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Development of human osteogenic cell sheets co-cultured with endothelial and pericyte-like cells.

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Abstract

In recent years the use of cell sheets for tissue engineering (TE) purposes has become a reality, with some studies showing that it is a reliable alternative for the traditional scaffold-based approaches by avoiding their associated shortcomings. This technology permits not only to eliminate the exogenous scaffolding biomaterials but also to create *ex vivo* tissue-like substitutes with organized cellular entities and cohesive cell-to-cell and cell-extracellular matrix (ECM) interactions. However, the progression of this technology is being limited by some of the barriers which have been hampering the evolution of other TE strategies, such as the lack of appropriate vasculature to supply thicker constructs *in vivo*. It is herein proposed the creation of a cell sheet construct, by co-culturing osteogenic, endothelial and pericyte-like cells, with the purpose of enhancing the vascularization of newly formed bone tissue and also the degree of maturation and stability of the vascular network.

The optimization of the culture conditions to fabricate osteogenic cell sheets derived from human bone marrow stromal cells (hBMSCs) was the first step of this work. This optimization allowed furthering evolving to the design of a co-culture system with human umbilical cord vein endothelial cells (HUVECs) and pericyte-like cells as a model for the vascularization of *in vitro* cultured constructs with potential for bone tissue engineering applications. Bone marrow was previously proposed as a source of perivascular-like cells, in particular those expressing CD146, therefore hBMSCs were isolated, cultured under different conditions and screened for the expression of CD146 as well as for the Mesenchymal Stem Cells (MSCs) markers CD90, CD73 and CD105. Flow cytometry results demonstrated that supplementation of standard hBMSCs culture medium with TGF-β1 promote an increase of CD146 expression in hBMSCs, from approximately 50% to more than 97%. Moreover, changes in CD146 expression are associated with different cellular morphologies. Immunocytochemistry assays performed on the co-cultures showed that induced CD146+ hBMSCs and HUVECs migrated and organized themselves over a thin, collagen-rich, osteogenic cell sheet, suggesting the existence of an efficient cross-talk involving all the co-cultured cell types. Further studies concerning the ability of these constructs to form functional, vascularized and osteo-committed tissue *in vivo* were performed using a well described protocol for subcutaneous cell sheets transplantation on mice. Immunohistochemistry analysis of transplanted cell sheets revealed the integration of HUVECs with host network vasculature as well as the osteogenic potential of the created construct, as shown by the expression of osteocalcin. Additionally, analysis of the diameter of CD146 positive blood vessels showed a higher mean vessel diameter for the experimental condition, reinforcing the advantage of the proposed model regarding blood vessels maturation and stability.

Resumo

Recentemente, o uso de c*ell sheets* tornou-se uma realidade na área da engenharia de tecidos, existindo alguns estudos que provam a viabilidade desta tecnologia como alternativa aos tradicionais métodos baseados no uso de *scaffolds. O uso de cell sheets* permite não só dispensar o uso de biomateriais de suporte mas também criar substitutos de tecidos, *ex-vivo*, com uma organização celular própria mantendo altamente coesivas as adesões célula-célula e as interacções com a matriz extracelular. Contudo, a evolução desta tecnologia tem sido limitado por algumas das barreiras que têm vindo a impedir o progresso das estratégias tradicionais de engenharia de tecidos, nomeadamente a ausência de uma vasculatura apropriada que permita a integração dos *constructs in vivo*. Deste modo, este trabalho propõe a criação de um *construct* baseado na tecnologia de *cell sheets*, obtido co-cultivando células osteogénicas, endoteliais e com marcadores pericíticos, com o objetivo de promover a vascularização de tecido ósseo novo e o grau de maturação e estabilidade da rede vascular formada.

Numa primeira fase deste trabalho começou-se por otimizar as condições de cultura celular para o fabrico de *cell sheets* osteogénicas a partir de células mesenquimais humanas derivadas da medula óssea (hBMSCs). Esta otimização permitiu evoluir em seguida para o *design* de um sistema de co-cultura de células endoteliais da veia do cordão umbilical (HUVECs) e células com potencial perivascular (CD146+), criando um modelo para a vascularização de *constructs* produzidos *in vitro* com potencial para aplicação em engenharia de tecido ósseo. A medula óssea foi anteriormente proposta como uma possível fonte de células com potencial perivascular, nomeadamente as que expressam o marcador celular CD146. Deste modo, as hBMSCs isoladas da medula óssea foram cultivadas em diferentes meios de cultura e caracterizadas tendo em conta a expressão de CD146 e dos marcadores associados a células mesenquimais, CD90, CD73 e CD105. Os resultados de citometria de fluxo demonstraram que a adição de TGF-β1 ao meio de cultura provoca um aumento de expressão do marcador CD146 nas hBMSCs, de aproximadamente 50% para um valor superior a 97%. Simultaneamente, verificou-se que alterações na expressão de CD146 estão associadas a alterações da morfologia celular. A análise das co-culturas por imunocitoquímica mostrou que as células induzidas a expressar CD146 e as HUVECs foram capazes de migrar naturalmente e de se organizar sobre uma superfície rica em colagénio composta por células osteogénicas confluentes, sugerindo a existência de um *cross-talk* eficiente entre todas as células que compõem o sistema de co-cultura. A capacidade do *construct* formar, *in vivo*, tecido osteo-comprometido, vascularizado e funcional foi avaliada após a transplantação subcutânea do *construct* em ratinhos. A análise de imunohistoquímica das *cell sheets* transplantadas revelou a

integração das HUVECs com a rede vascular do hospedeiro assim como o potencial osteogénico do *construct*, tal como comprovado pela detecção da expressão da osteocalcina. Adicionalmente, a análise do diâmetro dos vasos positivos para o marcador CD146 revelou um aumento da média do diâmetro dos vasos na condição experimental, o que confirma a hipótese colocada e reforça a relevância do modelo proposto na maturação e estabilização dos vasos sanguíneos formados.

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II – Material and Methods

Abbreviations

3D – Three dimensional
α-MEM – Minimum Essential Medium Alpha
α-SMA – Alpha smooth muscle actin
°C – Celsius degrees
AF – Alexa Fluor
AR-S – Alizarin Red-S
BMP – Bone morphogenetic protein
BSA – Bovine serum albumin
CAM – Cell adhesion molecule
CD – Cluster of differentiation
CECs – Circulating endothelial cells
CO_2 - Carbon dioxide
CS – Cell sheet
MSCs – Mesenchymal Stem Cells
DAB – 3, 3'- diaminobenzidine
DAPI – 4',6-diamidino-2-phenylindole
Dil-AcLDL – 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate Acetylated-low density lipoprotein
DNA – Deoxyribonucleic acid
ECs – Endothelial cells
ECM – Extracellular matrix
EGM-2 MV – Microvascular Endothelial Cell Growth Medium-2
EOCs – Endothelial outgrowth cells
EPC – Endothelial progenitor cells
FBS – Fetal bovine serum
FGF-β – Basic fibroblast growth factor
H&E – Hematoxylin and Eosin
hBMSCs – Bone Marrow-derived Mesenchymal Stromal/Stem Cells
HRP – Horseradish peroxidase
HUVECs – Human Umbilical Vein Endothelial Cells
IGF – Insulin-like growth factor
ISCT – International Society for Cellular Therapy
LCST – Lower critical solution temperature
MAPC – Multipotent adult progenitor cells
NG2 – Surfatide or nerve/glial antigen-2 (NG2) proteoglycan
NHS – Normal horse serum
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PDGF-β – Platelet-derived growth factor beta
PDGFR-β – Platelet-derived growth factor receptor beta
PIPAAm – Poly(N-isopropylacrylamide)
PVDF – Poly(vinylidene difluoride)
qRT-PCR – Quantitative reverse transcription PCR
RM – Regenarative Medicine
RT – Room temperature
Shh – Sonic hedgehog
TE – Tissue Engineering
TCPS – Tissue culture polystyrene
TGF-β1 – Transforming growth factor beta-1
TR – Thermoresponsive
vSMC – Vascular smooth muscle cells
VEGF – Vascular endothelial growth factor

I – INTRODUCTION

1 - Regenerative Medicine

The Regenerative Medicine (RM) field, in particular the area of tissue engineering (TE), holds a great promise for creating substitutes to repair congenital/trauma defects or diseased tissue. The utopian idea is to create whole organs or parts of organs in laboratory to replace and/or repair damaged parts of our body (1). Cell therapy, a RM approach, takes advantage of the systemic infusion of cells, namely mesenchymal stem cells (MSCs), to treat diseases or disorders using minimal invasive procedures. This concept has been proposed for a variety of applications namely to regenerate damaged tissue and to treat inflammation resulting from cardiovascular disease, myocardial infarction, brain, spinal cord, cartilage and bone injuries and Crohn's disease (2). A major issue regarding cell therapy is that only less than 1% of the total injected cells reach and home at the desired tissue due to the lack of MSCs homing receptors (3). On the contrary, the most common TE approach comprehends the seeding of cells, as an autologous approach, on a biodegradable scaffold with desired mechanical properties, and the *in vitro* culturing of the construct prior to *in vivo* implantation (4). Although it looks simple and valuable, this strategy also has some associated issues, namely cases of pathological fibrosis after scaffolds biodegradation, strong inflammatory reaction due to non-specific responses to the polymers and consequent significant pH drop in host tissues, and the existence of a necrotic core at the centre of large constructs (5, 6). A common issue both for cell therapy and TE in general concerns the *in vitro* cell expansion conditions before use. In the case of therapies using MSCs, the overall impact of *in vitro* culture conditions over those cells is largely unknown however, some studies have shown that confluence and high passage number have undesirable impact on MSCs function (7, 8). It is also possible that stem cells cultured *in vitro* acquire new DNA mutations that promote cell growth under *in vitro* conditions, which might increase the risk of tumour developing (benign or malignant) after transplantation (1).

Despite all the considered limitations and cautions, several clinical trials of stem cell therapies, namely for the treatment of muscular dystrophy (9) and to regenerate myocardium *de novo* after myocardial infarction (10) have shown good results. Furthermore, successful TE approaches were also reported after the transplantation of bioartifial bladders and trachea (1). A different TE perspective has arisen with the advent of the cell sheets (CS) engineering technology. CS engineering intends to be an alternative to the traditional TE approaches by avoiding their associated shortcomings, not only by eliminating the scaffolding structures but also by creating artificial tissue-like substitutes with organized cellular entities and cohesive

cell-to-cell and cell-extracellular matrix (ECM) interactions (5). This relatively new concept is taking advantage of temperature-responsive cultured dishes to create those intact CSs which have already demonstrated to possess great potential as cardiac (11), and skin (12) and corneal epithelium (13) grafts, as well as to regenerate urothelium (14) and periodontal ligament (15) without the use of biodegradable scaffolds.

2 - Bone Tissue Engineering

Bone tissue deficiencies, caused either by malformations, trauma, or medical treatments, have a great impact over the patient's life quality. Since the skeleton offers structure, posture and protection, damaged bones will hamper their physical function and appearance. Bone tissue engineering aims to be an alternative for skeletal reconstitution using metal plates or allogeneic and autologous bone grafts, decreasing the time and the associated costs, nonetheless it still remains a clinical challenge (16). Meijer *et al.* have considered that bone tissue engineering research comprises two promising approaches: the first one is based on three dimensional scaffolds that act as growth factors carriers, while the second one comprehends the combination of living osteogenic cells with three dimensional (3D) scaffolds (17). These structures are expected to provide a 3D environment where the cells are able to migrate, organize, differentiate and eventually, after implantation, successfully regenerate the desired tissue (18). However, despite the significant number of works (19-21) proving the feasibility of use the combination of scaffolds and MSCs in mouse models the fact is that few (22, 23) report the orthotropic application of bone-tissue engineering constructs in large animal models. The reality is that the four major causes for the stagnation of the bone tissue engineering field are: insufficient number of cells with osteogenic capacity; inappropriate scaffolds to seed the cells; uncontrolled delivery and unknown mechanisms of action of factors to stimulate osteogenic differentiation *in vivo*; and insufficient vascular supply (24). In fact, this last one is considered the principal cause of failure of bone tissue engineering constructs (25) and more considerations about this subject are discussed later (see I-3).

MSCs-based bone tissue engineering therapies rely on the capacity to pre-differentiate those cells *in vitro*, usually by the addition of dexamethasone, ascorbic acid and β- glycerophosphate. In addition to the time of expansion, cell's full differentiation has been achieved between 21 to 28 days, depending on the MSCs source, therefore, other strategies are being developed to decrease the time and increase the efficiency of osteogenic differentiation. Among them, the supplementation of culture media with growth factors (Platelet-derived growth factor (PDGF); Bone morphogenetic proteins (BMPs); transforming growth factor (TGF); Insulin-like growth factor (IGFs)) (26), gene delivery using gene therapy techniques to induce production of

growth factors (27), or the development of new bioreactor designs with capacity to enhance osteogenic performance of osteogenic cells (26), have been significantly explored.

2.1– Bone Tissue Biology and Regeneration Process

Bone is a remarkable organ with an interesting hierarchical structure. It combines an intimate interplay between an organic matrix and a mineralized phase with an extraordinary capacity to deal with mechanical stress (28). In bone it is also possible to find several distinct tissues and organs, including mineralized osseous tissue, marrow, endosteum and periosteum, nervous tissue, and cartilage (26). Moreover, it is constituted by three distinct cell types which contribute for a constant self-remodelling through a controlled balance between bone resorption and bone formation. In this process, osteoblasts are responsible for bone extracellular matrix production and mineralization, which become calcified and entrap the osteocytes (osteoblasts in a fully differentiated state), the most abundant cell type in bone, which play an important role in bone homeostasis. In opposition, osteoclasts, the third cell type, are highly specialized cells with special importance for bone resorbing (29). Adequate and balanced bone remodelling assures maintenance of skeletal integrity, healing, blood calcium regulation and accommodation of chances in bone stress profiles (30).

After wounding, the way by which multicellular organisms can restore the architecture and functions of injured tissues is called regeneration. This leads to the reactivation of complex development pathways restoring homeostasis of the damaged area (31). Bone fracture healing is a dynamic process where a diversity of molecular and cellular processes occur along the subsequent healing phases: haematoma formation, inflammation, angiogenesis, cartilage formation and bone remodelling (32). Being key players in the regulation of the inflammatory cascade it is clear the importance of several growth factors, such as interleukin (IL) 1 and 6, PDGF; TGFs; IGF and BMPs during bone healing. By releasing earlier BMPs, MSCs are also important players on that process (32). In addition to the inflammatory phase, the angiogenic response during healing is also a critical process. In fact, several studies have established a connection between inadequate or inappropriate bone vascularization and a decreased bone formation, resulting in the formation of fibrous tissue (33-35).

3 - Mesenchymal Stromal/Stem Cells: From one to multiple sources

Stem cells, in general sense, bare potential not only to repair and regenerate damaged or loss tissues, but also to treat several diseases including metabolic, degenerative and inflammatory ones (36). In mammals, several stem cells have been identified, such as muscle-derived stem

cells (37, 38), germline stem cells (39), epithelial stem cells (40, 41), neural stem cells (42) and hematopoietic stem cells (43). All of them are believed to be precisely located in a diversity of organs where they are maintained and regulated within a well-controlled microenvironment, the stem cell niches (44). At present, stem cells under investigation are: embryonic stem cells, embryonic stem cells created by somatic cell nuclear transfer and adult stem cells (45). However, lack of efficient strategies to control lineage-specific differentiation of embryonic stem cells has been directing the research focus to adult, proliferative but lineage restricted stem cells (45).

Multipotent mesenchymal stromal cells, generally called Mesenchymal Stem Cells (MSCs), have the ability to differentiate into more than one cell type of the body but committed to the mesenchymal lineage (36). Besides their potential to differentiate *in vitro* along adipocytic, osteoblastic and chondrocytic lineages (24, 46), this type of cells was also previously recognized as critical to the support of hematopoiesis (47), providing, within the bone marrow stroma, an environment for homing, maintenance, proliferation, and maturation of hematopoietic progenitors (48). An immunoprivileged status was also attributed to MSCs (46), meaning that these cells are able to evade immune recognition and modulate immune responses after *in vivo* transplantation, which might have significant impact over the translation into the clinic of allogeneic cell based therapies (49-51).

Unfortunately, there was a lack of common definition for these cells, which lead the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) to define the criteria to standardise the characterisation and nomenclature of MSCs populations. According to those, MSCs must be plastic-adherent in standard culture conditions using tissue culture flasks and must express CD105, CD73 and CD90 (≥95%) without traces (≤2%) of CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA class II antigens (52).

However, it is clear that over a MSC population selected by adherence to plastic, heterogeneity and variable expression of surface markers are inevitable (46). Moreover, accordingly to Horwitz *et al.* not all MSCs are true stem cells (considering the current definition of stem cells, i.e. a long term self-renewing cell that is capable of differentiation into specific, multiplecell types *in vivo*) (53), and the results of several studies suggest that cellular senescence is induced in MSCs due to long-term culture *in vitro* (54, 55), resulting in the loss of their proliferation and differentiation potential (56). Apart from the limitations described above, Jiang *et al.* reported the existence of a very small subset of mesenchymal cells from the bone marrow, termed multipotent adult progenitor cells (MAPC), which are capable of extensive self-renewal and possess pluripotency (57). Thus, it seems wise to consider MSCs as a mix of progenitor cells

with varying degrees of replicative/differentiation potential, rather than a homogeneous population of stem cells (45).

The existence of MSCs was first proposed in 1976 by Friedenstein who suggested the bone marrow as a source of adult stem cells (58). Twelve years after, Owen and Friedenstein proposed a model (59), later termed mesengenesis (60), of MSC differentiation into fibroblastic, reticular, adipogenic, osteogenic, and eventually other cell lineages. Since then, and for a long time, bone marrow stroma was the most commonly used MSCs' source (61). However, new sources of MSCs, with similar characteristics to bone marrow MSCs, have been discovered and proposed to fill the regenerative medicine requirements. Thus, MSCs have been isolated from multiple mouse and human organs and tissues, such as skeletal muscle, skin, pancreas, fat, dental pulp, placenta and umbilical cord, all of them with identical characteristics (62). Among these, MSCs from adipose tissue, the so called adipose-derived stem cells (ASCs), seem to be a good alternative to bone marrow-derived MSCs due to their easier accessibility, abundance and similar capacity to differentiate into adipogenic, osteogenic and chondrogenic cells (63, 64).

Despite all the generated knowledge along the years, one of the most exciting discoveries in the field of MSCs homing and origin was made by Crisan and colleagues which proposed a perivascular origin for MSCs in multiple human organs (65). It was demonstrated that human perivascular cells sorted from diverse human tissues and cultured over plastic adherent conditions are multilineage progenitor cells that exhibit MSCs features (for more considerations about this subject see I-5). In addition, da Silva Meirelles *et al.* directly correlated the amount of MSCs with lipoaspirates vascular density which is in agreement with previous considerations (66).

4 - The Vascularization Problem in Tissue Engineering

Most of the tissues in human body require a functional vascular network for the efficient delivery of oxygen and nutrients and removal of waste materials (4). In adults, the formation of quiescent and stable new blood vessels from pre-existent ones, i.e. angiogenesis, is a rare event, observed only in cases of pregnancy, female reproductive cycle and after prolonged and heavy physical exercise (67). For that reason and because passive diffusion of nutrients and the removal of metabolic waste are limited, the use of TE constructs thicker than approximately 150 µm (68) lead to loss of cell viability, due to nutrient deficiencies and/or hypoxia, and consequently, unsatisfactory tissue regeneration (5). During *in vitro* culture, it is possible to supply and diffuse nutrients through larger tissue engineered constructs using for example

perfusion bioreactors (69), however constructs vascularization is a demand to maintain viability and attain tissue regeneration after implantation. Interestingly, the signals released and respective response by the implanted cells, as a reaction to hypoxia, is sufficient for the blood vessels from the host start to invade new tissue and form a capillary-like network. A major issue is the time that such networks take to develop and invade dipper sections of the implanted construct, which significantly contributes to the limited efficiency of the process (70). In an attempt to overcome this limitation alternative paths, using different scaffolds designs or patterned techniques to re-create the microvasculature of normal tissues *in vitro* or angiogenic factors to potentiate angiogenesis *in vivo*, have been explored. In the field of bone tissue engineering one of the most common approaches is to co-culture endothelial or endothelial progenitor cells, and osteoblastic or osteoprogenitor cells on three-dimensional biomaterials in order to achieve a pre-vascularized construct prior to implantation. All of those strategies have used Human umbilical Vein Endothelial Cells (HUVECs), Human Dermal Microvascular Endothelial Cells (HDMECs) or Endothelial Outgrowth Cells (EOCs) in combination with osteoprogenitor cells from bone marrow, primary osteoblast cells or osteoblast-like cell lines, seeded on 3D scaffolds or arranged in co-culture spheroids (71-73). Independently of the combined source and type of cells, those works demonstrated that the proposed strategy has potential to improve vascularization *in vivo* however there are no solid evidences to consider it as the solution for what the TE field is claiming.

Moreover, the establishment of co-culture systems implies to consider several issues, such as the source(s) of cells and the culturing conditions, that from a clinical perspective are determinant to validate the proposed strategy (74). During *in vitro* culture stage, questions related with the choice of culture medium, the phenotypic characterization of all the cell types involved in the system (75), the ratio of different cells between the co-culture systems and the use of static or dynamic culture conditions are important considerations for co-cultures setting up (74). In the case of co-cultures aimed at attain a pre-vascularized bone TE constructs the obvious cells sources are osteoprogenitor cells derived from bone marrow and endothelial cells. Despite the source of osteoblasts seems evident, the choice for the correct source of endothelial cells has intrigued scientific community. Endothelial cells are ubiquitous in the entire vascular system and constitute the innermost layer of blood vessels (76), although they display remarkable heterogeneity in different organs and also within the same organ (77). HUVECs and human aortic endothelial cells are two commonly studied cell populations concerning the analysis of endothelial cell functions *in vitro* (78), however they might not be the ideal model since they are considered mature and fully differentiated endothelial cells close to senescence and derived from hypoxic and probably activated vessels (77). In this

context, a promise source of cells to enhance the neovascularization of tissue engineered constructs, called endothelial progenitor cells (EPC), has attracted general interest. EPCs have been described as capable to form vascular structures in pro-angiogenic matrices *in vitro*, as well as to contribute for vascularization *in vivo* (79). Although the concept of have an EPC that can differentiate into a true endothelial cells with high proliferation potential is attractive, its application revealed more difficult than originally anticipated (80). The main problem has been to define strategies to efficiently select and characterize those cells and subsequently understand their particular roles in vascularization. As reviewed by Ingram *et al.* in 2005, EPCs displays distinct phenotypes and can be classified by their specific antigens as hematopoietic derived EPCs, circulating endothelial cells (CECs) and EOCs (78). Also, after the first studies have described the cord and peripheral blood as possible sources of EPCs (81-83), Ingram and colleagues suggested that endotelial cells (ECs) surrounding umbilical veins and human aorta combine resident EPCs at different stages of maturation and levels of proliferative potential (84). The specific function of each progenitor cell type described above during new vessel formation is still to understand but has a remarkable importance for TE as it might be the key to create co-cultured TE constructs. Despite those questions, at present, there are arguments supporting the potential of EOCs for bone tissue engineering. These cells are able to grown in different types of scaffolds and to organize in pre-vascular structures when co-cultured with primary human osteoblasts at *in vitro (73, 85)* or *in vivo* levels (86).

Also, co-culture systems of osteoblasts and ECs appear to naturally fulfil all the requirements that a successful co-culture model for pre-vascularization should have, since several studies has proved that, in a co-culture environment, ECs stimulate the osteoblasts to upregulate vascular endotelial growth factor (VEGF) secretion (87), while at the same time ECs release several BMPs which contribute for bone formation and repair (74). These findings suggest that co-cultures provide a pro-angiogenic matrix based on components such as collagen and a network of signals based on intercellular cross-talks that lead to the activation of ECs triggering angiogenesis (79).

In sum, clinical application of co-cultured TE constructs outcomes is still dependent on several technical issues such as improvements of ECs or progenitor's isolation and culture protocols and on understanding the specific needs of each application.

5 – Pericytes Functions, Hallmarks and Origin

The cardiovascular system is the first functional organ system required for the development of mammalian embryo (88). Despite the huge contribution of ECs on that process, mural cells are

present since the first vascular network is formed (89). According to some reports, mural cells are also named vascular smooth muscle cells (vSMCs) while pericytes are described as a phenotypic variant of vSMC (89, 90). However, recent studies have questioned this simplified definition and, beyond their undeniable contribution for angiogenesis, vascular stabilization and blood flow regulation, pericytes have been looked as key players in response to injury and as precursors of MSCs (91, 92).

Regarding their localization, some studies have been correlating pericytes with the microvasculature (89, 90, 93) while others admit the presence of these cells around both capillaries (diameter < 10 μm) and arterioles (diameter from 10 to 100 μm) in all organs of human body, based on their ubiquitous expression of NG2 and CD146 (65). About this subject, Caplan and Correa have suggested the existence of a continuum of phenotypic similarities across various vessels types; pure pericytic cells are present in the microvessels while vSMCs, retaining the expression of some pericytic markers, can be found around larger vessels (91). Nevertheless, and independently of the pericytes origin, their recruitment appears to be regulated both by inductive and selective modes (89). Pericytes are induced to differentiate from immature mesenchymal cells surrounding blood vessels and/or are recruited, according to the selective model and as pre-existing mural cell or mural cells progenitors, from other locations (89). Taking into account new insights regarding pericytes multipotency and MSCs origin (65), it seems that the inductive model fails to explain the mechanism by which blood vessels become wrapped by pericytes. In what concerns the pericytes recruitment model, several *in vivo* experiments with knockout animals for PDGF-β or PDGFR-β have confirmed the involvement of PDGF-β (94-96). However, the absence of PDGF receptors on pericytes derived from sinusoidal vessels of liver represents evidence of the existence of other mechanisms in pericytes recruitment (97).

Once recruited into the correct place and at the appropriate time, pericytes play an important role in blood vessels maturation and stability through a fine balance between sphingosine-1-phosphate, TGF-β and angiopoietins (Ang1 and Ang2) secretion, and Ang1 and Ang2 Tie receptors expression (67). Nonetheless, vessels stability is not conferred by the mere presence of mural cells, but it was suggested to be highly dependent on the pericyte/endothelial interaction using the intermediate filament desmin, the so called Desmin Ensheathment Ratio (98).

The co-expression of several surface markers by pericytes and MSCs lead Caplan to suggest in 2008 (26), in part supported by other findings (65), that all the MSCs are pericytes, changing the MSCs application perspective. In fact, since then, pericytes are seen as the precursors of MSCs, and the perivascular space as a stem cell niche with cells carrying mesenchymal

differentiation capabilities and with implications in tissue response to injury (91). A controversy about the correct marker or the combination of markers for pure pericytes selection is installed. Pericytes were isolated from skeletal muscle based on alkaline phosphatase expression (99), but markers such as WAT7, CD146, NG2, α-SMA or PDGFR-β in the absence of hematopoietic, endothelial and myogenic cell markers, have been also considered to select pericytes from biological samples (91). Nevertheless, it seems that CD146, which is expressed by a subpopulation of bone marrow MSCs (100-102), is a useful marker to select MSCs with increased pericytic characteristics. The CD146 is a transmembrane glycoprotein, which belongs to a class of adhesion molecules (CAMs), and has important functions in early and late development. Moreover, CD146 has been suggested to play an important role in cancer, angiogenesis, cardiovascular diseases and placentation (103). The existence of potential recognition sites for protein kinases on its cytoplasmic domain suggests an involvement in signal transduction (104) however, the CD146 unknown ligand and the impact of CD146 signalling on cellular transcription is poorly understood (103). Studies performed by Yoshioka and colleagues suggest that CD146 mediate cell-endothelium adhesion and might play a role in neovascularization (105). Moreover, a significant number of studies in the field of cancer research have assigned to CD146 a critical role in tumour growth and metastasis, as well as in tumour angiogenesis, suggesting an anti-angiogenic effect in several types of tumour vessels by CD146 inhibition (106, 107). In summary, it seems that CD146 displays different expression patterns, structures and even biological functions depending on the circumstances, but these are certainly in the centre of the mechanisms of endothelial phenotype modulation and angiogenesis (108) and therefore constitute valuable elements to consider and empower bone TE vascularisation strategies.

6 – Cell sheet engineering technology

The use of scaffolds for the reconstitution of 3D tissues has been considered a potential solution and, at the same time, a limiting factor for further clinical applications. Because scaffolds are usually made of biomaterials with no or limited biological activity, those supports might function as a barrier for tissue regeneration (109). CS engineering technology has been proposed as a way to avoid this problem. The concept of this new technology can be placed in between the traditional regenerative medicine approaches, the systemic infusion of stem cells and the use of biodegradable scaffolds to create TE constructs.

6.1 - The intelligent cell detachment

CS engineering is taking advantage of thermoresponsive (TR) surfaces prepared by covalent grafting of a TR polymer, poly(N-isopropylacrylamide) (PIPAAm), into ordinary polystyrene surfaces such as Petri dishes. PIPAAm has a reversible temperature-dependent phase transition, in aqueous solutions, also called lower critical solution temperature (LCST), at approximately 32°C (110, 111) and can be used to produce PIPPAm-modified intelligent surfaces, for *in vitro* cell culture (112, 113). Under normal 37°C culture conditions, the relatively hydrophobic surface allows cells to attach, proliferate and differentiate if desired. However, changing temperature for values below LCST turns the surface hydrophilic and by hydration of the PIPPAm, the polymeric chains extend allowing the spontaneous detachment of cells (Figure I-1) (5, 114, 115). Besides the "deadhesion" being inherent to the TR surface, cellular detachment is an active process, dependent of ATP and led by intracellular signalling and cytoskeleton reconstitution (116).

TR surfaces as well as the CS concept are expanding. The original idea was proposed during the 90's in Japan by Okano and co-workers (117), but other groups were attracted by the potential of this approach and have been trying to create other responsive surfaces for non-invasive two or three-dimensional CS harvesting. Alternative TR culture surfaces, grafted with poly(N-isopropylacrylamide-co-acrylic acid)-b-poly(L-lactic acid) (118), were proposed as promoters of faster cell detachment, however osteoblastic cells growth and respective alkaline phosphatase activity are low (109). Other stimuli responsive polymers have also been used to fabricate "smart" surfaces with the capacity to regulate cell adhesion and detachment. Gold surfaces controlled on a voltage dependent manner (119), pH-dependent (120) and ionic strength (121) controlled surfaces are some examples. Nevertheless, these surfaces seem to be more useful for the understanding of cellular dynamics in anchorage-dependent cells than to produce CS with potential in TE.

A "next generation" of TR surfaces led by micropatterned (122-125) and biomolecule–immobilized (126, 127) cell culture dishes have also been developed. Micropatterning methods permitted to combine, within the same surface, two TR polymers with different LCSTs, allowing selective cell adhesion under temperature controlled environments. Furthermore the introduction of bioactive molecules onto the surface of a TR culture dishes constitute a first step to avoid the presence in culture of components from mammalian sources and allow the fabrication of CSs under serum-free conditions.

CS engineering avoids the traditional proteolytic treatment for cellular detachment, thus allowing preserving cell-cell interactions and maintaining their deposited ECM (114), which has

been considered and demonstrated as an achievement of great value for regenerative medicine applications (128).

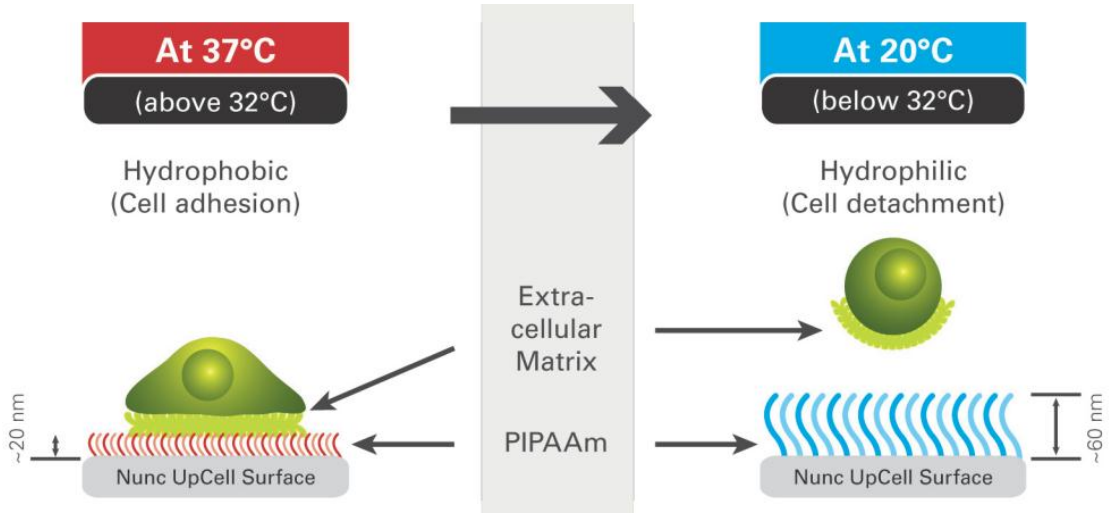

Figure 1 – Features and functioning of TR surfaces. Adapted from Thermo Scientific Nunc UpCell Surface manual.

6.2 – Applications in Regenerative Medicine

A major feature of CS engineering is the maintenance of the deposited ECM after cell detachment as a sheet. After CS harvesting by temperature decrease, the ECM is totally recovered with the intact sheet of cells, which is being presented as the main reason for its adherence onto other surfaces, including other CSs or host tissues (5, 6, 114, 116, 129). Because the parenchyma end epithelia of many tissues consists of several cell layers associated with ECM, the use of CSs as the starting unit to follow a bottom-up approach for tissue reconstitution (6), mainly for thick, cell-dense tissues such as heart, liver, muscle and kidney (116), where biodegradable scaffolds has been failing, seems to be promising. In addition to the preservation of the ECM, the non-invasive cell-sheet harvesting by temperature decrease preserves ion channels, growth factor receptors and cell-to-cell junctions, as well as the integrity of cell surface markers, therefore cells retain higher differentiation functions in comparison to similar cells recovered by trypsinization (5).

The first clinical application of CS engineering was in cornea replacement. Corneal epithelial stem cells were isolated, expanded in TR dishes and recovered by temperature decrease to treat patients with ocular trauma. As a result, significant improvement in visual acuity was observed in all cases, which represents an alternative strategy to the use of scaffolds or substrate carriers that, due to their opacity, hampers proper reconstitution of that specific

tissue (5, 13). Tissue regeneration by direct transplantation of single CSs has been also proposed for skin, periodontal ligaments and bladder, using the base strategy of autologous cells isolation and proliferation on TR dishes, harvesting and subsequent application in the host (Figure 2 A).

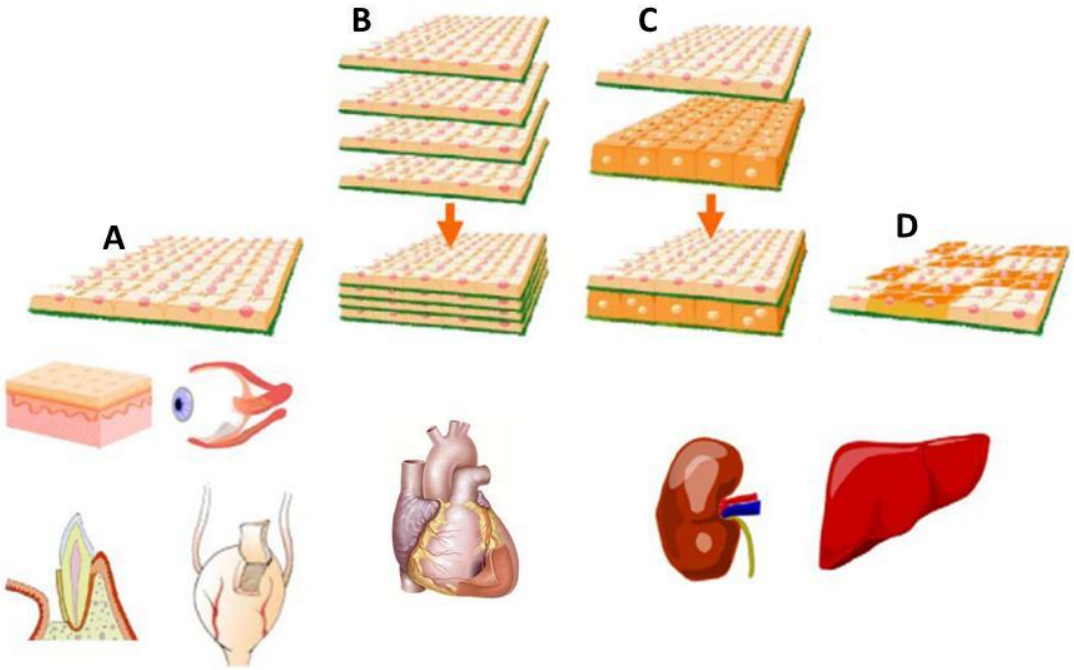

Figure 2 - Examples of CS engineering to create tissue-like substitutes. (A) Application of Single CS transplantation for cornea replacement and regeneration of skin, periodontal ligament and bladder. (B) Stacking of several CS for the improved performance of cardiac tissue. (C and D) Liver and kidney, tissues with laminar and higher-order structures, can also be recreated with CS engineering. Adapted from (5).

When replacement/regeneration of cell dense tissues is required, CSs can be manipulated to create 3D constructs with the desirable characteristics. A good example of CS engineering for complex tissue regeneration is the recreation of cardiac tissue by layering several cardiomyocyte CSs (11) (Figure 2 B). Once again, avoiding the inflexible and bulky properties of scaffolds, which significantly hamper the dynamic pulsation of cardiac myocytes, it was possible to recreate cell dense constructs *in vitro* with functional and synchronized pulsations. This phenomena is explained by the presence of gap junctions, specially connexin 43, formed during CSs adherence and by mediation through deposited ECM, which allows electrical communication between them similarly to what occurs in the heart (115). The four-layered construct fabricated with neonatal rat cardiac myocyte sheets was subcutaneously transplanted into rats and shown to be successfully integrated with the host tissue, as the formation of microvascular networks and maintenance of spontaneous beating proved (5).

Since this first attempt, improved, more complex, vascularized and thicker constructs for improving heart performance have been created. The successful implantation of 30 cardiac myocyte sheets stacked (approx. 1 mm) avoiding hypoxia, nutrient insufficiency or waste accumulation was achieved by the use of polysurgery (130). More recently, another approach was designed to treat congenital heart defects (129). Patterned TR surfaces permitted a controlled tissue organization and the creation of complex structures for vascular reconstruction.

It is expected that with the "next generation" of TR surfaces with dual phase transition temperatures it will be possible to create heterotypic CSs useful to reproduce higher order structures such as the liver (116). Using these surfaces the aim is to control the periphery contact between different cells on a CS co-culture system, mimicking the heterotypic cell–cell interactions occurring in *in vivo* structures (131).

Nevertheless, another important field where CS technology has been shown promising results is bone tissue regeneration. Both CS obtained by temperature decrease (132) and by mechanical means using a cell scraper (109, 133) have already demonstrated capacity to promote new bone tissue formation. However, a significant considering limitation of this approach for hard tissue regeneration concerns the mechanical properties of the construct.

It is clear that a TE construct must possess mechanical properties capable of withstand the mechanical environment of the defect to be regenerated. Osteogenic cell sheets certainly comply with those demands when aimed at regenerating flat bone defects, however a "next generation" of osteogenic cell sheets-based constructs are expected to allow the creation of thicker and stiffer bone tissue substitutes. These can potentially generalize the application of the cell sheet technology to other bone defects with other mechanical demanding.

7 - References

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II - MATERIAL AND METHODS

1. Cell culture

1.1 – Isolation and culture of hBMSCs

Bone marrow aspirates were collected from several patients (n=5) after informed consent and routine surgical procedures in Hospital da Prelada, Porto, Portugal. Adult bone marrow derived mesenchymal stromal cells (hBMSCs) were selected by gradient centrifugation and cultured in plastic adherent conditions as previously described (1). In detail, 5 mL of marrow were collected with bone marrow aspiration needles (Angiotech, USA) and transferred to a falcon tube containing 5 mL of sterile RPMI-1640 (Sigma, USA) medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 5% of Antibiotic-Antimycotic (Gibco, USA) and 5% of Heparin Winthrop (Glaxo Wellcome Production, France). Samples were transported under controlled temperature and processed within 2 hours after collection. Once in the lab, samples were carefully homogenized, layered onto histopaque-1077 (Sigma, USA) and centrifuged at 400 x g for 30 minutes, at 4°C. During centrifugation, erythtocytes and granulocytes sediment, while the mononuclear cells remain at the plasma/histopaque interface. The upper layer of the interface was carefully aspirated with a Pasteur pipette and transferred into a clean centrifuge tube. Cells were then washed by adding 10 mL of sterile cell culture tested phosphate buffer saline (PBS, Sigma, USA) and centrifuged at 250 x g for 10 minutes, room temperature (RT). The supernatant was discarded and the PBS washing step was repeated until a completely white pellet was achieved. The low speed centrifugation during washing steps removed most extraneous platelets before sediment mononuclear cells were resuspended and counted. Cells were then seeded at a density of 2×10^5 cells/cm^2 on plastic-adherent culture flasks (Falcon, England) using Minimum Essential Medium Alpha (α-MEM, GIBCO, USA) supplemented with 2 ng/mL of basic fibroblast growth factor (FGF-β, PeproTech, USA), 10% FBS and 1% antibiotic-antimycotic, and incubated at 37°C in humidified atmosphere with 5% CO_2. After 24 or 48 hours in culture, medium containing non-adherent cells was replaced by fresh α-MEM. Culture medium was replaced every 3 days and the adherent cells were grown to approximately 80% confluence or until the cells in the colonies become densely associated. At the time of passaging, cells were washed twice with PBS, 5 minutes each, and incubated with 0.05% Trypsin-EDTA (Gibco, USA) at 37ºC for at least 5 minutes. At the end of the incubation, trypsin

was inactivated with complete α-MEM and cell suspension was transferred to a falcon tube, centrifuged at 250 × g for 5 minutes RT, and the supernatant was discarded. Cells were then resuspended in complete α-MEM, counted using disposables counting chambers (Kova, UK) and sub-cultured at a cellular density of 3500 cells/cm^2. hBMSCs were used at P2 for cell sheets fabrication (See II-2) and from isolation day to P5 for flow cytometry analysis (See II-4.2).

1.2 – Osteogenic differentiation of hBMSCs

Osteogenic differentiation of expanded hBMSCs was achieved by culturing 3500 cells/cm^2 in osteogenic differentiation medium, composed of complete α-MEM supplemented with 10 mM β-Glycerophosphate (Sigma, USA) and 1×10^{-8}M dexamethasone (Sigma, USA), as previously defined (2), but with a higher concentration of ascorbic acid, 150 µg/mL (Sigma, USA). Cells were incubated at 37°C in humidified atmosphere with 5% CO_2 and the culture medium was replaced every 3 days.

The expression of osteogenic-related genes was assessed at day 7 of culture by real time qRT-PCR (See II-4.1) and the deposition and mineralization of the deposited ECM evaluated at days 7, 14 and 21 after alizarin red staining (see II.4.4).

1.3 – Induction of CD146+ phenotype

The induction of the CD146 phenotype in hBMSCs was carried out by culturing cells in complete α-MEM supplemented with 1 ng/mL of human transforming growth factor beta-1 (TGF-β1, ebiosciences, USA) for 7 days at 37°C in humidified atmosphere with 5% CO_2. Culture medium was replaced once during the time of culture. The expression of CD146 antigen was evaluated after 7 days in culture by flow cytometry (See II-4.2) and fluorescence immunocytochemistry (See II-4.3) procedures. Only cells above P5 were characterized and used in further experiments.

1.4 –Isolation and culture of endothelial cells (ECs)

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from umbilical cords as previously published (3). HDMECs were isolated from fresh plastic surgery discarded skin samples accordingly to published reports with some specific modifications (4-6). Both HUVECs and HDMECs were kindly provided by 3B's Research group colleagues that obtained the biological samples from healthy patients after informed consent and under cooperation

protocols previously approved by the "Ethical Committees of Hospital S. Marcos, Braga" and "Hospital da Prelada, Porto".

Isolated HUVECs were seeded at a density of 3×10^4 cells/cm^2 in plastic adherent culture flasks using medium 199 (M199, Sigma, USA) containing 20% FBS, 1% antibiotic-antimycotic, 0.34% glutaMAX (Gibco, Japan), 50 µg/mL of heparin (Sigma, USA) and 50 µg/mL Endothelial Cell Growth Supplement (ECGS, BD Biosciences, USA). HDMECs were seeded at a density of 40000 cells/cm^2 on 0.5% gelatin coated surfaces[1] and cultured with Microvascular Endothelial Cell Growth Medium-2 (EGM-2 MV, Lonza, USA). Both cell types were cultured at 37°C in humidified atmosphere with 5% CO_2, with culture medium replacement every 3 days and trypsinized before confluence was reached. At the passaging time HUVECs and HDMECs were washed twice with PBS, 5 minutes each time, and respectively incubated at 37°C with 0.05% Trypsin-EDTA and TrypLE Express (Gibco, Denmark). Cell morphology was controlled with the inverted microscope and when detachment was complete, trypsin was inhibited by adding complete M199 and TrypLE Express with EGM-2MV. Cell suspensions were centrifuged at 250 × g for 5 minutes RT, resuspended in the respective fresh culture medium, counted and seeded at the desired concentration.

1.5 – Co-culture of hMBSCs, ECs and pericyte-like (CD146+) cells

Co-cultures of hBMSCs, ECs and pericyte-like (CD146+) cells were performed either in plastic culture 13 mm coverslips (Sarstedt, USA) for immunocytochemistry analysis (see II-4.3), or in thermoresponsive (TR) dishes (Nunc, Denmark) to obtain cell sheets for histological characterization (see II-4.5) and *in vivo* procedures (see II-3) (Figure 1).

To set up the co-cultures, hBMSCs were seeded at a density of 34000 cells/cm^2 on coverslips or TR dishes and cultured with osteo-inductive culture medium (see II-1.2) during 7 days. A mixture of 80000 ECs and pericyte-like (CD146+) cells (per coverslip) or 400000 ECs and pericyte-like (CD146+) cells (per TR-dish), at a ratio of 4:1 was then seeded onto the confluent layer of hBMSCs cultured in osteogenic medium. Co-cultures were maintained for further 7 days at 37°C in humidified atmosphere with 5% CO_2 in either M199 (HUVECs) or EGM-2 MV (HDMECs), supplemented with the osteogenic factors (described in II-1.2). Culture medium was replaced twice during the 7 days of culture.

[1] A solution of 0.5% bovine gelatin (Sigma, USA) in distillated water was spread over the culture surface and left at 37°C for 30 minutes. Gelatin solution was then removed and the culture surface washed with

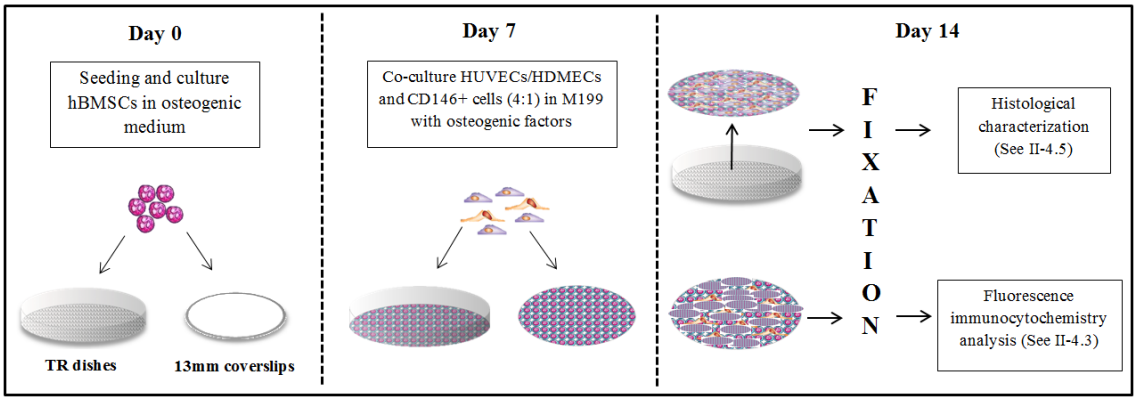

Figure 1 – Schematic representation of the steps to set-up the co-cultures.

2. Cell sheets fabrication

2.1 Monocultured and co-cultured cell sheets

With the purpose of producing monocultured osteogenic cell sheets for *in vivo* tests (See II-3), hBMSCs were differentiated in TR; 300,000 hBMSCs were cultured for 14 days at 37°C in humidified atmosphere with 5% CO_2 in osteo-inductive culture medium (See II-1.2). Culture medium was replaced every 3 days. To obtain co-cultured osteogenic cell sheets with ECs and pericyte-like (CD146+) cells, procedure described in section II-1.5 was followed to set up the culture.

After the 14 days of culture, both types of cell sheets were recovered from the TR dishes by temperature decrease. In detail, culture medium was removed from the TR dishes and replaced with 1 mL of PBS. Dishes were left at RT for at least 20 minutes. Temperature decrease promoted the recovery of a contracted but intact cell sheet. Cell sheets for characterization were further fixed for 1 hour with 3.7% formalin.

2.2 Stacked cell sheets

Co-cultured osteogenic cell sheets were stacked with monocultured osteogenic ones for *in vivo* tests (See II-3) as follows. At the time of recovery from the TR dishes, and when incubating with the PBS (See II-2-2.1) a poly(vinylidene difluoride) (PVDF) membrane (Millipore, USA), with 2 cm of diameter, was placed over an osteogenic cell sheet and incubated at RT for 15 minutes. After this time, CS border was carefully folded onto the membrane and the cell sheets, spontaneously detached from thermoresponsive dishes, were attached to the membrane which allowed its manipulation and stacking over the co-cultures, still in TR dishes.

The all construction was further incubated at RT for 15 minutes to allow the detachment of the co-cultured cell sheet from the TR dish and adhesiveness to the osteogenic one, forming a double cell sheet construct that combined two osteogenic cell sheets with the ECs plus pericyte-like (CD146+) cells cultured and organized in between.

3. *In vivo* transplantation of cell sheets

In vivo tests, according with a well described protocol for subcutaneous transplantation of cell sheets (7), were carried out to test the osteogenic potential and vascularization ability of the co-cultured cell sheets-based constructs. Two types of cell sheets were transplanted, single monocultured osteogenic cell sheets, as control, and co-cultured osteogenic cell sheets stacked with monocultured osteogenic ones, as experimental condition.

Male, 5 weeks old, nude mice (Charles River, USA) (Figure 2-A) were anesthetized 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 saline solution. After the confirmation of analgesia/anaesthesia, dorsal skin was cut opened using 3x3 cm cutting sides (Figure 2 B, C and D). Recovered cell sheets were placed on mouse subcutaneous dorsal flap and left to adhere to the connective tissue of dorsal skin for 5 minutes (Figure 2 E). After that time, the PVDF membrane was removed (Figure 2 F and G), skin flap was brought back to the original location and sutured. (Figure 2 H and I). Five experimental and five control mice were used per implantation time point. Animals were kept with food and water *ad libitum*.

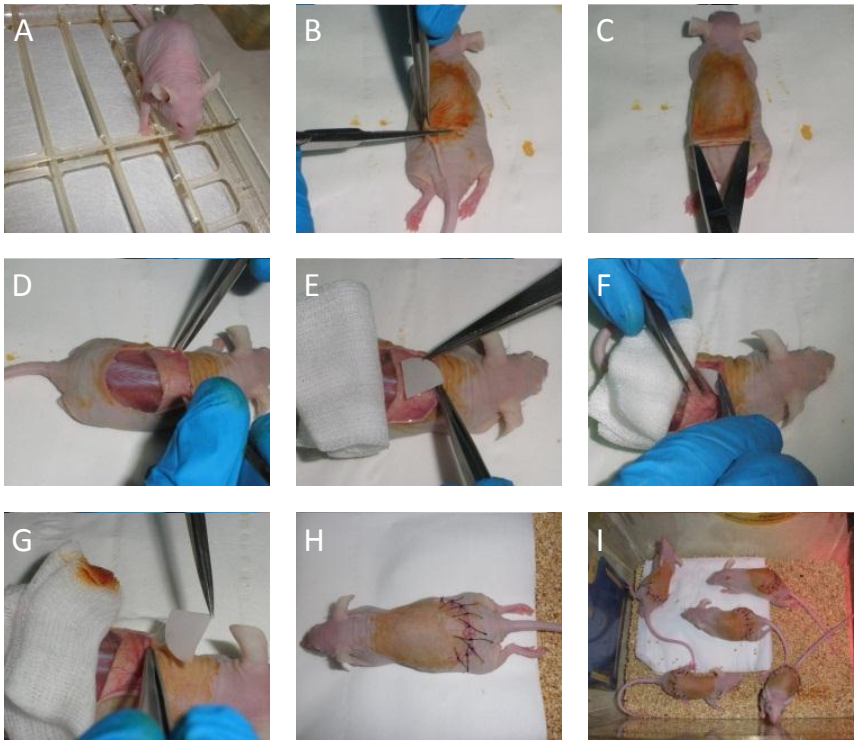

Figure 2 – Overview of the cell sheets implantation process

After 7 days and 21 days of transplantation, animals were euthanized with an intracardiac overdose of anaesthesia and implants were recovered for histological characterization (See II-4.5.3).

The skin flap was removed following the suture marks (Figure 3, A and B) and pinned on a piece of cork to prevent curling up (Figure 3, C and D). The skin flaps were then emerged in 3,7% formalin for 24 hours at 4ºC and processed as described in section II-4.5.1.

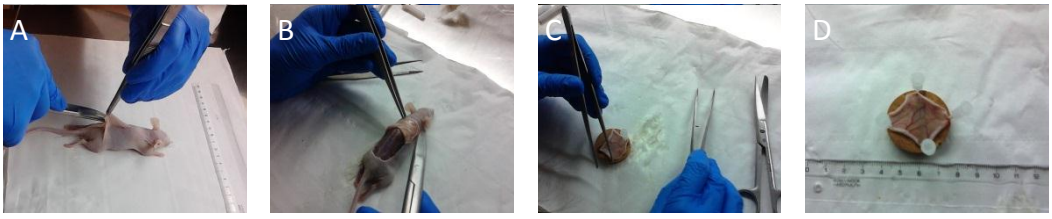

Figure 3 – Overview to the process of implanted cell sheets recovery

4. Characterization Techniques and Methodologies

4.1 - Flow cytometry Analysis

The three cell types used in co-culture procedures (see II-1.5) were analysed for the expression of several surface markers when cultured with different medium compositions and at different passages.

The mesenchymal phenotype of the selected and expanded hBMSCs was assessed for cells growing in monocultured in complete α-MEM supplemented with 2 ng/mL of FGF-β from isolation day to P6.

In order to evaluate the effect of osteogenic factors and the absence of TGF-β1 in co-culture medium on pericyte-like (CD146+) cells, flow cytometry was performed for those cells cultured for 7 days with M199 and EGM-2MV, both supplemented with osteogenic factors (see II-1.2).

The endothelial phenotype of HUVECs and HDMECs was screened for cells cultured in EGM-2MV or M199 and EGM-2MV supplemented with osteogenic factors. By the evaluation of those cellular markers in monoculture we expect that any phenotypic variation in the co-culture system is due to cellular interactions and independently of the cell culture medium.

4.1.1 – Preparation of cells suspensions

To perform flow cytometry analysis, a number of cells between 2 and 3×10^5, in a final volume of 100 µl, was added to each one of flow cytometry tubes (Falcon, USA). Depending on the marker of interest, two different staining procedures were used; the direct staining that comprehends the use of primary fluorophore-conjugated antibodies, or the indirect staining that combines the use of non-labelled primary antibodies and a complementary labelled secondary antibody (Table 2). The analysis of mesenchymal phenotype, was carried out on hBMSCs (n=3) cultured in complete α-MEM by screening the expression of CD146, CD105, CD90 and CD73. After induction of CD146+ phenotype (see II-1.3), several biological samples (n=3) were tested for the expression of CD146 antigen, as well CD105, CD90 and CD73, to confirm the expected phenotype.

Endothelial phenotype (HUVECs and HDMECs) was assessed for the expression of CD31 and CD34 on cells (n=2) growing in monoculture and with passages above 5.

4.1.2 – Flow cytometry using fluorochrome-conjugated antibodies

For direct staining, different primary fluorophore-conjugated antibodies were used at variable concentrations (Table 1). Antibodies were added to the 100 µl of cell suspension referred above (see II-4.2.1), and incubated for 20 minutes at RT protected from light. After the

incubation time, 2 mL of PBS were added to each one of the flow cytometry tubes that were then centrifuged at 250 × g for 5 minutes at RT. Supernatant was carefully removed and the cell pellet was resuspended in 500 µl of PBS. At this point, samples were ready to be analysed using a BD FACSCalibur flow cytometer (Enzifarma, Portugal). For each flow cytometry tube, 20.000 counts were analysed using CELLQuest software V3.3.

Table 1 - List of antibodies and dilutions used to perform flow cytometry analysis

Antibodies panel for Flow Cytometry	Dilution
CD34-PE conjugated (BD Biosciences, USA)	1:25
CD31-APC conjugated (R&D Systems, USA)	1:25
CD105-FITC conjugated (AbD Serotec, UK)	1:50
CD73-PE conjugated (BD Biosciences, USA)	1:100
CD90-APC conjugated (ebiosciences, USA)	1:50
CD146 unconjugated (abcam, UK)	1:100

PE: Phycoerythrin; APC: Allophycocyanin; FITC: Fluorecein Isothiocyanate

4.1.3 - Flow cytometry procedures using unconjugated primary antibodies

For indirect staining, 1 µl of the unconjugated primary CD146 antibody (P1H12) (mouse:anti-human, abcam, UK) was added to the 100 µl cell suspension referred above (see II-4.2.1). The mixture was homogenized and incubated for 45 minutes at 4°C. After the incubation time, 2 mL of PBS were added to the flow cytometry tube that was then centrifuged at 250 × g for 5 minutes RT. Supernatant was carefully discarded and 100 µl of AF488 conjugated secondary antibody (goat:anti-mouse, Molecular probes, USA), at a concentration 1:500, was added. The mixture was homogenised and incubated at 4°C during 45 minutes protected from light. The cell suspension was further diluted with 2 mL of PBS and centrifuged at 200 × g for 5 minutes RT. Supernatant was carefully discarded and 500 µl of PBS were added. At this point, samples were ready to be analysed using a BD FACSCalibur flow cytometer and CELLQuest software V3.3.

Additional flow cytometry tubes with BD™ CompBeads Negative Control (BD Bioscience, USA) and BD™ CompBeads Anti-Mouse Ig, κ particles, (BD Bioscience, USA) conjugated with the fluorochromes used on each condition were prepared in order to optimize fluorescence compensation settings for multicolour flow cytometric analyses.

4.2 – Fluorescence immunocytochemistry

The phenotype of hBMSCs cultures induced to express CD146 (pericyte-like (CD146+) cells), as well as of the cells in co-culture (See II-1.5) was assessed by fluorescence immunocytochemistry. For pericyte-like (CD146+) cells in monoculture, immunocytochemistry was performed after 7 days in culture as described in section II-1.3, while for co-cultures it was performed at days 2, 5 and 7 days of co-culture. After culture, cells were washed with PBS, twice, 5 minutes each, and incubated at RT for 30 minutes with 3,7% formalin. After fixation cells were thoroughly washed with PBS, again twice and 5 minutes each time, and incubated for 30 minutes at RT with 50 µl of a freshly prepared 3% bovine serum albumin (BSA, Sigma, Canada) solution in PBS (BSA/PBS). After this blocking step, cells were washed with PBS twice, 5 minutes each time, and incubated overnight at 4°C with 50 µl of CD146 primary antibody (P1H12, abcam, UK), diluted to 1:200 concentration with the 1% BSA/PBS blocking solution. In the following day, the PBS washing step was once again repeated and cells were incubated for 1 hour at RT with 50 µl of AF488 conjugated secondary antibody (Molecular Probes, USA) diluted to 1:500 in 1% BSA/PBS. After the final PBS washing, cell nuclei were counterstained after incubation with a 3 µg/mL DAPI (Sigma, USA) solution for 30 minutes RT, and the excess removed with a PBS washing.

Cells in co-cultures were incubated overnight with Dil-AcLDL (Molecular Probes, USA) at a final concentration 0.2 µg/mL before they were fixed. That procedure allowed ECs present in the co-culture to incorporate Dil-AcLDL and therefore to be identified together with the immunolabeled CD146 positive cells. After immunocytochemistry cells were observed using the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany), and images were acquired and processed using the AxioVision V.4 software.

4.3 – Alizarin Red Staining

The Alizarin Red-S (AR-S) staining method is a widespread technique used to evaluate calcium-rich deposition by cells in culture and in mineralized tissues. AR-S is an anthraquinone derivative which forms an AR-calcium complex in a chelation process (8). Although AR-S is not specific for Ca^{2+} – it reacts with a wide variety of cations - it binds selectively to calcium salts forming intense orange-red staining on mineralized areas. Nevertheless, staining efficiency can be reduced by issues such as the pH, the anion bonded to calcium and the used fixative (9).

The mineralization of the matrix deposited by hBMSCs under osteogenic conditions (See II-1.2) was assessed at days 7, 14 and 21 as previously described (10) (See annex for results). Cultures of hBMSCs were washed with PBS twice, 5 minutes each, and fixed for 30 minutes with 3,7%

formalin. Then, cells were washed again twice, 5 minutes each with PBS and incubated with a solution of 2% Alizarin Red[2] at a pH of 4.3 for 2 to 5 minutes to reveal calcium deposition.

AR-S staining was also performed on implanted cells sheets. Samples were deparaffinised in the automatic stainer Microm HMS740 (Thermo-Scientific, Germany) following the program described in Table 2 and a solution of AR-S (2%) were applied for 3 minutes. After, samples were washed in distillate water for 5 minutes, mounted and microscopically visualized. Images were captured using an Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and the AxioVision V4.8 software.

4.4 – Cell sheets characterization

4.4.1 - Cell sheets processing and H&E staining

The *in vitro* cultured cell sheets recovered by temperature decrease, carefully placed between two PVDF membranes (See II-2.2), and the explanted tissue samples (See II.3), were placed in a biopsy cassette (Bio-Optica, Italy) and processed using the spin tissue processor Microm STP120 (Thermo-Scientific, Germany) programmed as described in Table 3. When the processing step was complete, tissue samples were cut into smaller parts and, as for the *in vitro* cultured cell sheets, embedded in paraffin blocks using the embedding centre Microm EC350-1/CD350-2 (Thermo-Scientific, Germany). Paraffin embedded samples were cut into 5 µm sections with the microtome Microm HM355S (Thermo-Scientific, Germany), placed on glass microscope slides (Histostar, China) and left to dry at room temperature for 24 hours. Slides were then placed 5 minutes at 70°C to melt the excess of paraffin and then completely deparaffinised in the automatic stainer Microm HMS740 (Thermo-Scientific, Germany) following the program described in Table 2. At this point slides were ready to be used for H&E (Haematoxylin and Eosin) staining and for immunostaining (see. II-4.5.2).

H&E staining of the histological sections of the cell sheets was carried out by dipping the slides in Mayer's Haematoxylin[3], for 5 minutes, followed by a 10 minutes washing under tap water. Slides were mounted using a synthetic mounting medium (Histofluid, Germany) and analysed using a Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and the AxioVision V4.8 software.

[2] **2% Alizarin Red S solution:** 2 g of Alizarin Red S (Sigma, China) was dissolved in 100 mL of distillated water. pH was adjusted to 4.1~4.3 with 10% ammonium hydroxide.

[3] **Mayer's Haematoxylin Solution:** 50 g of aluminium potassium sulphate (Sigma, USA) was dissolved in 1 L of distillated water. After complete dissolution, 1 g of Mayer's haematoxylin (Sigma, USA) was added. The solution was well mixed before 0,2g of sodium iodate (Sigma, USA) and 20 mL of acetic acid were added. The solution was boiled and then allowed to cool down before use.

Table 2 – Automatic program used for samples deparaffinisation

Reagent	Time (Minutes:Seconds)
Xylene[2]	08:00
Xylene[2]	06:00
100 % ethanol	04:00
100 % ethanol	04:00
95% ethanol	04:00
70% ethanol	02:00
diH_2O	02:00

Table 3 – Automatic program used on spin tissue processor

Reagent	Immersion time (hours:minutes)	Stirring rate (rpm)
Formalin 4%	01:00	60
Formalin 4%	01:00	60
Alcohol 70%	01:30	70
Alcohol 80%	01:30	70
Alcohol 96%	01:30	70
Alcohol 100%	01:00	70
Alcohol 100%	01:00	70
Alcohol 100%	01:00	70
Xylene[4]	01:30	70
Xylene[2]	01:30	60
Paraffin	02:00	60
Paraffin	02:00	60

4.4.2 – Immunostaining of in vitro cultured cell sheets

After deparaffinisation, the histological sections of the *in vitro* cultured cell sheets were hydrated, immersed in citrate antigen retrieval solution[5], heated at 98°C for 20 minutes using a thermostatic water bath and finally left to dry at RT. The sections were then rinsed with distilled water three times, 5 minutes each, and incubated with a 3% peroxidase blocking solution[6] for 5 minutes. The immunostaining was performed using the R.T.U. Vectastain kit (Vector, USA) following manufacturers instruction. In detail, after a 5 minutes wash with PBS, the sections were incubated with a 2.5% horse serum blocking solution for 30 minutes at RT. Sections were then washed with PBS 3 times, 5 minutes each, and incubated overnight at 4°C with 100 µl of the selected antibodies (Table 4). All the antibodies were diluted to the final

[4] **Xylene substitute** (Clear-Rite, Thermo-Scientific, Germany)

[5] **Citrate buffer solution**: 2.94 g of tri-sodium citrate (Sigma, USA) was dissolved in 1 L of distillated water. The pH was adjusted to 6.0 using 1 N HCl solution. 500 µl of Tween20 (Sigma, Germany) was added and mixed.

[6] **3% Peroxidase blocking solution**: Hydrogen peroxide 30% was diluted in distilled water to a final concentration of 3%.

concentrations using 1,5% Horse Serum solution. From this point on, all the washing steps were performed using a solution of 0.1% PBS-Tween20[7], three times, 5 minutes each. After the washing step, sections were incubated with 2 drops (approx. 100 µl) of secondary biotinylated antibody for 30 minutes, washed again and incubated with 2 drops of streptavidin–HRP for 20 minutes. A final washing step was followed by the incubation with 150 µl of 3, 3'-diaminobenzidine (DAB) solution[8] in order to reveal the streptavidin binding sites, and until the desirable stain was achieved. Sections were washed in distilled water for 5 minutes and counterstained with haematoxylin (see II-4.5.1). Samples were left under running tap water for 10 minutes and then dehydrated in a series of ethanol solutions (70%, 90%, 100%). Samples were then dipped in xylene for at least 5 minutes and mounted with mounting medium Histofluid. Images were captured using the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and the AxioVision V4.8 software.

Table 4 – List of antibodies used to perform immunostaining on cell sheets

Antibodies panel for histogical staining	Dilution
CD146 (Mouse, abcam, UK)	1:200
CD31 (Mouse, Dako, USA)	1:30
Osteocalcin (Mouse, AbB Serotec, UK)	1:100
Collagen type-I (Mouse, abcam, UK)	1:200
Anti-mitochondria (Rabbit, Millipore, UK)	1:100

4.4.3 Immunostaining of implanted cells sheets

The methodology followed for the immunostaining of the histological sections of the implanted cells sheets was in conformity with section II-4-5.2 up to the endogenous peroxidase blocking step. Since the cell sheets were implanted in mice and some of the antibodies used to perform the immunostaining are created in mice, a mouse on mouse kit (M.O.M kit, Vector, USA) was used to diminish unspecific binding of secondary biotinylated antibodies. Before blocking the endogenous peroxidase, the histological sections aimed at intracellular antigens identification were treated with 0.2% Triton-X100[9] for 20 minutes. To better preserve the integrity of the samples, a 0.3% hydrogen peroxide solution in methanol was used, instead of the 3% hydrogen peroxide solution in water, for 30 minutes in order to

[7] **PBS-Tween20 (0.1%)**: 1 mL of Tween20 (Sigma, Germany) was added to 1 L of PBS (Sigma, USA) and mixed carefully.
[8] **DAB Peroxidase Substrate kit**: For a final volume of 5 mL, 2 drops of buffer stock solution, 4 drops of DAB stock solution and 2 drops of hydrogen peroxide solution were added to distillated water, sequentially, and mixed well.
[9] **0,2% Triton-X100**: 20 µl of Triton-X100 (Sigma, USA) were diluted in 10 mL of PBS (Sigma, USA) and mixed well.

block the endogenous peroxidases. Sections were then washed with PBS-Tween three times, 5 minutes each, and incubated for 1 hour at RT with the M.O.M. Mouse Ig Blocking reagent. After another washing step with PBS-Tween, three times, 5 minutes each, sections were incubated for 5 minutes with M.O.M. diluent. After removal of the excess of diluent, sections were incubated with the primary antibodies, diluted to desirable concentrations (Table 5) using the M.O.M. diluent, for 30 minutes at RT. Sections were then washed with PBS-Tween three times, 5 minutes each, and incubated for 10 minutes with M.O.M. biotinylated anti-mouse IgG reagent according to manufacturer instructions. Sections were washed again with PBS-Tween three times, 5 minutes each, and treated as described in section II-4.5.1 from the incubation with the streptavidin–HRP and using the R.T.U. Vectastain kit reagents.

4.4.4 – Fluorescence co-localization of CD146 and human mitochondria on implanted cells sheets

Co-localization of CD146 and human mitochondria was carried out following the general steps of immunostaining using enzymatic detection, including deparafinisation, antigen retrieval, permeabilization and mouse Ig's blocking (See II-4.4.3). This analysis was performed to identify human cells from our construct and also human pericyte-like (CD146+) cells. A limiting subject regarding the use of fluorescence microscopy on tissue samples is the tissue autofluorescence, either intrinsic or induced by fixation and tissue processing. Several reagents have been used to overcome this problem, including ammonia-ethanol, sodium borohydride and Sudan Black B (11). In this work we successful used Alizarin Red-S solution (2%), applied for 5 minutes after antigen retrieval, to reduce tissue autofluorescence. Primary antibodies CD146 and anti-mitochondria (Table 5) were incubated for 1 hour at RT and fluorescence secondary antibodies AF488-conjugated secondary antibody (donkey anti-rabbit, Molecular probes, USA) and AF594-conjugated secondary antibodies (goat anti-mouse, Molecular probes, USA) were also incubated for 1 hour at RT. DAPI was applied for 30 minutes at RT as nuclei counterstaining. Samples were mounted with Vectashield mounting medium for fluorescence (Vector Labs, USA) and images were captured using the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and the AxioVision V4.8 software.

5 - References

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Development of human osteogenic cell sheets co-cultured with endothelial and pericyte-like cells

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Abstract

In recent years the use of cell sheets for tissue engineering proposes has become a reality, with some studies showing that it is a reliable alternative for the traditional approaches. However, the progression of this technology is being limited by some of the barriers which have been hampering the evolution of other tissue engineering strategies, such as the lack of appropriate vasculature to supply thicker constructs *in vivo*. The creation of a cell sheet-based construct, by co-culturing osteogenic, endothelial and pericyte-like cells, with the purpose of enhancing the vascularization of newly formed bone tissue and also the degree of maturation and stability of the vascular network is herein proposed. Since it has been proposed that bone marrow can be a source of perivascular-like cells, in particular those expressing CD146, human bone marrow mesenchymal stromal cells (hBMSCs, n=3) were isolated, cultured under different culture conditions and characterized for the expression of CD146 as well as for the MSCs markers CD90, CD73 and CD105. Flow cytometry results demonstrated that supplementation of standard hBMSCs culture medium with TGF-β1 promote an increase of CD146 expression in hBMSCs, from approximately 50% to more than 97%. Moreover, changes in CD146 expression were associated with different cellular morphologies. Immunocytochemistry performed on the co-cultures showed that induced CD146+ hBMSCs and human umbilical vein endothelial cells (HUVECs) migrated and organized themselves over a thin, collagen-rich, osteogenic cell sheet, suggesting the existence of an efficient cross-talk involving all the co-cultured cell types. *In vivo* studies were performed to test the ability of these constructs to form functional, vascularized and osteo-committed tissue. Immunohistochemistry analysis of transplanted cell sheets revealed the integration of HUVECs with host network vasculature as well as the osteogenic potential of the created construct, as shown by the expression of osteocalcin and calcium deposition. Additionally, the analysis of the diameter of CD146 positive blood vessels showed a higher mean vessel diameter for the

experimental condition, reinforcing the advantage of the proposed model regarding blood vessels maturation and stability.

1 - Introduction

The interest on cell sheet engineering concept for regenerative medicine purposes has been increasing over the years. Gradually, this approach is being established as a reliable alternative for traditional tissue engineering (TE) and regenerative medicine methods, namely the use of biodegradable scaffolds to create a tissue substitute, and the injection of isolated cells (1). The revolutionary conception consisted on the use of poly(N-isopropylacrylamide) (PIPAAm), a thermoresponsive polymer which has been successfully used in biomedical applications (2-4), to produce thermoresponsive culture surfaces that allow cells recovery, within their own extracellular matrix (ECM) that can act as a natural glue, as a sheet with cohesive cell-cell and cell-ECM interactions (5).

Few years after the first steps of cell sheet engineering technology, it was clear the potential of building cell sheets-based constructs *in vitro* that mimic, as much as possible, the complex structures of human tissues. For the past 10 years, several works have shown the potential of this technology for cornea (6) and myocardial tissues reconstitution (7), hepatocyte transplantation (8), renal tube epithelial cell transfer (9) and for bone tissue engineering applications (10). Moreover, several reports have also proved the advantages of cell sheets stacking and of patterned thermoresponsive surfaces to obtain co-cultured cell sheets (11-14), to further enhance the similarities of the created constructs with *in vivo* tissues and, at the same time, to overcome the limited and non-functional vascularization of thick tissue engineering constructs after implantation (15, 16). In fact, like for traditional TE strategies, the pre-vascularization of cell sheets-based constructs was proposed as one possible way that to circumvent this problem (11, 12) taking advantage of co-cultures that combine endothelial cells with other progenitor or mature cells. According to Rouwkema and coleagues (17), this methodology can dramatically reduce the time needed to vascularize the implant when compared with approaches that depend on scaffold design and angiogenic factors delivery.

Despite endothelial cells lining blood vessels of the entire cardiovascular system (18), perivascular cells, specially pericytes, have great impact on vascularization, contributing for blood vessel stability, maturation of newly formed vessels and for the regulation of microvascular blood flow (19-21). Recent findings in this field have catapulted the number of works exploring pericytes progeny and multipotency, as well as their significance for the advance the TE field (22-25). Also, co-expression of several surface markers between pericytes and mesenchymal stem cells (MSCs) led Caplan to suggest in 2008 (26) that all the MSCs are pericytes, fact that later lead the same author to revise the initial concept of the mesengenic process (27). The CD146 is an important adhesion molecule for vascular endothelial cell activity and angiogenesis (28), which is co-expressed on a subpopulation of hBMSCs and in

some populations of pericytes (29). This transmembrane glycoprotein has important functions in early and late development and it has been suggested to play an important role in cancer, angiogenesis, cardiovascular diseases and placentation (30). Moreover, a significant number of studies in the field of cancer research have assigned to CD146 a critical role in tumour growth and metastasis, as well as in tumour angiogenesis, suggesting an anti-angiogenic effect in several types of tumour vessels lead by CD146 inhibition (31, 32).

The main goal of this work was to develop a three-dimensional osteogenic cell dense construct with an organizational pattern of endothelial and perivascular-like cells differentiated from hBMSCs, as a way to accelerate engineered construct perfusion *in vivo* contributing to its survival. To demonstrate our assumptions a co-culture system was created *in vitro.* A confluent layer of osteoblasts derived from hBMSCs was used as substrate for HUVECs and pericyte-like (CD146+) cells. The involved cross-talk between the three cell types led to the formation of organized colonies of HUVECs surrounded by pericyte-like (CD146+) cells. The *in vivo* transplantation of co-cultured constructs combining two osteogenic cell sheetswith HUVECs and pericyte-like (CD146+) cells in between permitted to demonstrate the active role of these cells in the formation of the new vasculature as well as its influence over its maturation and stability as shown by the increased number and blood vessel diameter at early time points. These findings and osteogenic character of the created construct seem good indicator of its potential for bone tissue engineering proposes, however it is our believe that it might be also considered as a suitable model for *in vitro* pre-vascularization of TE constructs.

2 - Material and Methods

2.1 - Cell Isolation and culture

Bone marrow aspirates were obtained after informed consent from patients undergoing hip replacement surgery, at Hospital da Prelada, Porto, Portugal. Human bone marrow-derived mesenchymal stem cells (hBMSCs) were isolated by gradient centrifugation as previously described (33). hBMSCs were cultured and maintained in complete α-MEM (Gibco, USA) supplemented with 2ng/mL FGF-β (PeproTech, USA). Cells were used to establish the co-cultures at passage between 2 and 3. Umbilical cords (UCs) from healthy donors obtained by caesarean section were provided by Hospital de Braga (Braga, Portugal) and delivered to the cell culture laboratory in transport buffer, containing 0.14 M NaCl, 0.004 MKCl, 0.001M phosphate buffer, pH 7.4, 0.011M glucose. The isolation of the human umbilical cord vein endothelial cells (HUVECs) was carried out as described in the literature by Jaffe and others

(34), and cells were used until passage 5. Biological samples were provided under a protocol approved by the Hospitals Ethical Committees and established with the 3B's Research Group.

2.2 - Induction of CD146+ phenotype

hBMSCs at passages 4 or lower were cultured for 7 days in complete α-MEM supplemented with 1ng/mL TGF-β1 (ebiosciences, USA). Culture medium was replaced twice during culture time and the differentiated pericyte-like (CD146+) cells were used as pericyte-like cells to establish the co-cultures.

2.3 - Co-cultured cell sheets fabrication

hBMSCS, at a density of 35.000 cells/cm^2, were cultured on thermo-responsive dishes (Nunc, Danmark) for 7 days in complete α-MEM (Gibco, USA) supplemented with osteogenic differentiation factors, 10mM β-Glycerophosphate (Sigma, USA), 150 µg/mL ascorbic acid (Sigma, USA) and 1×10^{-8}M dexamethasone (Sigma, USA). HUVECs and pericyte-like (CD146+) cells, in a total of 45.000cells/cm^2 and at a final ratio of 4:1, were then seeded onto the confluent layer of hBMSCs and cultured in Medium 199 (Sigma, USA) supplemented with the osteogenic differentiation factors described above. After 7 days, co-cultured cell sheets were retrieved by temperature decrease as previously described (35), fixed with 3.7% buffered formalin and paraffin embedded for histological characterization. For immunofluorescence characterization, the co-cultures were established on tissue culture polystyrene (TCPS) coverslips under the described conditions.

2.4 - Flow cytometry

Flow cytometry was performed using mouse anti-human antibodies CD146 (unconjugated, abcam, UK), CD31 (APC-conjugated, R&D Systems, USA), CD73 (PE-conjugated, BD biosciences, USA), CD90 (APC-conjugated, ebiosciences, USA) and CD105 (FITC-conjugated, AbD Serotec, UK). Experiments were performed using cells in different passages, from isolation day to P6, and isolated from different donors (n=3). hBMSCs, pericyte-like (CD146+) cells and HUVECs were trypsinized, counted and resuspended in 2% BSA (Sigma, Canada) solution in PBS (BSA/PBS) at a concentration of 2500 cells/µL. For indirect staining, cells were first incubated for 45 min at 4ºC protected from light with CD146 antibody (1:100). After a washing step with PBS, cells were incubated for 45 minutes protected from light at room temperature with AF488 conjugated secondary antibody (goat anti-mouse, Molecular probes, USA) at a

concentration 1:500. For direct staining, cells were incubated for 20 minutes at room temperature protected from light with the fluorescence-conjugated primary antibodies listed above. After a washing step, cells were resuspended in PBS and 20.000 counts were analysed using a FACSCalibur flow cytometer (BD Biosciences) and the CELLQuest software V3.3.

2.5 - Immunofluorescence

Monocultures of pericyte-like (CD146+) cells and co-cultures established on the TCPS were incubated for 30 minutes with 3% BSA/PBS at room temperature. Then, cells were washed with PBS and incubated overnight at 4ºC with mouse:anti-human CD146 antibody (1:100) diluted in 3% BSA/PBS. Cells were then washed in PBS and incubated for 1 hour at room temperature with AF488 conjugated secondary antibody (goat:anti-mouse, Molecular probes, USA), diluted in 3%BSA/PBS to a 1:500 concentration. Nuclei were counterstained with DAPI (3µg/mL) by incubation for 30 minutes at room temperature. The co-cultures were previously incubated overnight at 37ºC in humidified atmosphere and 5%CO_2 with Dil-AcLDL (Molecular Probes, USA), at a final concentration 0.2µg/mL, and before fixation to label HUVECs.

For the co-localization of human cells and pericyte-like (CD146+) cells on transplanted cell sheets at different implantation periods samples were treated for 5 minutes with alizarin red-S solution (2%) (Sigma, China) in order to quench auto-fluorescence. Mouse:anti-human CD146 and human specific anti-mitochondria (Milipore, USA) antibodies were incubated for 1 hour at room temperature followed by the incubation with AF488-conjugated secondary antibody (donkey anti-rabbit, Molecular probes, USA) and AF594-conjugated secondary antibodies (goat anti-mouse, Molecular probes, USA) at a concentration of 1:500 for 1 hour at room temperature. DAPI was used for 30 minutes as nuclei staining.

Samples were analysed using an Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and images were acquired and treated with AxioVision V.4.8 software.

2.6 - Immunohistochemistry

Immunohistochemistry was performed both for *in vitro* cultured and *in vivo* transplanted cell sheets using antibodies against mouse:anti-human CD146 (1:100) (abcam, UK), mouse:anti-human CD31 (1:40) (Dako, USA) and mouse:anti-human Osteocalcin (1:100) (AbD Serotec, UK). Histological cuts (5µm) were first deparaffinised with Clear-rite3 (Thermo-Scientific, Germany) and rehydrated in graded alcohol series, followed by antigen retrieval with 10mM sodium citrate buffer solution (pH 6) at 98ºC for 20 minutes. For intracellular antigens, sections were treated with 0.5% Triton X100 (Sigma, USA) in PBS for 10 minutes. Unspecific binding was

blocked using 2.5% normal horse serum (NHS) (Vector Labs, USA) for 30 minutes, flowed by 3 washing steps, 5 minutes each, with PSB-0.1%Tween20 (Sigma, Germany). Antibodies were diluted in 1.5% normal horse serum and incubated at room temperature for 1 hour. Sections were then washed 3 times in PBS-Tween, 5 minutes each, and the endogenous peroxidase activity was quenched with 0.3% in methanol (30 minutes). After a washing step, 3 times in PBS-Tween, 5 minutes each, sections were incubated for 30 minutes with biotinylated anti-mouse secondary antibody (Vector Labs, USA). After washing sections were incubated for 30 minutes with R.T.U. vectastain elite ABC reagent (Vector Labs, USA) before HRP enzymatic activity was revealed with DAB (Vector Labs, USA). Nuclei were counterstained with Mayer's haematoxylin followed by sections dehydration in graded ethanol series and mounted. In the case of *in vivo* transplanted cell sheets, mouse Ig blocking reagent (M.O.M. kit, Vector Labs, USA) was applied for 1 hour before primary antibody incubation in order to reduce background. Samples were analysed using the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and images were acquired and treated with AxioVision V.4 software.

2.7 - Cell sheet stacking and transplantation

Two types of cell sheets were transplanted, single monocultured osteogenic cell sheets, as control, and co-cultured osteogenic cell sheets stacked with a second monocultured osteogenic cell sheets, as experimental condition. To stack the two cell sheets, a poly(vinylidene difluoride) (PVDF) membrane (Millipore, USA) with 2 cm of diameter was placed over an osteogenic cell sheet and incubated at RT for 15 minutes. After this time, the cell sheets spontaneously detached from thermoresponsive dishes and were attached to the membrane which allowed its manipulation and stacking over the co-cultures, still in TR dishes. The all construction was further incubated at room temperature for 15 minutes to allow the detachment of the co-cultured cell sheet from the TR dish and adhesiveness to the osteogenic one, forming a double cell sheet construct that combined two osteogenic cell sheets with HUVECs plus pericyte-like (CD146+) cells in between.

The transplantation of *in vitro* cultured cells sheets was carried out as previously reported (36). Briefly, 5 weeks old male nude mice (Charles River, USA), n=5 per condition and timepoint, were anesthetized 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, dorsal skin flap 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 membrane was removed, the skin flap was brought back to the original location and sutured.

2.8 - Statistical analysis

Quantification of the mean diameters of CD146 positive blood vessels formed on control and experimental groups was replicated (n=3). The numeral analysis of the positive and negative CD146 blood vessels was performed on control and experimental conditions. The total number of blood vessels and the diameter of CD146 positive ones were counted when adjacent to skeletal muscle and around cell sheets (n=3) in every condition using AxioVison software (V. 4.8).

Data was analysed using *anova test* and results considered statistically different for *P* values lower than 0.05.

3 – Results

3.1 - Osteogenic cell sheets derived from hBMSCs

hBMSCs were cultured on PIPAAm culture surfaces for 14 days in osteogenic medium in order to obtain osteogenic cell sheets. After retrieval by temperature decrease (Figure 1 A), the nature of produced cell sheets was analysed by H&E staining and immunohistochemistry for osteocalcin and type-I collagen deposition. H&E performed on histological sections showed that hBMSCs form thin layers of contiguous cells (from 2.5 to 6 µm thickness)(Figure 1 B). Additionally, the positive staining for osteocalcin (Figure 1 C) and type-I collagen (Figure 1 D) after 14 days in culture with osteogenic medium demonstrated the deposition of extracellular matrix characteristic of the commitment of hBMSCs towards the osteogenic lineage. This was further confirmed by Alizarin Red-S staining at day 21 (Figure 1 F) that revealed an intense staining due to high calcium deposition.

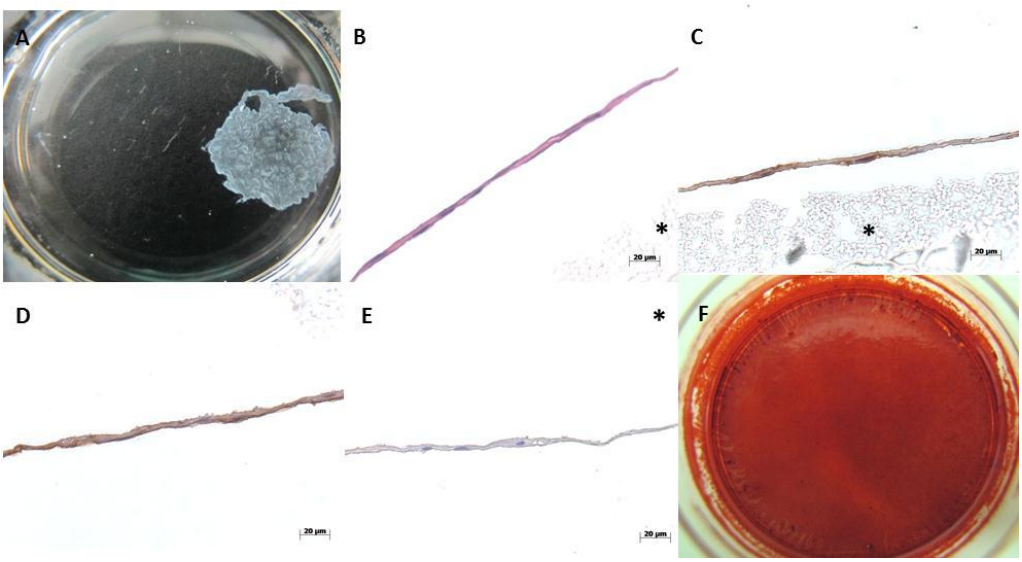

Figure 1 - Macroscopic view (A,F) and histological characterization (B-E) of hBMSCs cell sheets cultured for 14 days in osteogenic medium; (A) Osteogenic cell sheets derived from hBMSCs after detachment and contraction. (B) H&E staining; (C) immunostaining for osteocalcin and (D) type-I collagen; (E) Control for immunostaining; (F) Osteogenic character of CSs after 21 days in culture revealed by AR-S staining. * PVDF membrane used to protect CS during processing step.

3.2 - Effect of TGF-β1 on CD146 expression and cellular morphology

The expression pattern of some surface markers expressed by hBMSCs in multiple culture conditions was followed by flow cytometry using several antibodies, and immunocytochemistry for CD146.

Flow cytometry performed on the mononuclear fraction from marrow at the isolation day, revealed the presence of a small $CD146^{+}$ sub-population representing approximately 2.5% of total cellular content (figure 2A). The frequency of cell markers, such as CD105, CD73 and CD90 normally associated to the mesenchymal phenotype, in the mononuclear fraction of the marrow was less than 2% (data not shown). However, after selection by adhesion to TCPS these values increased and were kept stable along different passages. For a representative sample (P4) cultured in complete α-MEM, the percentage of pericyte-like (CD146+) cells was approximately 46% (Figure 2B), and more than 98% of the hBMSCs expressed the surface markers CD105, CD73 and CD90 (data not shown).

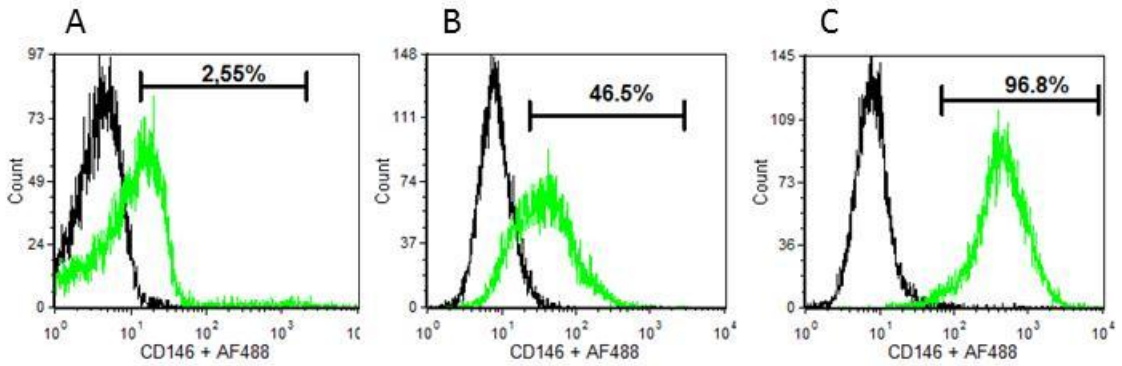

Figure 2 - Representative flow cytometry analysis of hBMSCs at different passages and cultured in different culture medium. (A) CD146 expression of bone marrow mononuclear fraction at isolation day; (B) CD146 expression on hBMSCs (P5) cultured in complete α-MEM; (C) CD146 expression on hBMSCs (P5) cultured 7 days in complete α-MEM supplemented with 1 ng/mL TGF-β1.

In what concerns the effect of the TGF-β1 over the hBMSCs surface markers expression, the number of cells expressing CD146 antigen, as well as the amount of CD146 antigen, increased after the treatment. In a representative population at P5, cultured for 7 days in α-MEM supplemented with 1ng/mL TGF-β1, more than 97% of the analysed cells were positive for CD146 (Figure 2C), and the population expressing CD105, CD73 and CD90 was maintained (≥98%) (data not shown). Immunocytochemistry of the derived pericyte-like (CD146+) cells after TGF-β1 treatment in monoculture showed the ubiquity of the CD146 antigen over their surface confirming flow cytometry results, as well as their characteristic "star morphology" with extended interactions between neighbour cells (Figure 3A). This pronounced morphological change after culture with TFG-β1 (pericyte-like (CD146+) cells) was also clearly observed by contrast phase microscopy in comparison to hBMSCs (Figure 3 B,C).

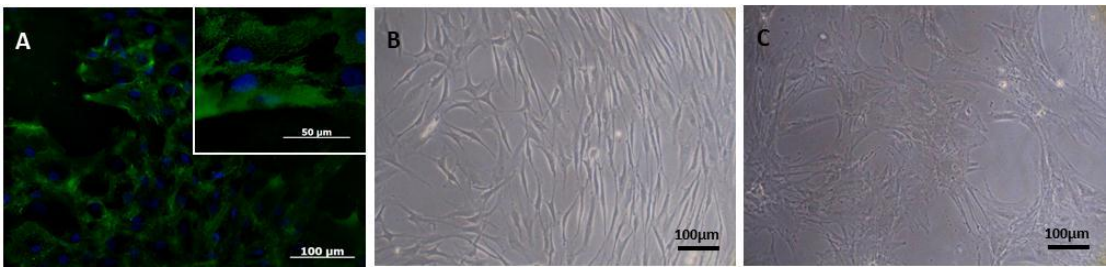

Figure 3 – Expression of CD146 (green) (A) and evolution of cell morphology of hBMSCs before (B) and after culture for 7 days in α-MEM + 1ng/mL TGF-β1 (C). For immunocytochemistry DAPI (blue) was used as nuclear staining. Right upper corner image represent a higher magnification (A)

3.3 - Characterization of co-cultured cell sheets

A new co-culture model, composed by 3 different cell types, with a characteristic cellular organization and distribution pattern was developed. HUVECs, incorporating DiL-AcLDL, were

disposed in round colonies from 2 day onward while pericyte-like (CD146+) cells, displaying an elongated morphology as depicted in Figure 4, were only observed from day 5 forward.

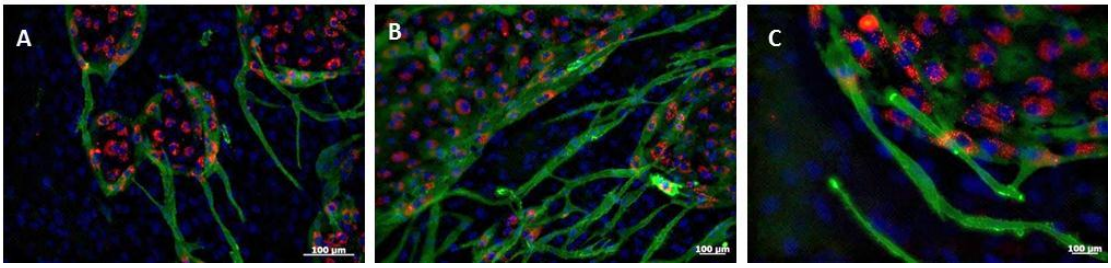

Figure 4 - Immunocytochemistry for CD146 (green) expression on hBMSCs, pericyte-like (CD146+) cells and HUVECs co-cultured for 7 days. Dil-AcLDL (red) was used to assess LDL uptake and identify HUVECs. DAPI (blue) was used as nuclear staining. A, B and C represent successive close ups.

Histologically, the co-cultured cell sheets showed a cellular organization similar to single osteogenic cell sheets but with increased thickness, approximately 12µm, and overlapped cells corresponding to colonies of endothelial and pericyte-like (CD146+) cells (Figure 5 A). These results were confirmed by the immunolocalization of CD31 (Figure 5 B) and CD146 (Figure 5 C) positive cells. As for the osteogenic cell sheets the deposition of osteocalcin (Figure 5 D) and type-I collagen (Figure 5 E) attested the osteogenic commitment of the *in vitro* system.

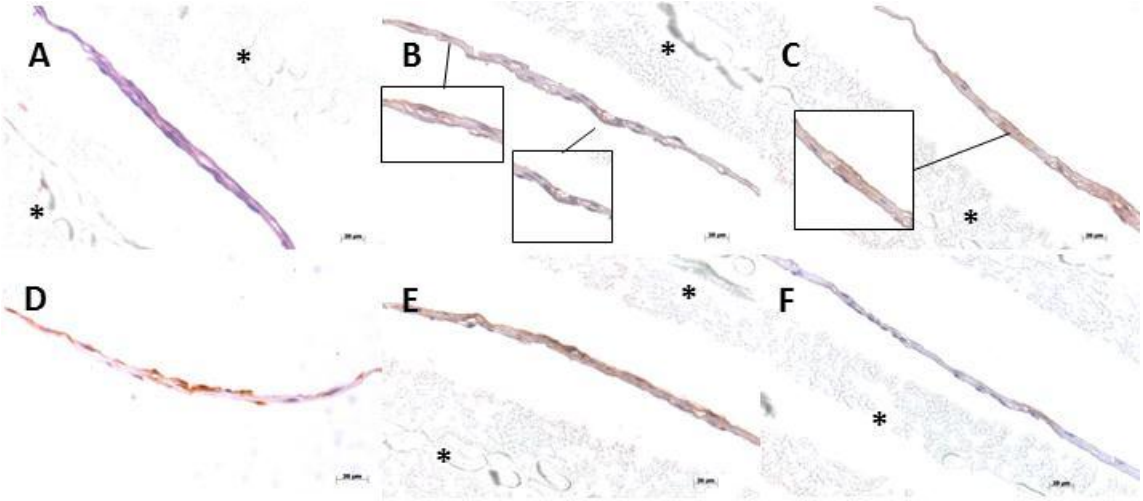

Figure 5 - Histological analysis of co-cultured cell sheets after H&E staining (A) and immunostaining for CD31 (B), CD146 (C); osteocalcin (D) and type-I collagen (E). Squares represent close up views of specific regions of the cell sheets showing expression of CD31 (B) and CD146 (C). Identification of positive signal was determined in comparison to immunocytochemistry negative control (F). * PVDF membrane used to protect cell sheet during processing.

The expression of CD31 and CD146 on HUVECs and pericyte-like (CD146+) cells monocultures maintained for 7 days in the culture medium used for the co-culture, the M199 supplemented with osteogenic factors, was analysed by flow cytometry. The maintenance of the CD146 and CD31 phenotype (>98%) on HUVECs was confirmed (Figure 6 A,B) however, a slight decrease of

CD146 expression was observed on pericyte-like (CD146+) cells, both for the number of cells expressing the antigen as well as for the amount of antigen (Figure 6 C) when compared with results of the same cells cultured in the presence of TGF-β1 (Figure 2 C).

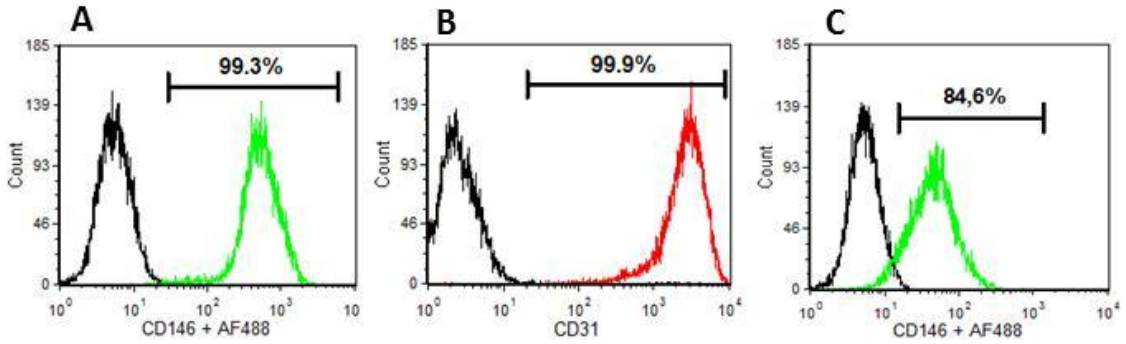

Figure 6 - Representative flow cytometry analysis of CD146 and CD31 expression on (A;B) HUVECs (P5) and (C) CD146 expression on pericyte-like (CD146+) cells (P5) cultured for 7 days in M199 supplemented with osteogenic factors. High expression (>98%) of CD31 and CD146 on HUVECs confirmed the absence of an effect of the osteogenic factors on their native phenotype. A reduction on CD146 expression of pericyte-like (CD146+) cells (84%) was verified after 7 days in culture without TGF-β1.

3.3 - Characterization of implanted cell sheets

The H&E staining of the histological sections of the implanted cell sheets showed its positioning adjacently to the mouse skeletal muscle and surrounded by mouse connective tissue (Figure 7 A-D).The presence of some blood vessels around and inside transplanted cell sheets growing were already visible after 7 days of implantation, both on control and experimental conditions, some of those containing erythrocytes which prove their inosculation with the host vasculature and its perfusion.

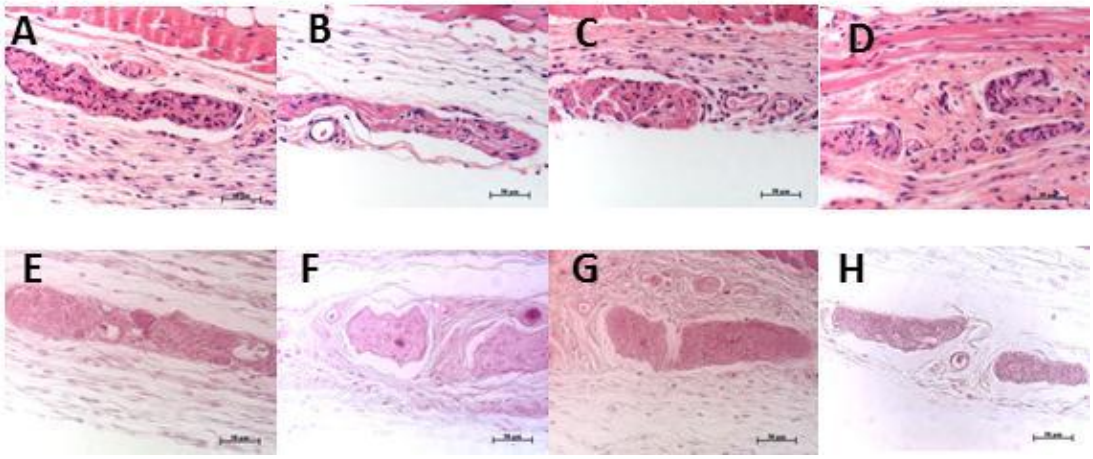

Figure 7 - H&E (A;B;C;D) and Alizarin Red-S (E;F;G;H) staining on cell sheets after 7 (A;C;E;G) and 21 days (B;D;F;H) of subcutaneous implantation. (A;B;E;F) control group (C;D;G;H) experimental group.

The osteogenic character of the retrieved samples was confirmed after revelation of calcium deposits by Alizarin red-S (Figure 7 E-H), and by the identification of osteocalcin (Figure 8).

The contribution of the transplanted HUVECs and pericyte-like (CD146+) cells for the development of new blood vessels, namely their integration with host vascular network, was confirmed by the presence of human CD31 positive cells on those vessels both at 7 and 21 days of implantation (Figure 9 A,F). In what concerns the contribution of the pericyte-like (CD146+) cells, it was interesting to find a differential expression of CD146 antigens on blood vessels as some were negative for this marker, both on experimental and control conditions (Figure9 B,G,D,I). In order to specifically identify human pericyte-like (CD146+) cells, co-localization for CD146 and human specific anti-mitochondria antibodies, was performed. The human pericyte-like (CD146+) cells were identified after 7 days of implantation (Figure 10).

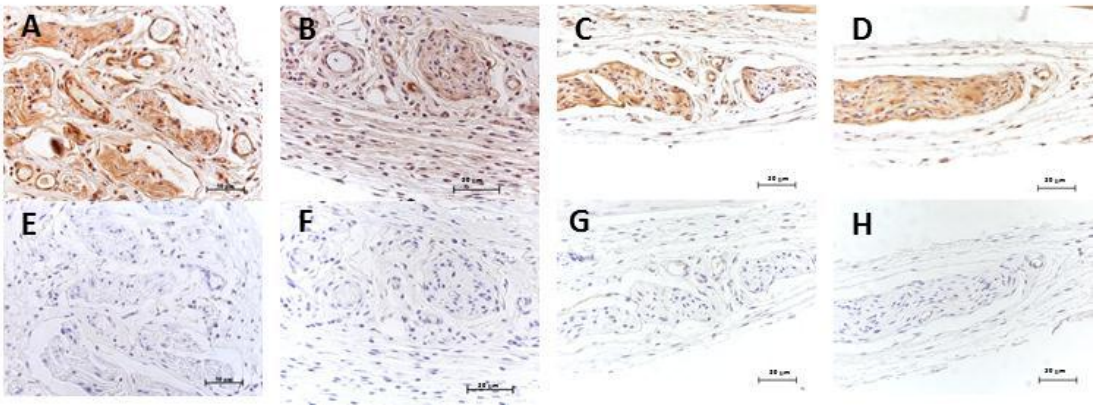

Figure 8 - Immunohistochemistry for Osteocalcin on experimental (A,C) and control (B,D) conditions at 7 (A,B) and 21 (C,D) days of implantation. (E,F,G,H) immunostaining negative control.

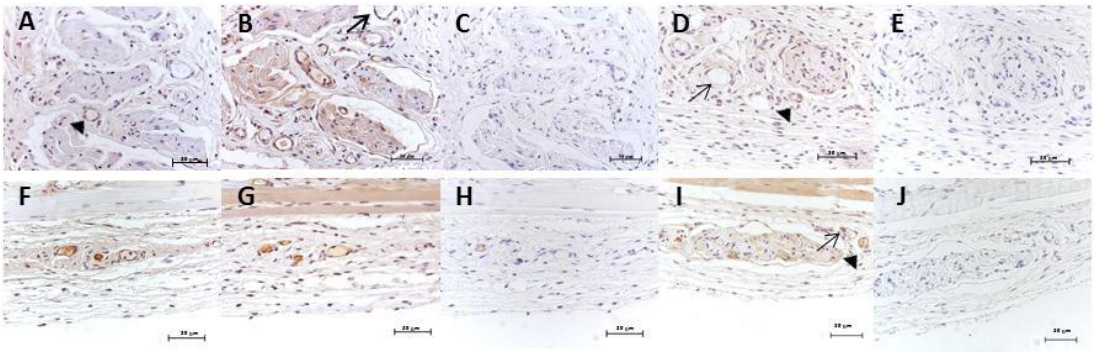

Figure 9 – Immunohistochemistry for CD31 (A,F) and CD146 (B,G,D, I) on experimental (A,B,C,F,G,H) and control (D,E,I,J) conditions at days 7 (A,B,C,D,E) and 21 (F,G,H,I,J) of implantation. (C,H,I,J) Immunostaining negative control. → negative blood vessels for CD146; ► positive blood vessel for CD146

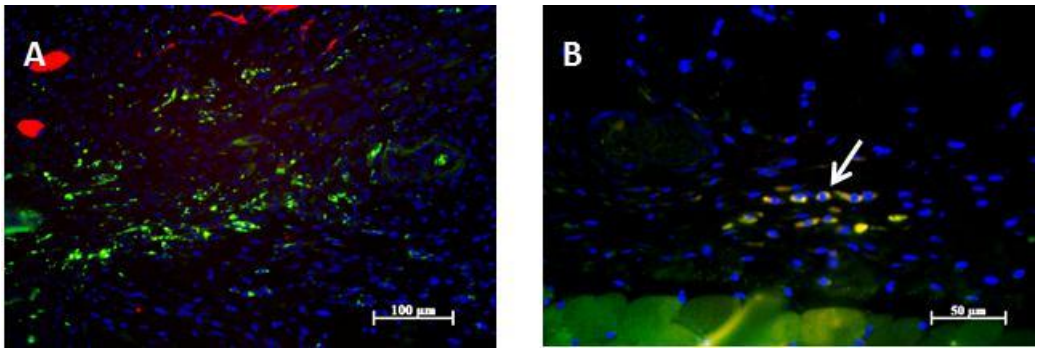

Figure 10 - Co-localization for CD146 and human-specific anti-mitochondria performed on experimental condition after 7 days of implantation. DAPI (blue) was used as nuclear staining. (A) Human cells (green) detected by human-specific anti-mitochondria antibodies. (B) Double labelled cells for CD146 (green) and anti-mitochondria (red) assembled in a blood vessel-like structure (arrow).

3.4 - Analysis of the number and diameter of blood vessels

The diameter of CD146+ blood vessels, as well as the total number of new blood vessels formed were assessed both for experimental and control conditions (Figure 11). Only the blood vessels formed between skin skeletal muscle and the mice connective tissue were considered. A significant increase of the diameter of blood vessels was observed in the experimental condition after 7 ($p \leq 0.01$) and 21 ($p \leq 0.05$) days of transplantation. Contrarily, no significant differences ($p > 0.05$) were observed between experimental and control conditions regarding the total number of blood vessels.

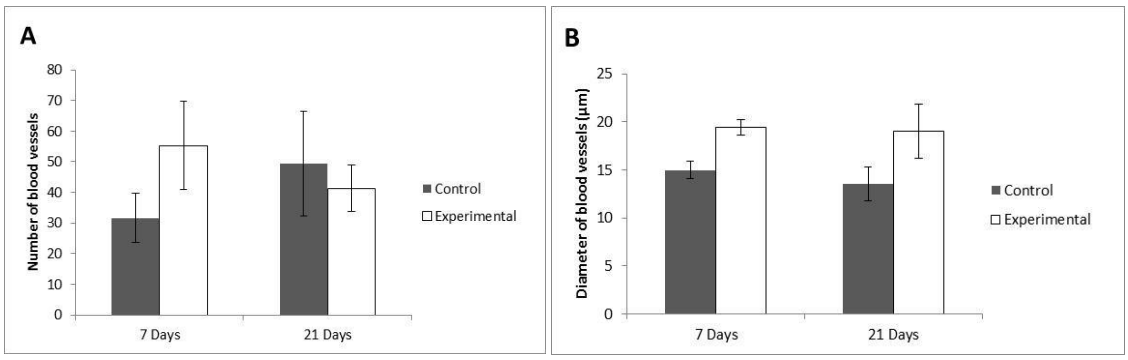

Figure 11 – Representation of the number of blood vessels and the mean diameter for control and experimental conditions at days 7 and 21 of implantation. *$p \leq 0.05$; **$p \leq 0.01$..

4 - Discussion

The main objective of this work was the development of a multi cell sheet stacked construct with an organizational pattern of endothelial and perivascular-like cells focusing the contribution of pericyte-like (CD146+) cells for the in vivo vascularization for bone TE

applications. Thus, to test our hypothesis, we started from the *in vitro* fabrication of a co-culture construct and its characterization in terms of osteogenic commitment and cell-to-cell interactions to *in vivo* subcutaneous tests to evaluate the capacity of our construct for new bone formation and vascularization ability.

As we previously reported (10), rat bone marrow-derived mesenchymal stem cells have shown the capacity to form osteogenic cell sheets with potential applications for bone TE. In this work human bone marrow mesenchymal stem cells were used to create an advanced model of pre-vascularized osteogenic cells sheets. Nevertheless, due to distinct osteogenic differentiation patterns between rat and human MSCs a different strategy to produce a workable cell sheet-based construct was used.

While creating osteogenic cell sheets there are two major aspects that are critical, the secretion of ECM that has to be sufficient to allow cell sheet detachment and confer robustness, and the mineralization degree that cannot hinder its detachment. hBMSCs cultured for 14 days on TR dishes using standard osteogenic differentiation medium gave rise to a fragile monolayer of cells, involved in an ECM composed by collagen type-I, the most abundant protein in the organic bone matrix synthesized by active osteoblasts, and osteocalcin, a small protein produced by mature osteoblasts during mineralization (37), that was not sufficient to allow cell manipulation. Ascorbic acid is known to stimulate proliferation rate and induce the secretion of ECM (38) therefore we were able to compensate the deficient integrity of the cell sheet by inducing ECM production through the supplementation of the osteogenic medium with ascorbic acid at a concentration 3 times higher than the standard conditions. A side effect of the increase in ascorbic acid concentration was the higher contractibility degree of the cell sheets after detachment. However, this behaviour is not expected to have consequences for further clinical application since the standard procedure to recover cell sheets involves the use of a PVDF membrane or gelatin coated manipulators that avoid that shrinking and which are only removed after the natural gluing of the cell sheet to the host tissues.

Our experiences reveal that the expression of CD146 surface marker on hBMSCs can be induced by cell culture conditions. According to Anfosso and colleges (39), CD146 can act as a signalling molecule in the dynamics of cytoskeleton rearrangement on HUVECs. The demonstrated changes on CD146 expression of pericyte-like (CD146+) cells and the consequent morphological variation observed when those cells were cultured in different culture media support this hypothesis and also reveal the inducible characteristic of this molecule. The addition of TGF-β1 to hBMSCs cultured in α-MEM lead to an increase on the CD146 expression and a consequent morphological alteration. Changes over CD146 expression

and cell morphology were further observed when the same cells were cultured for 7 days in co-culture medium consisting of M199 without TFG-β1 and supplemented with osteogenic factors. Considering that CD146 was also not involved on cell-cell adhesion between osteoblastic cells, originally with a sub-population expressing CD146, in the established co-culutres, we might speculate that the behaviour of the pericyte-like (CD146+) cells can be mediated by the same signalling cascade described on HUVECs (39) , where CD146 has a central role but is probably not the only molecule involved.

An evident conclusion of this work is related with the ability of the confluent layer of osteoblastic-like cells derived from hBMSCs to act as a remodelling and organizational structure for other cell types. To date, there are some works regarding the study of cellular interactions between osteoblasts and endothelial cells in co-culture systems, as well as their contribution for the vascularization of *in vitro* cultured constructs (40-46). As a result, there is a significant collection of new data concerning the molecular intervenient on cellular behaviour for a number of osteoblastic and endothelial cell types. Endothelial cells are able to migrate, proliferate and form round shaped colonies when cultured with osteoblastic cells (42) and some reports showed spontaneously self-assembly of EC in tubular-like structures when co-cultured with MSCs or osteoprogenitor cells on plastic culture surfaces (40, 41) or in co-culture spheroids (44). However, as far as we know, the behaviour of endothelial and pericyte-like (CD146+) cells, cultured together over a confluent layer of osteoblastic cells, was not documented before. Our *in vitro* results revealed the organization of endothelial cells in colonies in a relatively short period of time (at least two days) when cultured on an osteogenic cell sheet. Under the same conditions, pericyte-like (CD146+) cells altered their “star-shape” morphology and reorganized them-selves in cord-like structures. As discussed above, in addition to a potential effect of the co-culture medium over pericyte-like (CD146+) cells morphology, the presence of the ECM of the osteogenic cell sheet as substrate, the cell-cell connections and/or paracrine signalling pathways are likely to contribute to the observed behaviour. In fact, other works have already suggested the importance of the ECM produced by osteoprogenitor cells for the storage and release of chemotactic factors (47, 48) as well as in the establishment of homotypic and heterotypic gap junctions for cell-to-cell communication on endothelial and osteoblastic cells co-culture models (49, 50). Although it seems that pericyte-like (CD146+) cells are directed by the co-culture conditions, the nature of the mechanisms involved on this crosstalk were not addressed. Nevertheless, the dissimilarity on CD146 expression in co-cultures and in the CD146+ monocultures established in co-culture medium might be explained by the release of several growth factors and/or by cell-cell interactions that up-regulated CD146 expression of pericyte-like (CD146+) cells in co-culture.

Despite the fact that ECs are considered the most sensitive cellular element on a co-culture system with osteoblastic cells (18), the addition of osteo-inductive factors in the proposed model did not interfere with the endothelial phenotype neither with their survival. Our results do not support previous observations such as the formation of tubular-like structures by HUVECs co-cultured with osteoprogenitor cells (41), and the angiogenic structures on co-cultures of outgrowth endothelial cells and primary osteoblasts (42). Nonetheless, HUVECs also seems to be an endothelial cell type with less capacity to form highly organized structures of endothelial networks (18, 46). A preliminary study with human dermal microvascular endothelial cells (HDMECs), which is considered a promising source of endothelial cells for *in vitro* vessel networks forming (18), in the proposed co-culture system lead us to consider that other factors than the cell source are the responsible for the formation of tubular-like structures *in vitro.*

Extensively research in the field of vasculature stabilization led by pericytes has contributing for the discovery of possible new pericyte functions, including regulation of endothelial proliferation and differentiation, microvascular perfusion, permeability regulation through paracrine agents (51, 52) and regulation of epithelial proliferation and tissue regeneration (53). The capacity of HUVECs to migrate, organize and interact with pericyte-like (CD146+) cells in our co-culture model is evident. Results from other works suggest that the observed interaction between HUVECs and pericyte-like (CD146+) cells might be related with the release of some signalling molecules by HUVECs, such as PDGF-β, FGF and TGF-β or due to heterotypic cell-cell interactions (54, 55). The release of TGF-β by HUVECs is also a possible explanation for the maintenance of the high CD146 expression in co-cultures. According to Hirsch and D'Amore, TGF-β is released in a latent form and its activation is led by EC-pericytes contact (56), proving the existence of causative cell-cell interactions in our model. PDGF-β and FGF are chemoattractants for vSMCs and mesenchymal derived cells (57). Also, a study with EC from bovine aortas and presumptive mural cell precursors (10T1/2 cells) showed that the use of neutralizing antibodies for PDGF-β suppresses mural cells migration (54). Recently, Caplan and Correa (58) suggested a critical role for PDGF-β in the vascular-pericyte-MSC-osteoblast dynamics as a central connector between cellular components and osteoblast differentiation program.

In this work, results with CD146 in co-cultures and osteocalcin and type-I collagen expression in co-cultured cell sheets confirms changes in cells behaviour, such as migration and cytoskeleton reorganization, leading to a continuous stimulation of the osteoblastic phenotype.

The subcutaneous transplantation of single monocultured and co-cultured osteogenic cell sheets stacked with monocultured osteogenic ones showed the integration of HUVECs with host developed vasculature, thus demonstrating their active role in the angiogenic process that occurs after implantation of TE constructs. These cells are functional elements useful to accelerate the establishment of an efficient vasculature at the implantation site and within the construct. Additionally the integration of human cells with host connective tissue and the alignment of the transplanted human $CD146^{+}$ in a vessel-like structure were confirmed. Nevertheless, the specific contribution of pericyte-like (CD146+) cells was not evident using immunodetection methodologies, since CD146 antibodies cross-react with mouse antigens and are not specific for perivascular cells (it is also a marker of ECs). Furthermore, the diameter of the CD146 positive blood vessels was higher on the experimental condition, which might be directly correlated with the presence of pericyte-like (CD146+) cells and with vessels stability. According to a theoretical model proposed by Pries *et al.* (59), increased vessel diameter and wall mass are needed to ensure stable vascular adaptation. Also, regarding the importance of CD146 molecule for angiogenesis, the differential expression of CD146 on the blood vessels allow us to speculate that this could increase blood vessels stability.

In summary, this work proved the capacity of hBMSCs to form osteogenic cell sheets and its role in modulating the assembly of two cell types intimately related with *in vivo* vasculature, endothelial cells and perivascular-like (CD146+) cells. Moreover, the stacking of osteogenic cells sheets and its vascularization *in vivo* reinforced that the proposed model constitutes a suitable starting element to further develop thicker cell dense constructs. This can be easily achieved by combining several layers of cells, including pre-vascularized cell sheets, as a single cell-sheet based construct or with biomaterials to improve the vascularization of thicker constructs or the mechanical properties known to be a shortcoming of these systems for certain applications such as bone TE.

5 - Conclusions

The main objective of this work was to develop a multi cell sheet stacked construct with an organizational pattern of endothelial and perivascular-like cells differentiated from hBMSCs, focusing the contribution of pericyte-like (CD146+) cells for the improvement of *in vivo* vascularization of the construct contributing to its survival in bone TE applications.

hBMSCs showed the ability to form osteogenic cell sheet *in vitro* after induction of ECM deposition with high concentrations of ascorbic acid. The proposed co-culture system suggested that the presence of a collagen-based substrate containing the secretory osteoblastic cells is critical for the self-assembly of endothelial and pericyte-like (CD146+) cells. *In vivo*, our construct seems to contribute for an increase of blood vessels diameter and thus to the stabilization of the vascular network.

In this context, the conception of using bone marrow cells as a source of pericyte-like and osteogenic-derived cells to create a co-culture model combining these with endothelial cells appear to be a useful strategy for the *in vitro* pre-vascularization tissue engineering constructs and to improve its survival after implantation by promoting a stable and mature supplying vasculature.

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IV – GENERAL CONCLUSIONS

After the first enthusiastic thoughts about the potential of for TE cell-based therapies, almost 30 years ago, it is now clear the need for several improvements in this field. These start from the discovery of a true reliable cell source, easily accessible and with the ability to differentiate and grow *in vitro* without losing those characteristics, to the understanding of genetic and biochemical pathways involved on tissue replacement/regeneration, which can dictate the medical failure or success of TE substitutes. Nevertheless, the unequivocal existence of mechanisms to regenerate body parts in other animals, and also during human fetal development, is still giving hope for the reconstitution of tissues and/or organs through cellular autologous approaches.

If by one hand cell sheet technology brought some fresh ideas to the TE field which has been successfully used to circumvent some issues associated with scaffolds-based therapies, on the other hand it loses when mechanical properties are required and presents similar issues regarding the vascularization concerns restraining the required advances of the field. The efforts that have been done using cell sheet technology to improve *in vivo* vascularisation rely on the *in vitro* pre-vascularization of the constructs. Some approaches have been taking advantage of the technology flexibility to produce constructs of stacked cell sheets with "sandwiched" endothelial cells (1, 2) while others have been aiming to create tubes of endothelial cells by wrapping endothelial cell sheets around silicon tubes (3). In the herein presented work we combined our knowledge in cell sheet engineering and co-cultures to advance the state of the art of human osteogenic cell sheets for bone tissue engineering purposes. Our intent was carried out by the use of MSCs induced to express CD146 phenotype, and endothelial cells from human umbilical cord vein (HUVECs) co-cultured on a layer of bone marrow derived osteoblastic-like cells. The co-culture revealed an interesting pattern of cellular behaviour and distribution, where CD146+ and endothelial cells seemed to interact, although without the formation of tubular-like structures by endothelial cells. Preliminary assays using endothelial cells derived from, under the same conditions, resulted on similar organizational pattern. Hence, these results using distinct cell sources reinforce present concerns: functional endothelial cell sources for tissue engineering are still lacking and there is still a limited understanding about endothelial cells needs on *in vitro* cultured conditions.

Despite the lack of significant differences concerning the number of new blood vessels formed in the presence and absence of HUVECs and pericyte-like (CD146+) cells *in vivo*, as initially hypothesised as a suitable approach to improve construct vascularization, we found positive and negative blood vessels for CD146 and increased blood vessels diameter in our

experimental condition. These findings can be related with increased blood vessel stability, one of the most relevant roles attributed to the CD146 molecule (4).

This achievement constitutes the basis for a deeper understanding of the cellular and molecular mechanisms involved in the crosstalk between the co-cultured cells. The use of neutralizing antibodies for PDGF-β and/or TGF-β in *in vitro* studies as well as the improvement of the osteogenic cell sheet stacking technique for the creation of ticker constructs and the *in vivo* analysis of vessels stabilization using blood vessels disruption drugs which led to the regression of blood vessels less covered by pericytes, are examples of future approaches that could be followed.

In summary, although our results point for a slightly increase on the number of blood vessels and for their *in vivo* stabilization using the established co-culture system, it seems reasonable to affirm that there is a long way to go until a true reliable methodology for the promotion of vascularization of tissue constructs is achieved. However the study of perivascular cells acting mode and their cross-talk with endothelial cells will be the next years challenge for the TE field. A better control over the differentiation events of stem cells or either the creation of cells with the desirable properties by genetic engineering approaches could be also a major attainment for tissue engineering and regenerative medicine. Furthermore, avoiding cellular senesce *in vitro* using a controlled system for the safe activation of telomerase might be a significant advance for the substitution of large body parts.

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V – ANNEX

1 – Morphologic analysis of hBMSCs cultured into different media

As a preliminary experiment to the setting up of the co-cultures, hBMSCs were cultured in different media: i) α-MEM supplemented with FGF-β, ii) α-MEM supplement with osteogenic factors, iii) α-MEM supplemented with TGF-β1, and iv) M199 and v) EGM-2MV, both supplemented with osteogenic factors, for 7 days. In addition to the analysis of the expression of surface markers reported in chapter III, cell morphology was also assessed along the culture by optical microscopy in order to identify eventual morphological changes associated to the different medium composition. hBMSCs cultured with basal α-MEM display the typical fibroblastic-like morphology (Figure 1A) while in osteogenic medium reached confluence and started their osteogenic differentiation (Figure 1B). In what concerns the pericyte-like (CD146+) cells, obtained by induction with TGFβ1, its culture with M199 and EGM-2MV supplemented with osteogenic factors lead to clear morphological changes. The "star morphology" of pericyte-like (CD146+) cells changed in different modes when M199 or EGM-2MV were used, probably due to different supplements composing each medium, namely the presence of VEGF in the EGM-2MV.

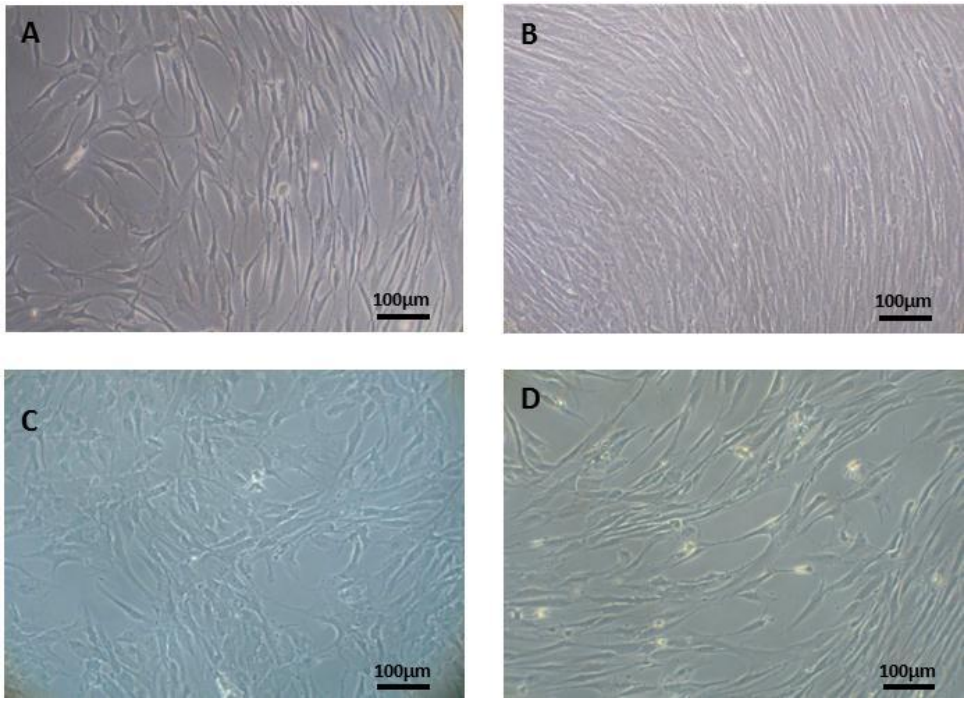

Figure 1 - hBMSCs cultured in different conditions. (A) hBMSCs (P1) in α-MEM+2 ng/mL FGF-β; (B) confluent hBMSCs (P3) cultured in osteogenic medium for 7days; (C) pericyte-like (CD146+) cells (P3) cultured in M199+osteogenic factors for 7 days; (D) pericyte-like (CD146+) cells (P3) cultured for 7 days with EGM-2+osteogenic factors.

2 – Morphological similarities between pericyte-like (CD146+) cells and reported pericytes

In our model, plastic adherent cells from bone marrow were induced to express the CD146 marker and used as pericyte-like cells. Other works have been selecting this specific cell type from the perivascular space of several organs. The similarities between the pericyte-like (CD146+) cells used in our work (Figure 2A) and previously reported pericytes (1) (Figure 2B) are evident (Figure A,B) supporting the hypothesis that bone marrow can naturally be a source of progenitors of pericyte cells with great importance for tissue engineering purposes.

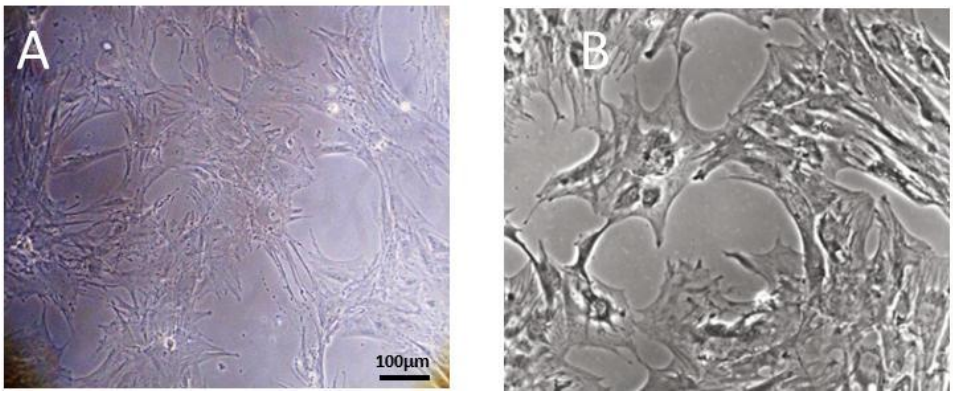

Figure 2 – Morphological similarities between pericyte-like (CD146+) cells cultured in our lab (A) and perivascular cells sorted from skeletal muscle ([B] ×100) (1).

3 – Calcium deposits at different days of hBMSCs culture in osteogenic medium.

Although the established co-culture comprised osteogenic cell sheets cultured for 14 day in osteogenic medium, a systematic analysis of calcium deposits was performed using AR-S staining at 7, 14 and 21 days of culture. After 7 days (Figure 3A), calcium deposition is barely seen, but an increase was observed after 14 (Figure 3B) and remarkably after 21 days (Figure 3C) of culture.

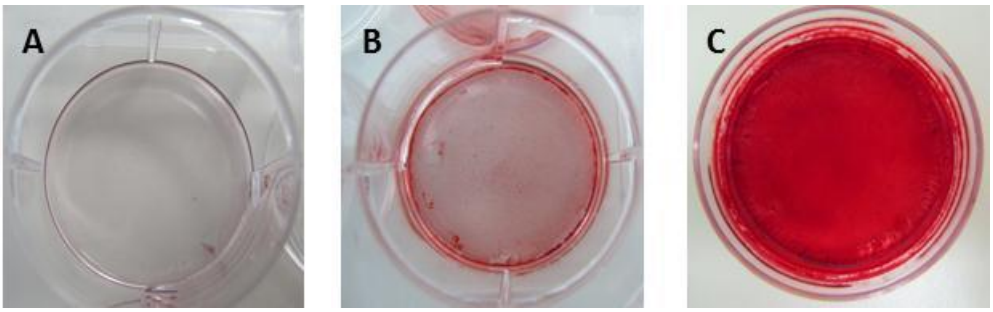

Figure 3 – Representative Alizarin red staining on hBMSCs cultured in osteogenic medium during (A) 7days, (B) 14 days and (C) 21 days.

4 – Fluorescence Immunocytochemistry

Preliminary studies using the same conditions, including the time in culture and the ratio of cells in the co-culture system, were performed with human dermal microvascular endothelial cells (HDMECs) instead of HUVECs. In this section is presented some of those results.

4.1 – Immunocytochemistry for CD146 on HDMECs in monoculture

As shown in chapter III CD146 molecule is highly expressed on HUVECs. Surprisingly, immunocytochemistry for CD146 performed on monocultured HDMECs (Figure 4 A,B) showed that endothelial cells from skin microvasculature behave as an heterogeneous population concerning the expression of this surface marker. Their endothelial phenotype was also confirmed by Dil-AcLDL uptake.

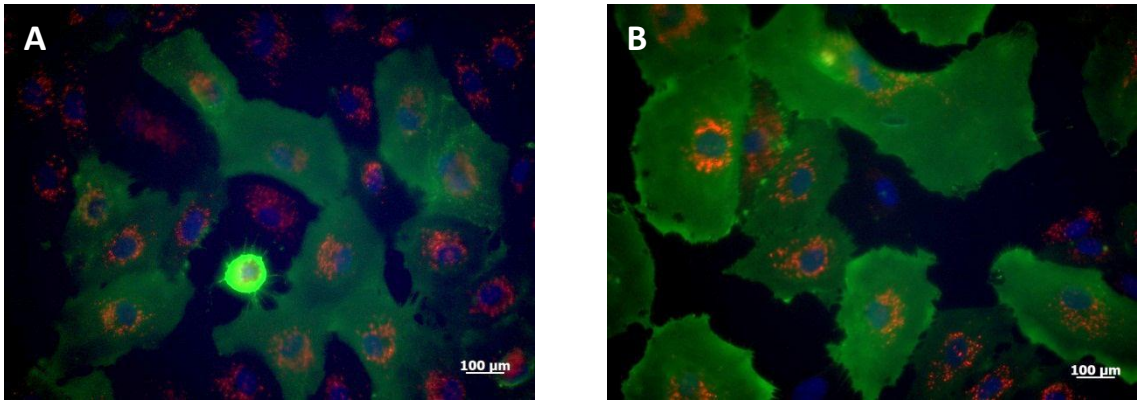

Figure 4 – Immunocytochemistry for CD146 performed on monocultured HDMECs capable of uptaking Dil-AcLDL (red). (A) Low magnification revealing heterogeneity of CD146 expression (green) on HDMECs. **(B)** High magnification image showing HDMECs morphologic profile and interconnections.

4.2 – Fluorescence immunohistochemistry for CD146 on co-cultures of osteoblastic cells, HDMECs and pericyte-like (CD146+) cells.

Immunocytochemistry for CD146 in co-cultures assembled with HDMECs instead of HUVECs revealed the same organizational pattern as those with HUVECs (Figure 5 A,B,C,D). Round shaped colonies of ECs and elongated pericyte-like (CD146+) cells seem to act in the same way as in the co-culture system with HUVECs. In some cases (Figure 5 D), is clear the interaction of pericyte-like (CD146+) cells with endothelial cells where the former wraps a small colony of HDMECs with no expression of CD146. This pattern is reminiscent of the *in vivo* behaviour and function of pericytes, which implies an intimate bidirectional cross-talk between those cells in co-culture.

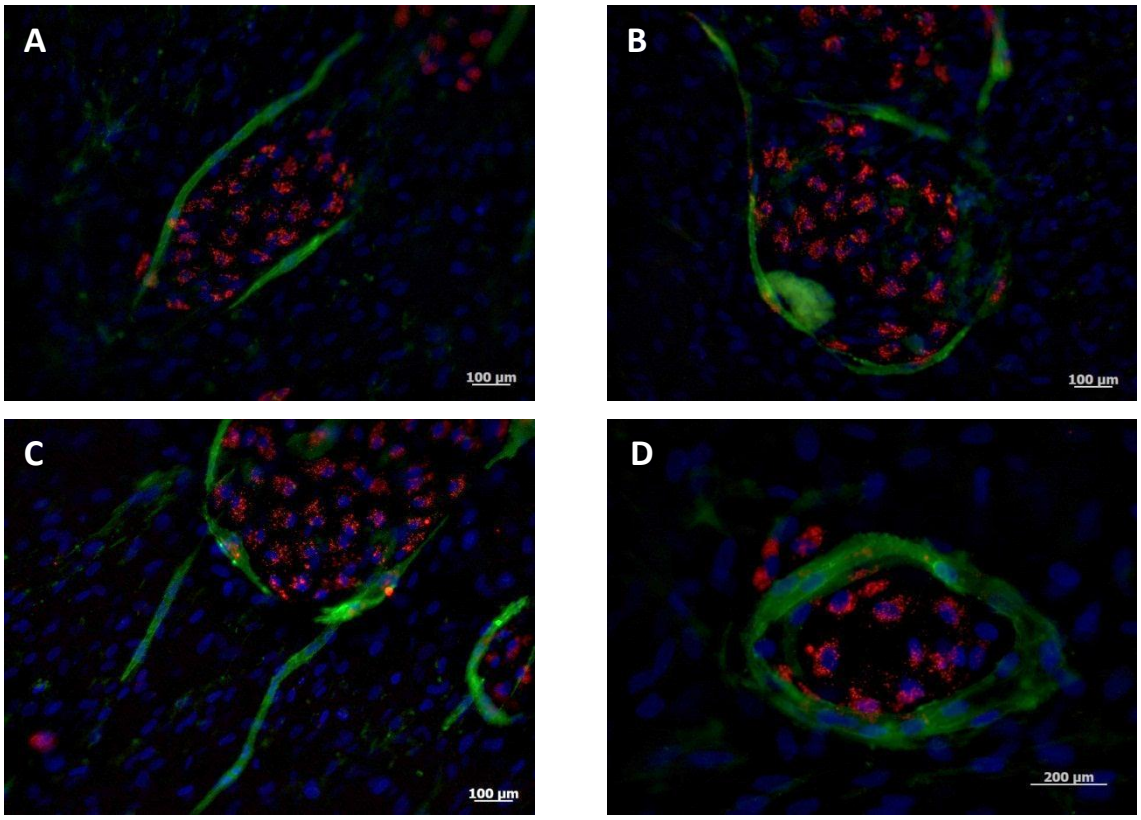

Figure 5 - Immunocytochemistry for CD146 (green) on hBMSCs, pericyte-like (CD146+) cells and HDMECs co-cultures using CD146, mouse:anti-human primary antibody and AF488-linked secondary antibody (green). Dil-AcLDL (red) was used to assess LDL incorporation. DAPI (blue) was used as nuclear counterstain. EGM-2MV supplemented with osteogenic factors was used. A, B, C, and D are successive magnifications.

5 - References

1. Crisan M*, et al.* (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3(3):301-313.