Universidade de Aveiro

Secção Autónoma de Ciências da Saúde

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Co-culture of SSEA4^{+}-hASCs derived Osteoblasts and Endothelial Cells

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Manuela Estima Gomes, Professora auxiliar convidada da Escola de Engenharia da Universidade do Minho e co-orientação da Doutora Odete Abreu Beirão da Cruz e Silva, Professora auxiliar da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

Dedico este trabalho aos meus Pais e ao meu Irmão.

o júri

presidente

Prof. Doutora Margarida Sâncio da Cruz Fardilha
Professora auxiliar convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

Prof. Doutora Maria Manuela Estima Gomes
Professora auxiliar convidada da Escola de Engenharia da Universidade do Minho

Prof. Doutora Odete Abreu Beirão da Cruz e Silva
Professora auxiliar com agregação da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

Prof. Doutor Alexandra Margarida Pinto Marques
Investigador Auxiliar da Universidade do Minho

Prof. Doutora Ana Gabriela Henriques
Professora auxiliar convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

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palavras-chave

Co-cultura; SSEA4; hASC; Tecido Adiposo; Osso; Osteoblastos; Células Endoteliais; Differenciação Osteogénica; Angiogénese.

resumo

A comunicação celular entre Osteoblastos (OBs) e Células Endoteliais (ECs) é bidireccional e ocorre a dois níveis: comunicação indirecta e comunicação directa, sendo esta última mediada por proteínas através de "gap junctional communication". O estudo *in vitro* dessas interacções é desenvolvido através de sistemas de co-cultura, onde pelo menos dois tipos de células são cultivados simultaneamente e submetidos ao mesmo microambiente, sendo espectável que este mimetize mais especificamente o ambiente encontrado *in vivo.* O tecido adiposo humano é actualmente reconhecido como uma fonte de células estaminais. Uma subpopulação específica de células estaminais isoladas a partir da Fracção Vascular do Estroma do Tecido Adiposo, positiva para o marcador associado de pluripotência SSEA4 – SSEA4^{+}-hASCs – demonstrou ter capacidade de diferenciação osteogénica e endotelial, possibilitando a obtenção de OBs e ECS a partir da mesma fonte inicial.

Esta tese reporta o estabelecimento de um modelo de co-cultura de SSEA4^{+}-hASCs precondicionadas para a linhagem osteogénica (SSEA4^{+}-hASCs OBs) e para células endoteliais derivadas da mesma subpopulação. Foi avaliada a forma como o meio de cultura e as proporções de cada tipo celular influenciam os fenótipos osteogénico e endotelial das células em co-cultura.

Os resultados demonstraram que o meio de cultura EGM suplementado com factores de diferenciação osteogénica permitiu a manutenção do potencial de diferenciação da subpopulação SSEA4^{+}-hASCs nas duas linhagens. Adicionalmente, os resultados evidenciam um potencial de mineralização e angiogénico mais consistente nas condições de co-cultura compostas por uma maior proporção de células endoteliais. Em conclusão, a condição de co-cultura com a proporção SSEA4^{+}-hASCs OBs: SSEA4^{+}-hASCs derived ECs de 1:3 em EGM OST revelou melhores resultados no que diz respeito ao fenótipo osteogénico e permitiu uma manutenção consistente da estabilidade fenotípica das células endoteliais. O modelo de co-cultura estabelecido tem grande potencial para permitir ultrapassar a limitação de vascularização apresentadas pelas estratégias actuais de Engenharia de Tecidos de Osso.

keywords

Co-cultures; SSEA4; hASC; Adipose Tissue; Bone; Osteoblasts; Endothelial Cells; Osteogenic Differentiation; Angiogenesis.

abstract

The crosstalk between Osteoblasts and Endothelial cells is bidirectional and occurs at two levels: indirect cell communication and direct cell communication mediated by proteins at gap junctions structures. The *in vitro* study of these interactions rely on co-culture systems where at least two cell types are cultured at the same time and submitted to the same microenvironment that is expected to mimic the *in vivo* settings. Human adipose tissue has been recognized as a potential source of stem cells for regenerative medicine, with multilineage differentiation potential. Recently, it was found that a specific subpopulation of adipose stem cells isolated from the Stromal Vascular Fraction of the Adipose Tissue, positive for the pluripotency associated marker SSEA4 – $SSEA4^{+}$-hASCs – can give rise to both osteoblastic and microvascular endothelial-like cells, enabling the obtention of these two cell types from the same cell source.

This thesis reports the establishment of a co-culture model for the simultaneous culture of two cell types – $SSEA4^{+}$-hASCs preconditioned osteoblasts ($SSEA4^{+}$-hASCs OBs) and $SSEA4^{+}$-hASCs derived endothelial cells. The influence of the cell culture media and the proportion of each cell type influenced the osteogenic and angiogenic outcomes from the system.

Our results show that EGM OST culture media allowed to the maintenance of the osteogenic and endothelial differentiation potential of this subpopulation. Concerning the osteogenic and angiogenic potential of the co-cultures, it was shown that both were more consistent on the conditions with higher proportion of ECs in the system.

In conclusion, the co-culture condition with the cell ratio OBs:ECs of 1:3 in EGM OST revealed the most efficient osteogenic outcome parameters as well as great stability of endothelial phenotype along the culture period.

The established co-culture system has a great potential to overcome the vascularization limitations of the current Bone Tissue Engineering strategies.

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ABBREVIATIONS

α-MEM	Alpha-Minimal Essential Medium
ALP	Alkaline Phosphatase
AR	Alizarin Red
ASC	Adipose Stem Cell
BAT	Brown Adipose Tissue
BBE	Bovine Brain Extract
BMSC	Bone Marrow-derived Mesenchymal Stem Cell
CAM	Cell-Adhesion Molecules
CD	Cluster Differentiation
CD31	See PECAM-1
cDNA	Complementary DNA
Cx43	Connexin 43
DAPI	4, 6-Diamidino-2-phenyindole dilactate
diH_2O	Distilled water
DMF	N,N-Dimethylformamide
EC	Endothelial Cell
ECM	Extracellular Matrix
EGM	Microvascular Endothelial Growth Medium
EGM OST	Microvascular Endothelial Growth Medium supplemented with osteogenic differentiation factors
EPC	Human Umbilical Cord blood Endothelial cells
ESC	Embryonic Stem Cells
FACS	Fluorescent-activated Cell Sorting
FBS	Fetal Bovine Serum
GDF-5	Growth and Differentiation Factor 5
GAPDH	Glycetaldehyde-3-phosphate Dehydrogenase
hEGF	Human Epidermal Growth Factor
HIF	Hypoxia-Inducible Factor
HUVEC	Human Umbilical Vein endothelial cells
hOEC	Human Outgrowth Endothelial cells
Ig	Immunoglobulin
MSC	Mesenchymal Stem Cell
OB	Osteoblast

OCN	Osteocalcin
OPN	Osteopontin
OST	Osteogenic Media
pNP	p-nitrophenol
pnPP	p-nitrophenyl phosphate
PECAM-1	Platelet Endothelial Cell Adhesion Molecule
PCR	Polymerase Chain Reaction
PTH	Parathyroid Hormone
R3-IGF-1	Human recombinant analog of insulin-like growth factor-I with the substitution of Arg for Glu at position 3
RT	Room Temperature
SSEA4^{+}-hASCs OB	SSEA4^{+}-hASCs derived Osteoblasts
SSEA4^{+}-hASCs ECs	SSEA4^{+}-hASCs derived Endothelial Cells
SVF	Stromal Vascular Fraction
TE	Tissue Engineering
TGF-β	Transforming Growth Factor-beta
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand factor
WAT	White Adipose Tissue

I. INTRODUCTION

1. INTRODUCTION

Adult stem cells are undifferentiated cells present mostly in a variety of tissues and organs and that predominantly show the property of self-renewal and capacity to differentiate into major specialized cell types. For this reason adult stem cells have been widely studied for application in the field of tissue engineering (TE) and regenerative medicine. Up to now several sources of adult stem cells have been identified and one of the most promising is adipose tissue. In fact it represents an abundant and accessible source of adult stem cells with the ability to differentiate along multiple lineage pathways including osteogenic and endothelial.

Tissue engineering strategies are generally based on a supportive material (scaffold) where stem cells are seeded and eventually cultured to obtain a functional tissue substitute. Nevertheless, the success of any TE strategy is largely dependent on controlling the biology of the cells at the site of repair or regeneration, since are the cells that constitute and co-ordinate the basic structure and function of tissues. Regarding bone tissue engineering, the establishment of a concise vascular supply of bone constructs is one of the major pitfalls and therefore a hurdle for the clinical application of such engineered constructs. The intraosseous vasculature is important for several bone physiological processes. Its role is evident on the skeletal development and growth by affecting both bone modelling and remodelling processes and is found essential to guarantee the viability and functionality of the construct upon implantation and hence, its success in the regeneration of the tissue defect.

Endothelial cells (EC) are the main cellular mediator of the vascular network formation and in the sequence of a well-developed vascular network, primary osteoblasts (OB) undergo calcification and differentiation into osteocytes, enabling a healthy bone formation. Therefore, culturing ECs as a homogenous population or combined with OBs is a promising approach to solve the lack of vascularization in bone tissue engineered constructs. This thesis is centred on a specific subpopulation of cells existing within stromal vascular fraction (SVF) of adipose tissue which can be differentiated into the osteogenic and endothelial lineages, and which was used to develop a co-culture model that has a great potential for application in bone tissue engineering approaches.

2. BONE TISSUE

2.1. OVERVIEW OF BONE BIOLOGY, STRUCTURE AND FUNCTION

Bone tissue is a dynamic highly vascularized tissue with the capacity of constant remodeling by undergoing local resorption and rebuilding. The matrix producing OB, the tissue resorbing osteoclast and the osteocytes, which accounts for 90% of all cells in the adult skeleton, are three of the main cell types that can be found in bone tissue. All perform crucial functions in bone homeostasis. [2]

Osteocytes are the most abundant cell type and rely enclosed within the lacuna-canalicular network of bone. They can be viewed as highly specialized and fully differentiated OBs. From each osteocyte a network of cytoplasmic processes extends through cylindrical canaliculi to blood vessels and other osteocytes. These cells are involved in the control of extracellular concentration of calcium and phosphorus, as well as in the adaptive remodeling behavior via cell-to-cell interactions in response to local environment. Considering that these cells are the principal cell in the adult bone, it appears that osteocyte veritable three-dimensional syncytium may drive the spatial and temporal recruitment of the cells that form and resorb bone.[3]

Osteoblasts are bone forming cells derived from mesenchymal stem cells (MSCs) present among the bone marrow stroma that have one of three fates: can incorporate in its own osteoid and differentiate into an osteocyte, remains quiescence into a lining cell, or undergo apoptosis. Functionally, OBs are the cells that produce and lay down the bone ECM and that regulate its mineralization/calcification. They are located at the bone surface together with their precursors, forming a tight layer of cells, and are highly anchorage dependent, relying on the extensive cell-matrix and cell-cell contacts to maintain their cellular function and responsiveness to metabolic and mechanical stimuli. [4-6] During its life-span, the OBs produce between 0.5-1.5µm of osteoid per day, the unmineralized organic matrix that subsequently undergoes mineralization giving the bone its strength and rigidity.[2]

Osteoclasts are multinucleated giant cells of the hematopoietic descent; their precursors are located in the monocytic fraction of the bone marrow and functionally, they are the main responsible for the bone resorption process, which is required for bone morphogenesis during development, for the continual repair of microdamage in the skeleton and for the adaptation of bone to mechanical load. Osteoclasts bind to bone matrix peptides via integrin receptors in their membrane linking to bone matrix peptides. This causes them to become polarized, with the bone resorbing surface developing a ruffled border that forms when acidified vesicles that contain matrix metalloproteinases are

transported via microtubules to fuse with the membrane. The protons and proteases components are secreted to demineralise and degrade the bone matrix, respectively.[2, 6, 7]

Osteodifferentiation occurs along successive stages leading mature OBs which initially involves three different phases: proliferation, matrix maturation and mineralization, each of them characterized by a specific pattern of gene expression. [8, 9] The first phase – proliferation phase – is essentially associated with cell growth and cell cycle regulatory genes, along with extracellular matrix (ECM) biosynthesis. At the post-proliferative period the ECM starts to be mineralized and the activity of alkaline phosphatase (ALP), which is responsible for promoting crystal formation in matrix vesicles by removing nucleation inhibitors, increases. Mineralization of ECM occurs after a period of matrix production and occurs in three steps: nucleation of calcium phosphate crystals, crystal growth by precipitation and conversion into octacalcium phosphate and finally hydroxyapatite.[10] Ultimately, OBs will acquire the typical osteocyte morphology, become entrapped in their own bone matrix, and progressively stop producing matrix. The life span of osteocytes is probably largely determined by bone turnover, when osteoclasts resorb bone and either "liberate" or destroy osteocytes.[10]

In adults, bone tissue exists in two forms, trabecular bone and compact bone, distinguishable by the spatial orientation of its mineral and organic constituents, and by their distinct characteristic locations in the skeleton. [8, 10]

The main functions of bone include structural body support, protection of vital organs, mineral reservoir acting in mineral homeostasis. Also have a front line role in hematopoiesis and endows the body with the capability of movement. [8]

Considering its functional relevance and the ever-growing incidence of skeleton/bone disorders, most of them resulting from the imbalance between breakdown and formation of new bone, it is evident how patients' quality of life can be affected. In fact, bone tissue lost occurs in a wide variety of clinical situations, for which currently used therapies are clearly insufficient.

2.2. BONE FORMATION PATHWAYS

Bone formation occurs as a result of coordinated cell proliferation, differentiation, migration, and remodelling of the ECM that occurs by two different processes: endochondral ossification and intramembranous ossification.[11] In both cases MSCs first have to aggregate to form mesenchymal condensations that are situated at the location of future skeletal elements and

prefigure its shape.[12] In most cases, the cells of the mesenchymal condensations will differentiate into chondrocytes, expressing characteristic cartilaginous matrix genes such as *type II collagen*, *type IX collagen*, *type XI collagen*, *aggrecan*, *chondromodulin-1* and *matrilin-3*, and that form the growth template of the future bone. [11] In the center of the condensations that will give rise to bone through endochondral ossification, mesenchymal cells differentiate into chondrocytes, forming a cartilaginous template, that once formed leads to further differentiation of the innermost chondrocytes into hypertrophic chondrocytes. The mesenchymal cells that remain in the periphery of the growth plate form a structure called perichondrium, whose cells start to differentiate into OBs to form, around the cartilaginous core, a mineralized structure called the bone collar. Once the hypertrophic chondrocytes are fully differentiated, they are already surrounded by a calcified ECM and dye from apoptosis. [13]

One of the most important functions of the mineralized ECM is to favor the vascular invasion from the bone collar through a vascular endothelial growth factor (VEGF)-dependent mechanism. This mechanism serve two different purposes: to attract chondroclasts that will degrade the ECM surrounding the hypertrophic chondrocytes, and to bring in osteoblast progenitors derived from the bone collar. The hypertrophic chondrocytes express and synthesize a number of VEGF isoforms, VEGF-A, VEGF-B, VEGFC, VEGF-D and also express neuropilin-1, VEGF receptor-2 and VEGF receptor-3, comprising an autocrine/paracrine system that will further encourage inwards penetration of the vascular endothelium.[14, 15] This sequence of events lead to the development of the primary ossification centres in which the cartilaginous ECM will then be replaced by a bone ECM, rich in type I collagen. The ossification process will move on centrifugally, consuming most of the cartilage template, recruited osteoblasts (OBs) replace the degraded cartilage with trabecular bone, and a bone marrow is thus formed.[16-18] These highly coordinated events lead to longitudinal bone growth and bone formation between epiphyses and metaphyses regions at each end of bones of bearing joints and long bones.[8, 13]

At the same time, there is a radial growth of the diaphysis, the region between the epiphyses and metaphyses, and of parts of the metaphyses by intramembranous bone formation with direct apposition of cortical bone by OBs from the inner layer of the periosteum. This type of ossification involves the condensation and differentiation of mesenchymal cells into OBs, which began to synthesise and secret osteoid.[19] Gradually, more osteoid is produced, followed by the complete mineralization. As a result, more osteoblasts become trapped within their lacunae and start the differentiation into osteocytes. This is closely coordinated with resorption of bone by osteoclasts on the inner cortical endosteal surfaces and lateral metaphyseal surfaces to maintain the relative proportions of the marrow cavity to the cortices and the overall shape of the bone as it grows.[19]

This third type of bone formation is known as appositional formation and occurs during bone enlargement and bone remodelling. [10]

Some common regulators of both types of ossification are angiogenic growth factors. In intramembraneous ossification there is an invasion of capillaries transporting MSCs which directly differentiate into OBs.[11] In endochondral ossification there is an intrinsic relationship between chondrogenesis and osteogenesis which is dependent on the vascularisation status of the cartilage template. MSCs/precursor cells undergo chondrogenic differentiation, become hypertrophic and initiate angiogenic growth factors secretion, namely several isoforms of VEGF [14] thus determining vascular vessel invasion responsible for the consequent recruitment of bone forming cells.[20, 21] Moreover, vasculature and more specifically ECs secrete several factors that control the recruitment, proliferation and differentiation of either osteoblasts or osteoclasts, highlighting the point that angiogenesis and osteogenesis are not dissociated processes.[22, 23]

Bone vasculature is also decisive for the remodelling process. This process comprises two phases: resorption of pre-existing bone by osteoclasts and *de novo* bone formation by OBs through transitory specialized anatomical structures, called basic multicellular units (BMUs). BMUs are located in the vicinity of blood vessels, which growing rate is coordinated with the advance of BMUs, this indicate the crucial role of vasculature in bone remodelling and consequently the importance of vascular cells, such as ECs and pericytes in this process.[24, 25]

2.3. BONE REPAIR

The intraosseous vascular system of adult bone tissue is mainly composed by diaphyseal or feeding arteries, superior and inferior metaphyseal arteries, superior and inferior epiphysial arteries and periosteal vessels.

When bone fracture occurs there is a disruption of the marrow architecture and the blood vessels structure within and around the fracture site is also compromised.

Fracture healing process results of the combination of both endochondral and intramembranous ossification, the first occurring usually external to periosteum in regions that are mechanically less stable and immediately adjacent to the fracture site, while the last one occurs internal to periosteum at the proximal and distal edges of the callus.[26, 27] The repair process itself is comprised of four overlapping phases: the initial inflammatory response, soft callus formation, hard callus formation, initial bone union and bone remodeling. The immediate inflammatory response leads to the

recruitment of MSCs that subsequent differentiate into chondrocytes that produce cartilage and OBs.[26, 27]

At the molecular level, fracture repair is driven by the three main classes of factors: (1) pro-inflammatory cytokines, (2) the transforming growth factor-beta (TGF-β) superfamily and other growth factors, and (3) the angiogenic factors.[28, 29] Understanding how those factors affect fracture or postsurgical healing is essential to the development of molecular approaches intended to enhance bone healing after surgery or traumatic injury.

Pro-inflammatory cytokines like interleukins (IL) -1 and -6 and tumor necrosis factor (TNF) -α have been shown to be involved in the initiation of the repair cascade.[30] Their expression profiles vary along the repair process, being secreted by different cell types according to the primary or later stages, playing a central role over the timing of the immune response, and the beginning of local tissue repair and regeneration. TNF-α, IL-1α and IL-6 expression is primarily localized inflammatory cells during the early periods of inflammation (within 24 hours of injury) and in mesenchymal and osteoblastic cells later during healing process, at the transition from chondrogenesis to osteogenesis during endochondral maturation.[31, 32] Moreover, carry out central functions in the induction of downstream responses to injury by having a chemotactic effect on other inflammatory cells, enhancing ECM production and stimulating angiogenesis in the injury site. [33]

During this early stage of the healing process, angiogenesis also takes place, being a prerequisite for further progression of the regeneration cascade. In fact, the formation of the callus is a physiological reaction that requires the existence of an adequate blood flow.[34, 35] Therefore, the reconstruction of an intact intraosseous vascular structure is an early event during fracture repair. [36, 37] The biochemical environment and particularly hypoxia also has an important role in this process because of the effect that the latter has in the secretion, by OBs, of signaling molecules that regulate ECs proliferation, differentiation and secretion of osteogenic growth factors, namely hypoxia-inducible factor (HIF).[38] As result, committed osteoprogenitor cells of the periosteum and undifferentiated multipotent MSCs are activated and differentiate into chondrocytes and fibroblasts that produce a semi-rigid soft callus, stabilizing the fracture and serving as a template for primary bone formation. [27] The new bone is known as the hard callus and is composed of woven bone.

In regions that are mechanically less stable, endochondral ossification occurs, enhanced by the soft tissues surrounding the fracture site. During this process, MSCs are recruited and begin to proliferate by day 3 after fracture. From day 7 to 21 the chondrogenic differentiation occurs

followed by its proliferation, resulting in the formation of the soft callus. These cells synthesize and release cartilage-specific matrix and once it achieves mechanical stability, the cartilage undergoes hypertrophy and mineralization in an organized special manner at the primary ossification centres. [39] VEGF-A is secreted by the hypertrophic chondrocytes inducing sprouting angiogenesis from the perichondrium, which then leads to the recruitment of OBs, osteoclasts and heamatopoietic cells. It also leads to the removal of the calcifying hypertrophic chondrocytes by chondroclasts and woven bone formation occurs after the MSCs recruitment and differentiation into OBs, which replaces the degraded cartilage.[34] Eventually, the soft callus is replaced by laminar bone and undergoes remodeling into the original cortical and/or trabecular bone configuration following the pathway observed in the growth plate.[16, 17, 40]

3. TISSUE ENGINEERING APPROACHES FOR BONE REGENERATION

Tissue engineering approaches are based on seeding and *in vitro* culturing of cells harvested preferentially from an autologous source, within a structural support material or scaffold prior to implantation. The effectiveness of those strategies are ultimately highlighted on the stability and functional restore of the damaged tissue, which will be in certain way influenced by the exogenous source of cells and by the capacity to control its proliferation and differentiation state. [7]

One of the major challenges in the development of tissue engineering therapies for bone regeneration is to achieve the vascularisation of the biomaterial-cells construct, which is found essential to allow the viability and functionality of the construct upon implantation and hence, its success in the regeneration of the bone defect.

When the engineered tissue construct is implanted, seeded cells are dependent on the local microvasculature and microcirculation to have access to substrate molecules (oxygen, glucose and amminoacids) and for clearance of metabolic products (CO_2, lactate and urea). In the absence of a concise vasculature structure, the movement of those molecules in not assured and pressure gradients that allow molecules movement between the vessel lumen and the cell membrane are not achieved. There are many factors that can affect the generation of these pressure gradients: tissue deformation (movement), mechanical loading, muscle contraction, gravitational pooling, starling flow, and arterial pulsation. When this happens, a compensatory process of molecule diffusion starts. However its efficiency is limited to short distances or for small molecules.[41]

Considering bone engineering constructs, vessels are initially confined to the outer surface of the implantation site, and the diffusion distance for oxygen and other metabolites from the edge to the

centre of the graft is considerably higher than the normal diffusion distance. For this reason the inner cells in the graft are subjected to a hypoxic and metabolic stress that can impair the success of the implantation because of cell necrosis in deeper regions.[42]

3.1. STEM CELLS OVERVIEW

Discovered in the last two decades, embryonic stem cells (ESCs) had been described as the most promising cell source for TE, determined by their strong differentiation capacity and plasticity which allowed differentiation towards of an unlimited number of specific cell types, derived from all three embryonic germ layers.[43, 44] Ethical and political debates emerged since the primordial of research on ESCs cells, and this prompted the research focusing on finding alternative stem cell sources, such as adult stem cells or progenitor cells, and on optimizing differentiation protocols for specific cell types converging into organotypic regeneration approaches. One of the primary hurdles is finding a safe source of cells, which in the end yield a functional tissue. [45, 46] For this purpose there are several criteria that should be considered and ideally satisfied: the accessibility and availability of the tissue samples, the proportion of cells of interest in the harvested tissue, its ability to differentiate into the desired cell type and their capacity to maintain phenotypical and functional attributes specific of each type of differentiated cells.[7]

By definition, stem cells are capable of both proliferate in an undifferentiated but pluri- or multi-potent state (self-renewal) and to differentiate into at least one mature cell type.[47] Adult stem cells represent a pool of multipotent somatic stem cells found in adult, neonatal and foetal tissues or organs that have the capacity to differentiate into cells of all three germ layers, mesodermal, endodermal and ectodermal. They were first identified in tissues with a high rate of cell turnover, such as bone marrow. In fact, for many years, bone marrow was considered as the major source of stem cells for bone TE applications and for therapeutic purposes [7, 48] serving as the prototypic example of an adult stem cell. Bone marrow contains an heterotypic adult stem cell population from which two main populations derive: haematopoietic stem cells (HSCs), which produce the blood-cell lineages, and MSCs, which provide the bone-marrow stromal niche and have the potential to produce several cell lineages, including adipogenic, osteogenic and chondrogenic lineages.[49, 50] Currently, it is known MSCs can also be obtained from the stromal fraction of lipoaspirates of adipose tissue, and these possess similar properties to bone marrow-derived MSCs (BMSCs) but are easier to obtain. [51] Further sources of human MSCs include the intestinal[52], limbal [53], knee-joint[54-57]and prostate [58] stroma, trachea[59], nasal mucosa [60], Wharton's jelly (WJ) [61], cord blood[62] and placenta.[63] The microenvironment of these niches supports

the conservation of stem cell characteristics and the maintenance of their pool for the lifetime of the organism. [64, 65]

According to the International Society for Cellular Therapy, human MSCs under standard culture conditions must satisfy at least three criteria: (1) they must be plastic-adherent; (2) they must express C105, CD73 and CD90 and not CD45, CD34, CD14, CD11b, CD79 or CD19 and HLA-DR surface molecules by flow cytometry; (3) they must be capable of differentiating into osteoblasts, adipocytes and chondroblasts.[66]

4. ADIPOSE TISSUE

4.1. PRINCIPLES OF ADIPOSE TISSUE PHYSIOLOGY

Adipose tissue is recognized as a highly active metabolic and endocrine organ. Is composed of one third of mature adipocytes that are organized in a multidepot structure and the remaining two thirds are a combination of small blood vessels, nerve tissue, fibroblasts, preadipocytes and adult MSCs[67]

Mature adipocytes exist as two cytotypes, white and brown adipocytes, respectively defining white adipose tissue (WAT) and brown adipose tissue (BAT).[68] They are distinguished by cell color, function, anatomical distribution and relative abundance in different developmental stages.[67, 69]

The major subdivisions of adipose tissue are the visceral adipose tissue, and the subcutaneous adipose tissue. Subcutaneous adipose tissue is divided into two portions: superficial and deep subcutaneous adipose tissue. The visceral adipose tissue is composed of either intra- or retroperitoneal adipose tissue, separating two distinct intra-abdominal compartments, being in the first that is located the majority of visceral fat.[67, 70]

The relative abundance and metabolic properties of WAT and BAT change according to ageing, gender, nutritional and pathological status of individuals. [71-73] There are marked regional differences in adipose tissue distribution between males and females as well as in the abundance of each type of adipose tissue with ageing. In neonates, BAT is most abundant, serving mainly as a thermogenic organ; with ageing, BAT is replaced by WAT, and consequently, in adults, BAT is present in tiny discrete collections.[73-75] WAT is not a highly vascularized as its brown counterpart; each fat cell in WAT is in contact with at least one capillary, providing a vascular network allowing its continued growth. WAT has long attracted attention because of its great and reversible capacity for expansion, apparently permanent throughout adult life.[76]

The stromal vascular fraction (SVF) of the adipose tissue represents a heterogeneous cell population surrounding adipocytes in fat tissue, and was reported to be a very convenient and nonrestrictive source of multipotent cells such as hematopoietic progenitors and spare mesodermal stem cells able to differentiate into endothelial, neurogenic, osteogenic, chondrogenic, and myogenic lineages. Additionally, other cell types were found in the SVF namely smooth muscle cells, pericytes, fibroblasts and circulating cell types such as leukocytes or EPCs.[77]

4.2. ADIPOSE-DERIVED STEM CELLS (ASCS)

Adipose tissue, because of its abundance and relatively less invasive harvest methods, represents a practical and appealing source of mesenchymal stem cells (MSCs) present within the SVF of subcutaneous adipose tissue, termed "adipose-derived stem cells (hASCs) [78]. It has been demonstrated by many groups that hASCs display multilineage plasticity *in vitro* and *in vivo*.[79-82]

Rodbell first reported [83-85] the initial methods for isolating cells from rodent adipose tissue in 1960's with the final purpose of separating the buoyant fraction of mature adipocytes from the SVF, which would be pelleted through density gradients. Since then, several groups modified the harvesting methods of human subcutaneous adipose tissue, taking advantage of the abundance, accessibility and of the overgrowing number of local excision procedures or minimal invasive liposuction surgeries that are performed. Nowadays, liposuction is considered a safe and well-tolerated procedure, with a complication rate of around 0.1% that routinely allows the removal of fat tissue between few hundred milliliters and several liters.

Zuk and his group [86, 87] were the first to report on the existence of ASCs in fat tissue. In their study, an enzymatic method with collagenase type I was used, followed by a selection of the hASC based on its capacity to adhere to culture flasks.[86] Until today, those are the basic principles of most of isolation procedures of hASCs. Few different isolation methods are described in the literature; Myazaky *et al* [88] and Yamamoto *et al [89]*, described two alternative isolation procedures that allowed the identification of different subpopulations within SVF of adipose tissue. Sengènes *et al* [90] used an isolation method based on a series of purifications and selections using immunomagnetic coated beads to obtain CD34^{+}/CD31^{+} cells directly from SVF. Since then, other groups used this selection technique, [91, 92] to isolate specific subpopulations of hASCs positive for different cellular marker such as CD34, CD105, and CD271, and compared their stemness and their osteogenic/chondrogenic differentiation potential. Those studies highlighted the concept that

within SVF of adipose tissue exist several subpopulations of hASCs that may possess intrinsic distinct differentiation potential. Recently, Mihaila et al evaluated the presence of a specific pluripotency marker SSEA4 within hACSs fraction of the SVF isolated from the human lipoaspirates, confirming its presence as well as the differentiation ability of this subpopulation into the osteogenic and endothelial lineages.[93]

In 2002, Zuk et al published the first results that demonstrated the resemblance of hASCs with human MSCs, as showed by the cluster differentiation (CD) and molecular profiles and by their differentiation potential. In fact, direct comparisons between hASC and MSC immunophenotypes are > 90% identical. [86, 87] Nevertheless, differences in surface protein expression have been noted between ASCs and MSCs. For example, the glycoprotein CD34 is present on hASCs early in passage but has not been found on MSCs.[94, 95] On the surface, hASCs also present consistent expression patterns of HLA-ABC, CD49e, CD51 and CD90 makers but with an expression level lower than 50% [96]

It has been also demonstrated that the levels of expression of several markers, namely CD166, CD29, CD90, CD44 and CD73 vary along the passages, significantly increase with the progression of passages, becoming stable after passage P2.[94, 97] Another interesting characteristic of ASCs is the expression of some pluripotency markers, besides SSEA4, in short term cultures, such as OCT-4, UTF-1, SOX2 and Nodal. [93, 98]

Despite the higher number of studies regarding ASCs immunophenotyping, there are still differences in the characterization information from different research groups, that can derive from the adipose tissue source itself and also from the antibodies and detection methods that were used, especially in what concerns the quantitative evaluation.

4.3. DIFFERENTIATION POTENTIAL OF ASCS

As it was previously mentioned, the stem cells from adipose tissue have the capacity to differentiate into several different cell types. It has been demonstrated that the hASCs have a neurogenic [86, 99], cardiomyogenic [51, 100], endothelial [100], hepatocytic [101, 102], adipogenic [86, 103, 104], osteogenic and chondrogenic lineage [87, 105-107]. The following sections will focus on the osteogenic and endothelial differentiation, as this was the subject of the present thesis.

4.3.1. OSTEOGENIC LINEAGE DIFFERENTIATION

Several studies have been published in the field of bone tissue engineering using ASCs. In general, these studies have demonstrated that stem cells obtained from the adipose tissue exhibit good attachment properties to most of the materials surfaces and the capacity to differentiate into osteoblast-like cells.[108, 109]

A large number of studies revealed that hASC undergo osteogenic differentiation within 2 to 4 weeks of culture, under the same specific culture conditions that induce osteogenic differentiation of BMSCs. [80, 82, 86, 87, 91, 104, 110, 111]

In fact, osteogenic differentiation is known better *in vitro* and as expected, the formation of mineralized osseous tissue requires the presence of specific inducers of osteogenesis in the medium. Commonly, a combination of dexamethasone, ascorbic acid and β-glycerophosphate is used as such inducer. [112]

Dexamethasone, a synthetic glucocorticoid, stimulates the cellular proliferation and supports osteogenic lineage differentiation, accompanied by an increase in the expression of ALP. Organic phosphates, such as β-glycerophosphate, and ascorbic acid also support ostegenesis being essential for the formation and mineralization of the ECM – modulating osteoblast function. The cultivation of BMSCs as well as ASCs in the presence of these factors determines the acquisition of the osteoblast' properties: cube or polygonal shape, active basophilia of the cytoplasm, and synthesis of intracellular substance with calcium salts.[112, 113]

In fact it has been demonstrated that in the presence of those supplements the gene expression pattern of cultured cells is characteristic of osteoblast like cells and includes *Run-x2*, BMP2, BMP2 receptor I and II.[87, 110, 114]

However, the mechanisms that drive the ASCs into the osteoblast lineage are still not completely clear. Nevertheless, some transcription factors such as *PPARγ* and *RUN-x2* have been suggested to play a criticial role in the commitment of bipotent stem cells with the capacity to differentiate into the osteoblastic and adipogenic phenotype.[114]

Further investigations have been carried out using BMP2 as differentiation factor as its presence increases the expression of *Run-x2* and Osteopontin, promoting osteogenic differentiation.[115] Another particularly important factor regarding osteogenic differentiation of ASCs is the growth and differentiation factor 5 (GDF-5), that appears to be more effective than BMP-2 in the induction

of the osteogenic differentiation, also increasing the expression of VEGF, necessary to promote angiogenesis.[116]

Lee et al. provided the first evidence of bone formation in *in vivo* following the *in vitro* differentiation of ASCs into the osteogenic lineage, exposing stem cells to lineage-specific induction factors.[117] Evidences of bone formation were obtained after 8 weeks of subcutaneous implantation in Lewis rats following an autologous approach.

4.3.2. ENDOTHELIAL LINEAGE DIFFERENTIATION

As it was mentioned, ASCs have a great differentiation potential over several cell lineage, including endothelial.[86, 118] This lineage is among the most recent findings regarding the research on the ASCs differentiation potential. In 2001, Reyes et al.[119] described for the first time the isolation and ex vivo expansion of cells from postnatal BM that can differentiate not only into cell from the mesenchyma (OBs, chondrocytes, adipocytes, stroma cells, and skeletal myoblasts), but also into cells of visceral mesodermal origin, such as endothelium.

Miranville *et al.* [120] and Sengènes *et al*.[90] isolated a specific subpopulation of the SVF that was phenotypically positive for CD34$^+$/CD31$^+$ cell markers, with the aim of studying neovascularization/angiogenesis in adipose tissue. They demonstrated that at least there is a subpopulation of ASCs that can be differentiated into the EC lineage and that can be used in experimental approaches to promote vascularization.

Planat-Benard *et al*.[76] studies also indicate that adipose lineage cells can function as bipotent cell progenitor's source for ECs and adipocytes, opening new perspectives on the application of hASCs for cell-based approaches aiming to promote angiogenesis.[82] Mihaila et al evaluated simultaneously the differentiation potential of a SSEA-4$^+$subpopulation derived from the adipose tissue towards endothelial and osteogenic lineages. The results show that these cells differentiate towards the endothelial lineage, maintaining consistent endothelial phenotype along passages, when cultured in EGM-2 MV. Furthermore, SSEA-4+hASCs showed a strong osteogenic potential. Thus, SSEA-4+ subpopulation exhibits a promising potential for the development of vascularized bone TE constructs.[93]

Despite the still limited number of studies regarding the endothelial differentiation potential of specific subpopulations of ASCs, the identification of suitable sources of ECs represents a challenge for therapeutic angiogenic purposes. Its application in regeneration of tissues is quite

important. However when considering experimental cell-based approaches that should be able to accelerate the establishment of a functional vascular network it is quite relevant to have an improved understanding of molecular events that occurs between heterotypic cell-to-cell populations that the would better mimic the *in vivo* settings.

5. RELEVANCE OF CELL-TO-CELL INTERACTIONS IN BONE TISSUE ENGINEERING

5.1. CELL-TO-CELL COMMUNICATION

The organization of cells in tissues and organs is controlled by molecular events that support cells' ability to recognize other cells and the ECM, and to communicate with their neighbors.

Cells can communicate through three mechanisms: direct interaction between membrane proteins (adhesive interactions and tight junctions) of two adjacent cells, indirect interaction at gap junction level, and by the secretion of diffusible factors, either produced by the cells or released from the ECM, that can activate specific receptors on the target cells.

Adhesive interactions are essential not only for embryonic development, but also in a variety of other biologic processes, including the differentiation and maintenance of tissue architecture and cell polarity, the immune response and the inflammatory processes, cell division and death, among others. [121, 122]

Cell-to-cell and cell-matrix adhesions are mediated by specialized membrane proteins called cell-adhesion molecules (CAMs). A large number of CAMs fall into four major families: the cadherins, the immunoglobulin (Ig) superfamily, the integrins and the selectins.

In the adult skeleton, bone remodeling occurs via repeated sequences of bone resorption and formation cycles, which requires the continuous recruitment and differentiation of bone marrow precursors. The cooperative nature of bone remodeling requires an efficient intercellular recognition and communication that allow cells to sort and migrate, synchronize their activity, equalize hormonal response and diffuse correctly locally generated signals. The same happens during skeletal development, in which cell-to-cell interactions are critical for aggregation and condensation of immature chondro-osteoprogenitor cells and mesenchymal precursors.

Cadherins are an integral part of *adherens junctions*, which along with tight junctions and desmosomes, constitute the anchoring junctions, which join cells through their cytoskeletons.[123]

Cells of the osteoblastic lineage express two major cadherins: N-cadherin and cadherin-11; earlier *in vitro* results had suggested that cadherin-11 was specifically involved in osteoblast differentiation.[124-126] However, targeted ablation of the cadherin 11 gene (*Cdh11*) does not completely affect the skeletal development, which raises the possibility of the existence of a compensation mechanism by another member of this superfamily – the most likely being N-cadherin. [127] The same does not happen when there is the disruption of cadherin function by expression of a dominant-negative mutant of the cadherin gene of N-cadherin in differentiated OBs. A delayed peak bone mass acquisition, impaired osteogenic differentiation and an osteogenic to adipogenic shift in BMSCs precursors was observed. [128]

A different type of intercellular junction is the *gap junction* which does not provide cell anchorage but allows direct communication via specialized intercellular channels, and allow the diffusion of small molecules with molecular weights approximately less than 1 kDa, including second messengers (i.e. calcium, cAMP, inositol triphosphate), metabolites and ions, among coupled cells. Gap junctions are arrays of hexameric transmembrane channels, called connexons, formed by protein subunits called connexins. Abundant gap junctions are present between osteocytic processes and periosteal fibroblasts, between osteocytes and OBs on the bone surface, and among OBs.[129-132] In the last decade important insights have been gained into the importance of gap juntions in processes such as response to growth factors and hormones [133-136], mechanical load[137] and interaction with other cell types.[1] At least three connexins are present in bone cells, each providing unique permeability, ion selectivity, and electric conductance. Connexin43 (Cx43) is the most abundant, and multiple evidences have established an important role for Cx43 in skeletal development and for the function and survival of OBs and osteocytes. Cx43 is thought to be involved in the tramsmission of hormonal signals, mechanical load, and growth factor cues among cells in order to coordinate the synthesis of new bone.[138, 139] Lecanda et al demonstrated that the genetic ablation of *gja1*, the gene encoding Cx43, in mice leaded to a severe delay in the ossification of both intramembranous and endochondral derived skeletal elements during embryonic development. Thus, the OBs isolated from the Cx43 null animals are dysfunctional, with reduced osteogenic and mineralizition capacity. The interference with Cx43 function or *Gja1* expression also alters the expression of osteoblastic genes and the ALP, bone sialoprotein, osteocalcin and parathyroid hormone (PTH) responsiveness.[140] Furthermore, chemical inhibition of gap junctional communication leads to delayed bone nodule formation. Accordingly, primary osteoblast cell cultures isolated from *Gja1* null mice exhibit delayed ability to form mineralized nodules *in vitro*.[141]

The *in vitro* study of these interactions rely on co-culture systems where at least two cell types are cultured at the same time and submitted to the same microenvironment that is expected to mimic the *in vivo* settings.[25, 142] Several studies were undergone to study the relevant cell-cell interactions for bone TE, since there are several mature cells like chondrocytes [28, 143], macrophages [144], ECs [145] and myeloma cells [146, 147], that can promote osteogenic differentiation of MSCs either by direct or indirect contact.

Different settings are also defined according to the type of cell interaction under investigation. Indirect communication between cells can be investigated using porous cell chambers resulting in its physical separation, using a conditioned medium or an ECM obtained from monoculture of one cell type, or the co-culture. Those systems are particularly useful for the identification of diffusible factors that are released from each cell type and that may influence several cellular functions as proliferation and differentiation.[148]

Co-culture systems of different cell types that establish direct communication are maintained by the release of soluble factors and by cell-to-cell contacts. However, in these systems it is difficult to discern the role of particular molecules in each cellular response, becoming necessary to isolate both and analyse the effect of the co-culture in the phenotypical pattern of the cell of interest.[149]

5.2. CELL-TO-CELL COMMUNICATION BETWEEN OSTEOGENIC AND ENDOTHELIAL CELL LINEAGES

Considering the intricate relation between angiogenesis and osteogenesis it is quite clear that the understanding of the interactions between ECs and OBs are one of the most relevant processes [150] that regulate bone homeostasis and regeneration.[38] The closer location of OBs and osteoprogenitor cells near ECs in blood vessels at the site of new bone formation corroborate this as a statement. However, it remains unclear the *in vivo* direct contact communication between those cell types.

The crosstalk between OBs and ECs is bidirectional and occurs at those two levels: by indirect cell communication [151] through the release of soluble factors with paracrine and autocrine action and by direct cell communication mediated by proteins at gap junctions structures.[1] One of the most studied growth factors is VEGF, a potent cytokine that induces vascular permeability and angiogenesis *in vivo*.[152] It is now well known that VEGF is an important mediator of the angiogenic process and a potent stimulator of major sketetal cell populations.[152, 153] Fibroblast growth factor (FGF) is another angiogenic growth factor, and functions as a paracrine agent

stimulating EC proliferation and migration and as an autocrine agent on OBs' proliferation and differentiation processes. ECs also secrete regulatory molecules that control the activity and differentiation status of OBs. BMPs and endothelins, particularly BMP-2 and endothelin-1, are the main intervenient in these regulatory mechanisms. PDGF-BB is released by ECs and acts during fracture repair because of its mytogenic effect over OBs. ECs also influence the expression of ALP gene, whose up-regulation is an indicator of the effect of ECs on osteogenic differentiation.[154]

Additionally, Conexin-43 have been referred as the gap junction protein intervenient in the indirect crosstalk between OBs and ECs.[1] As result of these interactions, the biological system should be self-sustainable as each cell type produced the suitable molecules that should hold a favorable environment for cell maintenance and for the formation of microvascular-like structures as it mimics the physiological environment in which the vascular network structures are formed.

Several co-culture model systems have been established for the study of cellular interactions between OBs and ECs either in two- and three-dimensions. In a 2D system, cells can be seeded directly on plastic culturing flasks or on plates coated with specific ECM proteins. In 3D systems, cells are seeded in a 3D structure, like a scaffold when considering biomaterial associated approaches as for example for bone TE. Many researchers are exploring this relationship and developing strategies to regenerate a vascularized bone construct based on the simultaneous culture of these two cell types derived from a common cell source as it has been described for adipose tissue and bone marrow. [155-157]

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II. MATERIALS AND METHODS

This chapter describes all experimental methods and techniques, as well as cells used under the scope of this thesis. Although chapter III also comprehends a material and methods section, further details, as well as the justification for the selection of the methodologies, markers and cells used are provided herein. Additionally, the layout of the present section provides a more comprehensive overview of the performed work.

1. HARVESTING, ISOLATION, SELECTION AND CULTURE OF STEM CELLS FROM HUMAN ADIPOSE TISSUE

All the human lipoaspirates samples used for the experiments described in this thesis were obtained with informed consent from healthy females, with an average age of 42 years old, undergoing plastic surgeries, and under a collaboration agreement between Hospital de Prelada, Porto, Portugal and the 3B´s Research Group.

Liposuction procedures are nowadays considered a safe and well-tolerated non-invasive procedure, with a complication rate of around 0.1% [1, 2], and have been increasingly used during the last decade by plastic surgeons to eliminate excess of fat tissue. The different techniques of liposuction, dry, wet, super wet and tumescent approach, mainly vary in the volume of infiltrate which ultimately defines the intensity of the bleeding during surgery, as well as the post-operative bruising and swelling. The use of larger volumes of subcutaneous infiltrate, such as in the super wet and tumescent techniques, generally leads to a blood loss decrease to less than 1% of the aspirate volume.[3] Although the lipoaspirate has been subjected to suction forces during aspiration, research shows that 98–100% of the adipose cells in the lipoaspirate are viable.[4, 5] Likewise a study performed by Fraser *et al.* has shown that the lipoaspiration procedure does not affect the adipose stem cells (ASCs) and apparently only the lipocytes are damaged by the mechanical stress applied during the liposuction.[6]

The first method to isolate cells from adipose stromal vascular fraction was proposed by Rodbell and Jones in the 1960s.[7-9] Minor modifications of the pioneer method used to isolate cells from rat tissue, allowed its successful application to human tissue.[10] Zuk *et al* were the first to report the existence of ASCs in the fat tissue, in 2001.[11] This group isolated the adult stem cells from human fat tissue using an enzymatic method, which is still the basis of the most methods used nowadays, followed by a "natural" selection of the cells based on the ability of the hASCs to adhere to the plastic of the culture flasks. At this moment, there are several reported methods for

isolating ASCs which present only minor difference s among them. The main differences between the reported methods in the literature rely on the collagenase concentration and on the incubation time that should enable an efficient digestion and that would lead the higher number of adherent cells.

The SVF is composed of heterogenic cell populations, such as adipocytes, endothelial cells and fibroblasts. For this reason, other isolation methods have been proposed aiming at either obtaining "purified" stem cells population or obtaining specific cells subpopulations. One of these methods is the immunomagnetic sorting technique that uses specific markers as isolation element. At the same time, scientific literature relating to the isolation, characterization, differentiation, expansion, immunophenotype, immunomodulatory properties, and preclinical use of stromal vascular fraction (SVF) cells and adipose derived stem cells (ASCs) had increased dramatically as adipose tissue represents a reliable and highly available source of stem cells, whose yield varies between $1x10^7$ and $6x10^8$ cells per 300mL of lipoaspirate.[12-17]

1.1. HARVESTING OF HUMAN ADIPOSE TISSUE STROMAL VASCULAR FRACTION

After the liposuction procedure, the adipose tissue settles into two layers – the upper one constituting the processed lipoaspirates layer, composed of suctioned adipocytes as well as surrounding endothelium and stroma; and the bottom layer or liposuction aspirate fluid, that consists of injected saline, erythrocytes, and denser pieces of the processed lipoaspirate layer.

Human ASCs isolated from both fractions, however, the percentage of cells that adhere to the culture flask is higher when harvested from the processed lipoaspirates layer.[18]

The adipose tissue samples used in this work were collected by tumescent and super wet liposuction procedures, placed in sterile containers containing a solution of PBS with 10% of antibiotic/antimycotic (Invitrogen, USA) and stored at 4ºC. All the samples were processed within 24 hours after collection. The followed procedure is described herein.

On the isolation day, lipoaspirate was incubated with 100mL of a 0.075% collagenase II A (Sigma Aldrich, Germany) solution in PBS, at pH 7.4 and 37°C for 45 min in a shaking water bath. After the incubation, the digested tissue was passed through 200μm filter mesh to eliminate remaining matrix components. The mature adipocytes and the cells from the connective tissue were separated by centrifugation at 1000xg, for 10 minutes at 4°C. The supernatant containing the adipocytes was removed and the cell pellet was resuspended in NH_4Cl-based erythrocyte lysis buffer composed of NH_4Cl 155mmol (Enzifarma, Portugal), K_2HPO_4 5.7 mmol (Riedel-de-Häen, Switzerland) and EDTA 0.1mmol (Sigma Aldrich, Germany). Cell suspension was incubated with the lysis buffer for

10 minutes at room temperature (RT) and then centrifuged at 800xg at 4ºC for 10 minutes. The resulting pellet, constituting the SVF, was resuspended in PBS, passed through a 100µm cell strainer used to select the SSEA4$^+$ sub-population (see section 1.2.2) or the ASCs. The use of the 100µm cell strainer avoided the entrapment of the immunomagnetic beads within any remaining matrix components.

1.2. IMMUNOMAGNETIC BEADS-BASED CELL SEPARATION

As mentioned before, in most of the studies describing the isolation of hASCs, it is used a method based on the enzymatic digestion of the tissue/lipoaspirates sample, followed by the seeding of the obtained heterogeneous cell suspension onto a tissue culture flask. However, other techniques can be used for the further selection/separation of specific subpopulations within this heterogeneous cell population, such as the fluorescent-activated cell sorting (FACS) and the methods (automated or not) based on the use of immunomagnetic beads.

In a previous work of the 3Bs Research Group, Tommaso *et al* used immunomagnetic beads coated with the antibodies for some mesenchymal stem cell markers, namely CD90, CD73, CD105 among others, and analysed the stemness of the subpopulations as well as their chondrogenic and osteogenic differentiation profiles.[19] This studies allowed to conclude about the existence of specific ASCs subpopulation with distinct differentiation potential. More recently, a work by *Mihaila* et al. described the isolation of SSEA4$^+$ hASCs using the same methodology and showed the ability of this subpopulation to differentiate into the endothelial and osteogenic lineage under optimized culturing conditions.[20] The main aim of the present thesis was therefore to establish optimal conditions for co-culturing SSEA4$^+$ hASCs pre-conditioned to the endothelial lineage with SSEA4$^+$ hASCs pre-conditioned to the osteoblastic lineage.

2. PREPARATION OF THE IMMUNOMAGNETIC BEADS

The immunomagnetic beads isolation/purification method used in this work was based on coating the commercially available magnetic beads (Dynal M-450 Epoxy beads, Dynal Biotech, USA) with the selected antibody for this study – SSEA4 (Abcam, USA). As mentioned above, the selection of SSEA4 was based on a recent study developed at the 3Bs research Group. .[20]
The coated beads were prepared according to the instructions provided by the manufacturer. Dynal M-450 Epoxy beads were washed in coupling buffer (0.1M sodium phosphate buffer: pH 7.4-8.0)

and coated with 10 µl of the SSEA-4 antibody in an optimized concentration of 4µg Ab/1x10^7 beads. The antibody-beads system was incubated overnight at RT, under gentle stirring with a sodium phosphate buffer.

After the SVF isolation, the SSEA-4 coupled beads were separated using the Dynal MPC® magnet. The supernatant was discarded, and the coupled beads were mixed with 1mL of 0.2 % (w/v) human serum albumin/PBS washing and storage buffer at pH 7.4. The washing procedure was repeated for three times. The coupled beads were again separated using the magnet and resuspended in the storage buffer at a concentration of 4 x 10^8 Dynabeads per ml.

2.1.1. SELECTION OF THE HASCS SUB-POPULATION EXPRESSING SSEA-4 (SSEA-4$^+$HASCS)

In order to select the sub-population of interest, expressing SSEA-4, the coated beads were mixed with 1ml of the SVF cell suspension and incubated for 30 minutes at 4°C under gentle stirring. Subsequently the cells bonded to the coated beads were separated from the rest of the cell suspension using the Dynal MPC magnet as previously described (See section 1.2.1). The selected cells, SSEA4$^+$hASCs, were then cultured in appropriate culture medium according to the lineage of interest (see section 1.3)

2.2. CELL CULTURE

2.2.1. HASCS AND SSEA4$^+$HASCS EXPANSION

For selection of hASCs, SVF was plated in 75cm^2 cell culture flasks with basal medium – Alpha-Minimal Essential Medium (α-MEM, Sigma Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% antibiotic/ antimycotic (Invitrogen, The Netherlands). The selected subpopulation SSEA4$^+$-hASCs was also plated in the same type of flasks using the same culture medium. Subsequently both hASCs and SSEA4$^+$hASCs were expanded up to passage 2, using the described medium, under humidified 5% CO_2 atmosphere at 37ºC. Culture medium was replaced each 3 days.

2.2.2. ENDOTHELIAL COMMITMENT

The recent study performed by Mihaila et al reported that the SSEA4-hASCs subpopulation achieved the endothelial differentiation when cultured in Microvascular Endothelial Growth Medium (EGM 2-MV, Lonza, USA) right after the isolation and selection, up to 14 days in culture.[20] The reported data showed a consistent expression of endothelial cell markers: CD31) and vWF, either at protein or molecular levels. Thus, the

The culturing conditions used to induce the endothelial differentiation of SSEA4$^+$hASCs were based on this study.

After the immunomagnetic selection of SSEA4$^+$hASCs subpopulation, cells were seeded, in 25cm^2 cell culture flasks and maintained in EGM -2 MV supplemented with SingleQuots containing several growth factors (hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-100, hydrocortisone) (see Table 1) and 5% FBS until reaching 70% of confluence, in a humidified atmosphere with 5% CO_2 at 37ºC. After reaching that confluence point, the cells were tripsinized and sub-cultured into 75cm^2 flasks. The co-culture experiment was set-up with SSEA4$^+$-hASCs derived ECs at passage 2. These cells will be referred as SSEA4$^+$hASCs-ECs in further sections of this thesis.

2.2.3. OSTEOGENIC COMMITMENT

It is well described in the literature the biochemical induction of the osteogenic phenotype of the bone marrow stromal cells using β-glycerophosphate, ascorbic acid and dexamethasone. Likewise, Mihaila et al demonstrated the osteogenic potential of the SSEA4$^+$-hASC subpopulation using culture medium supplemented with β-glycerophosphate, ascorbic acid and dexamethasone.

The osteogenic differentiation factors currently used are responsible for: Ascorbic acid was found to be essential for collagen synthesis and secretion; β-glycerophosphate provides an inorganic source of phosphate essential for the occurrence of mineralization; and dexamethasone has demonstrated to determine some morphological changes from an elongated to a more cubical cell shape and an increase of the expression/activity of the alkaline phosphatase enzyme.

In this thesis, it was performed a pre-conditioning of the SSEA4$^+$-hASCs to the osteogenic lineage. The selected SSEA4$^+$hASCs at passage 2, previously expanded in α-MEM (see section 1.3.1) was cultured at a density of 2000cells/cm^2 in alpha-medium supplemented with 50µg/mL ascorbic acid (Sigma Aldrich, Germany), 10^{-8}M dexamethasone (Sigma Aldrich, Germany), 10mM β-glycerophosphate (Sigma Aldrich, Germany), 10% FBS and 1% antibiotic/antimycotic (Osteogenic Media, OST) for 7 days. After this time of culture, cells were considered to be pre-conditioned to

the osteogenic lineage and used to establish the co-culture (see section 2). These cells will be referred as SSEA4+hASCs-OBs in further sections of this thesis.

Table 1. Summary of the constituents of each cell culture media used in this work: α-MEM for cell expansion of hASCs and SSEA4+-hASCs; EGM 2-MV for endothelial committement of SSEA4+-hASC fraction.

Culture Media	Commercial Name	Composition
ALPHA-MINIMAL ESSENTIAL MEDIUM (α-MEM)	Alpha-MEM	10% FBS 1% antibiotic/ antimycotic
ENDOTHELIAL GROWTH MEDIA (EGM)	EGM-2 MV Bullet Kit	No Bovine Brain Extract (BBE) Human Epidermal Growth Factor (hEGF) Hydrocortisone Gentamicin and Amphotericin-B (GA-1000) Fetal bovine serum (FBS) Vascular Endothelial Growth Factor Human Fibroblast Growth Factor-basic (hFGF-b) Human recombinant analog of insulin-like growth factor-I with the substitution of Arg for Glu at position 3 (R3-IGF-1) Ascorbic Acid

3. CO-CULTURE SYSTEM

3.1. OPTIMIZATION OF THE CULTURE CONDITIONS

SSEA4-hASCs, pre-conditioned in osteogenic (SSEA4+hASCs-OB) and endothelial differentiation (SSEA4+hASCs-ECs) conditions at passage 2 were used to establish the co-culture. Cells were cultured in EGM 2-MV, Osteogenic Media (as described in the section 1.3.2 and 1.3.3) and EGM 2-MV supplemented with the osteogenic factors (EGM OST) (see Table 2).

A preliminary analysis of each monoculture of SSEA4$^+$hASCs-OBs and SSEA4$^+$hASCs-ECs was performed in order to evaluate the cell behavior either in EGM, OST or EGM OST culture media. However, for the monoculture of SSEA4$^+$hASC derived ECs it was observed that after 7 days in culture, cells did not survive, probably due to the absence of endothelial growth factors in OST media. Nevertheless in order to analyse how would SSEA4$^+$hASCs-OBs influence the cell behavior of SSEA4$^+$-hASCs-ECs in co-culture conditions in the absence of endothelial growth factors, the OST culture media was still considered for the conditions to be analysed in the co-culture experiment. Concerning the SSEA4$^+$-hASCs-ECs it was also found relevant to analyse in more detail the maintenance of the endothelial phenotype of these cells in EGM OST. For that purpose immunocytochemistry analysis for the endothelial markers – CD31 and vWF – was performed. The results showed that after 14 days in culture, the endothelial phenotype was kept stable.

3.2. ESTABLISHMENT OF THE CO-CULTURE

As mentioned above, after optimization studies, the SSEA4$^+$hASCs pre-conditioned in osteogenic and endothelial differentiation were cultured in the pre-established ratios, namely, SSEA4$^+$hASCs-OBs:SSEA4$^+$hASCs- ECs, 1:3; 1:1; 3:1, either in osteogenic, EGM 2-MV or EGM OST medium as described in Table 3.

The effect of the relative proportion of cells in co-culture represents an important issue to be investigated. Several examples have been published regarding the co-culture ECs and Osteoblast-like cells using different cellular proportions: 4:1[21], 2:1 [22-25], 1:1 [26-30], 3:1 [31]. Morever, among these works only one mentions a preliminary cell ratio analysis to justify the selected ratio. [32] Although authors did not show the data, they stated that higher proportions of ECs resulted in the presence f both cell types after 1 week of culture. Lower proportions of ECs – ratios of 1:1, 1:5 and 1:1 of ECs:OBs resulted in substantial reduction of the ECs number after the same period.

The co-culture ratios of SSEA4$^+$hASCs-OBs – SSEA4$^+$-hASCs derived ECs were defined in order to evaluate the effect of the proportion of each cell type in the maintenance of the osteogenic and angiogenic potential of both cell lineages.

In detail, cell suspensions were prepared at appropriate ratio and 2000 cells/cm^2 were seeded in 24-well plates (BD Falcon, USA) in 1 ml of culture medium. Medium was changed twice a week. Monocultures of SSEA4$^+$hASCs-OBs (1:0) and SSEA4$^+$hASCs-ECs (0:1) were established as controls.

Table 2. Summary of the constituents of each cell culture media used for the co-culture system: EGM 2-MV; OST - α-MEM supplemented with osteogenic differentiation factors; EGM OST - resulting from the supplementation of EGM 2MV media with osteogenic differentiation factors at the same concentrations used for preparing Osteogenic media.

ENDOTHELIAL GROWTH MEDIA (EGM)	EGM-2 MV Bullet Kit	No Bovine Brain Extract (BBE) Human Epidermal Growth Factor (hEGF) Hydrocortisone Gentamicin and Amphotericin-B (GA-1000) Fetal bovine serum (FBS) Vascular Endothelial Growth Factor Human Fibroblast Growth Factor-basic (hFGF-b) Human recombinant analog of insulin-like growth factor-I with the substitution of Arg for Glu at position 3 (R3-IGF-1) Ascorbic Acid
OSTEOGENIC MEDIA (OST)	α-MEM	50µg/mL ascorbic acid 10^{-8}M dexamethasone 10mM β-glycerophosphate 10% FBS 1% antibiotic/antimycotic
EGM OST	EGM-2 MV Bullet Kit	All the supplements that constitute the EGM kit and all osteogenic differentiation factors, at the same concentrations.

Table 3. Co-culture set-up for cell ratio: all the proportions corresponds to the ratio SSE4^{+}hASCs OBs : SSEA4^{+}hASCs derived ECs.

EGM	Osteogenic Media	EGM OST
1:0	1:0	1:0
3:1	3:1	3:1
1:1	1:1	1:1
1:3	1:3	1:3
0:1	0:1	0:1

4. CHARACTERIZATION TECHNIQUES AND METHODOLOGIES

4.1. CELL MORPHOLOGY

All the established cultures were regularly monitored in terms of cells morphology by inverted light microscopy using an Axiovert 40 (Zeiss, Germany) microscope. Images of the co-cultures and respective controls were also acquired at days 7, 14 and 21 of culture.

4.2. CELL PROLIFERATION

Cell proliferation of the SSEA4^{+}-hASCs OBs and SSEA4^{+}-hASCs derived ECs either in monoculture or in co-culture was assessed by quantification of the double stranded (ds) DNA content along the culture using the Quant-iTTM Picogreen Kit (Invitrogen, The Netherlands) following the instructions of the manufacturer. The principle of this assay relies on the capacity of the Picogreen fluorophore to specifically bind to the double-stranded DNA, allowing its quantification after excitation of the fluorophore.

Cells were rinsed with sterile PBS and incubated with 200µl of sterile ultrapure water for 1h at 37ºC in order to induce an osmotic shock. To guarantee the lysis of the cells, thermal shock was also inflicted after the incubation at 37ºC by directly frozen the samples at -80ºC. Before starting the dsDNA quantification, samples were thawed and sonicated for 15 minutes in an ultrasonic bath.

Aliquots of 28,7µl of each sample were then placed into individual wells of 96-well plate. Standards ranging from 0 – 2 µg/mL of DNA were made from dilutions of a standard solution prepared from the DNA standard (component C) with a concentration of 2µg/mL of DNA in ultrapure water, to obtain the calibration curve. Aliquots of the prepared standards were placed into other wells. Tris-EDTA buffer was prepared according to the manufacturer's instructions using reagents provided in the kit and 100µl added to each of the samples and standards wells. The PicoGreen dye solution was also prepared according to the manufacturer's instructions – preparing a 200 times diluted solution – and 71,3µL were added to each well (samples and standards).

The 96-well plate was then incubated in the dark for 10 minutes at room temperature and the fluorescence measured at 485nm of excitation and 528 of emission in a Synergy HT microplate reader (Bio-Tek, USA). The prepared standard curve with serial dilutions of the dsDNA stock was used to extrapolate the samples dsDNA concentration.

4.3. ALKALINE PHOSPHATASE ACTIVITY QUANTIFICATION

Alkaline phosphatase (ALP) is an enzyme that hydrolyses phosphate esters and is involved in the initial processes of bone extracellular matrix mineralization. Consequently, it is widely regarded as an indicator of osteoblastic differentiation. The ALP enzymatic activity of SSEA4^{+}-hASCs OBs and SSEA4-hASCs derived ECS in monoculture and in co-culture was measured using a colorimetric assay based on the conversion of p-nitrophenyl phosphate (pNPP), colorless, into p-nitrophenol (pNP), yellow.

The ALP quantification was performed in the same cell lysates used for the DNA quantification and obtained by osmotic and thermal shocks (see section 3.2). The enzyme reaction was set up by incubating 20µl of sample with 60µL of a 0.2% w/v p-nytrophenyl phosphate (pnPP) in 1M Diethanolamine HCl solution with a pH of 9.8 for 45 minutes, at 37ºC. A calibration curve was prepared with the concentration standards ranging from 0 to 0,20 µmol/ml mixing a p-nytrophenol (pNP) stock solution (10 µmol/ml) in a solution of 2M NaOH/0.2mM EDTA solution in distilled water (STOP Solution).

The reaction was stopped by adding 80µl of a the STOP Solution. The optical density was determined at 405nm, with a reference filter at 602nm. The results were expressed in µmol of pNP hidrolized that correlates with the ALP activity and normalized against dsDNA values.

4.4. ALKALINE PHOSPHATASE STAINING

The membrane-bound ALP was stained to determine the covered area of the cells that exhibit the enzyme. The protocol followed produces a red reaction product that can be seen using brightfield microscopy.

SSEA4-hASCs OBs and SSEA4^{+}hASCs derived ECs in monoculture and in co-culture were seeded on TCPS slides (Sarstedt, Newton, NC, USA). After each time point cells were washed for three times with PBS (with no Ca^{2+} or Mg^{2+}) and subjected to a fixative 3.7% v/v solution of formaldehyde in PBS for 30 minutes at room temperature. After the removal of the fixative, cells were washed twice with PBS and kept in this buffer at 4ºC until they were subjected to the ALP staining. Briefly in an eppendorf tube it was prepared a solution composed of N,N-Dimethylformamide (DMF) (Merck, Germany) and Naphthol AS-MX phosphate, 0.25% (w/v),

buffered at pH 8.6 (Sigma Aldrich, Germany) which was added to the previously prepared solution of Tris-HCL 0,2M with distilled water, buffered at pH 8.3 with Sodium hidroxyde. The Fast Red Violet LB salt (alkaline solution, 0.104mg/mL) (Sigma Aldrich, Germany) was mixed at last. The final solution was added to the samples and they were kept in the dark for 45 minutes at room temperature. Finally the cells were washed with distilled water 3-4 times and the photographed under and inverted microscope Axiovert 40 CFL(Zeiss; Germany).

4.5. ALIZARIN RED STAINING

The Alizarin Red (AR, Merk, Germany) staining is one of the most used and reliable stainings to detect the mineralization of the matrix produced by osteoblastic cells in culture as it reacts specifically with calcium SSEA4^{+}-hASC OBs, SSEA4^{+}-hASC derived ECs and in co-culture were plated in 24 well plates at the cell density referred in the section 2.1. At each time point, all the samples were washed for three times with PBS and fixed with a 3.7% v/v solution of formaldehyde in PBS for 30 minutes, at RT. After removal of the fixative, cells were washed first with PBS and then with distilled water to remove any calcium residues that may exist in the PBS solutionThe fixed cells were then incubated with a 2% AR solution for 10 minutes, and finally washed with distilled water. Stained samples were observed and photographed using (Canon PowerShot G11, Canon, Japan).

4.6. GENE EXPRESSION ANALYSIS OF OSTEOCALCIN

4.6.1. MRNA EXTRACTION AND CDNA SYNTHESIS

The mRNA of SSEA4^{+}hASCs-OBs and SSEA4^{+}hASCs derived ECs monocultures and in co-culture at all the defined conditions was extracted with TriZol Reagent (Invitrogen, USA) following the procedure provided in the datasheet of the product. In detail, 800 µl of TriZol Reagent were added to each sample. After an incubation of 5 min, 160 µl of chloroform (Sigma Aldrich, Germany) were added, and agitated vigorously in the vortex for 15 seconds; the samples were then incubated for 15 minutes at 4°C and centrifuged at the same temperature and 13000 x rpm for 15 minutes at the same temperature. After the centrifugation, the aqueous part was collected and an equivalent volume of isopropanol (Sigma Aldrich, Germany) was added. Samples were incubated overnight at -20ºC. On the following day, all the samples were centrifuged at 13000

x rpm for 15 minutes at 4ºC. The supernatant was discarded and left at the bottom nearly 50µl of volume.

After this incubation period, were washed with 800 µl ethanol 70% prepared with RNase/DNase free water (Gibco, UK), centrifuged at 4ºC and 9000rpm for 5 minutes. The supernatant was carefully discarded and resuspended in 12µL of water RNase/DNase free. The RNA quantity and purity were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using the qScript cDNA Synthesis kit (Quanta Biosciences, USA), and the Mastercycler ep Realplex gradientS (Eppendorf, USA), using an initial amount of RNA of 1 µg in a total volume of 20 µl of RNAse free water (Gibco, UK). After the synthesis of the cDNA, the samples were used to quantify the relative expression of specific genes (see section 3.6.2).

4.6.2. QUANTITATIVE REAL TIME PCR

The expression of the markers of the osteogenic and endothelial phenotype was assessed on each sample after the mRNA extraction and the synthesis of the cDNA (See section 3.6.1) by Real Time RT-PCR. The reaction was carried out with 5ng of cDNA, the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences, USA) and the primers shown in Table 4. The primers were previously designed using the Primer 3 online software (v 0.4.0) and synthesized by MGW Biotech (Germany). For each sample, glycetaldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. A concentration of 300 nM was used for all the primers in a final volume of 20µL of sample.

The relative quantification of the gene expression was performed using Pffafl method.[33] The values were normalized against the housekeeping gene GAPDH and then against the control; monoculture of SSEA4^{+}hASCs-OBs (1:0) in osteogenic medium at day 7for Osteocalcin.

Table 4: Genes under evaluation, primers and PCR conditions: GAPDH=housekeeping gene, OCN =osteocalcin, Fwd=forward, Rev= reverse.

Name of gene	Product size / bp	Annealing temperature (ºC)	Primer pair sequence
GAPDH	87	60	**Fwd:** TGCACCACCAACTGCTTAGC **Rev:** GGCATGGACTGTGGTCATGAG
OCN	230	61.6	**Fwd:** CTG GAG AGG AGC AGA ACT GG **Rev:** GGC AGC GAG GTA GTG AAG AG

4.7. IMMUNOCYTOCHEMISTRY

The expression of the endothelial cell markers CD31 and vWF as well as the expression of the osteogenic markers Osteopontin and Osteocalcin of SSEA4$^+$hASCs-OBs and SSEA4$^+$hASCs- ECs monocultures and in co-culture at all the defined conditions, was assessed by immunocytochemistry. For this purpose, co-cultures were established on TCPS slides as described in section 2.1. At days 7, 14 and 21 all the samples were washed twice with PBS and fixed with 3,7% formalin 30 minutes at RT. All the samples were then washed twice with PBS and kept at 4ºC in PBS.

For the evaluation of the endothelial markers expression, which required intracellular staining, cells were washed with PBS and permeabilized with a 0.2% Triton solution for 5 minutes. For all the conditions, nonspecific antibody binding was blocked with an incubation with a 3% BSA in PBS (blocking solution) for 30 minutes. Cells were then incubated with the primary antibodies – mouse anti-human CD31 (Dako, Denmark), rabbit anti-human von Willebrand factor (vWF) (Dako, Denmark) antibodies – for one hour, RT and after appropriate dilution in 1.5% BSA/PBS (see table 4) A serie of washing cycle (3x5minutes) followed the incubation with the primary atobody. Then the samples were incubated with the secondary antibodies: goat anti-mouse Alexa Fluor 488 (Invitrogen, USA), and donkey anti-rabbit Alexa Fluor 594, (Invitrogen, USA) diluted 1:500 in 1.5% BSA/PBS for 1 hour at RT, after which a new washing cycle of 3x 5 minutes was perfomed.

For the evaluation of the osteogenic markers – Osteocalcin and Osteopontin – all samples were not permeabilized, as the intracellular staining was not desired. Instead after fixation were subjected to the blocking procedure by incubation for 30 minutes with the blocking solution a RT. Then, cells were incubated with mouse anti-human osteocalcin (OCN, AbD Serotec, UK) primary antibody. After the incubation time, cells were washed 3 times with PBS, 5 minutes each, and incubated for 1 hour with the appropriate secondary antibody – donkey anti-mouse Alexa Fluor 488 diluted 1:500 in 1.5% BSA/PBS.

Table 5. Primary and Secondary antibodies used on the immunocytochemistry protocol: optimal dilutions

Primary Antibody	Secondary Antibody	Dilution Primary antibody	Dilution Secondary antibody
CD31	Goat anti-mouse Alexa Fluor 594	1:50	1:500
vWF	Donkey anti-rabbit Alexa Fluor 488	1:200	3:500
Osteocalcin	Goat anti-mouse Alexa Fluor 594	1:50	1:500
Osteopontin	Donkey anti-rabbit Alexa Fluor 488	1:50	1:500

Cell nuclei were counterstained with 4, 6-Diamidino-2-phenyindole dilactate (DAPI, Sigma Aldrich, Germany), diluted 1:10000 in PBS, for 5 minutes and then washed 3 times, 5 minutes each. To preclude false positives induced by the nonspecific binding of secondary antibodies, all cell types were also treated in a similar manner, but incubated with PBS instead of the primary antibody.

Immunolabelling was qualitatively analysed under an optical Imager.Z1 fluorescence microscope (Axiovert 40 CFL; Zeiss, Germany) and photographed using an Axio Cam MRm camera (Zeiss, Germany). The results were processed using the AxioVision 4.8 software.

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III. OPTIMIZED CONDITIONS FOR CO-CULTURING PRE-OSTEOBLASTS AND ENDOTHELIAL CELLS DERIVED FROM SSE4+-HASCS

Optimized Conditions for Co-culturing Pre-Osteoblasts and Endothelial Cells derived from SSEA4$^+$-hASCs Subpopulation

ABSTRACT

Currently, the vascular supply of bone substitutes is identified as the main pitfall of bone tissue engineering (TE) and the major hurdle for the successful achievement of engineered constructs as its survival and integration *in vivo* depends on a complex interplay between numerous cell types and the scaffolding biomaterial. Cell-to-cell interactions at the implant location that mutually affect the differentiation and functional state of the others, either by direct contact or indirectly by the release of soluble factors, are also critical. The mutual crosstalk between endothelial and osteoblast cells have been of major interest when considering experimental cell-based approaches to improve osteogenic and angiogenic potential of bone TE approaches. As the success of tissue regeneration strategies depends in part in the reintroduction of in vitro expanded cells in a state that guarantees the functionality and differentiation of the cell types involved in the system, turning mandatory the optimization of the cell culture conditions.

The stromal vascular fraction (SVF) of adipose tissue is composed of a heterotypic cell population among which the SSEA-4$^+$ subpopulation (SSEA-4$^+$hASCs) was found to exhibit endothelial and osteogenic differentiation potential, enabling the obtention of both cell populations from the same cell source. In this work SSEA4$^+$-hASCs preconditioned Osteoblasts (OBs) were co-cultured with SSEA4$^+$-hASCs derived Endothelial Cells (ECs) for up to 21 days in a 2D system at different cell ratios (3:1-1:1-1:3) in different culture media – EGM, Osteogenic media (OST) and EGM supplemented with osteogenic differentiation factors (EGM OST) to analyse how these parameters would influence the maintenance of the osteogenic and endothelial differentiation potential of the SSEA4$^+$-hASCs population in co-culture. The results show both cell culture media and cell ratio are important parameters for co-cultures of SSEA4$^+$-hASCs derived OBs and ECs. In fact either EGM or EGM OST allowed to the maintenance of the osteogenic and endothelial differentiation potential of this subpopulation, however, in OST media, the endothelial phenotype of the SSEA4$^+$-hASCs derived ECs was not stable. Collectively, osteogenic and endothelial potential of the co-cultures of SSEA4$^+$-hASCs OBs and SSEA4$^+$-hASCs derived ECs increased along with the proportion of ECs in the system and that the presence of both endothelial and osteogenic differentiation factors in the culture media is of higher relevance.

1. INTRODUCTION:

During the last years of tissue engineering research great and important achievements have been obtained. However, to date, bone tissue engineering approaches have not yet resulted in successful clinical applications.[1] One of the major limitations to overcome concerns the insufficient vascular supply of the tissue engineered constructs, leading to conditions of hypoxia and necrosis in the central core of grafts with more than few micrometers in volume. The depletion of nutrients, oxygen and hundreds of soluble factors comprises one of the major hurdles to attain functional tissue engineered constructs, as the interstitial fluid diffusion and blood perfusion are not sufficient to cover the metabolic demands of cellular components of the structure and of the new formed tissue.[2] Furthermore, it is widely accepted that vascular development always precedes osteogenesis and the existence of reciprocal interactions between osteoblasts (OBs) and endothelial cells (ECs) are essential to their normal function. [3-6] For those reasons, research in bone tissue engineering is increasingly focusing on the study of the crosstalk between OBs and ECs as the outcome of the synergistic effect between these cell types is expected to translate into high functionality and increased success rates of an engineered construct after transplantation. Several studies demonstrated that in comparison to monocultures, osteogenesis and angiogenesis benefit from co-culture both *in vitro* [4, 7-9] and *in vivo.* [10-15] Emerging co-culture systems have been established aiming to understand the effect of osteogenic cells on the angiogenic potential of ECs from different origins: human umbilical vein endothelial cells (HUVECs), human umbilical cord blood endothelial cells (EPCs), human outgrowth endothelial cells (hOECs) and human dermal microvascular endothelial cells (HDMECs). ECs from all origins could migrate and lead to microvessel-like structures.[16-20] However, the limited availability and proliferation capability of mature ECs hinders their use in tissue engineering approaches.[21] Therefore, it became a priority to find a suitable source of ECs that allow overcoming those constrains.

Human adipose tissue has been recognized as a potential source of stem cells for regenerative medicine applications, including bone tissue engineering, especially due to a combination of several favorable characteristics: the accessibility of the source, the high cellular yield of human adipose-derived stem cells (hASCs) and their differentiation potential over multiple cell lineages.[22, 23]

The adipose stromal vascular fraction (SVF) is composed by an heterogeneous cell population composed of immuno cells, haematopoietic stem cells, adipocytes, endothelial cells and fibroblasts [24] which can negatively affect the proliferation and/or differentiation potential of ASCs. In fact, the major drawback of adipose tissue as a source of stem cells is the contamination by other cell types that occurs upon the isolation of these cells, using a technique based on enzymatic digestion

with collagenase, followed by the seeding of the heterogeneous cell populations.[25] The basic principle of selecting ASCs from the SVF fraction relies on their ability to adhere to the surface of the culture flasks, which does not avoid the adherence of other cell populations that easily stay in culture. For this reason Rada *et al* developed and optimized an isolation/selection method, based on the use of immunomagnetic beads coated with different antibodies, to obtain ASC populations with lower amounts of other cell types.[25] Following the same method, Mihaila *et al* explored the differentiation potential of a subpopulation defined by the expression of the pluripotency marker, SSEA4.[26] The later study revealed that under specific culture conditions SSEA4^{+}-hASCs subpopulation can give rise to both osteoblastic and microvascular endothelial-like cells, which comprises a step forward to create, from the same cell source, a bone tissue engineering (TE) construct.[26]

Taking into consideration the biology of bone tissue, it is understandable that the cellular interactions that regulate the homeostasis and regeneration of this tissue are of great importance to consider when designing a cell based TE approach. The close relationship between bone tissue and blood vessel is well studied. Bone formation is temporal and spatially accompanied by the development of the vascular system and at cellular level it is well documented the reciprocal crosstalk between osteoblasts and endothelial cells.[27-29]

The study of these cell interactions *in vitro* rely on co-cultures systems.[30] The establishment of co-cultures is far from being trivial. There are a number of theoretical and practical considerations to take into consideration.[31] It is understandable that the success of tissue regeneration strategies depends in part in the reintroduction of in vitro expanded cells in a state that guarantees the functionality and differentiation of both cell types involved in the system. For this reason, it is mandatory to proceed to a simultaneous optimization of the cell culture conditions that will lead to the best outcome results. In fact, the importance of cellular crosstalk in TE field is increasing the interest in developing co-culture models.

So far, several co-culture reports described different experimental set-ups regarding the cell source, which usually is not the same for osteoblasts and endothelial cells, culture media and the relative proportions of each cell type in the system, highlighting the lack of consensus concerning the optimal conditions for such a culture. Moreover these experimental set-ups compared the co-culture system with the monocultures and focused only on the evaluation either of osteogenesis or angiogenesis, not in both.

For instance, Villars *et al* co-cultured different cell ratios of bone marrow stromal cells (BMSCs)/HUVECs in proliferation media for OBs, demonstrating good results in terms of

osteogenic outcome, showing that ALP activity was increased when in co-culture compared with the monoculture.[4] Rouwkema and colleagues used the same cell source either for MSCs or endothelial cells. The tested culture media was osteogenic media, and they simultaneously defined different cell ratios. His analysis was focused only on the angiogenic outcome, finding an optimal CD31$^+$ staining for co-cultures with the same BMSCs/HUVECs proportions, although finding different conclusions concerning the formation of vessel-like structures, being more favorable in cocultures with higher BMSCs number.[9] Taking this into account, we aimed to evaluate both osteogenic and angiogenic outcome parameters for co-cultures of preconditioned SSEA4$^+$-hASCs to the osteogenic lineage and SSEA4$^+$-hASCs derived endothelial cells using different cell ratios and cell culture media. In detail, we aimed to study how these parameters would influence the maintainance of the osteogenic and endothelial differentiation potential of the SSEA4$^+$-hASCs in coculture. Therefore, the experimental set up included the co-culture of SSEA4$^+$-hASCs derived OBs and ECs at different cell ratios (3:1-1:1-1:3) in different culture media – EGM, Osteogenic media (OST) and EGM supplemented with osteogenic differentiation factors (EGM OST). The outcome parameters were cell differentiation and mineralization for osteogenesis and immunological staining of endothelial markers for angiogenesis.

2. MATERIALS AND METHODS

2.1. CELL CULTURE

Human abdominal subcutaneous fat tissue samples were obtained from healthy females, with an average age of 42years old, undergoing lipoaspiration procedures, after informed consent. The lipoaspirates were kindly provided by Hospital de Prelada, Porto, Portugal, under a collaboration agreement with 3B´s Research Group. The lipoaspirates were processed according to a standard isolation protocol optimized by Mihaila et al for the isolation of the subpopulation SSEA$^+$-hASCs from the SVF of adipose tissue. The selected cells, SSEA4$^+$hASCs, were cultured in basal medium – Alpha-Minimal Essential Medium (α-MEM, Sigma Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% antibiotic/ antimycotic (Invitrogen, The Netherlands) and in Microvascular Endothelial Growth Medium (EGM 2-MV, Lonza, USA) right after the isolation and selection, up to 14 days in culture, obtaining SSEA4$^+$-hASCs derived ECs. [32] A pre-conditioning to the osteogenic lineage of the SSEA4$^+$-hASCs cultures, previously expanded in α-MEM, was performed, by culturing those in alpha-medium supplemented with 50µg/mL ascorbic acid (Sigma Aldrich, Germany), 10^{-8}M dexamethasone (Sigma Aldrich, Germany), 10mM β-

glycerophosphate (Sigma Aldrich, Germany), 10% FBS and 1% antibiotic/antimycotic (Osteogenic Media, OST) for 7 days, with a cell density of 2000cells/cm^2 – SSEA4$^+$-hASCs OBs.

In order to establish the co-culture consitions, a preliminary analysis of each monoculture of SSEA4$^+$hASCs-OBs and SSEA4$^+$hASCs-ECs was performed to evaluate the cell behavior either in EGM 2-MV, OST or EGM 2-MV supplemented with the same osteogenic differentiation factors that constitutes the osteogenic media, at the same concentrations.

The SSEA4$^+$-hASCs OBs and SSEA4$^+$-hASCs derived ECs were seeded at a density of 2000cells/cm^2 following different cell ratios of SSEA4$^+$-hASCs OBs: SSEA4$^+$-hASCs derived ECs, namely – 3:1, 1:1 and 3:1. SSEA4$^+$-hASCs OBs and SSEA4$^+$-hASCs derived ECs monocultures were used as a control and were seeded at the same cell density.

2.2. CELL PROLIFERATION

Cell proliferation of the SSEA4$^+$-hASCs OBs and SSEA4$^+$-hASCs derived ECs either in monoculture or in co-culture was assessed by quantification of the double stranded (ds) DNA content along the culture using the Quant-iTTM Picogreen Kit (Invitrogen, The Netherlands) following the instructions of the manufacturer, in a lysed cell suspension obtained after osmotic and thermal shocks. Fluorescence was measured at 485nm of excitation and 528 of emission in a Synergy HT microplate reader (Bio-Tek, USA).

2.3. ALKALINE PHOSPHATASE ACTIVITY AND STAINING

The ALP activity quantification was performed in the same cell lysates used for the DNA quantification and obtained by osmotic and thermal shocks. The enzyme reaction was set up by incubating 20µl of sample with 60µL of a 0.2% w/v p-nytrophenyl phosphate (pnPP) in 1M Diethanolamine HCl solution with a pH of 9.8 for 45 minutes, at 37ºC. A calibration curve was prepared with the concentration standards ranging from 0 to 0,20 µmol/ml mixing a p-nytrophenol (pNP) stock solution (10 µmol/ml) in a solution of 2M NaOH/0.2mM EDTA solution in distilled water (STOP Solution). The reaction was stopped by adding 80µl of a STOP Solution. The optical density was determined at 405nm, with a reference filter at 602nm. The results were expressed in µmol of pNP hidrolized that correlates with the ALP activity and normalized against dsDNA values.

The cultures were also stainined for ALP was stained to determine the cells that were positive for ALP activity. SSEA4-hASCs pre-conditioned OBs and SSEA4^{+}hASCs derived ECs in monoculture and in co-culture were seeded on TCPS slides (Sarstedt, Newton, NC, USA). After each time point cells were washed for three times with PBS (with no Ca^{2+} or Mg^{2+}) and fixed in 3.7% v/v solution of formaldehyde in PBS for 30 minutes at room temperature. After the removal of the fixative, cells were washed twice with PBS. A solution composed of N,N-Dimethylformamide (DMF) (Merck, Germany) and Naphthol AS-MX phosphate, 0.25% (w/v), buffered at pH 8.6 (Sigma Aldrich, Germany) which was added to the previously prepared solution of Tris-HCL 0,2M with distilled water, buffered at pH 8.3 with Sodium hidroxyde. The Fast Red Violet LB salt (alkaline solution, 0.104mg/mL) (Sigma Aldrich, Germany) was mixed at last. The final solution was added to the samples and they were kept in the dark for 45 minutes at room temperature. Finally the cells were washed with distilled water 3-4 times and the photographed under a stereo microscope Stemi 1000 (Zeiss, Germany).

2.4. ALIZARIN RED STAINING

SSEA4-hASCs OBs and SSEA4^{+}hASCs derived ECs in monoculture and in co-culture were fixed at the different time points with 10% formalin solution and washed, firstly with PBS and then with distilled H_2O (diH_2O). The cells were then incubated for 10 minutes with a 2% Alizarin Red solution (Merck, Germany) in diH_2O, at a pH of 4.1-4.3. After incubation, cells were again washed with diH_2O and the staining observed under a

2.5. REAL TIME-RT PCR

The mRNA of SSEA4^{+}hASCs-OBs and SSEA4^{+}hASCs derived ECs monocultures and in co-culture at all the defined conditions was extracted with TriZol Reagent (Invitrogen, USA) following the procedure provided in the datasheet of the product. In detail, 800 µl of TriZol Reagent were added to each sample. After an incubation of 5 min, 160 µl of chloroform (Sigma Aldrich, Germany) were added, and agitated vigorously in the vortex for 15 seconds; the samples were then incubated for 15 minutes at 4°C and centrifuged at the same temperature and 13000 x rpm for 15 minutes at the same temperature. After the centrifugation, the aqueous part was collected and an equivalent volume of isopropanol (Sigma Aldrich, Germany) was added. Samples were incubated overnight at -20ºC. On the following day, all the samples were centrifuged at 13000 x rpm for 15 minutes at 4ºC and then washed with 800 µl ethanol 70% prepared with RNase/DNase

free water (Gibco, UK), centrifuged at 4ºC and 9000rpm for 5 minutes. The supernatant was carefully discarded and resuspended in water RNase/DNase free. The RNA quantity and purity were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using the qScript cDNA Synthesis kit (Quanta Biosciences, USA), and the Mastercycler ep Realplex gradientS (Eppendorf, USA), using an initial amount of RNA of 1 µg in a total volume of 20 µl of RNAse free water (Gibco, UK).

2.5.1. QUANTITATIVE REAL TIME PCR

The expression of the markers of the osteogenic and endothelial phenotype was assessed on each sample after the mRNA extraction and the synthesis of the cDNA by Real Time RT-PCR. The reaction was carried out with 5ng of cDNA, the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences, USA). The primers were previously designed using the Primer 3 online software (v 0.4.0) and synthesized by MGW Biotech (Germany) (supplementary Table 1). For each sample, glycetaldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. A concentration of 300 nM was used for all the primers in a final volume of 20µL of sample. The relative quantification of the gene expression was performed using Pffafl method.[33] The values were normalized against the housekeeping gene GAPDH and then against the control; monoculture of SSEA4^{+}hASCs-OBs (1:0) in osteogenic medium at day 7, and the monoculture of SSEA4^{+}hASCs-derived ECs (0:1) in EMG 2-MV at day 7, respectively for Osteocalcin expression.

2.6. IMMUNOCYTOCHEMISTRY

The expression of the endothelial cell markers CD31 and vWF as well as the expression of the osteogenic marker Osteopontin and Osteocalcin of SSEA4^{+}hASCs-OBs and SSEA4^{+}hASCs-ECs monocultures and in co-culture at all the defined conditions, was assessed by immunocytochemistry. For this purpose, co-cultures were established on TCPS slides as described for the co-culture procedure. At days 7, 14 and 21 all the samples were washed twice with PBS and fixed with 3,7% formalin 30 minutes at RT. All the samples were then washed twice with PBS and kept at 4ºC in PBS.

For the evaluation of the endothelial markers expression, which required intracellular staining, cells were washed with PBS and permeabilized with a 0.2% Triton solution for 5 minutes. For all the

conditions, nonspecific antibody binding was blocked with an incubation with a 3% BSA in PBS (blocking solution) for 30 minutes. Cells were then incubated with the primary antibodies – mouse anti-human CD31 (1:50 Dako, Denmark), rabbit anti-human von Willebrand factor (1:200 vWF Dako, Denmark), mouse anti-human Osteopontin and rabbit antihuman Osteocalcin (1:50 AbD Serotec, UK) – for one hour, RT and after appropriate dilution in 1.5% BSA/PBS. A serie of washing cycle (3x5minutes) followed the incubation with the primary antibody. Then the samples were incubated with the secondary antibodies: donkey anti-rabbit Alexa Fluor 488 (green) (Invitrogen, USA), and goat anti-mouse Alexa Fluor 594 (red), (Invitrogen, USA) diluted 1:500 in 1.5% BSA/PBS for 1 hour at RT, after which a new washing cycle of 3x 5 minutes was perfomed. Cell nuclei were counterstained with 4, 6-Diamidino-2-phenyindole dilactate (DAPI), at a 1:10000 dilution in PBS, for 10 minutes and then washed 3 times. Negative control samples were prepared by replacing the primary antibody incubation by PBS. Immunolabelling was qualitatively analyzed under the Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany) and photographed using the Axio Cam MRm camera (Zeiss, Germany) and the AxioVision 4.8 software (Zeiss, Germany).

3. RESULTS

3.1. DEFINITION OF CO-CULTURE CONDITIONS

SSEA4$^+$-hASCs-OBs and SSEA4$^+$-hASCs derived ECs behavior was evaluated in three different culture media aiming to define the best conditions to maintain or enhance the osteogenic and endothelial phenotype of the co-culture subpopulations.

A preliminary analysis of each monoculture of SSEA4$^+$hASCs-OBs and SSEA4$^+$hASCs derived ECs was performed in order to evaluate the cell behavior either in EGM, OST and EGM OST. Both cell monocultures could normally proliferate in all cell culture media except for the monoculture of SSEA4$^+$hASC derived ECs that in OST media that after 7 days in culture cells did not survive (data not shown). Despite the results obtained with the SSEA4$^+$-hASCs derived ECs in OST media, this culture media was maintained in the co-culture in order to analyze how would SSEA4$^+$-hASCs OBs influence the cell behavior of SSEA4$^+$-hASCs derived ECs in co-culture conditions, in the absense of supplied endothelial growth factors. In addition to cell survival, the maintenance of the endothelial phenotype was also considered a crucial issue. Therefore, the expression of CD31 and vWF was assessed in the SSEA4$^+$-hASCs derived ECs cultured in EGM OST. The results showed that after 14 days in culture, the endothelial phenotype was stable (data not shown).

3.2. CO-CULTURE PERFORMANCE

3.2.1. CO-CULTURE MORPHOLOGY

Co-cultures were established by seeding the two cell populations in direct contact at the cell ratios of 3:1 - 1:1 and 1:3 (SSEA4$^+$-hASCs OBs:SSEA4$^+$-hASCs derived ECs). Both cell populations were cultured in monoculture and in co-culture in the selected culture media. Cell morphology was followed by light microscopy observation; At day 7 which showed cells in co-culture presented morphologies similar to monocultures in all co-culture conditions. (Figure 1) Along the following 2 weeks, cells proliferated becoming integrated therefore the morphological differences between the fibroblastic between the fibroblastic-like morphology of SSEA4$^+$-hASCs OBs and the cobblestone morphology characteristic of SSEA4$^+$-hASCs derived ECs were less evident as the cell density increased in every co-culture condition. SSEA4$^+$-hASCs derived ECs in monoculture demonstrated the culture medium dependent behavior observed on the preliminary analysis – characterized by cell death in the SSEA4$^+$-hASCs OBs monoculture after 7 days of culture in OST media.

3.2.2. CELL PROLIFERATION

Regarding cell proliferation (Figure 2), SSEA4$^+$-hASCs OBs proliferation occurred in all culture media and showed the higher proliferation rate when cultured in OST media. Lower rate was found when these cells were cultured in EGM OST. The SSEA4$^+$-hASCs derived ECs monocultures showed a significantly high proliferation in EGM, although these cells were also able to proliferate in the presence of osteogenic differentiation factors. In OST media SSEA4$^+$-hASCs derived ECs did not survive, as previously mentioned.

As a whole, the proliferation rate regarding the co-cultures in EGM increased with higher proportions of ECs in the system (3:1<1:1<1:3), showing with values for DNA content at day 21 clearly increasing. The values varied from 4ng/ml for the 3:1, 7ng/ml for the 1:1 and 14ng/ml for the 1:3 cell ratios, being the last one comparable to the monoculture of SSEA4$^+$-hASCs derived ECs. In what concerns the OST media, the proliferation rate of the co-cultures increased untill day 14, followed by a decrease of the cell proliferation in the last week. This decrease is more evident with the increase of SSEA4$^+$-hASCs derived ECs in the system (3:1<1:1<1:3). SSEA4$^+$-hASCs derived ECs death was confirmed by the complete absence of any DNA content in all the time points. In EGM OST, SSEA4$^+$-hASCs OBs have a reduced proliferation rate compared with the results for the other culture media. Regarding the cell ratio in EGM OST, the proliferation rate for the cell ratio with the highest amout of SSEA4$^+$-hASCs derived ECs (1:3) is higher (6ng/ml) then

the cell ratio with higher amount of SSEA4^{+}-hASCs OBs (3:1), however, at the cell ratio with the same proportion of both SSEA4^{+}-hASCs OBs and SSEA4^{+}-hASCs ECs (1:1) at day 21 the proliferation rate reaches values comparable to the monoculture SSEA4^{+}-hASCs derived ECs in EGM.

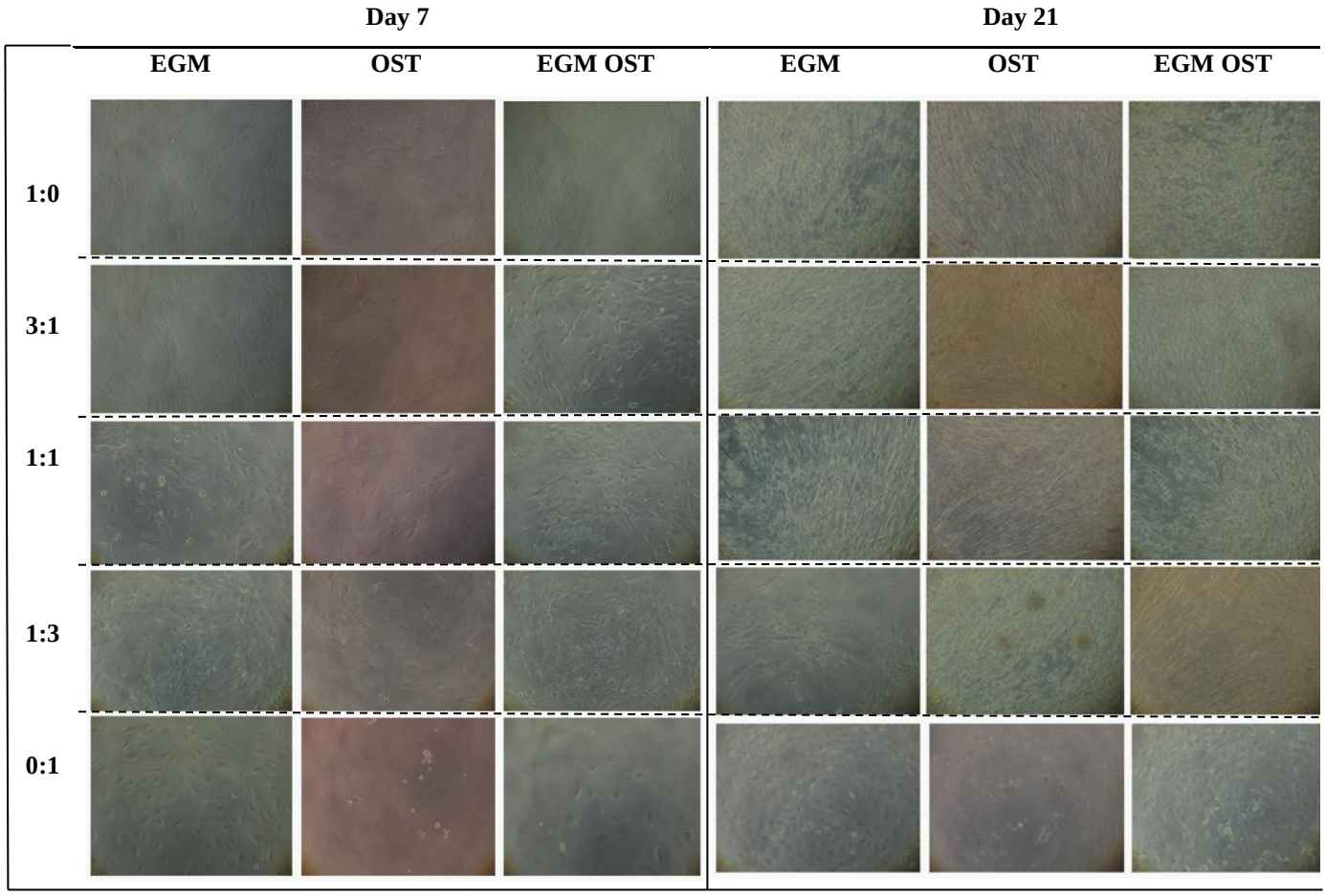

Figure 1. Optical micrographs showing the morphology of SSEA4+-hASCs OBs and SSEA4+-hASCs derived ECs in monoculture and in co-culture, cultured in EGM, OST and EGM OST after 7 and 21 days after cell seeding. At day 7 is still possible to detect the cell morphology differences between both cell types in co-culture (3:1 , 1:1 and 1:3) in EGM and EGM OST; however, not visible in OST media.

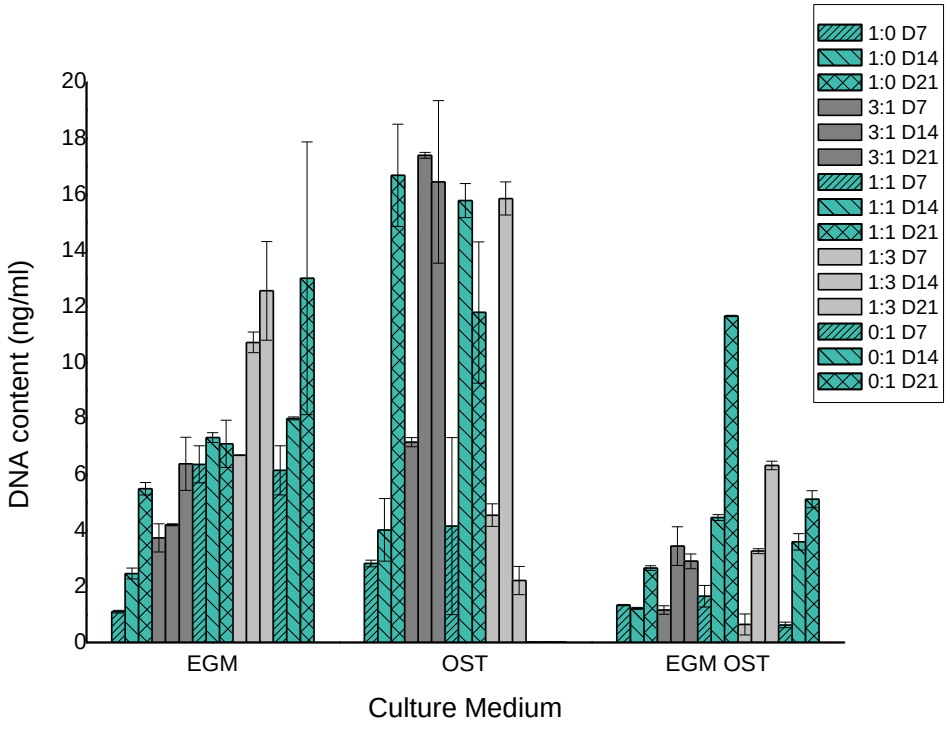

Figure 2. Amount of dsDNA that correlates with cell number quantified along culture – 7, 14 and 21 days – in monocultures of SSEA4+-hASCs committed into the osteogenic lineage and SSEA4+-hASCs derived ECs in three different culture media – EGM, OST and EGM OST. and in co-culture of SSEA4-hASCs committed cells onto endothelial and osteogenic lineage.

3.2.3. OSTEOGENIC AND ANGIOGENIC OUTCOME PARAMETERS

The influence of the simultaneous culture of SSEA4-hASCs OBs and SSEA^{+}-hASCs derived ECs and the effect of the culture media over the osteogenic and endothelial phenotype was assessed with basis on: (1) the ALP activity (2) extracellular matrix mineralization and (3) expression of OCN, OPN, CD31 and vWF.

The qualitative analysis of the activity of ALP in all monocultures of SSEA4^{+}-hASCs OBs and SSEA4^{+}-hASCs derived ECs and co-cultures was carried out after 21 days of culturing (Fig. 3A). It was possible to detect a more intense positive staining for the co-culture conditions in OST media, comparable to the monoculture of SSEA4^{+}-hASCs OBs in the same culture media. Independently of the SSEA4^{+}-hASCs OBs:SSEA4^{+}-hASCs derived ECs ratios less intense staining was visualized for the remaining co-culture conditions. Either for EGM or EGM OST, the co-culture ratio has the predominant effect in terms of intensity of the staining. A parallel comparison can be made between the cell ratios 3:1 and 1:3 in EGM and 1:3 and 3:1 in EGM OST to the monocultures of SSEA4^{+}-hASCs derived ECs, showing staining intensities similar to each other. A more intense

staining was visible for the co-culture ratio 1:1 in EGM and EGM OST, however, not so pronounced as the same co-culture ratio in OST media.

These results were further complemented in terms of ALP activity quantification by colorimetric assay (Fig 3B), performed on days 7, 14 and 21 after the seeding. ALP quantification was performed in the same cell lysates used for the DNA quantification. The present data show that the activity of the ALP was higher in all co-culture conditions in OST media, where the cell ratio 1:1 presented the higher value. However the cell ratio did not influence considerably the ALP activity as the co-culture ratios of 3:1 and 1:3 show quite comparable results. In EGM media, either the cell ratio 3:1 and 1:3 presented similar results in terms of the ALP activity, but when comparing with the same ratios in EGM OST, an increase is evident for both cases, reaching levels of 40 nmol/ng/hr in the case of the co-culture condition of 1:3 in EGM OST, not so different from the levels of the same cell ratio in OST media. For the monocultures of SSEA4^{+}-hASCs derived ECs in EGM and EGM OST, low values of ALP activity were detected.

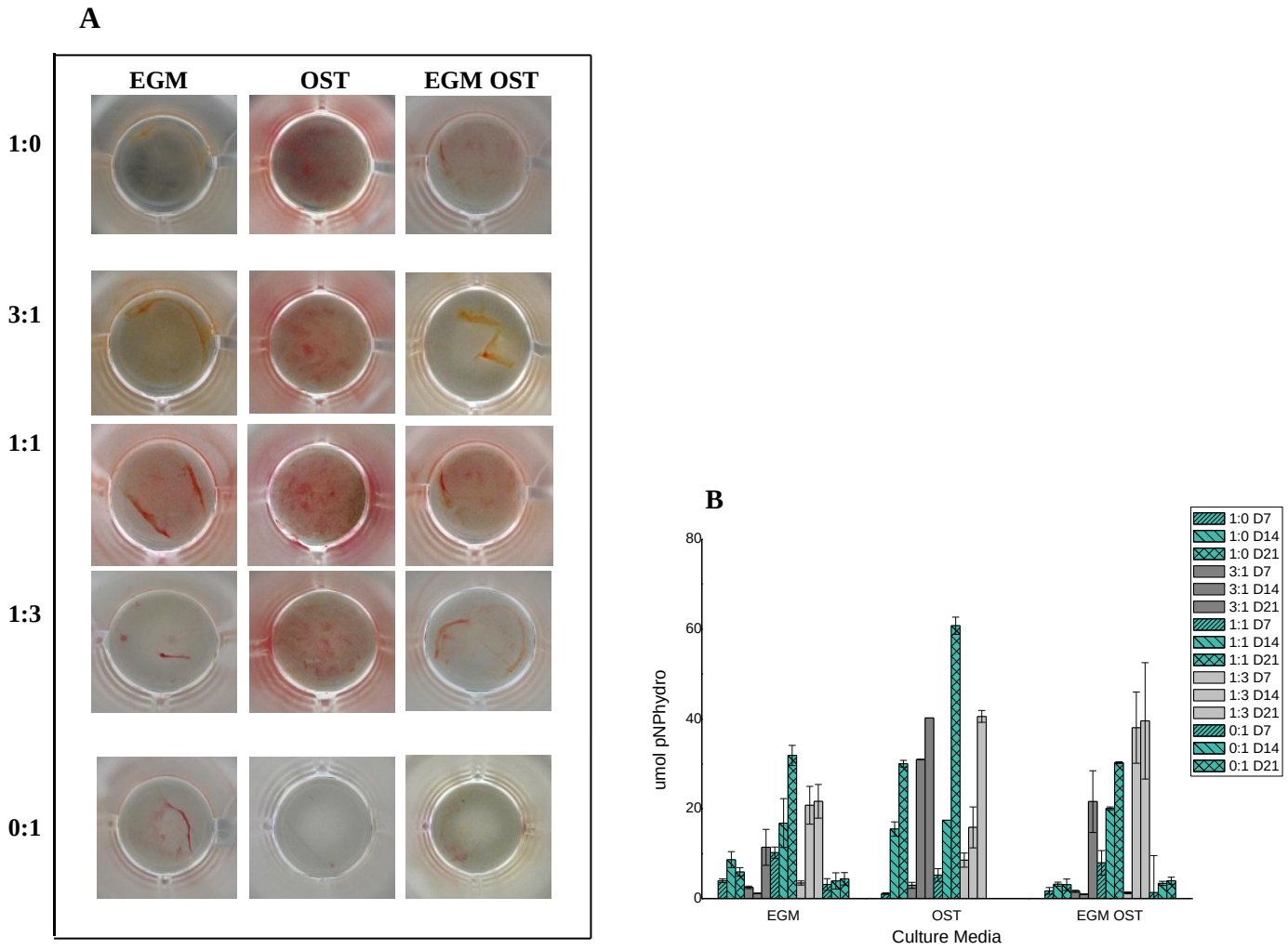

Figure 3. (A) Alkaline Phosphatase Staining at days 14 and 21 of culture in co-culture at different cell ratios – 3:1| 1:1| 1:3 – in three culture media – EGM, OST and EGM OST. Positive Control – monoculture of SSEA4+-hASCs committed into the osteogenic lineage; Negative control: SSEA4+-hASCs derived ECs. (B) Amount of hydrolised p-nitrophenol phosphate that correlates with the ALP activity quantified along culture – 7, 14 and 21 days – in monocultures of SSEA4+-hASCs committed into the osteogenic lineage and SSEA4+-hASCs derived ECs and in co-culture at different cell ratios – 3:1| 1:1| 1:3 – in three different culture media – EGM, OST, and EGM OST.

Mineralization of the deposited extracellular matrix and consequently the detection of the calcium deposits of each co-culture condition were evaluated by Alizarin Red staining after 21 days in culture. The Alizarin Red data evidences a moderate mineralization for both cell ratios 3:1 and 1:3 either in OST or EGM OST, but more pronounced for both cell ratios cultured in EGM OST rather than in EGM. Considering the OST media, the mineralization pattern was more pronounced than in other culture media, where the cell ratio did not seem to originate big differences in the staining, being comparable to the mineralization pattern of the SSEA4^{+}-hASCs OBs in OST media. Thus no or minor signs of mineralization could be observed for the monoculture of SSEA4^{+}-hACSs derived ECs.

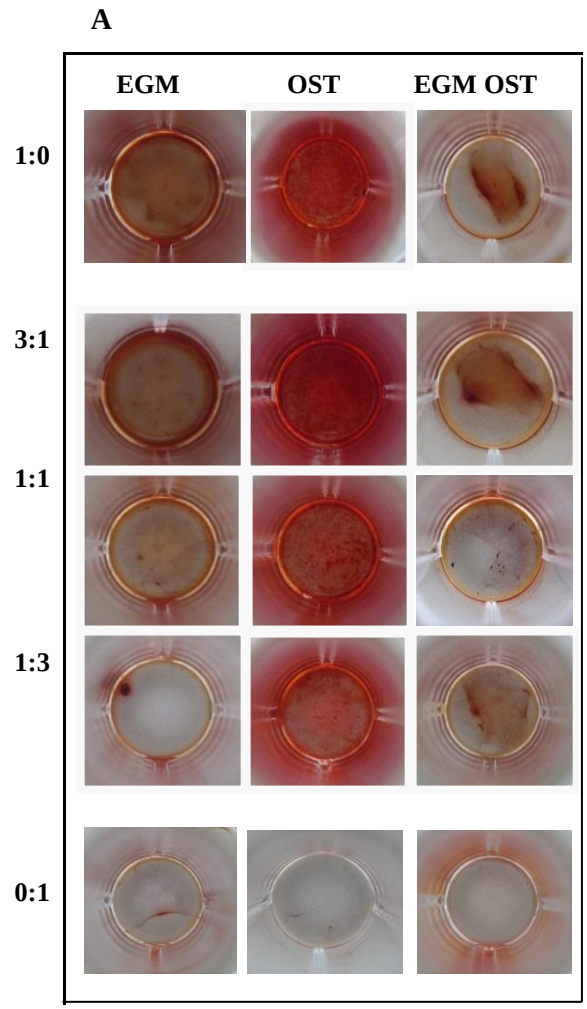

Figure 4. (A) Alizarin Red staining results after 21 days of culture, to perform the evaluation of calcium-rich deposits and mineralization of the extracellular matrix in the systems: in monocultures of SSEA4+-hASCs committed into the osteogenic lineage and SSEA4+-hASCs derived ECs (as controls, positive and negative, respectively) and in co-culture at different cell ratios – 3:1| 1:1| 1:3 – in three different culture media – EGM, OST, and EGM OST.

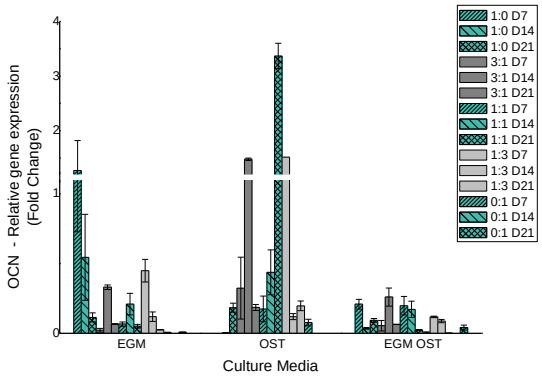

Figure 5. Osteocalcin relative expression calculated after quantitative Real Time RT-PCR. Results were first normalized against GAPDH and then against the monoculture of SSEA4^{+}-hASCs OBs after 7 days of culture and are presented as fold-change.

At the molecular level, the osteocalcin expression relative to SSEA4^{+}-hASCs OBs at day 7 of culture was screened using real time RT-PCR (Fig 5) after 7, 14 and 21 of co-culture. The expression levels of OCN gene did not follow the same pattern for the different culture media. It was observed a generalized downregulation of the OCN gene in all the co-culture conditions in EGM and in EGM OST, as well as for the monocultures of SSEA4^{+}-hASCs OBs in OST and EGM OST. In EGM media it was observed an a fold increase of about 1.2 for the monoculture of SSEA4^{+}-hASCs OBs at day 7 that was not verified along with the culture time. Not so different results were found for the coculture ratio 3:1 and 1:3 in OST media, at days 14 and 7 respectively. The higher fold change was found for the 1:1 cell ratio in OST at day 21, presenting a fold change between 3 and 4.

The results from the immunocytochemistry evaluation for the osteogenic differentiation markers (Fig.5A1 and A2) – Osteopontin and Osteocalcin – revealed in general a significant synthesis and deposition of OPN in all co-culture conditions. The staining pattern was positive for the co-cultures all defined culture media – EGM, OST and EGM OST. Concerning the OCN protein staining no positive result was found after 21 days in culture. The immunostaining results for endothelial markers: vWF and CD31 (Fig.4B) revealed that the number of endothelial colonies in the co-culture conditions, distributed with the cobblestone rearrangement characteristic to these cells. The size of the colonies was directly proportional to the number of ECs in the system. No positive staining was found for the co-cultures in OST media or in the SSEA4^{+}-hASCs OBs monocultures in EGM, OST and EGM OST.

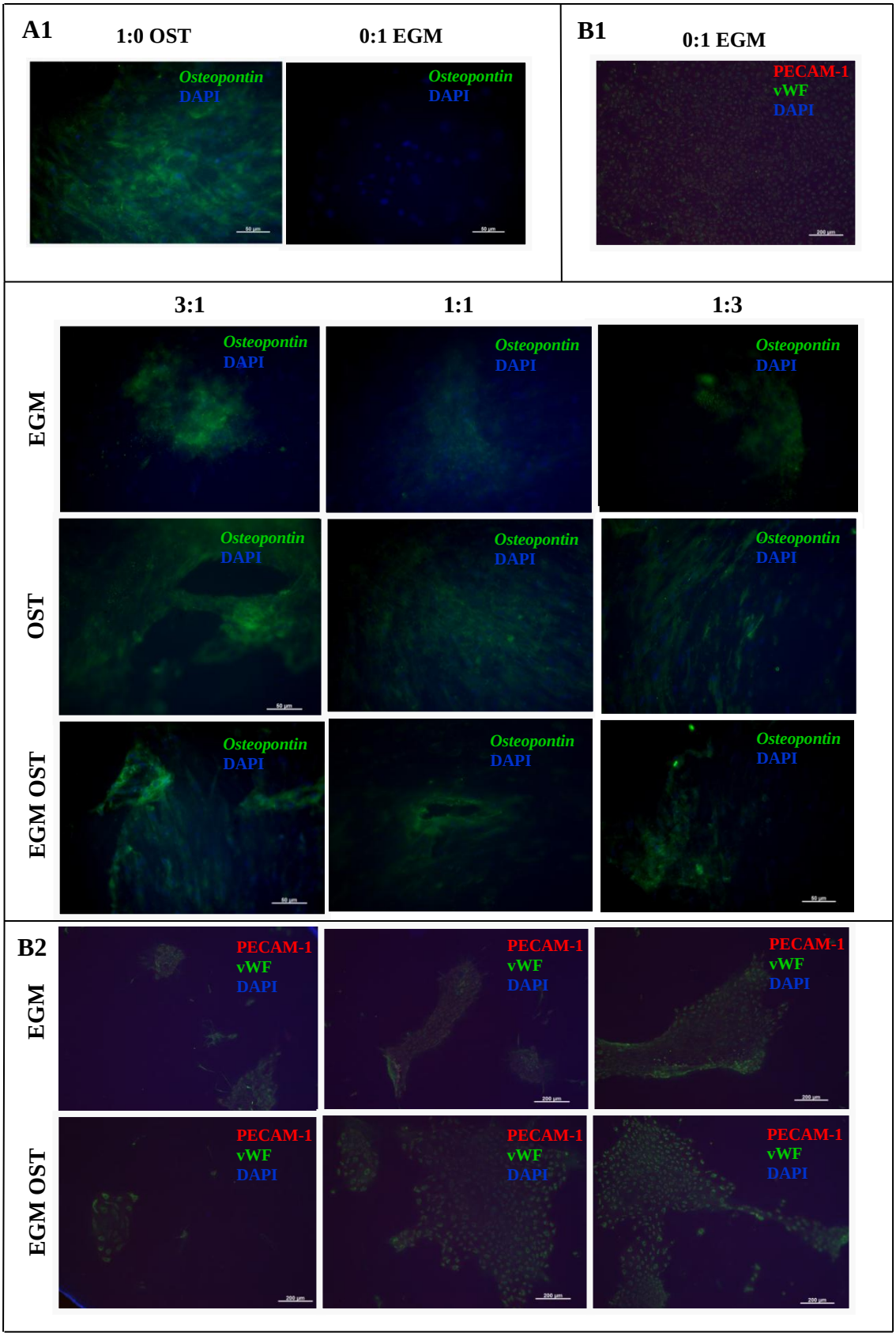

Figure 6. (A1 and A2) Immunostaining of SSEA4^{+}-hASCs OBs and ECs in monoculture and in co-culture for Osteopontin (green) and Osteocalcin (red) markers. (B1 and B2) SSEA4^{+}-hASCs derived ECs in monoculture and SSEA4^{+}-hASCs OBs and ECs in co-culture in EGM and EGM OST for the analysis of vWF (green) and PECAM-1 (red); No signs of staining were found for the conditions in OST media.

4. DISCUSSION

In bone, the reciprocal crosstalk between osteoblasts and endothelial cells is well recognized. Factors produced by endothelial cells may therefore affect osteoblast function or differentiation and vice versa. [5, 34] Osteoblasts are also able to produce paracrine factors that influence endothelial cell function. [35]

Aiming at the establishment of a co-culture system of SSEA4+-hASCs OBs and SSEA4+-hASCs derived ECs for purposes of bone tissue engineering is imperative to define the ideal culture conditions to respective promote and maintain the osteogenic and endothelial phenotypes.

The selection of the subpopulation of interest was based on the study of Mihaila *et al* that proved the endothelial differentiation capacity of the SSEA4+-hASCs subpopulation by culturing these cells in EGM, and the osteogenic differentiation by culture this subpopulation under standard osteogenic conditions.[26] As reviewed by Grellier *et al* there are some studies on the reciprocal regulation and functional relationship between bone and endothelial cells which may be greatly influenced by the culture conditions.[19] For these reasons, a compromise media, which can assure the possibility to sustain all the cellular subpopulations involved, has to be delineated. In a preliminary study, and due to the influence that those results would had on the following analysis both cells types – SSEA4^{+}-hASCs OBs and SSEA4^{+}-hASCs derived ECs were cultured as monoculture in EGM, OST and EGM OST. Based on this analysis, it was realized that the SSEA4+-hASCs derived ECs were the most sensible cell subpopulation. This might be due to its dependence on the exogenous growth factors present in the EGM-2MV culture media to sustain the endothelial phenotype.[26] Taking this into account, the co-culture system was set in EGM, commercially available as support media for microvascular endothelial cells, in a standard osteogenic media known to induce osteogenic differentiation of hASCs-SSEA4^{+} subpopulation and in EGM, supplemented with osteogenic differentiation factors, hypothesizing that the SSEA4+-hASCs derived ECs were able to proliferate maintaining the endothelial phenotype and that the pre-conditioned hASCs-SSEA4+ (SSEA4+-hASCs OBs) continued the osteogenic differentiation process. The data obtained from the preliminary study allowed moving forward and analyzing quantitatively the influence of the cell culture media and the cell ratio on the angiogenic and osteogenic outcomes of the co-culture system.

Several studies so far provided some insights about the relation between OBs and ECs in co-culture; however, there is still limited information about cell proliferation rate of cells in co-culture. The analysis of cell proliferation showed a higher rate for all co-culture conditions when compared with the corresponding monocultures of SSEA4^{+}-hASCs OBs and SSEA4^{+}-hASCs derived ECs in the same culture media. The cell proliferation results for the EGM culture media showed that the

proliferation rate in the co-culture 1:3 is similar to the values of the monoculture of SSEA4$^+$-hASCs derived ECs. Moreover, the proliferation rate regarding the co-cultures in EGM increased along with higher proportions of ECs in the system (3:1<1:1<1:3), which was expected as EGM corresponds to the optimal culture media for the population SSEA4$^+$-hASCs derived ECs.[26] Considering the OST media, it was expected a less pronounced proliferation rate comparing to the EGM media as it is proved that the cell proliferation rate decreases during the osteogenic differentiation.[36] In fact this was verified for the co-culture ratio 3:1, however considering the proliferation results for the co-culture ratio 1:1, 1:3 and the monoculture of SSEA4-hASCs derived ECs, the decreasing observed between day 14 and 21 may be explained by the cell death of the SSEA4$^+$-hASCs derived ECs, corroborated by the immunostaining negative results for all the co-culture conditions and monocultures in OST media. Additionally, the production, deposition and mineralization of the extracellular matrix might also explain the observed proliferation rate decrease between day 14 and 21. In fact, there is both *in vitro* and *in vivo* [39] evidence that deposition of a collagenous matrix in a transitional stage, characterized by deposition of an insoluble type I collagen-rich extracellular matrix and an initial rise in ALP activity, may regulate or at least have a permissive effect on the third stage of maturation of the osteoblast phenotype. The third stage is characterized by further increase in ALP activity, expression of osteocalcin, and mineral deposition. The levels of osteocalcin are low at early stages and increase with increasing age, while osteopontin follows the inverse pattern as corresponds to an early marker for osteogenic differentiation.[38] The Alizarin Red assay results showed intense mineralization of the co-culture conditions in OST culture media, corroborating that SSEA4$^+$-hASCs OBs are differentiating towards the osteogenic lineage as indicated by the upregulation of the osteocalcin gene for the co-culture conditions in OST media. However the gene expression was not traduced into protein deposition as the immunocytochemistry staining did not show positive results for osteocalcin marker either in SSEA4$^+$-hASCs OBs monoculture or in co-culture conditions.

In EGM OST, the data shows that the monoculture of SSEA4$^+$-hASCs OBs and the co-culture ration 3:1 show a proliferation rate lower than in the EGM media. This result points towards the potential stimulatory effect of SSEA4$^+$-hASCs derived ECs to stimulate the proliferation of the SSEA4$^+$-hASCs OBs. On the other hand, SSEA4$^+$-hASCs OBs could have curtail the ECs capability for expansion corroborating the results of the immunostaining for the endothelial markers: CD31 and vWF, which revealed few and small endothelial colonies in culture. At the same time, the monoculture of SSEA4$^+$-hASCs derived ECs did not show cell proliferation results similar to the monoculture EGM media, which may suggest an effect of the presence of the osteogenic differentiation supplements over the cellular proliferation, namely the β-glicerophosphate. In fact a recent study revealed the synergistic effect β-glicerophosphate in the

endothelial progenitor cell ossification. The presence of this supplement in the media may be affecting the proliferation of both SSEA4$^+$-hASCs derived OBs and ECs population, despite the presence of the endothelial growth factors in the media. This statement is corroborated by the number of the endothelial cell colonies revealed by the immunostaining analysis for endothelial markers that was reduced comparing to the EGM media. For the case of the co-culture ratio 1:3, the proliferation rate value at day 21 was similar to the monoculture of ECs in EGM at day 7, which may result of the influence of the presence of the osteogenic differentiation factors, as referred previously.[37] However, when considering the immunostaining results for the endothelial markers, the number of endothelial colonies is higher comparing with the cell ratios with lower percentage of SSEA4$^+$-hASCs ECs in EGM OST. By the other hand, the co-culture ratio 1:1 revealed an increased proliferation rate when compared with the monoculture of SSEA4$^+$-hASCs OBs and SSEA4$^+$-hASCs derived ECs in EGM OST, and similar to the monoculture of SSEA4$^+$-hASCs derived ECs in EGM. The immunocytochemistry analysis revealed the deposition of Osteopontin either in 3:1, 1:1 or 1:3 cell ratios. Overall, the intensity of the immunostaining for osteopontin in EGM and EGM OST was more discrete when compared to the SSEA4$^+$-hASCs OBs monoculture or to the co-cultures in OST, but more intense for the co-culture conditions in EGM OST, namely for the ratios 1:1 and 1:3, with higher percentage of ECs. These results, along with the ALP activity analysis and the mineralization results of Alizarin Red assay confirms the maintenance of the differentiation potential of the SSEA4$^+$-hASCs OBs to the osteogenic lineage in EGM OST and revealed the cell ratio 1:3 had the most efficient osteogenic outcome parameters as well as great stability of endothelial phenotype along the culture period.

Collectively the present findings demonstrate that both cell culture media and cell ratio are important parameters for co-cultures of SSEA4$^+$-hASCs derived OBs and ECs. Therefore this work constitutes a step forward in the pursuing for a better understanding of the interaction between OBs and ECs derived from the same SSEA4$^+$-hASCs subpopulation, and for the use of this model in bone tissue engineering applications.

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IV. GENERAL CONCLUSIONS

1. GENERAL CONCLUSION

Bone formation is temporal and spatially accompanied by the development of the vascular system and at cellular level it is well documented the reciprocal crosstalk between OBs and ECs. The study of these cell interactions *in vitro* rely on co-cultures systems. Furthermore, the co-culture of ECs with OBs is one of the most promising approaches to promote the vascularization of bone TE constructs, a current major hurdle in this field. However, primary cells are difficult to obtain. Recently, a work developed in the 3B's Research Group suggests that a hASCs subpopulation, positive for SSEA4 marker, can be used to obtain endothelial cells presenting a stable phenotype. Besides, the same subpopulation can also be differentiated into osteoblasts. Therefore, the main aim of this thesis was to establish the optimal culture conditions, concerning the cell culture media and the cellular proportions for the co-culture of SSEA4$^+$-hASCs pre-conditioned OBs and SSEA4$^+$-hASCs derived ECs, aiming to analyze their influence the maintenance or improvement the osteogenic and endothelial phenotypes in the system. The complexity of the system allowed to conclude that a compromise has to be considered when defining the cell culture media to the co-culture system. In the defined system, the osteogenic phenotype of the SSEA4$^+$-hASCs preconditioned OBs was improved and the endothelial phenotype of the SSEA4$^+$-hASCs derived ECs was kept stable in the culture media EGM OST. The co-culture condition with the cell ratio SSEA4$^+$-hASCs OBs:SSEA4$^+$-hASCs derived ECs of 1:3 in EGM OST revealed the most efficient osteogenic outcome parameters as well as great stability of endothelial phenotype along the culture period. The established co-culture system has a great potential to overcome the vascularization limitations of the current Bone Tissue Engineering strategies.