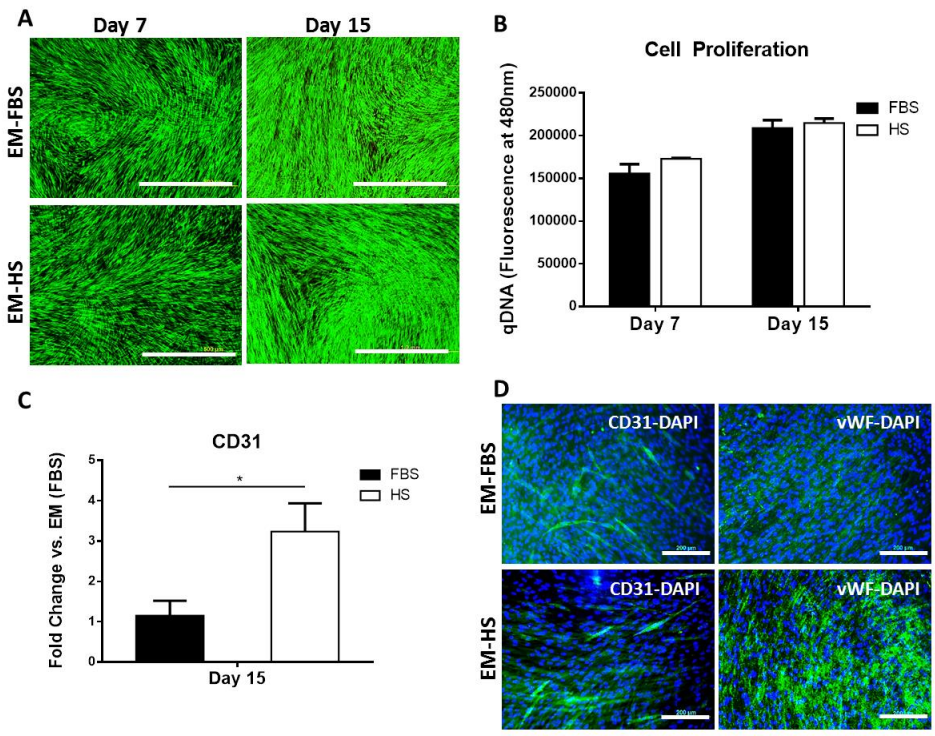


Figure 6.5. DPPSC endothelial differentiation under xeno-free conditions. A-B) Viability and proliferation of DPPSC cultured with FBS or HS supplemented endothelial medium after 7 and 15 days of culture (N=3) by **(A)** Live/Dead staining and **(B)** CyQUANT Cell Proliferation Assay Kit. Scale bars: 1 mm. **C)** Gene expression after 15 days of culture by qRT-PCR of the endothelial marker CD31 relative to cells cultured in EM-FBS at day 7 (N=3). Data were normalized to the expression of housekeeping gene RPLP0. **P<0.05.* **D)** Protein expression by CD31 and vWF immunostainings in DPPSC treated with FBS or HS supplemented endothelial medium after 15 days of culture.

CD31 and vWF are stained green and nuclei are stained blue with DAPI. Scale bars: 200 µm.

6.3.2 Effect of BaG in DPPSC differentiations

In order to assess the effect of BaG in the osteogenic and endothelial differentiations of DPPSC, we used the ionic S53P4 glass dissolution in both osteogenic and endothelial mediums (OM-BaG, EM-BaG) during 15 days of culture in each case.

Moreover, we performed all the experiments using the xeno-free mediums previously described (OM-HS, EM-HS). All the results were compared with the corresponding xeno-free medium without BaG extracts (OM, EM).

6.3.2.1 Effect of BaG in DPPPSC osteogenic differentiation

Firstly, in order to evaluate the survival and the proliferation of DPPSC in the presence of BaG ions in the osteogenic medium (OM-BaG), Live/Dead staining and CyQUANT Cell Proliferation assay were conducted after 7 and 15 days of culture as indicated in Figure 6.6. Based on the staining, OM-BaG supported the viability of the cells. No dead cells were detected in any of the conditions at neither of the time points studied and the morphology also appeared similar in both conditions (Figur 6.6A). The same phenomenon was also observed in the cell proliferation assay. However, at day 7 of differentiation, there was a significant decrease in cell proliferation in OM-BaG cultures compared to OM cultures. Otherwise, there were no differences in cell proliferation between OM-BaG and OM cultures at day 15 (Figure 6.6B).

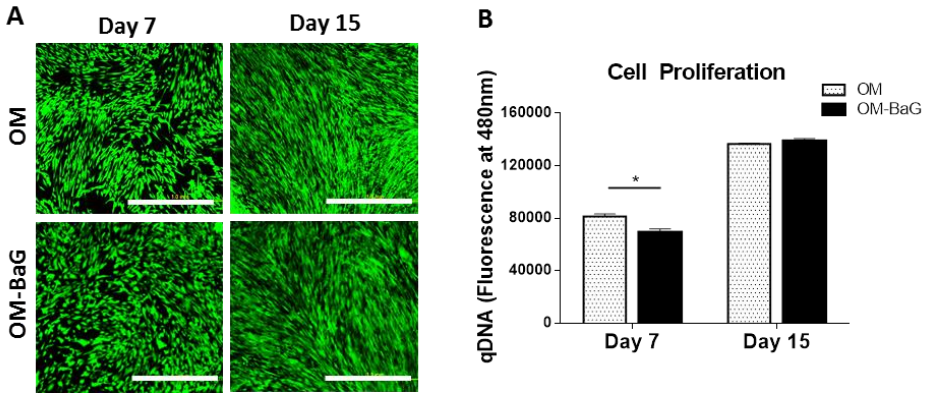


Figure 6.6. Viability and proliferation of DPPSC cultured on BaG extract osteogenic medium. (A) The viability of DPPSC in BaG extracts was analysed by Live/Dead staining after 7 and 15 days of osteogenic culture (N=3). Scale bars: 1 mm. **(B)** The proliferation of DPPSC in BaG extracts was analysed by CyQUANT Cell Proliferation Assay Kit at 7 and 15 days of osteogenic culture (N=3). *$P<0.05$.

The effect of the BaG-conditioned medium on the osteogenic differentiation of DPPSC was evaluated by qRT-PCR, IF and Alizarin Red S at 7 and 15 days of osteogenic induction (Figure 6.7). qRT-PCR showed that OM-BaG upregulated the expression of the early osteogenic markers OSX and DLX5 at day 7 and the expression of the later marker OC at day 15 (Figure 6.7A). Moreover, the IF stainings revealed that, in a similar manner in both culture conditions at day 7, COL1 was still predominantly located intracellularly and at day 15, was secreted to the ECM (Figure 6.7B). Nevertheless, the OM-BaG seemed to induce higher production of OC at day 7 than the OM control although after 15 days the protein expression was similar in both culture conditions (Figure 6.7C).

Alizarin Red S staining was conducted in order to see whether the BaG extracts can induce mineralization of DPPSC after 15 days of osteogenic induction. Results showed that OM-BaG induced extensive mineral formation compared to the OM control. The difference was significant as seen after quantification of the staining (Figure 6.7D).

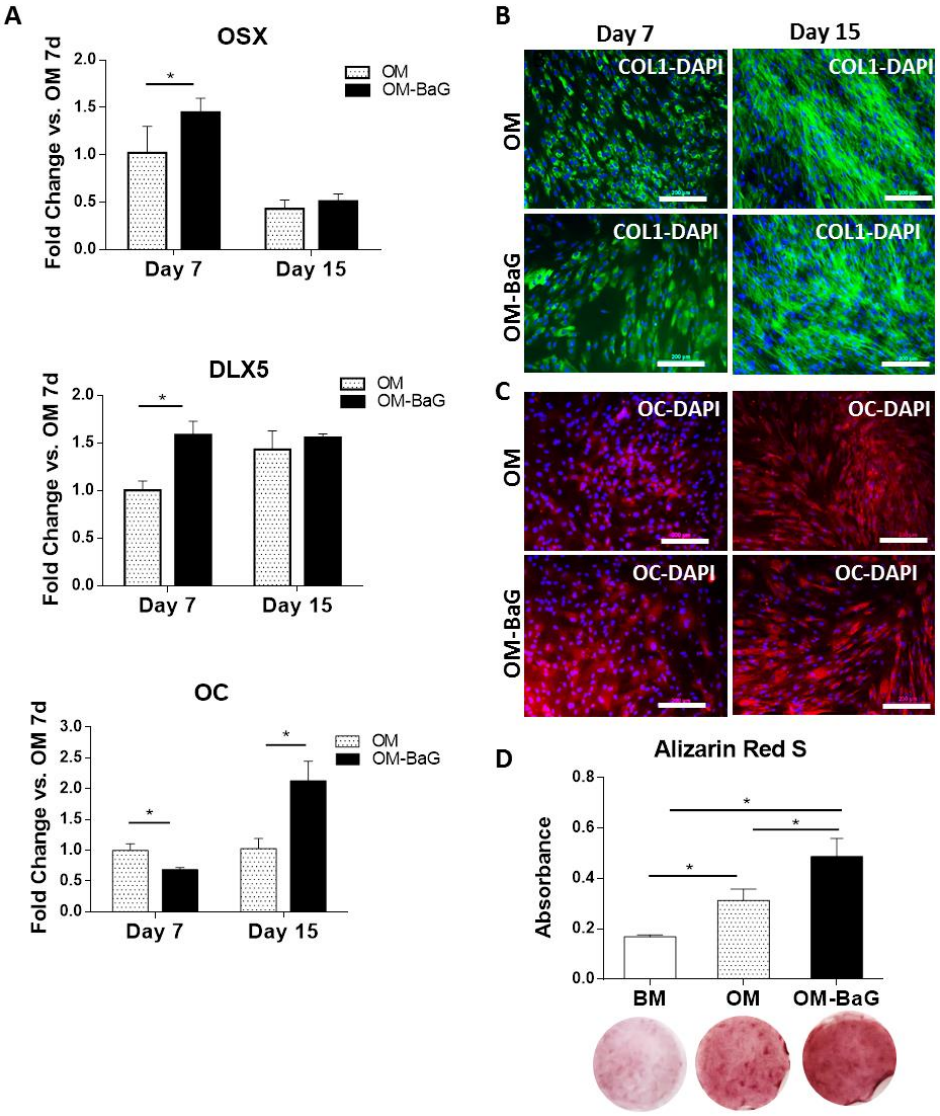


Figure 6.7. Effect of BaG extracts in the osteogenic differentiation of DPPSC. A) Relative expression of osteogenic marker genes OSX, DLX5 and OCN analysed by qRT-PCR at 7 and 15 days of differentiation relative to cells cultured in OM at day 7 (N=3). Data were normalized to the expression of housekeeping gene RPLP0. **P<0.05*. **B-C)** Protein expression by COL1A1 and OC immunostainings after 7 and 15 days of culture (N=2). **(B)** COL1 is stained green, **(C)** OC is stained red and nuclei are stained blue with DAPI in both IFs. Scale bars: 200 µm. **D)** Mineral formation after BaG extract treatment by Alizarin red S staining after 15 days of osteogenic culture (N=3). Images of the whole

wells in 24-well plates (1.5 cm of diameter) and quantification by cetylpyridinium chloride extracts absorbance (544nm). *$P<0.05$.

6.3.2.2 Effect of BaG in DPPPSC endothelial differentiation

The effect of BaG extracts in endothelial medium (EM-BaG) was analysed during 15 days of DPPSC culture and compared to the effects of EM control.

Live/Dead staining demonstrated that DPPSC cultured in EM-BaG survived as well as DPPSC in EM control. Moreover, at day 7 of differentiation the cell amount of EM-BaG cultures increased. The same phenomenon was also observed by CyQUANT Cell Proliferation Assay, which revealed that the increase of cell amount in EM-BaG cultures was also statistically significant (Figure 6.8).

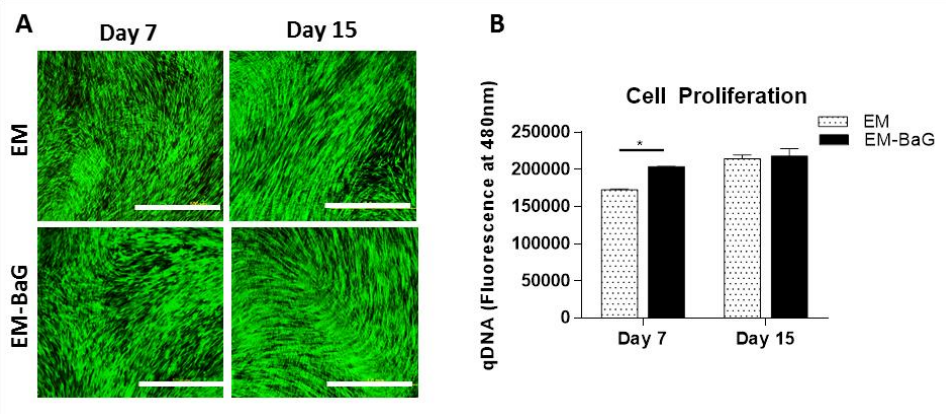


Figure 6.8. Viability and proliferation of DPPSC cultured on BaG extract endothelial medium. (A) The viability of DPPSC in BaG extracts was analysed by Live/Dead staining after 7 and 15 days of endothelial culture. Scale bars: 1 mm. **(B)** The proliferation of DPPSC in BaG extracts was analysed by CyQUANT Cell Proliferation Assay Kit at 7 and 15 days of endothelial culture. *$P<0.05$.

Afterwards, the endothelial differentiation potential of DPPSC cultured in EM-BaG was evaluated during 15 days of culture (Figure 6.9). In general, it was observed an upregulation of the endothelial markers (FLK1, vWF, CD31) by qRT-PCR at 7 and 15 days of culture in EM-BaG compared to cultures in the standard EM (Figure 6.9A).

At protein level, CD31 and vWF IF images showed that BaG extracts increased the expression of both endothelial proteins, mainly in vWF staining, which higher secretion of this endothelial marker to the ECM (Figure 6.9B).

Moreover, we tested the VEGF secretion in both endothelial media and, although there were no significant differences between the two conditions, the results seemed to indicate that after 15 days of differentiation, DPPSC in EM-BaG secreted more quantities of VEGF than DPPSC cultured in standard EM (Figure 6.9C).

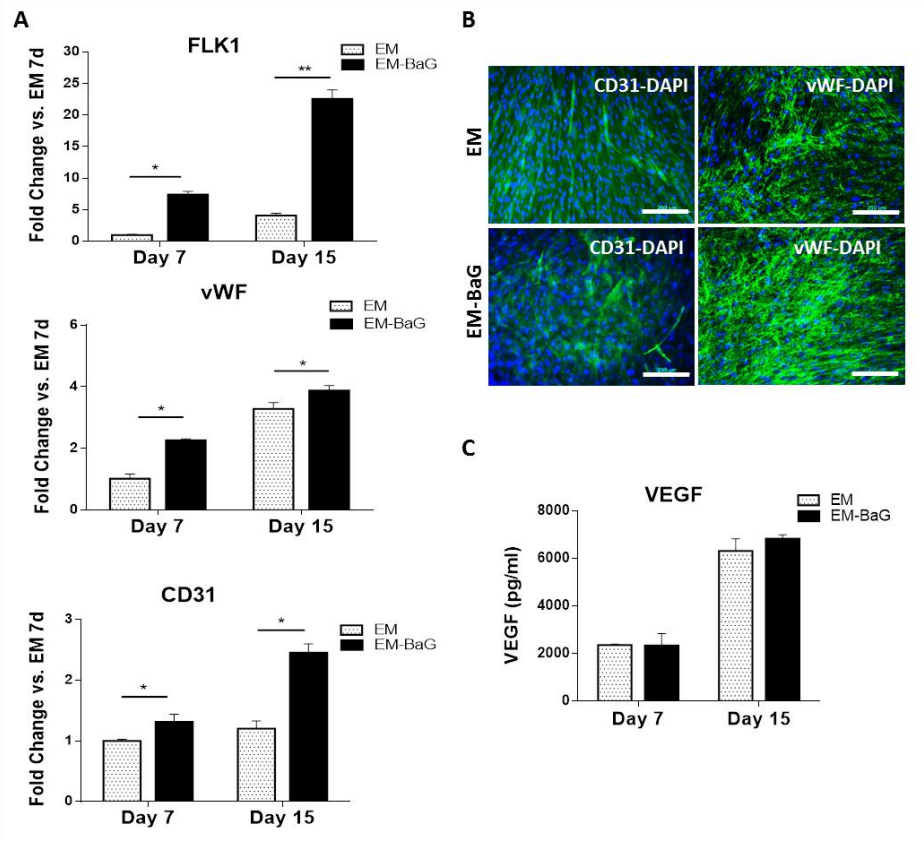


Figure 6.9. Effect of BaG extracts in the endothelial differentiation of DPPSC. A) Gene expression of endothelial marker genes FLK1, vWF and CD31 analysed by qRT-PCR after 7 and 15 days of endothelial differentiation relative to cells cultured in EM at day 7 (N=3). Data were normalized to the expression of the housekeeping gene RPLP0. **P<0.05.* **B)** CD31 and vWF production by immunostainings after 15 days of culture.

CD31 and vWF are stained green and nuclei are stained blue with DAPI. Scale bars: 200 µm. **C)** Levels of vascular endothelial growth factor (VEGF) production measured by enzyme-linked immunosorbent assay in the media of cultures at days 7 and 15 of differentiation. *$P<0.05$; **$P<0.01$.

6.3.3 Vascularization of engineered bone *in vitro*

As we have previously shown, DPPSC show a good osteogenic and endothelial potential, even better in xeno-free culture conditions and in the presence of BaG. Hence, we suggested the combination of both osteogenic and endothelial xeno-free DPPSC differentiations as a new strategy to induce vascularization of the bone-like tissue *in vitro*. Moreover, we evaluated the effect of BaG extracts in different co-culture mediums.

To achieve this objective, we studied different direct and indirect co-culture models including DPPSC. A diagram of the experimental design with DPPSC co-cultures is provided in Figure 6.10. Moreover, firstly, we performed a preliminary study evaluating the effect of co-culture DPPSC with primary endothelial cells (HUVEC cells).

In each 2D co-culture system, we evaluated the effect of four different mediums: Osteogenic Medium (OM), Endothelial Medium (EM), a mixture of Osteogenic and Endothelial Medium (OM/EM) and Basal Medium (BM). In the case of 3D co-cultures, we only used EM and EM-BaG, because these mediums demonstrated the highest osteogenic and endothelial potential in the 2D systems.

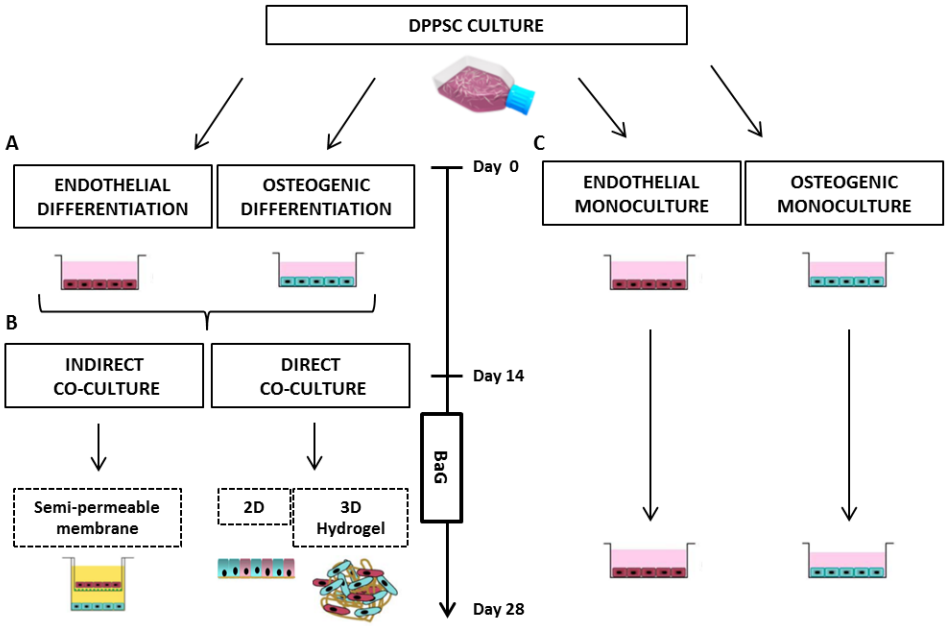


Figure 6.10. Experimental design of co-culture systems used for pre-vascularized bone-like tissue with DPPSC. A) DPPSC from the same donors, were expanded and differentiated into endothelial or osteogenic cells during 14 days. **B)** Indirect or Direct co-culture systems were realized with bone-like and endothelial-like pre-differentiated DPPSC and cultured during 14 days more in different medium conditions. In direct co-cultures BaG extracts were added to the media. **C)** At the same time, DPPSC were differentiated in monocultures during 28 days, which are used as control cultures. BaG extracts conditioned medium was added from day 14 until the end of the co-culture and monoculture systems.

6.3.3.1 Co-cultures of HUVEC and Bone-like DPPSC

Firstly, we evaluated the direct and indirect 2D co-cultures methods using bone-like DPPSC (DPPSC cultured during 14 days in OM) as osteoblastic cells and the primary endothelial cell line HUVEC as endothelial cells.

The indirect communication between HUVEC and bone-like DPPSC was investigated using semi-permeable membranes (porous cell chambers) that physically separate the cells. HUVEC were cultured above (in the chamber) and

bone-like DPPSC below (on the plastic surface). At the same time we cultured DPPSC alone in a monoculture with OM as a control condition during 28 days.

Gene expression after 14 days revealed that indirect co-cultures of HUVEC and bone-like DPPSC with EM or OM/EM increased the expression levels of the endothelial markers (FLK1, CD31) compared to monocultured DPPSC in OM (Figure 6.11A). In contrast, the osteogenic markers (COL1A1, ALP, OSN) were maintained or upregulated only with the effect of OM and BM (Figure 6.11B).

On the other hand, we performed direct co-cultures of HUVEC with bone-like DPPSC (Figure 6.12). qRT-PCR results demonstrated that the EM and the OM/EM upregulated the expression of the endothelial markers (FLK1, CD31) (Figure 6.12A). Moreover, these medium conditions also increased the expression levels of the osteogenic markers (ALP, OSN) reaching the highest levels in EM co-cultures (Figure 6.12B). Afterwards, we also evaluated the vessel-like structures formation by IHC of CD31 endothelial marker. Results clearly showed that HUVEC-bone-like DPPSC direct co-cultures under EM conditions were able to form micro capillary-like structures (Figure 6.13).

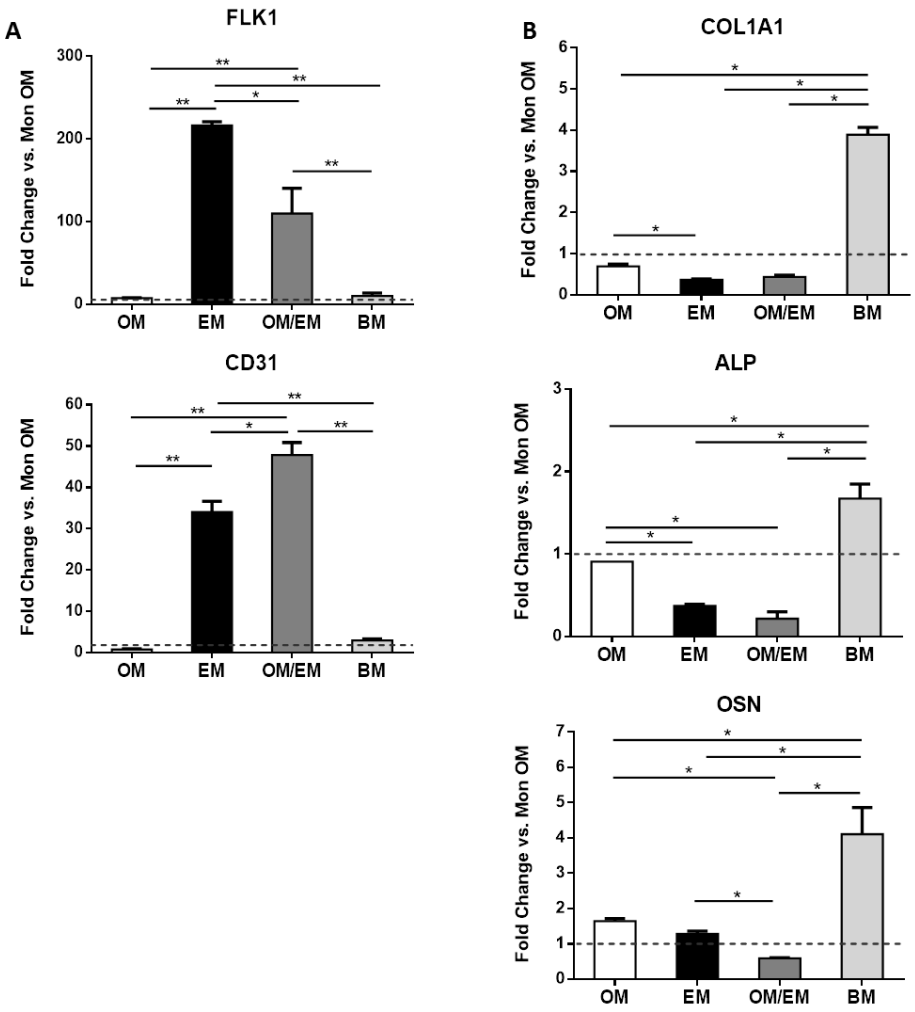


Figure 6.11. Indirect co-cultures of HUVEC and bone-like DPPSC. Gene expression after 14 days of culture by qRT-PCR of **(A)** endothelial markers (FLK1,CD31) and **(B)** osteogenic markers (COL1A1, ALP, OSN) with different medium conditions relative to DPPSC in OM monoculture (dotted line) (N=3). Data were normalized to the expression of the housekeeping gene RPLP0. OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: mixture of Osteogenic and Endothelial Medias; BM: Basal Medium. **P<0.05;* ***P<0.01.*

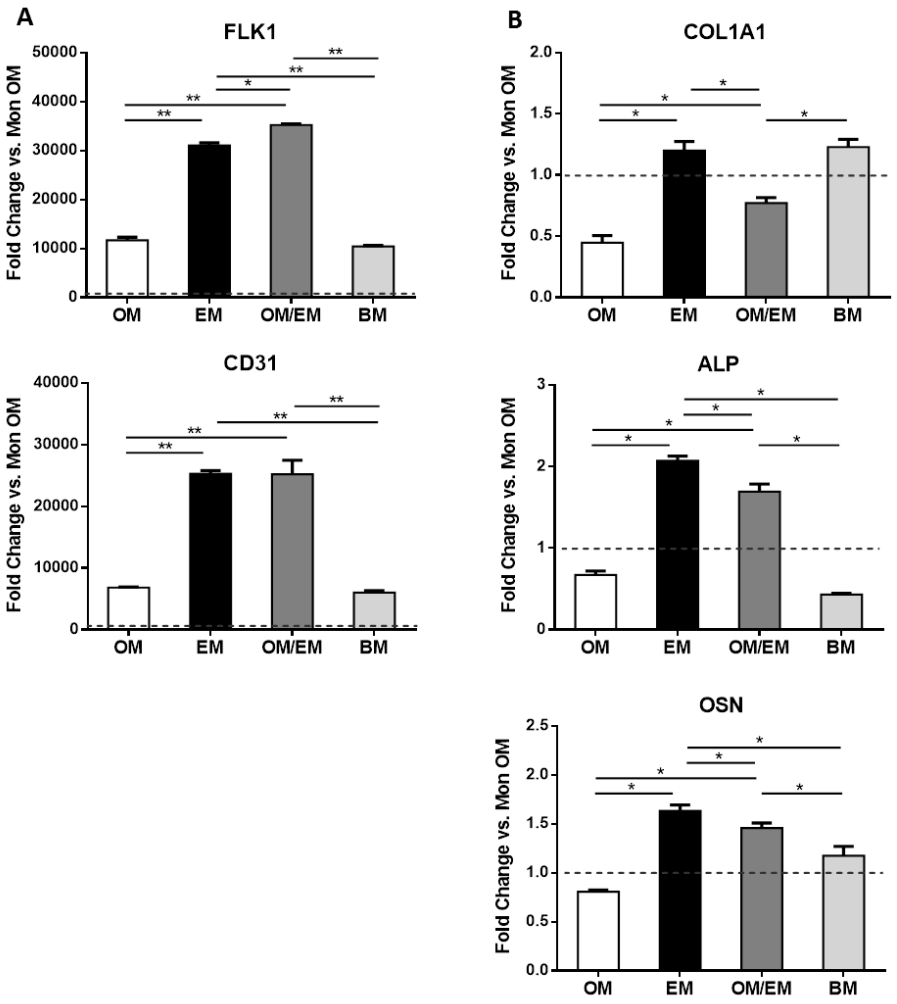


Figure 6.12. Direct co-cultures of HUVEC and bone-like DPPSC. Gene expression after 14 days of culture by qRT-PCR of **(A)** endothelial markers (FLK1,CD31) and **(B)** osteogenic markers (COL1A1, ALP, OSN) with different medium conditions relative to DPPSC in OM monoculture (dotted line) (N=3). Data were normalized to the expression of the housekeeping gene RPLP0. OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: mixture of Osteogenic and Endothelial Media; BM: Basal Medium. *$P<0.05$; **$P<0.01$.

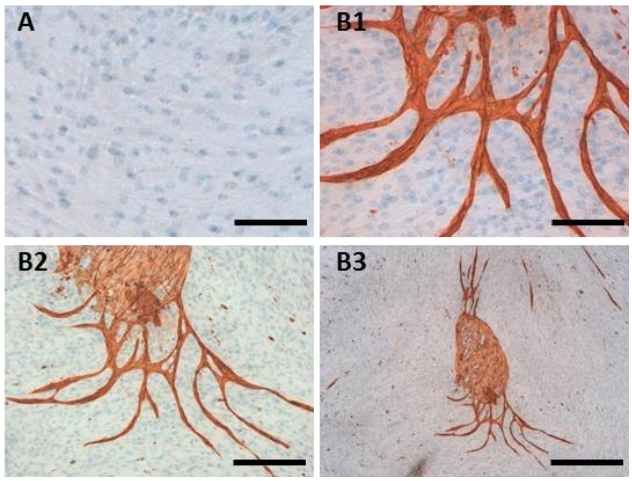


Figure 6.13. Microvessel-like structures in direct co-cultures of HUVEC and DPPSC. CD31 immunohistochemistry after 14 days of culture of **(A)** DPPSC in OM monoculture; **(B1-3)** Co-cultures of HUVEC and DPPSC in EM. Scale bars: 200 µm (A, B1), 500 µm (B2), 1 mm (B3). Representative images from 1 of 3N.

6.3.3.2 Indirect DPPSC co-cultures

The indirect cell interaction between endothelial-like DPPSC (DPPSC cultured in EM during 14 days) and bone-like DPPSC (DPPSC cultured in OM during 14 days) was also investigated using porous cell chambers that physically separate the cells. Hence, we put the endothelial-like DPPSC above (in the chamber) and the bone-like DPPSC below (in the plastic surface). At the same time we cultured DPPSC alone in monoculture with OM as a control condition during 28 days (Figure 6.14).

Gene expression after 14 days of culture in different medium conditions showed that only DPPSC co-cultures in EM increased the expression levels of the endothelial markers (FLK1, CD31) compared to monocultured DPPSC in OM (Figure 6.14A). In contrast, the expression of the osteogenic markers (COL1A1, ALP, OSN) was upregulated with the effect of OM and BM (Figure 6.14B).

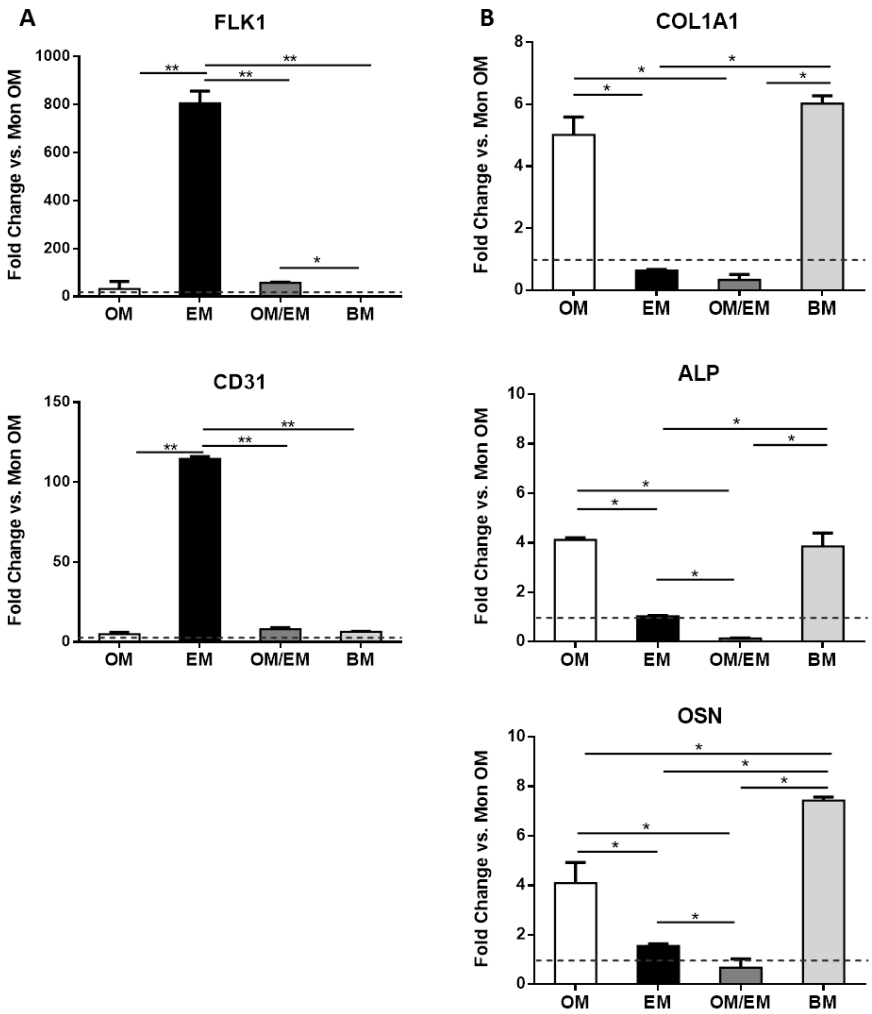


Figure 6.14. Indirect co-cultures of endothelial-like and bone-like DPPSC. Gene expression after 14 days of culture by qRT-PCR of **(A)** endothelial markers (FLK1,CD31) and **(B)** osteogenic markers (COL1A1, ALP, OSN) with different medium conditions relative to DPPSC in OM monoculture (dotted line) (N=3). Data were normalized to the expression of the housekeeping gene RPLP0. OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: mixture of Osteogenic and Endothelial Media; BM: Basal Medium. *$P<0.05$; **$P<0.01$.

6.3.3.3 Direct DPPSC co-cultures

For direct co-cultures of endothelial-like DPPSC and bone-like DPPSC, the effects of the xeno-free culture media used in the indirect co-cultures were also compared with the effects of each medium conditioned with BaG extracts after 14 days of co-culture.

At the same time, DPPSC were differentiated alone during 28 days in xeno-free OM and EM monocultures with and without BaG as a control conditions.

- Endothelial characteristics of direct DPPSC co-cultures

The formation of angiogenic structures depending on the type of culture medium used was investigated by IF analyses for the endothelial markers vWF (Figure 6.15) and CD31 (Figure 6.16) after two weeks of co-culture.

IF images showed that vWF was expressed in a similar manner in co-cultures with or without BaG extract medium. However, co-cultures in EM with BaG extract resulted in a considerable formation of vWF network (Figure 6.15A). Moreover, both monocultures in the presence of BaG extracts increased the expression of vWF (Figure 6.15B).

On the other hand, co-cultures in OM or BM and monocultures in OM were characterized by the absence of CD31 expression (Figure 6.16). Thus, there was CD31 expression only in co-cultures and monocultures in EM or OM/EM, characterized by an interconnected endothelial cell structure. Moreover, as seen in Figure 6.16A, the expression of CD31 was increased in co-cultures with EM and OM/EM under the effect of BaG extracts resulting in a considerable formation of angiogenic-like structures.

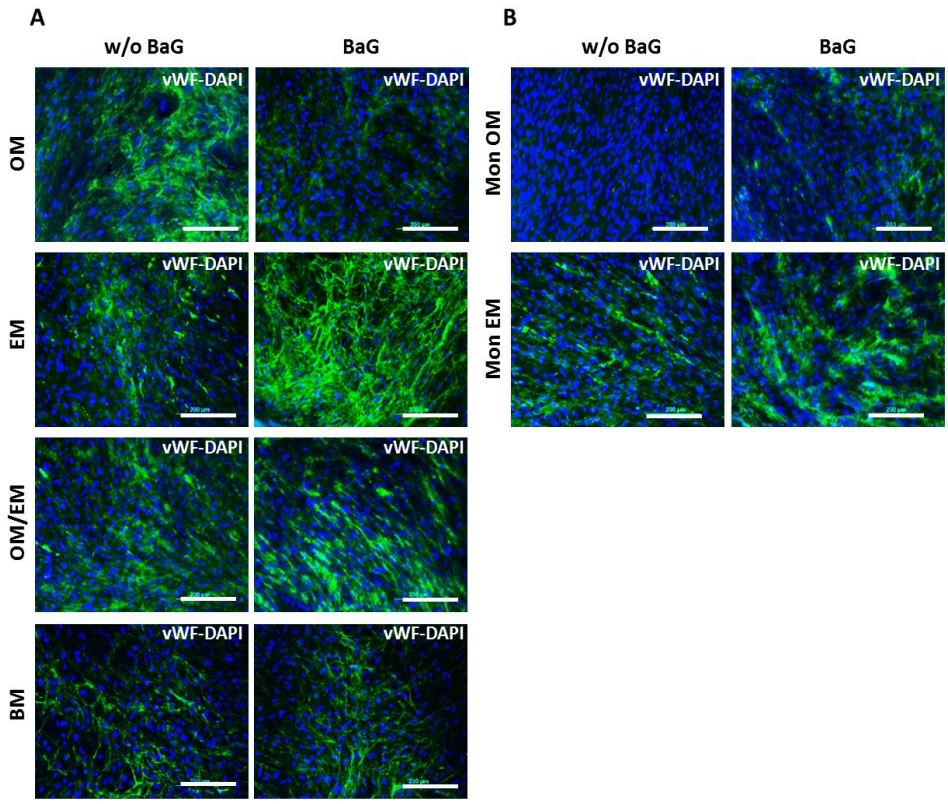


Figure 6.15. Effect of BaG extracts in the endothelial characteristic of direct DPPSC co-cultures. vWF production by IF analyses after 14 days of **(A)** co-cultures in different media and **(B)** monocultures in OM and EM. vWF is stained green and nuclei are stained blue with DAPI. Scale bars: 200 µm. w/o BaG: media without BaG extracts; BaG: BaG extracts conditioned media; OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: a mixture of Osteogenic and Endothelial Media; BM: Basal Medium; Mon OM: DPPSC monoculture in OM; Mon EM: DPPSC monoculture in EM. Representative images from 1 of 3N.

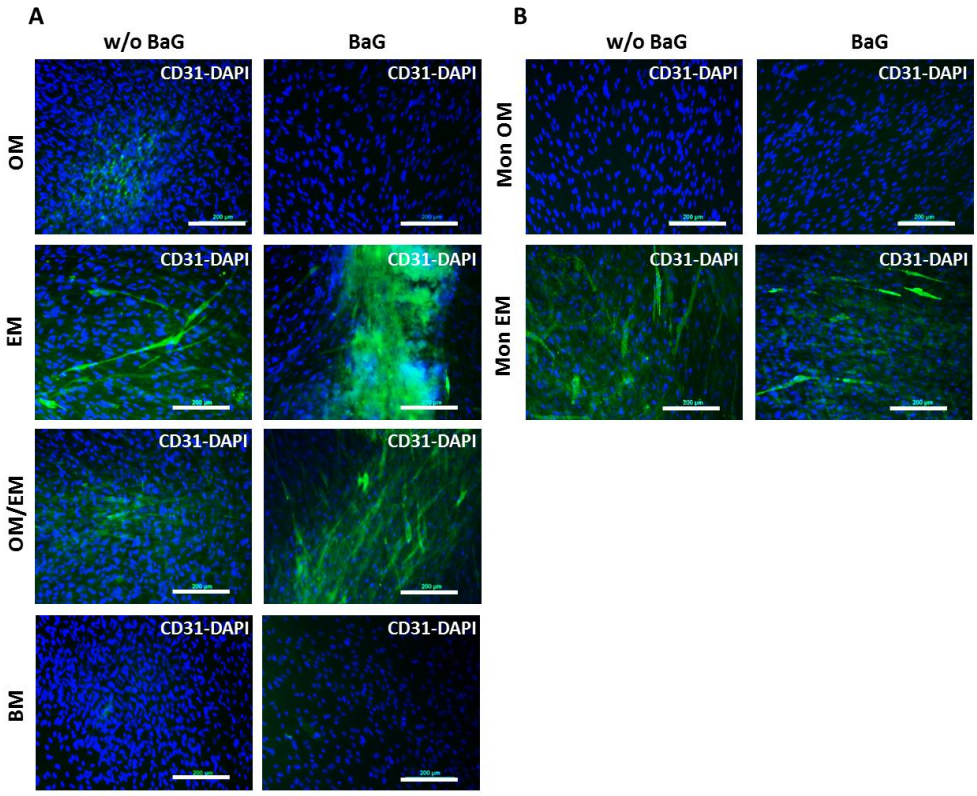


Figure 6.16. Effect of BaG extracts in the endothelial characteristics of direct DPPSC co-cultures. CD31 production by IF analyses after 14 days of **(A)** co-cultures in different mediums and **(B)** monocultures in OM and EM. CD31 is stained green and nuclei are stained blue with DAPI. Scale bars: 200 µm. w/o BaG: media without BaG extracts; BaG: BaG extracts conditioned media; OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: a mixture of Osteogenic and Endothelial Media; BM: Basal Medium; Mon OM: DPPSC monoculture in OM; Mon EM: DPPSC monoculture in EM. Representative images from 1 of 3N.

Therefore, micro-vessel like structures were observed in EM and OM/EM in the presence of BaG extracts as seen with less magnification (Figure 6.17A). To assess in more detail how the medium with BaG extract affects the formation of angiogenic structures by endothelial-like DPPSC in co-culture with osteogenic-like DPPSC, the release of the angiogenic growth factor VEGF was investigated after 14 days of co-culture. Results showed that the production of

VEGF was increased in EM and EM/OM co-cultures and in EM compared to OM monocultures (Figure 6.17B).

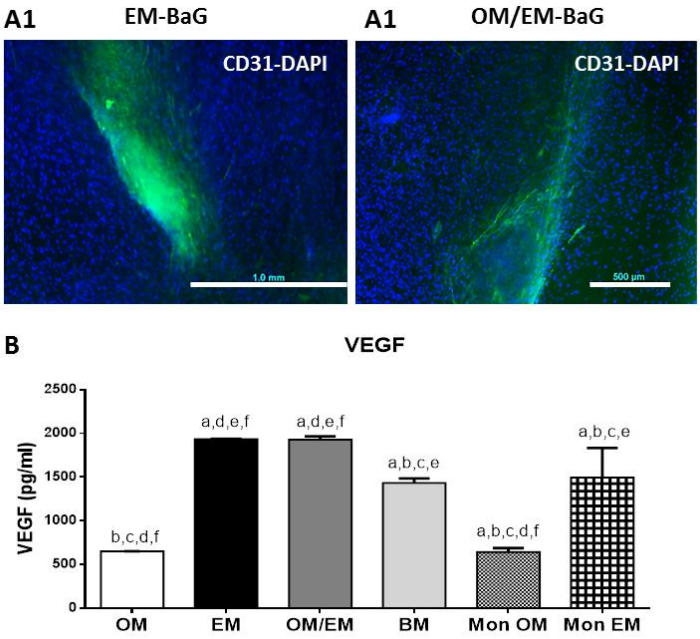


Figure 6.17. Effect of BaG extracts in the endothelial characteristics of direct DPPSC co-cultures. **A)** CD31 production by IF analyses after 14 days of co-cultures in (A1) EM and (A2) OM/EM with BaG extracts. Scale bars: 1 mm (A1); 500 µm (A2). **B)** Levels of VEGF production measured by enzyme-linked immunosorbent assay in the BaG extract media of co-cultures and monocultures after 14 days of differentiation. OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: a mixture of Osteogenic and Endothelial Media; BM: Basal Medium; Mon OM: DPPSC monoculture in OM; Mon EM: DPPSC monoculture in EM. *(a) Significant difference versus OM; (b) Significant difference versus EM; (c) Significant difference versus OM/EM; (d) Significant difference versus EM; (e) Significant difference versus Mon OM; (f) Significant difference from Mon EM. P<0.05.* Representative images from 1 of 3N.

Furthermore, the endothelial characteristics of DPPSC co-cultures were evaluated by qRT-PCR after 14 days of culture (Figure 6.18). In general, an up-regulation of the endothelial markers (FLK1, vWF) was observed in DPPSC co-cultured either in EM or OM/EM BaG conditioned media (Figure 6.18A).

Concretely, the expression levels of FLK1 increased in co-cultures and monocultures in EM with the highest levels in BaG extracts conditioned media. On the other hand, the expression levels of vWF were upregulated in EM and EM/OM co-cultures also with higher levels in BaG extracts conditioned media.

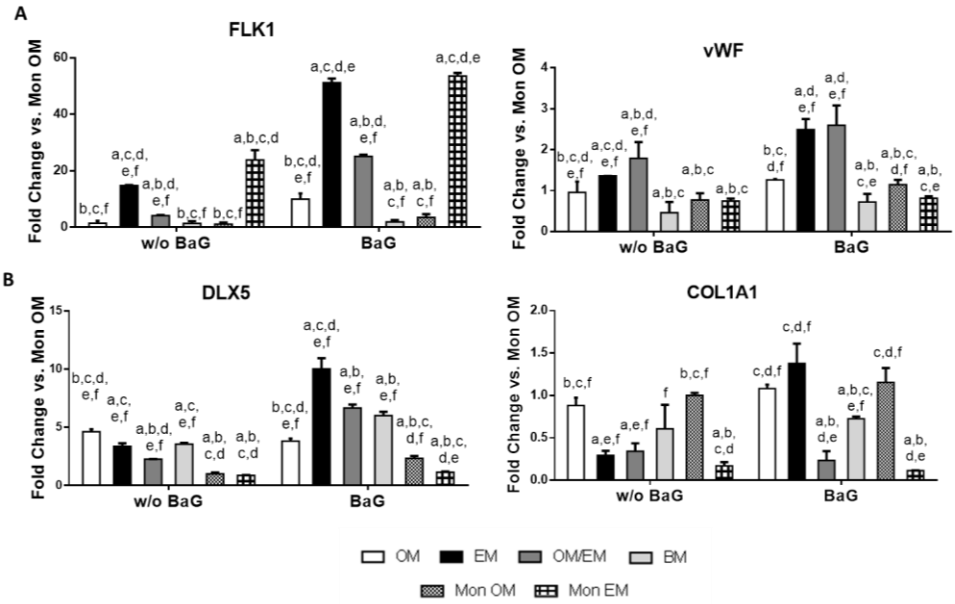


Figure 6.18. Effect of BaG extracts in the endothelial and osteogenic characteristics of direct DPPSC co-cultures. Gene expression after 14 days of culture by qRT-PCR of **(A)** endothelial markers (FLK1, vWF) and **(B)** osteogenic markers (DLX5, COL1A1) with different medium conditions relative to cells monocultured in OM (N=3). Data were normalized to the expression of the housekeeping gene RPLP0. w/o BaG: media without BaG extracts; BaG: BaG extracts conditioned media; OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: a mixture of Osteogenic and Endothelial Media; BM: Basal Medium; Mon OM: DPPSC monoculture in OM; Mon EM: DPPSC monoculture in EM. *(a) Significant difference from OM; (b) Significant difference from EM; (c) Significant difference from OM/EM; (d) Significant difference from EM; (e) Significant difference from Mon OM; (f) Significant difference from Mon EM. P<0.05.*

- Osteogenic characteristics of direct DPPSC co-cultures

The previously described results focused on the endothelial response of DPPSC co-cultures. In addition, the direct co-culture of DPPSC might also result in

effects on the osteogenic differentiation. By qRT-PCR, standard co-cultures without BaG showed the highest expression of the osteogenic markers (DLX5, COL1A1) in OM and BM conditions. However, there were changes in the expression profile of the osteogenic genes in response to the BaG treatment. In general, BaG extracts upregulated the expression of the osteogenic markers in all co-cultures, reaching the highest levels in EM condition (Figure 6.18B).

Moreover, in order to see whether the BaG extracts can induce mineralization of the co-cultures, Alizarin Red S staining was conducted after 14 days of co-culture. Figure 6.19 shows that co-cultures in EM conditioned with BaG extracts induced extensive mineral formation. Moreover, the difference was significant compared to the other co-culture and monoculture conditions as seen after stainings quantification.

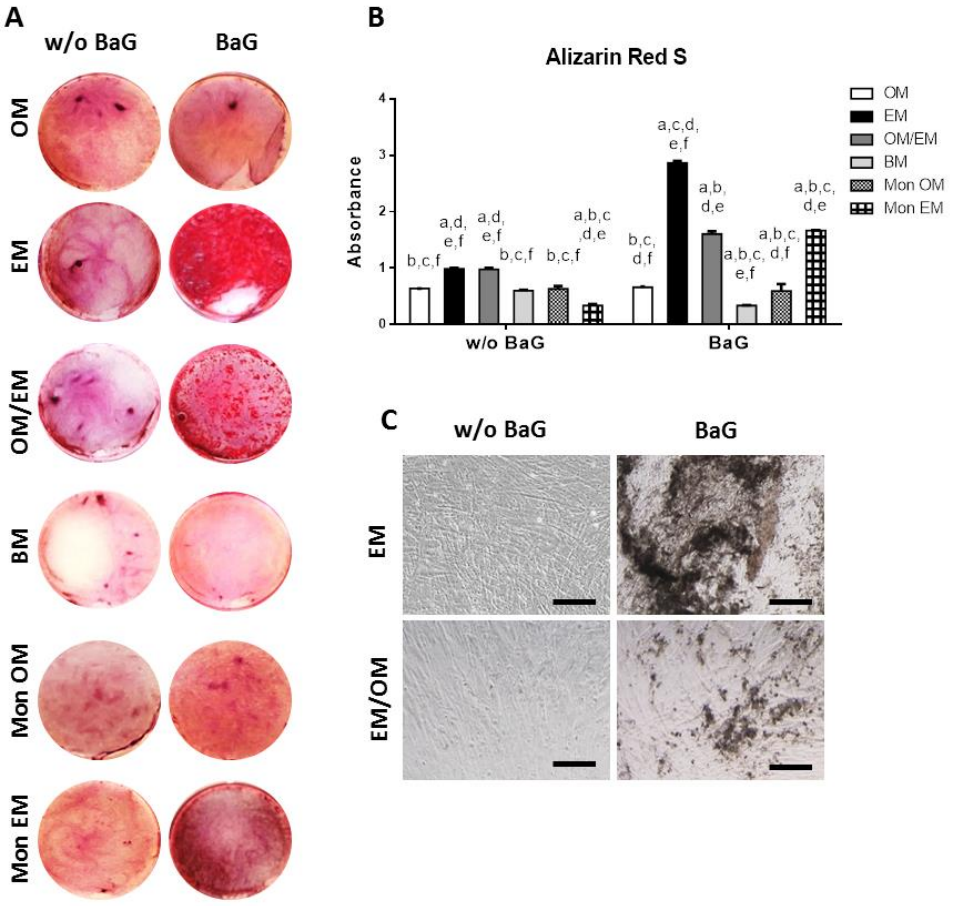


Figure 6.19. Effect of BaG extracts in the mineralization of direct DPPSC co-cultures. A-B) Mineral formation by Alizarin red S staining after 14 days of culture. **(A)** Images of the whole wells in 24-well plate (1.5 cm of diameter) and **(B)** quantification by cetylpyridinium chloride extracts absorbance (544nm). **C)** Images by optic microscopy with calcifications in dark. Scale bars: 200 µm. w/o BaG: media without BaG extracts; BaG: BaG extracts conditioned media; OM: Osteogenic Medium; EM: Endothelial Medium; OM/EM: a mixture of Osteogenic and Endothelial Media; BM: Basal Medium. *(a) Significant difference from OM; (b) Significant difference from EM; (c) Significant difference from OM/EN; (d) Significant difference from EM; (e) Significant difference from Mon OM; (f) Significant difference from Mon EM. P<0.05.* Representative images from 1 of 3N.

6.3.3.4 3D DPPSC co-cultures

The respective 2D co-culture studies provided detailed information of cellular events governing the differentiation of bone-like DPPSC those are in contact with endothelial-like DPPSC. Conversely, 3D culture might offer a physiologically optimized environment for cell survival which favors cell-to-cell interaction, cell functionality and a rapid formation of functional blood vessels. Hence, DPPSC monocultures and co-cultures in a 3D peptide-hydrogel system were analysed. For these experiments, we decided to use PuraMatrix hydrogel and EM or EM-BaG culture conditions, because co-cultures in EM demonstrated the highest osteogenic and endothelial potential in the 2D co-culture experiments.

Firstly, cell viability of PuraMatrix encapsulating DPPSC as monocultures were evaluated after 5 days of culture in OM or EM. Results showed that both culture conditions supported the viability of the cells as indicated in Figure 6.20. In addition, DPPSC in EM survived and grew faster than did DPPSC in OM. On the other hand, images showed changes in the morphology of DPPSC. While DPPSC in EM acquired a more elongated morphology, DPPSC in OM remained in a more rounded form. In fact, in EM, some interconnected endothelial cell structures were found to resemble vessel-like structures.

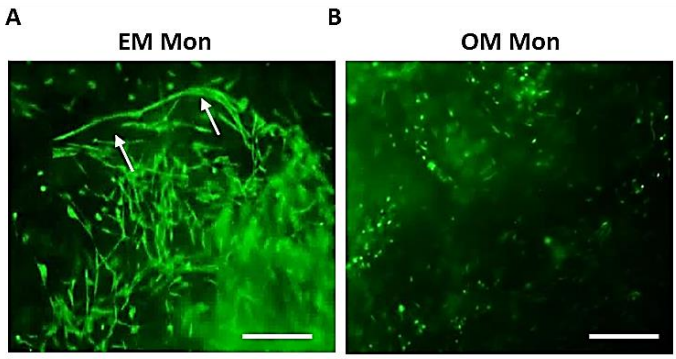


Figure 6.20. Viability of DPPSC after encapsulation in PuraMatrix during 5 days of culture by Calcein AM assay. A) DPPSC monoculture in endothelial medium (EM Mon). White arrows indicate the formation of interconnected endothelial cell structures; **B)** DPPSC monoculture in osteogenic medium (OM Mon). Scale bars: 200 µm. Representative images from 1 of 3N.

Secondly, in order to study cell alignment of DPPSC co-cultures in 3D conditions, a phalloidin IF staining was realized after 14 days of culture in EM with and without BaG extracts (Figure 6.21). Images at different magnifications showed that an important amount of cells were elongated and aligned in an interconnected cell structure, forming a complex network with a series of branching points. However, only under the effect of BaG, DPPSC seemed to form tubular-like structures with the presence of an internal lumen (Figures 6.21 and 6.22).

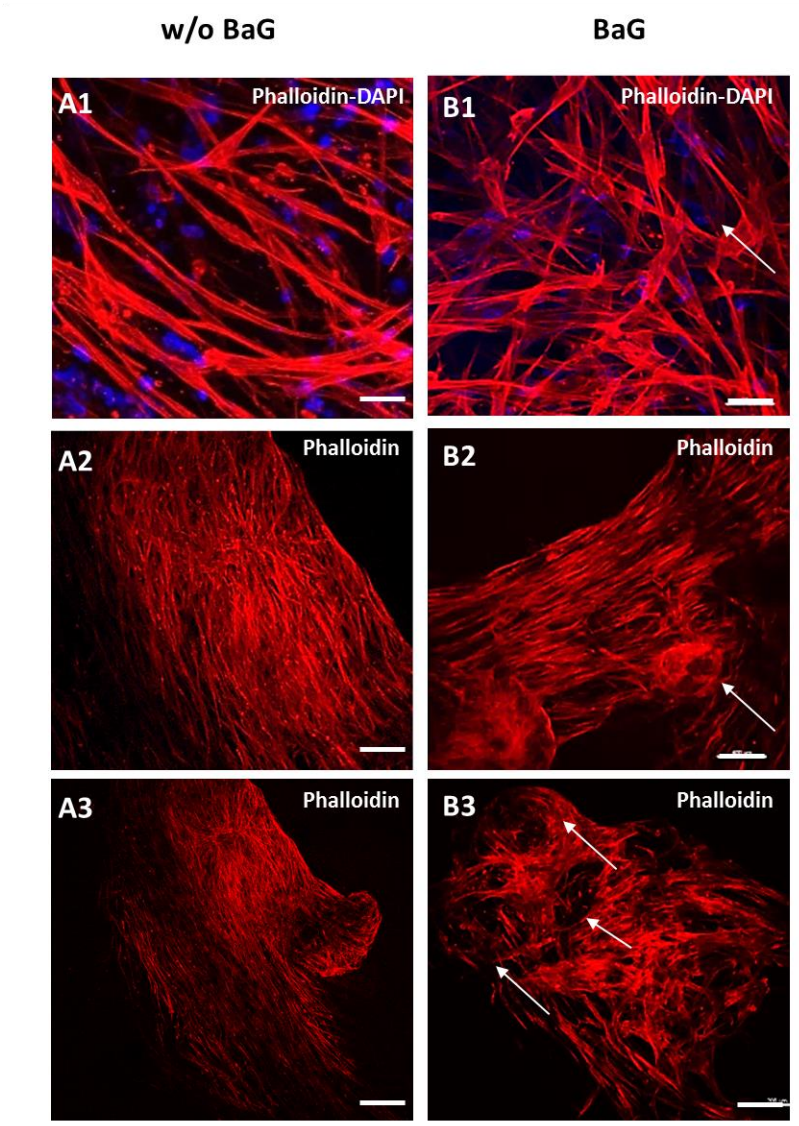


Figure 6.21. Cell alignment and tubular-like structures of DPPSC co-cultures encapsulated in PuraMatrix. A1-3) Phalloidin IF of 3D DPPSC co-cultures after 14 days of culture in EM without BaG. **B1-3)** Phalloidin IF of 3D DPPSC co-cultures after 14 days of culture in EM treated with BaG extracts. White arrows show tubular-like structures with the formation of an internal lumen. Phalloidin is stained red and nuclei are stained blue with DAPI. w/o BaG: EM without BaG extract; BaG: EM conditioned with BaG extracts. Scale bars: 10 µm (A1, B1), 200 µm (A2, B2, A3, B3). Representative images from 1 of 2N.

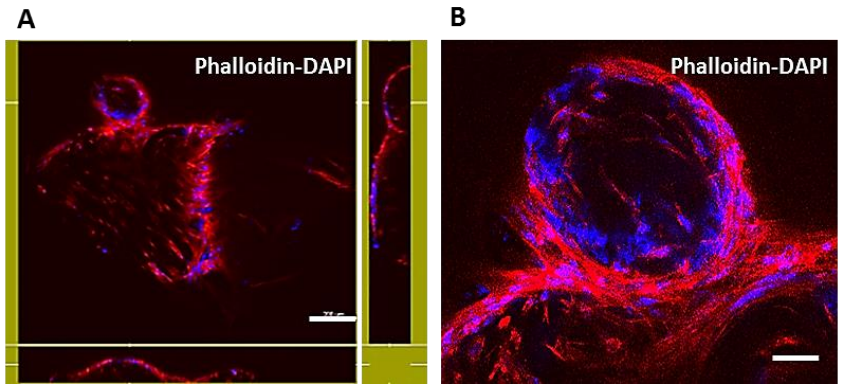


Figure 6.22. Tubular-like structures with the formation of an internal lumen in 3D DPPSC co-cultures. DPPSC co-cultures encapsulated in PuraMatrix after 14 days in EM treated with BaG. **A)** 3D composite image with lateral views. **B)** Magnification of a vascular lumen structure. Phalloidin is stained red and nuclei are stained blue with DAPI. Scale bars: 200 µm (A), 100 µm (B). Representative images from 1 of 2N.

6.4 DISCUSSION

6.4.1 HS is a suitable alternative to FBS for the differentiation of DPPSC under xeno-free culture conditions

A critical factor to facilitate the translation of DPPSC from basic biology to clinical application is the development of appropriate GMP cell culture protocols under xeno-free culture conditions.

Previous studies reported the osteogenic and the endothelial potential of DPPSC induced by OM or EM supplemented with FBS, which poses risk of transferring animal derived infections and related immunoreactions [5, 47]. Our present data demonstrate, for the first time, that OM and EM supplemented with HS maintain cell viability and proliferation rate in DPPSC, comparable to the ones measured in culture conditions using FBS. Furthermore, the expression of bone and endothelial related markers, as well as the functional assays, indicated that the use of HS is adequate for both differentiation protocols. In fact, DPPSC cultured in OM-HS and EM-HS seem to have higher differentiation potential than DPPSC cultured in OM-FBS and EM-FBS in most of

the experiments. Therefore, the use of HS could be considered to be a safer alternative for DPPSC osteogenic and endothelial cultures.

These results are in accordance with other studies that report the culture and the multilineage differentiation potential of other DPSC populations under xeno-free culture conditions. In some of these reports, allogenic HS maintains the proliferation and the differentiation capability of DPSC as effectively as FBS [11, 48].

Moreover, in a previous study of our group it has been demonstrated that in medium conditions supplemented with HS, the expression of the pluripotency markers OCT3/4 and NANOG in DPPSC increases while the SOX2 expression decreases [13]. It is known that high levels of OCT3/4 induce meso-endoderm differentiation, while low levels of OCT3/4 specify ectoderm differentiation; NANOG represses ectoderm differentiation; and SOX2 represses meso-endoderm differentiation [49]. Therefore, we then hypothesized that by isolating and culturing DPPSC in HS supplemented medium we could improve the mesoderm differentiation in DPPSC probably due to the up-regulation of OCT3/4 and NANOG and the downregulation of SOX2 before differentiation. The present data seem to confirm this hypothesis and indicate that HS is a suitable alternative to FBS for the expansion and the differentiation of DPPSC in xeno-free culture conditions, which is compatible with GMP-approved protocols.

6.4.2 BaG extracts provide an effective way to differentiate DPPSC towards osteogenic and endothelial lineage *in vitro*

BaG and their dissolution ions have been shown to be advantageous biomaterials for BTE due to their osteogenic and angiogenic potential [15]. Moreover, reactions on the BaG surface induce the release of critical concentrations of soluble ions such as Si, Ca, and P, which has been shown to lead to favorable intracellular and extracellular responses promoting rapid bone formation [17]. In the present study we have investigated, for the first time, the effect of S53P4 BaG extracts in the osteogenic and endothelial differentiation of DPPSC under xeno-free conditions.

Some authors described that 45S5 BaG dissolution products with no added osteogenic supplements induce osteoblast differentiation [20, 50]. However, recent studies with ASC revealed that S53P4 BaG extracts could not induce mineralization without the supplementation with ascorbic acid, β-glycerol phosphate and dexamethasone [26]. This lack of osteogenic differentiation might be explained by the few amount of phosphorous in the ionic composition of S53P4 (Table 6.1), an essential substrate for mineralization. For these reasons, we only evaluated the effect of BaG extracts using differentiation media.

Our results demonstrated that BaG conditioned media maintained the viability of DPPSC during osteogenic and endothelial differentiations and moreover, enhance both differentiations processes. However, at the beginning of the process (day 7), BaG extracts produced different effects on cell proliferation depending on the type of differentiation. In the osteogenic induction the proliferation rate was decreased while in the endothelial induction it was increased compared to cells in control media.

There is evidence that BaG ions stimulate and control the cell cycle of osteoblasts towards osteogenic differentiation [23]. Similar to our results, the combination of BaG ions and OM supplements has been shown to enhance mineralization and OC and COL1 expression in MC3T3-E1 cells and fibroblasts [51, 52]. In addition, a recent study with ASC has reported a similar effect also with OM and S53P4 BaG extracts in xeno-free conditions [26].

Controversial results also exist in concern to proliferation: the proliferation of MC3T3-E1 cells and human osteoblasts was increased when cultured in 45S5 extracts [52, 53]. Whereas in SAOS-2 cells treated with MBG85 extracts or ASC treated with S53P4 extracts, the proliferation was also decreased [26, 54]. The reason for this large variation in the studies conducted with BaG extracts could be explained by the experimental setup differences between the various studies (e.g. cell types, glass compositions, medium supplements…).

On the other hand, our results in DPPSC endothelial differentiation are in agreement with other studies that have reported a mitogenic response and an increase of angiogenic markers through both direct and indirect contact of

human endothelial cells with BaG [21]. Moreover*, in vivo* studies have confirmed the ability of certain BaG to stimulate neovascularization [55].

In the present study, we used a small concentration of S53P4 BaG (0.08% w/v) for stimulating the endothelial potential of DPPSC. Previous studies have shown that the angiogenic potential of BaG was dose dependent. Hence, concentrations of 45S5 BaG superior than 0.1% w/v inhibited VEGF secretion from fibroblasts possibly due to cytotoxic effects related to either increased concentration of ion dissolution products or increased medium pH associated with higher concentrations of BaG [56]. Taken together, our results demonstrated that S53P4 BaG extracts at 0.08% w/v could potentially provide a fast and effective way to differentiate DPPSC towards both the osteogenic and endothelial lineage *in vitro*.

6.4.3 DPPSC co-cultures can enhance angiogenesis and osteogenesis, supporting the formation of vascular-like structures

The intimate functional relationship between bone vascular endothelium and osteoblasts might be crucial to the necessary cell behavior for bone development and remodeling [36, 57]. This fact was substantiated by histological findings indicating that osteoblasts and osteoprogenitors cells are always located adjacent to endothelial cells of blood vessels at the site of new bone formation [57]. Therefore, the communication between osteogenic and endothelial cells is the theoretical basis of the *in vitro* co-culture systems for bone tissue pre-vascularization.

In the present study, different co-culture systems with pre-differentiated DPPSC were investigated in terms of angiogenic and osteogenic activation depending on the culture medium.

It is known that during co-culture, endothelial cells secret soluble factors, such as BMP2, bFGF, ET1 or IGF, which affect the migration, proliferation and differentiation of the osteogenic cells [58-61]. Moreover, at the same time, osteogenic cells produce high levels of VEGF, which has been shown to increase the proliferation, differentiation and angiogenesis of the endothelial cells [62].

However, some studies revealed significant differences in the stability and function of the co-cultured cells depending on the culture medium, which seems to correlate with the secretion of the endothelial and the osteogenic factors in the context of specific culture conditions [63].

Therefore, optimal conditions for inducing both angiogenic and osteogenic properties without negative effect in the differentiation of the other cell type should be considered in each co-culture system [46]. In general, it has been reported that BM and OM are beneficial for the expansion and the differentiation of osteoblast-like cells. In contrast, survival and maintenance of phenotype of endothelial cells are highly dependent on EM. Therefore, there is no consensus on the optimal media composition for the co-culture of both cell types [29, 63]. Our results of indirect co-cultures either with HUVEC or endothelial-like DPPSC demonstrated that the osteogenic differentiation of bone-like DPPSC was enhanced in BM or OM, correlating with a higher release of osteogenic diffusible factors by the endothelial cells cultured in these media. According to these results, Dariima *et al.* demonstrated that indirect co-cultures of endothelial cells and osteoblasts in OM enhanced the osteoblast function [64].

On the other hand, our results showed that, in EM, the expression of osteogenic markers in both indirect co-cultures was downregulated, while the expression of the endothelial genes was up-regulated. It might be at least partly explained by a new process of differentiation of bone-like DPPSC towards the endothelial lineage instead of bone lineage due to the effect of EM. In contrast, the situation was completely different when HUVEC and bone-like DPPSC were co-cultured in direct contact. Results showed that co-cultures in EM, EM/OM or BM enhanced both endothelial and osteoblastic differentiations, with higher expression levels than in indirect co-cultures. Moreover, HUVEC in contact with bone-like DPPSC were able to form microvessel-like structures through the osteoblastic cells in EM. These results suggest that during direct co-cultures, there was an intense crosstalk between bone-like DPPSC and HUVEC that must therefore not only stimulate osteoblastic functions of DPPSC, but also angiogenic functions of HUVEC.

Vascularized BTE by DPPSC co-culture systems

There are number of reports of direct co-cultures with ASC pre-differentiated to bone-like cells with HUVEC cells that generally showed similar results. They demonstrated and increase in proliferation, VEGF production and high expression of bone differentiation markers and mineralization by EM, BM or EM/BM [34, 65-67]. Moreover, some of them demonstrated the formation of microcapillary-like structures [34, 67].

An optimal approach for clinical translation of BTE constructs would be the obtainment of both endothelial and osteoblast cells from a single individual in a minimally invasive extraction manner. However, there are few reports with co-cultures of cells originated from a single stem cell population [41, 42]. Previously, we demonstrated the capacity of DPPSC to differentiate towards the osteogenic and the endothelial lineage, even better in xeno-free culture conditions and with the effect of S53P5 BaG ions. Therefore, in the present study, 2D and 3D direct co-cultures of bone-like and endothelial-like DPPSC were investigated as a new strategy to induce vascularized bone tissue *in vitro*. Results of 2D DPPSC direct co-cultures demonstrated that the expression of endothelial markers and the formation of angiogenic structures were improved in the presence of EM (either in EM or EM/OM conditions) with the effect of BaG, correlating with a higher release of angiogenic factors such as VEGF by bone-like DPPSC in these EM co-cultures. Moreover, simultaneously, the differentiation of DPPSC toward the osteogenic lineage and the mineralization was also more effective in EM. Therefore, it suggests that the interaction between both cell types was dependent on culture medium.

In addition, the dissolution of S53P5 BaG ions in the EM enhanced more the pre-vascularization of DPPSC constructs, and, consequently, accelerates their mineralization through a direct co-culture system. It would be possible than DPPSC pre-differentiated during 15 days into endothelial cells before co-culture, may be partially differentiated cells and, therefore, need the effect of BaG to achieve a complete differentiation.

It is known that a 3D hydrogel scaffold might offer an optimal environment for cell-to-cell communication, cell migration and cell functionality that promote the formation of vessel-like structures [36]. Hence, 3D DPPSC co-cultures with EM and BaG were finally performed in a peptide-hydrogel system

(PuraMatrix). Confocal microscopy images of DPPSC co-cultures with BaG ions dissolution demonstrated the formation of tube-like structures characterized by the definition of a vascular-like lumen. Fuchs *et al.*, confirmed that when co-cultured with human osteoprogenitors on plastic surface, HUVEC are organized in vascular-like structures, but these do not exhibit a lumen in the centre. In contrast, associated in 3D silk fibroin scaffolds, these endothelial cells formed capillary-like networks that appear to be functional as indicated by the presence of a lumen [68]. Therefore, the formation of a vascular lumen is a critical feature of angiogenically activated cells and a prerequisite for establishing a functionally active blood supply [36]. In line with our results, Dissanayaka et *al.* demonstrated a coordinated migration of undifferentiated DPSC and HUVEC in PuraMatrix, which supported cell survival, cell migration, and capillary network formation in the absence of exogenous growth factors [69].

Based on our co-culture results, there is evidence of a direct interaction between bone-like DPPSC and endothelial-like DPPSC supporting the formation of vascular-like structures that consequently, enhance the osteogenic process *in vitro*. Moreover, 3D hydrogel co-cultures demonstrate the formation of active vascular-like structures that appeared to be functional as indicated by the presence of a lumen.

6.5 CONCLUSIONS

The present data assess the osteogenic and the endothelial capacity of DPPSC in different culture conditions for pre-vascularized BTE approaches.

Specifically, these results show that HS could be a suitable xeno-free alternative for DPPSC osteogenic and endothelial monoculture differentiation as well as for DPPSC co-cultures. Moreover, results suggest that S53P4 BaG extracts could provide an effective way to enhance both osteogenic and endothelial process in DPPSC, supporting the formation of vascular-like structures in DPPSC co-cultures.

Therefore, the co-culture of pre-differentiated DPPSC in combination with BaG under xeno-free medium conditions provides a new promising system for the *in vitro* vascularization of BTE constructs.

6.6 REFERENCES

[1] Committee for Advanced T, Secretariat CATS, Schneider CK, Salmikangas P, Jilma B, Flamion B, et al. Challenges with advanced therapy medicinal products and how to meet them. Nature reviews Drug discovery. 2010;9:195-201.

[2] Giancola R, Bonfini T, Iacone A. Cell therapy: cGMP facilities and manufacturing. Muscles, ligaments and tendons journal. 2012;2:243-7.

[3] Unger C, Skottman H, Blomberg P, Dilber MS, Hovatta O. Good manufacturing practice and clinical-grade human embryonic stem cell lines. Human molecular genetics. 2008;17:R48-53.

[4] Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, et al. Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science. 2012;125:3343-56.

[5] Atari M, Caballe-Serrano J, Gil-Recio C, Giner-Delgado C, Martinez-Sarra E, Garcia-Fernandez DA, et al. The enhancement of osteogenesis through the use of dental pulp pluripotent stem cells in 3D. Bone. 2012;50:930-41.

[6] Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. Journal of dental research. 2002;81:531-5.

[7] Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, et al. N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem cells. 2007;25:197-202.

[8] Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Molecular therapy : the journal of the American Society of Gene Therapy. 2004;9:747-56.

[9] Aldahmash A, Haack-Sorensen M, Al-Nbaheen M, Harkness L, Abdallah BM, Kassem M. Human serum is as efficient as fetal bovine serum in supporting proliferation and differentiation of human multipotent stromal (mesenchymal) stem cells in vitro and in vivo. Stem cell reviews. 2011;7:860-8.

[10] Lindroos B, Boucher S, Chase L, Kuokkanen H, Huhtala H, Haataja R, et al. Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy. 2009;11:958-72.

[11] Khanna-Jain R, Vanhatupa S, Vuorinen A, Sandor G, Suuronen R, Mannerstrom B, et al. Growth and Differentiation of Human Dental Pulp Stem Cells Maintained in Fetal Bovine Serum, Human Serum and Serum-free/Xeno-free Culture Media. Journal of Stem Cell Research & Therapy. 2012;2.

[12] Lu J, Hou R, Booth CJ, Yang SH, Snyder M. Defined culture conditions of human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2006;103:5688-93.

[13] Martínez-Sarrà E. Characterization of Dental Pulp Pluripotent-like Stem Cells (DPPSC) and their mesodermal differentiation potential. Barcelona: Universitat Internacional de Catalunya; 2017.

[14] Jones JR. Review of bioactive glass: From Hench to hybrids. Acta Biomaterialia. 2013;9:4457-86.

[15] Rahaman MN, Day DE, Sonny Bal B, Fu Q, Jung SB, Bonewald LF, et al. Bioactive glass in tissue engineering. Acta Biomaterialia. 2011;7:2355-73.

[16] Will J, Gerhardt LC, Boccaccini AR. Bioactive glass-based scaffolds for bone tissue engineering. Advances in biochemical engineering/biotechnology. 2012;126:195-226.

[17] Hoppe A, Guldal NS, Boccaccini AR. A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics. Biomaterials. 2011;32:2757-74.

[18] Hench LL. The story of Bioglass®. Journal of Materials Science: Materials in Medicine. 2006;17:967-78.

[19] Hench LL. Bioceramics. Journal of the American Ceramic Society. 1998;81:1705-28.

[20] Tsigkou O, Jones JR, Polak JM, Stevens MM. Differentiation of fetal osteoblasts and formation of mineralized bone nodules by 45S5 Bioglass® conditioned medium in the absence of osteogenic supplements. Biomaterials. 2009;30:3542-50.

[21] Gorustovich AA, Roether JA, Boccaccini AR. Effect of bioactive glasses on angiogenesis: a review of in vitro and in vivo evidences. Tissue engineering Part B, Reviews. 2010;16:199-207.

[22] Drago L, De Vecchi E, Bortolin M, Toscano M, Mattina R, Romano CL. Antimicrobial activity and resistance selection of different bioglass S53P4

formulations against multidrug resistant strains. Future microbiology. 2015;10:1293-9.

[23] Rahaman MN, Day DE, Bal BS, Fu Q, Jung SB, Bonewald LF, et al. Bioactive glass in tissue engineering. Acta biomaterialia. 2011;7:2355-73.

[24] Carmeliet P. Manipulating angiogenesis in medicine. Journal of internal medicine. 2004;255:538-61.

[25] Lindfors NC, Hyvonen P, Nyyssonen M, Kirjavainen M, Kankare J, Gullichsen E, et al. Bioactive glass S53P4 as bone graft substitute in treatment of osteomyelitis. Bone. 2010;47:212-8.

[26] Ojansivu M, Vanhatupa S, Bjorkvik L, Hakkanen H, Kellomaki M, Autio R, et al. Bioactive glass ions as strong enhancers of osteogenic differentiation in human adipose stem cells. Acta Biomater. 2015;21:190-203.

[27] Waselau M, Patrikoski M, Juntunen M, Kujala K, Kääriäinen M, Kuokkanen H, et al. Effects of bioactive glass S53P4 or beta-tricalcium phosphate and bone morphogenetic protein-2 and bone morphogenetic protein-7 on osteogenic differentiation of human adipose stem cells. Journal of Tissue Engineering. 2012;3:2041731412467789.

[28] Unger RE, Dohle E, Kirkpatrick CJ. Improving vascularization of engineered bone through the generation of pro-angiogenic effects in co-culture systems. Advanced drug delivery reviews. 2015;94:116-25.

[29] Jin G-Z, Han C-M, Kim H-W. In vitro co-culture strategies to prevascularization for bone regeneration: A brief update. Tissue Engineering and Regenerative Medicine. 2015;12:69-79.

[30] Rouwkema J, Rivron NC, van Blitterswijk CA. Vascularization in tissue engineering. Trends in biotechnology. 2008;26:434-41.

[31] Weinandy S, Laffar S, Unger RE, Flanagan TC, Loesel R, Kirkpatrick CJ, et al. Biofunctionalized microfiber-assisted formation of intrinsic three-dimensional capillary-like structures. Tissue engineering Part A. 2014;20:1858-69.

[32] Montano I, Schiestl C, Schneider J, Pontiggia L, Luginbuhl J, Biedermann T, et al. Formation of human capillaries in vitro: the engineering of prevascularized matrices. Tissue engineering Part A. 2010;16:269-82.

[33] Fuchs S, Hofmann A, Kirkpatrick C. Microvessel-like structures from outgrowth endothelial cells from human peripheral blood in 2-dimensional and 3-dimensional co-cultures with osteoblastic lineage cells. Tissue engineering. 2007;13:2577-88.

[34] Grellier M, Ferreira-Tojais N, Bourget C, Bareille R, Guillemot F, Amedee J. Role of vascular endothelial growth factor in the communication between human

osteoprogenitors and endothelial cells. Journal of cellular biochemistry. 2009;106:390-8.

[35] Dariima T, Jin G-Z, Lee E-J, Wall IB, Kim H-W. Cooperation between osteoblastic cells and endothelial cells enhances their phenotypic responses and improves osteoblast function. Biotechnology Letters. 2013;35:1135-43.

[36] Grellier M, Bordenave L, Amedee J. Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. Trends in biotechnology. 2009;27:562-71.

[37] Chan BP, Hui TY, Yeung CW, Li J, Mo I, Chan GCF. Self-assembled collagen–human mesenchymal stem cell microspheres for regenerative medicine. Biomaterials. 2007;28:4652-66.

[38] Coelho MJ, Cabral AT, Fernande MH. Human bone cell cultures in biocompatibility testing. Part I: osteoblastic differentiation of serially passaged human bone marrow cells cultured in alpha-MEM and in DMEM. Biomaterials. 2000;21:1087-94.

[39] Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. The Journal of clinical investigation. 1973;52:2745-56.

[40] Fuchs S, Dohle E, Kolbe M, Kirkpatrick CJ. Outgrowth endothelial cells: sources, characteristics and potential applications in tissue engineering and regenerative medicine. Advances in biochemical engineering/biotechnology. 2010;123:201-17.

[41] Tao J, Sun Y, Wang Q, Liu C. Induced Endothelial Cells Enhance Osteogenesis and Vascularization of Mesenchymal Stem Cells. Cells Tissues Organs. 2009;190:185-93.

[42] Papadimitropoulos A, Scherberich A, Guven S, Theilgaard N, Crooijmans HJ, Santini F, et al. A 3D in vitro bone organ model using human progenitor cells. European cells & materials. 2011;21:445-58; discussion 58.

[43] Liu J, Chen W, Zhao Z, Xu HHK. Reprogramming of mesenchymal stem cells derived from iPSCs seeded on biofunctionalized calcium phosphate scaffold for bone engineering. Biomaterials. 2013;34:7862-72.

[44] Levenberg S. Engineering blood vessels from stem cells: recent advances and applications. Current Opinion in Biotechnology. 2005;16:516-23.

[45] Ma J, van den Beucken JJ, Yang F, Both SK, Cui FZ, Pan J, et al. Coculture of osteoblasts and endothelial cells: optimization of culture medium and cell ratio. Tissue engineering Part C, Methods. 2011;17:349-57.

[46] Liu Y, Chan JK, Teoh SH. Review of vascularised bone tissue-engineering strategies with a focus on co-culture systems. Journal of tissue engineering and regenerative medicine. 2015;9:85-105.

[47] Núñez-Toldrà R, Dosta P, Montori S, Ramos V, Atari M, Borrós S. Improvement of osteogenesis in dental pulp pluripotent-like stem cells by oligopeptide-modified poly(ß-amino ester)s. Acta Biomaterialia. 2017.

[48] Pisciotta A, Riccio M, Carnevale G, Beretti F, Gibellini L, Maraldi T, et al. Human serum promotes osteogenic differentiation of human dental pulp stem cells in vitro and in vivo. PloS one. 2012;7:e50542.

[49] Thomson M, Liu SJ, Zou LN, Smith Z, Meissner A, Ramanathan S. Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell. 2011;145:875-89.

[50] Gong W, Huang Z, Dong Y, Gan Y, Li S, Gao X, et al. Ionic Extraction of a Novel Nano-sized Bioactive Glass Enhances Differentiation and Mineralization of Human Dental Pulp Cells. Journal of Endodontics.40:83-8.

[51] Varanasi VG, Owyoung JB, Saiz E, Marshall SJ, Marshall GW, Loomer PM. The ionic products of bioactive glass particle dissolution enhance periodontal ligament fibroblast osteocalcin expression and enhance early mineralized tissue development. Journal of Biomedical Materials Research Part A. 2011;98A:177-84.

[52] Varanasi VG, Saiz E, Loomer PM, Ancheta B, Uritani N, Ho SP, et al. Enhanced osteocalcin expression by osteoblast-like cells (MC3T3-E1) exposed to bioactive coating glass (SiO2–CaO–P2O5–MgO–K2O–Na2O system) ions. Acta Biomaterialia. 2009;5:3536-47.

[53] Xynos ID, Edgar AJ, Buttery LDK, Hench LL, Polak JM. Ionic Products of Bioactive Glass Dissolution Increase Proliferation of Human Osteoblasts and Induce Insulin-like Growth Factor II mRNA Expression and Protein Synthesis. Biochemical and biophysical research communications. 2000;276:461-5.

[54] Alcaide M, Portolés P, López-Noriega A, Arcos D, Vallet-Regí M, Portolés MT. Interaction of an ordered mesoporous bioactive glass with osteoblasts, fibroblasts and lymphocytes, demonstrating its biocompatibility as a potential bone graft material. Acta Biomaterialia. 2010;6:892-9.

[55] Andrade AL, Andrade SP, Domingues RZ. In vivo performance of a sol-gel glass-coated collagen. Journal of biomedical materials research Part B, Applied biomaterials. 2006;79:122-8.

[56] Day RM, Boccaccini AR, Shurey S, Roether JA, Forbes A, Hench LL, et al. Assessment of polyglycolic acid mesh and bioactive glass for soft-tissue engineering scaffolds. Biomaterials. 2004;25:5857-66.

[57] Rivron NC, Liu JJ, Rouwkema J, de Boer J, van Blitterswijk CA. Engineering vascularised tissues in vitro. European cells & materials. 2008;15:27-40.

[58] Bouletreau PJ, Warren SM, Spector JA, Peled ZM, Gerrets RP, Greenwald JA, et al. Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. Plastic and reconstructive surgery. 2002;109:2384-97.

[59] Tsuboi R, Sato Y, Rifkin DB. Correlation of cell migration, cell invasion, receptor number, proteinase production, and basic fibroblast growth factor levels in endothelial cells. The Journal of cell biology. 1990;110:511-7.

[60] Veillette CJ, von Schroeder HP. Endothelin-1 down-regulates the expression of vascular endothelial growth factor-A associated with osteoprogenitor proliferation and differentiation. Bone. 2004;34:288-96.

[61] Fiedler J, Brill C, Blum WF, Brenner RE. IGF-I and IGF-II stimulate directed cell migration of bone-marrow-derived human mesenchymal progenitor cells. Biochemical and biophysical research communications. 2006;345:1177-83.

[62] Clarkin CE, Emery RJ, Pitsillides AA, Wheeler-Jones CP. Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-mediated crosstalk to endothelial cells. Journal of cellular physiology. 2008;214:537-44.

[63] Kolbe M, Xiang Z, Dohle E, Tonak M, Kirkpatrick CJ, Fuchs S. Paracrine effects influenced by cell culture medium and consequences on microvessel-like structures in cocultures of mesenchymal stem cells and outgrowth endothelial cells. Tissue engineering Part A. 2011;17:2199-212.

[64] Dariima T, Jin GZ, Lee EJ, Wall IB, Kim HW. Cooperation between osteoblastic cells and endothelial cells enhances their phenotypic responses and improves osteoblast function. Biotechnology letters. 2013;35:1135-43.

[65] Guillotin B, Bareille R, Bourget C, Bordenave L, Amédée J. Interaction between human umbilical vein endothelial cells and human osteoprogenitors triggers pleiotropic effect that may support osteoblastic function. Bone. 2008;42:1080-91.

[66] Kang Y, Kim S, Fahrenholtz M, Khademhosseini A, Yang Y. Osteogenic and angiogenic potentials of monocultured and co-cultured human-bone-marrow-derived mesenchymal stem cells and human-umbilical-vein endothelial cells on three-dimensional porous beta-tricalcium phosphate scaffold. Acta Biomaterialia. 2013;9:4906-15.

[67] Rivron NC, Raiss CC, Liu J, Nandakumar A, Sticht C, Gretz N, et al. Sonic Hedgehog-activated engineered blood vessels enhance bone tissue formation. Proc Natl Acad Sci U S A. 2012;109:4413-8.

[68] Fuchs S, Jiang X, Schmidt H, Dohle E, Ghanaati S, Orth C, et al. Dynamic processes involved in the pre-vascularization of silk fibroin constructs for bone regeneration using outgrowth endothelial cells. Biomaterials. 2009;30:1329-38.

[69] Dissanayaka WL, Hargreaves KM, Jin L, Samaranayake LP, Zhang C. The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo. Tissue engineering Part A. 2015;21:550-63.

CHAPTER 7:
GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 GENERAL CONCLUSIONS

DPPSC for bone regeneration and biomaterials evaluation:

1. DPPSC and DPMSC are different populations obtained from the same tissue origin, the dental pulp of the third molars. However, DPPSC are more favorable to differentiate toward chondrogenic and osteogenic lineage, whereas DPMSC seem to have more adipogenic potential.

2. DPPSC in osteogenic medium can differentiate into bone – like tissue *in vitro* expressing typical osteogenic markers and exhibiting high matrix mineralization and alkaline phosphatase activity.

3. DPPSC can differentiate into bone – like tissue showing high osteogenic potential and adhesion capacity on different biomaterials, such as metals (Ti alloy disks) or natural scaffolds (CCC).

4. DPPSC do not show chromosomal alterations, neither during culture expansion nor during osteogenic differentiation on culture plates or biomaterials.

Improvement of osteogenesis in DPPSC by oligopeptide-modified poly (β-amino ester)s:

5. Arginine/aspartic acid- modified pBAEs show high cell-specificity and transfection efficiency for co-deliver siRNAs and plasmids in DPPSC.

6. The silencing of pluripotent genes (OCT3/4, NANOG) at day 7 of DPPSC osteogenic differentiation enhances the expression level of the osteogenic markers at the end of the differentiation process.

7. The delivery of anti-OCT3/4 siRNA in combination with RUNX2 plasmid in DPPSC accelerates the expression of key osteogenic markers, which increases the functional quality of differentiated cells producing higher matrix mineralization and ALP activity.

8. The double pRUNX2-siOCT3/4 transfection by means of oligopeptide modified pBAEs does not induce chromosomal instability or negative impact in DPPSC viability during osteogenic differentiation.

Vascularized Bone Tissue Engineering by DPPSC co-culture systems:

9. DPPSC can differentiate toward osteogenic and endothelial lineage in xeno-free media supplemented with human serum.

10. S53P4 BaG extracts enhance the osteogenic and endothelial differentiation process in DPPSC, supporting the formation of vascular-like structures in DPPSC co-cultures.

11. The indirect co-culture of bone-like DPPSC, either with HUVEC or endothelial-like DPPSC enhances the quality of the osteogenic function under basal or osteogenic medium conditions.

12. The direct co-culture of bone-like DPPSC with HUVEC in endothelial or mixture medium, promotes both endothelial and osteogenic differentiations. Moreover, HUVEC are able to form microvessel-like structures through bone-like DPPSC.

13. The direct co-culture of bone-like DPPSC with endothelial-like DPPSC in endothelial medium conditioned with BaG extracts enhances the expression of endothelial markers and the formation of angiogenic structures, correlating with a higher release of angiogenic factors such as VEGF. At the same time, these medium conditions can also promote the osteogenic differentiation and the mineralization of DPPSC.

14. The direct co-culture of bone-like DPPSC with endothelial-like DPPSC within a peptide-hydrogel in endothelial medium conditioned with BaG extracts, allows cell-to-cell communication and cell migration enhancing the formation of vascular-like structures that appeared to be functional as indicated by the presence of an internal lumen.

7.2 FUTURE PERSPECTIVES

The results obtained in the present doctoral thesis propose the use of DPPSC for different BTE approaches: to evaluate the biological properties of biomaterials before *in vivo* applications, to enhance bone regeneration by non-viral gene therapy methods and to promote vascularization in osteogenic constructs by different co-culture systems. However, further studies testing the *in vivo* biocompatibility of these approaches will be necessary to confirm their potential application in BTE.

Regarding the study of p(BAE)s transfections, results report a rapid and simple strategy to direct osteogenic differentiation of DPPSC, that may have promising applications in bone repair. Nevertheless, it will be necessary to demonstrate its suitability and efficacy *in vivo*. The clinical translation of such approach may require the development of new ways of administering both DPPSC and nucleic acids in a controlled manner. This could be performed by an *ex vivo* treatment: the isolation of DPPSC from the dental pulp of patients followed by 7 days of differentiation and an appropriate transfection to adjust the expression of selected transcription factors. Such bone material may be grown on pre-designed scaffolds, which shall be later implanted in patients to correct bone defects. This strategy will allow a better quality control of the transformed cells, which might increase safety. Another approach would be the encapsulation of DPPSC within an injectable hydrogel which could incorporate nanoparticles containing the appropriate nucleic acids that could guide the differentiation of the stem cells. Although this situation may simplify the procedure it would be less controllable.

Another clinically relevant aspect that needs to be tackled is the lack of vascularization in BTE. The co-culture of pre-differentiated DPPSC combined with the effect of BaG in xeno-free medium conditions provides a promising system for the *in vitro* vascularization of BTE constructs. Nevertheless, further studies need to be done to optimize these co-culture systems and to demonstrate their efficacy during bone healing process *in vivo* with the anastomosis of the pre-formed microcapillaries to the host vasculature. First of all, it would be important to identify the endothelial cells and the osteoblastic

cells in the 3D co-cultures in order to corroborate the formation of vascular structures. Hence, it would be important to optimize the IF protocol in these scaffolds to use different antibodies against endothelial and osteogenic markers. Other options could be the previous transfection of the endothelial cells by a vector containing GFP or the use of other scaffolds more appropriates for these analyses, such as microspheres. Moreover, it would be of great benefit to fabricate polymeric scaffolds containing BaG that could allow the seeding of cells on them and the controllable release of the BaG ions in order to enhance the osteogenic and the endothelial potential of the co-culture system prior and after their implantation in the defect site. Finally, it would be also important to check the genetic stability of DPPSC in these co-culture systems *in vitro.*

APPENDIX - SUPPLEMENTARY DATA

APPENDIX

SUPPLEMENTARY TABLES AND FIGURES

DONOR	GENDER	AGE (years)	MOLAR	DATE of EXTRACTION
1	XY	16	18,28	27/04/2012
2	XY	16	38,48	27/04/2012
3	XX	14	38,48	18/10/2013
4	XY	16	38,48	25/10/2013
5	XX	15	38,48	10/01/2014
6	XX	15	38	10/01/2014
7	XY	21	38	06/02/2015
8	XX	17	38	01/12/2015
9	XY	17	38	17/06/2016
10	XX	18	48	17/06/2016

Table S1: Clinical information on the patients and third molars used in this study.

APPENDIX

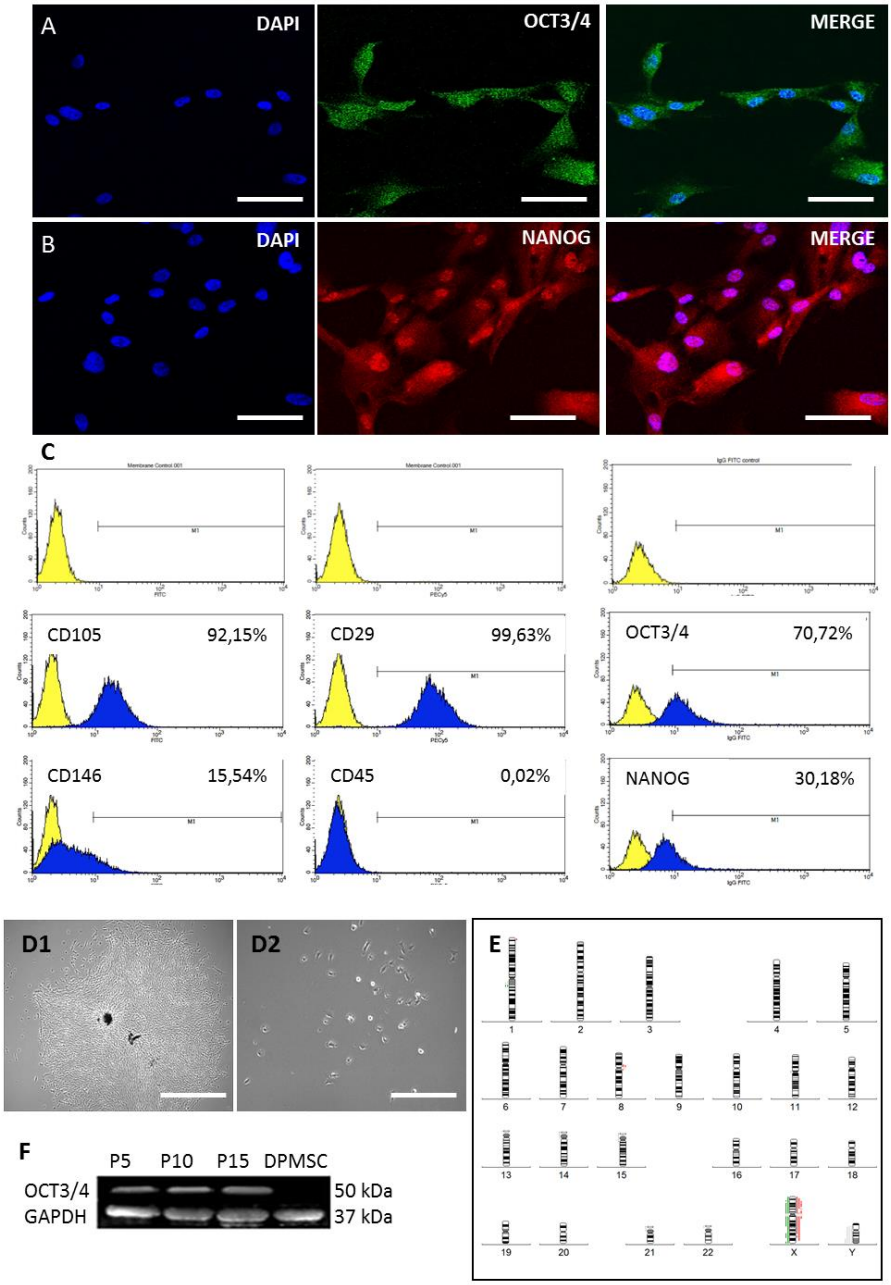


Figure S1. Characterization of undifferentiated DPPSC. A-B). IF analyses of the pluripotency markers **(A)** OCT3/4 (in green) and **(B)** NANOG (in red) in undifferentiated DPPSC (passage 10). DAPI (in blue) was used as a nucleus control in both analyses. Scale

bars: 50 µm. **C1)** FACS analysis of membrane markers: CD105 (92.15%), CD29 (99.63%), CD146 (15.54%) and CD45 (0.04%) in DPPSC. **C2)** FACS analysis of pluripotency nuclear markers: OCT3/4 (76.72%) and NANOG (30.18%) in DPPSC. **D1-2)** Cell morphology of DPPSC observed with optical microscopy. **D1)** DPPSC clone in a primary culture. **D2)** DPPSC after 3 culture passages. DPPSC are characterized as small-sized cells with large nuclei and low cytoplasm content. Scale bars: 200 µm. **E)** Genetic stability in DPPSC during culture expansion. Short-CGH summary of DPPSC at passages 5,10 and 15 (N=6). **F)** Pluripotency expression in DPPSC until passage 15 by Western blot analysis of OCT3/4 expression; DPMSC was used as a negative control and GAPDH as a housekeeping control.

APPENDIX

ETHICS COMITEE APPROVAL

CER Comitè d'Ètica de Recerca | Universitat Internacional de Catalunya

CARTA APROVACIÓ PROJECTE PEL CER

Codi de l'estudI: BIO-ELB-2013-03
Versió del protocol: 1.1
Data de la versió: 21/11/13
Títol: "Determinación del protocolo idóneo de diferenciación osteogénica de las DPPSC para la evaluación de distintos biomateriales"

Sant Cugat del Vallès, 25 de novembre de 2013

Investigadora: Raquel Núñez Toldrà

Títol de l'estudi: "Determinación del protocolo idóneo de diferenciación osteogénica de las DPPSC para la evaluación de distintos biomateriales"

Benvolgut(da),

Valorat el projecte presentat, el CER de la Universitat Internacional de Catalunya, considera que, des del punt de vista ètic, reuneix els criteris exigits per aquesta institució i, per tant, ha

RESOLT FAVORABLEMENT

emetre aquest CERTIFICAT D'APROVACIÓ per part del Comitè d'Ètica de la Recerca, per que pugui ser presentat a les instàncies que així ho requereixin.

Em permeto recordar-li que si en el procés d'execució es produís algun canvi significatiu en els seus plantejaments, hauria de ser sotmès novament a la revisió i aprovació del CER.

Atentament,

Dr. Josep Argemí
President CER-UIC

APPENDIX

STAGE CERTIFICATE

UNIVERSITY OF TAMPERE

FACULTY OF MEDICINE AND LIFE SCIENCES

1/1

CERTIFICATE OF ATTENDANCE

Tampere, 28.2.2017

To whom it my concern,

With this letter I certify that Raquel Núñez Toldrà, from Universitat Internacional de Catalunya, has joined the Adult Stem Cell Group at the BioMediTech Institute, University of Tampere to carry out a PhD stage between 15.02.2016 and 15.05.2016.

Particularly, the purpose of her stay at BioMediTech has been to study the osteogenic and angiogenic differentiation potential of dental pulp pluripotent-like stem cells (DPPSC) in bone repair, using different 2D and 3D co-culture systems.

Sincerely,

Susanna Miettinen
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