Universidade do Minho
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CO-CULTURES AND CELL SHEET ENGINEERING AS RELEVANT TOOLS TO IMPROVE THE OUTCOME OF BONE TISSUE ENGINEERING STRATEGIES

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Tese de Doutoramento em Engenharia de Tecidos,
Medicina Regenerativa e Células Estaminais

Trabalho efectuado sob a orientação do
Professor Rui L. Reis
e da
Doutora Alexandra P. Marques

Junho de 2011

“It’s been a long and lonely trip but I'm glad I took it because it was well worth it. I got to read a couple of books and do some research before I reached my verdict. Never thought that I was perfect. Always thought that I had a purpose.”

Sage Francis

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I would to begin by acknowledging Professor Rui Reis for the opportunity that he has given me to develop my work at the 3B's Research group. Besides his obvious scientific input he has been an example for me to push myself further and to always look at scientific excellence despite the circumstances. More importantly, he has showed me how hard work is the best weapon to fight for national and international recognition. His contribution was essential in my growth, both scientific and as a person.

To Doctor Alexandra Marques, the most powerful driving force behind the conclusion of my PhD. I am truly thankful for all the time you lost teaching and correcting me, fighting for me and for my work and many other things that I should mention but have no space. All of this work has as much of your sweat and tears as of my own. There are no words that can express my gratitude for that. It is an honor that I've been supervised by someone of your talent. This thesis is dedicated to you.

I would also like to acknowledge Professor Teruo Okano, for allowing me to work at the Institute of Advanced Biomedical Engineering and Science, in Tokyo. It is truly a privilege the time that I spent learning from you and your staff. I was, and still am, truly impressed with how your scientific motivation was rooted on your concern and urgency to help suffering people. Professor Masayuki Yamato also deserves to be mentioned here for all of his care in making sure I had everything I needed in the lab and outside the lab. You are a good friend and one of my most important examples as a researcher.

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To everyone in the 3B's that helped me directly in my work or just as friends, a big thank you. Many of you have left, some of you are still in the lab. But I will remember you all.

To the ladies with whom I share my daily trips to the lab, I would like to acknowledge you for also sharing many of my problems.

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The decisive contribution of my family and friends (at Porto, at Boca and elsewhere) to the conclusion of my work also needs to be acknowledged.

Finally, I want to acknowledge my first and permanent mentor, Carl Sagan.

ABSTRACT

Taking into consideration the complex biology of bone tissue it is quite clear that the understanding of the cellular interactions that regulate the homeostasis and regeneration of this remarkable tissue is essential for a successful Tissue Engineering strategy. The *in vitro* study of these cellular interactions relies on co-culture systems, a tremendously useful methodology where two or more cell types are cultured at the same time. Such strategy increases the complexity of typical monoculture systems, allowing the *in vitro* settings to closely mimic the *in vivo* environment. Moreover, 2D co-culture systems have been extensively used by cell biologists to study cell interactions as an attempt to understand specific cellular mechanisms and signalling pathways. The interaction between osteoblasts/ osteoprogenitor cells and different cell types relevant within the bone Tissue Engineering context, namely mononuclear cells from peripheral blood and umbilical cord blood and fibroblasts, has been addressed in the first part of this thesis. The different co-cultures showed that mononuclear cells from peripheral blood were capable of accelerating the osteogenic differentiation of human bone marrow stromal cells by producing BMP-2. On the other hand, osteoblasts cultured on carrageenan membranes were capable of supporting the culture of endothelial progenitors cells present in the mononuclear fraction of umbilical cord blood that contributed to the *in vivo* angiogenesis after implantation in an inflammatory setting. Furthermore, fibroblasts, which are key players in the formation of fibrotic tissue after a biomaterial implantation, were shown to decrease the osteogenic activity of osteoblasts through gap junctional communication.

A serious limitation of the paradigmatic use of scaffolds for bone Tissue Engineering is the lack of oxygen and nutrient supply to the cells in the core of the engineered construct leading to cell necrosis at the bulk of the constructs. Furthermore, foreign body response to the implanted biomaterial is a frequent reaction of the host and has

as a consequence the formation of fibrotic tissue surrounding the implant. The use of cell sheet engineering for bone Tissue Engineering can potentially avoid those shortcomings. One of the explored strategies comprised the production of osteogenic cell sheets using this technology. Its potential for *in vivo* bone formation was analyzed and the formation of vascularized bone tissue with a marrow was originally demonstrated by implanting a single osteogenic cell sheet in a nude mice model. Furthermore, in order to promote vascularization, co-cultured osteogenic cell sheets with endothelial cells were also created. Endothelial cells, stacked in between osteogenic cell sheets, were proven to contribute to new vessel formation and increased bone formation *in vivo*

This thesis demonstrates that monocytes/macrophages from peripheral blood can accelerate the osteogenic differentiation of osteoprogenitor cells while fibroblasts, have a deleterious effect on the osteogenic phenotype of osteoblasts. In addition, within an inflammatory host reaction, cells from the mononuclear fraction of umbilical cord blood were capable of contributing to new blood vessel formation after co-culture with osteoblasts. Moreover, when using the cell sheet technology to fabricate a bone tissue engineering construct, endothelial cells were also shown to improve *in vivo* bone formation.

RESUMO

Considerando a complexa biologia do tecido ósseo torna-se claro que compreender as interacções celulares que regulam a homeostasia e regeneração deste tecido notável, é de extrema importância numa estratégia de Engenharia de Tecidos. O estudo *in vitro* destas interacções celulares baseia-se em sistemas de co-cultura, uma metodologia de grande utilidade onde dois ou mais tipos de células diferentes são cultivados simultaneamente e no mesmo espaço. Estes sistemas representam um aumento de complexidade, em relação aos sistemas de monocultura, que permite que as condições *in vitro* mimetizem melhor o ambiente *in vivo*. Além disso, sistemas de cultura 2D têm sido muito utilizados por biólogos no estudo de interacções celulares numa tentativa de compreender os mecanismos e vias de sinalização envolvidas. A interacção entre osteoblastos ou células osteoprogenitoras com células mononucleares do sangue periférico e do cordão umbilical, e com fibroblastos para aplicações em Engenharia de Tecido Ósseo foi estudada na primeira parte desta tese. As diferentes co-culturas permitiram mostrar que as células mononucleares do sangue periférico são capazes de acelerar a diferenciação osteogénica de células do estroma de medula óssea humana através da produção de proteína morfogenética do osso 2 (*BMP-2*). Por outro lado, foi também demonstrado que osteoblastos cultivados em membranas de carragenano suportam a cultura de células progenitoras endoteliais presentes na fracção mononuclear de sangue do cordão umbilical humano e que estas, por sua vez, contribuem para a angiogénese *in vivo* e em condições inflamatórias. Além disso, foi mostrado que fibroblastos, células-chave na deposição de tecido fibrótico após a implantação de um biomaterial, inibem a actividade osteogénica de osteoblastos através da comunicação por *Gap Junctions*.

A utilização paradigmática de matrizes de suporte para a Engenharia de Tecido Óssea tem como limitação grave a falha de fornecimento de oxigénio e nutrientes às células no interior da matriz construída levando à necrose dessas mesmas células. Além

disso, a resposta do hospedeiro à implantação de corpos estranhos, como matrizes tri-dimensionais, tem como consequência frequente a formação de tecido fibrótico à volta do implante. A aplicação da Engenharia de *cell sheets* na Engenharia de Tecido ósseo tem a expectativa de ultrapassar estas limitações. Uma das estratégias exploradas teve como base a produção de *cell sheets* osteogénicas utilizando esta tecnologia. O potencial destas *cell sheets* para induzir a formação de osso foi analisada *in vivo* tendo sido demonstrada a formação de osso vascularizado com uma estrutura medular organizada após a implantação de uma única *cell sheet* num modelo de ratinho nude. Além disso, numa tentativa de promover a vascularização, foram criadas *cell sheets* osteogénicas co-cultivadas com células endoteliais. Estas, quando cultivadas entre *cell sheets* osteogénicas sobrepostas, contribuíram para a formação de novos vasos *in vivo* bem como para o aumento da formação de novo osso.

Esta tese demonstra que monócitos/macrófagos do sangue periférico podem acelerar a diferenciação osteogénica de progenitores osteogénicos enquanto os fibroblastos exercem um efeito negativo no fenótipo osteogénico de osteoblastos. Mais ainda, no contexto de uma reacção inflamatória do hospedeiro, ficou comprovado que células da fracção mononuclear do sangue do cordão umbilical participam na formação de novos vasos após co-cultura com osteoblastos. Além disso, as células endoteliais adultas, quando combinadas com *cell sheets* osteogénicas, promovem a formação de osso *in vivo*.

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SHORT *CURRICULUM VITAE*

Rogério Pedro Lemos de Sousa Pirraco was born in 1982 in Porto, Portugal. At the present he lives in Porto and is a PhD student in the 3B's Research Group (Biomaterials, Biodegradables and Biomimetics) of University of Minho, located in Caldas das Taipas. This is a research unit of Excellence integrated in the University of Minho, directly funded by the Portuguese Foundation for Science and Technology (FCT) and part of the Portuguese Government Associated Laboratory ICVS/3B's. Regarding his education background, in 2005 Rogério Pirraco concluded his four years graduation in Applied Biology at the University of Minho, Portugal. Rogério has started his contact with research during the last year of his graduation, when he developed his training period in the 3B's Research Group in the area of cell co-culture models for bone and cartilage Tissue Engineering. In 2006, he was admitted as a PhD student in the 3B's Research Group of the School of Engineering of the University of Minho, where he developed his work concerning co-cultures of cells for Tissue Engineering purposes, cell sheet engineering using thermally responsive surfaces and stem cell culture. During this time, he spent two periods of 6 months in Professor Teruo Okano's Institute of Advanced Biomedical Engineering and Science of the Tokyo's Women Medical University in Tokyo, Japan, where he acquired knowledge in cell sheet development and manipulation and its application in animal models. His know-how on animal models and animal experimentation was further consolidated by attending an Animal experimentation course certified by FELASA (Level C) and by Portuguese Regulatory Veterinary Entity (Direcção Geral de Veterinária). Rogério Pirraco was also a part of the organizing committee of the European chapter meeting of TERMIS-EU 2008, held in Porto, Portugal. As a result of his work, Rogério has attended the most important international meetings in the Tissue Engineering field both with posters and oral communications. He has published several works in refereed journals, in books and in international conference proceedings. In 2007, in the meeting of the European

chapter of the Tissue Engineering and Regenerative Medicine International Society (TERMIS), held in London, he received the prize for “Outstanding Student Contribution”. Since the beginning of 2010 he is the chair of the Students and Young Investigators Section of the European Chapter of TERMIS (TERMIS-SYIS EU).

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Chapter 2

Materials and Methods

LIST OF ABBREVIATIONS

A

A/b Antibiotic/antimicotic
AcLDL Acetylated low density lipoprotein
AG 3,6-anhydro-D-galactose
AGA 18-alpha-Glycyrrhetinic acid
ALP Alakline Phosphatase
APC Allophycocyanine
ATP Adenosine tri-phosphate
ALP Alkaline Phosphatase

B

BMP-2 Bone Morhopgenetic Protein
BMSCs Bone Marrow Stromal Cells
BMU Bone multicellular units
BSA Bovine Serum Albumin

C

cAMP Cyclic adenosine monophosphate
CBMNCs Cord Blood Mononuclear Cells
CPDA Citrate phosphate dextrose adenine
CRG Carrageenan
CS Cell Sheet
CVCs Calcifying vascular cells

D

DAB 3,3'-diaminobenzidine
DAPI 4',6-diamidino-2-phenylindole
DiI 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
DMEM Dulbecco's Modified Eagle's Medium
DMSO Dimethylsulfoxide

E

EB Electron beam
ECs Endothelial cells
ECGS Endothelial Cell Growth Supplement
ECH Epichlorohydrin
ECM Extracellular matrix
EPC Endothelial progenitor cell
ESCs Embryonic Stem Cells

F

FBS Fetal Bovine Serum
FGF Fibroblasts growth factor
FITC Fluorescein Isothiocyanate
FSP Fibroblast Surface Protein

G

GAG Glycosaminoglycans
GJ Gap junction
GjC Gap junctional Communication

H

HA Hydroxyapatite
hASCs Human adipose derived stem cells
hBMSCs Human bone marrow stromal cells
HCl Hydrogen Chloride
hDFb Human dermal fibroblasts
H&E Hematoxilin and Eosin
hDMECs Human dermal microvascular cells
hMSCs Human mesenchymal stem cells
hESCs Embryonic Stem Cells
hOB Human osteoblasts
hOEC Human outgrowth endothelial cells
hPBMNCs Human peripheral blood mononuclear cells
HRP Horseradish peroxidase
HUVECs Human umbilical vein endothelial cells

I

IP3 Inositol triphosphate
IPAAm Isopropylacrylamide

K

KCl Potassium chloride
KOH Potassium hydroxide

L

LCST Lower critical solution temperature

M

MEM Minimal Essential médium
MN Mononuclear
MNC Mononuclear cells
MSCs Mesenchymal Stem Cells

N

NaOH Sodium hydroxide

NiTi Nickel-titanium

P

PBS Phosphate Buffered Saline

PCL Poly(ε-caprolactone)

PCR Polymerase chain reaction

PE Phycoerythrin

PDGF Platelet-derived growth factor

PLGA Poly-lactic-glycolic acid

PLGA-BG Polylactide-co-glycolide and bioactive glass

PIPAAM Poly(N-isopropylacrylamide)

PU Polyurethane

PVDF Poly(vinylidene difluoride)

Q

qRT-PCR Quantitative Real-time PCR

R

RTU Ready to use

S

SCs Stem Cells

SPCL Starch-poly(ε-caprolactone)

STRO-1 Stromal Cell Precursor Surface Antigen-1;

T

TCPS Tissue culture polystyrene

TE Tissue Engineering

TERM Tissue Emgineering and Regenerative Medicine

TGF Transforming growth factor

TNF Tumor necrosis factor

U

UCB Umbilical cord blood

V

VEGF Vascular Endothelial Growth Factor

vWF von Willebrand factor

X

XPS x-ray photoelectron spectroscopy

INTRODUCTION TO THE STRUCTURE OF THE THESIS

The whole strategy of this PhD was based on the assumption that the use and development of co-culturing systems could provide a superior way to understand and to modulate mechanisms related with bone biology. This understanding and the ability to manipulate cell behavior would then have as a result models or tools that could be applied in Bone Tissue Engineering in order to overcome some of the current shortcomings of this field. Since the ultimate aim is related with bone therapies, the connecting thread of the works described in this thesis is osteogenic or osteoprogenitor cells(Ost/OstP). In fact, the interactions of Ost/OstP with different types of cells were addressed in order to be able to take advantage of those interactions to increase the osteogenic potential of Ost/OstP or Ost/OstP-based constructs.

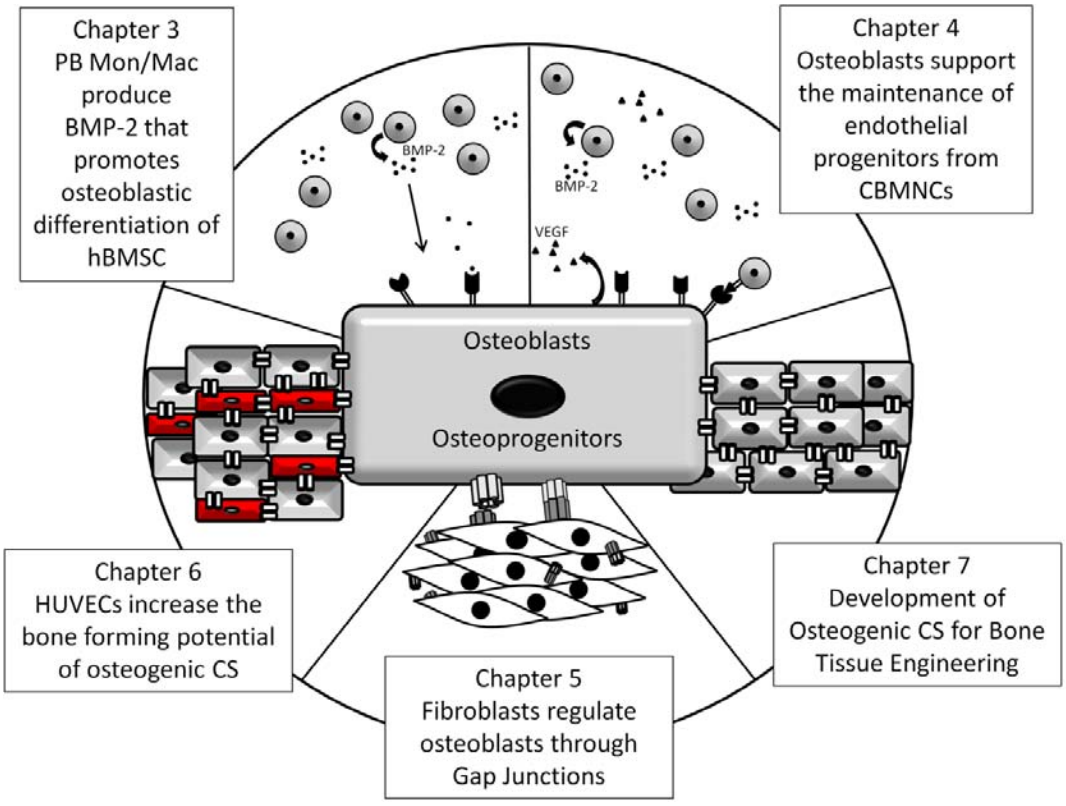

Figure I - Scheme depicting the interactions of osteoblasts or osteoblast progenitors with other cells addressed in the works described in the thesis. *PB: peripheral blood; mon/mac: monocytes/macrophages; BMP-2: bone morphogenetic protein 2; hBMSC: human bone marrow stromal cells; CBMNCs: cord blood mononuclear cells; HUVECs: human umbilical vein endothelial cells; CS: cell sheets; VEGF: vascular endothelial growth factor.*

All together, the defined lines of work, based on four co-culture models and one homotypic model (figure I), were able to provide new insights over different cellular mechanisms that influence the osteogenic process and consequently bone regeneration. Under this context, and aiming at framing the explored approaches, an introduction is provided in Chapter 1.

A detailed description of the materials and methods used along the experimental work is provided in Chapter 2. This chapter intends to present a clear, although detailed and practical presentation of all the techniques used to achieve the reported results. Although Chapter 2 contains some information repeated in the subsequent chapters, it allows for a better comprehension of the overall methodology followed and provides the justification for those options.

The following 5 chapters of the thesis are based on either already published or submitted manuscripts, in the respective formats of the Journals. Each one of them comprises the specific background of the study, a brief description of the methodologies used, and the presentation and discussion of the results.

The first work, described in chapter 3, studied how BMP-2-producing mononuclear cells isolated from human peripheral blood, more exactly monocytes/macrophages, could affect the osteogenic differentiation of human bone marrow stromal cells in a direct co-culture system. The eventual supportive effect of osteoblasts over the survival of endothelial progenitors of the mononuclear fraction from cord blood was verified in chapter 4.

In chapter 5, a direct co-culture model of osteoblasts and fibroblasts was established to characterize the potential interactions between osteoblasts and fibroblasts after the implantation of a tissue-engineered construct. Gap junctional communication between those two types of cells was verified and its effects over osteoblasts behavior were assessed.

Cell sheet engineering is established as a very promising approach for bone regeneration being described in chapter 6, while the final work, presented in chapter 7 takes advantage of the higher complexity provided by co-cultures and combines it with cell sheet engineering to demonstrate how addressing the vascularization issue can further promote the osteogenic potential of cell sheets.

The implications and final remarks of the described works are summarized in the last chapter, Chapter 8.

Chapter 1

BACKGROUND

Chapter 1

BACKGROUND

Chapter based on Pirraco RP, Marques AP, Reis RL Cell interactions in bone tissue engineering. J Cell Mol Med.14:93-102. 2009.

1.1 Introduction

Taking in account the functional importance of bone tissue, one may infer that any bone injury poses a high impact on the quality of life of an individual. In the US alone, there are about 8 million bone fractures/year of which 5-10% represent cases of healing delay or non-union fractures (1-2) that require the enhancement of the bone tissue innate regenerative capacity. In order to tackle these issues, Tissue Engineering (TE) presents itself as a phenomenal tool. TE has been defined(3) as an interdisciplinary area that combines the knowledge of the Engineering and Life Sciences fields for the creation of functional constructs that improve, maintain or restore the function of a given tissue. The current TE paradigm encompasses the application of three basic elements: appropriate cells, a 3D polymeric matrix that supports cell growth, and growth factors that provide cells an adequate chemical environment (4-5). Nonetheless, in order to apply these principles to such a special tissue like bone it is imperative to be sensitive to bone biology aiming at understanding how its correct function is achieved. Clearly, fundamental in this process is to recognize the complex biochemical environment that surrounds cells in bone tissue and within which cellular interactions play a pivotal role. The importance of cell-to-cell interactions in the context of bone tissue engineering is addressed by looking at what has been done to study those interactions and by analyzing several co-culture models in the frame of the different strategies used in Bone TE. Finally, the possibility of the application of the state-of-the-art technology of cell sheet engineering to bone regeneration is also discussed. This technology has been successfully applied to treat

several tissue disorders and there is currently a strong focus in attempting to use cell sheet engineering to engineer thick tissues.

Therefore, this chapter intends not only to establish the theoretical background of this PhD thesis but also to frame the described strategies inside the current problems of bone tissue engineering and regenerative medicine.

1.2 Bone Biology

In the adult skeleton, bone tissue presents two different architectural forms: the trabecular bone (6-9), with 50-90% porosity, represents approximately 20 % of the skeleton and can be found in the metaphysys of long bones and in vertebral bodies; and the cortical bone (6-9), an almost solid form of bone with a low porosity that represents 80% of the skeleton.

Osteoblasts, osteocytes and osteoclasts are the three main cell types that can be found in bone tissue, having each one of these defined functions crucial for bone homeostasis (10-14). Osteoblasts, anchorage dependent cells, are highly responsive to mechanical and chemical stimulus that are relayed through multiple cell-to-matrix and cell-to-cell interactions(15-16). These interactions are mediated through specific receptors and transmembranous proteins such as integrins, cadherins and connexins leading to bone's extracellular matrix production and mineralization (15-19). As matrix is deposited and calcified, some osteoblasts become entrapped in it and achieve their fully differentiated state, becoming osteocytes. These are the most abundant cells in bone. They are smaller and rounder than osteoblasts and present a high number of filopodia that permit homotypic connections as well as interactions with bone-lining osteoblastic cells. These connections form a 3D network which is believed (13, 20-21) to function as a mechanical stimuli transduction system and as a general regulator of bone homeostasis. Nonetheless, osteoclasts are also seen as critical players in this regulatory process. They are multinucleated and highly specialized cells, derived from

the hematopoietic lineage, that have the function of resorbing bone by creating a tight seal resorption pit at the bone surface(22-24). In these pits, osteoclasts create an acidic environment and secrete lytic enzymes that become activated at low pH.

The exact mechanisms involved in bone homeostasis are still far from being well understood; however, two routes are currently accepted to be responsible for skeletal formation and maintenance: bone modeling and bone remodeling(25-27). The modulation of skeleton geometry during growth, in order to reach the optimal geometry to fulfill the requirements of bone function, is referred to as bone modeling(25-27). This process progresses by selective bone resorption or formation at specific sites. Osteoclastic activity is regulated independently of osteoblastic activity, i.e., bone formation never occurs where bone is being resorbed (25-27). In opposition, in bone remodeling(25-27), osteoclastic activity is strictly coupled with osteoblastic activity. Bone forming and bone resorbing activities occur in a coordinated manner, so that the amount of produced bone balances the amount of resorbed bone (27). This coupling of bone formation and bone resorption is spatially enclosed within specialized anatomic structures called basic multicellular units (BMUs) (27-30). These temporary structures, mainly formed by osteoblasts and osteoclasts, exert their action in three sequential phases that, overall, constitute a bone remodeling cycle: activation (of remodeling activity in the target area), resorption and formation (27-30). It is currently accepted (27, 29-30) that BMUs progress through the bone in a three dimensional way, forming tunnels in the cortical bone or trenches in the trabecular bone. Spatial control of the BMUs, in terms of target area selection and movement, is thought to be controlled by the osteocytes(27, 29-30) since the sensitivity of these cells to mechanical stimulus in bone tissue, as well as their organization in a network represent significant properties for the perfect conduction of the bone remodeling process (27, 29-30). Bone vasculature is also decisive in the remodeling process. In addition to the demonstration of an intimate relation between new blood vessels and osteogenesis(31-33), it was

already proved that every BMU is located in the vicinity of a blood vessel(34) which grows at the same rate the BMU advances(34-35). These facts indicate the crucial role of vasculature in bone remodeling and consequently the importance of vascular cells such as endothelial cells and pericytes. The most likely role of blood vessels as a source of biochemical signals and cells, and as major players in the coupling of bone resorption and formation during bone remodeling is explored elsewhere(36).

1.3 Cellular interactions in Bone Tissue Engineering

1.3.1 The relevance of cell–cell interactions in bone tissue engineering

Taking in consideration the biology of bone tissue it is quite clear that the understanding of the cellular interactions that regulate the homeostasis and regeneration of this remarkable tissue is essential to a successful Tissue Engineering strategy. The study of these cellular interactions *in vitro* relays on co-culture systems, a tremendously useful methodology where two or more cell types are cultured at the same time. It increases the complexity of typical cell culture systems, allowing the *in vitro* settings to closely mimic the *in vivo* environment. 2D co-culture systems have been extensively used by cell biologists to study cell interactions as an attempt to understand specific cellular mechanisms and pathways.

The self-renewal and multilineage differentiation abilities of stem cells (SCs) render these cells as the potential ultimate source of cells to create tissue-like constructs for TE, including bone TE. Subsequently, several studies (37-43) regarding cellular interactions relevant for bone TE have been focusing, in addition to osteoblasts, to different populations of SCs obtained from various origins and species. However, these studies have been performed mainly to assess the differentiation potential of SCs towards the osteogenic lineage by establishing several co-culture systems. Mature cells like chondrocytes (37-39), macrophages (40), endothelial cells(42) or myeloma cells(41) have all been proven to promote the osteogenic differentiation of

mesenchymal SCs (MSCs), either by direct or indirect interactions. Co-culture with osteoblasts was also reported as a method to direct the differentiation of Embryonic Stem Cells (ESCs), either from mouse or human origin, into the osteogenic lineage (44-45). However, a major issue that many tissue engineers fail to address is the atypical osteoblastic function of osteoblastic cells in culture when compared with osteoblasts *in vivo*. This is reflected on the quality of bone formed by osteoblasts *in vitro* (46-48), either in a 3D or a 2D environment, which is undoubtedly a major drawback when envisaging a possible clinical application.

Another very important issue that greatly affects the performance of a TE construct is the vascularisation since an insufficient blood supply in implanted engineered tissues will determine their failure (49-52). Vascularisation is regarded as essential in TE in general, and in bone TE in particular(53-54). In the usual bone TE strategy, where bone cells are seeded and cultured in scaffolds before implantation, a recurrent finding points out to cell death at the bulk of the scaffold by hypoxia (49-50, 55). It became clear that the production of a vascular network that could perfuse the engineered constructs is essential. So far the proposed solution relies on the creation of a blood vessel network within an engineered tissue prior to transplantation by incorporating cells that will lead to blood vessel formation within the scaffold matrix (56-59) and subsequent engraftment with the host tissue. Moreover, the extent of knowledge regarding the interactions between endothelial cells and bone forming or osteoprogenitor cells indicates the existence of reciprocal interactions between both types of cells that are essential to their normal function (60-69). It is therefore logic that the interaction of endothelial cells and osteogenic or osteoprogenitor cells is a critical issue to be explored by bone tissue engineers.

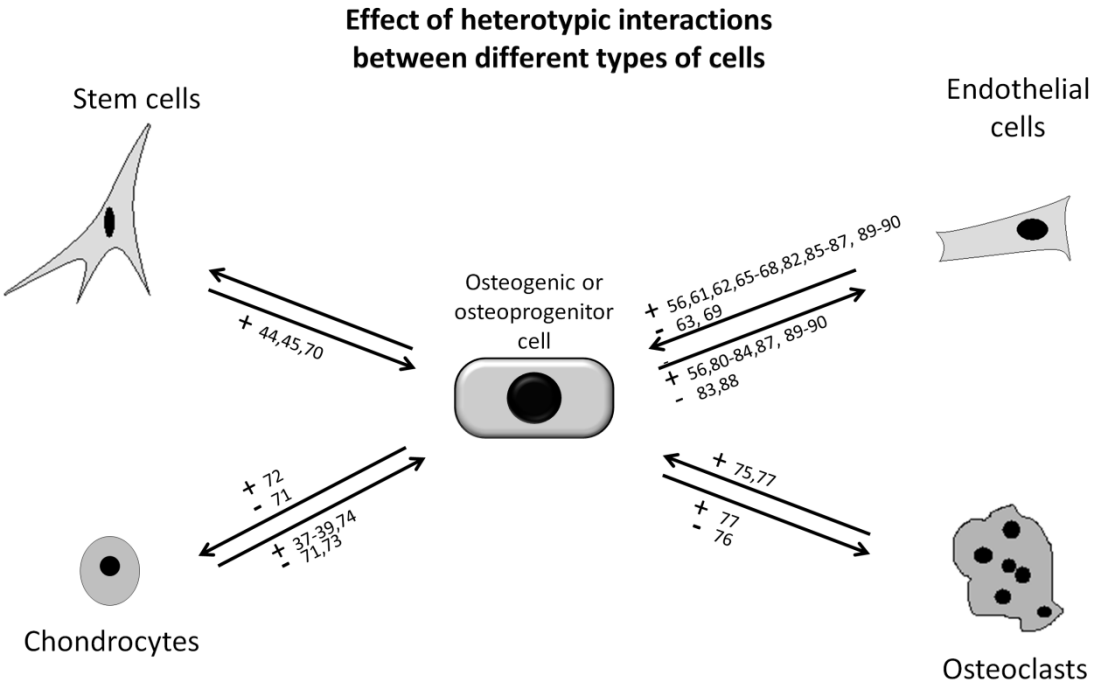

Figure 1.1 - Positive (+) and negative (–) effects of cell-to-cell interactions between osteoblasts or osteoprogenitor cells and stem cells(44-45, 70), chondrocytes (71-74)(37-39), osteoclasts(75-77) and endothelial cells(56, 61-63, 65-68, 78-90). Cell differentiation, function and proliferation were the reviewed parameters

As described in the previous section, bone modelling and remodelling processes rely in the specific crosstalk between osteoblasts and osteoclasts. It is for example well established that osteoblasts are deeply involved in the formation of osteoclasts as well as in their correct functioning(47, 91-95) but the effects of osteoclasts over osteoblastic function are still poorly understood. Studies done with osteoclast deficient mice models have shown not only a lack of bone resorption activity but also deficient osteoblastic activity, which is reflected in the quality of the formed bone(96-97). Dai *et al* (96) showed that implanting bone buds from osteoclast deficient mice into wild type mice resulted in the recovery of the buds' normal development. This work presents a major indication that engineering bone tissue for clinical use may require the use of osteoclasts along with osteoblasts. Therefore, compelling evidence suggests that osteoclasts have functions, namely regulatory functions, other than just bone resorption, which are reviewed elsewhere (75). Additionally, the vital importance of cell to cell interactions for bone homeostasis has been also reinforced by other works. Indications regarding the production of pro-osteogenic factors by osteoclasts were

given in a study that proved that osteoclast conditioned medium induced bone nodule formation by murine MC-3T3-E1 pre-osteoblasts(98). Increasing attention has also been given to the signalling action between ephrinB2 cell surface protein, present in osteoclasts, and its receptor EphB4, present in osteoblasts(76). It was shown that ephrinB2-EphB4 signalling is bidirectional and links two key molecular mechanisms of differentiation, one osteogenic and the other osteoclastogenic(76). This is of major importance since it describes a mechanism where both cells influence the differentiation of one another, possibly in simultaneous, by cell-cell contact.

Additional cues to create a successful bone TE construct might be provided by the endochondral ossification process in which chondrocytes produce a cartilaginous anlage that is mineralized by osteoblasts and osteocytes later in the process. A logic analysis would consider the high probability of chemical factors being produced by chondrocytes that enhance osteogenic activity and mineralization. Nonetheless, a study by Jiang *et al* (71) showed that in spite of an increase in alkaline phosphatase (ALP) activity in osteoblasts co-cultured with chondrocytes, their mineralization ability was diminished when compared to controls. This negative effect seemed however restricted to fully differentiated osteoblasts, which is in accordance with other works in the literature that describe chondrocytes as having a positive effect on the osteogenic differentiation of MSCs (37-39). Thus, in a Bone Tissue Engineering strategy based in the use of chondrocytes, it may be beneficial the use of MSCs or others osteoblastic precursors instead of fully differentiated osteoblasts(99). Hence, creative ways to ensure the clinical success of engineered constructs are needed. The study of the interactions between cells types relevant for bone TE is a powerful tool in the design of tissue engineered bone constructs. But how is this knowledge applied by bone tissue engineers?

1.3.2 Co-culture models in bone tissue engineering

Angiogenesis

As mentioned before, vascularization is a critical issue for TE constructs aiming at regenerating vascularized tissues. It has been demonstrated that the in vivo implantation of a cellularized construct without the production of an adequate vascular network leads to cell necrosis in the bulk of the construct (49-52). 3D co-cultures established in scaffolds have been proposed by tissue engineers as a powerful tool to overcome this question (56, 79-90). Two works reported that co-culturing endothelial cells with human bone marrow stromal cells (hBMSCs) in poly-lactic-glycolic acid (PLGA) scaffolds increased bone formation after implantation in a critical size rat calvaria defect compared to implanted scaffolds only seeded with hBMSC (85-86). These studies emerged as a proof-of-concept that co-culturing endothelial cells with osteogenic or osteogenic progenitor cells in a 3D scaffold can be an adequate strategy for targeting the vascularization of tissue engineered bone. Several other works (56, 79-90) have been reporting the co-cultivation of endothelial cells with osteogenic or osteoprogenitor cells for bone TE purposes. Rouwkema *et al* (82) showed that co-culturing human umbilical vein endothelial cells (HUVECs) with hMSCs in a spheroid aggregate model upregulated ALP expression of the hMSCs in comparison with monocultured spheroids. On the other hand, Kyriakidou *et al* (87) found that co-culturing HUVECs with a human osteoblast-like cell line, MG-63, in porous poly(ε-caprolactone) (PCL) scaffolds under dynamic conditions favored the proliferation of both types of cells but not their function. ALP activity and extracellular matrix (ECM) production of the osteoblast-like cells did not show a significant enhancement in comparison to the controls. More interestingly, Yu *et al* (89-90) reinforced the potential of osteoblast/endothelial cell 3D co-cultures for bone tissue engineering purposes. ECs and osteoblasts differentiated from the bone marrow of BALB/c mice were co-cultured in hydroxyapatite-PCL (HA-PCL) scaffolds and implanted in a critical size bone defect in the femur of mice of the same strain (89). The authors observed a dramatic

increase in vascularization and bone formation in the co-culture groups in comparison with scaffolds only seeded with osteoblasts. In addition, necrosis was found to occur in the osteoblasts groups but not in the co-culture groups. Similar results were found by the same group in a work that was carried out under the same conditions but in rats(90). Besides the enhanced bone formation and vascularization without signs of necrosis, the co-culture grafts presented better mechanical properties than those only seeded with osteoblasts.

A significant effort has also been applied to understand the effect of osteogenic or osteoprogenitor cells on the angiogenic potential of endothelial cells. Wenger *et al* (88) established a 3D collagen-based co-culture system of HUVECs and human osteoblasts (hOB). In this model, cells were grown in spheroid aggregates (the result of cell's self-aggregation in non-adhering conditions), either homotypic, only with HUVECs, or heterotypic, HUVECs plus hOB, and then embedded in collagen gels. It was verified that the presence of osteoblasts within the heterogeneous aggregates, diminished the formation of sprouts under angiogenic stimulus as compared with homogeneous spheroids suggesting an inhibitory effect of osteoblasts over endothelial cells activity. In contrast, Rowekema *et al* (82) developed a spheroid model where HUVECs were cultured with hMSCs. In this case, HUVECs were capable of forming a pre-vascular network which was further developed after *in vivo* subcutaneous implantation in nude mice. Using polyurethane (PU) cylindrical scaffolds, Hofmann *et al* (81) performed *in vitro* co-cultures of HUVEC and hOB, having platelet released growth factors (PRGF) as the only supplementation of the culture medium. Osteoblasts-seeded scaffolds were found to support cell proliferation and vessel formation by HUVECs in contrast with scaffolds loaded only with HUVECs. The contradictory results of some of these works suggests that this strategy is highly dependent on culture conditions such as cell seeding density, 2D or 3D culture and differentiation state of osteogenic cells.

The origin of endothelial cells appears also as an important issue. Stahl *et al* (83) established a co-culture model similar to Wenger *et al* (88) but of HUVECs and human umbilical cord blood endothelial progenitor cells (EPCs) and primary osteoblasts. The authors verified that osteoblasts inhibited the sprouting of HUVECs but not of EPCs. Similar results were reported in works that established co-cultures of human primary osteoblasts and HUVECs, Human Outgrowth Endothelial Cells (hOECs) and Human Dermal Microvascular Endothelial cells (hDMECs) in 2D conditions, 3D aggregates and 3D scaffolds (80, 84). hDMECs were cultured in the presence and absence of hOB on porous discs of three different materials, beta-tricalcium phosphate (beta-TCP), HA, nickel-titanium (NiTi) and, additionally, on silk fibroin nets (84). For all the materials, hDMECs only formed microcapillary-like structures in the presence of osteoblasts, independently of the angiogenic supplementation. When hOECs and HUVECs were cultured with and without osteoblasts either in 2D conditions or in a 3D spheroid aggregate co-culture model, a pre-vascular network was only formed by hOECs and in the presence of osteoblasts(80). In accordance, Santos *et al* (56) co-cultured hDMECs with primary human osteoblasts in starch-poly(3-caprolactone) (SPCL) fiber mesh scaffolds. Results showed that hDMECs self-assembled in a microcapilary-like structure, with a lumen where cells where positive for collagen IV, a marker of endothelial basement membrane. No such results were encountered in the control groups where hDMECs were cultured alone. Moreover, osteoblasts were found to produce more vascular endothelial growth factor (VEGF) and their collagen I mRNA levels were significantly higher when in co-culture. This is a proof of osteoblast-endothelial cell bi-directional communication. Reinforcing this, similar results were reported (79) when hOB were co-cultured with hOECs in SPCL scaffolds and then subcutaneously implanted in SCID mice. The constructs with both types of cells revealed improved vascularization in comparison with constructs only seeded with hOECs. Thus, these works have proven that osteoblasts provide endothelial cells sufficient stimuli for them to form a network of micro-vessel like structures. More

importantly, in some cases, the formation of that network was achieved without the addition of angiogenic factors to the culture medium (84). This is probably the best demonstration on how co-cultures provide a higher complexity culture system, with self-regulation, that can be of much use in Tissue Engineering approaches

Overall these approaches represent the growing awareness of bone tissue engineers to the significance of working on the vascularization issue prior to bone tissue formation which, is a natural evolution taking in account the (lack of) results delivered by typical TE approaches so far.

Osteochondral strategies

There are clinical conditions where cartilage defects progress to the underlying sub-chondral bone, affecting, at the same time, bone and cartilage tissues (100). In these cases, the application of osteochondral TE constructs is seen as a valid solution to regenerate both bone and cartilage tissues(101-102). To date, the proposed osteochondral TE constructs consist on co-culturing osteogenic and chondrogenic cells in 3D supports (72-74). However, there are few works in the literature using osteochondral constructs that address bone formation, most of them focusing, instead, on the cartilage part.

Spalazzi *et al* (72) studied the interactions between bovine osteoblasts and chondrocytes in Polylactide-co-glycolide and bioactive glass (PLGA-BG) composite scaffolds and films. The authors observed that in comparison with control groups, only seeded with osteoblasts or chondrocytes, co-culture constructs enhanced the ability of chondrocytes to maintain their normal morphology for a longer time period. The presence of osteoblasts and respective layered matrix also increased chondrocyte proliferation and matrix production. Similar results were observed by Mahmoudifar *et al* (74) after co-culturing, under dynamic conditions, human foetal chondrocytes and osteoblasts in PLGA scaffolds. The presence of osteoblasts was found to improve

cartilage formation in terms of glycosaminoglycans (GAG) content and total collagen deposition in comparison with no osteoblasts-containing controls. In contrast, the authors did not find significant benefit, in terms of cartilage formation, in co-culturing the chondrocytes with either bone chips or cartilage chips as possible sources of growth factors. Although the authors acknowledge that mineralization occurred in the osteoblast seeded part of the scaffold, no further analysis, like for the work by Spalazzi *et al* (72), were performed concerning the osteogenic performance of the construct. The effect of chondrocytes over osteogenic cells in an osteochondral construct was explored in a work by Cao *et al* (73). Human osteogenic cells, previously differentiated from stromal cells of the iliac crest, were co-cultured with human chondrocytes, obtained from the rib cartilage, in PCL scaffolds. The presence of chondrogenic cells had a positive effect on the production of the early osteogenic marker ALP but a negative effect on the expression of the later marker, osteocalcin. Nevertheless, the mineralization of the osteochondral construct was supported by the osteogenic cells. In contrast, Jiang *et al* (71) reported that culturing bovine chondrocytes and osteoblasts in micromass culture had a negative effect both on the chondrocyte and osteoblastic phenotype. Moreover, mineralization in the co-cultures was significantly diminished in comparison with monoculture controls.

These results demonstrated that chondrocytes and osteoblasts can modulate both cell types' phenotype in tissue engineered constructs. However, how will tissue engineers manipulate these interactions, either by controlling the culture conditions or the 3D scaffold, in order to obtain suitable osteochondral constructs is still to be seen.

Macrophages, Monocytes and osteoclasts

Macrophages, which are key players in an inflammatory process within the periprosthetic milieu, produce several cytokines and other soluble factors that affect all the other neighboring cells (103-105) and have been often associated with implant

failure. In this sense, co-cultures of monocytes and macrophages, with osteoblasts have been used in the biomaterial field as a way to elucidate the macrophages biological response to orthopedic biomaterials particulates in a more complex system that includes bone-forming cells, osteoblasts (106-108). The translation of the results from these works into the TE field is scarce; however, co-culturing monocytes/macrophages or monocyte/macrophages-derived osteoclasts and bone cells in 3D biodegradable matrices might be an interesting approach towards engineering high quality bone(47). Bone tissue engineers have not explored much this approach, existing therefore few works(77, 109) that co-cultured monocytes and stromal cells in 3D scaffolds. Domaschke *et al* (77) studied the in vitro remodeling of mineralized collagen I scaffolds using co-cultures of ST-2, a mouse bone marrow stromal cell line, and human monocytes. In this work, it was demonstrated that the co-cultures induced both osteoclastogenesis and osteoblastogenesis from progenitor cells of both lineages in a process, according to the authors, comparable to bone remodeling that involved the resorbing of the scaffold by the osteoclasts and matrix mineralization by the osteoblasts.

It seems obvious that exploring this model that focuses on the balance between the degradative osteoclastic and the constructive osteoblastic activities, as well as co-culturing monocytes/macrophages and bone cells to highlight the initial bone healing mechanisms, may be of extreme use for developing successful bone tissue engineering strategies.

Stem Cells

Nowadays, stem cells, which are capable of differentiate into the most varied lineages of cells, are seen as an exceptionally promising tool in TE. In bone TE there has been the tendency to differentiate those cells into the osteogenic lineage using exogenous factors, before scaffold seeding, probably due to time and cost efficiency. Nonetheless,

stem cells differentiation can be also directed towards the osteogenic lineage *in situ* using co-culture systems in a 3D environment. Kim *et al* (70) showed that human ESCs committed to the osteogenic lineage by co-culture with primary cells from bone explants and seeded in poly(D,L-lactic-co-glycolic acid)/hydroxyapatite composite scaffolds were able to support new bone formation in an immunodeficient mouse model. Although not presenting the controls with hESCs not subjected to co-culture with the bone primary cells, this work demonstrates the potential of the use of co-cultures to direct the fate of stem cells. There is no doubt that using 3D co-cultures to induce the differentiation of stem cells towards the osteogenic lineage will be a potentially successful path for future bone tissue engineers.

The study of cellular interactions can provide not only the understanding of the major drawbacks that tamper bone TE evolution but also the solution to overcome those obstacles. The jump from 2D cultures to 3D cultures in the TE field was an acknowledgement that culture systems needed to emulate more closely in vivo systems. In fact, it was suggested that many of the lessons brought by 3D research in TE could be adopted to other field such as cancer research(110).

1.4 Cell Sheet Engineering

Professor Teruo Okano's laboratory first proposed the use of temperature-responsive surfaces for cell culture in 1990 (111). These dishes allow to culture cells and, after confluency, to recover them and their ECM as a sheet simply by lowering the temperature. These surface were obtained by uniformly spread isopropylacrylamide (IPAAm) monomers dissolved in 2-propanol over tissue culture polystyrene (TCPS) dishes(111-112). As IPAAm monomers polymerize, they are irradiated with an electron beam (EB) for covalent grafting to TCPS. EB methodology, although requiring the use of complex apparatus, offers many advantages over other techniques of graft polymerization. It allows a uniform covalent polymer grafting over TCPS using few

steps and it is suitable for large-scale production. It has been reported, for instance, the use of plasma polymerization to produce poly(N-isopropylacrylamide) (PIPAAm)-grafted surfaces. Although the grafting was successful for surfaces such as glass(113), silicon(114) or TCPS(115-116) using only one step, this method is not suitable for large scale application due to issues related to the continuous treatment required and size of the apparatus. Furthermore, it was shown by XPS analysis(117) that following cell sheet recovery, there was a greater quantity of residual ECM proteins left on PIPAAm-TCPS dishes polymerized with plasma than on dishes polymerized with EB irradiation.

Using EB methodology, the thickness of the grafted PIPAAm is controlled by the concentration of the monomers and radiation energy. In fact, the thickness of the grafted polymer is critical for the adhesion behavior of cells. It was determined that for optimal cell adhesion and detachment by temperature decrease, the PIPAAm layer on a TCPS surface should have a thickness of 20 nm and a density range of 0.8-2.2 μg/cm ^{2}(118-119). It was also shown that increasing thickness and density of the grafted PIPAAm the surfaces did not present the hydrophobic/hydrophilic property alterations above and below the low critical solution temperature (LCST) (118), i.e., surfaces became more hydrophobic. This is related with the mobility of PIPAAm chains. For thicker layers of grafted PIPAAm, there is a greater mobility of PIPAAm chains and even above LCST the hydrophobic environment in the vicinity of polystyrene surface is not enough to promote dehydration and aggregation of the outermost chains. However, for thin or ultra-thin PIPAAm layers the mobility of the PIPAAm chains is more restricted and the chains at the interface with substrate, which have decreased hydration due to the hydrophobicity of the polystyrene, manage to drive dehydration of the outermost chains(118). This results in a slightly hydrophobic surface for cells to adhere. It is then clear that controlling the thickness of grafted PIPAAm is a critical issue for obtaining the desired temperature-dependent switchable attach/detach behavior of cultured cells.

PIPAAm-TCPS dishes are hydrophilic below PIPAAm LCST, 32ºC, and therefore cell repellent. When the temperature rises above 32ºC, PIPAAm chains dehydrate, collapse and cells are allowed to adhere and proliferate(118-119). Furthermore, after culturing cells in the dishes, when temperature falls below the LCST, attached cells detach spontaneously without the use of proteolytic enzymes. This spontaneous detachment is in fact an active detachment since it involves ATP spending as was demonstrated in works using inhibitors of ATP synthesis and tyrosine kinases (119). Other works focused on detachment mechanisms at cytoskeletal and intracellular signaling levels (120-122). From all of these works we can conclude that intracellular signal transduction is necessary for cells to detach from temperature responsive TCPS dishes. Moreover, cell detachment behavior from temperature responsive TCPS dishes is profoundly cell type-dependent. The nature and amount of ECM produced by cells is a critical factor for cell detachment from temperature responsive dishes. Since different cell types produce different types of ECM, the time required for detachment will vary or might not take place at all (123).

1.4.1 Applications in Regenerative Medicine

Many hopes have been put in the tissue engineering and regenerative medicine (TERM) field to solve many of the problems in organ regeneration. Several obstacles, however, still subsist due to current techniques limitations. Promising results have been achieved by injection of cell suspensions in the damaged site (124-131) or by transplantation of TE substitutes composed by biodegradable scaffolds and cells (132-133). Nevertheless, each of these techniques has their own important drawbacks. Migration of injected cells and consequent low efficiency in delivering the cells to the desired anatomic site, is a recurrent problem (134-135). Biodegradable scaffolds with cultured cells, on their hand, have the limitation of oxygen and nutrient supply to the cells in the core of the construct. This results in cell necrosis at the bulk of the construct

(50-52). Furthermore, foreign body response to the implanted biomaterial is a frequent reaction of the host and has as a consequence the formation of fibrotic tissue surrounding the implant (136).

The use of cell sheets in the TERM field, circumvent most of the problems described above. Cells are recovered from temperature sensitive dishes as a cohesive sheet where all cell-cell and cell-ECM connections are maintained (137-138). The ECM in the cell sheets acts as a natural glue that enables cell sheets to be applied in virtually all anatomic sites. Also, cells sheets comprise only cells and their matrix, so, besides the absence of a biomaterial, using autologous cells avoids an immune rejection of implanted cell sheets. Following all these advantages, Professor Okano's group has applied cell sheet engineering to the regeneration of various tissues as discussed below.

In the ophthalmology field, clinical trials to treat unilateral or bilateral total corneal stem cell deficiencies due to alkali burns or Stevens-Johnson syndrome were carried out. Using autologous limbal stem cells (for the unilateral cases) (139) or autologous oral mucosa epithelial cells (for the bilateral case) (140) and cell sheet engineering, patients with such conditions were successfully treated.

For the treatment of oesophageal ulcerations a method was developed where endoscopic submucosal dissection was combined with endoscopic transplantation of autologous oral mucosal epithelial cell sheets (141-142). These type of ulcerations are a major postoperative burden for many patients that can result in stenosis (141). Using the developed method patients who underwent oesophageal endoscopic mucosal resection due to oesophageal carcinoma were treated by endoscopic transplantation of autologous oral mucosal epithelial cell sheets(142). This was the first reported case of regenerative medicine applied to endoscopic treatment.

Cell sheet engineering application in periodontology has also been tested using animal models (143-146). In a recent work using beagle dogs as animal model, autologous periodontal ligament cell sheets were layered and, using a woven polyglycolic acid

support, transplanted to dental root surfaces having three-wall periodontal defects. Bone defects were filled with porous β-tricalcium phosphate. (146) Both new bone and cementum connecting with well-oriented collagen fibers was found only after cell sheet transplantation, confirming the usefulness of cell sheet engineering for periodontal regeneration in clinical settings.

Recently, Okano's group proposed cell sheet engineering as a potential supply therapy for diabetes type I (147). In this work, rat pancreatic islet cells were cultured on laminin 5-coated temperature-responsive culture dishes. Cells sheets were recovered and implanted subcutaneously in rats. Pancreatic cell sheets remained biofunctional, producing insulin and glucagon after 7 days of transplantation. This opens a new perspective for the treatment of type I diabetes and other islet-dependent diseases.

Myocardial tissue regeneration was also vastly studied by Okano's group using cell sheet engineering(148-158). The reconstruction of cardiac tissues has been tackled by producing cell sheets of mesenchymal stem cells(154), cardiac myocytes (148-152) and skeletal myoblasts(155-156, 158). In addition, the spontaneous beating of cardiomyocyte cell sheets synchronizes after CS layering (151, 157) thus rendering a cardiac patch that might be transplanted to a defective heart.

The diffusion limitations associated with increasing thickness of engineered tissues were possible to be circumvented by polytransplantation of cell sheets(150). Furthermore, Sasagawa *et al*(159) successfully pre-vascularized *in vitro* a myoblast cell sheet construct, by culturing HUVECS between myoblasts cell sheets, leading to the formation of microvessels containing red blood cells after implantation in the subcutaneous tissue of nude rats.

All the work developed by Okano's group in engineering thick tissues, and in particular cardiac tissue reconstruction, strongly suggests that cell sheet engineering is an extremely promising, ever evolving technology for that purpose. Although bone is a support tissue with very specific mechanical properties i.e., different from cardiac tissue, the achievements of these works comprise a significant starting point for

overcoming the main obstacle to engineer thick tissues, the lack of vascularization. Thus, the way for bone TE using cell sheet engineering is open.

1.4.2 Cell Sheet Engineering and Bone Tissue Engineering

The use of cell sheets for bone tissue engineering purposes has been addressed in several works. Zhou and colleagues (160) combined osteogenic cell sheets, made form porcine BMSCs, wrapped around PCL–calcium phosphate scaffolds. Post-subcutaneous implantation analysis of the construct showed some degree of new bone formation but mainly at the periphery of the scaffolds. The same pattern of new calcified tissue, around the scaffold, was achieved by Gao *et al* (161), using a coral scaffold, and Akahane *et al*. (162), using a hydroxyapatite ceramic scaffold. In the latter case, the cell sheets were also ectopically implanted without any scaffold (162) and new bone formation, albeit disorganized, was verified. In the three approaches, new bone tissue was fairly disorganized, poorly vascularised and limited to the surface of the scaffolds around which the cell sheets were wrapped. Ma *et al* (163) rolled cell sheets composed of BMSCs in a cylinder shape and implanted subcutaneously in nude mice. Once again, new bone formation was evident; however, the size and morphology of the construct inhibited the vascularisation of the construct. Akahane *et al* (164) proposed the injection of osteogenic cell sheets as a non-invasive method for hard tissue reconstruction. From the same group, Nakamura et al (165) wrapped an osteogenic cell sheet around a rat femur defect and found that, compared with the controls without cell sheet, the cell sheet groups showed improved regeneration and bone union.

The works described above showed the high potential of using cell sheets for bone tissue engineering. However, for all the studies, cell sheets were detached using a cell scraper. Cell sheets obtained using thermo-responsive dishes have unique features as they are harvested as confluent cells as sheets together with their ECM. The ECM

contains adhesive proteins that will act as a natural glue that, besides facilitating cell sheet adhesion to anatomic sites, also allows the union between the cell sheets after stacking. Using this principle, the production of three dimensional bone tissue, by the sequential layering of cell sheets without the use of scaffolds, constitutes an extremely promising approach for bone TE purposes.

1.5 Final Remarks

To manipulate cellular function through the use of co-cultures is nothing less than trying to mimic what happens in vivo. As this is one of the precepts of TE it should be largely embraced by tissue engineers. Combining co-cultures with 3D culture is the way to achieve higher complexity models of superior quality.

Nonetheless, the major hurdle in bone TE, is and will continue to be, the vascularization issue. The question “OSTEOGENESIS AND THE VASCULATURE: SHOULD THE SCAFFOLD COME FIRST?” as presented by Mikos *et al* [57] makes more sense than ever. The tendency in the next few years will be to make endothelial cells an indispensable component of any bone TE strategy though osteoblasts or osteoprogenitor cells will continue to be essential. Perhaps the default strategy in the future will be to co-culture, in 3D, endothelial and osteogenic cells.

Moreover, other shortcomings brought by the use of scaffolds limit the number successful clinical applications in the field of bone TE. The application of cell sheets avoids those shortcomings while presenting encouraging prospects for the creation of thick tissues and for dealing with the vascularization issue of TE constructs. It is then clear that cell sheet engineering presents an enormous potential as a strategy for bone tissue engineering.

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Chapter 2

MATERIALS AND METHODS

Chapter 2

MATERIALS AND METHODS

The works presented in this thesis are focused on the study of the interactions between different cell types for possible applications in Bone Tissue Engineering. An indirect co-culture system was established in order to assess the effect of soluble factors produced by human peripheral blood monocytes/macrophages over the osteogenic differentiation of human bone marrow stromal cells. In a step forward, mononuclear cells from umbilical cord blood were co-cultured with osteoblasts differentiated from human adipose derived stem cells in carrageenan membranes as a way to enhance post-implantation neovascularization. The effect of human dermal fibroblasts (hDFb) over human mesenchymal stem cell-derived osteoblasts with the involvement of Gap junctional communication was also assessed in a direct co-culture. In addition to the addressed heterotypic cellular interactions, the enormous advantage of the cell sheet engineering technology of being able to preserve cell-cell and cell-extracellular matrix interactions was also explored in the context of bone tissue engineering.

A detailed description of i) the materials used in the experimental work and of ii) the methodologies that led to the results displayed in the following chapters are provided in this chapter. As the approaches followed to attain specific results to support the proposed hypotheses of this PhD work required a strong application of both *in vitro* and *in vivo* methodologies, this description was sub-divided within these two main items. A characterization section encloses all the techniques used, some of them common for the *in vitro* and *in vivo* assays.

2.1. Materials

2.1.1 Carrageenan

Natural-based polymers have been increasingly proposed for biomedical applications due to their ability to elicit favorable biological responses and their high degree of processing flexibility(1). Carrageenan (CRG) in particular is a polyssacharide

isolated from red seaweed that has been used mainly in the food industry as a thickener(2). Recently, carrageenans of the *kappa* type have been proposed for regenerative medicine purposes (3-5) due to their favorable mechanical properties and an anticoagulant effect (6). Therefore we considered this polymer as a suitable material to produce the membranes to support the co-cultures of osteoblasts and mononuclear cells from cord blood in chapter 4.

The standard name carrageenan is used to name a class of galactan polysaccharides that behave as intercellular matrix materials, like agars, in specific species of red seaweeds, including the genus Gigartina, Chondrus, and Eucheuma a marine algae included in the class Rhodophyta (7-9). The CRGs are grouped into several sub-types according to their sulphation patterns and distribution of 3,6-anhydro-D-galactose (AG) residues (8-9). *Kappa*, *iota* and *lambda* CRGs are obtained by treatment in alkaline conditions(10), and are the molecular types with high relevance in industry. The commercial forms of these CRG types are rarely pure and usually contain varying amounts of the other CRG types, whose exact amount depends on the weed source and/or on the extraction procedure(9, 11).

CRGs are also classified as hydrocolloids, due to their structure comprising highly sulphated, alternating α(1→3) and β(1→4)-linked galactose residues, disaccharides repeating units. In addition, the galactose units linked β-(1→4) in this general structure often occur as 3,6-AG. The numbers and positions of the sulphate ester groups have an impressive effect on the tertiary structure and the subsequent interactions that occur for the different CRG types. *Kappa* is sulphated only at C4 in the 1,3-linked galactose ring, while *iota* has an additional sulphate at C2 in the 1,4-linked-3,6-anhydro-d-galactose ring. Due to these structural properties, the formation of double-helical segments is allowed leading to a proper arrangement as gel structures at appropriate conditions of salt concentration and temperature(9). *Lambda*-CRG has a higher average level of sulphation per disaccharide residue than *iota*- and *kappa*-CRG. The β-(1→4)-D galactose ring is not conformationally locked in the -3, 6-anhydro form,

and carries sulphate groups at C2 and C6. In addition, sulphation in the α(1→3)-D-galactose-1 residue occurs at C2. The removal of the anhydrous bridge confers a linking effect that inhibits double-helix formation, thus *lambda*-CRG does not undergo conformational ordering, and consequently does not form gels.

The gelling CRG viscoelasticity is sensitive to both the nature and concentration of cations, being lambda the least and kappa the most one salt sensitive. Through CRGs blending, the viscoelasticity of the gel phase can be varied, in order to suit a wide range of applications. Commercially available CRGs are essentially ground, dehydrated gels containing approximately 7% water. Generally, the higher the 3,6-AG content and the lower the ester sulphate level, the less readily will the CRG hydrate. Hydration and solubility of *kappa* and *iota* CRG can be optimized by ion exchanging the CRG leading to a material predominantly in the sodium form (8).

2.1.2 CRG Membranes preparation

The type *iota-kappa* (hybrid) CRG extracted from the red algae *Chondrus crispus* by CEAMSA (Porrino, Spain) was used to produce the membranes. This hybrid CRG presents several processing advantages like a higher degree of flexibility, when compared to *kappa* CRG, and a higher robustness when compared to pure *iota CRG.* Other advantage is related with the fact that is naturally a hybrid polymer, meaning that it is not a blend, and thus behaves as a monocomponent system, concentrating in a single molecule, properties of both *kappa* and *iota CRGs.* The ionic environment chosen for processing this hybrid also conferred specific features to the developed systems. The potassium chloride (KCl) solutions allowed preserving the structures processed by solvent casting and potassium hydroxide (KOH) provided the alkaline environment needed for the crosslinking reaction while its dissociation gave the potassium necessary for the stability of the membranes (12-13). The cations affect the balance of attractive and repulsive forces between the molecules, so that optimum gel

strength will occur at certain levels of each cation. In the case of *kappa*-CRG, alkaline ions bind to the helix of the hydrocolloid, causing the partially neutralization of sulphate groups, leading to the aggregation of the double helixes and thus increasing gel rigidity. The hybrid *iota-kappa* CRG was dissolved in distilled water to a final concentration of 2% w/v. While hot, 25-35 mL of the solutions were placed in square petri plates and left to dry at room temperature for 48 hours. In order to preserve its structural features while cutting was taking place, the formed membranes were placed into a low-hydrating solution of 20% (v/v) ethanol plus 3% (w/v) KCl (Sigma, USA) and cut into small round samples of 1.1 cm diameter. The average thickness was of 1mm (wet). The CRG membranes were crosslinked in a water solution of 50% (v/v) 2-propanol (Fluka, Czech Republic), and 0.32M potassium hydroxide (KOH, Fluka, Czech Republic) and 4.63 mM of epichlorohydrin (ECH) (Aldrich, USA) for 24h, at 37ºC under 100 rpm agitation. The samples were then washed twice with a 17% ethanol (v/v) and 0.1M sodium hydroxide (NaOH, Panreac, Spain) solution, aiming at blocking the partially reacted ECH and twice with water at 50Cº for 1h (while monitoring and neutralizing the pH).

Generally it is well accepted that the crosslinking of natural polymer alone or with other components induces higher resistance to degradation(14) as well as new physico-chemical properties that can be modulated by the degree and type of crosslinking agent and by modification of reaction parameters (15-16). The crosslinking approach chosen was based on the works published by Guner and co-workers(17) for dextran using different agents, and by Keppeler and co-workers (18) with several modifications dependent on the structures to be produced and the raw material used. The CRG crosslinking reaction was preceded in the presence of KOH, which is the acceptor for hydrogen chloride (HCl) - product of the dehydrochlorination of chlorihydrine. Some consider that adding ECH to CRG macromolecules, in the base-aqueous medium, a molecular reaction occurs via the side-chain alkoholate formation

stage (18). The chlohydrine fragments formed can be easily transformed to epoxy groups by dehydrochlorination in the presence of KOH.

CRG membranes were then sterilized overnight with 70% ethanol. Before cellular assays, the membranes were maintained in sterile phosphate buffered saline (PBS, Sigma, USA) with 3% (w/v) KCl and washed twice in sterile PBS immediately before cell seeding.

2.1.3 *Temperature-responsive dishes*

Temperature-responsive dishes for cell culture allow cells to be cultured and then, after reaching confluency, to harvest all the cells and their extracellular matrix deposited during culture as a single contiguous cell sheet by lowering temperature. For the original fabrication of these dishes, isopropylacrylamide (IPAAm) monomers are dissolved in 2-propanol at a concentration of 55% w/w and then 70 µL of the solution is uniformly spread over tissue culture polystyrene (TCPS) dishes(19-20). For polymerizing and covalent grafting onto TCPS dishes, these are irradiated with a 0.25 MGy electron beam (EB). PIPAAm-grafted dishes were rinsed with cold-distilled water to remove ungrafted monomer, and dried in nitrogen gas. Dishes were finally sterilized with ethylene oxide gas prior to experimental use.

2.2. *IN VITRO* Methodologies

2.2.1 Tissue samples procurement

All the human samples were obtained after written protocols were established between the 3B's Research Group and the involved institutions. The protocols also required in all cases approval from the respective ethical committees. This resulted in strict rules for assuring the patient's compliance for the collection of samples as well as patient's anonymity.

In what concerns the rat bone marrow collection, the procedure was carried out after approval of the laboratory ethical committee and according to international animal welfare regulations.

Given that osteoprogenitors and osteogenic cells have the central role in the works described in this thesis, mesenchymal progenitor cells were the cells of choice to undergo osteogenic differentiation. Bone marrow stromal cells (BMSCs) have been proposed as the gold standard for bone Tissue Engineering due to their immuno-privileged phenotype and their high osteogenic differentiation potential (21), true for cells both from human and rat origin. Human adipose derived stem cells (hASCs) share those molecular and functional characteristics of BMSCs (22), though adipose tissue is increasingly seen as more accessible and easier to harvest than bone marrow, rendering it a better source for tissue engineering purposes.

Human monocytes/macrophages were isolated from human peripheral blood, a readily available and abundant source of these cells (23). The use of primary monocytes/macrophages, obtained from different donors, instead of using a cell line intends to provide a more realistic approach that is more relevant for the extrapolation of the results to the clinical setting. This is also true for the selection of human skin for the isolation of dermal fibroblasts.

Under a different context, umbilical cord blood (UCB) was used as a source of endothelial progenitors aiming at eventually composing a bone tissue engineering construct. Despite the potential lower accessibility, UCB contains more progenitors than peripheral blood (24) and therefore the higher larger available amount of peripheral blood is easily fight by this knowledge. As the role of transplanted endothelial progenitors in a bone tissue engineering approach is still far from being revealed, mature human umbilical cord vein endothelial cells (HUVECs) were also used to pre-vascularize engineered constructs. These cells have a widespread use in research since they are functional endothelial cells that are relatively easy to isolate and culture (25).

Human BMSCs Isolation

Human bone marrow samples were obtained from bone marrow aspirates provided after routine surgical procedures in Hospital da Prelada, Porto, Portugal. All the sampled were collected before cut of the femoral head in hip prosthesis placement in order to avoid potential contamination of with osteoblasts. Bone marrow aspiration needles (Angiotech, USA) were used to collect 5 mL of marrow. These were transferred to a 50 mL falcon tube containing 10mL sterile Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 5% of antibiotics (A/b, Invitrogen, USA) and 400 units of heparin (Sigma, USA) per mL. Transport was carried out under controlled temperature conditions and samples were processed within 2 hours after collection. Samples were centrifuged in PBS and ressuspended again in PBS for mononuclear cell isolation (section 2.2.2).

Peripheral Blood Mononuclear Cells (PBMNCs) Isolation

Buffy coats from human peripheral blood were obtained from the Portuguese Institute of Blood (IPS) after voluntary donations. The samples provided were from healthy donors and were discarded by the institute due to insufficient volume for further use of the institution. Total blood was collected in citrate phosphate dextrose adenine (CPDA) I and processed into buffy coats in the day before use. Transport was carried out under controlled temperature conditions and samples were processed right after arrival. Prior to differential centrifugation to isolate the mononuclear cell fraction described in section 2.2.2, buffy coats were diluted 1:2 in PBS (Sigma, USA) in order to avoid gradient overload.

hASCs Isolation

Discarded subcutaneous adipose tissue from lipoaspiration procedures in Hospital da Prelada, Porto, Portugal, was collected to sterile containers with 100 mL of PBS (Sigma, USA) with 10% antibiotics (Invitrogen, USA). Lipoaspirates were washed with PBS, in order to discard the majority of blood, and then digested with 0.05% collagenase II (Sigma, USA) at 37ºC for 45 minutes. Following that, the digested tissue was passed through a strainer and centrifuged at 1000g for 10minutes, at 4ºC. The obtained pellet was ressuspended in PBS and centrifuged again at 800g for another 10minutes, at 4ºC. The resulting pellet was then ressuspended in Minimum Essential Medium alpha-modification (alpha-MEM, Invitrogen, USA), supplemented with 10%FBS and 1% Antibiotic-antimycotic, filtered with 100 µm cell strainer (BD Biosciences, USA) and plated in tissue culture polystyrene flasks, using the referred culture medium. After 24 hours, colonies of spindle-shaped adhered cells started to appear.

Cord Blood Mononuclear Cells (CBMNCs) Isolation

Cord blood was collected during programmed caesareans in Hospital de São Marcos, Braga. Cord was clamped in the proximity of the abdomen of the newborn and cut. Cord blood was collected from the vein, before placenta removal, directly to the collection bag containing CPDA I (Fenway Europe sprl, Belgium) as anticoagulant. Transport was carried out under controlled temperature conditions and samples were processed within 2 hours after collection. The blood was then diluted 2:1 in PBS (Sigma, USA) for posterior mononuclear cell isolation as described in section 2.2.2.

hDFb Isolation

hDFb were isolated from discarded skin tissue specimens obtained from healthy patients undergoing abdominoplasties at Hospital da Prelada, Porto. Transport was carried out under controlled temperature conditions and samples processed less than

24 hours after collection. Skin samples were washed with sterile phophate-buffered saline (Sigma, USA) and cut into small pieces ($2mm^2$), and digested with a 2.4 U/mL Dispase II (Sigma, USA) solution at 4ºC, overnight. After the incubation the epidermis was removed, and the remaining dermal components were further incubated with a 0.1% collagenase type IA (Sigma, USA) solution for 3 hours at 37ºC, under gently agitation. The digested dermis was then passed through a 100µm cell strainer (BD Biosciences, USA), centrifuged at 400 G for 10 minutes and the pellet ressuspended in low-glucose DMEM medium (Sigma, USA) supplemented with 10% FBS ((Invitrogen, USA) and 1% Antibiotic/Antimycotic (Invitrogen, USA). Spindle-shaped cells were adhered after 24 hours of culture.

Rat BMSCs Isolation

Male Wistar rats with 4 weeks of age (Charles River, Yokohama, Japan) were euthanized with CO_2 following animal safety regulations. Subsequently, rats were cleaned with 70% ethanol, placed in a flow chamber and femurs were surgically removed. The femoral condyles were removed with the joints to expose the marrow. The epiphysis were cut and a needle, coupled to a syringe containing DMEM medium supplemented with 10% FBS and 1% antibiotics, was inserted in the marrow cavity which was flushed several times, with the culture medium, into a falcon tube. The recovered marrow was then repeatedly pipetted to disaggregate any cell clumps before mononuclear cell isolation as described in section 2.2.2.

2.2.2 Mononuclear Cell Isolation

Cell populations present in blood, both peripheral and from umbilical cord, encompass mononuclear cells, erythrocytes and platelets. The populations of interest for the works in question are present in the mononuclear fraction so in order to avoid interference from the remaining cellular components, Histopaque, a density gradient, was used to separate that fraction.

The cell suspensions isolated as described above, with the exceptions of hDFb and hASCs, were placed over Histopaque 1077 (Sigma, USA) or Histopaque 1083 (Sigma-Aldrich Japan, Japan), respectively for human and rat cells. For this, 5 mL of Histopaque at room temperature were placed in 15 mL conical falcon tubes and 3 mL of the cell suspensions were carefully layered over it. The tubes were then centrifuged at 400 G for 30 minutes, at room temperature. Cells in the interface between plasma and Histopaque were recovered using a pasteur pipette, washed three times in PBS and counted using a Neubauer chamber. Mononuclear cells were then ready for downstream characterization and culture.

2.2.3 Cell Culture

Osteogenic Differentiation

The differentiation of BMSCs and ASCs towards the osteogenic lineage is extensively described in the literature(26-27) and has been demonstrated to be induced by supplementing specific culture medium with ascorbic acid, beta-glycerophosphate and dexamethasone (28). As ascorbic acid, proven to increase matrix production (29), has a very short half-life in the medium (30), a salt of ascorbic acid, ascorbate-2-phosphate, is used. Beta-glycerophosphate is involved in matrix mineralization by providing phosphate ions (31) while dexamethasone is a corticoid that commits osteoprogenitor cells to the osteogenic lineage (32-33)

Therefore, mesenchymal progenitors from either human bone marrow, rat bone marrow or human adipose tissue cells were cultured in DMEM (Sigma, USA) medium supplemented with10 mM beta-glycerophosphate (Sigma, USA), 50 µg/mL of ascorbic acid (Sigma, USA or Wako, Japan) and 10^{-8}M of dexamethasone (Sigma, USA or DEXART, Fuji pharma, Japan) (osteogenic medium).

Cell Sheets Fabrication

Cell sheet engineering, as vastly stated in the background introduction to this thesis, allows recovering cultured cells as sheets with intact cell-cell junctions and intact ECM through the use of thermoresponsive dishes(20). The intact ECM can then act as a natural glue that easily adheres to tissues, new culture surfaces or other cell sheets. Besides this, the existing volume of work regarding the vascularization of 3D multilayered cell sheet constructs, demonstrate the significant potential of this technique for bone tissue engineering applications.

Osteogenic Cell sheets

Isolated rBMSCs were seeded in 100 mm diameter tissue culture polystyrene (TCPS) dishes and cultured in basal medium DMEM (low glucose; Wako Pure Chemical Industries, Tokyo, Japan), supplemented with 10% FBS (Japan Bioserum Co.Ltd, Hiroshima, Japan) and 100 units/ mL of penicillin–streptomycin (Sigma Japan, Japan) at 37 ºC and in a 5% of CO_2 humidified atmosphere. After 24 hours of culture, non-adherent cells were removed and the adherent cells were then cultured until semi-confluence was achieved. Cells were detached using a 0.25% trypsin-EDTA solution (Gibco BRL LifeTechnologies, Carlsbad, USA) and seeded in 35 mm of diameter thermo-responsive dishes (CellSeed, Japan) at a concentration of 2.5 x 10^5 cells per dish. Cultures were maintained for 21 days in osteogenic medium to commit cells into the osteogenic lineage.

Osteogenic Cell Sheets with Endothelial Cells

The use of endothelial cells in bone tissue engineered constructs has been proven to be promising in promoting *in vivo* vascularization and construct survival (34-38). Mature and functional endothelial cells can be isolated in significant numbers from the vein of umbilical cords in a relatively simple procedure. Therefore, these cells widely

used in research models involving angiogenesis/vascularization were chosen as initiators of the pre-vascularization of cell sheet-based constructs.

HUVECs were purchased from Lonza Japan (Tokyo, Japan), seeded in 75 cm^2 Primaria culture flasks (BD Biosciences, USA) and cultured in Endothelial Cell Growth Medium MV2 (ECGM, Promocell, Heidelberg, Germany), 3 days before the rBMSC seeded in thermoresponsive-dishes completed 21 days of culture. HUVECs at passage 3 were seeded in half of the dishes at a density of 1 x 10^5 cells per dish and co-cultures were maintained in ECGM supplemented with 10^{-8} M dexamethasone (FujiPharma, Japan), 50 µg/mL ascorbic acid (Sigma, Japan) and 10 mM beta-glycerophosphate (Sigma, Japan) for 3 additional days until transplantation.

Cell Sheets Recovery from thermo-responsive dishes

In order to recover the cells from the thermoresponsive dishes, culture medium was removed and replaced by 1 mL of PBS (Sigma, Japan). A poly(vinylidene difluoride) (PVDF, Immobilon-P, Millipore Corporation, USA) membrane with a diameter of 2 cm was placed over the cells in the thermoresponsive dishes and incubated at 20 ºC for 10 minutes. After this time, cell sheets spontaneously detached from thermoresponsive dishes attached to the PVDF membranes.

Cell Sheets Stacking

Osteogenic cell sheets were stacked immediately before transplantation. The first cell sheet was recovered from thermo-responsive dish using a PVDF membrane after temperature reduction. The cell sheet and membrane were then stacked on top of a second cell sheet, homotypic (control) or co-cultured with HUVECs, the temperature was reduced and the stacked cell sheets were recovered from the dish and transplanted as described in section 2.3.2.

Co-cultures

Heterotypic cell interactions involve direct cell contact or the production of soluble factors that will have a paracrine action on target cells, or frequently both(39). To study *in vitro* the direct cell interactions, direct co-culture models where more than one type of cells are cultured together can be used (39). The disadvantage of these models is that paracrine signalling is difficult to block which makes it complex to isolate the effect of direct contact communication. To study the indirect paracrine signalling between two different types of cells there are two different ways that avoid their physical contact, the use of conditioned medium or the use of co-culture inserts. The first one involves to culture one cell type in medium previously conditioned by the other cell type while the second involves culturing both cell types in the same medium at the same time but in different surfaces (39). The use of culture inserts is more accurate since it allows bi-directional communication between the cell types.

hBMSCs and monocytes/macrophages co-cultures

hBMSCs in passage 1 (Section 2.2.1) were plated in 24-well culture plates, 30 000 per well, and cultured in 600 uL of osteogenic medium for 24 hours before establishing the co-culture with monocytes/macrophages. Monocytes/macrophages were obtained by cell adhesion selection (40) from the hPBMNCs isolated after differential centrifugation of the buffy coats (Sections 2.2.1). In detail, 50 000 hPBMNCs were plated in 6.5mm Transwells® inserts(Corning, USA), with 0.4µm pore polycarbonate membrane, and left to adhere for 24 hours to select the monocytes/macrophage sub-population. After that time, non adherent cells were discarded and the indirect contact co-cultures were set by placing the inserts on the 24-well culture plates wells with the hBMSCs seeded 24 hours before. Both cell types were cultured in osteogenic medium, for 2, 5 and 7 days.

Bone Morphogenetic Protein-2 Role

The study of the role of specific molecules in a defined pathway, either *in vitro* or *in vivo*, has been successfully addressed by using respective antagonists capable of blocking their action. The use of an anti-Bone Morphogenetic Protein (BMP)-2 antibody is described in the literature as a way to block the action of soluble BMP-2 (41-42). The concentration of blocker with maximum effect was optimized by testing the effect of four dilutions (1:50, 1:100, 1:200 and 1:400) over hBMSC proliferation in the presence of monocytes/macrophages. As the blocking effect was maximum, and similar, for the 1:50 and 1:100 dilutions, anti-BMP-2 antibody was added to the medium of half of the hBMSCs in co-culture at a concentration of 1:100. Monocultures of hBMSCs in osteogenic medium were set as controls of the assay.

hASCs-derived osteoblasts and CBMNCs co-cultures

Isolated hASCs in passage 3 were cultured for 21 days in osteogenic medium in order to obtain osteoblasts. The osteoblasts derived from hASCs were seeded on the CRG membranes in a concentration of $82x10^3$ cells/cm^2 and cultured for 5 days in complete M199 medium plus 10% FBS and 1% A/b supplemented with osteogenic factors (as described in section 2.3.1), 50 µg/mL of endothelial cell growth supplement (ECGS, BD Biosciences, USA) and 50 µg/mL of heparin (Sigma, USA). After this time, the isolated CBMNCs were seeded over the hASCs-derived osteoblasts adhered onto the CRG membranes at a concentration of $82x10^4$ cells/cm^2 and maintained in culture for further 7 and 21 days. Monocultures of hASCs-derived osteoblasts and of monocytes/macrophages in the membranes were set as controls.

Human Bone Marrow MSCs-derived osteoblasts and hDFb co-cultures

Human bone marrow-derived mesenchymal stem cells (hMSCs) were purchased from Lonza (Lonza, Switzerland). Cells in passage 1 were cultured in DMEM (Sigma, USA) osteogenic medium for three weeks until osteogenic cells were obtained. A hMSC-

derived osteoblast cell suspension, with a cell density of 5 x 10^3 cells/mL, was prepared, transferred to 24-well culture plates (1mL per well) and incubated for 24 hours. After this time of culture, hDFb were prepared and added to the wells previously seeded with osteoblasts at a cell density of 2.5 x 10^3 cells/mL (1 ml per well) thus establishing the direct contact co-culture model. As the isolated fibroblasts had a higher proliferation rate than hMSCs-derived osteoblasts the cell number ration in the co-cultures was defined having that in consideration. Monocultures of osteoblasts were kept as controls in parallel with the co-culture for 2, 7 and 12 days. All cells were used in passage 3.

Involvement of Gap Junctional Communication

Gap junctional communication (GjC) is known to be involved in bone cellular differentiation (43-44), in cellular communication between endothelial cells and osteoblasts (45), in mechanical signal transduction between bone cells (44) and in immune response and tissue inflammation and repair (46). The most important gap junctional protein present in osteoblasts (47) is connexin 43 (Cx43) and GjC through this protein modulates osteoblast gene expression (48). Cx43 is also widely expressed in fibroblasts (49) and is involved, for instance, in skin wound healing increasing tissue repair rate (50-51).

The assessment of the involvement of GjC between the osteoblasts and fibroblasts in co-culture was targeted by using a chemical inhibitor of Gj communication, 18-alpha-Glycyrrhetinic acid (AGA) (Sigma, USA). This chemical has been described to effectively inhibit gap junctional communication between osteoblasts and other cell types and is therefore suitable to study this cell-cell communication mechanism (52-54).

AGA was dissolved in fresh dimethylsulfoxide (DMSO, Sigma, USA) and diluted to a working solution of 25 mM with PBS (Sigma, USA). The co-cultures were prepared as described above but AGA was added to the medium attaining a final concentration of

100 µM. This concentration was defined after previous toxicity testing. Osteoblasts were cultured in the presence of 0, 10, 50, 100 and 500 µM of AGA and viability and calcein-AM transfer between cells were evaluated. The concentration of 100 µM of AGA that presented maximum inhibition of calcein-AM transfer while having no effect over cell viability. Therefore, AGA was added to the cultures 24 hours before the establishment of the co-cultures and of the parachute assay. In the case of the co-cultures, AGA was added to the culture medium every renewal, ie, every other day. Controls were made by adding either DMSO or AGA to the hMSC-derived osteoblasts monocultures in the same concentrations.

Parachute Technique

The visualization of GjC was achieved using the parachute technique where one type of cell is labelled with a Gj-permeable fluorescent dye and seeded (parachuted) over a second cell type. The assumption of occurring GjC between the cells is confirmed if the dye staining the parachuted cells permeate to the other cell type (55).

hDFb and hMSCs-derived osteoblasts were prepared separately by seeding 7 $x10^4$ cells per 24 well coverslip, 24 hours before the assay. At the time of the assay, hMSCs-derived osteoblasts or hDFb were labelled with 2 µM of calcein-AM (Invitrogen, USA) for 15 minutes at 37ºC. Cells were then thoroughly washed with PBS (Sigma, USA), seeded over the previous culture of the other cell type (7 $x10^3$ cells/well) and cultured for 15 minutes at 37ºC. Cultures were observed with the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) to assess if the Gj-permeable calcein was transferred from the “parachuted” cells to the cells in the monolayer.

2.3. *IN VIVO* Assays

2.3.1 Sub-cutaneous implantation of CRG membranes with co-cultures

Twenty-four 4-week-old female Balb/C nude mice with an average weight of 20 g (Charles River Laboratories Inc. USA) were anaesthetized with a mixture of ketamine

(1.2 mg/mouse s.c., Imalgene® 1000, Merial, Lyon, France) and medetomidine (20 µg/mouse s.c., Domitor®, Orion Corp., Finland) prepared in physiological serum. After the confirmation of analgesia/anaesthesia one incision was performed (reaching a maximum of 1.5 cm each) in the intrascapular region. Two craniolateral oriented pockets were created by blunt dissection and the implants (2 per animal except for the controls) were subcutaneously inserted. The incision was sutured and the mice transferred to heated recovery compartments and, when the recovery from analgesia/anaesthesia was confirmed, returned to their respective cages and kept under food and drink *ad libitum*. The number of animals per conditions, as well the times of implantation are summarized in table 2.1. After each implantation time, animals were euthanized with and intracardiac overdose of anaesthesia for implant retrieval and subsequent characterization.

Table 2.1 - Number of animals used per condition in the *in vivo* assays of the different works

Constructs	Conditions	Number of Animals	Implantation Time
CRG Membranes	7 days of in vitro co-culture	3	7 Days
		3	21 Days
	21 days of in vitro co-culture	3	7 Days
		3	21 Days
	Osteoblasts Monoculture for 7 days (Control)	3	7 Days
		3	21 Days
	Osteoblasts Monoculture for 21 days (Control)	3	7 Days
		3	21 Days
Cell Sheets	Osteogenic Cell Sheets	3	7 Days
		3	3 Weeks
		3	6 Weeks
	Silicone Membranes (Control)	3	7 Days
		3	3 Weeks
		3	6 Weeks
	Stacked Osteogenic Cell Sheets with HUVECs	5	7 Days
	Stacked Osteogenic Cell Sheets (Control)	3	

2.3.2 Cell Sheets Transplantation

The cells sheets were transplanted into a mouse subcutaneous dorsal flap model. Male or female 6 weeks old nude mice (Charles River Japan, Yokohama, Japan) were respectively used for the transplantation of osteogenic cell sheets and osteogenic cell sheets with HUVECs. Animals were anesthetized with a constant flux of 4% of isofluorane. Dorsal skin was cut opened using 3x3cm cutting sides and recovered cell sheets were placed on mouse subcutaneous dorsal flap and left to adhere to the connective tissue of dorsal skin for 5 minutes. After that time, the PVDF membranes were peeled off from the adhered CSs and silicone membranes were placed over the cell sheets to prevent the contact between the cell sheets and the muscular tissue. Control mice were also defined by implanting only silicone membranes, as controls of the osteogenic cell sheets, or double osteogenic cell sheet constructs, as controls of the osteogenic cell sheets with HUVECs. Skin incisions were closed using 5-0 nylon sutures. Animals were kept with food and water *ad libitum*. The number of animals per conditions, as well the times of implantation are summarized in table 2.1. After each implantation time animals were euthanized with CO_2 and implants were recovered for characterization.

2.4 Characterization Techniques and Methodologies

2.4.1 Flow Cytometry Analysis

The phenotype of the isolated hPBMNCs, hCBMNCs and hBMSC was screened by flow cytometry using the markers depicted in table 2.2, known to be expressed or not by the targeted cells. Flow cytometry allows a fast and accurate screening, within a population, of the number of cells simultaneously positive for a defined panel of markers. Panels encompassing CD14, CD45 and CD34 markers were used to characterize the mononuclear population of cells from the peripheral and cord blood (56). The expression of CD73, CD90 and CD105 markers in more than 95% of the

population permits to confirm them as mesenchymal stem cells (57) while CD31 marker identify cells both from the monocytic and endothelial lineages (58).

Table 2.2 - Panels of antibodies used to characterize, by flow cytometry, the different isolated cell populations

Cells	Antibodies Panel
hPBMNCs	CD14-PE (BD Pharmingen, USA) CD45-FITC (BD Pharmingen, USA) CD34-PE (BD Pharmingen, USA) CD105-FITC (eBiosciences, USA) CD90-APC (eBiosciences, USA)
hCBMNCs	CD14-PE (BD Pharmingen, USA) CD45-FITC (BD Pharmingen, USA) CD34-PE (BD Pharmingen, USA) CD105-FITC (eBiosciences, USA) CD90-APC (eBiosciences, USA) CD73-PE (BD Pharmingen, USA)
hBMSCs	CD45-FITC (BD Pharmingen, USA) CD73-PE (BD Pharmingen, USA) CD34-PE (BD Pharmingen, USA) CD31-APC (R&D Systems, USA) CD105-FITC (eBiosciences, USA) CD90-APC (eBiosciences, USA)

PE: Phycoerythrin; FITC: fluorescein isothiocyanate; APC: allophycocyanin

The cells to be analyzed were ressuspended in cold PBS with 2% (w/v) bovine serum albumin (BSA, Sigma, USA) solution in PBS (BSA/PBS). Each cell suspension (100 µL), containing $2x10^5$ cells, was incubated with the antibodies at the concentration advised by the manufacturer (table 2.2). After incubation for 20 minutes at room temperature, in the dark, cells were washed with PBS/BSA and ressuspended in PBS with 1% of formaldehyde. Cells were analyzed in a BD FACScalibur flow cytometer (BD

Biosciences, USA). Cells of interest were gated in a forward versus side scatter dot plot with a linear scale. Isotype controls were made to discern non-specific from specific staining. A minimum of 10 000 gated events were acquired and displayed in dot plots created using the Cyflogic software (version 1.2.1, CyFlo Ltd, Finland).

2.4.2 Proliferation Assessment

Cell proliferation can be reliably assessed by dsDNA quantification. However, it is a non-specific assay which means that, in the case of direct co-cultures, the dsDNA from the two cell types was not distinguishable. Therefore, in the case of co-cultures of hMSCs-derived osteoblasts with hDFb and in monoculture, cell number along the time of the cultures was additionally followed using a cell tracking fluorescent marker, which permitted to quantify independently the cell number of the different cell types in the direct co-culture.

PicoGreen Quantification Assay

Proliferation of cells in the co-cultures of hMSCs-derived osteoblasts with hDFb and in monoculture was assessed after total dsDNA quantification along the culture time. For this, the PicoGreen Quantification Kit (Invitrogen, USA) was used. Picogreen is a fluorescent staining that binds to dsDNA and therefore permit to measure the proportional fluorescence enhancement (59). A protocol was defined according to manufacturer instructions and applied after obtaining a lysate, by consecutive osmotic and thermal shocks, of the cells of interest. Standard samples were prepared from a 2ug/mL standard solution of dsDNA by serial dilutions. The samples and standards were mixed with the PicoGreen reagent, previously diluted 200-fold in Tris-EDTA buffer, on a 1:1 ratio in a 96-well white opaque plate (Corning, USA) and incubated for five minutes protected from light. Fluorescence was then read at 480 nm of excitation and 520 nm of emission in a Synergy HT microplate reader (Bio-Tek, USA). A standard

curve was made with the standards readings for extrapolation of the samples dsDNA concentration.

Image Analysis

In the beginning of each experiment a hMSCs-derived osteoblasts cell suspension was prepared and incubated with a 2 μM solution of 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Sigma, USA) in culture medium for 15 minutes at 37 ºC. After, cells were washed 3 times in warm medium and seeded for the experiments as described above. After each time point, three samples of each condition were fixed with formalin and stained with nuclear stain 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA). Fluorescent micrographs of three independent fields of each one of the analyzed samples were taken using the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and the Axiovision software (Zeiss, Germany). The micrographs were analysed using the image-processing software ImageJ (Wayne Rasband, NIH, USA). The fraction of hMSCs-derived osteoblasts in the co-cultures was determined by dividing the number DiI positive cells by the total number of cells given by DAPI staining. The direct correlation between the dsDNA values and the cell number (60) allowed to extrapolate the dsDNA values corresponding to the fraction of hMSCs-derived osteoblasts in culture.

2.4.3 Alkaline Phosphatase Activity Quantification

Alkaline phosphatase (ALP) is a common hydrolase thought to be involved int he mineralization process by osteogenic cells(28). This enzyme activity quantification has been extensively used to confirm the osteogenic differentiation or osteogenic activity (28). The analysis was performed in the same cell lysates used for the DNA quantification and obtained by osmotic and thermal shocks, and following an adapted *p*-nitrophenol assay (28, 61). In this assay, *p*-nitrophenil phosphate was used as an ALP substrate. ALP, if present, hydrolysed *p*-nitrophenil, colourless, into *p*-nitrophenol

(yellow). In each assay 20 µL of lysate were incubated with 80 µL of *p*-nitrophenil phosphate solution (0.2% w/w, in diethanolamine, Sigma, USA) in a transparent 96 well microplate, at 37ºC, for 45 minutes. The reaction was stopped using 80 µL of a 2M NaOH (Sigma, USA) and 0.4 mM EDTA (Sigma, USA) solution. The optical density of the samples was read at 405 nm. A calibration curve was previously prepared using the *p*-nitrophenol (Sigma, USA) standards of 0, 0.05, 0.10, 0.15 and 0.20 µmol/mL and used to extrapolate the ALP activity values. These values were then normalized against dsDNA results obtained within the same experiments.

2.4.4 Alizarin Red Staining

Alizarin Red S, an anthraquinone derivative, is used to identify calcium deposits in *in vitro* cultures and tissue mineralization(62). Calcium forms an Alizarin Red S-calcium complex in a chelation process, and the end product is birefringent (62). Despite the non specific character of the reaction, the elements such as magnesium, manganese, barium, strontium, and iron that might interfere are usually present in sufficient concentrations. The identification of mineralized tissue was carried out both in *in vitro* and *in vivo* samples. In the case of *in vitro* cultured cells, these were fixed in 3.7% (v/v) buffered formalin (Sigma, USA) for 20 minutes at room temperature, washed in PBS to remove the formalin and washed with ddH_2O. For tissue sections, these were depparafinized and washed with ddH_2O.

A solution of 0.1 % w/v of alizarin red S (Sigma, USA) was prepared in ddH_20 and the pH was adjusted to 4.6 by using a 10% ammonium hydroxide (Sigma, USA) solution. The samples, both in vitro and in vivo, were incubated in this solution until a red colour/purple colour developed indicating the calcified tissue. After this, sections were quickly dipped in hematoxylin, washed in running tap water, dehydrated, mounted and visualized with a visible light microscope. *In vitro* samples in tissue culture well plates were further washed in ddH_2O and visualized with a visible light microscope.

2.4.5 Acetylated Low Density Lipoprotein Uptake

Monocytes/macrophages and endothelial cells possess scavenger receptors at their surface specific for acetylated low density lipoprotein (AcLDL) (63). When taken up by those cells, AcLDL accumulates intracellularly, which allows to target and to identify specifically endothelial and macrophagic cells. This technique was used to identify cells of these lineages adhered on the CRG membranes.

After the different time points the CRG membranes with the cells were incubated with 10 µg/mL of the fluorescent complex DiI-AcLDL (Invitrogen, USA) for 15 minutes, at 37ºC. Cells were then washed with warm medium and fixed with 3.7% (v/v) buffered formalin (Sigma, USA) for 20 minutes at room temperature. Cells were then counterstained with DAPI and visualized using a Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany).

2.4.6 Immunostaining

The principle of immunostaining techniques that use antibodies to target specific markers is the same as for flow cytometry. Both can be used to identify specific cell types or the particular expression of that marker, although immunostaining of cultured cells, immunocytochemistry, or of fixed tissues, immunohistochemistry, is only quantitative if coupled to image analysis methods.

Immunocytochemistry

After each culture time point, the samples were fixed in a 3.7% (v/v) buffered formalin solution for 30 minutes at room temperature. After fixation, cells were washed three times in PBS and either kept at 4ºC or directly used for immunocytochemistry. Non-specific binding was blocked by incubation with a 3% (w/v) BSA/PBS solution for 30 minutes. Cells were then incubated with the primary antibody diluted in 1% (w/v) BSA/PBS for 1 hour at room temperature at a pre-determined concentration (Table

2.3). Cells were then washed three times with PBS and incubated for 1 hour at room temperature with an appropriate AlexaFluor (Invitrogen, USA) secondary antibody at 1:500 dilution. Cells were again washed in PBS and counterstained with DAPI (Invitrogen, USA) or Hoechst 33342 (Wako, Japan). Cells were analyzed in an Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) or in an Eclipse TE2000-U (Nikon, Japan).

Table 2.3 - Antibodies used to perform immunocytochemistry to characterize different cultured cells

Antibody	Antibody Dilution	Screened Cells
Mouse anti-human CD14-FITC (BD Pharmingen, USA)	1:15	hPBMNCs
Mouse anti-human STRO-1(Invitrogen, USA)	1:100	hBMSCs
Mouse anti-human CD31 (Dako Cytomation, Denmark)	1:30	hASCs-derived osteoblasts and CBMNCs in co-culture
Rabbit anti-human vWF (Dako Cytomation, Denmark)	1:100	
Mouse anti-human FSP (Sigma, USA)	1:500	hDFb
Rabbit anti-human Connexin 43 (ABM, Canada)	1:100	hBMSCs-derived osteoblasts and hDFb in co-culture
Mouse anti-human CD31 (Dako Cytomation, Denmark)	Ready to use	HUVECs

FITC: fluorescein isothiocyanate; STRO-1: Stromal Cell Precursor Surface Antigen-1; vWF: von Willebrand Factor, FSP: Fibroblast surface protein

Immunohistochemistry

Sections were depparafinized, immersed in an antigen retrieval solution (table 4) and heated in the microwave for 2 minutes at maximum power. After cooling, sections were incubated with a peroxidase inhibitor solution (table 2.4) for 10 minutes, washed with

PBS and incubated with a blocking solution (table 2.4) for 20 minutes to impede non-specific protein interactions. Sections were then washed in PBS-tween 20 (PBS-T) and incubated with the primary antibody, at a pre-determined concentration (table 2.5) overnight at 4ºC. Sections were washed in PBS-T and incubated for 45 minutes at room temperature with a byotinilated universal secondary antibody for 30 minutes (Table 4). After washing, cells were incubated first with a streptavidin- horseradish peroxidase (HRP) conjugate (Table 2.4) and then with enzyme substrate 3,3'-diaminobenzidine (DAB) chromogenic substrate (Table 2.4).

Stained sections were mounted and analysed with an Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany), or with an Eclipse E800 microscope (Nikon, Tokyo, Japan).

Table 2.4 - Reagents used along the immunohistochemistry procedure for different sets of experiments

Reagent	CRG-Based Implants	Cell Sheets-Based Implants
Antigen Retrieval Solution	10mM Sodium Citrate, 0.05% Tween 20, pH 6.0	Target Retrieval Solution, pH 6 (Dako)
Peroxidase Inhibitor	3% H_2O_2 in H_2O	Peroxidase Blocking (Dako)
Blocking Solution	3% BSA	Serum (Dako)
Secondary Antibody	Universal (Vector Labs)	Universal (Dako)
Streptavidin- HRP conjugate	Vector ABC system (Vector Labs)	Dako LSAB2 kit (Dako)
Enzyme chromogen-substrate	DAB (Vector Labs)	DAB (Dako)

Table 2.5 - Antibodies used to perform immunohistochemistry to characterize different samples.

Antibody	Dilution	Screened Implants
Mouse anti-human CD31 (Dako Cytomation, Danmark)	1:30	Carragenan-based
Mouse anti-human osteocalcin (AbD Serotec, UK)	1:100	Carragenan-based
Rabbit anti–mouse/rat osteocalcin (Millipore Corporation, Billerica, USA)	1:200	Cell Sheets/Single Osteogenic Cell Sheet
Goat anti-mouse/rat SRY (Santa Cruz Biotechnology, USA)	1:100	Cell Sheets/ Stacked Osteogenic Cell Sheets with and without HUVECs
Mouse anti-human CD31 (Dako Cytomation, Danmark)	RTU	Cell Sheets/ Stacked Osteogenic Cell Sheets with and without HUVECs
Rabbit anti-human/mouse/rat collagen I (Abcam, USA)	1:200	Cell Sheets/ Stacked Osteogenic Cell Sheets with and without HUVECs
Rabbit anti-human/rat/mouse osteopontin (Abcam, USA)	1:100	Cell Sheets/ Stacked Osteogenic Cell Sheets with and without HUVECs
Rabbit anti-human/mouse/rat osterix (Santa Cruz Biotechnology, USA)	1:100	Cell Sheets/ Stacked Osteogenic Cell Sheets with and without HUVECs

RTU: ready to use

2.4.7 Hematoxylin and Eosin Staining

Retrieved tissues after implantation were fixed in 4% paraformaldehyde at 4ºC, for 12 hours, and then embedded in paraffin (Microm, Germany) and sectioned. The thickness of the sections varied between 3 µm for the implanted CRG membranes and 10 µm for the cell sheets.

Standard hematoxylin and eosin stainings were used to make a general morphological assessment of the sectioned tissues. Sections were deparaffinized in xylene (Thermo Scientific, USA) and hydrated by incubation with increasingly diluted ethanol solutions (75%, 90% and 95%). Sections were then washed in water and incubated with hematoxylin for 5 minutes. After this, sections were washed under running tap water for 10 minutes and finally ddH_2O. After the incubation with eosin for 1 minute, sections were dehydrated with ethanol, washed with xylene and mounted.

2.4.8 Gene Expression Analysis

Quantitative Real Time Polymerase Chain Reaction was used to evaluate the expression of several osteogenic related genes. The whole procedure consists on: extraction of RNA, cDNA production from the extracted RNA and real-time quantification of the amplification of specific sequences using specific primers.

RNA extraction and cDNA production

Messenger RNA (mRNA) of cells (section 2.2.3) and tissues (section 2.3) was extracted, after each time-point, using Tri-reagent (Sigma, USA) according to manufacturer instructions. 800 µl of the reagent were added per $1X10^6$ cells or 50 mg of tissue, and samples were homogenized by vigorous pipetting. Following 5 minutes incubation, 160 µl of chloroform (Sigma, USA) were added to each sample; samples were then incubated for 15 min at 4ºC and centrifuged at 13000rpm and 4ºC for 15min. After the centrifugation, the aqueous part of each sample was collected and an equal volume of isopropanol (Sigma, USA) was added. Following an overnight incubation at -20ºC, samples were centrifuged at 9000 rpm and 4 ºC for 10 minutes. Supernatants were discarded and pellets were washed in ethanol, centrifuged at 9000rpm and 4ºC for 5 minutes and re-suspended in 12 µl of RNase/DNase free water (Gibco, UK). RNA quantity and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using iScript cDNA synthesis Kit (BioRad, USA) and the thermoblock of the MiniOpticon Real-Time PCR Detection System (BioRad, USA). An initial amount of 2 µg of mRNA was used in a total volume of 20 µl of RNase/DNase free water.

Quantitative Real Time PCR

Osteocalcin and osteopontin transcripts were quantified in the cDNA samples using a quantitative Real Time PCR reaction. For each sample GAPDH was used as the

housekeeping gene. The primers were designed using the Primer 3 software (v 0.4.0) and synthesized by MWG Biotech (Germany) (Table 2.6).

Table 2.6 - List of the primers and respective sequences used to perform qRT-PCR analysis of the expression of different genes.

Gene	Primers Forward (F) and Reverse (R) Sequences (5'-3')	Controls
hGAPDH	F: ACA GTC AGC CGC ATC TTC TT R: GAC AAG CTT CCC GTT CTC AG	Monocultures of hBMSCs with and without inhibitor; monocultures of hMSC-derived osteoblasts
hOsteocalcin	F: GTG CAG AGT CCA GCA AAG GT R: TCC CAG CCA TTG ATA CAG GT	
hOsteopontin	F: CCC ACA GAC CCT TCC AAG TA R: GGG GAC AAC TGG AGT GAA AA	
hGAPDH	F: ACA GTC AGC CGC ATC TTC TT R: GAC AAG CTT CCC GTT CTC AG	Monocultures of hASCs-derived osteoblasts
hOsteocalcin	F: GTG CAG AGT CCA GCA AAG GT R: TCC CAG CCA TTG ATA CAG GT	
hOsteopontin	F: CCC ACA GAC CCT TCC AAG TA R: GGG GAC AAC TGG AGT GAA AA	
Collagen I	F: AGC CAG CAG ATC GAG AAC AT R: ACA CAG GTC TCA CCG GTT TC	
VE-Cadherin	F: CAC AGT GCT GGC CAT GGA R: GTC CTG CGG ATG GAG TAT CC	Monocultures of CBMNCs
CD31	F:AAG GCC AGA TGC ACA TCC R:TTC TAC CCA ACA TTA ACT TAG CAG G	
vWF	F: CCC TGG GTT ACA AGG AAG AAA AT R: AGT GTC ATG ATC TGT CCT CCT CTT AG	
mOsteocalcin	F:CTGAGTCTGACAAAGCCTTC R:GCTGTGACATCCATACTTGC	Stacked osteogenic cell sheets implanted without HUVECs
mCollagen I	F:GAGCGGAGAGTACTGGATCG R:GCTTCTTTTCCTTGGGGTTC	
mOsterix	F:GCTCCTCGGTTCTCTCCATCTG R:TGTGTTGCCTGGACCTGGTG	
mGAPDH	F:ACGGCCGCATCTTCTTGTGCA R:AATGGCAGCCCTGGTGACCA	
hGAPDH	F:TGCACCACCAACTGCTTAGC R:GGCATGGACTGTGGTCATGAG	
hVE-Cadherin	F:CACAGTGCTGGCCATGGA R:GTCCTGCGGATGGAGTATCC	

A concentration of 200 nM of primer was used in a final volume of 25 µl of sample. The Real Time PCR reaction was done using the iQ Syber Green SuperMix (BioRad, USA), in a MiniOpticon Real-Time PCR Detection System (BioRad, USA).

The relative quantification of gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Perkin Elmer User Bulletin No.2). All values were first normalized against GAPDH values and then against a chosen control (Table 2.6)

2.4.9 Calcium Quantification

The retrieved cell-sheet implants (Sections 2.3.2) were used for calcium quantification. Halves of the recovered implants were weighted and incubated in 0.5M HCl (Sigma, USA) to remove and dissolve the calcium. Calcium quantification in the obtained solutions was performed using the o-*cresolphtalein*-complexon method with the Roche Cobas kit (Roche Diagnostics, Germany). In a 96 well plate, 175 µL of reagent 1 (ethanolamine, 1M, pH10.6) were mixed with 10 µL of each sample and incubated for 5 minutes at room temperature. 70 µL of reagent 2 (*o*-cresolphtalein-com plexon 0,3 mM, hydroxy-8-quinoleine 13,8 mM, HCl 122 mM) were added at each well and incubated at 37ºC for 2 minutes. The absorbance of the samples was read at 570 nm in a microplate reader Synergy-HT (Bio-Tek, Germany). The calcium concentrations were extrapolated from the calibration curve obtained using serial dilutions of a 200 mg/L calcium chloride solution and then normalized using the initial tissue mass.

2.4.10 Micro-computed Tomography

In order to investigate the 3D structure of the mineralized tissue formed after transplantation, non-destructive techniques, X-Ray and Micro-Computed Tomography (SkyScan, Kontich, Belgium), were used. Recovered implants, after paraffin embedding, were cut in half and scanned in a high-resolution mode of 11.32 µm x/y/z and an exposure time of 1900ms. The energy of the scanner used was 50 keV with a

171 mA current. The μCT scans were followed by a 3D reconstruction of serial images and quantification of the mineralized tissue using a minimum threshold value of 70.

2.5. Statistical Analysis

Data was obtained from the minimum of 3 separate experiments with 3 replicates for each condition and averaged. Standard deviation is reported as a measure of sample deviation. Statistical analysis was performed using either student *t*-test for n=4 or one way ANOVA test. The first was applied to test the difference between the means of two independent groups subjected to different treatments. When more than two groups and/or treatments were compared at the same time, one-way ANOVA test was applied using Tukey-Kramer method as a *post-hoc* pairwise comparison test. For both tests, values were considered statistically significant for $p \leq 0.05$.

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Chapter 3

EFFECT OF MONOCYTES/MACROPHAGES ON THE EARLY OSTEOGENIC DIFFERENTIATION OF HUMAN BONE MARROW STROMAL CELLS

Chapter 3

EFFECT OF MONOCYTES/MACROPHAGES ON THE EARLY OSTEOGENIC DIFFERENTIATION OF HBMSC

Abstract

Heterotypic cell interactions are essential for the homeostasis of bone tissue, in particular the widely studied interaction between osteoblasts and osteoclasts. Closely related with osteoclasts are monocytes/macrophages. These have been proven before to produce osteogenic factors, namely bone morphogenetic protein (BMP-2), and to play a key role in bone metabolism. The mechanisms through which monocytes/macrophages interact with osteoblasts are however still not well understood.

The aim of this work was to assess the influence of human peripheral blood monocytes/macrophages over the early osteogenic differentiation of human bone marrow stromal cells (hBMSCs), in the presence of dexamethasone-supplemented osteogenic medium. The co-cultures were performed using porous transwells that allowed the interaction between both cell types through the production of paracrine factors. The potential effect of BMP-2 produced by monocytes/macrophages was addressed by adding to the co-cultures an anti-BMP-2 antibody.

It was found that hBMSCs cultured in the presence of monocytes/macrophages had a higher proliferation rate than hBMSCs cultured alone. The quantification of the early osteogenic marker alkaline phosphatase (ALP) revealed higher activity of this enzyme in cells in the co-culture throughout the time of culture. Both of these effects were inhibited when an anti-BMP-2 antibody was added to the cultures. Moreover, qRT-PCR for osteocalcin and osteopontin transcripts showed overexpression of both markers, although more evident for osteocalcin. Once again, the effect of monocytes/macrophages over hBMSC osteogenic differentiation was completely inhibited in the co-cultures by blocking BMP-2.

The present work confirmed that monocytes/macrophages produce BMP-2 that promotes osteogenic differentiation and proliferation of hBMSC cumulatively to dexamethasone-supplemented medium. This potentially implies that monocyte/macrophages have a stronger role in bone homeostasis than supposed so far.

Chapter based on Pirraco RP, Reis RL, Marques AP, Effect of Monocytes/Macrophages on the early osteogenic differentiation of hBMSC, (2010) submitted.

3.1 Introduction

It is well established that heterotypic cellular interactions in bone microenvironment are essential for bone homeostasis. The demonstration that cells of the osteoblastic lineage control the formation and activity of bone resorbing cells, the osteoclasts (1) is even more relevant for biologists that intend to understand bone biology and in particular bone remodeling and regeneration(2-4). Bone formation and bone resorption, essential for a normal bone turnover, are coupled processes (5-6) that co-exist within a delicate balance, controlled by factors such as hormones(7) and other growth factors (8). Osteoclasts, being multinucleated and highly specialized cells derived from the hematopoietic lineage that have the function of resorbing bone, are seen as critical players in this well balanced regulatory process (2-3, 9). Also from the hematopoietic lineage, and closely related to osteoclasts, are circulating peripheral blood monocytes which can easily reach almost any tissue in the human body(10). While in physiological conditions monocytes differentiate into resident macrophages after tissue infiltration (10), upon injury or infection, monocytes are recruited to the injury site and differentiate into activated macrophages (10) participating in the tissue response. In the particular case of bone tissue, after acquiring an inflammatory phenotype, for instance at a fracture site, macrophages can differentiate into osteoclasts (11-12).

The role of monocytes/macrophages in bone remodeling and regeneration certainly does not simply rely on this phagocytic lineage interplay. Monocytes/macrophages are linked to the production of several cytokines and growth factors like tumor necrosis factor (TNF)-alpha, transforming growth factor (TGF)-beta, platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and others (13-14). Additionally, it has been proven that macrophages produce several osteogenic factors such as for instance 1,25-dihydroxyvitamin D3 (15) and BMP-2 (16). All these molecules have a potential effect on other cells present at the fracture healing site, namely mesenchymal stem cells (MSCs) and osteoblastic progenitors (13). Furthermore it is well known that

activated monocytes/macrophages contribute to *in vitro* vascular calcification by means of affecting the osteoblastic differentiation of calcifying vascular cells (CVCs), a subpopulation of osteoblast-like cells derived from the artery wall (17). Recently, some works focusing on bone resident macrophages proposed a complex bone regulatory role for those cells (18-19). These works suggested that macrophages can be key players in bone homeostasis, acting both in bone resorption by regulating osteoclasts activity, and in osteoblast mineralization ability through cytokine production. However, the precise effect of monocytes/macrophages over mesenchymal progenitor cells has been subject of very few studies, which either focus on the immunomodulatory effect of the MSCs (20-22) or on the osteoclastic differentiation potential of macrophages (12, 23-24).

BMP-2 is part of the super family of TGF-β and is proven to induce bone formation *in vitro* and *in vivo* (25-28). Its potential for bone regeneration is attested by several clinical studies involving this protein (26). Champagne et al (16) demonstrated, *in vitro*, that macrophage cell lines influence the differentiation behavior of human MSCs by producing BMP-2, which in turn triggered its osteogenic differentiation. This was an important finding since it eventually implicates macrophages in the process of bone healing.

The objective of the present study was therefore to assess the effect of human peripheral blood monocytes/macrophages over the osteogenic differentiation of human bone marrow stromal cells (hBMSCs) in order to understand this cross-talk and to complement what was previously reported with murine and human macrophage cell lines. For that, an indirect co-culture system, using culture inserts, was set with hBMSCs and human peripheral blood-derived monocytes/macrophages under standard osteogenic conditions in the presence of an anti-BMP-2 antibody. The use of osteogenic culture conditions intended to further explore the effect of the culture

medium, and in particular of the dexamethasone supplement, over the role of monocytes/macrophages in the osteogenic differentiation of hMSCs.

3.2 Materials and Methods

3.2.1 Cells Isolation and Culture

Human bone marrow aspirates (n=4) were obtained from routine hip revision surgeries in Hospital da Prelada, Porto, Portugal, after informed consent. Buffy coats (n=4) were obtained from the Portuguese Blood Institute (IPS, Porto, Portugal). All samples were acquired under cooperation agreements with the 3B's Research Group, University of Minho, Portugal.

Mononuclear cells from Human bone marrow samples were obtained after homogenization of the samples and differential centrifugation using Histopaque 1077 (Sigma, USA). Cells were then cultured at 37ºC, in a humidified atmosphere with 5 % CO2, in Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA) supplemented with 10% of FBS (Invitrogen, USA) and 1% of antibiotics (A/B, Invitrogen, USA). After human bone marrow stromal cells (hBMSCs) reached confluence at passage 1, 30 000 cells were plated in 24-well culture plates and cultured in 600 uL of osteogenic medium composed of DMEM plus 10% FBS and 1% of A/B, and supplemented with 10 mM beta-glycerophosphate (Sigma, USA), 50 µg/mL of ascorbic acid (Sigma, USA) and 10^{-8}M of dexamethasone (Sigma, USA) (29) for 24 hours before establishing the co-culture with monocytes/macrophages.

Human peripheral blood mononuclear cells (hPBMNCs) were isolated from buffy coats following the differential centrifugation procedure using Histopaque 1077 described above. Monocytes/macrophages were obtained by cell adhesion selection (30). Briefly, 50 000 hPBMNCs were plated in 6.5mm Transwells® inserts, with 0.4µm pore polycarbonate membrane (Corning, USA), and left to adhere for 24 hours to select the monocytes/macrophage sub-population. After that time, non adherent cells were

discarded and the indirect contact co-cultures were set by placing the inserts in the 24-well culture plates wells with the hBMSCs seeded 24 hours before. Both cell types were cultured in osteogenic medium, for 2, 5 and 7 days. Anti-BMP-2 antibody (Abcam, UK) was added to the medium of half of the hBMSCs in co-culture at a dilution of 1:100 obtained after optimization (data not shown). Monocultures of hBMSCs in osteogenic medium were set as controls of the assay.

3.2.2 Flow Cytometry

After isolation, hPBMNCs were screened by flow cytometry for the following markers: CD14-PE, CD45-FITC and CD34-PE (all from BD Pharmingen, USA), and CD105-FITC and CD90-APC (eBiosciences, USA). hBMSCs, at passage 1, were tested for the expression of CD45-FITC, CD73-PE and CD34-PE (all from BD Pharmingen, USA), CD31-APC(R&D Systems, USA), and CD105-FITC and CD90-APC (eBiosciences, USA) markers. Cells were ressuspended in cold PBS with 2% BSA and 100 uL of cell suspension with $2x10^5$ cells, and incubated with the antibodies at the concentration advised by the manufacturer. After incubation for 20 minutes at room temperature in the dark, cells were washed with PBS/BSA and ressuspended in PBS with 1% of formaldehyde. Tubes were analyzed in a BD FACScalibur flow cytometer (BD Biosciences, USA). Cells of interest were gated in a forward versus side scatter dot plot with a linear scale. Isotype controls were made to discern non-specific from specific staining. A minimum of 10 000 gated events were acquired and displayed in dot plots created using the Cyflogic software (version 1.2.1, CyFlo Ltd, Finland).

3.2.3 dsDNA quantification

Proliferation of hBMSCs was assessed after total dsDNA quantification along the different culture time points. The test was performed using the PicoGreen

Quantification Kit (Invitrogen Corporation, California, USA), according to manufacturer instructions, in a lysed cell suspension obtained after osmotic and thermal shocks. Fluorescence was read in a microplate reader (Bio-Tek, USA) at 485 ex/ 525 em.

3.2.4 Alkaline phosphatase (ALP) activity quantification

The ALP activity quantification was performed in the same cell lysates used for the DNA quantification obtained by osmotic and thermal shocks and following an adapted p-nitrophenol assay (29). Briefly, 20 uL of lysate were incubated with 80 uL of p-nitrophenol phosphate solution (0.2% w/w, in diethanolamine, Sigma, USA) at 37ºC, for 45 minutes. The reaction was stopped using 80 uL of a 2M NaOH and 0.4 mM EDTA solution. The optical density of the samples was read at 405 nm. A calibration curve was previously prepared using the p-nitrophenol standards (Sigma, USA) and used to extrapolate the ALP activity, which was then normalized against dsDNA results.

3.2.5 Immunocytochemistry

After 24 hours of hPBMNCs culture, the polycarbonate membranes were cut off from the inserts and the selected monocytes/macrophages were stained for CD14-FITC monocytic lineage marker using a mouse anti-human monoclonal antibody (BD Pharmingen, USA) in a concentration of 1:15. Non-specific binding was blocked by incubation with a 3% bovine serum albumin (BSA, Sigma, USA) solution in PBS for 30 minutes. After 1 hour incubation at room temperature, Cells were washed in PBS and counterstained with DAPI (Invitrogen, USA) nuclear staining.

The presence of the stromal progenitor marker STRO-1 in the hBMSCs cultured for 24 hours was screened using a mouse anti-human monoclonal antibody (Invitrogen, USA), diluted 1:100. Briefly, after fixation with formalin, cells were incubated with a 3% BSA solution in PBS for 30 minutes to block non-specific binding. Cells were then

incubated with the primary antibody for 1 hour at room temperature and washed in PBS. Alexafluor 488 secondary antibody (Invitrogen, USA) was incubated with cells for one hour at room temperature. Cells were then washed in PBS and counter-stained with DAPI (Invitrogen, USA) nuclear staining.

The expression of CD14 and STRO-1 in the monocytes/macrophages and hBMSCs was analysed using the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany).

3.2.6 PCR analysis

RNA extraction and cDNA production

mRNA of hBMSCs was extracted, after each culture time-point, using Tri-reagent (Sigma, USA) according to manufacturer instructions. Briefly, 800 µl of TriZol were added per $1X10^6$ cells and samples were homogenized by vigorous pipetting. Following 5 minutes incubation, 160 µl of chloroform (Sigma, USA) were added to each sample; samples were then incubated for 15 min at 4℃ and centrifuged at 13000rpm and 4℃ for 15min. After the centrifugation the aqueous part of each sample was collected and an equal volume of isopropanol (Sigma, USA) was added. Following an overnight incubation at -20℃, samples were centrifuged at 9000 rpm and 4 ºC for 10 minutes. Supernatants were discarded and pellets were washed in ethanol, centrifuged at 9000rpm and 4℃ for 5min, and re-suspended in 12 µl of RNase/DNase free water (Gibco, UK). RNA quantity and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using iScript cDNA synthesis Kit (BioRad, USA) and the MiniOpticon Real-Time PCR Detection System (BioRad, USA). An initial amount of 2 µg of mRNA was used in a total volume of 20 µl of RNase/DNase free water.

Quantitative Real Time PCR

Osteocalcin and osteopontin transcripts were quantified in the cDNA samples using a quantitative Real Time PCR reaction. For each sample GAPDH was used as the housekeeping gene. The primers were designed using the Primer 3 software (v 0.4.0, Whitehead Institute, USA) and synthesized by MWG Biotech (Germany) as follows: osteocalcin forward 5' GTG CAG AGT CCA GCA AAG GT 3' and osteocalcin reverse 5' TCC CAG CCA TTG ATA CAG GT 3'; osteopontin forward 5' CCC ACA GAC CCT TCC AAG TA5 3' and osteopontin reverse 5' GGG GAC AAC TGG AGT GAA AA 3', GAPDH forward 5' ACA GTC AGC CGC ATC TTC TT 3' and GAPDH reverse 5' GAC AAG CTT CCC GTT CTC AG 3'. A concentration of 200 nM of primer was used in a final volume of 25 µl of sample. The Real Time PCR reaction was done using the iQ Syber Green SuperMix (BioRad, USA), following manufacturer's instructions, in a MiniOpticon Real-Time PCR Detection System (BioRad, USA).

The relative quantification of osteocalcin and osteopontin expression was performed using the $2^{-\Delta\Delta CT}$ method (Perkin Elmer User Bulletin No.2). All values were first normalized against GAPDH values and then the co-cultures values against monocultures values.

3.2.7 Statistical analysis

Data was obtained from 4 separate experiments with 3 replicates for each condition and averaged. Standard deviation is reported as a measure of sample deviation. Statistical analysis was performed using student *t*-test for n=4 and values were considered statistically significant for $p \leq 0.05$. In the case of the Quantitative Real Time RT-PCR results, the statistical analysis was performed in the ΔCt values (31).

3.3 Results

3.3.1 Cell characterization

The isolated hPBMNCs were screened by flow cytometry for the expression of CD14, CD45, CD31, CD34, CD90 and CD105 markers (figure 3.1). Isolated cells were negative for CD90 and CD34 while a small population (approximately 3 %) was positive for CD105. Pan-leukocyte marker CD45 was present in more than 96 % of the cells. CD14-positive cells were found to be around 3% of the analyzed cells while CD31-positive cells comprised 53% of the population.

In what concerns to hBMSCs, the mononuclear fraction obtained from the bone marrow was analysed before the seeding to perform the co-cultures. Cells were negative for CD45, CD34 and CD31. On the other hand, more than 99% of cells expressed the mesenchymal stem cell markers CD73, CD90 and CD105 (figure 3.1).

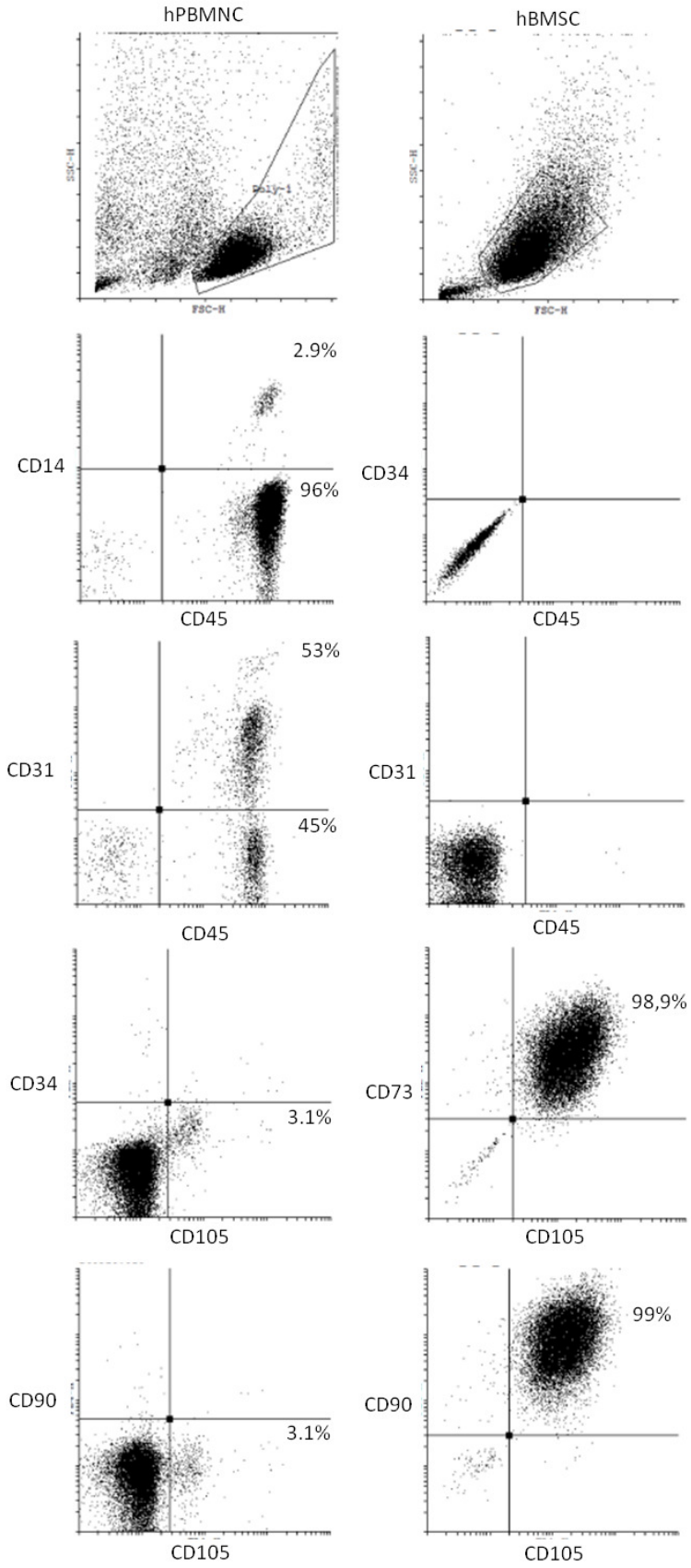

Figure 3.1 – Flow cytometry analysis of hPBMNCs and hBMSCs prior to seeding. In the case of hPBMNCs, cells were gated to exclude dead cells, platelets and debris of erythrocytes.

The selected populations, monocytes/macrophages and hBMSCs were characterized 24 hours after seeding, respectively for the expression of CD14 (Figure 3.2A) and STRO-1 (Figure 3.2B) markers.

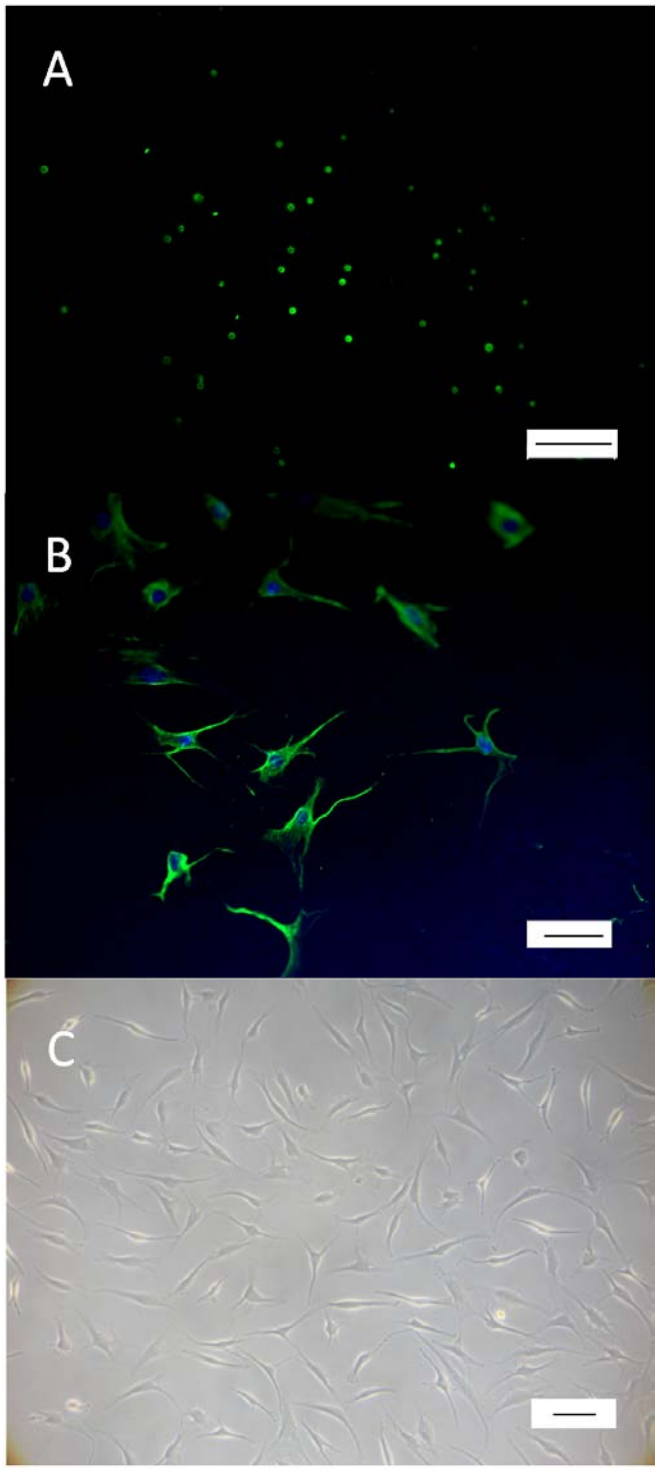

Figure 3.2 – Characterization of Monocytes/macrophages (A) and hBMSCs (B,C) cultures after seeding. Monocytes/Macrophages cultures are positive for CD14 (green) (A). The hBMSCs express the stromal progenitor marker STRO-1 (green) (B) and present the typical fibroblast-like morphology (C). Cell nuclei were counterstained with DAPI (blue) (B). Bar = 100 um

The immunocytochemistry results showed that the cell population isolated from the bone marrow, albeit with different intensities between cells, was positive for STRO-1 mesenchymal stem cell. In addition it also displayed a spindle shape morphology, as observed by optical microscopy (Figure 3.2C), that is typical of mesenchymal stem or progenitor stromal cells. The cells selected from the hPBMNCs were, as expected, positive for CD14.

3.3.2 Proliferation of hBMSC

The effect of monocyte/macrophages on the proliferation of hBMSCs was assessed by means of quantifying the dsDNA of the hBMSCs cultures at different time points (Figure 3.3). Results indicated that hBMSCs cultured in the presence of monocytes/macrophages proliferated more than cells in the control conditions from day 2 onward ($p<0.02$) This effect was eliminated when the co-cultures were established in the presence of the anti-BMP-2 antibody. For this condition the proliferation rate of the hBMSCs was comparable to what was determined for the hBMSCs monocultures

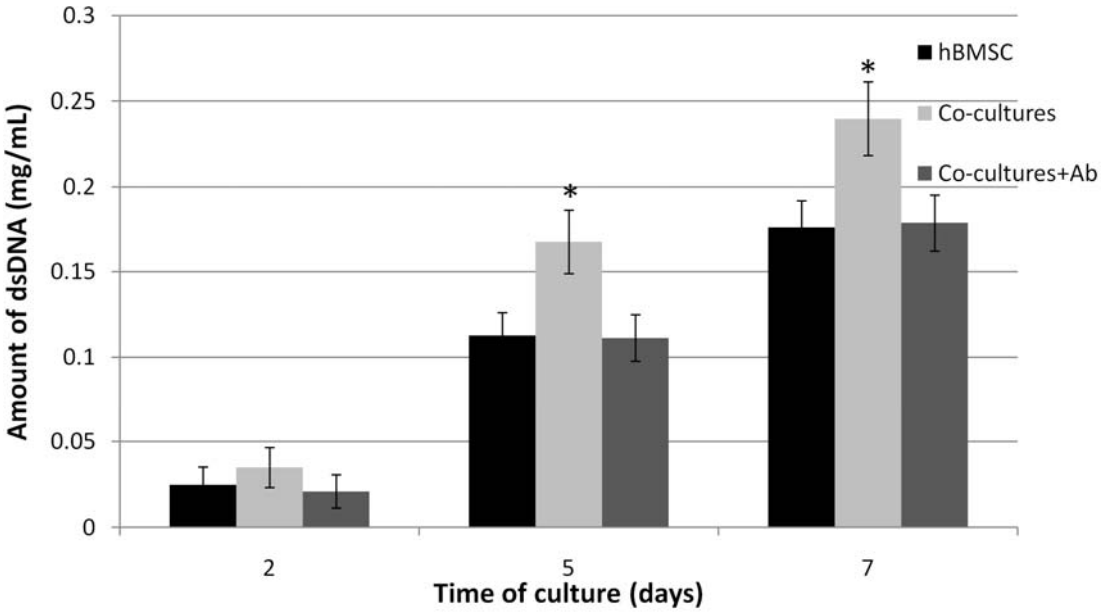

Figure 3.3 – Amount of dsDNA that correlates with cell number quantified along culture in hBMSCs (hBMSC) and in co-culture with monocytes/macrophages with (Co-Cultures+Ab) and without (Co-Cultures) anti-BMP-2 antibody. *$p<0.02$ relating to hBMSCs cultures for the same time point.

3.3.3 Osteogenic differentiation of hBMSC

The influence of monocytes/macrophages over the osteogenic differentiation of hBMSCs was assessed following different approaches: (i)the quantification of the ALP activity, considered an early osteogenic marker and the relative quantification of the (ii) osteopontin and (iii) osteocalcin transcripts.

The activity of the ALP enzyme at the second and seventh day of culture was significantly higher (p<0.02) in the hBMSCs co-cultured with monocytes/macrophages than in the hBMSC monocultures and in the co-culture with monocytes/macrophages in the presence of anti-BMP-2 antibody. At day 5, however, the opposite was observed; the ALP activity in both hBMSCs monocultures and co-cultures in the presence of anti-BMP-2 antibody had significantly higher (p<0.02) values than in the co-cultures. Moreover, it is worth noting that the presence of anti-BMP-2 antibody in the culture at day 2 was not sufficient to reverse the increased ALP activity observed in the co-culture of hBMSCs and monocytes/macrophages. The ALP activity in the hBMSCs co-cultured with monocytes/macrophages in the presence of anti- BMP-2 antibody was significantly higher (p<0.05) than in the hBMSCs in monoculture. (Figure 3.4)

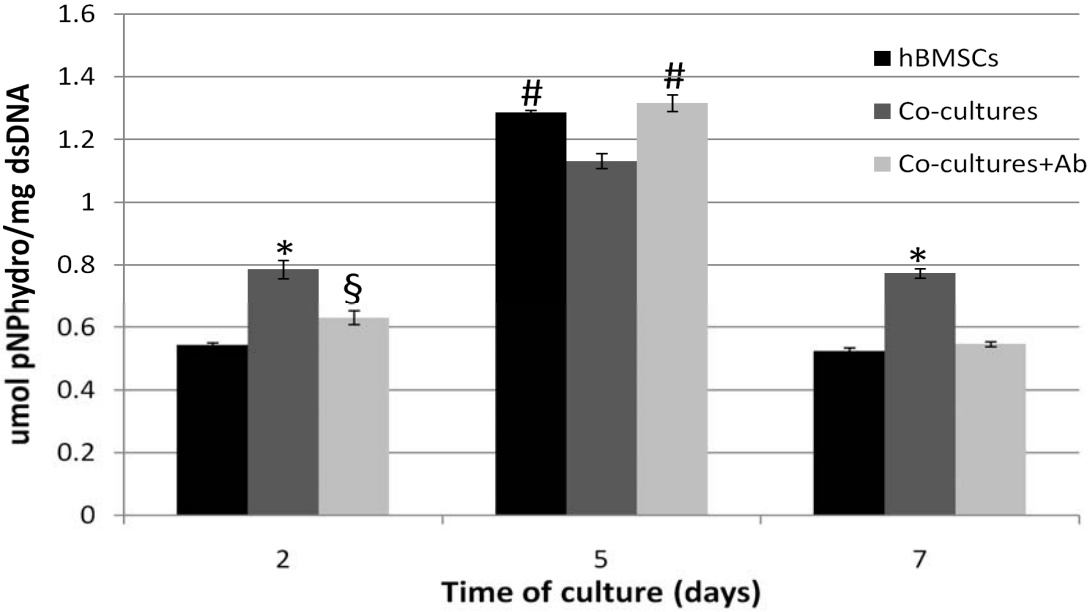

Figure 3.4 - Amount of hydrolysed p-nitrophenol phosphate that correlates with the ALP activity quantified along culture in hBMSCs (hBMSCs) and in co-culture with monocytes/macrophages with (Co-Cultures+Ab) and without (Co-Cultures) anti-BMP-2 antibody. Results were normalized against dsDNA values. § p<0.05 and * p<0.02 relating to hBMSCs for the same time point; # p<0.02 relating to Co-cultures for the same time point.

The osteogenic differentiation of the hBMSCs was screened at the molecular level by quantifying the osteopontin and osteocalcin expression relative to hBMSCs monocultures using real time RT-PCR (Figure 3.5).

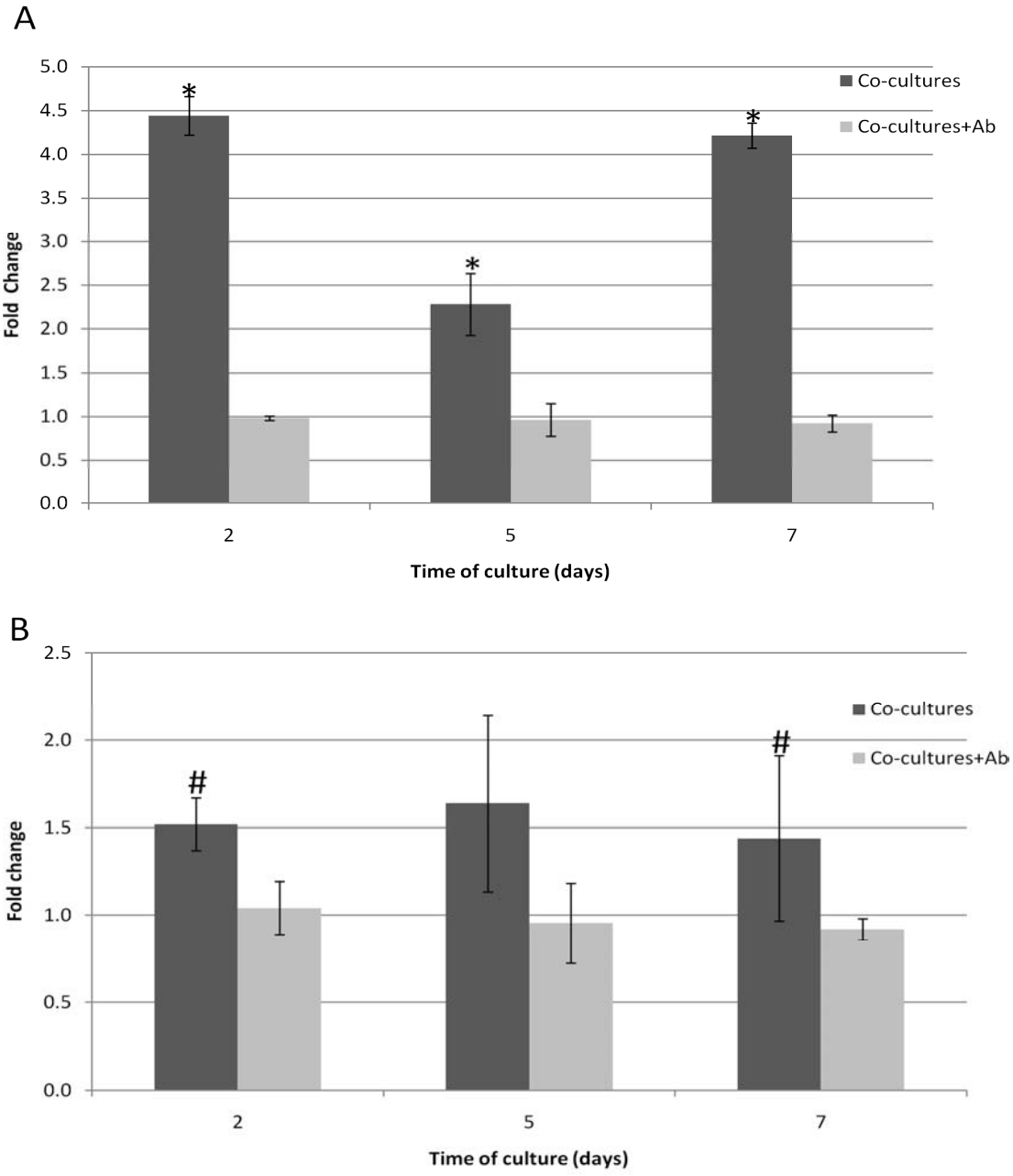

Figure 3.5 – Osteocalcin (A) and osteopontin (B) relative expressions calculated after quantitative real time RT-PCR. Results were first normalized against GAPDH and then against hBMSCs cultures and are presented as fold-change hBMSC monocultures. * $p<0.02$ and # $p<0.05$ statistically different ΔCt values in comparison to hBMSCs cultures at the same time point.

The real time RT-PCR results showed that for day 2 and day 7 of culture, the expression of osteocalcin in hBMSCs in co-cultures have a fold change of approximately 4 (p<0.02) in comparison to the monocultures condition. A similar trend was observed at day 5 though the fold change was around 2. In opposition, the expression of osteocalcin in the hBMSCs co-cultured in the presence of anti-BMP-2 antibody was not different from what was observed for the hBMSCs monoculture. (Figure 3.5A) On what concerns to the expression of osteopontin, an overexpression of this gene (fold change of 1.5) was observed in the hBMSCs in co-cultures in comparison to the hBMSCs in monoculture, for all the times of culture. Nonetheless, these observations were only statistically significant (p<0.05) at days 2 and 7. As for the osteopontin expression, the presence of anti-BMP-2 antibody in the cultures annulled the overexpression of the osteopontin gene as demonstrated by the fold change of 1 when comparing the co-cultures established in the presence of anti-BMP-2 antibody with the hBMSCs monoculture. (Figure 3.5B)

3.4 Discussion

The study of cellular interactions after bone injury and during regeneration is of extreme importance for Tissue Engineering in the sense that it can help researchers to better design engineered constructs and to predict their outcome when applied in the clinics. While after bone injury, monocytes are recruited to the injury site and differentiate into inflammatory macrophages, in normal physiologic conditions, its recruitment to bone leads to their differentiation in bone-specific macrophages(18-19). Monocytes/macrophages are also known to produce several osteogenic factors (15-16, 32-34) and several studies (16, 18) suggested its strong influence over the osteogenic function or the differentiation, of osteoblasts. Among those different osteogenic factors, is BMP-2(16, 18).

Based on this knowledge we hypothesized herein that peripheral blood monocytes/macrophages would also have a significant effect, , in particular through BMP-2, over the osteogenic differentiation of hBMSCs, the most studied cellular population that has been proposed for the construction of bone tissue engineering products(35). Furthermore, the magnitude of the monocytes/macrophages effect was further addressed by determining if the BMP-2 and the typical dexamethasone-containing osteogenic medium would have a synergistic effect, over a short period of time, on hBMSCs osteogenic differentiation.

In order to guarantee the validity of the proposed research strategy, our first concern was to use a progenitor cell population from the bone marrow, intimately linked with bone tissue. More than 95% of the isolated hBMSCs expressed CD73, CD90 and CD105 and were negative to CD14, CD34 and CD31 (<2%) conforming to the accepted mesenchymal stem cell markers pattern. After seeding cells in the cell culture plate wells, positivity for STRO-1 marker and spindle-shape morphology confirmed that cells maintained their mesenchymal phenotype (36). The marker profile of hPBMNCs, also confirmed the normal expression pattern of the peripheral blood leukocyte population ($CD45^+$) with 3% of the population expressing the monocytic marker CD14(37). Furthermore the hPBMNCs were negative (<2%) for CD34, CD31 and CD90 and a low number (approximately 3%) was positive for CD105. The confirmation of the monocyte/macrophage lineage of the cells co-cultured with the hBMSCs was demonstrated by the expression of CD14 in the cells selected by adherence. The different intensities of expression observed after the immunocytochemistry reflect the different expression pattern of CD14 in monocytes and macrophages (38).

Cell proliferation results showed that the presence of monocytes/macrophages had a positive effect on hBMSC proliferation and that this effect was inhibited by means of adding anti-BMP-2 antibody. By Real Time RT-PCR we detected the expression of BMP-2 by monocytes/macrophages (results not shown). This suggests that BMP-2

produced by monocytes/macrophages is involved in promoting hBMSCs proliferation. Reports in the literature concerning the effect of BMP-2 over bone marrow stromal cells proliferation are contradictory (39-41), which indicates that this effect may be highly dependent on the type of culture model used. Some works using hBMSCs showed a diminished proliferation when cells were cultured in the presence of BMP-2 (42-43). It is also well understood how cell proliferation and differentiation are inversely regulated (44). This seems to indicate that, in our model, BMP-2 acts synergistically with other soluble factor in the culture resulting in hBMSCs proliferation.

ALP activity in the co-cultures was inhibited to the level of monocultures, at day 2 and 7 of culture, after BMP-2 blocking, which reveal the direct effect of BMP-2 over ALP activity, as published by others (42). The fact that for day 5 the opposite result was verified, less ALP activity in the co-cultures, could be an artifact of the experiment related to the used culture model. This is further reinforced by the similar trend observed for the osteopontin expression. Nevertheless, the analysis of the expression of osteocalcin and osteopontin markers confirmed that the overexpression of those markers in the presence of monocytes/macrophages was eliminated by blocking BMP-2. Once again, this is coherent to what is described as the effect of BMP-2 over the expression of osteogenic markers in hBMSCs (42). Osteocalcin and osteopontin are associated with the osteogenic differentiation and mineralization processes of hBMSCs (39, 41-42), thus an overexpression of these markers was expected.

It is also known that hBMSCs produce BMP-2 during osteogenic differentiation (45). In the present work, the control condition, hBMSCs monocultures in osteogenic medium, clearly demonstrates how significant the presence of monocytes/macrophages is. In addition, the effect of monocyte/macrophage-produced BMP-2 was evaluated while cells were already being directed to osteogenic lineage by dexamethasone-containing osteogenic medium. Therefore, a synergistic effect to our knowledge unreported so far, of BMP-2 and the osteogenic factors in the culture medium cannot be discarded. Both

BMP-2 (46-47) and dexamethasone (48-49) ultimately upregulate a cascade of osteoblastic transcription factors among which is Runx2, although involving two different molecular pathways. In the case of dexamethasone, it has been proposed that its osteogenic effect is accomplished through a glucocorticoid-receptor mediated mechanism that modulates Runx2 phosphorylation via mitogen activated protein kinase phosphatase-1 (MKP-1) (48). On the other hand, it is well established that BMP-2 upregulates Runx2 via the Smad pathway (50). Therefore, it is possible that the mechanisms triggered by both dexamethasone and BMP-2 have a cumulative effect on the osteogenic differentiation of hBMSC. The mechanisms orchestrating the osteogenic differentiation of hBMSC are as complex as important to be addressed even more if taking in account a possible synergistic effect of two known osteogenic factors. It is thus our consideration that the mechanisms proposed here should be the subject of a more pervasive study.

The mechanisms orchestrating the osteogenic differentiation of hBMSC are as complex as important to be addressed, even more if taking in account a possible synergistic effect of two known osteogenic factors. Furthermore, this knowledge is potentially advantageous for the design of bone Tissue Engineering (TE) strategies. The prospective advantage of the presence of specific sub-populations of macrophages for the positive outcome of implanted scaffolds was previously hypothesized (51). This concept was also the basis to demonstrate that peripheral blood-derived macrophages influence the degradation and remodelling of scaffolds made of extracellular matrix-based natural materials (52). Therefore, it is our belief that promoting the chemotaxis of such sub-population of cells to the implantation site, might be a valuable approach to promote the success of scaffold-based bone TE strategies.

3.5. Conclusions

The present report confirms that peripheral blood monocytes/macrophages are capable of deeply influencing hBMSC metabolism. It is also strongly suggested that this mechanism is regulated by BMP-2 production by monocytes/macrophages. BMP-2 effect over hBMSC differentiation was cumulative to the one exerted by dexamethasone-containing osteogenic medium and, additionally, promoted hBMSC proliferation. Synergistic effects between dexamethasone- and BMP-2-induced effects may explain this result but a more profound analysis, both in molecular terms and experimental time-spanning, is needed.

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Chapter 4

OSTEOBLASTS SUSTAIN THE FUNCTIONALITY OF ENDOTHELIAL PROGENITORS FROM THE MONONUCLEAR FRACTION OF UMBILICAL CORD BLOOD

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Abstract

Vascularization is currently the most pressing issue in Tissue Engineering (TE) as to assure that engineered constructs are adequately perfused after *in vivo* transplantation is essential for the construct's survival. The combination of endothelial cells with current TE strategies seems the most promising approach but doubts persist on which type of endothelial cells to use. Umbilical cord blood (UCB) cells have been suggested as a possible source of endothelial progenitors.

Osteoblasts obtained from human adipose-derived stem cells (hASCs) were co-cultured with the mononuclear fraction of human UCB for 7 and 21 days on carrageenan membranes. The expression of vWF and CD31, and the ability of uptaking DiI-AcLDL allowed detecting the presence of endothelial and monocytic lineages cells in the co-culture for all times of culture. In addition, the molecular expression of CD31 and VE-cadherin increased after 21 days of co-culture. The functionality of the system was assessed after transplantation in nude mice. Although an inflammatory response developed, blood vessels with cells positive for human CD31 were detected in around the membranes. Furthermore, the number of blood vessels in the vicinity of the implants increased when cells from the mononuclear fraction of UCB composed the transplants in opposition to transplants only with osteoblasts.

These results show how endothelial progenitors present in the mononuclear fraction of UCB can be sustained by osteoblasts co-culture and contribute to angiogenesis even in an *in vivo* setting of inflammatory response.

Chapter based on Pirraco RP, Ferreira B, Santos TC, Frias AM, Marques AP, Reis RL, Osteoblasts sustain the functionality of Endothelial progenitors from the mononuclear fraction of Umbilical Cord Blood, (2011) submitted.

4.1 Introduction

It is well established that vascularization is a critical issue when engineering a tissue (1-2). Improper inosculation and blood perfusion after transplantation will determine the failure of an implant especially if its thickness prevents, from oxygen and nutrient supplying, the transplanted cells. This is a present issue in bone Tissue Engineering (TE) where typically a 3-dimensional scaffold is seeded with osteogenic or osteoprogenitor cells, and transplanted *in vivo* for subsequent bone formation (1-2). The current limited clinical potential of this approach is directly related with deficient vascularization *in vivo* which results in necrosis at the bulk of the scaffold (1-5). Therefore, several strategies are being employed to try to circumvent this important challenge, one of which comprises the use of endothelial cells to accelerate inosculation and the implant's vascularization after transplantation.

Endothelial progenitor cells (EPCs) is one type of endothelial cells that has been recently in focus. These cells were first isolated from the mononuclear fraction of human peripheral blood by Asahara et al(6) but were, since then, also proven to exist in the bone marrow (7) and umbilical cord blood (UCB) (8). Studies have shown that EPCs transplantation can increase angiogenesis and vessel formation in different ischemia models (9-12). UCB is considered the best source of EPCs since it contains 10-times more CD34+ cells, one of the markers used to isolate EPCs, than peripheral blood (13). Furthermore, other blood mononuclear cells such as monocytes (14-15) and lymphocytes (16) were shown to have a positive and essential role in the *in vitro* formation of EPCs colonies pointing to a potential advantage of not specifically isolating CD34+ cells or other subpopulations to obtain EPCs.

The intimate relationship between bone tissue and blood vessels is well studied. Bone formation is always accompanied by blood vessel development both in embryogenesis and post-natal growth (17). Bone multicellular units (BMU), responsible for bone remodeling, are always neighboring a blood vessel that grows as the BMU advances

(18-19). At cellular levels, endothelial cells have been proven to modulate the activity of osteoblasts and osteoblasts progenitors by direct and indirect interactions (20-27). On the other hand, osteoblasts are capable of controlling angiogenesis through the production of factors such as vascular endothelial growth factor (VEGF) (28-30) that is in turn upregulated when osteoblasts are in contact with endothelial cells (31). More importantly, osteoblasts have been proven to support the maintenance of cells such as hematopoietic stem cells in several *in vitro* models (32-33) by direct contact (32).

Carrageenans (CRGs) are a class of galactan polysaccharides that behave as extracellular matrix materials, like agars, in specific species of red seaweeds, including *Gigartina, Chondrus, and Eucheuma* (34-36). These galactan polysaccharides have been used in many industrial and some pharmaceutical applications (35, 37) and have been recently proposed for biomedical applications (38-40). The CRGs are grouped into several sub-types according to their sulphation patterns and distribution of 3,6-anhydro-D-galactose residues (34-35). In the present work, a kappa-iota hybrid CRG derived from the seaweed *Chondrus crispus* was used. This hybrid CRG presents several processing advantages like a higher degree of flexibility, when compared to kappa CRG, and a higher robustness when compared to pure iota CRG (41-42). Being a natural occurring hybrid polymer, it behaves as a monocomponent system and not like a blend, concentrating in a single molecule, characteristics of both kappa and iota types.

Therefore, the aim of this study was to define a co-culture system, using CRG membranes as carriers, composed by osteoblasts and cells from the mononuclear fraction of UCB by taking advantage of the osteoblast capacity to generate angiogenic conditions. The capacity of human osteoblasts cultured on CRG membranes to support the *in vitro* survival of human endothelial progenitors present in the mononuclear fraction of UCB was addressed. The developed systems were then transplanted into nude mice in order to infer about their *in vivo* functionality and therefore to allow us to conclude about the potential use of the mononuclear cells from the UCB, without

further selection, as a source of endothelial progenitors supported by co-culture with osteoblasts.

4.2 Materials and Methods

4.2.1 Carrageenan Membranes production

Carrageenan type iota-kappa (hybrid) extracted from the red algae *Chondrus crispus* by CEAMSA company (Spain) was used to produce the membranes. Carrageenan (CRG) was dissolved in distilled water to a final concentration of 2% w/v. While hot, 25-35 mL of the solutions were placed in square Petri plates and left to dry at room temperature for 48 hours. To preserve its structural features while cutting, the formed membranes were placed into a low-hydrating solution of 17% (v/v) ethanol plus 3% (w/v) potassium chloride (KCl) (Sigma, USA) and cut into small round samples of 1.1 cm diameter and 1mm thickness. The CRG membranes were crosslinked in a water solution of 50% (v/v) 2-propanol (Fluka, Czech Republic), and 0.32M potassium hydroxide (KOH, Fluka, Czech Republic) and 4.63 mM of Epichlorohydrin (ECH, Aldrich, USA) for 24h, at 37ºC under 100 rpm agitation. The samples were then washed twice with a 17% ethanol (v/v) and 0.1M NaOH (Panreac, Spain) solution, aiming at blocking the partially reacted ECH and twice with water at 50Cº for 1h. Each group of CRG membranes used on each assay was then sterilized overnight with 70% ethanol. Before cellular assays, the membranes were maintained in sterile PBS (Sigma, USA) with 3% (w/v) KCl and washed twice in PBS immediately before cell seeding.

4.2.2 Cell Isolation

Umbilical cord blood and adipose tissue were obtained under cooperation agreements established with 3B's Research Group and after approval of the respective Ethical

Committees. All samples were obtained after patient's compliance and within total anonymity.

Cord Blood Mononuclear cells (CBMNCs)

Cord blood was collected during programmed caesareans in Hospital de São Marcos, Braga, Portugal. Cord was clamped in the proximity of the abdomen of the newborn and cut. Cord blood was collected from the vein, before placenta removal, directly to the collection bag containing CPDA I (Fenway Europe sprl, Belgium) as anticoagulant. Transport was carried out under controlled temperature conditions and samples were processed within 2 hours after collection. The blood was then diluted 2:1 in PBS (Sigma, USA) and placed over Histopaque 1077 (Sigma, USA) for density gradient centrifugation. For this, 5 mL of Histopaque at room temperature were placed in a 15 mL conical falcon tube and 3 mL of the cell suspension were carefully layered over it. The tubes were then centrifuged at 400 G for 30 minutes at room temperature. Cells in the interface between plasma and Histopaque were recovered using a pasteur pipette, washed three times in PBS (Sigma, USA), counted and used for establishing the co-culture.

Human Adipose Derived Stem Cells (hASCs)

Discarded subcutaneous adipose tissue from liposuction procedures was collected to sterile containers with 100 mL of PBS with 10% antibiotics (Invitrogen, USA), at Hospital da Prelada, Porto, Portugal. Lipoaspirates were washed with PBS, in order to discard the majority of blood, and then digested with 0.05% collagenase II (Sigma, USA) at 37ºC for 45minutes. Following that, the digested tissue was passed through a strainer and centrifuged at 1000g for 10minutes, at 4ºC. The obtained pellet was ressuspended in PBS and centrifuged again at 800g for another 10minutes, at 4ºC. The resulting pellet was then ressuspended in Minimum Essential Medium alpha-modification (alpha-MEM, Invitrogen, USA), supplemented with 10%FBS (Gibco, USA)

and 1% Antibiotic-antimycotic (Gibco, USA), filtered with a 100 µm pores size cell strainer (BD Biosciences, USA) and plated in tissue culture polystyrene flasks, using the referred culture medium. After 24 hours, colonies of spindle-shaped adhered cells started to appear

4.2.3 Flow Cytometry

After isolation, CBMNCs were screened by flow cytometry for the following markers: CD14-PE, CD45-FITC, CD34-PE and CD73-PE (all from BD Pharmingen, USA), and CD105-FITC and CD90-APC (eBiosciences, USA). Cells were ressuspended in cold PBS with 2% (w/v) BSA (Sigma, USA)) and 100 µL of cell suspension, with $2x10^5$ cells, was incubated with the antibodies at the concentration advised by the manufacturer. After incubation for 20 minutes at room temperature in the dark, cells were washed with PBS/BSA, ressuspended in PBS with 1% of formaldehyde (Sigma, USA) and analyzed in a BD FACScalibur flow cytometer (BD Biosciences, USA). Cells of interest were gated in a forward versus side scatter dot plot with a linear scale. Isotype controls were made to discern non-specific from specific staining. A minimum of 10 000 gated events were acquired and displayed in dot plots created using the Cyflogic software (version 1.2.1, CyFlo Ltd, Finland).

4.2.4 Co-culture set-up

The isolated hASCs at passage 3 were cultured in alpha-MEM supplemented with osteogenic factors, 10^{-8} M of dexamethasone (Sigma, USA), 10 mM beta-glycerophosphate (Sigma, USA), and 50 µg/mL of ascorbic acid (Sigma, USA) for 3 weeks in order to induce differentiation into the osteogenic lineage. After differentiation, cells were seeded on the membranes in a concentration of $82x10^3$cells/cm^2 and cultured for 5 days in M199 medium (Sigma, USA), commonly used to culture primary endothelial cells, supplemented with 10% FBS, 1% antibiotic/antimycotic, osteogenic

factors, 50 µg/mL of endothelial cell growth supplement (ECGS, BD Biosciences, USA) and 50 µg/mL of heparin (Sigma, USA). After this time, the mononuclear cells isolated from cord blood were seeded on the membranes at a concentration of $82x10^4$ cells/cm^2 and cultured for further 7 and 21 days. Monocultures of ASCs-derived osteoblasts in the membranes were set as controls.

4.2.5 DiI-AcLDL uptake assay

After each time point, co-cultures were incubated with 10 µg/mL of the complex DiI-AcLDL (Invitrogen, USA) for 4 hours, at 37ºC and 5% CO_2. Cells were then washed with warm medium and fixed with 3.7% (v/v) buffered formalin (Sigma, USA) for 20 minutes at room temperature. Cells were then counterstained with DAPI (Invitrogen, USA) and visualized using an Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany).

4.2.6 Immunocytochemistry

After each culture time point, the samples were fixed in a 3.7% (v/v) buffered formalin solution for 30 minutes at room temperature. After fixation, cells were washed three times in PBS and used immediately for immunocytochemistry. Non-specific binding was blocked by incubating the membranes with a 3% (w/v) bovine serum albumin (BSA, Sigma, USA) solution in PBS (Sigma, USA) for 30 minutes. Cells were then incubated with the primary antibody diluted in 1% BSA/PBS for 1 hour at room temperature. Mouse anti-human CD31 (1:30, Dako, Denmark) and mouse anti-human vWF (1:100, Dako, Denmark) primary antibodies were used. Cells on the membranes were then washed three times with PBS (Sigma, USA) and incubated for 1 hour at room temperature with an appropriate AlexaFluor (Invitrogen, USA) secondary antibody diluted 1:500. After this time cells were washed in PBS, counterstained with

DAPI, and analyzed in an Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany).

4.2.7 Quantitative Real Time RT-PCR

Messenger RNA (mRNA) of cells was extracted, after each *in vitro* time-point, using Tri-reagent (Sigma, USA) according to manufacturer instructions. 800 µl of the reagent were added per $1X10^6$ cells and samples were homogenized by vigorous pipetting. Following 5 minutes incubation, 160 µl of chloroform (Sigma, USA) were added to each sample which were then incubated for 15 min at 4ºC, and then centrifuged at 13000rpm and 4ºC for 15min. After centrifugation the aqueous part was collected and an equal volume of isopropanol (Sigma, USA) was added. Following an overnight incubation at -20ºC, samples were centrifuged at 9000 rpm and 4 ºC for 10 minutes. Supernatants were discarded and pellets were washed in ethanol, centrifuged at 9000rpm and 4ºC for 5min and re-suspended in 12 µl of RNase/DNase free water (Gibco, UK). RNA quantity and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using iScript cDNA synthesis Kit (BioRad, USA) and the thermoblock of the MiniOpticon Real-Time PCR Detection System (BioRad, USA). An initial amount of 2 µg of mRNA was used in a total volume of 20 µl of RNase/DNase free water.

Osteocalcin and osteopontin transcripts were quantified in the cDNA samples using a quantitative Real Time PCR reaction. For each sample GAPDH was used as the housekeeping gene. The primers were designed using the Primer 3 software (v 0.4.0) and synthesized by MWG Biotech (Germany) as follows: Human GAPDH forward 5' ACA GTC AGC CGC ATC TTC TT3' and reverse 5' GAC AAG CTT CCC GTT CTC AG 3'; human osteocalcin forward 5' GTG CAG AGT CCA GCA AAG GT 3' and reverse 5'TCC CAG CCA TTG ATA CAG GT 3': human osteopontin forward 5'CCC ACA GAC

CCT TCC AAG TA 3' and reverse 5' GGG GAC AAC TGG AGT GAA AA 3'; human collagen I forward 5' AGC CAG CAG ATC GAG AAC AT 3' and reverse 5' ACA CAG GTC TCA CCG GTT TC 3'; human VE-cadherin forward 5' CAC AGT GCT GGC CAT GGA 3' and reverse 5' GTC CTG CGG ATG GAG TAT CC 3'; human CD31 forward 5' AAG GCC AGA TGC ACA TCC 3' and reverse 5' TTC TAC CCA ACA TTA ACT TAG CAG G 3'; human vWF forward 5' CCC TGG GTT ACA AGG AAG AAA AT 3' and reverse 5' AGT GTC ATG ATC TGT CCT CCT CTT AG 3'. A concentration of 200 nM of primer was used in a final volume of 25 µl of sample. The Real Time PCR reaction was done using the iQ Syber Green SuperMix (BioRad, USA), following manufacturer's instructions, in a MiniOpticon Real-Time PCR Detection System (BioRad, USA).

The relative quantification of gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Perkin Elmer User Bulletin No.2). All values were first normalized against GAPDH values and then against the respective monoculture.

4.2.8 In vivo assay

Four-weeks-old female Balb/C nude mice with an average weight of 20 g (Charles River Laboratories Inc. USA) were anaesthetized with a mixture of ketamine (1.2 mg/mouse s.c., Imalgene® 1000, Merial, Lyon, France) and medetomidine (20 µg/mouse s.c., Domitor®, Orion Corp., Finland) prepared in physiological serum. After the confirmation of analgesia/anaesthesia one incision was performed, reaching a maximum of 1.5 cm each, in the intrascapular region. Two craniolateral oriented pockets were created by blunt dissection and the implants were subcutaneously inserted. Two CRG membranes were implanted in each animal and 3 animals were used per condition. The incisions were sutured and the mice transferred to heated recovery compartments. When the recovery from analgesia/anaesthesia was confirmed they were returned to their respective cages and kept under food and drink *ad libitum*.

After 7 and 21 days post implantation, animals were euthanized with an intracardiac overdose of anaesthesia for implants retrieval and processed for characterization.

4.2.9 Histology

Standard hematoxylin and eosin (H&E) stainings were used to make a general morphological assessment of the sectioned tissues. Briefly, sections were deparaffinized in xylene (Thermo Scientific, USA) and hydrated by incubation with increasingly diluted ethanol solutions. Sections were then washed in water and incubated with Hematoxylin (Sigma, USA) for 5 minutes. After this, sections were washed under running tap water for 10 minutes and finally ddH_2O. After the incubation with eosin (Sigma, USA) for 1 minute, sections were dehydrated with ethanol, washed with xylene and mounted with Histofluid (Marienfeld, Germany).

4.2.10 Immunohistochemistry

After deparaffinization, sections were incubated with antigen retrieval solution (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) and heated in the microwave for 2 minutes at maximum power. After cooling, sections were incubated with 3% H_2O_2 for five minutes to inhibit non-specific peroxidase activity and washed with PBS. The next step was to block non-specific protein interactions by incubating with a 3% (w/v) BSA solution for 30 minutes. Sections were then washed in PBS-tween 20 (PBS-T, Sigma, USA) and incubated with the mouse anti-human CD31 (1:30, Dako, Denmark) primary antibody overnight at 4ºC. Sections were then washed in PBS-T and incubated for 45 minutes at room temperature with a biotinylated secondary antibody (Vector Labs, UK). After washing, cells were incubated first with Streptavidin-HRP (Vector Labs, UK) and then with 3,3'-diaminobenzidine (DAB) chromogenic substrate (Vector Labs, UK). Cells were then observed in an in an Axioplan Imager Z1 microscope (Zeiss, Germany).

4.2.11 Blood vessel quantification

After H&E staining, sections were observed in an Axioplan Imager Z1 microscope and analyzed for blood vessels using Axiovision software (Zeiss, Germany). The number of perfused blood vessels was counted in the periphery of the implants within a 200 µm distance. Three different sections with two cuts per sample (n=6) were used in this procedure.

4.2.12 Statistical Analysis

In vitro data was obtained from 4 independent experiments with 3 replicates for each condition and averaged. Standard deviation is reported as a measure of sample deviation. Statistical analysis of blood vessel quantification was performed using one way ANOVA test with Tukey-Kramer method as a post-hoc pairwise comparison test. The statistical analysis of the Quantitative Real Time RT-PCR results was performed using the t student-test for n=4. Values were considered statistically significant for $p<0.05$.

4.3 Results

4.3.1 CBMNCs Phenotype

The expression profile of CD14, CD45, CD34, CD90, CD105 and CD73 markers in the isolated human CBMNCs was determined by flow cytometry (figure 4.1). The isolated cells expressed the hematopoietic marker CD34 (1.8%), the monocytic marker CD14 (4.94%), the CD90 (6.27%) and the CD105 (7.8%) surface proteins. In addition, 13.8% of the population was positive for the expression of CD73 while 4.9% expressed the endothelial marker CD31.

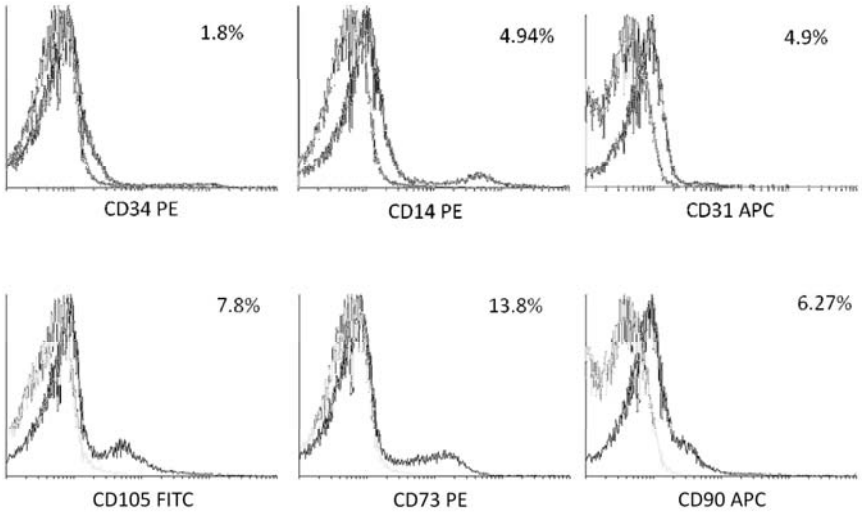

Figure 4.1 - Flow cytometry analysis of CBMNCs immediately after isolation and prior to seeding. Cells were gated to exclude dead cells, platelets and debris of erythrocytes. The black lines represent stained cells; grey lines are the controls. The percentage of positive cells for each marker is indicated in the graph

4.3.2 Co-cultures Characterization

The uptake of the DiI-AcLDL was assessed after 7 and 21 days of *in vitro* co-culture. Cells with internalized DiI-AcLDL were observed throughout the surface of the membranes and without any specific organization (figure 4.2). The morphology and distribution of cells did not change throughout the time of co-culture. A similar cellular distribution was observed when cells in the co-culture were stained for CD31 and vWF markers (figure 4. 2). As for the DiI-AcLDL, the patter of expression of CD31 and vWF was similar for all the tested co-culture times.

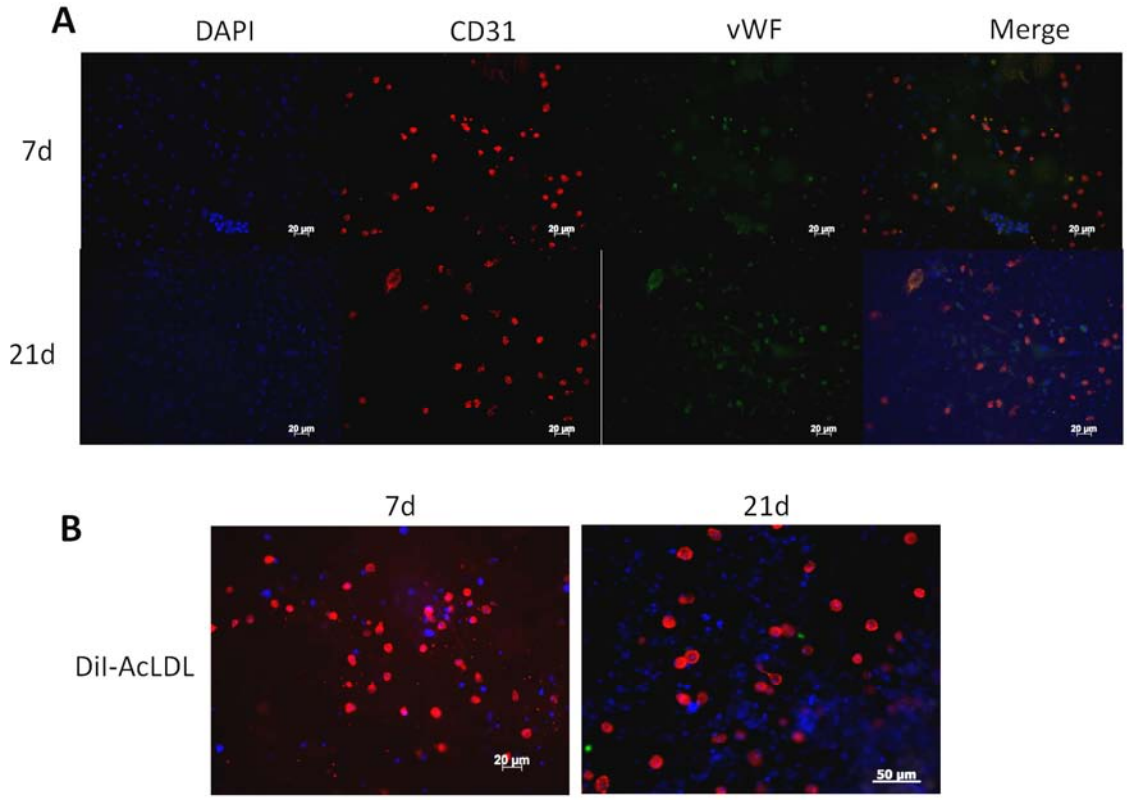

Figure 4.2 – Co-cultures of osteoblasts and CBMNCs on the carrageenan membranes after 7 and 21 days. (A) Endothelial cell markers, CD31 (red) and vWF (green), were detected by immunocytochemistry. (B) DiI-AcLDL (red) staining identifies cells from the phagocytic and endothelial lineages. Nuclei were stained with DAPI (blue)

The quantification of the transcripts for the genes of interest was also performed after 7 and 21 days of co-culture (figure 4.3). The expression level of osteogenic genes osteopontin, osteocalcin and collagen I, did not change in relation with monocultures for the same time period. On the contrary, the expression of vWF, VE-cadherin and CD31 genes significantly increased when compared to monocultures for the same time period. The variation of the expression of vWF for 7 days of co-culture was of 2.8 fold while for 21 days of co-culture the fold change was of 2.7. In the case of the VE-cadherin gene, for 7 days of co-culture the expression varied 3.0 fold while for 21 days of co-cultures there was a change of 13.5 fold. Regarding the CD31 gene expression a positive variation of 3.3 fold was detected after 7 days of co-culture, while for 21 days of co-culture, the variation was of 9.3 fold.

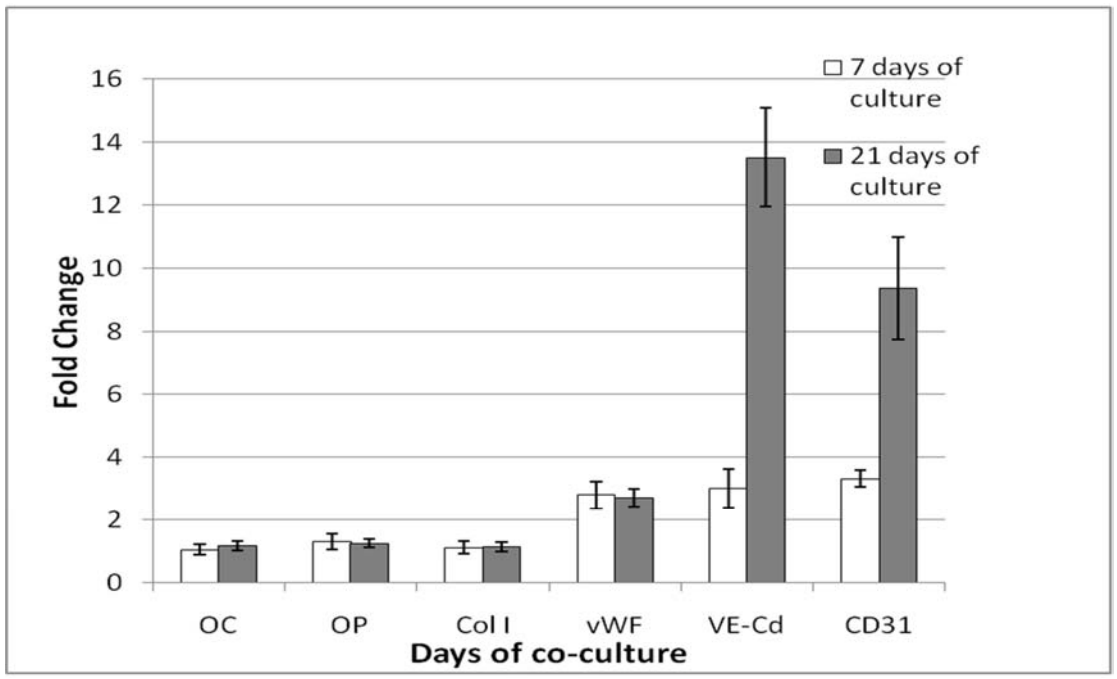

Figure 4.3 – Quantitative Real Time RT-PCR of osteopontin (OP), osteocalcin (OC), collagen I (COL I), vWF, VE-cadherin (VE-Cd), and CD31 transcripts in co-cultures of osteoblasts and CBMNCs on carrageenan membranes after 7 and 21 days.

4.3.3 In vivo performance

The nature of the host reaction was analyzed based on the histological and immunohistological characterization of the tissues surrounding the different implants recovered (figure 4.4). The histological evaluation of the obtained sections showed no particular differences between the co-cultured (Figures 4.4A-H) and monocultured (Figure 4.4I-L) carrageenan membranes for 7 and for 21 days in vitro, for each implantation period. A significant inflammatory infiltrate surrounded the membranes at day 7 of implantation of both systems co-cultured in vitro for 7 (figure 4.4A) and 21 (figure 4.4C) days.

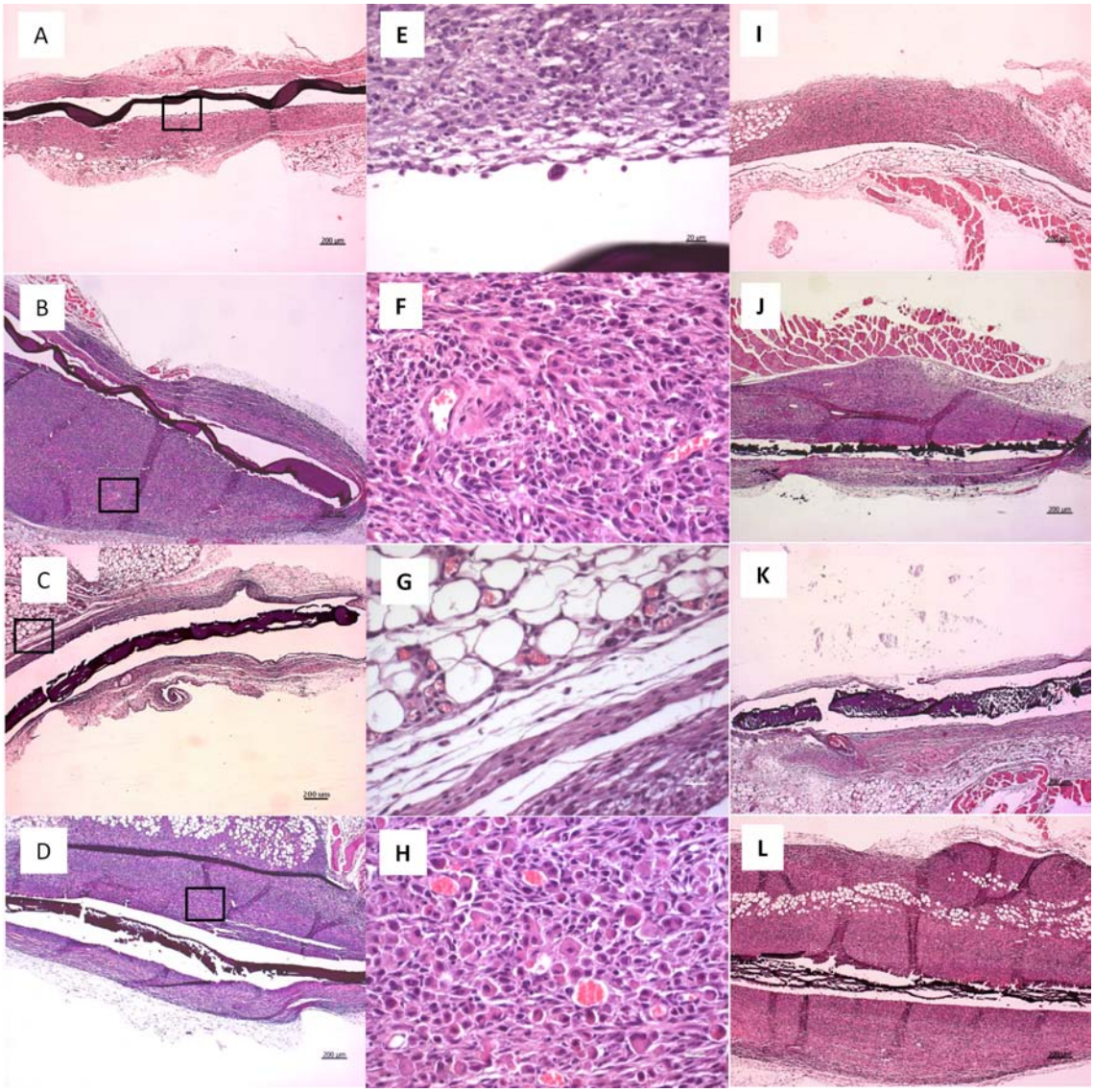

Figure 4.4 – Histological characterization of carrageenan membranes with co-cultured hASCs-derived osteoblasts and CBMNCs (A-H) and monocultures of hASCs-derived osteoblasts (I-L) in vitro for 7 (A, B, E, F, I, J) and 21 (C, D, G, H, K, L) days, and after 7 (A, C, E, G, I, K) and 21 (B, D, F, H, J, L) days of implantation. Implants sections were stained with hematoxylin-eosin (A-L)

This infiltrate was characterized by the presence of some polymorphonuclear neutrophils, of a significant number of macrophages, either resident or recruited from circulation, and some foreign body giant cells (FBGCs) and lymphocytes (figure 4. 4E, G). The inflammatory infiltrate around the membranes co-cultured in vitro for 21 days was surrounded by vascularized adipose tissue (figure 4.4C, G).

After 21 days of implantation of the membranes, the extension of the inflammatory infiltrate clearly increased (figure 4.4B, D, J, L) as compared with the early implantation time period. The presence of many mononuclear cells similarly to the early time period

(Figure 4.4F, H) was observed. Moreover, macrophages fused together forming FBGCs, lymphocytes maintained their apparent activation status some plasma cells were identified.

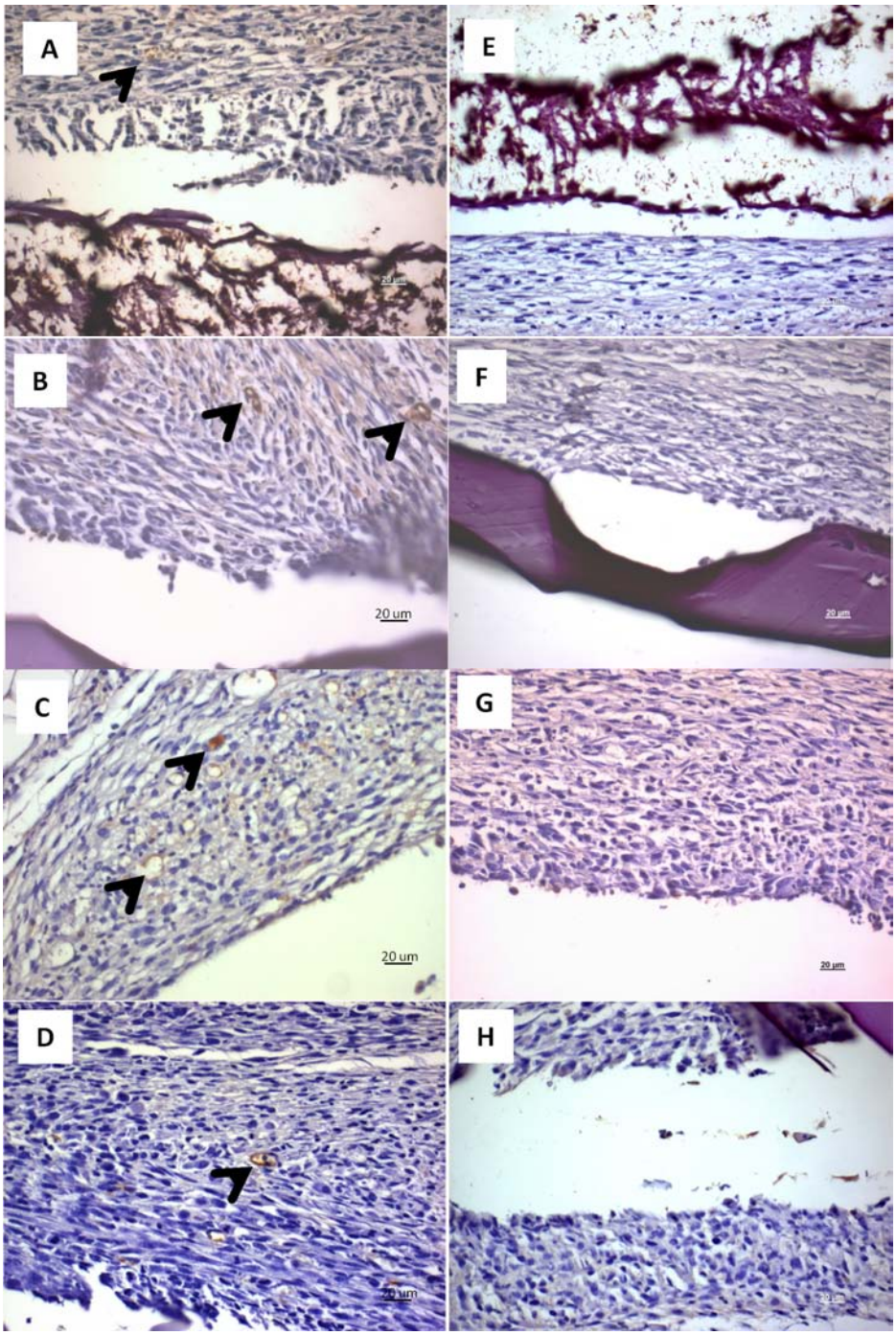

Figure 4.5 – Immunohistological characterization of carrageenan membranes with co-cultured hASCs-derived osteoblasts and CBMNCs (A-D) and monocultures of hASCs-derived osteoblasts (E-H) in vitro for 7 (A, B, E, F) and 21 (C, D, G, H) days, and after 7 (A, C, E, G) and 21 (B, D, F, H) days of implantation. Immunostaining was performed to localize human CD31 marker. Sections were counterstained with hematoxylin.

Perfused blood vessels around the implants with the co-cultures were found to possess cells positive for human CD31 marker (Figure 4. 5A-D) while this was not the case of the implants with the monocultures of osteoblasts (Figure 4.5 E-H).

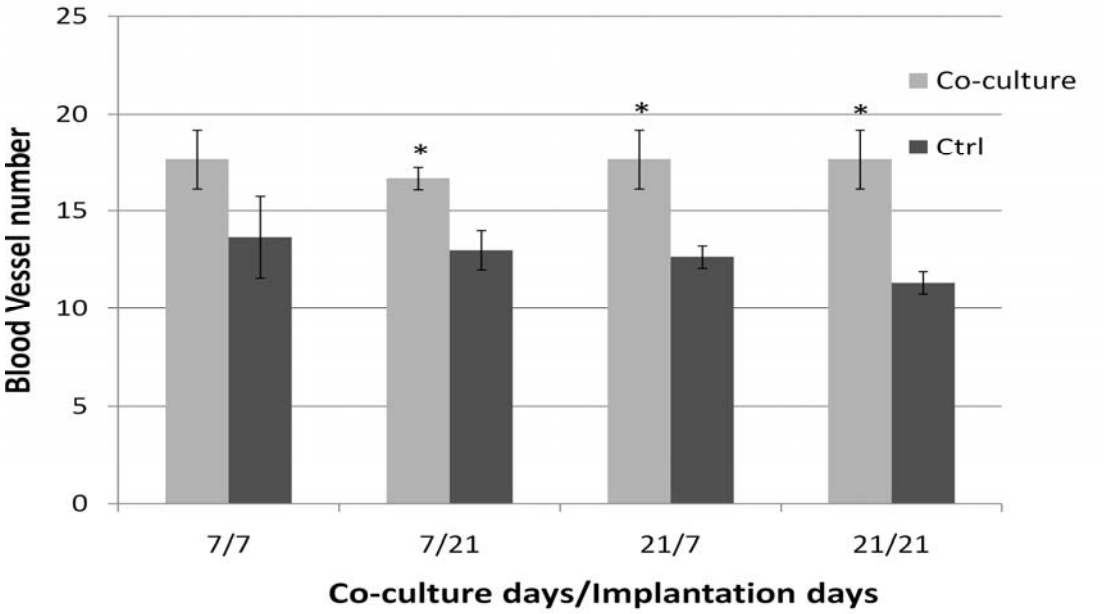

Figure 4.6 – Quantification of blood vessels within the 200 µm around the surface of the implanted membranes. Implanted carrageenan membranes with the co-cultures (Co-Culture) and with osteoblast monocultures (Ctrl) were compared. *statistical significance for $p \leq 0.05$

Blood vessels were quantified in the periphery of the implants within a 200 µm distance for all the conditions, with and without UCB MNC (figure 4.6). The number of vessels did not significantly vary with the in vitro culture and implantation times. However, the number of blood vessels was always greater when MNCs from cord blood were present. This difference was statistically significant when comparing the co-culture conditions and their respective monoculture control.

4.4. Discussion

The need to address the vascularization problem in TE is widely recognized as essential for a successful strategy. More so when engineering bone tissue, which normally involves the use of complex 3D structures prone to necrosis if not correctly perfused (1-5). The use of endothelial cells in bone tissue engineered constructs has

been proven to be promising in promoting *in vivo* vascularization and construct survival (31, 43-46). The cell source is however still an issue due to the limited number of autologous cells obtained. UCB has been shown to contain endothelial progenitors that can be used to promote the vascularization of bone tissue engineered constructs (45). Furthermore, current cord blood cryopreservation techniques allow the preservation of the potential of endothelial progenitors to be isolated from this source (47-48). Reports showing the positive influence of diverse populations of the UCB mononuclear fraction in the behavior of endothelial progenitor cells (EPCs) have also reinforced the potential of this cells source (16, 49). Mononuclear fractions of peripheral blood depleted from CD14+ monocytes lose the ability to form colony forming unit-endothelial cells (CFU-EC) (49). In fact, CD14 positive cells have been suggested to be pro-angiogenic (16) and capable of differentiating into endothelial cells (15). Furthermore, CD4+ T cells were proven to be essential to the CFU-EC formation by CD14+ cells (16). Using MNCs from cord blood for vascularization purposes could then be an advantageous alternative to the typically used isolation of its specific sub-populations (6).

Several co-culture constructs of EPCs with MSCs/osteoblasts have been described for bone tissue engineering (43, 45-46). Although most of them successfully lead to increased vascularization of their constructs because of EPCs, the isolation and culture of these cells is a long and complex process. This work therefore proposes the use of the complete mononuclear fraction of UCB in order to be able to define a co-culture system carrying functional endothelial progenitors.

The present work allowed verifying that osteoblasts obtained from adipose-derived stem cells support the survival of endothelial progenitors present in the mononuclear fraction of UCB. Taichman and colleagues (33) previously demonstrated how osteoblasts can support the maintenance of hematopoietic progenitors *in vitro*. Later Jung et al(32) proved that this supportive effect of osteoblasts required direct cell-cell contact. We hypothesized that osteoblasts can have a similar action on the endothelial

progenitors present in the mononuclear fraction of the UCB. Throughout the culture times, the monolayers of osteoblasts supported the culture of CD31 and vWF positive cells and of cells capable of uptaking DiI-AcLDL. These properties are commonly used to define EPCs, however, blood monocytes have been shown to present these markers under angiogenic conditions (49-50). In addition, over expression of the endothelial genes vWF, CD31 and VE-cadherin was detected in comparison with monocultures of MNCs under the same culture conditions and more significant in the case of CD31 and VE-cadherin genes after 21 days of co-culture. This result for VE-cadherin is particularly remarkable as its expression is strongly linked to the maintenance of new blood vessels integrity (51). Although striking, this promotion of the expression of endothelial genes is not unexpected. In fact osteoblasts produce factors, such as vascular endothelial growth factor (VEGF) (30), that are capable of inducing the endothelial phenotype from progenitors (6). Furthermore, the ECM deposited by osteoblasts is composed by fibronectin which may support the adherence of endothelial progenitors from the CBMNCs (52). Fuchs and colleagues (46) have in fact demonstrated that outgrowth endothelial cells, a type of EPCs, have increased endothelial activity when co-cultured with osteoblasts.

Contrarily to the endothelial lineage related genes, the expression of osteogenic genes osteopontin, osteocalcin and collagen I, did not change throughout the time, which is not unexpected as the osteoblastic supporting cells were fully differentiated at the time of the co-culture set-up. Co-culture systems established with mesenchymal stem cells or other osteoblast progenitors instead of osteoblasts are probably more prone to be influenced by the other cell types. This was demonstrated by Fedorovich and colleagues (53) in a co-culture model where EPCs promote the osteogenic differentiation of MSCs, while these promote the proliferation of the EPCs. In our strategy, totally differentiated cells were the ones we wanted to test.

Carrageenans are natural sulphated polyssacharides that have a widespread use in the food industry (54). Recently, kappa-carrageenans have been proposed for

regenerative medicine purposes (38-40) presumably due to their favorable mechanical properties and an anticoagulant effect (55). We hypothesized that membranes produced using a kappa-iota hybrid type of this natural polymers could act as carriers to test the functionality of the developed co-cultures *in vivo*. To our surprise, a very strong and progressive inflammatory reaction was detected after implantation of the CRG membranes. Furthermore, the amount and nature of the inflammatory infiltrate did no vary with the implant type, CRG membranes only with osteoblasts and co-cultured with CBMNCs, which seems to indicate that the cellular component did not significantly influence the inflammatory reaction. Solutions of carrageenan from kappa, iota and lambda types have been used to induce acute inflammation in animal models (56-58). Moreover, as Tobacman thoroughly reviewed (54), the ingestion of carrageenan, used in the food industry, is associated with the onset of several lesions in the gastro-intestinal tract in several animal models. Its use as a food additive is in fact very controversial due to health concerns (54, 59-60). Additionally, an acute inflammatory response, characterized by an abundant neutrophilic exudate and high levels of c-reactive protein, was reported after implanting gelatin/kappa-carrageenan (55%/45% w/w) sponges. The intensity of the reaction was nevertheless milder when the weight percentage of carrageenan was lowered to 9% suggesting a dose-dependent inflammatory effect of this polyssacharide. A similar acute inflammatory response that progressed to chronic inflammation was observed after implanting our membranes made of hybrid carrageenan. Although these results impair their potential use for tissue engineering or regenerative medicine purposes, the functionality of the defined co-culture system was nevertheless demonstrated.

A link between chronic inflammation and angiogenesis is well described in the literature (61-63) as there are many factors secreted by inflammatory cells that can act on EPCs (64-65). Furthermore, differentiation of EPCs and their contribution to angiogenesis in pathological situations is also been observed experimentally (7, 66). In our model, human CD31-positive cells were found forming the wall of perfused blood vessels in

the vicinity of the implant for all the times of culture. This suggests that an endothelial progenitor population present in the co-cultures was able to respond to the stimuli present at the implantation site and to differentiate and to contribute to angiogenesis in the inflammatory setting. These results also demonstrate the usefulness of the mononuclear fraction of UCB as a source of endothelial cells or endothelial progenitors without previous selection. In addition to the proved active participation of transplanted CBMNCs in new blood vessels formed, their impact over the number of vessels formed around the implants was also demonstrated. A significantly higher number of vessels formed around the implants containing the CBMNCs than around the implants only with osteoblasts.

Overall, this work suggests that osteoblasts can support the maintenance of endothelial progenitors present in CBMNCs. Furthermore, the latter can be used to increase vascularization *in vivo* in an inflammatory setting. This can potentially be very interesting for future bone Tissue Engineering approaches although more studies have to be made especially using biomaterial carriers/supports capable of eliciting host reactions with different degrees of severity.

4.5. Conclusions

The current work showed that osteoblasts sustain *in vitro* the survival of endothelial progenitors present in the mononuclear fraction of UCB in a direct co-culture system established using carrageenan membranes as supports. The functionality of the cells from UCB co-cultured with osteoblasts was demonstrated in an *in vivo* inflammatory setting. The presence of human CD31-positive cells in blood vessels around the implant as well as the increased number of blood vessels when the mononuclear fraction of UCB was used, confirms the existence of a sub-population of that fraction that can contribute to angiogenesis. This is an indication of the potential use of the

mononuclear cells from the UCB, without further selection, as a source of endothelial progenitors supported by co-culture with osteoblasts.

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Chapter 5

FIBROBLASTS REGULATE OSTEOBLASTS THROUGH GAP JUNCTIONAL COMMUNICATION

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ABSTRACT

Fibroblasts are present in most tissues of the body playing an active role in the regulation of the homeostasis of such tissues. While the fibroblasts heterotypic interactions are fairly acknowledged in the regeneration of tissues such as skin and periodontal ligament, their role in bone regeneration is far from being understood. We hypothesized that fibroblasts could influence bone cells, and since connexin 43 is the predominant connexin in both fibroblasts and osteoblasts, we speculated that those heterotypic interactions would occur through gap junctional communication (GjC). IN order to test that, direct co-cultures of human mesenchymal stem cell (hMSCs)-derived osteoblasts and human dermal fibroblasts (hDFb) were established in the presence and absence of the GjC inhibitor α-glycyrrhetinic acid. The existence of the communication between hMSCs-derived osteoblasts and hDFb via this pathway was demonstrated by the transference of the gap junction (Gj) permeable dye calcein-AM from the donor cells to the recipient cells, independently of their type. The amount of transferred dye was decreased when gap junctional communication inhibitor was used. While the proliferation of the hMSCs-derived osteoblasts was not affected by the presence of the hDFb, the level of osteogenic markers such as alkaline phosphatase activity and osteocalcin transcripts in hMSCs-derived osteoblasts was severely diminished. This effect was partially reversed by adding the inhibitor of GjC to the co-cultures, which strongly suggests that fibroblasts regulate osteoblasts behaviour partially through GjC. This information can be critical to predict the outcome of strategies aiming to promote bone regeneration as for example in bone Tissue Engineering approaches.

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5.1 Introduction

Cell-cell interactions are fundamental for the normal function of every system of the human body. Tissue function and repair depends on a complex network of cellular communication that occurs at different levels and is based on the production of soluble factors and on the direct cell-cell contact (1-4). In fact, the detailed knowledge of these interactions and respective communication mechanisms is of extreme importance in Tissue Engineering and therapeutic approaches as it contributes to a more precise prediction of the outcome of an implanted Tissue Engineered construct. In this sense, co-culture systems established with different types of cells are useful tools to study such interactions. Some co-culture models were also defined and have been used to predict and to modulate the outcome of osteochondral tissue engineered constructs (5-6). These works and others (1, 3, 7-9) have shown that direct cell-cell communication between different types of cells present on the bone microenvironment, such as chondrocytes and endothelial cells, is essential for both bone development and bone remodelling.

Fibroblasts are key players in regenerative processes in different types of tissues. In the case of ligaments, such as the periodontal ligament (10) or anterior cruciate ligament (11), fibroblasts interactions with osteoblasts are essential for tissue development, function and regeneration. Another example is the case of skin wound healing where fibroblastic interaction with keratinocytes is essential to restore epithelial functions thus promoting wound closure and tissue repair (12). In the case of tissue engineering constructs, where biomaterials are combined with cells, proteins and/or other biological agents, all the system will be subjected to an environment specific of a foreign body reaction after implantation (13). How the immune system interacts with these cells and how biomaterials influence these interactions is still far from being understood. Nonetheless it is known that fibroblast stimulatory potential correlates to the in vivo fibrotic response after biomaterials implantation (14). Thus, interactions

between fibroblasts and osteoblasts, transplanted or native, can be of great importance to determine the outcome of an implanted bone tissue construct. Some studies have been made concerning the effect of fibroblast-secreted soluble factors, namely prostaglandins (PG), in osteogenic cells (15-16). In these works, the authors proved that the conditioned medium from cultures of fibroblasts from rat skin or from human periodontal ligament inhibited the mineralization of rat bone marrow stromal cells in osteogenic medium. This effect was reverted when indomethacin or anti-PG antibodies, were added to the fibroblast cultures. To our knowledge direct interactions between osteoblasts and fibroblasts through gap junctions have not been described in the literature. The majority of the works regarding heterotypic cellular interactions in bone focus on the communication between osteoblasts and osteocytes (1, 7, 17) and between osteoblasts and endothelial cells (3, 18-20). Gap junctional communication (GjC) is known to be involved in bone cellular differentiation (1, 8, 20), in cellular communication between endothelial cells and osteoblasts(3), in mechanical signal transduction between bone cells (8) and in immune response and tissue inflammation and repair (2). The most important gap junctional protein present in osteoblasts (21) is connexin 43 (Cx43) and GjC through this protein modulates osteoblast gene expression (22). This protein is also widely expressed in fibroblasts (23) and is involved, for instance, in skin wound healing(24-25). In the latter case, modulation of connexin 43-mediated GjC has increased tissue repair rate (24-25).

The aim of this work was primarily to verify the existence and the nature of direct interactions between human mesenchymal stem cell (MSC)-derived osteoblasts and human dermal fibroblasts (hDFb). By hypothesising that these two cell types communicate through GjC, the importance of these mechanisms was analysed by assessing the effects of hDFb over the osteogenic phenotype of the hMSC-derived cells.

5.2 Materials and Methods

5.2.1 Co-culture of osteoblasts and fibroblasts

Human bone marrow mesenchymal stem cells (hMSCs) were purchased from Lonza (Lonza, Switzerland). Cells were cultured in osteogenic medium composed of DMEM plus 10% FBS (Invitrogen, USA) and 1% of antibiotics (Invitrogen, USA), and supplemented with 10 mM beta-glycerophosphate (Sigma, USA), 50 µg/mL of ascorbic acid (Sigma, USA) and 10^{-8}M of dexamethasone (Sigma, USA) for three weeks.

Human dermal fibroblasts (hDFb) were isolated from fresh skin specimens (discarded tissue) from informed healthy patients undergoing plastic surgery at Hospital da Prelada, Porto, Portugal and under a protocol previously approved by the Ethical Committee. After being washed with sterile phosphate-buffered saline (PBS; Sigma, USA), the skin was cut into small pieces ($2mm^2$) and digested with 2.4u/mL Dispase II (Sigma, USA) solution at 4ºC overnight. The epidermis was removed, and the remaining dermal parts were further incubated with 0.1% collagenase type IA (Sigma, USA) for 3hours at 37ºC under gently agitation. The digested dermis was then passed through a 100µm cell strainer (BD Biosciences, USA), centrifuged and the cell suspension ressuspended in low-glucose DMEM medium (Sigma, USA) supplemented with 10% FBS and 1% Antibiotic/Antimycotic (both Invitrogen, USA).

A hMSC-derived osteoblast cell suspension, with a cell density of 5×10^3 cells/mL, was prepared, transferred to 24-well culture plates (1mL per well) and incubated for 24 hours. After this time of culture, hDFb were prepared and added to the wells previously seeded with osteoblasts at a cell density of 2.5×10^3 cells/mL (1 ml per well) thus establishing the direct contact co-culture model. Monocultures of osteoblasts were kept as controls in parallel with the co-culture for 2, 7 and 12 days. All cells were used in passage 3.

5.2.2 Alizarin Red Staining and Immunocytochemistry

Before establishing the co-culture model, the hMSCs-derived osteoblasts and the isolated hDFb were characterized as follows. After culture of hMSC in osteogenic medium for three weeks, cells were fixed and mineralization was assessed using alizarin red staining. Briefly, a solution of 0.1 % of alizarin red (Sigma-Aldrich, USA) was prepared in ddH_20 and the pH was adjusted to 4.6 by using a 10% ammonium hydroxide solution.

After isolation from dermis, hDFb were screened for the presence of the Fibroblast Surface Protein (FSP) marker using a monoclonal mouse anti-FSP antibody (Sigma, USA) at a concentration of 1:500. Briefly, after fixation with formalin, cells were incubated with 3% BSA in PBS for 30 minutes to block non-specific binding. Cells were then incubated with the primary antibody for 1 hour at room temperature and washed in PBS. Alexafluor 488 (Invitrogen, USA) secondary antibody in a concentration of 1:500 was incubated with cells for one hour at room temperature. Cells were then washed in PBS and counter-stained with DAPI nuclear staining.

Both types of cells were screened for connexin 43 before setting up the co-cultures, following a similar immunocytochemistry protocol as described above. A rabbit anti-human connexin 43 antibody (ABM, Canada) was used as primary antibody (1:100 dilution) and a Alexafluor 488 (Invitrogen, USA) goat anti-rabbit antibody was used as secondary antibody (1:500 dilution).

5.2.3 Gap junctional communication (GjC) assessment

The visualization of GjC was achieved using the parachute technique (26). Briefly, either hDFb or hMSCs-derived osteoblasts were prepared by seeding 7 x10^4 cells per 24 well coverslip, 24 hours before the assay. At the time of the assay, hMSCs-derived osteoblasts or hDFb were labelled with 2 µM of calcein-AM for 15 minutes at 37ºC.

Cells were then thoroughly washed with PBS, seeded over the previous culture of the other cell type (7 x10^3 cells/well) and cultured for 15 minutes at 37ºC. Cultures were observed with the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) to assess if the Gj-permeable calcein was transferred from the "parachuted" cells to the cells in the monolayer.

The assessment of the involvement of GjC between the osteoblasts and fibroblasts in co-culture was targeted by using a chemical inhibitor of Gj communication, 18-alpha-Glycyrrhetinic acid (AGA, Sigma, USA). AGA was dissolved in fresh DMSO and diluted to working solution of 25 mM with PBS. The co-cultures were prepared as previously described but 18-alpha-glycyrrhetinic acid was added to the medium attaining a final concentration of 100 µM. The used concentration was determined after previous toxicity testing (results not shown). 18-AGA was added to the cultures 24 hours before the establishment of the co-cultures and of the parachute assay. In the case of the co-cultures, 18-AGA was added to the culture medium every renewal, i.e., every other day. Controls were made by adding either DMSO or AGA to osteoblasts monocultures in the same concentrations.

5.2.4 Cell number quantification

Cell proliferation was assessed through total dsDNA quantification in hMSCs-derived osteoblasts monocultures and in co-cultures along the culture time. The test was performed using the PicoGreen Quantification Kit (Invitrogen Corporation, California, USA), according to the manufacturer instructions in a lysed cell suspension obtained after osmotic and thermal shocks. Fluorescence was read in a microplate reader (Bio-Tek, USA) at 485 ex/ 525 em.

In parallel, the number of hMSCs-derived osteoblasts in monoculture and in co-culture with hDFb was followed in time using a cell tracking fluorescent marker. In the

beginning of each experiment an osteoblast cell suspension was prepared and incubated with a 2 μM solution of 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Sigma, USA) in culture medium for 15 minutes at 37 ºC. After, cells were washed 3 times in warm medium and seeded for the experiments as described above. After each time point, three samples of each condition were fixed with formalin and stained with DAPI (Sigma, USA), a nuclei marker. Fluorescent micrographs of three independent fields of each one of the analyzed samples were taken and analysed using the image-processing software ImageJ (Wayne Rasband, NIH, USA). The fraction of osteoblasts in the co-cultures was determined by dividing the number DiI positive cells by the total number of cells given by DAPI staining. The direct correlation between the dsDNA values and the cell number (27) allowed to extrapolate the dsDNA values corresponding to the fraction of osteoblasts in culture.

5.2.5 Alkaline phosphatase (ALP) activity quantification

The ALP activity quantification was performed in the cell lysates obtained by osmotic and thermal shocks and following an adapted p-nitrophenol assay.(28) Briefly, 20 uL of sample were incubated with 80 uL p-nitrophenol phosphate solution (0.2% w/w, in diethanolamine, Sigma, USA) at 37ºC, for 45 minutes. The reaction was stopped using 80 uL of a 2M of NaOH and 0.4 mM of EDTA solution. The optical density of the samples was read at 405 nm. A calibration curve was previously prepared using the p-nitrophenol standards (Sigma, USA) and used to extrapolate the ALP activity. Results were normalized against dsDNA results as described above.

5.2.6 PCR analysis

RNA extraction and cDNA production

mRNA was extracted using TriZol (Invitrogen, USA) reagent according to manufacturer instructions. Briefly 800 µl of TriZol were added per $1X10^6$ cells and samples were homogenized by vigorous pipetting. Following 5 minutes incubation, 160 µl of chloroform (Sigma, USA) were added to each sample; samples were then incubated for 15 min at 4ºC and centrifuged at 13000rpm and 4ºC for 15min. After the centrifugation the aqueous part of each sample was collected and an equal volume of isopropanol (Sigma, USA) was added. Following an overnight incubation at -20ºC, samples were centrifuged at 9000 rpm and 4 ºC for 10 minutes. Supernatants were discarded and pellets were washed in ethanol, centrifuged at 9000rpm and 4ºC for 5min, and ressuspended in 12 µl of RNase/DNase free water (Gibco, UK). RNA quantity and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using iScript cDNA synthesis Kit (BioRad, USA) and the MiniOpticon Real-Time PCR Detection System (BioRad, USA). An initial amount of 2 µg of mRNA was used in a total volume of 20 µl of RNase/DNase free water.

Quantitative Real Time PCR

Osteocalcin and osteopontin transcripts were quantified in the cDNA samples using a quantitative Real Time PCR reaction. For each sample GAPDH was used as the housekeeping gene. The primers were designed using the Primer 3 software (v 0.4.0) and synthesized by MWG Biotech (Germany) as follows: osteocalcin forward 5' GTGCAGAGTCCAGCAAAGGT 3' and osteocalcin reverse 5' TCCCAGCCATTGATACAGGT 3'; osteopontin forward 5' CCC ACA GAC CCT TCC AAG TA5 3' and osteopontin reverse 5' GGG GAC AAC TGG AGT GAA AA 3';

GAPDH forward 5' ACAGTCAGCCGCATCTTCTT 3' and GAPDH reverse 5' GACAAGCTTCCCGTTCTCAG 3'. A concentration of 200 nM was used for all the primers in a final volume of 25 µl of sample. The Real Time PCR reaction was done using the iQ Syber Green SuperMix (BioRad, USA) following manufacturer instructions in a MiniOpticon Real-Time PCR Detection System (BioRad, USA).

The relative quantification of osteocalcin and osteopontin expression was performed using the $2^{-\Delta\Delta CT}$ method (Perkin Elmer User Bulletin No.2). All values were first normalized against GAPDH values and then co-culture values against osteoblasts monocultures without inhibitor values at each time point of culture.

5.2.7 Statistical analysis

Data was obtained from 4 separate experiments with 3 replicates for each condition and averaged. Standard deviation is reported as a measure of sample deviation. Statistical analysis was performed for dsDNA and ALP quantification using one way ANOVA test with Tukey-Kramer method as a post-hoc pairwise comparison test. In the case of the Quantitative Real Time RT-PCR results, the statistical analysis was performed in the ΔCt values (29) using the *t student-test* for n=4. Values were considered statistically significant for $p \leq 0.05$.

5.3 Results

5.3.1 Human bone marrow MSCs-derived osteoblasts and hDFb characterization

Isolated cells from human dermis were screened for Fibroblast Surface Protein. Results showed that this marker was uniformly distributed throughout the culture showing that isolated cells were a homogeneous fibroblast population Furthermore, looking at light microscopy, one could see that cells presented a typical fusiform morphology (Figure 5.1A).

hMSCs-derived osteoblasts showed an expected compact polygonal morphology after 3 weeks of culture in osteogenic medium. Furthermore, their ability to mineralize the deposited matrix was assessed after Alizarin Red staining. The intense red colour shows that the matrix was heavily mineralized after 3 weeks of culture in standard osteogenic medium (Figure 5.1B).

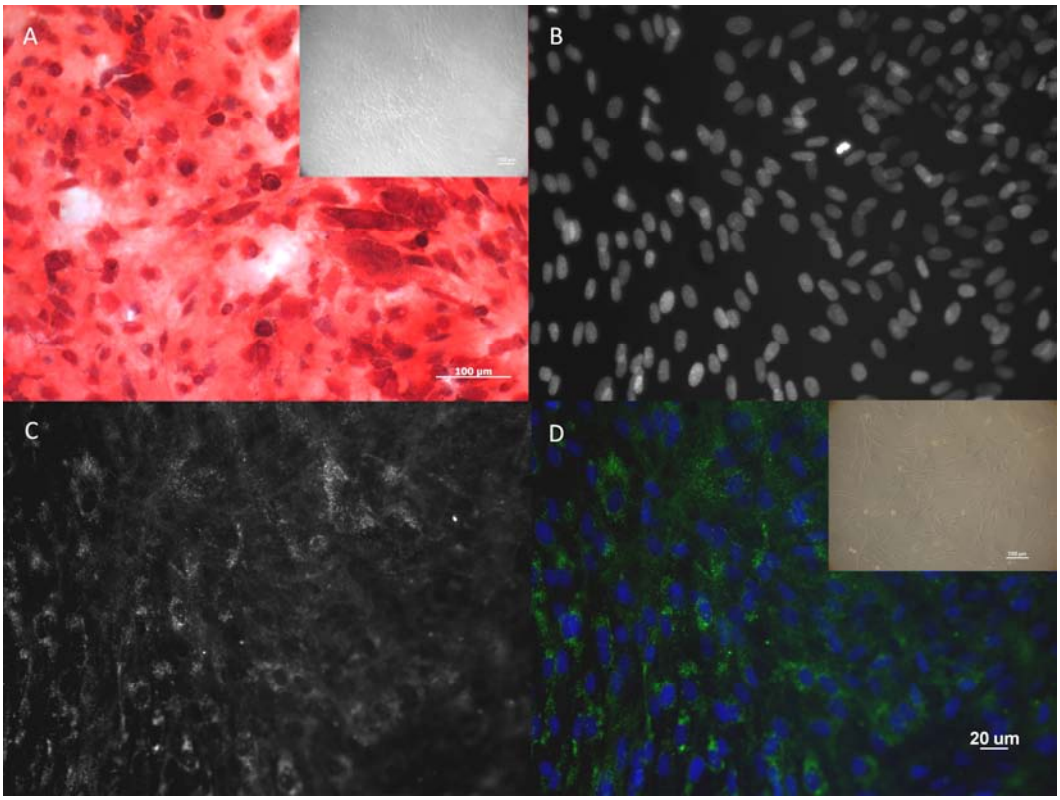

Figure 5.1 - Characterization of hMSCs-derived osteoblasts (A) and hDFb (B, C, D) before co-culture set-up. Mineralized matrix (red) after Alizarin Red staining in cultures of MSC-derived osteoblasts cultured for 3 weeks in standard osteogenic medium (A). hDFb are stained with mouse anti-human Fibroblast Surface Protein (C) and counterstained with DAPI (B). A merged picture is presented in D. Representative phase contrast micrographs of each cell type are presented as inserts.

5.3.2 Assessment of Cx43 presence and GjC between osteoblasts and fibroblasts

The presence of Cx43 protein in both fibroblasts and osteoblasts cultures was assessed by immunocytochemistry (figure 5.2). Micrographs show that this gap junctional protein was ubiquitously expressed throughout the culture of both types of cells. The fluorescence microscopy results showed no significant differences in terms of the presence of this surface protein in both types of cells.

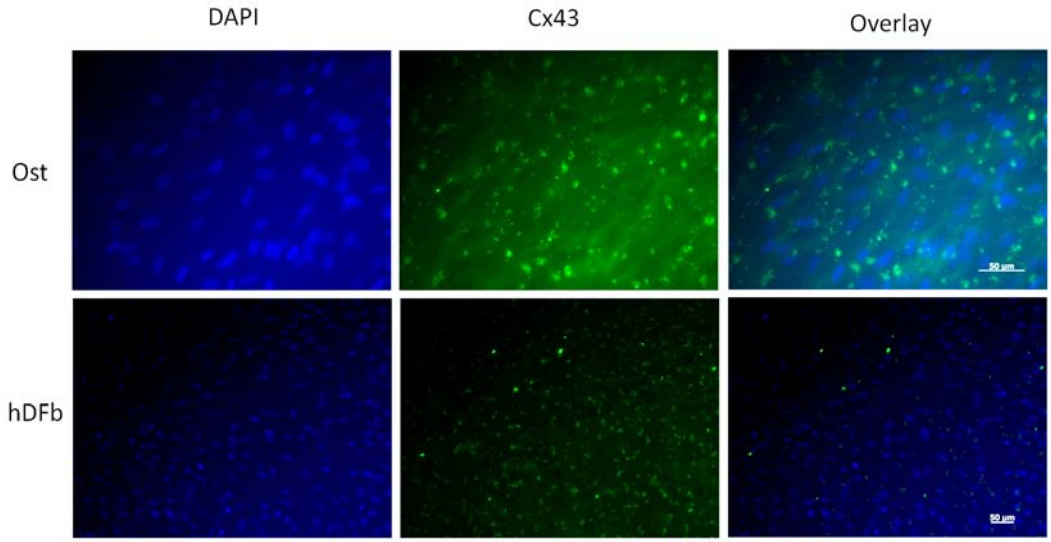

Figure 5.2 - Immunocytochemistry against gap junction protein Connexin 43 (Cx43, green) in both hMSCs-derived osteoblasts (Ost) and human dermal fibroblasts (hDFb) cultures. Cell nuclei were counterstained with DAPI (blue)

Furthermore, Cx43 presence was also assessed during the co-cultures. Like for the hMSCs-derived osteoblasts and hDFb monocultures, the gap junctional protein was present in a uniform manner in the co-cultures of hMSCs-derived osteoblasts and hDFb (figure 5.3).

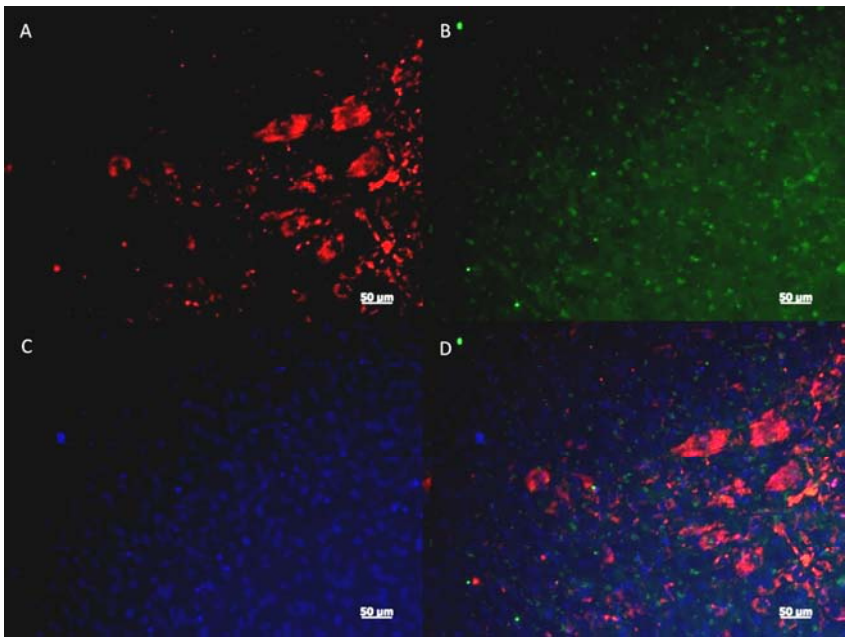

Figure 5.3 - Micrographs depicting co-cultures of hMSCs-derived osteoblasts and human dermal fibroblasts. Previously labeled hMSCs-derived osteoblasts are positive for DiI (red, A) and Connexin 43 (green, B) while hDFb only present signal for connexin 43 (green, B). Cell nuclei were counterstained with DAPI (blue, C). A merge picture of A, B and C is presented (D).

Heterotypic gap junctional communication between hMSCs-derived osteoblasts and hDFb was assessed by using the gap junctional permeable fluorescent dye calcein-AM. Looking at the fluorescence images it was clearly seen that after a short amount of time, 15 minutes, both hMSCs-derived osteoblasts and hDFb were capable of establishing heterotypic gap junctional communication, allowing calcein-AM to be transferred from the just seeded cells, round and brighter cells, to the adhered monolayer (figure 5.4 A and B). This observation was true both when fibroblasts or osteoblasts were the "parachuted" cells. When AGA was added to the culture medium, the transference of calcein-AM between cultured and parachuted cells was inhibited (figure 5.4 C and D).

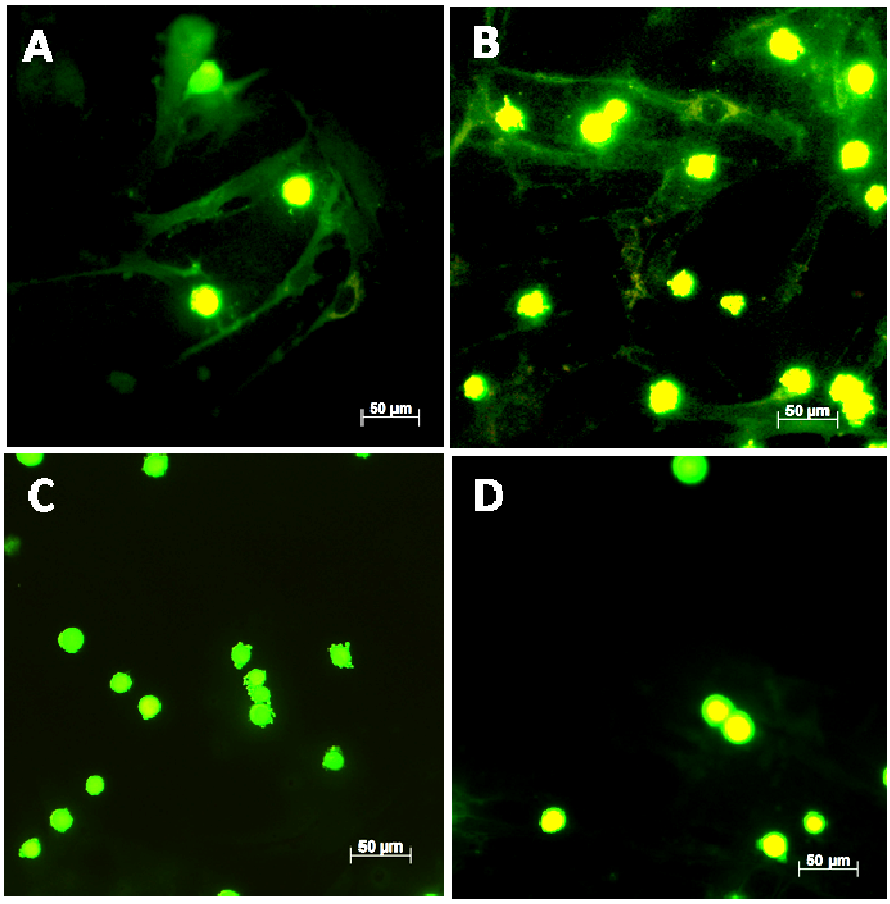

Figure 5.4 - Micrographs of the parachute technique to prove gap junctional communication between hMSCs-derived osteoblasts and hDFb. Either hMSCs-derived osteoblasts or hDFb were stained with calcein-AM and seeded over hDFb or hMSCs-derived osteoblasts, respectively. The transference of the gap junctional permeable dye was observed after 15 minutes of incubation at 37ºC from the just seeded and labeled hDFb (A, C) or hMSCs-derived osteoblasts (B, D) to the adhered monolayer. The addition of AGA to the cultures inhibited the transfer of the fluorescent dye (C, D).

5.3.3 Cell proliferation

hMSCs-derived osteoblast proliferation was measured by extrapolating the amount of dsDNA corresponding to the fraction of hMSCs-derived osteoblasts in co-culture from the total dsDNA quantified. The fraction of hMSCs-derived osteoblasts in co-culture resulting from the DiI-positive cells in the co-cultures was calculated by analysing the fluorescence images as described above. The extrapolated dsDNA results that directly correlate with the number of cells were plotted against culture time (Figure 5.5).

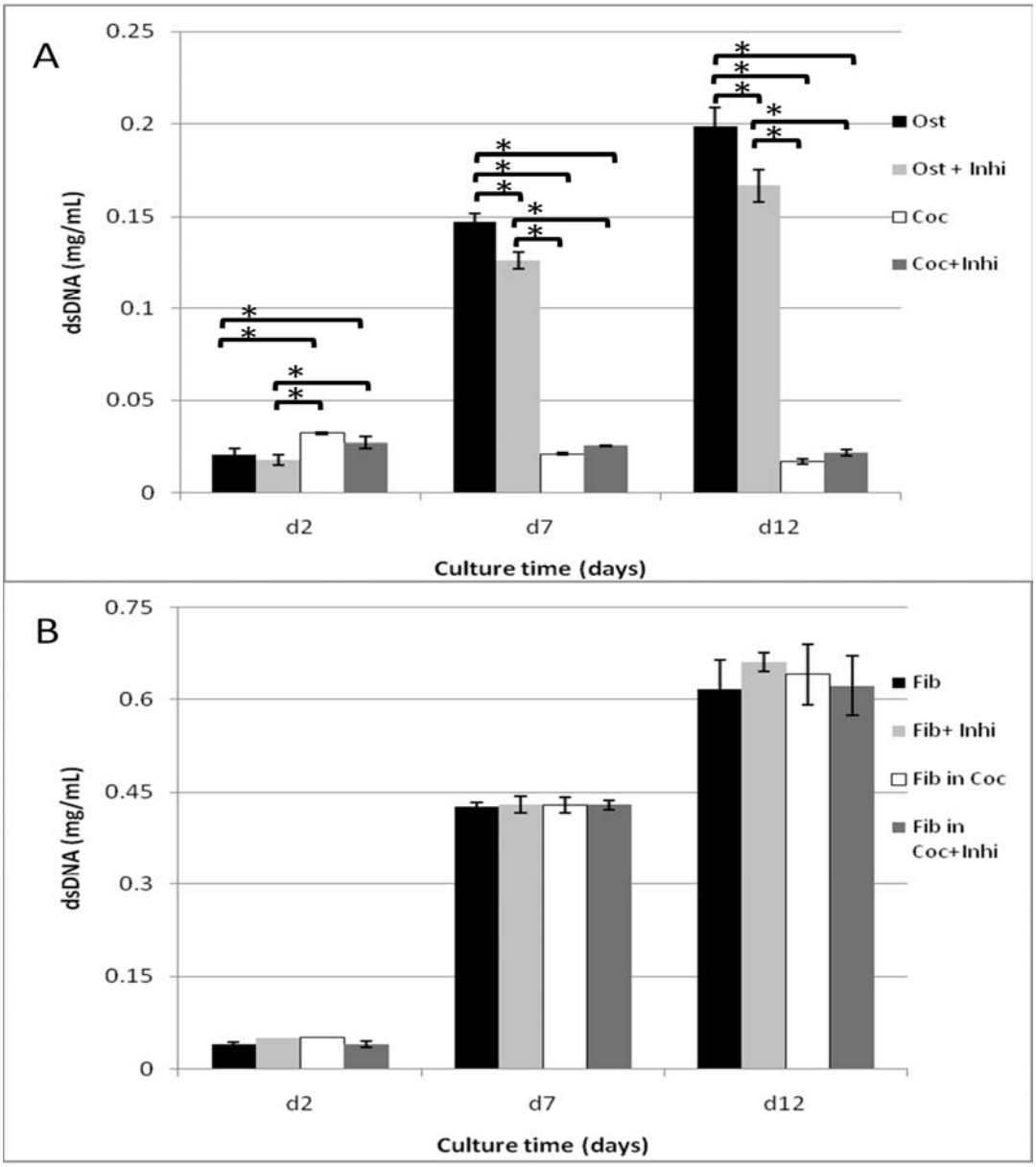

Figure 5.5 - Amount of dsDNA that correlates with the cell number quantified along culture of A) hMSCS-derived osteoblasts in monoculture (Ost) and in co-cultures (Coc),and B) hDFb in monoculture (Fib) and in co-cultures (Fib in Coc) both with and without gap junctional inhibitor (+Inhi). *p<0.05 in one way ANOVA test.

The dsDNA values at days 2, 7 and 12 for the hMSCs-derived osteoblasts monocultures in the presence and absence of GjC inhibitor were statistically different ($p<0.05$) from the ones in the co-cultures, also with and without inhibitor. Additionally, dsDNA values for both co-culture groups were constant throughout the time of culture meaning that no proliferation of hMSCs-derived osteoblasts in those conditions occurred. No differences were detected for the dsDNA amounts when comparing the co-cultures in the presence and absence of inhibitor ($p>0.05$) suggesting that the observed effect over hMSCs-derived osteoblasts did not involve GjC. Some inhibition, at days 7 and 12, was found when 18-AGA was added to the osteoblast monocultures ($p<0.05$), as expected. (Figure 5.5A)

Regarding dsDNA quantification for hDFb (figure 5.5B) no statistical significant difference was found between any of the tested conditions along the time of culture ($p>0.05$) indicating a normal proliferation rate for hDFb.

5.3.4 Osteoblastic activity

The analysis of the osteoblastic activity as assessed by the quantification of the ALP activity in co-cultures in comparison to the monocultures showed that the activity of this enzyme was significantly inhibited ($p<0.05$) in the presence of fibroblasts. When the co-cultures were performed in the presence of the GjC inhibitor, the values of ALP activity increased significantly ($p<0.05$) in comparison to what was observed in the co-cultures in the absence of AGA. Moreover, for day 7 of culture the ALP activity was similar in the co-cultures with AGA and in the monocultures of hMSCs-derived osteoblasts in the presence of the GjC inhibitor ($p>0.05$). The addition of GjC inhibitor to the monocultures ($p<0.05$) of hMSCs-derived osteoblasts induced a statistical significant decrease in the ALP activity at all times of culture (figure 5.6).

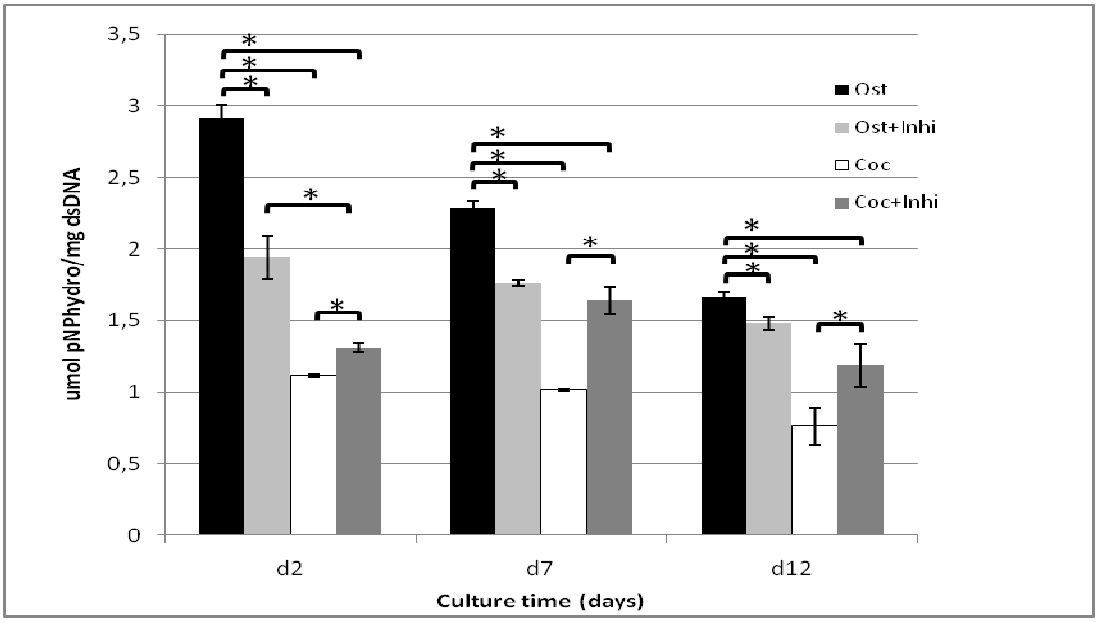

Figure 5.6. Amount of hydrolysed p-nitrophenol phosphate that correlates with the ALP activity quantified along culture in osteoblasts monocultures (Ost) and in co-cultures with hDFb (Coc), both with (+Inhi) and without gap junctional inhibitor. *p<0.05 after one way ANOVA test.

The expression of the osteogenic markers osteopontin and osteocalcin was quantified using quantitative real time RT-PCR (figure 5.7). The expression of the osteopontin gene in the co-culture with and without GjC inhibitor, in comparison to hMSCs-derived osteoblasts monocultures, was not changed. Osteopontin expression was detected for hDFb cultures for all the times of culture although at much lower levels than in the hMSCs-derived osteoblasts monocultures (figure 5.7B). As for osteocalcin, underexpression (p<0.05) of this gene was observed when hMSCs-derived osteoblasts were cultured in the presence of hDFb which was reversed (p<0.05), when co-cultures were established in the presence of GjC inhibitor.

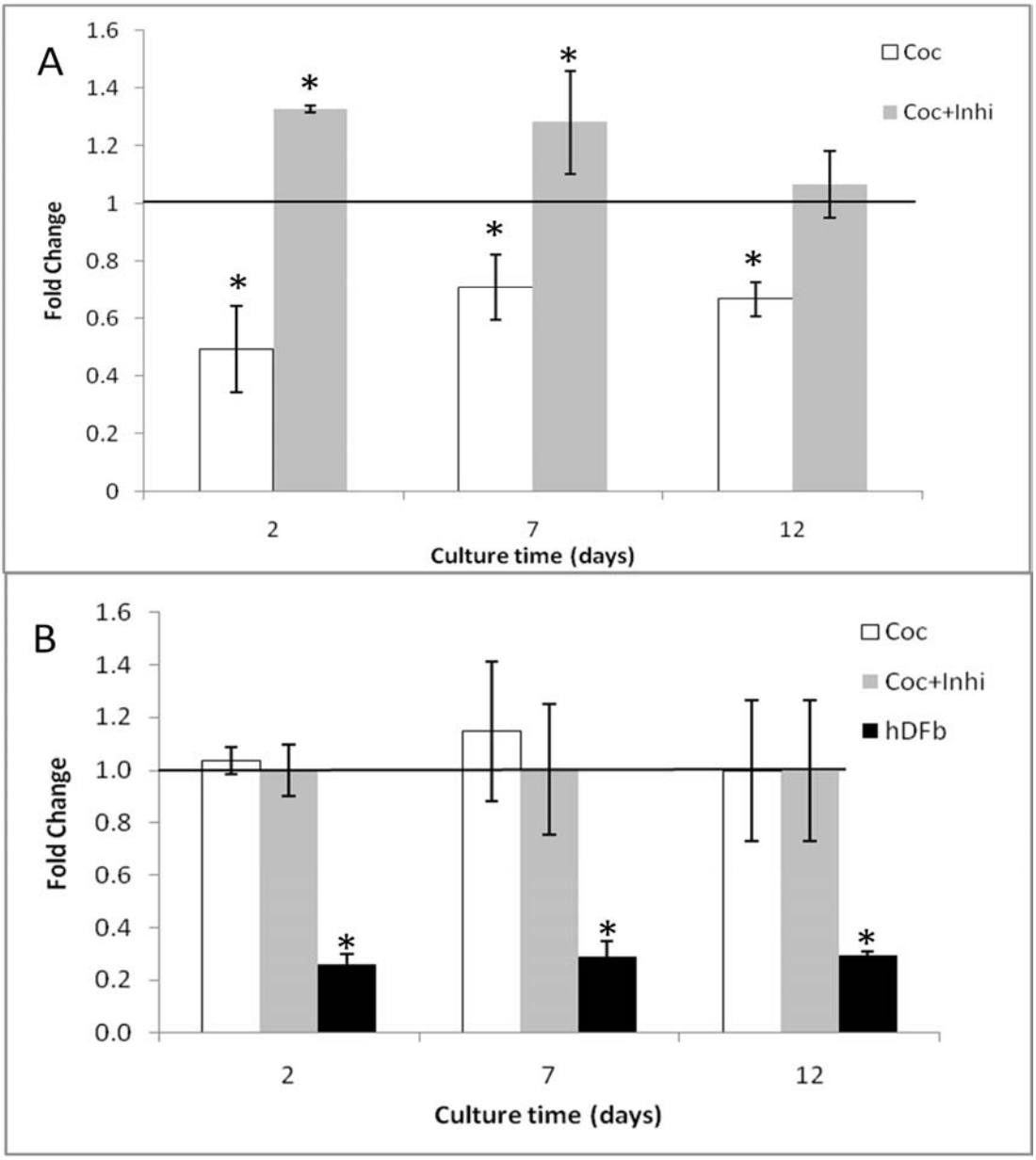

Figure 5.7. Osteocalcin (A) and osteopontin (B) relative expression in co-cultures (Coc), co-cultures with gap junctional inhibitor (Coc+Inhi) and fibroblasts (hDFb) calculated after quantitative real time RT-PCR. Results were first normalized against GAPDH and then against hMSCs-derived osteoblast monocultures at each day of culture. *p<0.05 against the ΔCt of the monocultures after *t*-test.

5.4 Discussion

Fibroblasts are found in most tissues of the body. They play key roles in regulating extracellular matrices and, depending on the tissue they belong to, in their regeneration (10-12, 30). In the particular case of bone tissue, fibroblasts have been linked to the

release of osteoclastogenic factors such as RANKL(31-33), osteoprotegerin(31-32) and cathepsin K(32), thus rendering them a significant role in periprosthetic loosening of hip arthroplasty by controlling osteoclastogenesis. As described before, fibroblasts are also linked to the production of prostaglandins that inhibit the mineralizing activity of BMSC during osteogenic differentiation. (15-16). In this work we hypothesized that such regulatory functions by fibroblasts might not be restricted to osteoclastogenesis nor to the production of soluble factors. We therefore considered GjC as a way fibroblasts could influence osteoblasts. To test this hypothesis we established a co-culture system of hMSCs-derived osteoblasts and hDFb in the presence and absence of AGA, a chemical GjC inhibitor. The effect of hDFb over hMSCs-derived osteoblasts was assessed in terms of cell proliferation and maintenance of the osteoblastic phenotype. In a first phase, gap junctional communication between those types of cells was proven. Co-cultures were then set up in normal and GjC-inhibiting conditions and cell proliferation, as well as the production of ALP and expression of osteopontin and osteocalcin, was analyzed along the culture.

Gap junctions (Gjs) are channels formed between the cell plasma membrane by the assembly of connexins (1, 8, 34-35). These channels allow the intercellular exchange of ions and metabolites known to have significant effects on the metabolism of the cells (1-2, 8, 34-35). Various studies of GjC in osteoblasts (3, 21, 36-37) and fibroblasts (23, 38) showed that the predominant connexin involved in homotypic GjC in both types of cells is the same, Cx43. Besides the homotypic GjC existing in osteoblasts and fibroblasts, there are examples of heterotypic GjC communication between osteoblastic cells and osteocytes(1, 17) and endothelial cells(3, 39). In the present work, heterotypic communication between hMSCs-derived osteoblasts and hDFb through Gjs was proven to occur. The use of the parachute technique, first employed by Ziambaras et al (26) to assess the effect of cyclic stretch on GjC between osteoblasts, but widely used thereafter to identify GjC,(40) permitted to verify the transference of the calcein-AM dye

from donor cells to recipient cells, independently of their type. The heterotypic GjC between hMSCs-derived osteoblasts and hDFb was further analysed in a co-culture system using a GjC inhibitor, AGA (41). The dsDNA results clearly demonstrated that hMSCs-derived osteoblasts in the co-cultures have their proliferation impaired in the presence of fibroblasts. This result is mainly due to the higher hDFb proliferation rate. Even when one tries to adjust the initial proportion of the two types of cells, hMSCs-derived osteoblasts were limited in terms of physical space and nutrient supply thus having their proliferation inhibited. This effect masked a possible inhibition of osteoblasts' proliferation derived from the presence of AGA in the co-cultures and consequential GjC disruption(42) since no significant difference ($p>0.05$) is found in dsDNA values for co-cultures with and without the GjC inhibitor. On the other hand, this inhibitory effect by AGA is evident when comparing the results of the monocultures of hMSC-derived osteoblasts cultured with and without AGA ($p<0.05$). Since no statistical difference ($p>0.05$) was found between the dsDNA values of hDFb for all the conditions and time points we can conclude that these were not affected by the presence of osteoblasts or by the presence of 18-AGA.

The regulation of bone cell differentiation as well as the modulation of gene expression in bone cells has been also connected to Gap junctional communication (36, 43-44). This issue was addressed in our work by quantifying the activity of ALP and by assessing, at the molecular level, eventual changes over the expression of osteocalcin and osteopontin in the hMSCs-derived osteoblasts. ALP activity of the hMSCs-derived osteoblasts was greatly affected by the presence of fibroblasts in the cultures. This effect was partially reversed by adding AGA to the co-cultures; in the presence of AGA the ALP activity values were always significantly higher ($p<0.05$) than those determined for the co-cultures in the absence of AGA indicating that GjC is implicated on the hDFb's inhibitory effect. Nevertheless, for the second and twelfth day of culture the ALP activity in the co-cultures with AGA was significantly lower ($p<0.05$) than that of monocultures in the presence of AGA, which can be explained by the negative effect of

fibroblasts on osteoblast mineralizing ability through the production of soluble factors (11, 15-16). In other hand, ALP values for monocultures in the presence of AGA were always significantly inferior ($p<0.05$) to the ones for control monocultures. This is an expected outcome from the inhibition of homotypic GjC in osteoblasts cultures.

As stated before, the modulation of GjC in osteoblasts results in the alteration of the expression of genes important for their osteogenic behaviour (36, 43-44). The relative expression of osteogenic-related genes osteocalcin and osteopontin was also addressed. In the presence of fibroblasts the expression of osteocalcin was downregulated between 1.5 fold for day 7 and 2 fold for day 2 ($p<0.05$). Although it is rather difficult to define these values as down regulation, the fold-change values for co-cultures without the GjC inhibitor were always significantly lower ($p<0.05$) than for cultures in the presence of inhibitor. Since this effect of fibroblasts was reverted when AGA was added to the co-cultures a correlation was thus established between GjC and the expression of this important osteogenic gene. Surprisingly, no difference ($p>0.05$) in fold change was observed in co-cultures with or without AGA for osteopontin expression. Although osteopontin is an osteoblast related gene it is also expressed by other cells (45) and it is involved in several processes such as inflammation, cancer progression and others(46-48). Among the osteopontin-producing cells are the fibroblasts (49-50). In our experiments, osteopontin expression was detected in the fibroblasts monocultures although at lower levels ($p>0,05$) than in the hMSCs-derived osteoblasts. Therefore, in the case of the co-cultures, both hMSCs-derived and hDFb osteopontin mRNA transcripts were quantified and the results cannot be specifically attributed to the hMSCs-derived osteoblasts.

To our knowledge this is the first work showing fibroblasts-osteoblasts interactions through GjC. As mentioned above, heterotypic GjC involving osteoblasts have a great phenotypic impact in the latter. Taylor et al (51) reported that osteocytes under fluid shear were capable of controlling osteoblasts' ALP activity through GjC. Villars and colleagues (39) demonstrated that GjC are required for human vascular endothelial

cells (HUVECS) to promote the differentiation of osteoprogenitor cells. More recently, Ciovacco et al (52) observed that the positive effect of megakaryocytes over osteoblast proliferation is further enhanced when GjC between these two cell types is inhibited. Therefore, it is well established that heterotypic GjC involving osteoblasts and other cell types can modulate the behaviour of the latter. We were able to demonstrate that in a direct co-culture system, hMSCs-derived osteoblasts and hDFb communication involves GjC. Moreover, hDFb were shown to exert a negative effect over the hMSCs-derived osteoblasts traduced in diminished ALP activity and downregulation of osteocalcin expression. Though, the detailed molecular mechanisms of the fibroblastic effect over osteoblasts through GjC can only be the subject of speculation. The involvement of secondary messengers such as Ca^{2+}, IP_3 or cAMP that can permeate through Gjs and act on the recipient cell (53-55) or the modulation by Gjs of the propagation of kinase signalling triggered by extracellular cues among adjacent cells, are all possible pathways to explore in order to obtain a complete picture of the heterotypic action of fibroblasts over osteoblasts through GjC.

5.5 Conclusions

In this work it was originally demonstrated not only the existence of Gap Junctional Communication between hMSCs-derived osteoblasts and hDFb but also the negative effects of fibroblasts over the osteoblasts using that pathway. These conclusions can be very relevant for a bone Tissue Engineering strategy when a construct with *ex-vivo* cultured osteoblast is implanted, as immediately afterwards it is expected an inflammatory reaction with the involvement of fibroblasts. The present work represents therefore an advance in predicting the outcome of such devices and constitutes a first step for exploring the communication mechanisms existing between fibroblasts and osteoblastic cells.

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Chapter 6

DEVELOPMENT OF OSTEOGENIC CELL SHEETS FOR BONE TISSUE ENGINEERING APPLICATIONS

Chapter 6

DEVELOPMENT OF OSTEOGENIC CELL SHEETS FOR BONE TISSUE ENGINEERING APPLICATIONS

Abstract

The use of scaffolds in combination with osteogenic cells has been the gold standard in Bone Tissue Engineering strategies. These strategies have, however, in many cases failed to produce the desired results due to issues such as the immunogenicity of the biomaterials used and cell necrosis at the bulk of the scaffold related to deficient oxygen and nutrients diffusion. Here, we originally propose the use of cell sheet (CS) engineering as a possible way to overcome some of these obstacles. Osteogenic CSs were fabricated by culturing rat bone marrow cells in thermo-responsive culture dishes. The CSs were recovered from the dishes using a low temperature treatment and then were implanted subcutaneously in nude mice. New bone formation was verified from day 7 post transplantation using x-ray, μ-CT and histology. It was also verified the presence of a vascularized marrow in the new formed bone after 6 weeks of transplantation. Osteoid deposition in the interior of the new bone suggests bone remodelling. Furthermore, osteocytes could be found in the new formed bone, supporting the conclusion that healthy bone tissue was formed after ectopically transplanting of osteogenic CSs. These results therefore confirm the great potentiality of CS engineering to be used in bone tissue engineering applications.

Chapter based on Pirraco RP, Obokata H, Iwata T, Marques AP, Tsuneda S, Yamato M, Reis RL, Okano T, Development of Osteogenic Cell Sheets for Bone Tissue Engineering Applications (2011) Tissue Engineering Part A 17:1507-1515

6.1 Introduction

The number of people that require medical intervention in order to solve bone related illnesses such as non-union fractures or loss of bone tissue due to cancer tends to grow due to the constant aging of population (1-3). Currently, the gold-standard strategies to address issues like critical bone defects are the use of autologous bone graft, allografts and materials like ceramics and metals (1, 3-4). All of these strategies have significant problems; examples are availability of tissues, donor site morbidity issues, immunogenic and integration in the host tissue, that limit their application range and their overall performance(1, 5). It has been accepted for a few years that new strategies are needed in order to address the challenges posed in this field. Tissue Engineering (TE)-based strategies have been trying to solve many of the above referred to problems. These approaches typically involve the use of different cell types suitable for bone TE, growth factors and 3D biodegradable scaffolds (4, 6). Such approaches, however, face in many cases serious problems such as the immune response to the implanted construct, inadequate biodegradability rate and the lack of vascularization which leads to cell necrosis in the bulk of the construct (2, 7-10).

Cell sheet engineering technique using thermo-responsive dishes might constitute a useful alternative to solve some of the mentioned issues. This technique, as proposed by Professor Okano's group allows for the recovery of the cells within its own matrix to be used as intact single or multilayered cell sheets to engineer transplantable tissues (11-13).So far this technology was proposed for the treatment of several tissues such as cornea(14), myocardium(15), periodontal ligament(16) and bladder(17) but never for bone. The peculiar mechanical and biological properties of bone tissue make the translation of the obtained outcomes from the regeneration of the above mentioned tissues into bone rather complicated. Others have previously attempted to produce cell sheets for the regeneration of bone tissue (18-20). Zhou and colleagues (20) combined osteogenic cell sheets made form porcine bone marrow stromal cells wrapping them around polycaprolactone–calcium phosphate scaffolds. Analysis post-subcutaneous

implantation of the construct showed some degree of new bone formation but mainly at the periphery of the scaffolds. The same pattern of new calcified tissue, around the scaffold, was achieved by Gao *et al* (19), using a coral scaffold, and Akahane *et al*. (18), using a hydroxyapatite ceramic scaffold. In the latter case, the cell sheets were also ectopically implanted without any scaffold (18) and new bone formation, albeit disorganized, was verified. In the three cases, new bone tissue was fairly disorganized, poorly vascularised and limited to the surface of the scaffolds on where the cell sheets were wrapped. In contrast with the above referred works, where cells were detached using a cell scraper, the use of thermo-responsive dishes allows for the use of an intact cell-cell and cell-matrix architecture, due to the well developed culture dish recovery methods (12-13).

In this work, we aimed at studying the *in vivo* bone formation potential of osteogenic cell sheets recovered non-invasively by temperature decrease. Osteogenic cell sheets were developed from rat bone marrow stromal cells, cultured in thermo-responsive dishes, and then characterized. The developed sheets were subsequently transplanted subcutaneously to the dorsal flap of nude mice. Implants were recovered at different time points post-transplantation and characterized. New bone formation was apparent from 7 days post-implantation. Six weeks after implantation thick, vascularised new bone with what appears to be bone marrow was clearly observed.

6.2 Materials and Methods

6.2.1 Temperature-responsive culture surfaces

Thermo-responsive dishes (CellSeed, Tokyo, Japan) were prepared as previously described (21). Briefly, N-isopropylacrylamide monomer in 2-propanol solution was spread onto 35 mm diameter culture dishes (BD Biosciences, Franklin Lakes, NJ). Dishes were then irradiated by electron beam, resulting in both polymerization and covalent grafting of the poly(N-isopropylacrylamide) (PIPAAm) onto the cell culture surfaces. PIPAAm-grafted dishes were rinsed with cold-distilled water to remove

ungrafted monomer, and dried in nitrogen gas. Dishes were finally sterilized with ethylene oxide gas prior to experimental use.

6.2.2 Cell sheets fabrication

Bone marrow was flushed from the femurs of 4 weeks old male Wistar rats (Charles River, Yokohama, Japan). After vigorous pipetting to disaggregate any clumps, the suspension was placed over Histopaque 1083 (Sigma-Aldrich, Tokyo, Japan) and centrifuged at 2500RPM for 25 minutes. The mononuclear cell fraction was recovered after centrifugation and washed in phosphate buffered saline (PBS, (Sigma-Aldrich, Tokyo, Japan)) to remove any remaining Histopaque. Cells were then seeded in 100 mm of diameter dishes and cultured in basal medium (DMEM (low glucose; Wako Pure Chemical Industries, Tokyo, Japan), supplemented with 10% fetal bovine serum (Japan Bioserum Co.Ltd, Hiroshima, Japan) and 100 units/ mL of penicillin–streptomycin (Sigma-Aldrich Japan, Tokyo, Japan)) at 37 ºC and in a 5% of CO_2 humidified atmosphere. After 24 hours of culture, non-adherent cells were removed from the culture and the adherent cells were then cultured until semi-confluence was achieved. Cells were detached using a 0.25% trypsin-EDTA solution (Gibco BRL LifeTechnologies, Carlsbad, USA) and seeded in 35 mm of diameter thermo-responsive dishes at a concentration of 2.5 x 10^5 cells per dish. Cultures were maintained for three weeks in osteogenic medium (basal medium supplemented with 10^{-8} M dexamethasone (Sigma-Aldrich, Tokyo, Japan), 50 µg/mL ascorbic acid (Sigma-Aldrich, Tokyo, Japan) and 10 mM beta-glycerophosphate (Sigma-Aldrich, Tokyo, Japan).

6.2.3 Recovery of cells from thermoresponsive dishes

In order to recover the cells from the thermoresponsive dishes, culture medium was removed from the culture dishes and replaced with 1 mL of PBS. A poly(vinylidene difluoride) (PVDF) (Immobilon-P, DURAPORE®, Millipore Corporation, Billerica, USA)

membrane with 20 cm of diameter was placed over the cells in the thermoresponsive dishes and incubated at 20 ºC for 10 minutes. After this time, cell sheets spontaneously detached from thermoresponsive dishes. Some of the recovered cell sheets were fixed in 10% formalin (Wako Pure Chemicals, Osaka, Japan) for posterior histological characterization.

6.2.4 In vivo transplantation

The transplantation of the cells sheets was carried out as reported previously (22). Briefly, 6 weeks old male nude mice (Charles River Japan, Yokohama, Japan) (6 animals per transplantation time) were anesthetized with a constant flux of 4% of isofluorane. Dorsal skin was cut opened using 3x3cm cutting sides. Recovered cell sheets were placed on mouse subcutaneous dorsal flap and left to adhere to the connective tissue of dorsal skin for 5 minutes. After that time, the PVDF membranes were removed and silicone membranes were placed over the cell sheets to prevent the contact between the cell sheets and the muscular tissue. Control mice (3 animals per transplantation time) were also prepared by implanting only silicone membranes. Skin incisions were closed using 5-0 nylon sutures. Animals were kept with food and water *ad libitum*. After 7 days, 3 weeks and 6 weeks of transplantation, animals were euthanized with CO_2 and implants were recovered for histological characterization.

6.2.5 Histological characterization

After fixation, both in vitro recovered cell sheet and implanted samples were embedded in paraffin, without demineralization, and 5 µm thick sections were made. Hematoxylin and eosin staining was performed following standard protocols.

To assess mineral deposition Alizarin Red staining was performed. Briefly, a solution of 0.1 % of alizarin red (Sigma-Aldrich, Tokyo, Japan) was made in ddH_2O and the pH was adjusted to 4.6. Sections were deparaffinized and 1 mL of alizarin red solution was added to each slide. Sections were observed in the microscope until correct amount of

colour developed. Pink/purple colour was considered positive for mineral deposition. Sections were then washed in ethanol and xylene. Micrographs of the sections were taken after both stainings.

Immunostaining for osteocalcin was performed by incubating both cell sheet and implant sections with a 1/200 anti–osteocalcin antibody (Millipore Corporation, Billerica, USA) overnight, at 4 ºC, and then for one hour, room temperature, with a biotinylated secondary antibody (DakoCytomation, Glostrup, Denmark). Sections were incubated with Streptavidin-HRP (DakoCytomation, Glostrup, Denmark) solution for 20 minutes and then treated with DAB chromogenic substrate solution (DakoCytomation, Glostrup, Denmark) for 3 minutes.

Stained sections were analysed with an Eclipse E800 microscope (Nikon, Tokyo, Japan).

6.2.6 Micro-computed Tomography

To investigate the 3D structure of the mineralized tissue formed after transplantation, non-destructive techniques, X-Ray and Micro-Computed Tomography (SkyScan, Kontich, Belgium) were used. Recovered implants, after paraffin embedding, were cut in half and scanned in a high-resolution mode of 11,32 µm x/y/z and an exposure time of 1900ms. The energy of the scanner used was 50 keV with 171 mA current. The Micro-CT scans were followed by a 3D reconstruction of serial images.

6.3 Results and discussion

6.3.1 Osteogenic Cell Sheets

The herein used cell sheet engineering methodology allowed fabricating and recovering intact rat bone marrow-derived cell sheets composed by a dense collagenous matrix where cells are embedded (figure 6.1A). By micrograph observations, the estimated thickness of the cell sheets after recovery was of about 30 µm (figure 6.1). Although this value does not reflect the real thickness of the sheet in

vitro due to the phenomena of contraction of the sheet that happens after the release from the dish, it corresponds to the thickness of the implanted cell sheets.

The presence of what appeared to be mineral deposits was clearly macroscopically identified in the sheets before its recovery from the thermoresponsive dishes. In fact, after the recovery of the cell sheets from the dishes several mineral nodules were attached to the dish, which was expected to happen considering the osteogenic differentiation culture protocol used.

Alizarin Red staining was used to confirm the presence of mineral deposition in the cultured cell sheets (figure 6.1B). Significant mineral deposition in the cultured cell sheets was observed as evidenced by the intensity of the staining. Immunohistochemistry against osteocalcin was used to validate the osteogenic nature of the cultured cell sheets. Osteocalcin is a non-collagenous matrix protein whose secretion correlates with matrix mineralization and therefore it is a marker for the osteogenic phenotype (23-25). The results showed that the cultured cell sheets were highly expressing this protein, once again demonstrating the osteogenic nature of the developed cell sheets (figure 6.1C).

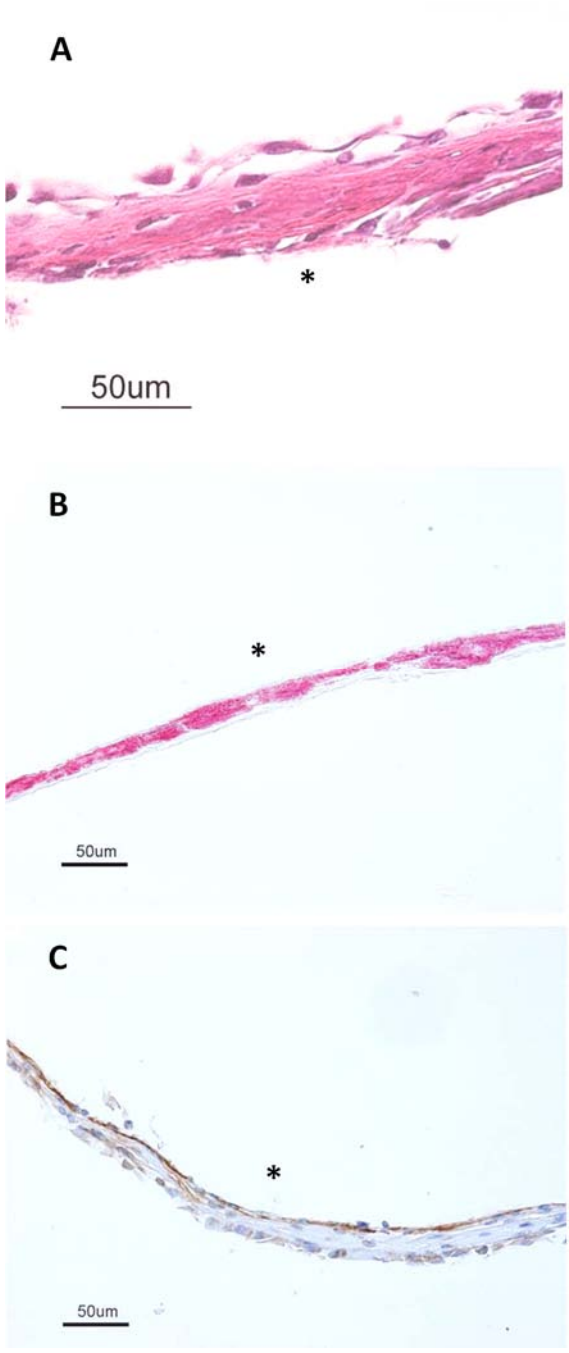

Figure 6.1 – *In vitro* cultured cell sheets after low-temperature recovery from thermoresponsive dishes characterized using (A) H&E staining, (B) alizarin red staining (mineral deposition in purple) and (C) immunocytochemistry for osteocalcin (expression in brown). Asterisks mark the basal side of the cell sheets.

6.3.2 In Vivo *Bone Formation*

Evidences of in vivo new bone formation after transplantation of the osteogenic cell sheets were confirmed by X-ray and µ-CT analysis (figure 6.2). After 7 days of transplantation the amount of dense tissue is already significant and a notorious

increase in the density of the neo-bone was observed as the transplantation time increases.

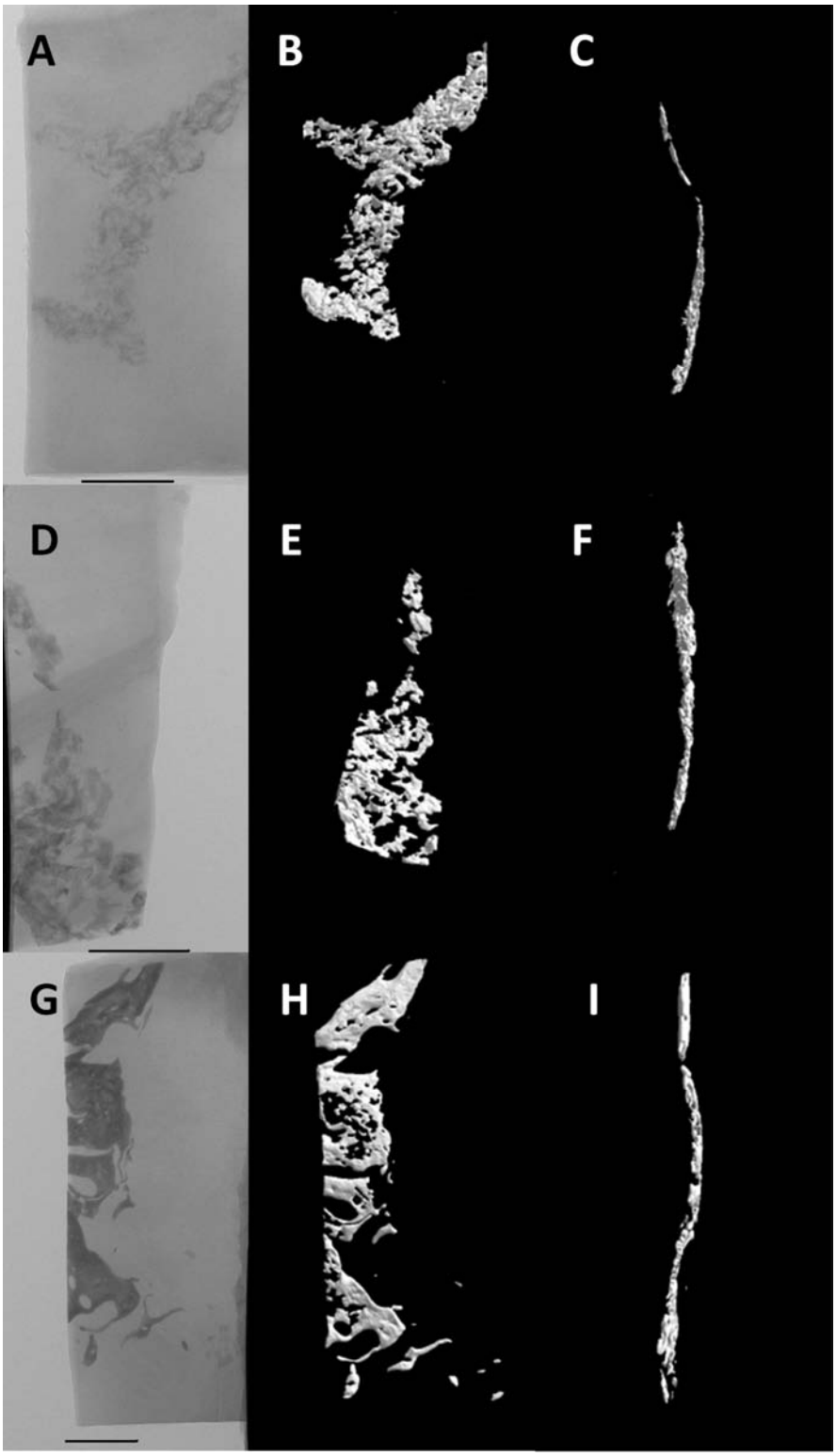

Figure 6.2 – Representative x-ray (A, D and G) and μ-CT images (side [C, F and I] and front view [B, E and H]) of the transplants (each divided in half after recovery) after (A-C) 7 days, (D-F) 3 weeks and (G-I) 6 weeks of implantation. Bars in the x-ray images represent 2 mm and are valid for all the images.

These results were further confirmed by Alizarin Red/H&E staining (figure 6.3). Mineralized tissue was observed at the implant site just seven days post-transplantation. The amount of new- mineralized bone increased through-out the time of transplantation until it reached a maximum 6 weeks post-transplantation.

The new bone developed in what appears to be separated patches along the width of the subcutaneous dorsal flap. Six weeks after transplantation numerous contiguous bone patches, with a maximum thickness of 250 µm and a maximum width of 1 mm (figure 6.3) were clearly observed. In fact, crossing these results with the µ-CT 3D reconstruction (figure 6.2C), the patches observed in the histology were ramifications of the same bone mass. These bone masses had a flat form that may result either from the original form of the cell sheets, or from the local mechanical environment present in the mouse's subcutaneous site of transplantation, which determined the form of the new tissue (26-27), or from both.

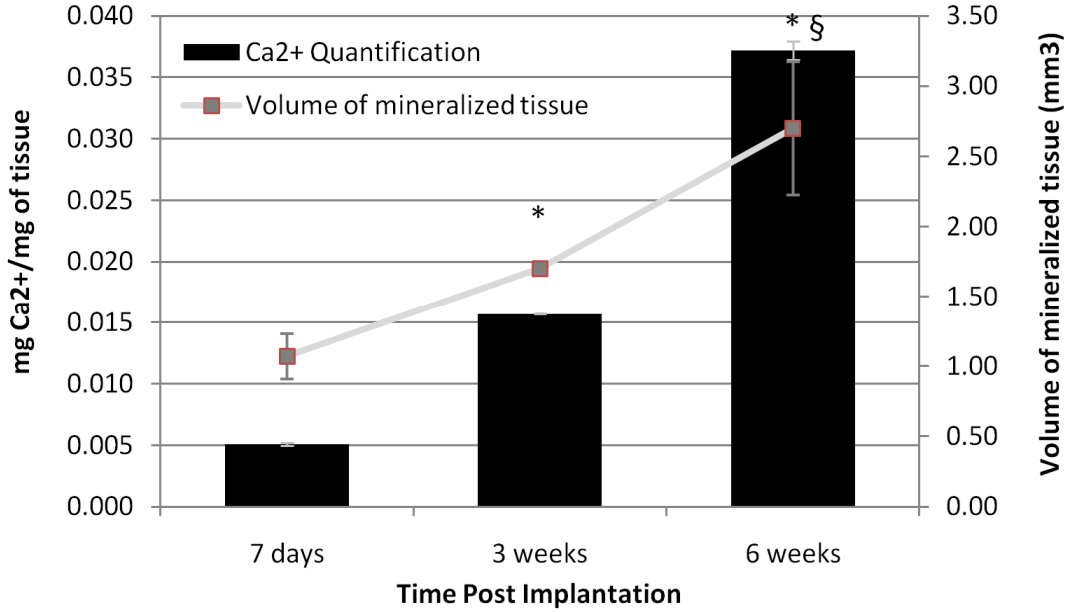

Figure 6.3 – Amount of Calcium, quantified by the o-cresolphtaleine-complexone method (bars), and volume of mineralised tissue measured by µ-CT (line), of the retrieved samples at different times post-implantation. *p<0.05 relating to "7 days" values and § p<0.05 relating to "3 weeks" values for both Ca^{2+} quantification and mineralized tissue volume.

Osteocalcin immunohistochemistry results showed that this protein was expressed at different locations even after only 7 days of transplantation (figure 6.4A). These positive

sites seem to correlate to sites where new bone was being formed. With the increase of new bone formed with the transplantation time, it was clear that the majority of the cells that were positive for this protein were concentrated around the new tissue (figures 6.4B and 6.4C). This is an expectable outcome because this protein is directly related to matrix mineralization and, consequently to new bone formation (23-25).

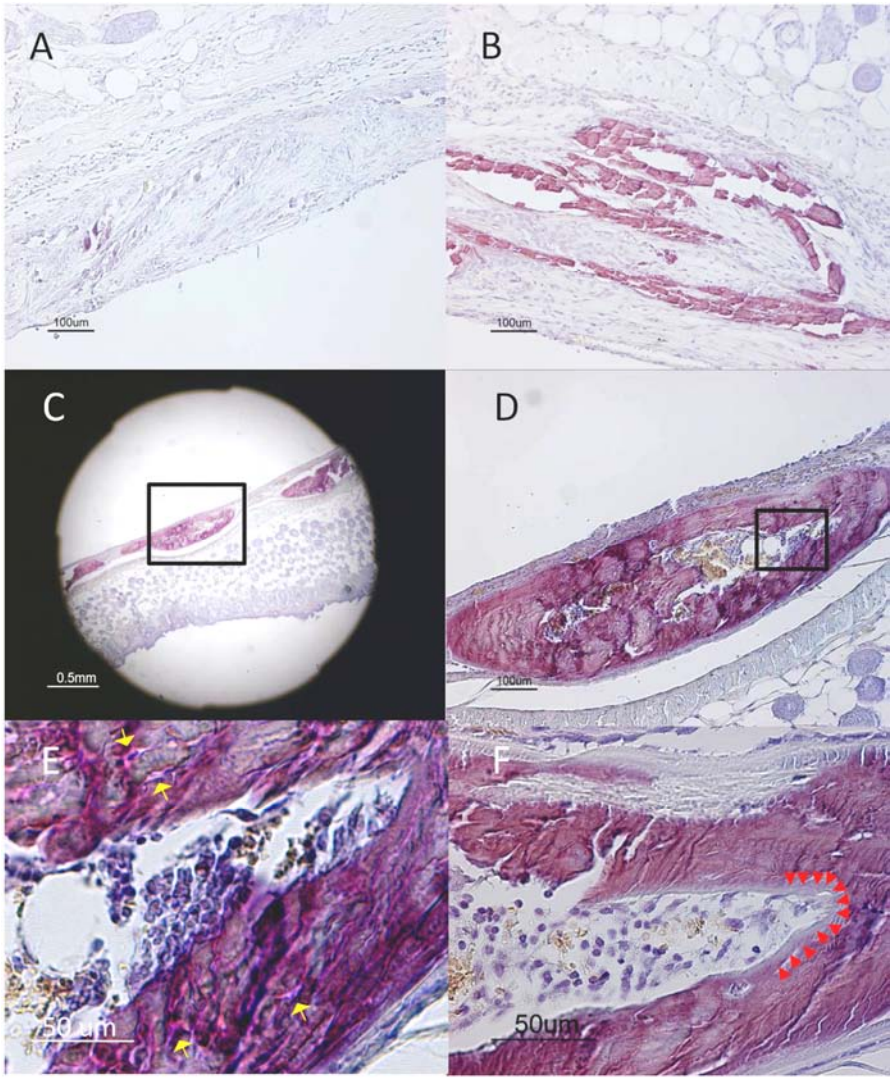

Figure 6.4 - Alizarin red and Haematoxylin staining of the transplants sections recovered after (A) 7 days, (B) 3 weeks and (C, D, E and F) 6 weeks of implantation. D and E are sequential close-ups of image C. Yellow arrowheads in E mark osteocytes and red arrowheads in F mark osteoid deposition. Mineral deposition is in purple.

The most striking feature observed in the new-bone at 6 weeks post-transplantation was the void largest patches (figures 6.3D and 6.3E). This fact may indicate that bone remodelling was occurring, following the normal bone tissue metabolism where bone is constantly resorbed and formed (28-30). In fact figure 6.3F clearly shows osteoid deposition. Moreover, numerous cells could be found in those void marrow spaces, among which red blood cells that seems to indicate vascularisation of the new formed bone. This fact reinforces the hypothesis that the new formed bone is being remodelled, since it is proven the existence of a direct connection between vasculature and bone remodelling (31-35). Also very important to notice was the presence of osteocytes, as it is visible in figure 6.3E since, besides being the most common cells in bone tissue, these cells are believed to be regulators of bone homeostasis (36-38).

Previous works that used osteogenic cell sheets in *in vivo* settings(18-20), either with or without scaffolds, have successfully produced new bone tissue. However, the new formed tissue was in most cases either disorganized (18) or incapable of growing in the interior of the scaffold (19-20). In the latter situations, the limitations imposed by the use of scaffolds, namely cell migration to the interior of the scaffold and oxygen and nutrient diffusion limitations (7-10), impaired those strategies to achieve more successful outcomes. However, in the work of Akahane *et al* (18), cell sheets are also implanted without any scaffold and new bone tissue was formed. In this work, cell sheets were recovered using a cell scraper. Although cell-to-cell junctions are preserved using this method, the use of thermo-responsive dishes guarantees that both cell-matrix junctions and the ECM itself are preserved (12-13). The ECM can then serve as a natural glue that enable these sheets to be applied virtually in any anatomic site when implanted. Maeda and colleagues (39) recently developed a device that allows a minimally invasive endoscopic transplantation of cell sheets fabricated in thermo-responsive dishes. Further developments of this method can bring the way to, using a minimally invasive surgery, deliver cell sheets as *de facto* sheets for many applications, in opposition to the hypothesis of just injecting them, as suggested by

Akahane and colleagues (18). The herein proposed methodology might be applied in flat bones defects, which, due to the nature of the cell sheets, is the most obvious choice. However, methods such as the overlay of several cell sheets combined with polysurgery (40) can be the answer to create thicker and bigger bone tissue. This will be addressed in future studies.

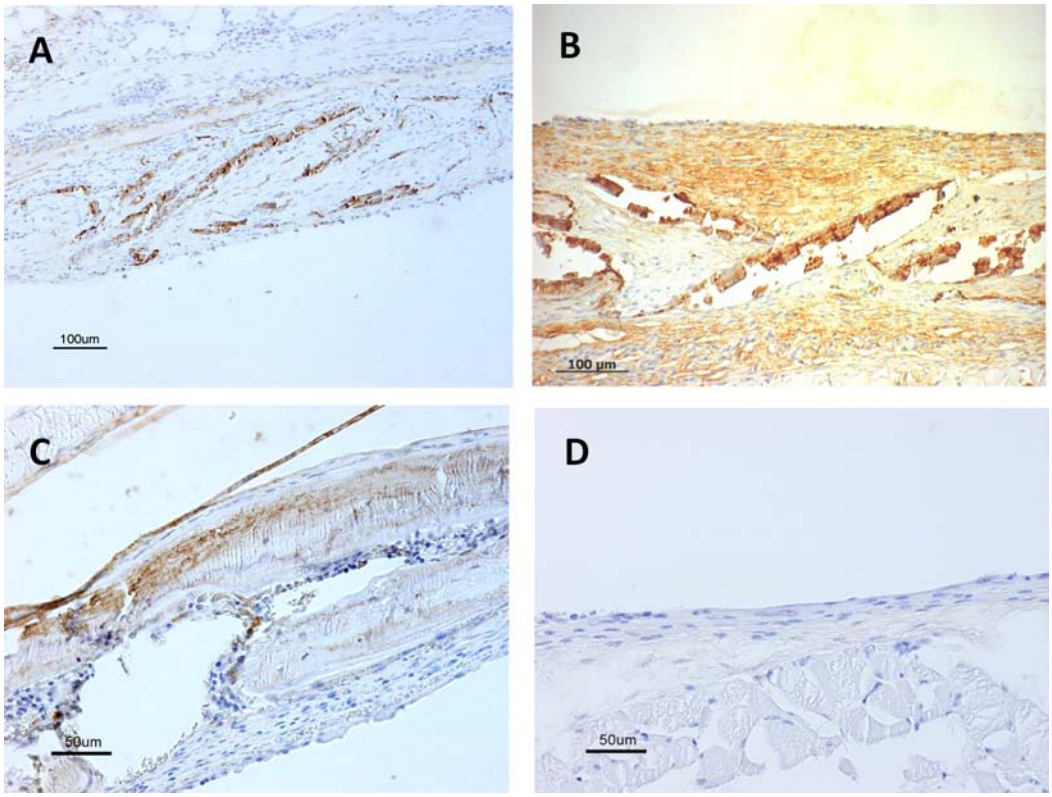

Figure 6.5 – Immunocytochemistry for osteocalcin in the transplants sections recovered after (A) 7 days, (B) 3 weeks and (C) 6 weeks of implantation. (D) corresponds to the negative control section obtained from the nude mice where only the silicon membranes were implanted for 6 weeks.

6.4 Conclusions

As already demonstrated for other tissues, cell sheet engineering is also a very promising technique for bone tissue engineering applications. This work demonstrated that new bone tissue, with very interesting characteristics, namely the presence of osteocytes, vascularisation and bone marrow formation, was formed from a single osteogenic cell sheet. 3D neo-bone tissue was obtained in vivo without the use of any

biodegradable scaffold and a new window of opportunity with great potential is open in the bone tissue engineering field.

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Chapter 7

ENDOTHELIAL CELLS ENHANCE THE *IN VIVO* BONE FORMING ABILITY OF OSTEOGENIC CELL SHEETS

Chapter 7

ENDOTHELIAL CELLS ENHANCE THE *IN VIVO* BONE FORMING ABILITY OF OSTEOGENIC CELL SHEETS

Abstract

Addressing the vascularization problem is of vital importance when engineering three-dimensional tissues. The application of endothelial cells in tissue engineered constructs is being increasingly used in order to obtain pre-vascularized constructs and to enhance *in vivo* neo-vascularization.

In this work, rat bone marrow stromal cells were cultured in thermo-responsive dishes under osteogenic conditions to obtain homotypic or heterotypic, with human umbilical vein endothelial cells, cell sheets. Cells were retrieved as sheets from the dishes after incubation at 20°C. Monoculture osteogenic CS were stacked on top of homotypic or heterotypic CS, and subcutaneously implanted in the dorsal flap of nude mice for 7 days. The implants characterization clearly showed mineralized tissue formation for both conditions. Transplanted osteogenic cells were found at the new tissue site demonstrating the CS bone-inductive effect. Perfused vessels, positive for human CD31, confirmed the contribution of human umbilical vein endothelial cells (HUVECs) for the neo-vascularization of co-culture CS constructs. Furthermore, calcium quantification and expression of osteocalcin and osterix genes were higher for the CS constructs with HUVECs demonstrating a more robust osteogenic potential of these constructs.

This work originally demonstrates the potential of using ECs, combined with osteogenic CS, to increase the *in vivo* vascularization of CS-based 3D constructs for bone tissue engineering purposes. .

Chapter based on Pirraco RP, Iwata T, Yoshida T, Marques AP, Yamato M, Reis RL, Okano T, Endothelial cells enhance the *in vivo* bone forming ability of Osteogenic Cell Sheets, (2011) submitted

7.1 Introduction

The use of scaffolds in combination with osteogenic cells has been the gold standard in Bone Tissue Engineering strategies (1-4). These imply that the engineered constructs would then be transplanted to patients in order to restore or improve the function of bone tissue. Bone engineered constructs have however often failed to produce the desired results due to issues such as the immunogenicity of the biomaterials used, and cell necrosis at the bulk of the scaffold related to deficient oxygen and nutrients diffusion (5-9). Oxygen and nutrient supply is in fact a critical issue when aiming at artificially creating thick tissues such as bone (1-2). The consequence of this is that successful production of tissue engineered products has been quite limited to thin tissues such as skin (9). This scenario illustrates how vascularization is a major hurdle of bone tissue engineering. An approach that has been increasingly studied to overcome this issue is the *in vitro* pre-vascularization of the constructs (6, 9-11). This strategy relies on combining, with the engineered construct, endothelial cells that will self-organize in pre-vascular structures, which will anastomose with the host vasculature after implantation. Nonetheless, the success of this approach to solve the vascularization issue of tissue engineered constructs strongly depends on a fast and functional anastomosis of the pre-vascular network with the host's vasculature (9).

Cell sheet (CS) engineering as proposed by Okano et al (12-14) is based on the use of thermo-responsive poly-N-isopropylacrylamide (PIPPAam)-grafted culture surfaces that allow the recovery of confluent cells in the form of sheets. The principle of the method is based on the hydrophilic/hydrophobic nature of PIPPAam, below/above its low critical solution temperature (LCST), respectively forming cell adhesive and cell repellent (12-14) surfaces. Cell adhesion is therefore controllable by means of modulating the temperature. Using this strategy, Okano's group has been proposing solutions for the regeneration of cornea(15), myocardium(16), periodontal ligament(17) and bladder(18), among others. Recently, we have proposed the use of osteogenic CS

produced using thermo-responsive dishes for bone tissue engineering applications (19). In that work, using a single CS we successfully obtained, ectopically, vascularised bone with marrow. Osteogenic CS obtained by mechanical retrieval with cells scrapers, alone or combined with scaffolds, were also tested for bone tissue engineering purposes but the general outcome was a poor amount of new bone tissue formed (20-22). Nakamura et al (23) previously demonstrated, however, the full regeneration of a rat critical size defect by using CS recovered by cell scraper. These works further show how scaffold–free, CS-based constructs can be applied for bone regeneration.

The recovery of CS using thermo-responsive methods presents several advantages over the use of enzymatic and mechanical dissociation methods (24). It was demonstrated that mechanical recovery of cells by scraping depletes ECM proteins from the resulting sheets (24). On the contrary, recovery of cells from thermoresponsive dishes preserves ECM proteins (13-14), which act as a natural glue thus avoiding the use of sutures after transplantation (13-14). This advantage has been also explored to construct 3D CS-based tissues, as the adhesion of the successive CS occurs naturally. The vascularization of the 3D CS-based tissues is an essential issue since necrosis affects non-vascularized transplanted tissues thicker than 100 µm (25). This was previously addressed by Shimizu and colleagues (25) by multi-transplanting rat cardiomyocytes CS. Using this strategy, the authors were able to create 1 mm-thick myocardium with an organized vascular network. The pre-vascularization of CS-based 3D constructs has nevertheless been the focus recent works (26-27). The combination of several myoblasts sheets (recovered from PIPPAam dishes) and human umbilical vein endothelial cells (HUVECs) using a CS stacking methodology resulted in the generation of a pre-vascularized cell dense tissue (27). This work is a first proof-of-concept on how CS engineering has the potential to produce 3D pre-vascularized tissues.

Considering the need to promote the formation of vascularized neo-bone tissue the aim of the present work was to further develop our previously (19) proposed CS-based

strategy for bone tissue engineering. With this purpose the potential of combining osteogenic CS with endothelial cells to increase the vascularization of cell constructs was assessed, and the influence of endothelial cells over the amount of bone tissue formed was demonstrated. Rat osteogenic CS were obtained using thermo-responsive dishes and stacked with HUVECs in between. The constructs were then implanted subcutaneously in the dorsal flap of nude mice and characterized in terms of involvement of the transplanted cells on both bone tissue formation and vascularization.

7.2. Materials and Methods

7.2.1 Temperature-responsive culture surfaces

Thermo-responsive dishes (CellSeed, Tokyo, Japan) were prepared as previously described (28). Briefly, N-isopropylacrylamide monomer in 2-propanol solution was spread onto 35 mm diameter culture dishes (BD Biosciences, Franklin Lakes, NJ). Dishes were then irradiated by electron beam, resulting in both polymerization and covalent grafting of PIPAAm onto the cell culture surfaces. PIPAAm-grafted dishes were rinsed with cold-distilled water to remove ungrafted monomer, and dried in nitrogen gas. Dishes were finally sterilized with ethylene oxide gas prior to experimental use.

7.2.2 Cell sheets fabrication

Bone marrow was flushed from the femurs of 4 weeks old male Wistar rats (Charles River, Yokohama, Japan). After careful pipetting to disaggregate any clumps, the suspension was seeded in 75 cm^2 Primaria culture flasks (BD Biosciences, USA) and cultured in basal medium (α-MEM with Glutamax I, low glucose (Gibco, USA), supplemented with 10% fetal bovine serum (Japan Bioserum Co.Ltd, Japan) and 100 units/mL of penicillin–streptomycin (Sigma-Aldrich Japan, Japan) at 37 ºC and in a 5% of CO_2 humidified atmosphere. After 24 hours of culture, non-adherent cells were

removed and the adherent cells were then maintained in culture until semi-confluence was achieved. Rat bone marrow stromal cells (rBMSCs) were detached using a 0.25% trypsin-EDTA solution (Gibco BRL LifeTechnologies, USA) and seeded in 35 mm of diameter thermo-responsive dishes at a concentration of 2.5 x 10^5 cells per dish. Cultures were maintained for 21 days in osteogenic medium (basal medium supplemented with 10^{-8} M dexamethasone (Dexart, Fuji Pharma, Japan), 50 µg/mL ascorbic acid (Sigma-Aldrich, Japan) and 10 mM beta-glycerophosphate (Sigma-Aldrich, Japan). HUVECs were purchased from Lonza (Lonza, Japan), seeded in 75 cm^2 Primaria culture flasks and cultured in Endothelial Cell Growth Medium MV2 (ECGM, Promocell, Germany).

7.2.3 Establishment of co-cultures

HUVECs at passage 3 were seeded on the thermoresponsive-dishes with the rBMSCs, at a density of 1 x 10^5 cells per dish, 3 days before those completed the 21 days of osteogenic differentiation. Co-cultures were maintained in ECGM supplemented with 10^{-8} M dexamethasone, 50 µg/mL ascorbic acid (Sigma-Aldrich, Tokyo, Japan) and 10 mM beta-glycerophosphate (Sigma-Aldrich, Japan) for 3 days until transplantation.

7.2.4 Recovery of cells from thermoresponsive dishes

In order to recover the cells from the thermoresponsive dishes, culture medium was removed from the culture dishes and replaced by 1 mL of PBS (Sigma-Aldrich, Japan). A poly(vinylidene difluoride) (PVDF) (Immobilon-P, Millipore Corporation, USA) membrane with 2 cm of diameter was placed over the rBMSCs monocultures and incubated at 20ºC for 10 minutes. After this time, CS spontaneously detached from thermoresponsive dishes. CS-membrane constructs were stacked over the rBMSCs/HUVECs co-cultures and the procedure to detach the adhered CS repeated. Controls consisted of two stacked monocultured osteogenic CS.

7.2.5 In vivo *transplantation*

The transplantation of the stacked cells sheets was carried out as reported previously (27). Briefly, 6 weeks old female nude mice (Charles River, Japan) were anesthetized with a constant flux of 4% of isofluorane. Dorsal skin was cut opened using 3x3cm cutting sides. Recovered CS constructs were placed on mouse subcutaneous dorsal flap and left to adhere to the connective tissue of dorsal skin for 5 minutes. After that time, the PVDF membranes were removed and silicone membranes were placed over the CS to prevent the contact between the CS and the muscular tissue. Control mice were prepared by implanting constructs composed of two stacked osteogenic monocultured sheets. Skin incisions were closed using 5-0 nylon sutures. Animals were kept with food and water *ad libitum* for all the experiment duration. After 7 days of transplantation, animals were euthanized with CO_2 and implants were recovered for histological characterization. Since the implanted cells were from male rats and the hosts were female, we were able to track by immunohistochemistry the implanted cells by screening for the sex-determining region Y protein, exclusively present in the male cells.

7.2.6 Histological characterization

Both *in vitro* recovered CS constructs and implanted samples were fixed in 4% paraformaldehyde (Wako Pure Chemicals, Japan) and embedded in paraffin, without demineralization to obtain 5 µm thick sections. Hematoxylin and eosin staining was performed following standard protocols.

Immunostaining was performed in both *in vitro* recovered CS constructs and implanted samples using the following antibodies: goat anti-mouse/-rat SRY (Santa Cruz Biotechnology, USA), rabbit anti-human/-mouse/-rat collagen I (Abcam, USA), rabbit anti-human/-rat/-mouse osteopontin (Abcam, USA), rabbit anti-human/-mouse/-rat osterix (Santa Cruz Biotechnology, USA) and mouse anti-human CD31 (DakoCytomation, Danmark). Briefly, sections were incubated with the primary

antibody overnight, at 4 ºC, and then for one hour, at room temperature, with the biotinylated secondary antibody (DakoCytomation, Denmark). Sections were incubated with Streptavidin-HRP (DakoCytomation, Denmark) solution for 20 minutes and then treated with DAB chromogenic substrate solution (DakoCytomation, Denmark). Stained sections were analysed with an Eclipse E800 microscope (Nikon, Japan).

To assess mineral deposition, Alizarin Red staining was performed both on the dishes with the differentiated rBMSCs and on the deparaffinized histological sections. Samples were incubated with alizarin red S solution (Sigma-Aldrich, Tokyo, Japan) and observed in the microscope until correct amount of colour developed. Cells were then counterstained with haematoxylin and washed in ethanol and xylene.

7.2.7 PCR analysis

RNA extraction and cDNA production

mRNA was extracted from tissues using TriZol (Invitrogen, USA) reagent according to manufacturer instructions. Briefly 800 µl of TriZol were added per 50 mg of tissue and samples were homogeneized by vigorous pipetting and maceration. Following 5 minutes incubation, 160 µl of chloroform (Sigma, USA) were added to each sample; samples were then incubated for 15 min at 4℃ and centrifuged at 13000rpm and 4℃ for 15min. After the centrifugation the aqueous part of each sample was collected and an equal volume of isopropanol (Sigma, USA) was added. Following an overnight incubation at -20℃, samples were centrifuged at 9000 rpm and 4 ºC for 10 minutes. Supernatants were discarded and pellets were washed in ethanol, centrifuged at 9000rpm and 4℃ for 5min, and ressuspended in 12 µl of RNase/DNase free water (Gibco, UK). RNA quantity and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using iScript cDNA synthesis Kit (BioRad, USA) and the MiniOpticon Real-

Time PCR Detection System (BioRad, USA). An initial amount of 2 µg of mRNA was used in a total volume of 20 µl of RNase/DNase free water.

Quantitative Real Time PCR

Mouse osteocalcin, collagen I and osterix transcripts were quantified in the cDNA samples using a quantitative Real Time PCR reaction. For each sample GAPDH was used as the housekeeping gene. The primers were designed using the Primer 3 software (v 0.4.0) and synthesized by MWG Biotech (Germany) as follows: osteocalcin forward 5' CTGAGTCTGACAAAGCCTTC 3' and osteocalcin reverse 5' GCTGTGACATCCATACTTGC 3'; collagen I forward 5' GAGCGGAGAGTACTGGATCG 3' and collagen I reverse 5' GCTTCTTTTCCTTGGGGTTC 3'; osterix forward 5' GCTCCTCGGTTCTCTCCATCTG 3' and osterix reverse 5' TGTGTTGCCTGGACCTGGTG; Mouse GAPDH forward 5' ACGGCCGCATCTTCTTGTGCA 3' and mouse GAPDH reverse 5' AATGGCAGCCCTGGTGACCA 3';

A concentration of 200 nM was used for all the primers in a final volume of 25 µl of sample. The Real Time PCR reaction was done using the EvaGreen SuperMix (BioRad, USA) following manufacturer instructions in a MiniOpticon Real-Time PCR Detection System (BioRad, USA).

The relative quantification of the referred gene transcripts expression was performed using the $2^{-\Delta\Delta CT}$ method (Perkin Elmer User Bulletin No.2). All values were first normalized against GAPDH values and then the CS with HUVECs values against the CS alone values at each time point of culture.

7.2.8 Calcium Quantification

Halves of the recovered implants were weighted in and incubated in HCl 0,5M to remove and dissolve the calcium. Calcium quantification in the obtained solutions was performed with the o-*cresolphtalein*-complexon method using the Roche Cobas kit

(Roche Diagnostics, Germany) following manufacturer's instructions. The absorbance of the samples was read at 570 nm in a microplate reader Synergy-HT (Bio-Tek, Germany). The calcium concentrations were extrapolated from the calibration curve obtained using serial dilutions of a calcium chloride solution and then normalized with the initial tissue mass.

7.3 Results

7.3.1 In vitro *Cell Sheets characterization*

Osteogenic differentiation of rBMSCs in the thermo-responsive dishes was confirmed by the intense and uniform alizarin red staining for calcium deposition observed in the culture in osteogenic medium (figure 7.1A, left dish) in contrast with absence of staining detected in basal medium culture (figure 7.1A, right dish).

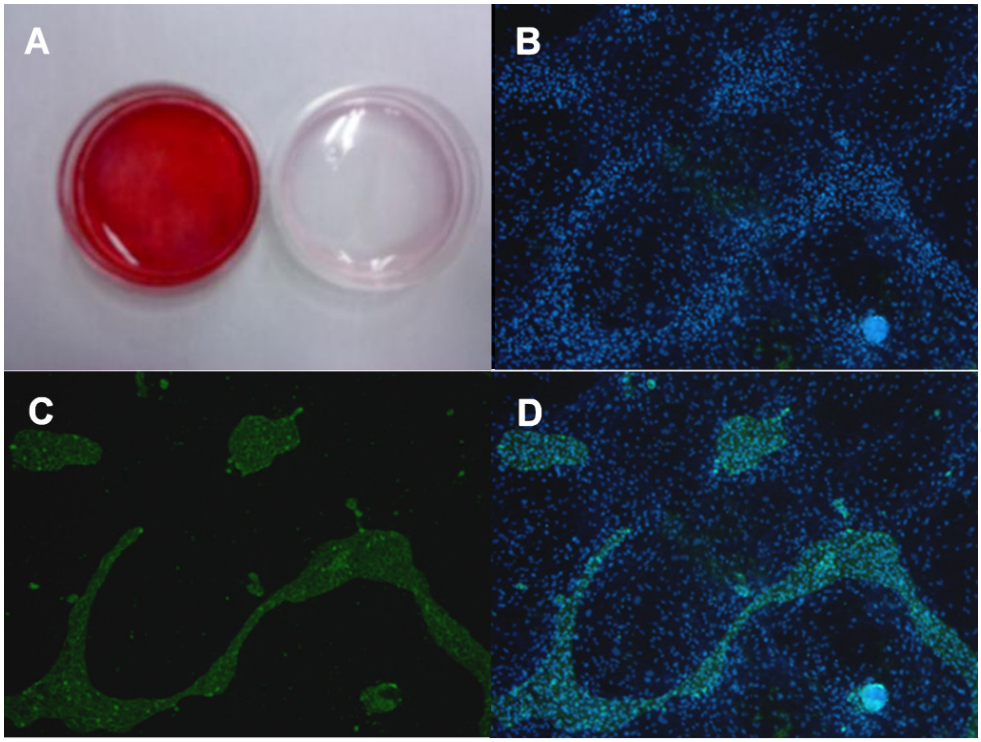

Figure 7.1 – Alizarin Red staining (A) and CD31 immunostaining (C and D) of osteogenic cell sheets co-cultured with HUVECs just before retrieval from thermo-responsive dishes. rBMSCs after 21 days of osteogenic differentiation (A, left side dish) and cultured in basal medium (A, right side dish). The nuclei of all cells are stained blue with DAPI nuclear staining (B) and HUVECS (CD31 positive) distribution is depicted in green. A merge micrograph of B and C is presented (C).

The presence of HUVECs in co-culture with the rBMSCs was assessed by immunohistochemistry against human CD31 (figure 7.1C, D). HUVECs seeded on the

rBMSCs cultured under osteogenic conditions, after 3 days, were organized in round or elongated colonies on top of the rBMSC.

After recovery from the thermoresponsive dishes following low temperature treatment, the organization of the HUVECs remained intact as observed after immunostaining for human CD31 in a transversal section of the co-cultured CS (figure 7.2A).

After recovery from dishes following low temperature treatment, homotypic and heterotypic CS were histologically characterized. H&E staining revealed that the CSs were composed by a thick collagenous matrix (figure 7.2B). Matrix mineralization in the sheets was confirmed by Alizarin Red staining (figure 7.2C). The osteogenic character of the CSs was further corroborated by the positive immunostaining for the matrix protein osteopontin (figure 7.2D) and for the transcription factor osterix (figure 7.2E). The male origin of the cells forming the sheets was also confirmed with the positivity for SRY marker (figure 7.2F).

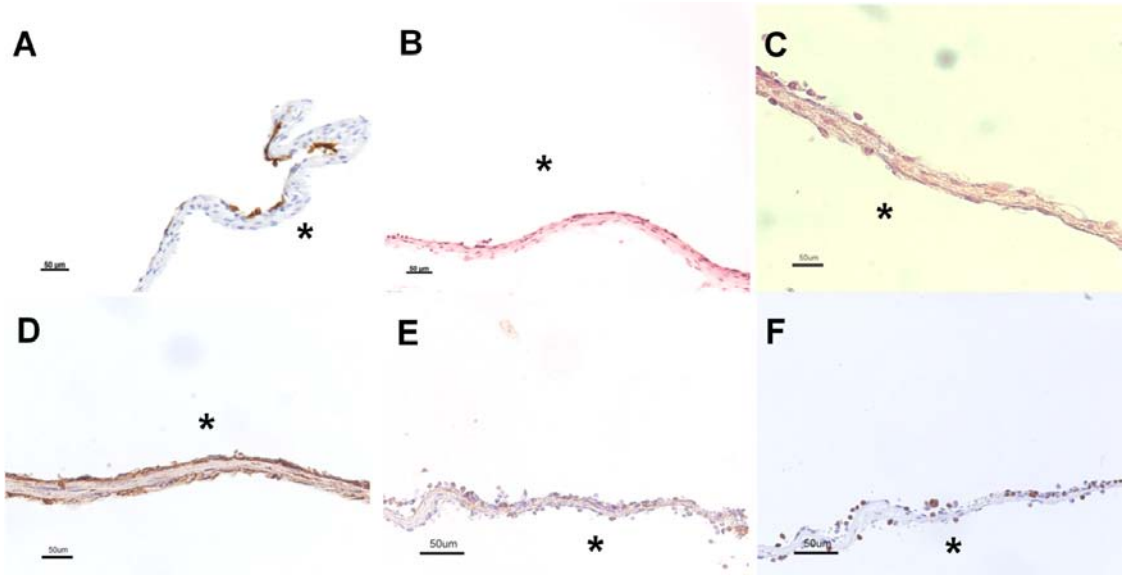

Figure 7.2 - Histological characterization of osteogenic cell sheets after retrieval from thermo-responsive dishes. Cell sheets were stained with H&E (B) and alizarin red (C) and immunostained for CD31 (A) osteopontin (D), osterix (E) and SRY (F). Asterisks (*) indicate basal side of the cell sheets.

7.3.2 Implants characterization

The characterization of the implanted co-culture and control constructs was performed 7 days post-implantation. The deposition of the neo-tissue formed was assessed by H&E staining (figure 7.3A and 7.3B). The new tissue developed in patches along the dorsal flap of the mice; it seems that the patches are larger in the co-cultured (figure3A) than in the control (figure 3B) condition. The deposition and mineralization of an osteogenic matrix was confirmed by the presence of the bone protein osteopontin (figure 7.3C and 7.3D) surrounding the mineralized tissue, as determined by alizarin Red staining (figure 7.3E and 7.3F).

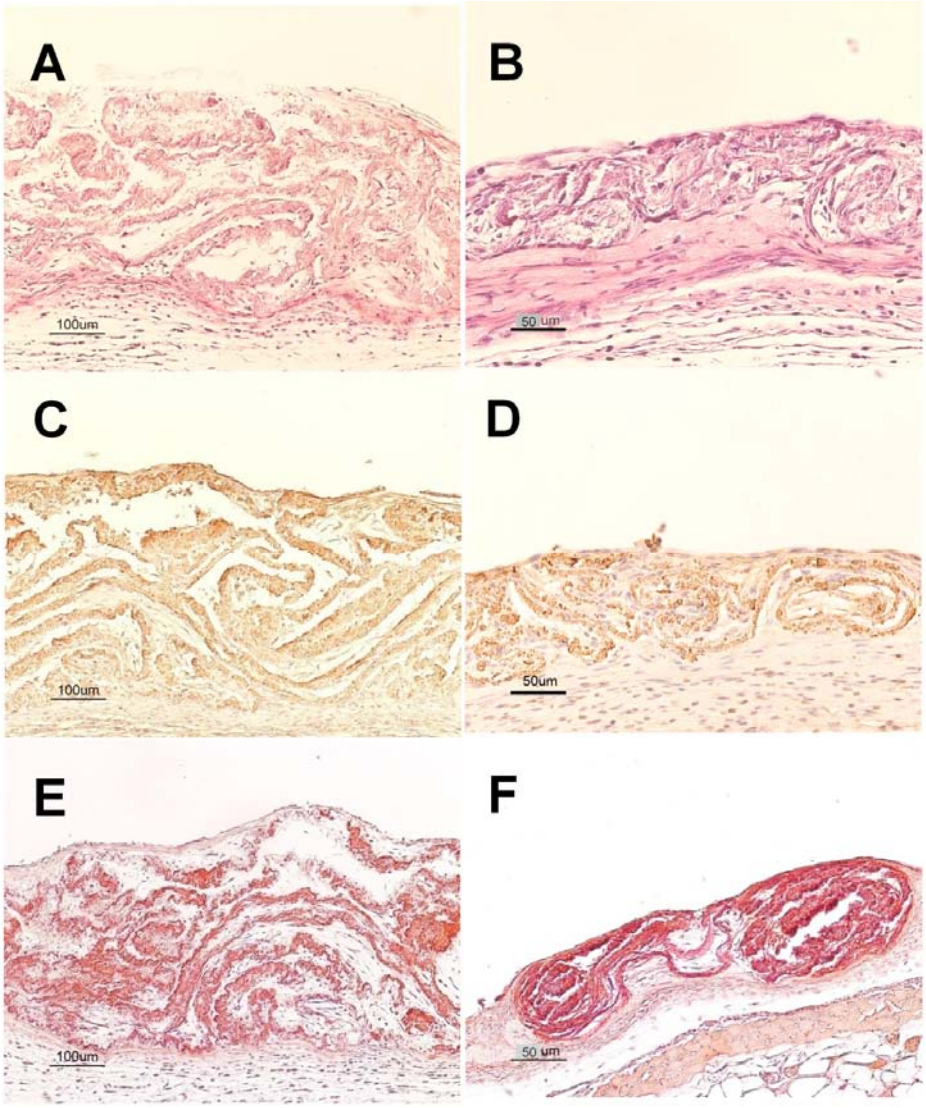

Figure 7.3 - Histological characterization of retrieved implants 7 days after transplantation of the co-culture (A,C, E) or monoculture (B, D, F) constructs. Retrieved constructs were stained with H&E (A, B) and alizarin red (E, F) and immunostained for osteopontin (C,D).

Comparing the SRY and the osterix stainings that respectively identify only rat male cells (figure 7.4A and 7.4B) and both mouse and rat cells (figure 7.4C and 7.4D), it was possible to conclude that osteogenic cells, other than the transplanted donor cells, are present and contributing to the formation of mineralized tissue. Furthermore, transcripts of bone-related genes were quantified for the retrieved implants (figure 7.4E). Osteocalcin and osterix were significantly upregulated, respectively 19.6 and 8.7 times after the implantation of the co-cultured constructs and in relation to the construct without HUVECs. No statistical significant difference was detected for the collagen I transcripts quantity between co-culture constructs and controls.

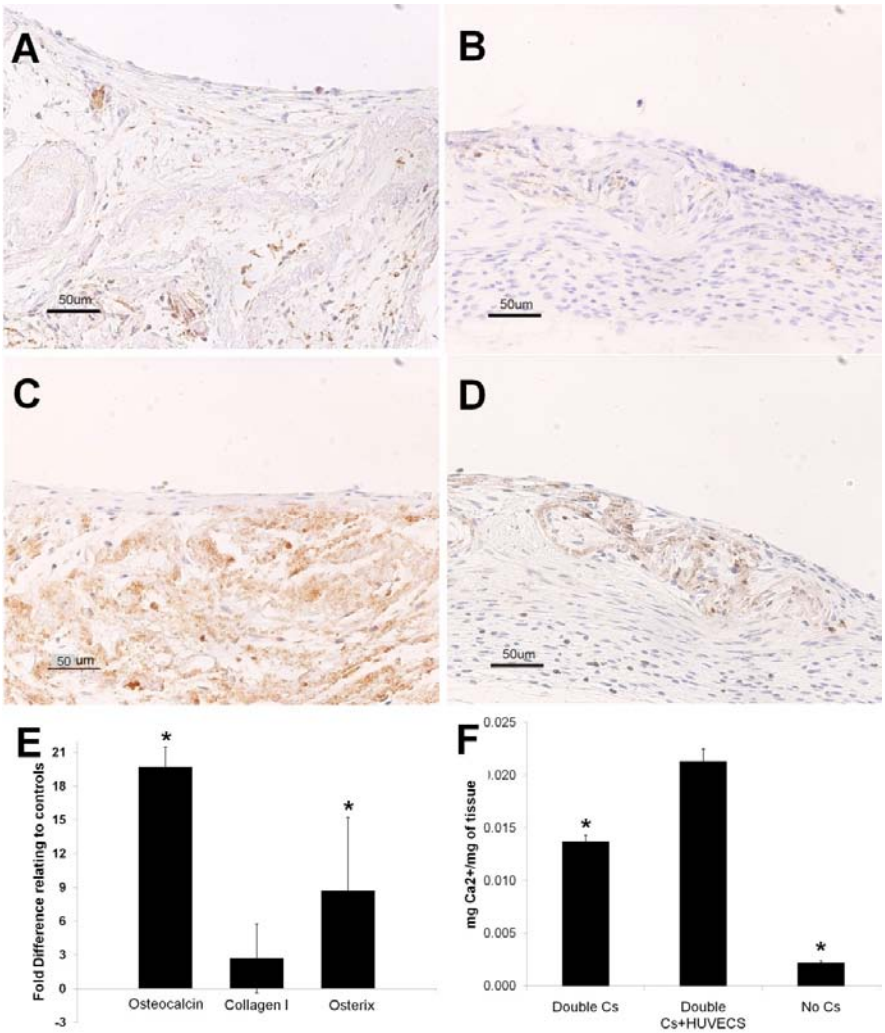

Figure 7.4 - Immunohistological characterization of retrieved implants after co-culture cell sheet (A, C) or monoculture cell sheet (B, D) constructs implantation. Retrieved constructs were immunostained for SRY (A, B) and osterix (C, D). Quantitative RT-PCR analysis (E) and calcium quantification (F) was performed in retrieved constructs one week after implantation. In E, osteocalcin, osterix and collagen I transcripts were quantified in both co-culture cell sheet constructs and control cell sheet constructs (* $p<0.05$ relating to control values). Calcium was quantified using the o-*cresolphtalein*-complexon method and values were normalized with tissue mass (*$p<0.05$ relating to co-culture cell sheets).

The amount of calcified tissue produced after the transplantation of the co-cultured and control constructs was determined through calcium quantification (figure 4F). The calcium values per amount of retrieved tissue detected for the co-culture constructs were significantly higher than for the controls.

The presence of human endothelial cells in the retrieved co-cultured implants was confirmed by immunostaining for human CD31 (figure 7.5). Perfused vessels formed by endothelial cells positive for human CD31 were clearly identified.

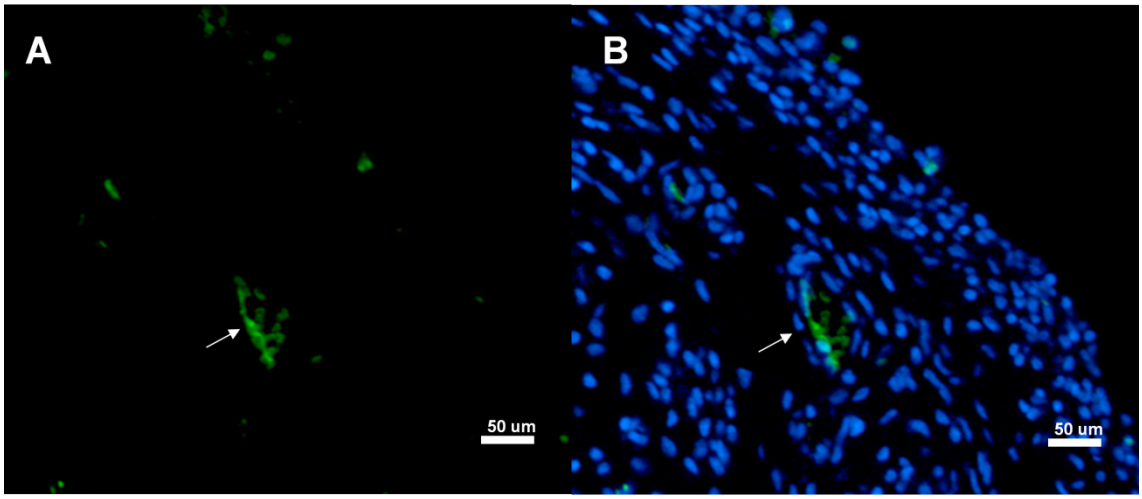

Figure 7.5 – Immunohistological characterization of retrieved implants after co-culture cell sheet constructs implantation. Implants were immunostained for human CD31 (E, F) and counterstained with DAPI (F). White arrows indicate perfused blood vessel.

7.4 Discussion

In a recent work we reported (19) the development of osteogenic CS using thermo-responsive dishes. A single osteogenic CS was able to induce the formation of vascularized new bone tissue, with marrow, after 6 weeks of implantation in nude mice. Even if considered as not sufficiently robust, the use of osteogenic CS represents not only a valuable approach for bone replacement but also for bone regeneration (19, 23, 29) In this sense, we intended to move forward into the creation of a 3D CS-based 3D construct and speculated that by adding endothelial cells within the stacked osteogenic CS would increase the amount of new tissue formed by promoting *in vivo* vascularization of the construct.

Monocultured and co-cultured with HUVECs, rat osteogenic CS were produced using thermoresponsive surfaces and the CS technology. As previously reported (19), mineralized CS, positive for matrix protein osteopontin and for bone related transcription factor *osterix,* all strong indicators of its osteogenic nature*,* and composed by a thick collagenous matrix were obtained by culturing rBMSCs in osteogenic conditions for 21 days.

The perfusion of tissue engineered constructs after transplantation is one of the most critical aspects to a successful regenerative approach (6, 9-11). Strategies for improving the vascularization of tissue engineered constructs consist on the use of growth factors or on the constructs *in vitro* or *in vivo* pre-vascularization (9, 30). The use of angiogenic growth factors is proven to increase the vascularization of implanted constructs (9, 30) but the difficulty in controlling their delivery often results in disorganized and leaky vessels (9, 30). In the case of *in vivo* prevascularization, the need to perform multiple surgeries is seen as a serious drawback of the approach (9). For these reasons, most of the current research is focusing on ways to *in vitro* pre-vascularize constructs using endothelial cells in order to accelerate its perfusion after transplantation (6, 9-11). Similar efforts have been intense in the CS engineering field (26-27, 31). Sasagawa et al (27) seeded HUVECs between myoblast CS, building 5-layers constructs, to obtain in vitro a capillary-like network that anastomosed when implanted in nude rats allowing the survival of the construct. Another work (26) showed that the positioning of endothelial cells within two fibroblast CS constructs was determinant for tubular formation and influences its lumen area *in vitro*. Thus, it is increasingly clear that stacking several CS results in a suitable 3D environment for endothelial cells to form a pre-vascular network *in vitro*. In the herein presented work it was shown that HUVECs, seeded over rBMSCs 3 days before the end of the osteogenic differentiation period, self-organized either in small round colonies or in elongated aggregates resembling a network. Since these co-cultured CS were not cultured *in vitro* after stacking with a second osteogenic CS, which could provide a 3D

matrix for HUVECs re-organization, the formation of capillary-like structures, as seen in other works (26-27, 31), was not expected.

The transplantation of the stacked CS constructs to nude mice clearly resulted in mineralized tissue formation after 7 days, both in the presence and absence of HUVECs. The new tissue was strongly positive for alizarin red and for bone matrix protein osteopontin, which confirms its mineralization and osteogenic nature. Moreover, it was organized in patches throughout the flap, in accordance to what was observed at later time points of implantation of a single CS. (19).

The capacity of an implanted construct to induce host to produce new tissue is critical in a tissue engineering strategy. The contribution to neo-tissue formation of the transplanted cells was discriminated from the contribution of host's cells by immunostaining for SRY. The SRY protein is encoded by a gene located in the short arm of the Y chromosome (32) and therefore can be used to identify cells from male origin and, in this case, to distinguish transplanted cells from cells from the host female mice. Therefore, the comparison of the SRY and osterix stainings, the latter identifying both mouse and rat cells, permitted to verify the presence of host's osteogenic cells in addition to the transplanted ones, within the new formed tissue. This proveswithout any doubt that the implanted CS constructs are inducing new bone formation through the recruitment of host's bone forming cells.

Although *in vitro* pre-vascularization ensures a faster perfusion of the construct after implantation, one of its pitfalls comprehends the complexity and slow determination of the ideal *in vitro* culture conditions and construct 3D design for allowing endothelial cells to form networks while maintaining at the same time the function of tissue-specific cells (6, 9). CS engineering, as CS are easy to obtain and to manipulate for assembling 3D constructs therefore allowing a fast optimization of protocols for pre-vascularization, presents advantages. Nonetheless, in this work besides producing a pre-vasculature, we intended to take advantage of the mutual beneficial effects of the crosstalk between endothelial cells and osteoblasts (6), and of the intimate relation between blood vessels

and bone formation (6, 33) to prove that the mere presence of HUVECs within the CS-based construct would be advantageous for new tissue formation. For this reason, the CS were only stacked right before implantation in determent of the pre *in vitro* culture of the 3D constructs. To achieve this, two assumptions had to be verified: the presence of HUVECs in new vessels, and more mineralized tissue formed after implantation of HUVEC-containing CS-based constructs.

In fact, the transplanted human endothelial cells were distributed in the new tissue, forming vessels which were perfused with erythrocytes thus showing HUVECs contribution to new tissue vascularization. This is reinforced by the fact that human VE-Cadherin transcripts were detected by qRT-PCR in the samples with HUVECs (results not shown). Furthermore, calcium quantification allowed to conclude that a higher amount of mineralized tissue was formed in the co-culture constructs. Based on these results we are able to confirm the postulated hypothesis and to state clearly that HUVECs positively contributed to the osteogenic potential of the developed 3D CS-based constructs. This conclusion was further reinforced by quantitative RT-PCR analysis of the transcripts of bone related proteins osteocalcin, osterix and collagen I. The expression of osteocalcin and osterix was significantly increased for co-cultured CS implants, which is also consistent with the higher amount of mineralized tissue formed. The similarity regarding the collagen I transcripts quantification between conditions can be explained by the fact that this protein is also present in tissues other than bone, such as the connective tissue found in the analyzed dorsal flaps (34-35).

Although it was demonstrated that the transplanted HUVECs participate in the neovascularization of the construct we might speculate that in addition to a faster perfusion of the implant, the osteogenesis promoting effect of the endothelial cells (6, 33) also led to a higher amount of bone tissue formed. Future studies will clarify on the exact role of endothelial cells on the formation of the new mineralized tissue.

One aspect aspect that was not addressed in this work is the possibility of further culturing in vitro the stacked cell sheet constructs to build a de facto prevascular

network. A 3D vessel network was previously achieved in vitro in vitro by culturing five stacked myoblast cell sheets interspersed with HUVECs. Moreover, HUVECs were cultured between two myoblast cell sheets were capable of forming a network after 3 days of co-culture (27). Although a similar approach was not followed in the present work, given those previous results using similar number of cell sheets, it can be speculated that the in vitro formation of a capillary-like network would promote the vascularization of the construct and, consequently, the rate of new bone formation. This might also be of interest when stacking increasing number of osteogenic cell sheets combined with endothelial cells ultimately to obtain the thickest viable construct possible to consider the application of one or several scaffold-free osteogenic cell sheet constructs in a large bone defect.

7.5 Conclusions

Herein we have demonstrated that when combined with osteogenic CS, endothelial cells participate in the neo-vascularization of the new tissue formed in an ectopic implantation model. More importantly, double osteogenic CS constructs, with endothelial cells in between, led to a faster, and in increased amounts, formation of bone tissue as compared with control constructs without HUVECs. This, together with the presence of HUVECs in new vessels, confirmed its positive contribution to the osteogenic potential of the developed 3D CS-based constructs. It was also demonstrated that the transplanted CS-based constructs induce bone formation through the recruitment of host's bone forming cells.

This work highlights the versatility of CS engineering for tissue engineering applications, in particular for vascularized bone tissue engineering.

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Chapter 8

Final Remarks

Chapter 8

FINAL REMARKS

The limitations of current strategies of Bone Tissue Engineering require a more thorough characterization of the mechanisms of cellular interaction between osteogenic cells and other cells of interest. This type of studies must mimic the *in vivo* setting in a closer way than has been achieved so far. Co-culture systems give researchers the chance to reproduce more accurately *in vitro* highly complex situations found in a living organism. This allows for obtaining more accurate results and consequently a faster advance of the knowledge which ultimately and optimally will translate better therapies to address the regeneration of bone defects.

Monocytes/macrophages are immune cells present during host response, always developed after the implantation of a biomaterial/construct. These cells were shown to produce BMP-2 that can accelerate the osteogenic differentiation of osteoprogenitor cells. Although only a part of the whole host response scenario, it strongly suggests that this characteristic of monocytes/macrophages can be used to potentiate bone constructs outcome. The contrary happens with fibroblasts which are deeply involved in the formation of the fibrotic capsule that is often a mark of host response. The demonstration of the way fibroblasts inhibit the osteogenic activity of osteoblasts will allow for bone tissue engineers to consider this fact and to potentially address it by controlling this inhibitory effect.

In addition to the host reaction issue another serious concern in current bone Tissue Engineering approaches, the vascularization hurdle, was also addressed in this thesys. The source of endothelial cells to assemble bone tissue engineered constructs is critical. Endothelial progenitor cells have been proposed as a possible source for creating vascularised complex 3D tissue-like structures. However, the steps for their isolation are time consuming and a significant controversy regarding their specific markers impair their future use. Moreover, the beneficial interactions between

osteoblasts and endothelial cells have been demonstrated as being a valuable tool for successfully engineer bone tissue. An *in vitro* co-culture system established on carrageenan membranes permitted to sustain the endothelial potential of the cells of mononuclear fraction of umbilical cord blood for subsequent implantation. More importantly, those mononuclear cells were capable of contributing to angiogenesis *in vivo* in an inflammatory setting which is common after implanting tissue engineered devices. Once again, this can potentially be very interesting for future bone Tissue Engineering approaches, although more detailed studies have to be made especially using biomaterial carriers/supports capable of eliciting host reactions with different degrees of severity.

A strategy to potentially avoid the use of biomaterials is the use of cell sheets. The potential of cell sheet engineering for bone Tissue Engineering applications was successfully demonstrated in different studies presented herein. Single osteogenic cell sheets were capable of promoting new bone formation that significantly increased along the implantation time. The new formed bone was vascularised, had a cellularized marrow and osteocytes which suggests that it is mature bone. Despite this result, in order to pursue an increased amount of newly formed bone and promote its vascularization, stacked osteogenic cell sheets co-cultured with endothelial cells were created. The verification of this hypothesis was originally achieved *in vivo*. A significant amount of bone tissue was formed and was greater when endothelial cells were present. More importantly, the endothelial cells were present in perfused vessels which suggest their contribution to the vascularization of the new bone tissue. In sum, the potential of using scaffold-free cell sheets for bone tissue engineering purposes and the advantage of co-culturing endothelial cells with the cell sheets for increased *in vivo* vascularization was confirmed.

The results obtained in this thesis demonstrate not only the usefulness of using co-culture models to characterize heterotypic cell interactions but also the establishment of cell sheet engineering as a valid technology for bone tissue engineering purposes.