Exploring the crosstalk between mesenchymal stem cells and endothelial cells in bone tissue engineering

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Abstract

Bone is one of the few organs that retains the potential for regeneration in adult life, as it possesses the ability to heal and to remodel. However, in several clinical settings bone is not able to heal by itself and further treatment is needed, such as in: bone fusion, reconstruction of a shattered bones, filling of bone defects caused by cysts or tumors, and stimulation of bone growth to help anchor an artificial joint or other implant. For most of these injuries, available treatments are still inadequate. Therefore, new strategies need to be considered.

In view of this evidence, in this thesis a bone tissue engineering strategy, based on an injectable system in combination with mesenchymal stem cells (MSCs) and endothelial cells (ECs), was developed. The proposed system could potentially act as a bone filler to stimulate the repair and to enhance the bone function.

As a starting point, it was investigated if a synergistic coculture system of MSCs with ECs could affect the number and state of differentiation of harvested MSCs. ECs have been previously shown to induce osteogenic differentiation *in vitro* and *in vivo,* when in direct contact with bone marrow stromal cells. However, in contrast with previously published reports on the interrelationship between these cell types, the present investigation was carried out for longer times, namely 3 weeks instead of 1 to 6 days, and different ratios of both cell types were considered. Additionally, several media were tested and the osteogenic commitment of MSCs in a non-osteogenic medium, in the presence of ECs, was carefully analyzed. Collectively, it was demonstrated that, by coculturing MSCs with human umbilical vein endothelial cells (HUVECs), osteogenic differentiation was promoted (and even enhanced, depending on the relative cell ratios used), and MSCs proliferation was significantly increased as well. Hence, ECs appear to not only modulate MSC phenotype but also their proliferation rate.

As a strategy to develop an injectable system able to deliver MSCs in a clinical setting they were immobilized within microspheres of alginate modified with the arginine-glycine-aspartic acid (RGD) peptide sequence. This hydrophilic polymeric network provided a 3D support for MSCs. Immobilized cells showed metabolic activity with a sustained viability, short cell extensions and, when induced, they were able to differentiate into the osteogenic lineage.

Since bone is a highly vascularized tissue the stimulation of vascularization is a

requirement of paramount importance in bone regeneration strategies. ECs play a key role in this process since they constitute the lining of blood vessels, and bone cells have been previously shown to influence ECs by secreting for instance vascular endothelial growth factor (VEGF), which is essential in angiogenesis. Therefore, the ability of MSCs immobilized within RGD-alginate microspheres to influence neighboring ECs was also investigated. MSCs produced VEGF that diffused through the hydrogel network into the surrounding medium, enhancing the ability of neighboring ECs to form tube-like structures in a reconstituted basement membrane (Matrigel).

An alternative way to promote angiogenesis consists in promoting the *in vitro* prevascularization of a biomaterial. Cell-based therapies offer an attractive approach for tissue revascularization. However, and despite the pressing clinical needs for effective revascularization strategies, the successful immobilization of viable vascular cells within 3D matrices has been difficult to achieve. The *in vitro* potential of RGD-alginate hydrogel to immobilized and deliver HUVECs was evaluated. HUVECs were able to proliferate and maintained a higher viability for at least 48h post-immobilization within the alginate matrix. Additionally, entrapped cells were able to create a 3D organization into cellular networks and, when put in contact with Matrigel, they migrated out of the RGD-matrix. Moreover, since this biomaterial was prepared from soluble precursors that promote its crosslinking *in situ*, this strategy was shown to support the *in vitro* formation of cellular networks by migrating cells and also to allow delivery of ECs at the injury site by minimally invasive methods. Overall, the potential of injectable alginate hydrogels as an *in situ* gel forming delivery systems for vascular cells was demonstrated.

In summary, this thesis was focused on the need to enhance vascularization in bone regeneration strategies and its main findings are based in the close relationship between osteoprogenitor cells and endothelial cells, namely: i) ECs enhance MSCs differentiation and proliferation; ii) immobilized MSCs within RGD-modified alginate stimulate tube-like structures formation by ECs; iii) RGD-alginate provides an adequate 3D environment to support ECs adhesion, survival, migration and organization. Moreover, the obtained results demonstrate that RGD-modified alginate hydrogel is a good candidate for minimally invasive bone tissue regeneration strategies, suitable to immobilize and deliver bone and endothelial cells.

Resumo

O osso é um dos poucos órgãos que conserva potencial de regeneração na idade adulta graças à sua capacidade de cicatrização e remodelação. Em certas condições clínicas, porém, o osso é incapaz de cicatrizar por si próprio, exigindo um tratamento auxiliar adequado. São disso exemplo a fusão óssea, a reconstrução de ossos planos, o preenchimento de defeitos causados por quistos ou tumores e a estimulação do crescimento ósseo para facilitar a ancoragem de articulações artificiais ou outros implantes. Os tratamentos disponíveis para a maioria destas lesões continuam a ser inadequados, razão pela qual novas estratégias devem der equacionadas.

Considerando estas evidências, a presente tese procurou desenvolver uma estratégia de engenharia de tecidos do osso baseada num sistema injectável em combinação com células estaminais mesenquimais (MSCs) e endoteliais (ECs). Actuando como um material de preenchimento, o sistema proposto pode potencialmente estimular a reparação do osso, incrementando também a função do mesmo.

Como ponto de partida avaliou-se se um sistema sinergético de co-cultura de MSCs com ECs pode influenciar o número e estado de diferenciação das primeiras. Ensaios anteriores têm demonstrado que ECs induzem a diferenciação osteogénica *in vitro* e *in vivo*, quando em contacto directo com células estromais de medula óssea. No entanto, e contrariamente aos estudos publicados acerca da inter-relação entre os referidos tipos celulares, a corrente investigação foi realizada durante períodos de tempo mais alargados (3 semanas em detrimento de 1 a 6 dias), tendo ainda contemplado diferentes proporções de ambos os tipos celulares em análise. Em paralelo, diferentes meios de cultura foram testados e o comprometimento osteogénico de MSCs na presença de ECs num meio não-osteogénico detalhadamente analisado. De uma forma geral, foi demonstrado que co-cultivando MSCs com células endoteliais de veia umbilical humana (HUVECs) a diferenciação osteogénica é promovida (ou até melhorada dependendo das proporções relativas de células usadas), sendo ainda a proliferação de MSCs significativamente aumentada. Assim, as ECs parecem não só modular o fenótipo das MSCs mas também a sua taxa de proliferação.

Com o intuito de desenvolver um sistema injectável capaz de fornecer MSCs em cenários clínicos, estas células foram imobilizadas em microesferas de alginato modificado com a sequência peptídica arginina-glicina-aspartato (RGD). A rede polimérica hidrofílica assim constituída proporcionou um suporte 3D para as MSCs. As células imobilizadas mostraram uma actividade metabólica com viabilidade sustentada, extensões celulares curtas e, quando induzidas, capacidade de diferenciação na linhagem osteogénica.

Sendo o osso um tecido altamente vascularizado, estimular a vascularização é um requisito de capital importância em estratégias de regeneração óssea. As ECs desempenham um papel crucial neste processo, uma vez que são parte constituinte da parede interna dos vasos sanguíneos. Por outro lado, a influência das células do osso nas ECs foi previamente demonstrada mediante a secreção de, por exemplo, factor de crescimento endotelial vascular (VEGF), elemento essencial na angiogénese. Por conseguinte, a possibilidade de MSCs imobilizadas em microesferas de alginato modificado com RGD influenciarem ECs vizinhas foi igualmente aferida. Nas nossas condições experimentais, as MSCs produziram VEGF que, ao difundir-se pela rede de hidrogel até ao meio circundante, incrementou a capacidade das ECs vizinhas formarem estruturas tubulares numa membrana basal reconstituída (Matrigel).

Uma forma alternativa de potenciar a angiogénese consiste em promover *in vitro* a pré-vascularização do biomaterial. As terapias baseadas em células constituem uma abordagem atractiva para a revascularização de tecidos. No entanto, e apesar da crescente urgência clínica em sistemas de revascularização profícuos, a imobilização eficiente de células vasculares viáveis em matrizes tridimensionais tem sido difícil de alcançar. Neste contexto, o potencial do hidrogel de alginato modificado com RGD imobilizar e fornecer HUVECs foi avaliado. As HUVECs foram capazes de proliferar e manter uma elevada viabilidade durante pelo menos 48h após imobilização na matriz de alginato. Cumulativamente, as células aprisionadas conseguiram criar redes celulares com organização tridimensional, tendo ainda migrado para o exterior da matriz RGD quando colocadas em contacto com o Matrigel. Para além disso, dado que este biomaterial foi preparado a partir de componentes precursores solúveis que determinam o seu *crosslinking in situ*, a estratégia seguida revelou sustentar a formação *in vitro* de redes celulares por células migrantes, permitindo ainda a aplicação de ECs no local da lesão através de procedimentos minimamente invasivos. Globalmente, a potencialidade de hidrogéis injectáveis de alginato como sistemas formados *in situ* para a distribuição de células vasculares foi demonstrada.

Em síntese, focando-se na necessidade de melhorar a vascularização nas estratégias de regeneração óssea, as principais conclusões desta tese decorrem da estreita interligação entre células osteoprogenitoras e endoteliais, nomeadamente: i) as ECs aumentam a diferenciação e proliferação de MSCs; ii) MSCs imobilizadas em alginato modificado com RGD estimulam a formação de estruturas tubulares por parte de ECs; iii) o alginato modificado com RGD proporciona um ambiente 3D adequado à adesão, sobrevivência, migração e organização de ECs. Finalmente, os resultados obtidos revelam que o hidrogel de alginato modificado com RGD representa um bom candidato para a imobilização e distribuição de células do osso e endoteliais subjacentes a estratégias de regeneração óssea minimamente invasivas.

Résumé

Le tissu osseux est l'un des rares organes capable de se reconstruire, même à l'âge adulte. En effet, l'os peut se ressouder après une fracture ou peut être renouvelé lorsqu'il vieillit. Cependant, dans le cas de pertes osseuses trop importantes (maladie congénitale, traumatisme, exérèse de kyste ou tumeur), il est nécessaire d'avoir recours à un traitement. De nos jours, les techniques de reconstruction proposées ne sont pas adéquates et il apparaît donc essentiel de considérer de nouvelles stratégies.

Cette thèse porte sur une stratégie d'ingénierie du tissu osseux basée sur le développement d'un biomatériau injectable qui serait associé à des cellules souches mésenchymateuses (MSCs) et des cellules endothéliales (ECs). A terme, ce système pourrait à la fois combler une perte osseuse et stimuler la réparation naturelle de l'os.

Dans un premier temps, ce travail a étudié l'hypothèse selon laquelle un système de coculture synergique entre les MSCs et les ECS peut stimuler la prolifération et la différenciation des MSCs. Il a déjà été démontré que les ECs pouvaient induire la différenciation ostéogénique *in vitro* et *in vivo*, lorsqu'elles étaient en contact direct avec les cellules stromales de moelle osseuse. Cependant, contrairement à des travaux publiés précédemment portant sur l'interrelation entre les ECs et les MSCs, cette étude a été réalisée sur des durées plus longues, à savoir trois semaines au lieu de 1 à 6 jours. De plus, des densités cellulaires différentes des deux types de cellules ont été considérées. Par ailleurs, plusieurs milieux de culture ont été testés et la capacité de différenciation ostéogénique des MSCs dans un milieu non ostéogénique mais en présence des ECs, a été soigneusement analysée. Les résultats ont démontré qu'en coculture avec les cellules endothéliales de veine ombilicale (HUVECs), la différenciation ostéogénique des MSCs est engagée (voire même véritablement stimulée, selon les ratios de cellules utilisés), et la prolifération des MSCs augmente de manière significative. Par conséquent, les ECs semblent non seulement moduler le phénotype des MSCs, mais aussi leur taux de prolifération.

Dans un contexte clinique et afin de développer un système injectable capable de délivrer des MSCs à un site de lésion, ces cellules ont été immobilisées à l'intérieur de microsphères d'alginate sur lequel a été greffé de manière covalente la séquence peptidique arginine-glycine-aspartique (RGD). Ce réseau polymère hydrophile fourni un support 3D pour les MSCs. Les analyses montrent que l'activité métabolique des cellules immobilisées augmente au cours du temps. Les cellules semblent capables de

se fixer à la matrice 3D en créant de courtes extensions cellulaires. Enfin, lorsque l'on induit leur différenciation, les MSCs s'engagent dans la lignée ostéogénique.

Le tissu osseux étant un tissu très vascularisé, la stimulation de la vascularisation est fondamentale lorsque l'on envisage une stratégie de régénération osseuse. Les ECs sont connues pour jouer un rôle clé dans ce processus de vascularisation car elles constituent la paroi interne des vaisseaux sanguins. Il a été aussi démontré que les cellules osseuses sont capables de sécréter un facteur de croissance essentiel à l'angiogenèse, le VEGF (vascular endothelial growth factor), et pourraient ainsi avoir une influence sur les ECs. Par conséquent, la capacité des MSCs à stimuler les ECs après leur co-immobilisation dans les microsphères d'alginate fonctionnalisé avec la séquence peptidique RGD a été également étudiée. Les MSCs produisent du VEGF qui diffuse à travers le réseau d'hydrogel, passe dans le milieu environnant et stimule la capacité des ECs à former des structures tubulaires au sein d'une membrane basale reconstituée (Matrigel).

Une autre façon de promouvoir l'angiogenèse consiste en la prévascularization *in vitro* d'un biomatériau. Des études de thérapies cellulaires offrent une approche intéressante pour la revascularisation des tissus. Cependant, et malgré les besoins cliniques urgents de stratégies de revascularisation efficaces, l'immobilisation de cellules vasculaires viables dans des matrices 3D a été difficile à réaliser. Ici, la faculté de l'alginate fonctionnalisé à immobiliser et délivrer des HUVECs *in vitro* a été évaluée. Les HUVECs ont été capables de proliférer et de maintenir leur activité métabolique pendant au moins 48h après leur immobilisation au sein de la matrice d'alginate. En outre, les cellules ont été en mesure de créer une organisation 3D ainsi que des réseaux cellulaires et de migrer vers une couche de Matrigel lorsqu'elle existe. Les microsphères étant préparées à partir de solutions aqueuses capables de réticuler *in situ*, cette stratégie paraît adaptée à la formation de réseaux cellulaires, la migration des cellules ainsi que la délivrance des ECs au site de la lésion tout en utilisant une procédure chirurgicale peu invasive. Les hydrogels injectables tels que l'alginate constituent un système de délivrance *in situ* de cellules vasculaires prometteur.

En résumé, cette thèse s'est intéressée à la nécessité d'améliorer la vascularisation dans les stratégies de régénération osseuse et ses principales conclusions sont basées sur la relation étroite entre les cellules ostéoprogénitrices et les cellules endothéliales, à savoir: i) les ECs favorisent la différenciation et la prolifération des MSCs ; ii) les MSCs immobilisées dans l'alginate fonctionnalisé avec la séquence RGD stimulent la formation de structures tubulaires par les ECs ; iii) la matrice d'alginate

modifié avec la séquence RGD fournit un environnement 3D adéquat permettant l'adhésion, la survie, la migration et l'organisation des ECs. Finalement, une matrice d'alginate modifié avec la séquence RGD apparaît être une stratégie d'ingénierie du tissu osseux appropriée, permettant l'immobilisation et la délivrance de cellules osseuses et endothéliales ainsi qu'une procédure chirurgicale peu invasive.

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Chapter I

General Introduction

1. BONE

1.1. An Overview

Bone is a highly specialized form of connective tissue that constitutes part of the endoskeleton of vertebrates and is associated to several important functions: internal support, protection, shape, blood production, mineral storage and homeostasis, movement, detoxification and sound transduction. It can be classified according to its shape: long bones (e.g. thigh, leg, arm), short (e.g. wrist, ankle), flat (e.g. cranium) and irregular (e.g. spine bones) [1]. Bone is composed of an organic matrix that is reinforced by hydroxyapatite, salt deposits of calcium and phosphate. This organic matrix consists 95% of collagen type I (95%) and 5% of proteoglycans and several noncollagenous proteins [2].

Morphologically bone is not a uniform solid material but is composed of two types of tissues: cortical or compact and sponge or trabecular bone (Fig. 1). Cortical bone is rigid and dense, being found mainly in the outer regions of long bones or shells of other bones. Trabecular bone, which has a high porosity and is less organized, consists of a network of flat or needle-shaped trabeculae. The differences in morphology of the two types of tissue are associated to their major functions. In on hand, cortical bone provides the mechanical and protective functions and, on the other hand, cancellous bone provides the metabolic functions [2].

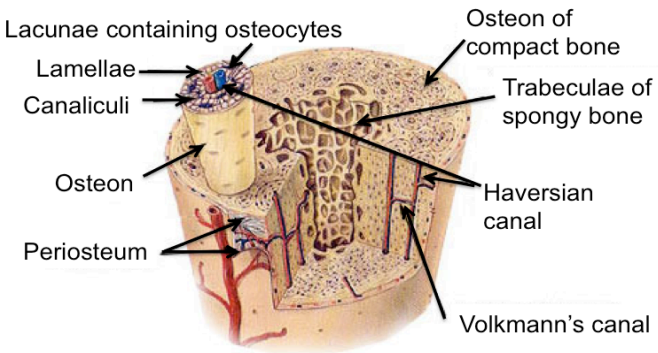


Figure 1. Schematic representation of the two morphological tissues (cortical and trabecular bone) that coexist in a long bone [3].

Cortical bone consists of repeating units, Harversian systems or osteons. Each osteon has concentric layers of mineralized matrix called lamellae, which are deposited around a central canal, the haversian canal, containing blood vessels and nerves [1]. Blood vessels connect to near osteons through anastomosing vessels in Volkmann's canals.

Bone is a living tissue comprising, apart from blood and nerves, four types of cells that contribute to its homeostasis: osteoblasts (bone forming cells), osteocytes (mature bone cells), osteoclasts (resorb or break down bone) and bone lining cells (cover inactive bone surface). Cellular communication between the bone cells, namely osteoblasts, osteocytes and (pre-)osteoclasts is essential for bone remodeling and therefore bone integrity [4]. Is the coordinate action of resorptive and formative cell populations that lead to new bone formation and, consequently, skeleton integrity [5]. Noteworthy, in the last decade several reports have come out demonstrating that bone homeostasis is also under the influence of central and peripheral neural control, establishing a link between the nervous system and bone [6].

Osteoblasts are fully differentiated cells responsible for bone development, growth, function, repair and maintenance [7]. They secrete type I collagen and noncollagenous proteins (bone sialoprotein, osteocalcin, osteonectin and osteopontin). Typically, an osteoblast is a protein producing cell with a prominent Golgi apparatus and a well developed rough endoplasmic reticulum [2].

Osteocytes are mature osteoblasts entrapped in the bone matrix and are responsible for its maintenance, constitute 90% to 95% of all bone cells in adult bone and are the longest lived bone cells. These cells have been found to have several functions, such as acting as an orchestrator of bone remodeling through regulation of both osteoclast and osteoblast activity and also by soluble factors production [8, 9].

Bone lining cells are flat, elongated, inactive cells (with few cytoplasmic organelles) that cover bone surfaces that are not undergoing bone formation or resorption [2].

Osteoclasts are large multinucleated bone-resorbing cells that are unique in their ability to degrade both the inorganic calcium matrix and the organic collagen matrix [2]. Bone resorption is a process highly dependent on a series of external stimuli, such as, matrix type, remodeling status, hormones involved in calcium homeostasis, genotype and inflammation [10].

Two different mechanisms are responsible for the development of bones: intramembranous (e.g. calvarial bones and mandibles) and endochondral ossification (e.g. long bones of the axial and appendicular skeleton) [11]. Both processes involve coordinated growth, differentiation, function and interaction of different cell types.

Intramembranous ossification involves the formation of bone directly, without intermediate formation of cartilage. Mesenchymal cells aggregate and differentiate into osteoblasts that deposit bone matrix rich in type I collagen, which becomes mineralized [12, 13].

The endochondral ossification process involves the recruitment, proliferation and differentiation of mesenchymal cells into chondrocytes, forming a cartilage template of the future long bone [13]. Chondrocytes in the center become hypertrophic, while cells in the perichondrium differentiate into osteoblasts forming a bone collar [12]. Then, the hypertrophic cartilage core is invaded by blood vessels, eroded and replaced by bone and marrow (the primary ossification center). This process continues at the growth plate, mediating longitudinal bone growth. Later, vessels invade the avascular epiphyseal cartilage and initiate a secondary center of ossification. Ultimately, the growth plates close and longitudinal growth stops.

1.2. An Amazing Organ - Regeneration of a Bone Fracture

Bone fracture healing is a remarkable process, which ends with bone anatomy and complete regeneration within 6-8 weeks [14]. Although bone formation is a complex process, the three-dimensional arrangement of cells and matrices is straightforward. Moreover, fracture healing is a process that recapitulates certain aspects of skeletal growth and depends on the cooperation of several elements, namely: i) specific cell types (Fig. 2); ii) extracellular matrix; iii) soluble molecules and iv) mechanical environment [15, 16].

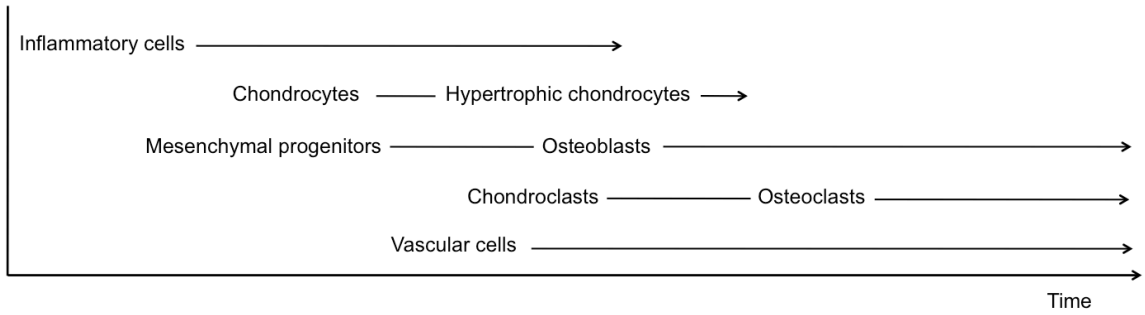


Figure 2. Cellular participants in the fracture repair processes [16].

Fracture healing can be divided into direct (primary) and indirect (secondary) fracture healing [13]. Direct healing occurs only when there is anatomic reduction of the fracture fragments due to rigid internal fixation and decreased intrafragmentary strain and there is no callus formation. Indirect healing involves the combination of intramembranous and endochondral ossification and there is callus formation. Noteworthy, the indirect healing is associated with the majority of bone repair processes, where intramembranous ossification occurs few days after fracture and endochondral ossification that occurs close to the fracture site over a period of up to 28 days [17] .

After bone fracture there is a disruption of the marrow architecture and of blood vessels within and around the fracture site. The repair of a fracture by callus formation is a well-organized process that can be divided in four different stages, which are hematoma formation, soft callus formation (chondrogenesis), hard callus formation (osteogenesis) and bone remodeling (Fig. 3) [18].

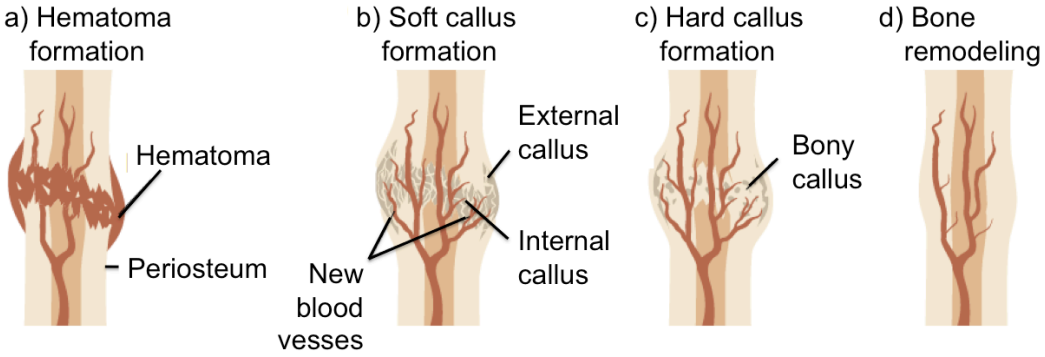


Figure 3. Stages of fracture repair: (a) hematoma formation and inflammation; (b) soft callus formation; (c) hard callus formation; (d) bone remodeling [18].

After injury of the musculoskeletal system, disruption of blood vessels leads to activation of the coagulation cascade and formation of hematoma, which encloses the fracture area, serves as a source of signaling molecules (interleukins, tumor necrosis factor-a, fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor, vascular endothelial growth factor and transforming growth factor) and provides pathways for cellular migration (Fig. 3a) [19]. The first few days post-fracture are characterized by an inflammatory phase. Inflammatory cells, fibroblasts, and stem cells are recruited to the site, and new blood vessels are formed from the pre-existing ones (angiogenesis) (Fig. 3b). Meanwhile, undifferentiated mesenchymal cells along with the periosteum form a large cartilaginous mass both outside the cortices, an external callus, and within the cortices, an internal callus (Fig. 3b) [20]. The main role of this region is to mechanically stabilize the fracture site and is composed of fibrous tissue and new cartilage [14]. Subsequently, the internal callus becomes mineralized with calcium hydroxyapatite. Capillaries from adjacent bone invade the calcified cartilage, increasing the oxygen tension. The hard callus is formed of woven bone and cartilage and connects the two fracture ends (Fig. 3c). In the final phase, bone remodeling, the large fracture callus is replaced by secondary lamellar bone (Fig. 3d). The size of the callus is reduced to that of the pre-existing bone at the damage site, the vascular supply reverts to a normalized state and the bone cortex acquires the pre-existing architecture [18].

1.3. A Complex Organ - Different Elements in Bone Regeneration

Bone regeneration is a complex physiological process that involves the coordinated participation of several cell types (e.g. haematopoietic, immune, mesenchymal stem, nerve and endothelial cells), growth factors/cytokines (e.g. fibroblast growth factor, transforming growth factor, bone morphogenetic protein, insulin-like growth factor, platelet-derived growth factor, vascular endothelial growth factor) and extracellular proteins. These factors regulate the cascade of molecular events by influencing different steps in the osteoblast and chondroblast pathway such as migration, proliferation, chemotaxis, differentiation, inhibition, and extracellular protein synthesis [13]. Table 1 lists key molecules and cells involved in bone repair.

Table 1 – Key molecules and cells involved in bone repair [15].

Key factors	Functions	*In vivo* and *in vitro* effects
Extracellular messengers		
IL-1, IL-6, TNFa	Elicit inflammation and migration	*In vitro* they inhibit osteoblastic differentiation, but *in vivo* TNFa is crucial for bone repair; the role of IL-6 is controversial (anti-or pro-osteogenic probably, depending on soluble IL-6 receptor)
SDF1	Chemotactic factor	Allows MSCs homing both *in vitro* and *in vivo*
TGFß	Mitogenic factor, osteogenic factor	Can induce osteoblast differentiation at the early stage of immature cells but can also inhibit osteogenesis in committed cells
BMP2	Osteogenic factor	Osteochondrogenic factor; might initiate bone formation and bone healing and can induce expression of other BMPs
BMP4	Osteogenic factor	Osteochondrogenic factor *in vivo* and *in vitro*
BMP7	Osteogenic factor	Osteogenic factoring *in vivo* and *in vitro*; active on more mature osteoblasts
Noggin	BMP2, 4 and 7 specific inhibitor	Suppresses osteoblastic differentiation
FGFb	Angiogenic and mitogenic factor, osteogenic factor (controversial)	Mutations induce chondrodysplasia and craniosynostosis; can stimulate Sox9; might be a negative regulator of postnatal bone growth and remodeling
IGF-I, II	Mitogenic factors, osteogenic factors	Stimulate growth plate formation, endochondrate ossification and bone formation by osteoblasts
VEGF	Angiogenic and vasculogenic factor	Most potent angiogenic and vasculogenic factor; crucial at the onset of bone formation
PlGF	Angiogenic and vasculogenic factor	Induces proliferation and osteogenic differentiation of MSCs; crucial for vascularization
PDGF	Mitogenic and chemotactic factor	Highly mitogenic factor and chemotactic for MSCs, osteoblasts and perivascular cells
Wnts	Mitogenic and osteogenic factors	Depending on Wnt type, crucial for osteoprogenitor proliferation; can also inhibit final osteoblast maturation
DKK1	Inhibitor of Wnt signaling	Strongly inhibits osteogenesis of MSC and osteoprogenitor cells; can stimulate terminal maturation
Ihh	Osteochondrogenic factor	Pivotal role for growth plate and endochondral formation; can inhibit osteoblast differentiation; might induce PTHrP expression
PTHrP	Osteochondrogenic factor	Pivotal role for growth plate and endochondral formation; can induce or inhibit osteogenesis
OPG	Decoy receptor of RANKL, inhibition of RANKL	Strongly inhibits bone resorption and has a pivotal role in bone remodeling
RANKL	Induces osteoclastogenesis	Strongly stimulates bone resorption and has a pivotal role in bone remodeling
M-CSF	Induces osteoclastogenesis	Crucial for osteoclastogenesis

Gastrointestinal serotonin	Neurotransmitter inhibiting osteogenesis	Expressed by enterochromatin cells, inhibits bone formation and is repressed by Lrp5
Intracellular messengers		
MAPKs	Transduce osteogenic signaling by phosphorylation	Crucial for regulation of intracellular signaling induced by osteogenic factors (still controversial)
PKA/CREB	Transduce osteogenic signaling	Can transduce osteogenic signaling (still controversial); possible indirect effect
ß-Catenin	Osteogenic transducer factor	Pivotal role in transducing osteogenic signal from Wnt and is negatively regulated by GSK3b
Runx2	Early osteogenic transcription factor	Master regulator of early osteogenesis; runx2$^{-/-}$mice die, with no bone formation
Osterix	Late osteogenic transcription factor	Master regulator of late osteogenesis, inhibiting chondrogenesis
Dlx5	Osteogenic homeobox protein	Induces osteoblast maturation but inhibits osteocyte formation
Msx2	Osteogenic homeobox protein	Induces proliferation of immature cells; responses depend on Dlx5 quantity
NF-kB	Inflammation transducer factor, inhibits osteogenesis	Inhibits the differentiation of MSCs and committed osteoblastic cells
Cells		
MSCs	Origin of osteoblasts	Can form bone *in vivo* and osteoblasts *in vitro*
Osteoblasts	Osteogenic professional cells	Generate bone formation
Endothelial cells	Blood vessels formation	Stimulate osteogenic differentiation and blood vessel formation
Nerve cells	Neurotransmitter production (NPY)	Bone homeostasis; *in vitro* enhance osteoprogenitor cells differentiation

Abbreviations: IL – interleukin; TNF – tumour necrosis factor; SDF – stromal derived factor; TGF – transforming growth factor; BMP – bone morphogenetic protein; FGF – fibroblast growth factor; IGF – insulin-like growth factor; VEGF – vascular endothelial growth factor; PlGF – placental growth factor; PDGF – platelet-derived growth factor; Ihh – indian hedgehog; PTHrP – parathyroid hormone-related protein; OPG – osteoprotegerin; RANKL – receptor activator of nuclear factor kappa-B ligand; M-CSF – macrophage colony-stimulating factor; MAPKs – mitogen-activated protein kinase; NF-kB – nuclear factor-kB; MSCs – mesenchymal stem cells; NPY – neuropeptide Y.

1.4. A Problem to be solved

Bone is one of the few organs that retains the potential for regeneration in adult life, as it possess the ability to heal and to remodel [21]. This ability is mainly due to the coordinated interaction of several elements. For instance, many local and systemic regulatory factors, cytokines and hormones, as well as extracellular matrix interact with several cell types. However, in several clinical settings bone is not able to heal by itself, such as fusion of bones, reconstruction of shattered bones, stimulation of bone growth to help anchor an artificial joint or other implant and filling of bone defects caused by trauma, tumours, infections, biochemical disorders and abnormal skeletal development [22, 23]. The current standard treatment for some of these problems is based on the use of bone-grafting materials that are divided into two groups: natural and synthetic bone grafts [24]. Natural bone grafts include auto-, allo- (human donors) and xenografts (other species). Autologous bone graft is considered the “gold standard” for bone repair and regeneration by many surgeons, mainly due to lack of immunogenic reaction and optimal biological performance in terms of osteogenicity, osteoinductivity and osteoconductivity [23]. However they present several limitations, namely the reduced availability and the risks associated to the harvesting, an additional surgical procedure, donor site morbidity, post-operative pain and infection [25]. On the other hand, allografts and xenogratfs are widely available and there is no need for an additional surgery. However, in both situations there is the risk of immunoreaction and since they pass through several processing techniques, their osteoinductive and osteoconductive potential are reduced [23, 25, 26]. To overcome the above limitations of natural gratfting, a large number of synthetic grafts have been designed over the past decades. They can be divided in different groups according to the base material: metals (e.g. titanium and its alloys, steel, cobalt-chromium), ceramics (e.g. calcium phosphate, alumina, glass ceramics), polymers (e.g. polylactic acid, polyglycolic acid, poly(methyl metacrilate), polyurethane, polyethylene) and composites of these three groups (e.g. hydroxypatite reinforced with high density polyethylene, metallic implants coated with calcium phosphate) [23, 24]. These synthetic materials present several advantages, such as large availability, lack of antigenic response and can be easily tailored to a specific application. Still, the biological performance of these synthetic grafts regarding initiation and support of bone growth are inferior to those of natural grafts [23]. One way to overcome this consists on adding cells and/or growth factors to those materials, giving

rise to more biological candidates capable to restore, maintain or improve bone function [17].

2. BONE TISSUE ENGINEERING

2.1. Helping Bone to Regenerate

Since the early 1990s several approaches have been developed that are of particular interest to restore, maintain and improve bone function that include the combination of engineering and life sciences to incorporate the biological performance that is generally missing in synthetic bone substitutes [27]. Tissue engineering constitutes a promising approach by using three-dimensional structured biomaterials as extracellular matrix in combination with stem cells as cell source and/or biologically active molecules (Fig. 4) [26]. Biomaterials scaffolds are investigated to not only to serve as cell carriers to providing mechanical support, but also to actively influence cell behavior, such as cell adhesion, proliferation, differentiation and function [28].

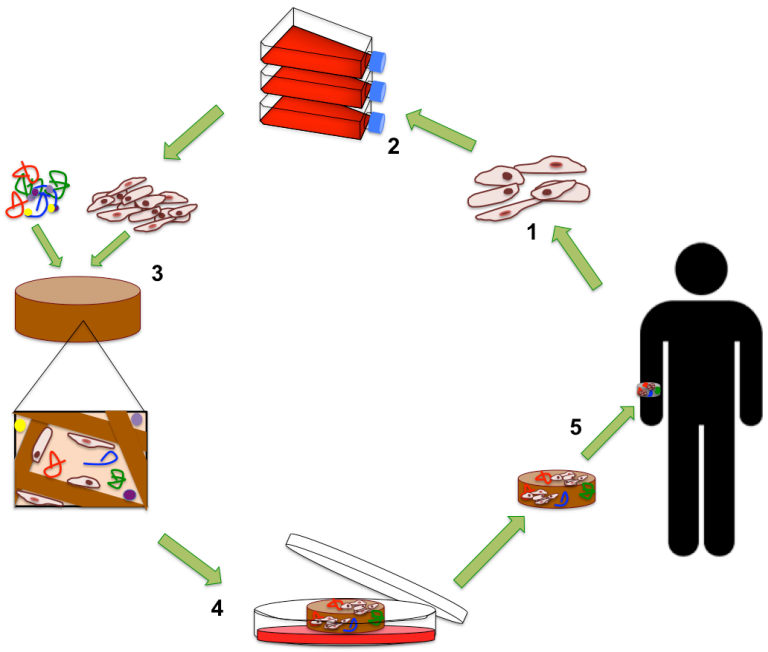


Figure 4. The typical engineering approach. 1) Cell removal; 2) Cell expansion in culture; 3) Cell seeding onto an appropriate scaffold incorporating suitable growth factors and cytokines; 4) Cell culture; 5) Re-implantation of engineered tissue in the damaged site.

2.2. Mesenchymal Stem Cells – An Outstanding Cell Population

The ideal cell source for bone tissue engineering should be easily expandable to higher passages, non-immunogenic and have a protein expression pattern similar to bony tissue. Cell sourcing and its characterization is one of the concepts with higher priority in the field of tissue engineering. More precisely, and according to Johnson and colleagues, stem cells science is the second most strategic concept and remains a critically important focus for the tissue engineering field [29].

A vibrant cell population is a mandatory first element for an unimpeded bone repair process, such as, multipotent mesenchymal cells or mesenchymal stromal cells (MSCs). MSCs have been utilized to enhance fracture healing in a number of *in vitro* and *in vivo* studies [19]. The cell-based concept to repair bone defects was first established using fresh autologous bone marrow combined with porous calcium phosphate ceramics [30].

MSCs were first reported in the 60's by Friedenstein *et al.* as fibroblast-like cells that could be isolated from the bone marrow through their capacity to adhere to plastic in culture and to differentiate into adipocytes, chondrocytes and osteocytes [31]. Nowadays, MSCs have been isolated from several tissues, including adipose tissue, liver, muscle, amniotic fluid, synovium, placenta and umbilical cord [32]. However, the principal source of MSCs for most preclinical and clinical studies is the bone marrow.

The true identity of MSCs has often been confused by different laboratories, which employ different isolation and *in vitro* culture methods, combined with different sources. To address this inconsistency between nomenclature and biological properties and to clarify terminology, the International Society for Cellular Therapy (ISCT) provided the following minimum criteria for defining multipotent mesenchymal stromal cells [33]:

a) Plastic-adherent under standard culture conditions;
b) Positive for expression of CD105, CD73 and CD90, and absent for expression of hematopoietic cell surface markers, namely CD34, CD45, CD11a, CD19 and HLA-DR;
c) Under specific stimulus, cells should differentiate into osteocytes, adipocytes and chondrocytes *in vitro*.

Despite of some questions that remain unanswered there is substantial evidence supporting the use of MSCs as a biological therapeutic for a diverse range of clinical

applications:

a) *Easily accessible isolation*: MSCs can be obtained from clinically available sources, such as, bone marrow, adipose tissue and cord blood, which are considered candidates for MSCs production for clinical applications [34, 35].
Bone marrow aspirate is considered to be the most accessible and enriched source of MSCs and is the principal source of MSCs for most pre-clinical and clinical studies [36, 37]. However, the harvest of bone marrow is a highly invasive procedure and the life span and differentiation potential of MSCs from this source decline with increasing age [34].
Adipose tissue has great potential as stem cell source for tissue engineering applications, mainly due its high availability. Adipose stem cells (ASCs) can be obtained as a "waste" from surgical procedures and/or harvested from humans through liposuctions. The major disadvantage of this source is contamination by other cells types, which can negatively influence the proliferation and/or differentiation of ASCs. To overcome this, new isolation methods have emerged, for instance based on the use of immunogenic beads [38].
The umbilical cord blood is a source of MSCs with low immunogenicity and with some immunosuppressive functions [39]. This source has the advantage of being widely available (normally it is discarded) and presents no risk for the mother or the newborns.

b) *High expansion potential in culture*: For large-scale production of MSCs, successive passages are needed to obtain a highly enriched population of MSCs. Additionally, the ideal culture conditions should be capable to maintain MSCs with (a) phenotypic and functional characteristics similar to those exhibited in the original niche, (b) indefinite proliferation and (c) the capacity to differentiate into multiple lineages [40]. In the past decade several studies emerged concerning optimal culture conditions for clinical scale production of MSCs [41-45].
Comparing the previous MSCs sources mentioned, bone marrow, adipose tissue and cord blood, they present different success rate in terms of MSCs isolation and proliferation capacities. For example, MSCs from cord blood present the lowest isolation efficiency, but the highest expansion potential [34].

c) *Presumptive plasticity*: Some studies showed that MSCs in the adult organism could differentiate *in vitro* into unexpected directions, beyond the well-know ability of these cells to give rise to mesodermal cell lineages (osteoblast, chondroblast and adipocyte) and cross the germinal boundaries (cardiomyocytes, hepatocytes, endothelial cells and neuronal cells) [46-50]. However, due to lack of experiment reproducibility (isolation, media and culture period) the plasticity of MSCs is still a debatable topic [47, 51, 52].

d) *Immunosuppressive properties and allogeneic transplantation*: Great interest is now being raised in the capacity of MSCs to modulate the immune response. In fact, MSCs do not induce considerable alloreactivity due to several unique characteristics, namely: inhibition of T-cell proliferation; modulation of B-cell proliferation, migration and immunoglobulin production; inhibition of natural killer cells proliferation; possible interference with dentritic cell functions (inhibition of cell maturation); and influence the function of neutrophils and macrophages [35, 37, 52, 53]. The poorly immunogenicity and the available data supports the concept that the transplantation of these cells into an allogeneic host may not require immunosuppression [54-56]. In fact, allogeneic MSCs could be used as therapeutic agents to combat graft versus host disease and Crohn's disease, both involving the strong immunoregulatory capacity of these cells. A company (Osiris Therapeutics Inc., USA) is currently evaluating the use of adult MSCs as therapeutic agents in both diseases, in phase III clinical trials [37, 53].

e) *Paracrine-mediated effects*: MSCs have the capacity to secrete a broad spectrum of bioactive macromolecules (growth factors and chemokines) capable of creating a regenerative microenvironment that may enhance the normal wound healing, stimulate proliferation and differentiation of tissue–intrinsic progenitor cells, decrease inflammatory and immune reactions [52, 53, 57].

f) *Homing and migratory behavior to sites of tissue injury*: MSCs have exhibited capacity to leave the bone marrow, circulate in the blood and home to injured tissues [58, 59]. Therefore, the use of MSCs has been particularly hailed due to

its presumed capacity to selectively migrate to sites of inflammation/injury when injected intravenously [37, 52]. However, the molecular mechanisms underlying MSCs migration are not yet fully understood.

g) *Ethical considerations*: Although embryonic stem cells (ESCs) have greater potential than adult stem cells, their use is still limited by lack of understanding about how to specifically regulate ESCs differentiation, their association with tumorigenicity and several ethical issues that are raised: destruction of embryos, creation of embryos for research (payment to oocyte donors and medical risks of oocyte retrieval) [37, 39, 60]. Therefore, adult stem cells represent a less controversial cell source (isolated from adult tissues) and their use in clinical practice has also been better documented and investigated.

2.3. Key Partakers in Bone Regeneration

The bone regeneration process involves the coordinated participation of haematopoietic and immune cells in conjugation with vascular and bone cell precursors [61]. During this process mesenchymal stem cells and endothelial cells are key partakers [62]. The important relationship between blood vessels and bone formation was first mentioned in 1763 by Albrecht von Haller who wrote: “the origin of bone is the artery carrying the blood and in it the mineral elements” [63]. However, in the following years, more attention was dedicated to the role of osteoblasts in bone formation. It was just in the 20th century that this relationship was revived, after the suggestion that tissues produce substances that initiate osteogenesis, such as a vascular stimulating factor (VSF), that appears in bone damage sites [64]. Nowadays, it is known that an adequate or proper bone vascularization is needed for bone formation and bone mass increase [18, 65, 66]. The inhibition of angiogenesis during fracture repair leads to the formation of a fibrous tissue. Moreover, the lack of blood supply is one of the risk factors during bone healing. Other risks include poor apposition of fractured bone ends, interposition of soft tissues or necrotic bone between bone fragments, inadequate immobilization, infection, drug use, advanced age and systemic disorders [67, 68].

As previously mentioned, bone is a highly vascularized tissue where blood

vessels are in intimate association with bone tissue. This tight correlation has been recently explored in several works [69-90]. Endothelial cells (ECs), the cells lining blood vessels, have been shown to produce a number of local and systemic mediators that influence the proliferation, differentiation and function of osteoprogenitor cells. On the other hand, osteoprogenitor cells secrete factors that enhance differentiation and survival of endothelial cells. Accordingly, Wang demonstrated that 1,25-Dihydroxyvitamin D_3 enhances the production of vascular endothelial growth factor (VEGF) by osteoblasts, thereby stimulating VEGF receptors of ECs which, in turn, produce osteotropic growth factors (insulin-like growth factor I (IGF-1) and endothelium-1 (ET-1)) [87]. Lamparter showed that angiotensin II, generated by ECs, stimulates proliferation and collagen synthesis of non mature osteoblasts [91]. Villars and colleagues also demonstrated that bone marrow stromal cells (BMSCs) express and synthesize VEGF, that human umbilical vein endothelial cell (HUVEC) conditioned medium has a proliferative effect on BMSCs and that early osteoblastic marker levels increase when these are co-cultured with HUVECs in direct contact [81]. Later, this group suggested that the gap junction connexin 43, which is expressed in both cells, has a significant contribution in osteoblastic differentiation [92]. Deckers *et al.* studied the expression of osteoblast-derived VEGFs and their receptors and proposed that they modulate endothelial and osteoclast functions as well as osteoblast differentiation [93]. Moreover, Kaigler *et al.* demonstrated that BMSCs secrete sufficient quantities of VEGF to enhance survival and differentiation of endothelial cells *in vitro* and afterwards established that ECs significantly increased BMSC osteogenic differentiation *in vitro*, only in direct contact, and that ECs also expressed bone morphogenetic protein 2 (BMP-2) [77, 94]. Noteworthy, *in vivo* studies where both cells were co-transplanted on a biodegradable polymer resulted in greater bone formation than when osteoprogenitor cells were implanted alone: Kaigler *el al.* used a poly(lactic-co-glycolic acid) scaffold and Grellier *et al.* immobilized the cells within a RGD-modified alginate [71, 77]. Overall, two main dialogues were identified: a paracrine effect through VEGF, BMP-2, IGF, ET-1 production and a juxtacrine mechanism by gap junctional activity between the two cell types [89, 95]. The available data so far strongly supports a relevant role of vascular cells in bone tissue regeneration.

Furthermore, an appropriate vascular network is necessary to support the metabolic needs of a forming tissue mass. Vascularization of engineered tissue constructs may improve the success rate after transplantation, since the survival of implanted cell relies on nutrients and waste diffusion between them and the

vasculature in the surrounding host tissue [96-99]. Therefore, attempts to mimic vascularization in engineered bone tissues that would lead to more clinically relevant cell-based therapies are being developed. These strategies are based on growth factor release, scaffold design and engineering, decellularized matrices and culture of endothelial cells in the 3D scaffold [100]. The design of matrices able to deliver growth factors in a controlled way instead of a bolus delivery or systemic administration present several advantages; for instance, they allow a longer exposure period to low and localized doses of the growth factor [101]. Different biomaterials have been shown to provide varied rates of success for localized and sustained release delivery of growth factors, such as for instance injectable alginate hydrogel, poly(lactide-co-glucolide) microspheres, collagen scaffolds, and fibrin matrix [102-105].

Scaffolds may be also designed to direct vascularization through microchanneling, molecular gradients or micro-patterning that would allow to control oxygen and nutrient gradients, cell alignment and concentration gradients of growth factors [98, 100, 106]. There are several micropatterning techniques that were developed to regulate angiogenesis on biomaterials: photolithography, microcontact printing, micromolding and three dimensional laser photolithography [107].

In the past few years a growing interest in *in vitro* prevascularization of different biomaterials for bone regeneration has emerged into the limelight [75, 76, 82, 84, 85]. This approach is based on the concept that a network of microvessels may be engineered *in vitro* by combining scaffolds with endothelial cells, providing a starting point for vascularization. Aiming bone regeneration several studies have been conducted based on cocultures of bone and endothelial cells. Unger and co-workers evaluated the vascularization potential on four different biomaterials previously studied for bone tissue engineering: porous calcium phosphate (hydroxyapatite), porous nickel-titanium and silk fibroin mesh [85]. Cocultures of microvascular endothelial cells (MECs) and primary osteoblasts self-assembled on these biomaterials, and MECs formed microcapillary-like structures without adding any exogenous angiogenic factor. Hofmann and colleagues were able to improve vascularization (formation of vessel-like structures) by seeding HUVECs with primary osteoblasts on a polyurethane scaffold [76]. Also using primary osteoblasts but with outgrowth endothelial cells (endothelial progenitor cells) Fuchs *et al.* were able to enhance pre-vascularization on a silk fibroin mesh and on a starch polycaprolactone scaffold [82, 84].

Although vascularization remains an unsolved challenge in tissue engineering, encouraging developments have been reported on the *in vitro* growth and maintenance of self-assembled biological vascular networks [108, 109].

3. INJECTABLE CELL-SYSTEMS FOR BONE REGENERATION

3.1. Cell Delivery

As the field of tissue engineering progressed, improvements in the fabrication of more refined and tailored cell based therapies are emerging to replace, repair or enhance the function of damaged tissues or organs. The ability to combine cells with biomaterials to create “living cell medicines” has open new doors.

The success of cell delivery systems is dependent on the use of a biomaterial vehicle/scaffold that should allow controlling three-dimensional shape, guide tissue development and permit the convenient delivery of cells into the patient [110, 111]. Therefore, such biomaterials should allow adhesion, spatial distribution, protection and guidance of cells [110]. Due to its great potential, therapeutic cell transplantation has become more and more important in regenerative medicine in this last decade [112].

There are several strategies for cell delivery in skeletal tissue engineering, depending on the clinical application: injectable delivery, delivery with soft scaffolds, load-bearing cell-seeded replacement biomaterials, delivery of moulded transplants, encapsulation for immunoprotection, and use of shape memory constructs for minimally invasive surgery [110]. For clinical applications, cell delivery through a minimally invasive approach may be a desired method, since these systems offer specific advantages over preformed scaffolds, such as ease of application, confined delivery and improved patient compliance and comfort [113].

3.2. Injectable Bone Substitute Materials

With the development of minimally invasive surgical methods, directly injectable biomaterials have been investigated in the search for the least possible invasive method [114]. Therefore, injectable bone substitutes must encompass a range of desirable properties to make it an ideal candidate: ability to mold to the shape of the bone defect; polymerize when injected into the site; ease of preparation and handling;

injectibility; no toxicity; low cost; adequate mechanical properties; appropriate cohesion; excellent osteoconductivity, osteoinductivity, biocompatibility and bioactivity [114, 115].

The burgeoning number of cutting edge discoveries is leading the design of minimally invasive, biomimetic and biodegradable cell-laden vehicles, such as microcarriers, microspheres for cell immobilization and *in situ* gelling biomaterials that can be injected into the target injured tissue, promoting cell integration and, consequently, tissue repair and regeneration (Fig. 5) [111, 116].

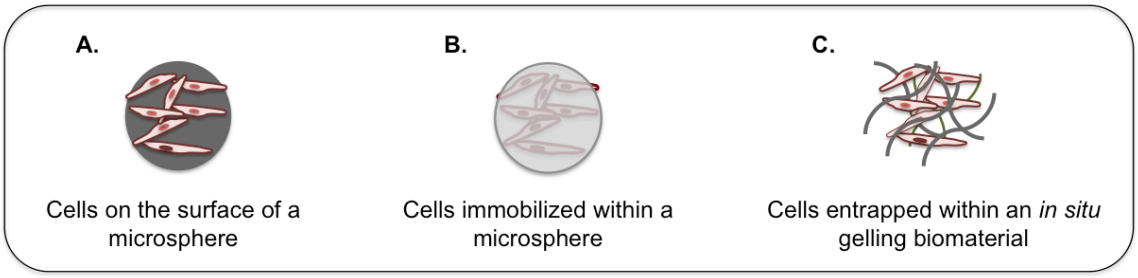


Figure 5. Injectable bone substitute biomaterials for cell delivery. A) microcarriers; B) microspheres for cell immobilization; C) *In situ* gelling biomaterials.

3.2.1. Microcarriers for cell delivery

Microcarriers serve as a cell-seeded system that can be delivered directly into the site of injury through injection or arthroscopically or used as templates to propagate large numbers of cells that can be recovered for subsequent analysis or transplantation [117]. Microcarriers offer the advantage of providing a large area for monolayer cell growth during propagation in a homogenous suspension culture system comparing with culture flasks. Therefore, these systems are space saving and cost effective with respect to the use of culture media and expensive additives such as growth factors and serum. Microcarriers can be combined with other technologies to attain a higher performance. For example, Kedong and co-workers combined a glass coated styrene copolymer microcarrier with a rotating wall vessel bioreactor to attain higher cell proliferation in a coculture system [118].

The use of microcarriers culture in tissue engineering has only been scarcely explored, although there are commercially available microcarriers that can potentially be used for that purpose (Table 2) [117, 119, 120].

Table 2 – Selection of commercially available microcarriers [117, 119, 120].

Name	Material	Surface coating	Surface charge	Diameter (μm)
Cytodex 1	Dextran	None	+	131-220
Cytodex 2	Dextran	Collagen	+	135-200
Cytodex 3	Dextran	Collagen	+	133-215
Biosilin	Polystyrene	n/a	-	160-300
P 102-L	Polystyrene	Polystyrene	None	125-212
RapidCell	n/a	Glass	-	150-210
ProNectin®F	Polystyrene	Fibronectin	None	125-212
FACT III	Polystyrene	Gelatin	+	125-212
Plastic plus	Polystyrene	None	+	125-212
Collagen	Polystyrene	Gelatin	None	125-212
Plastic	Polystyrene	None	None	125-212
Glass	Polystyrene	High silica	-	125-212
Hillex®II	Polystyrene	Trimethyl ammonium	+	160-180
Cultispher G	Gelatin	None		130-280
Cultispher S	Gelatin	None		130-280

Abbreviation: n/a, not available

Microcarriers composed of crosslinked dextran matrix (under the tradename of Cytodex) are the most commonly used for a wide range of cells. There are three different types of dextran-based microcarriers: type 1 - matrix with N, N-dimethylaminoethyl groups; type 2 - matrix with a surface layer of N,N,N-trimethyl-2-hydroxyaminopropyl groups; and type 3 - matrix with a thin layer of collagen type I. Frauenschuh and co-workers studied the three types of dextran-based microcarriers for MSC expansion and conclude that type 1 was the most suitable for MSC attachment and proliferation during a cultivation period of 28 days [121]. These results were later corroborated in a study where the seeding efficiency of MSCs was evaluated on nine different commercially available microcarriers: dextran-based types 1 and 3 and seven polystyrene-based microcarriers, including five with different surface coatings (fibronectin, gelatin, high silica trimethyl ammonium) and two without coating (under the tradename ProNectin F, FACT III, Plastic plus, Collagen, Plastic, Glass, Hillex II) [122]. Schop *et al.* investigated the best culture conditions for MSCs growing in type 1 dextran-based carrier and concluded that by adding 30% fresh medium with new microcarriers every three days, MSCs showed the longest continuous proliferation [119].

Although seeding efficiency studies are essential, good harvest processes that lead to high number of viable MSCs capable of retaining their differentiation potential are mandatory. For that reason macroporous microcarriers are not suitable since it becomes difficult to enzymatically separate cells within the pores. Alternatively, nonporous microcarriers should be considered for MSC expansion and further detachment. Weber and co-works investigated MSC behavior in five nonporous microcarriers: type 1 and 3 dextran-based microcarriers, two based on polystyrene (under the tradename of Biosilon and P 102-L) and another one with a glass surface (Rapidcell) [120]. Although they did not find considerable differences on MSCs growth rate among the five carriers, the harvest efficiency was quite different. Dextran-based microcarriers presented the lowest efficiency using the five enzymes tested for cell detachment. To avoid the use of proteolytic enzyme treatment and to enhance the harvest yield, Yang and colleagues conjugated the commercial type 3 dextran-based microcarrier with a thermosensitive polymer, poly-N-isopropylamide [123]. By simply reducing the temperature below 32ºC, MSCs detached and presented lower apoptosis and death rates than the ones treated with trypsin.

Due to harvest difficulties, alternatives to dextran-based microcarriers need to be considered for MSCs adhesion and proliferation, such as the use of gelatin-based microcarriers (e.g. Cultipher-G and Cultipher-S) [124-126]. These microspheres can be dissolved with trypsin-ethylenediamine tetraacetic acid (EDTA) or collagenase, and almost all MSCs can be recovered without loosing their differentiation potential [127]. Moreover, to attain readily available constructs, Lippens and colleagues proposed an interesting approach by examining the cryopreservation process of gelatin-based cell-microcarrier constructs [126]. They were able to achieve promising results in the pursuit for readily available tissue constructs for fast intervention in bone defects.

Beyond the materials used in commercially available microcarriers, other biomaterials have been proposed as cell-seeded systems. These biomaterials present several appropriate features that would allow to control cell quality and quantity and, therefore, to ensure intact cell phenotype during delivery, namely: chemical composition, surface topography, degree of porosity, charge density and sterilization [117]. Several different types of biomaterials have been investigated for microcarrier technology, such as: ceramics (e.g. calcium titanium phosphate), bioceramic microparticles in a polymeric matrix (e.g. hydroxyapatite particles in a poly(lactide-co-glycolide) matrix), and polymers (polycaprolactone with interconnected pore channels; D,L-lactide-co-glycolide acid microspheres with a biomimetic surface of fibronectin) [128-

[132].

In general, microcarriers are spherical units with various substrate matrices that can be kept in stirred bioreactors. In these systems, cells grow mainly on the spherical surface as monolayers or in the pores in the case of macroporous structures [112]. As a result, a large amount of microcarriers with cells has to be introduced in the body in order to obtain a sufficient cell number. To overcome this drawback, Su and co-authors developed a novel open and hollow shell-structure cell microcarrier based on gelatin for cell transplantation, which allowed cells to attach and proliferate on both outer and inner surface of the microcarrier [112]. This strategy aims to decrease the number of microcarriers necessary to achieve an adequate amount of cells and, subsequently, the volume in the injection system. In another alternative approach, bioactive calcium phosphate microparticles with the internal portion removed were studied [133]. These 3D spherical substrates provide more space for cells to adhere and proliferate than on the conventional filled microspheres.

Previously its was demonstrated that microcarriers support MSCs growth in the 3D matrix, by allowing cell adhesion and proliferation. However, in order to be further applied as injectable systems, an adequate vehicle is required to deliver the cell-loaded microcarriers into the defect and to keep them in place. For example, Lippens and coworkers successfully injected MSCs-loaded gelatin-based (CultiSpher-S) carriers within a chemically modified form of a polyoxythylene-polyoxypropylene copolymer based hydrogel (under the tradename of Pluronic F127) and implanted the system in noncritical size unicortical defects, resulting in a faster bone repair [134]. Oliveira *et al.* studied, different polymeric solutions as injectable matrices able to carry hydroxyapatite microspheres through an orthopedic device during an injection procedure: sodium carboxymethylcellulose, hydroxypropylmethylcellulose and Na-alginate [129, 135]. Alginate was the material that presented the best performance.

Overall, 3D microcarriers present advantages for application in tissue engineering because of their unique properties of scaling up 3D cell culture and loading capacity for drugs, growth factors or/and cells [121]. Biocompatible microcarriers loaded with cells offer the advantage of being able to readily place a large number of cells directly (*in situ*) and controllably in an injectable way [136]. Furthermore, microcarriers may also provide more than structural support for cells since they may work as regulators of cell function, metabolism and differentiation [137].

3.2.2. Microspheres for cell immobilization

Traditionally, cell immobilization, or encapsulation as it is often called, consists in physically protecting a cell mass from an outside environment within the confines of a semipermeable membrane that is designed to circumvent immune rejection and also protect the cells from mechanical stress (Fig. 6) [138]. This immune protection will eliminate or reduce the use of long-term therapies of modulating and/or immunosuppressive agents, which have potentially severe side-effects [116]. To ensure cell survival, the membrane confining the cells needs to be permeable to nutrients and molecules diffusion (oxygen and growth factors) and allow the elimination of cell secretions and catabolic products, while keeping components of the immune system out [116, 139]. Most of the research and applications in this field rely on the immunoprotective role of encapsulation technologies. The most widely used systems for that purpose consists in hollow polymeric systems (e.g. microcapsules) in which the active component is the polymeric selective membrane and the bulk of the system is filled with liquid.

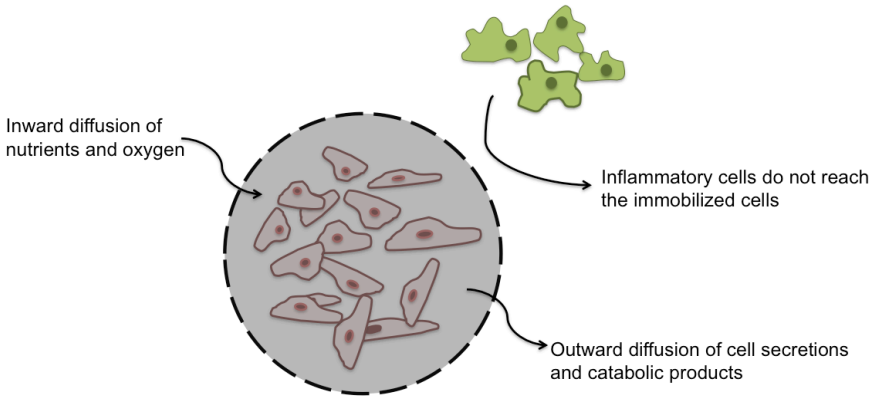


Figure 6. Cell immobilization technology consists in immobilizing cells within a polymeric semipermeable membrane (in this case, the bulk of the system is liquid) that is designed to circumvent immune reactions. The matrix allows the bi-directional diffusion of nutrients, oxygen, waste and the catabolic products [138].

Although extremely promising, there are several challenges to overcome for the application of cell immobilization technology: lack of adequate clinical-grade polymers, production of uniform microspheres, use of polycations, forethoughts before transplantation (assessment of the exact dosage and molecular-weight cutoff), selection of the suitable cell types for immobilization (appropriate cell source),

transplantation site, regulatory and ethical issues [140]. Moreover, to achieve a consistent clinical success critical aspects need to be taken into consideration: biocompatible, mechanically and chemically stable polymers with a suitable permeability providing immune protection; functional performance (allow the paracrine interaction between the cells within the microsphere and the host environment); biosafety; long-term cell survival (includes adhesion, proliferation and differentiation) and long-term survival of the graft (allow cell integration and consequently tissue repair) [116, 140].

One consideration of primary importance for cell immobilization technology to succeed is the source of the matrix material. Since, for cell immobilization strategies, scaffolds must be formed under mild and cytocompatible conditions, the number of suitable materials and formulation are significantly limited [141]. For that reason, the majority of biomaterials used in microsphere formation are hydrogels. Hydrogels offer unique characteristics for the designing of a suitable environment, providing a 3D structural support for cells and also a variety of biological cues that allow the maintenance of normal cellular functions [141-143]. Different natural polymeric hydrogel-based materials are available for cell immobilization and the most frequently used are agarose, alginate, collagen, fibrin, gelatin and hyaluronic acid [142]. Nevertheless, other polymers have been applied for the production of microspheres: polyelectrolytes of anionic nature (cellulose sulfate, chondroitin sulfate, polyacrylic acid), cationic nature (poly-L-lysine, poly-L-ornithine, chitosan, poly(methylene-co-guanidine), polyvinylamine) as well as non-anionic polymers (polyvinyl alcohol, poly(hydroxyethyl methacrylate-co-methyl methacrylate), polyethylene glycol) [139]. More recently several synthetic-based hydrogels have been produced, which include natural polymers that have been modified or synthetically built. These biomaterials can be organized into three categories: i) naturally derived hydrogels (DNA-based hydrogels, protein-based gels, polysaccharide-based gels, synthetic ECM analogs); ii) synthetic hydrogels (polyethylene glicol-based hydrogels, polyfumarate-based hydrogels, phosphoesters); iii) and biomimetic hydrogels (incorporation of proteins or small oligopeptides) [141].

Noteworthy, among the above-cited materials, alginate is the most dominantly used (Fig. 7). Alginate is a natural polymer that is commonly applied in many tissue engineering applications due to its biocompatibility, low toxicity, relatively low cost and mild gelation behavior using divalent cations [144-147].

The more recent cell immobilization strategies for tissue regeneration purposes differ significantly from previous strategies aiming at immunoprotection. Immunoisolation is no longer required since the systems are usually designed to

degrade *in vivo*, and be replaced by newly formed tissue. Also, tissue engineering strategies require adequate cell support for cell attachment, proliferation and differentiation and hence usually 3D polymeric systems are used instead of semipermeable membranes.

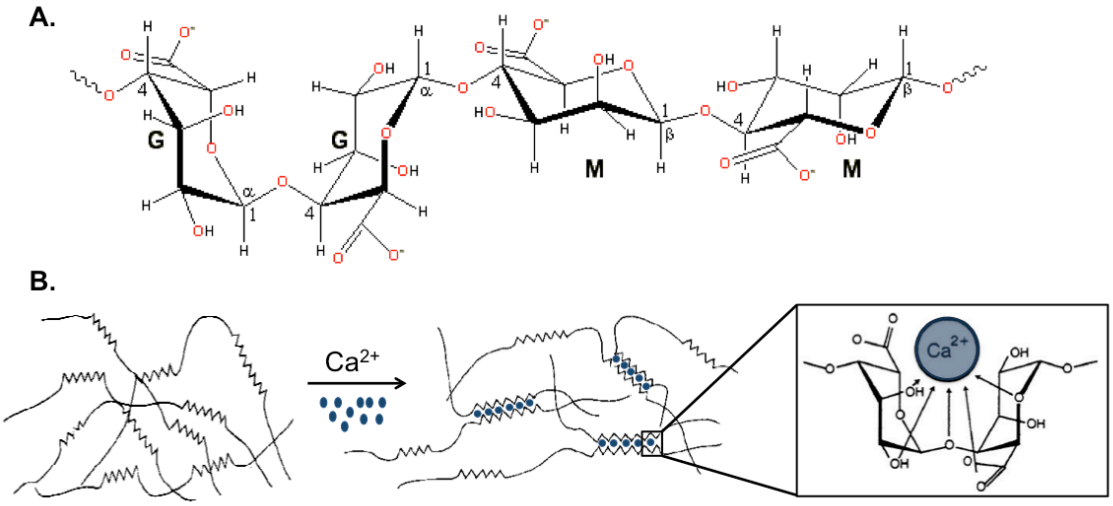


Figure 7. A) Structure of alginate showing both β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers. Alginate is a natural biopolymer extracted from brown algae. Alginate polymers are a family of linear unbranched polysaccharides containing different amounts of G and M units, which may constitute a homopolymeric block (M block or G block) or a heteropolymeric block (M and G blocks). B) Alginate can be ionically crosslinked by the addition of divalent cations in aqueous solution, such as calcium (Ca^{2+}) that exchange with the sodium ions from the guluronic units. The stacking of these G groups gives origin to the characteristic egg-box structure [148].

Cell delivery using microspheres for tissue engineering applications has been explored in several works aiming MSCs transplantation and/or bone regeneration. Alginate has been the polymer most widely investigated for that purpose. Although being originally a non cell-interactive polymer, alginate can be covalently modified to promote cell adhesion through the incorporation of peptides containing the amino acid sequence arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD) [149, 150]. Evangelista demonstrated that RGD-modified alginate hydrogel, in contrast with unmodified alginate, was able to promote a much higher level of metabolic activity, adhesion and differentiation of immobilized MC3T3-E1 pre-osteoblastic cells [149]. Moreover, its molecular weight can be modified through irradiation and oxidation to modulate its viscoelastic properties, as well as its degradation rate [150-152]. The lower viscosity of the irradiated polymer results in reduced shear forces during the cell-polymer mixing

process and, consequently, in higher cell viability [153]. Markusen *et al.* encapsulated MSCs in alginate beads covalently modified with RGD peptides with a sustained viability (>80%), but they did not observe cell proliferation during the two weeks experience [154]. Also, Grellier *et al*. explored the ability of this matrix to promote bone tissue regeneration *in vivo* after immobilization of human osteoprogenitors combined with endothelial cells [71]. Moreover, human mesenchymal progenitor cells from bone marrow were successfully immobilized within Ca-alginate microspheres and were also able to differentiate along the chondrogenic lineage [155]. Alternatively, Zhang and colleagues have generated small cell-loaded alginate microspheres (~100 μm), with good morphology and high cell-loading efficiency, using the electrostatic spray method [156]. Later they studied the effect of rapid cryopreservation of this cell-loaded alginate system to achieve readily available constructs. They concluded that water in these small capsules vitrified leading to intact capsules after cryopreservation, showing that this process could be applied in cells that are sensitive to freezing and high cryoprotectants [157]. Small microspheres (30-60 μm) were also produced using an alginate-barium system based on atomization processes that allow the administration of these microcapsules via injection and aerosol [158].

Alginate, which is negatively charged, can also be combined with positively-charged polymers, such as polylysine or chitosan, to prepare more stable microspheres. MSCs isolated from adipose tissue have been recently immobilized within an alginate-calcium core that was later complexed with chitosan and further cross-linked with genipin to increase the capsular strength [159]. MSCs within this matrix were able to promote increased VEGF expression and endothelial cell growth when cultured with the conditioned medium from the encapsulated MSCs, showing the angiogenic potential of this system. In another work, MSCs were immobilized in alginate-polylysine microspheres for long periods (70 days) maintaining their mesenchymal surface markers, viability and the capability to differentiate into all three mesodermal lineages [160]. Noteworthy, *in vivo* immobilized MSCs are significantly hypoimmunogenic and, since these cells have immunosuppressive properties, a semi-permeable membrane may not be necessary [55, 56]. Trouche and co-workers optimized MSCs delivery in alginate microspheres with and without a semipermeable membrane of polylysine and reported a higher viability of MSCs within microspheres without the semipermeable membrane [161]. However, when non-autologous MSCs are used, a protective membrane may be advantageous for immunoisolation. Ding *et al.* used alginate-poly-L-lysine microcapsules to immobilize BMP-2 gene-transfected MSCs that

were shown to be capable of constitutive synthesis and of delivering biologically active BMP-2 protein, and thus with potential utility for bone regeneration [162].

As previously pointed out, although alginate is the most widely used biomaterial for this purpose, there are other polymers that have been investigated for MSC immobilization. Agarose spheres containing immobilized matrix molecules (fibronectin and fibrinogen) that mediate cell-matrix interaction, allowed the survival of transplanted MSCs [163]. Self-assembled collagen microspheres were able to provide a protective, growth- and migration-supporting matrix to MSCs [164]. Gelatin microspheres were studied as temporary encapsulation system to protect marrow stromal osteoblasts from short-term environment effects caused by an injectable carrier (poly(propylene fumarate) [165]. Preosteoblast within pectin microspheres were investigated as an injectable biomaterial for bone tissue engineering [166]. Within this matrix cells maintain their viability, metabolic activity and differentiation capacity.

MSCs were also successfully photoencapsulated in poly(ethylene glycol) (PEG) incorporating the RGD sequence and were able to differentiate into the osteogenic lineage in the presence of dexamethasone released from PEG capsules [167] [168]. It is also possible to obtain individually preserved nanoencapsulated MSCs by polyectrolyte layers of poly-L-lysine and hyaluronic acid using the electrostatic layer-by-layer assembly technique, due to its mild conditions, nanoscale precision, tenability and the ability to modify surface characteristics [169].

Based on the previous examples, for bone tissue engineering applications microspheres present promising results. In general, this system allows cell survival, adhesion, proliferation and differentiation. Other advantage of this technology relies in the protection against shear stress and in avoiding cell loss by uncontrolled cell migration [155]. The 3D polymeric system should be permeable to paracrine interaction between the cells within the microsphere and the cells from host environment. By changing parameters associated with the cell immobilization system, such as size, the performance of the microspheres can be easily improved in terms of nutrients and oxygen supply. Furthermore, the number and location of injected cells can be easily controlled through the number of microspheres administrated and their site of injection [160].

3.2.3. *In situ* gelling biomaterials for cell entrapment

The development of biomaterials with the capacity to be transformed from liquid to solid *in situ* under mild conditions creates new possibilities in bone tissue engineering. Moreover, the conjugation of these matrices with cells and biomimetic peptides represents significant advances in tissue engineering strategies.

Recently, Low and co-workers reviewed the progress made towards the development of injectable calcium phosphate cements (CPCs) [114]. These compounds can be applied as granules, blocks, hydraulic cements or as injectable pastes, and encompass several ideal properties, such as nontoxicity, biocompatibility and bioactivity [114]. For instance, these cements have been used for augmentation of fractures involving crushed cancellous or osteoporotic bone, for bone defect filling or for gap filling around metal implants [170, 171]. As it is injected, the cement interdigitates with adjacent bone and forms a solid structure. CPCs have also been studied *in vitro* and *in vivo* and also in small clinical studies for vertebroplasty, as an alternative for the most widely used bone void filler – the polymethyl methacrylate (PMMA) cement [172]. To enhance the performance and expand the use of CPCs, research has been carried out to reinforce this compound with other materials, like fibers and polymeric additives [114]. The natural polymer chitosan can be incorporated into CPCs yielding higher flexural strength, toughness and strain to failure [173]. The CPC powder is mixed with the chitosan liquid and forms a paste that can be surgically applied via minimally invasive techniques and becoming a scaffold *in situ*. Moreau and colleagues investigated this strategy for MSCs delivery, and they were able to demonstrate that CPC-chitosan supports MSC attachment, proliferation and differentiation into the osteogenic lineage [174].

In the last few years, the concept of molecular self-assembly of peptides has lead to the development of new injectable biomaterials with potential in the field of bone tissue engineering [175, 176]. After injection, these peptides self-assemble to form micrometer-long nanofibrils and these fibrils give rise to dynamic 3D scaffold gels [177]. These designer self-assembling peptide nanofibers have been shown to be an excellent biological material for 3D cell cultures and to be capable of stimulating cell migration into the scaffold [178]. Ozeki and co-authors suggested that the RAD16 (16 amino acids sequence; also called PuraMatrix) could be used as a matrix for osteogenic differentiation of bone marrow stromal cells *in vitro* and *in vivo* [179]. In other works using the same material, it was shown that MSCs could differentiate into mature

osteoblasts, to form mineralized matrices and to promote bone regeneration in the 3D structure [180, 181].

Hydrogels present great potential as *in situ* injectable biomaterials for cell delivery [143]. The polymeric network mimics many features of the extracellular matrix and can be formed *in situ* by either chemical or physical crosslinking methods [182, 183]. The structural integrity of hydrogels depends on crosslinks between polymer chains via various chemical bonds and physical interactions that are responsible for the formation of a gel [184]. The gelation reaction leads to the formation of a 3D network that is often employable as an injectable system where cells suspended in a liquid precursor solution are delivered *in vivo* to the site of interest in a minimally invasive way [141]. For example, Wang and colleagues developed a thermosensitive hydrogel composite that is injectable at low temperature, with a rapid gelling period, and is capable of releasing insulin-like growth factor and support MSCs adhesion and growth [185]. Another thermosensitive hydrogel based on poly(organophosphazene)-RGD conjugate mixed with MSCs was able to enhance bone formation in a nude mice model [186]. Klouda *et al.* developed a thermoresponsive, *in situ* crosslinkable hydrogel based on N-isopropylacrylamide that maintained MSCs viability for over 3 weeks and allowed osteogenic differentiation [187]. Poly(propylene fumarate-co-ethylene glycol) based hydrogels modified in bulk with a covalently linked RGDS peptide allowed the adhesion and proliferation of marrow-derived osteoblasts [188].

In an alternative hydrogel strategy, Fonseca and co-workers developed a sophisticated 3D cell culture microenvironment of modified alginate through partial crosslinking with a matrix metalloproteinase (MMP) cleavable peptide (proline-valine-glycine-leucine-isoleucine-glycine, PVGLIG) combined with RGD peptides and used it in the preparation of injectable, *in situ* crosslinkable hydrogel-like matrices (Fig. 8) [189]. MSCs were successfully immobilized within this matrix and shown to be capable of forming cellular networks.

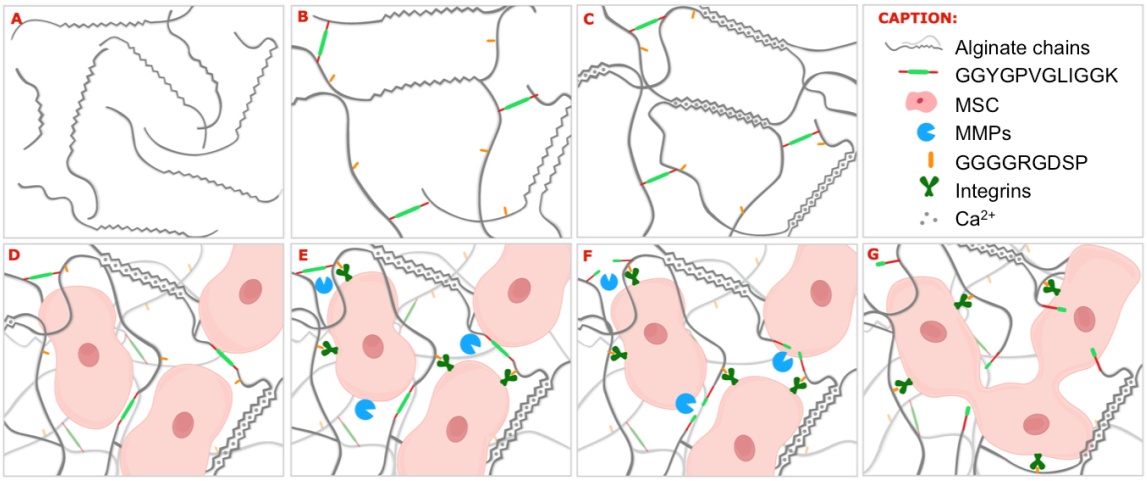


Figure 8. Schematic representation of functionalized alginate preparation and cell-hydrogel interactions: (A) soluble Na-alginate chains; (B) alginate chemically modified with cell-adhesion (RGD) and MMP-sensitive (PVGLIG) peptides; (C) crosslinking of alginate chains with calcium ions; (D) 3D culture of MSC within modified-alginate hydrogels; (E) integrin-mediated cell adhesion to RGD motifs present in the matrix; (F) cleavage of MMPs-sensitive peptide crosslinks by cellular proteases; (G) entrapped cells create pathways within the hydrogel, becoming less constrained, interacting with the matrix and surrounding cells [189].

The development of *in situ* gelling systems has received large attention over the past decade. More importantly, an increased number of *in situ* gel forming systems have been investigated for tissue engineering [190]. The interest has been sparked by the advantages shown by these *in situ* forming polymeric delivery systems, such as ease of administration, improved patient compliance and comfort. Moreover, they are also attractive for use in sterile conditions due to their reduced invasiveness associated with application. In addition, *in situ* cell immobilization is also beneficial for filling an irregular defect.

4. AIMS & OUTLINE OF THE THESIS

Aims

In this thesis, the putative crosstalk between mesenchymal stem cells (MSCs) and endothelial cells (ECs), key partakers in bone regeneration, was explored in the framework of a bone tissue engineering strategy.

The first aim consisted in investigating whether a coculture system of MSCs and ECs (human umbilical vein endothelial cells, HUVEC) could modulate the phenotype and proliferation of harvested MSCs. Complementing previous studies, different relative cell ratios were analyzed for longer, therapeutically relevant, culture periods in the work herein described. Moreover, MSCs osteogenic commitment, morphology, metabolic activity and gene expression of some osteogenic markers were assessed as well.

Secondly, a delivery system for MSCs based on alginate coupled with the arginine-glycine-aspartic acid (RGD) peptide, using a binary composition of high and low molecular weight alginate, was explored. To scrutinize the behavior of immobilized cells several parameters were considered, namely: metabolic activity, viability, morphology and osteogenic differentiation. Furthermore, it was evaluated whether and how MSCs immobilized in such 3D matrix influence the ability of neighboring ECs to form tube-like structures

Finally, the *in vitro* potential of the injectable RGD-alginate hydrogel as an *in situ* forming matrix to deliver ECs was studied. Multiple experimental approaches were employed to investigate how this microenvironment could influence the behavior of vascular cells, namely their ability to promote the outward migration of viable and proliferative cells, whose capacity to form 3D arrangements is preserved

Outline of the Thesis

One of the most promising tissue engineering approaches for the repair and regeneration of bone defects relies on the *in vitro* culture of three dimensional (3D) scaffolding materials seeded with autologous cells, followed by implantation into the patient [22, 191, 192]. Displaying multiple attractive features, such as easy accessibility, expansion potential, immunosuppressive properties, paracrine-mediated effect and capacity to differentiate into bone forming cells (osteoblasts) mesenchymal stem cells are often the selected cell population for such biomaterial-based approach [37, 108, 193, 194], Essentially, MSCs' fate decision can be regulated by several elements, including exogenous soluble growth factors, cytokines, hormones and chemicals, external mechanical forces and direct contact with other mature cell population [195, 196]. As pointed out in section 2.2 of the general introduction, endothelial cells (ECs) have been shown to express factors that induce osteogenic differentiation *in vitro,* when in direct contact with bone marrow stromal cells [73, 77]. However, this dialogue could influence not only the state of MSC differentiation, but also MSC proliferation. Intending to shed light on this issue, in **Chapter II** it was investigated if a synergistic coculture system of MSCs and ECs could affect the number and differentiation state of harvested MSCs. In contrast to other reports on this topic, the present investigation was carried out for a longer period of time (3 weeks instead of 1 to 6 days), and varying ratios of both cell types were considered [70, 72, 73, 78-81, 92, 197]. Additionally, several media were tested and MSCs osteogenic commitment in a non-osteogenic medium in the presence of ECs was carefully analyzed.

The success of biomaterials-based strategies is largely dependent on the success of the cell-material interface. The ability to functionalize a bioinert material like alginate with biomimetic peptides (integrin-binding arginine-glycine-aspartic acid (RGD) sequence) found in many extracellular matrix proteins, is sufficient to transform this polysaccharide into a material supporting cell adhesion, proliferation and organization [149, 150]. As highlighted in section 3 of the introduction, hydrogels represent an important class of biomaterials in biotechnology and medicine due to their excellent biocompatibility and minimum inflammatory response [198]. Moreover, these hydrophilic polymeric networks provide a 3D support for cells, mimicking many features of extracellular matrix and can be used for cell immobilization under mild conditions (e.g., in alginate microspheres) or as *in situ* gelling biomaterials for cell delivery [144-146, 182, 183]. **Chapter III** characterizes in detail the viability, metabolic activity and osteogenic

potential of MSCs immobilized within RGD-alginate microspheres and cultured under basal and osteogenic induction conditions.

The capacity of a biomaterial scaffold to allow proper vascularization and, therefore, vascular cell survival and organization, is of paramount importance to its good performance. In **Chapter III** the ability of MSCs immobilized within RGD-alginate microspheres to influence angiogenesis was also investigated. Immobilized MSCs significantly enhanced the ability of neighboring ECs to form tube-like structures in a reconstituted basement membrane (Matrigel). Since the two cell types were not in direct contact, the presence of vascular endothelial growth factor (VEGF) in the culture medium was also assessed, to verify if MSCs were able to produce this signaling protein and if it was able to diffuse through the hydrogel network into the surrounding medium.

Besides the strategies based on promoting angiogenesis from the host, in the past few years a growing interest has emerged in strategies for *in vitro* prevascularization of biomaterials [75, 76, 82, 85]. Although vascularization remains an unsolved challenge in tissue engineering, encouraging developments have been made to grow and maintain, *in vitro*, these self-assembled biological vascular networks [108, 109]. Polymers with the ability of forming 3D hydrogel networks offer an exciting possibility for the *in situ* delivery of vascular cells, allowing their organization into a 3D architecture and assisting cells in repopulating the surrounding tissue after transplantation [184]. For example, an alginate matrix can be made from soluble precursor solutions that crosslink *in situ*, which allows its application at the injury site by minimally invasive methods [199]. In **Chapter IV** the applicability of optimized injectable RGD-alginate hydrogels as vehicles for the minimally invasive delivery of endothelial cells was evaluated. Several techniques were employed to investigate the outcome of these microenvironments on vascular cell behavior, namely their ability to promote the outward migration of viable and proliferative cells that maintain the potential to form tubular-like structures.

Finally, **Chapter V** provides concluding remarks, integrating insights from the preceding chapters and discusses promising avenues of future research.

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Chapter II

Phenotypic and proliferative modulation of human mesenchymal stem cells via crosstalk with endothelial cells *

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ABSTRACT

The purpose of this work was to investigate if a coculture system of human mesenchymal stem cells (hMSC) with endothelial cells (human umbilical vein endothelial cells, HUVEC) could modulate the phenotype and proliferation of harvested MSCs. In addition to previous investigations on the crosstalk between these two cell types, in the present work different relative cell ratios were analyzed for long, therapeutically relevant, culture periods. Moreover, MSCs osteogenic commitment was assessed in a non-osteogenic medium and in the presence of HUVECs through magnetic cell separation, cell quantification by flow cytometry, morphology by fluorescent microscopy, metabolic activity and gene expression of osteogenic markers. Collectively, the present findings demonstrate that, by coculturing MSCs with HUVECs, there was not only the promotion of osteogenic differentiation (and its enhancement, depending on the relative cell ratios used), but also a significant increase on MSCs proliferation. This augmentation in cell proliferation occurred independently of relative cell ratios, but was favored by higher relative amounts of HUVECs. Taken together, this data suggests that HUVECs not only modulate MSC phenotype but also their proliferation rate. Therefore, a coculture system of MSCs and HUVECs can a have a broad impact on bone tissue engineering approaches.

Keywords: Cell-cell contact; Bone regeneration; Coculture; Osteogenic differentiation; Tissue engineering.

INTRODUCTION

Mesenchymal stem cells (MSCs) represent an adherent, fibroblast-like population present not only in bone marrow, but also in a number of other tissues, including blood, adipose tissue, muscle and dermis [1]. In bone tissue engineering approaches, bone marrow MSCs have demonstrated great potential for the treatment of bone loss, fracture non-union and osteogenesis imperfecta [2, 3]. The success of bone regeneration strategies is in part based in the reintroduction of *in vitro* expanded cells in a state that guarantees their differentiation into functional bone matrix-producing cells [4]. MSCs differentiated into the osteogenic lineage represent thus an appealing source of adult stem cells for bone tissue engineering applications [5].

However, these approaches find limitations in the considerably low percentage of MSCs in adult bone marrow, and the vast numbers required for therapeutic approaches [1, 6]. The identification of mechanisms involved in stem cells self-renewal and differentiation is important for the design of new strategies to expand stem cells in vitro and modulate their phenotype. MSCs fate decision can be regulated by exogenous soluble growth factors, cytokines, hormones and chemicals and by external mechanical forces [7]. Another approach to guide MSCs fate relies in the coculturing with other mature cell populations [8]. Ball and co-authors demonstrated that MSC are profoundly influenced by other cell types in direct contact [8]. Among these, endothelial cells (ECs) have been shown to express several factors, such as BMP-2, that induce osteogenic differentiation *in vitro,* when in direct contact with bone marrow stromal cells [9, 10]. Moreover, Villars and colleagues (2002) showed that intercommunication between ECs and osteoblast-like cells is dependent not only on diffusible factors but also on gap junctions (connexin 43) that play a crucial role in cell differentiation, which points to the need of cell-cell contact [11, 12]. Overall, two main dialogues were identified: a paracrine effect through VEGF, BMP-2, IGF production and a juxtacrine mechanism by gap junctional activity between the two cell types [13, 14].

Although several works have been evaluating the influence of a number of parameters for large-scale production of MSCs, such as cell source, age, density, passage number, medium composition, presence of growth factors and serum choice, the concept of using cocultures for MSCs expansion, in particular with ECs, seems to constitute a promising approach for bone regeneration applications and is far from being completely investigated [1, 6, 15-19].

The objective of this work was to investigate if a synergistic coculture system of

MSCs with endothelial cells could affect the number and state of differentiation of harvested MSCs. In contrast to other papers, in this cell-cell communication topic, the investigation was carried out for a longer time, 3 weeks instead of 1 to 6 days and different ratios of both cell types were considered [10-12, 20-25]. Additionally, several media were tested and MSCs osteogenic commitment in a non-osteogenic medium in the presence of ECs was carefully analyzed. The present results demonstrated that, independently of the relative cell ratio used, the coculture system promotes a significant increase in MSCs proliferation and differentiation. Furthermore, these results indicate that, for tissue engineering applications requiring a large number of MSCs committed into the osteogenic lineage, a coculture system with endothelial cells could be advantageous.

MATERIALS AND METHODS

Cell cultures

Human mesenchymal stem cells from bone marrow (MSCs, Lonza) were cultured and expanded in MSCGM (MSC growth medium, Lonza). For the experiments, cells were used at passage 8 and cultured in basal medium: Dulbecco's modified Eagle medium with low glucose (DMEM, Gibco), supplemented with 10% v/v inactivated fetal bovine serum (FBS, Gibco) and 1% v/v penicillin/streptomycin (P/S, Gibco). The culture medium was changed every other day.

Human umbilical vein endothelial cells (HUVECs, ScienCell) were cultured in M199 medium (Gibco), supplemented with 10% of inactivated fetal bovine serum (FBS) (Gibco), 0.1 mg/mL of heparin (Sigma) and 0.03 mg/mL of endothelial cell growth supplement (ECGS) (Sigma-Aldrich). HUVECs were used until passage 8 and harvested on 0.1 % w/v gelatin-coated tissue culture. The medium was renewed every 2 to 3 days until the cells reached confluence.

For cocultures, a pre-selection of the most adequate medium was performed. Several commercial media were tested, including Iscove Modified Dulbecco Medium (IMDM), M199, DMEM and a mixture 1:1 of these last two media. Cell behavior was analyzed in terms of metabolic activity and total protein content along the time.

After medium selection, MSC and HUVEC monocultures were seeded at a density of $3x10^3$ cells/cm^2 and cultured for 21 days. For cocultures both cell types were seeded at $1.5x10^3$ cells/ cm^2 (1:1), resulting in a final cell density of $3x10^3$ cells/cm^2. For

all the studies the total initial cell density was of $3x10^3$ cells/cm^2. For control experiments MSCs monocultures were seeded with a lower cell density (1500 cells/cm^2, the same MSC density used in the cocultures).

Cell metabolic activity

To measure metabolic activity a resazurin-based assay was performed. This method is based on the conversion of resazurin to resorufin by metabolically active cells that result in the generation of a fluorescent product. At different time points the medium was removed from the wells and resazurin (Sigma) was added in fresh medium at a final concentration of 10% (v/v). Then, cells were incubated at 37ºC for 4h, after which 200 µL/well were transferred to a 96 well black plate and fluorescence was measured (530 nm_{Ex}/590 nm_{Em}) in a Spectra Max Gemini XS (Molecular Devices).

Protein concentration

The bicinchoninic acid protein assay (BCA, Pierce) was used to detect and quantify the total protein content, as recommended by the manufacturer. Briefly, three samples of cell lysates for each condition, obtained by brief sonication in ice with 1% v/v Trition X-100, were incubated with the bicinchoninic acid (BCA) working reagent for 1 h at 37ºC. Then, the absorbance was read at 540 nm in a microplate reader (SLT Spectra).

Effect of relative cell ratios on MSC behavior

The effect of MSCs and HUVECs relative ratios on MSCs behavior was evaluated by testing the following cell combinations: 25%HUVEC-75%MSC, 50%HUVEC-50%MSC and 75%HUVEC-25%MSC. MSCs monocultures were used as a control and were seeded at a cell density of $3x10^3$ cells/cm^2. Independently of the cell ratio, cocultures were also prepared at the same total density of $3x10^3$ cells/cm^2, and the percentage for each cell type was calculated with reference to that density. The studies were carried on for 21 days.

To induce the formation of mineralized extracellular matrix, studies were performed in the presence of osteogenic stimuli (further on designated as osteogenic medium): basal medium (DMEM and M199 (1:1)) supplemented with 10 mM β-glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma) and 50 µg/mL ascorbic acid (Fluka).

Cell separation and quantification

At different time points, cells were detached from the well plate and an aliquot of cell suspension was transferred to a Neubauer chamber for total cell counting.

To quantify and further perform gene analysis on each cell type after coculture, cells were separated by magnetic cell sorting using magnetic beads coupled with an antibody against CD31 (Invitrogen) [12]. For this purpose, cells in coculture were harvested by trypsin and then incubated with anti-CD31 magnetic beads (at a ratio of 10:1 beads/HUVECs) for 30 min at 4ºC under gentle stirring. Afterwards, the supernatant fraction containing the cocultured MSCs (CoMSCs) was separated from the bead fraction, which contained the cocultured HUVECs (CoHUVECs), using a magnet device (Invitrogen).

Cell morphology

To analyze cell morphology, after fixation with 4% w/v paraformaldehyde and permeabilization with 0.1% v/v Triton X-100, cells were stained for F-actin (Alexa Fluor 488 phalloidin; Molecular Probes) and for nuclei (4',6-Diamidino-2-phenylindole dihydrochloride, DAPI; Sigma) and then visualized under an inverted fluorescence microscope (Axiovert 200M, Zeiss).

Flow cytometry analysis

Flow cytometry studies were performed on MSCs monocultures and on MSCs/HUVECs cocultures at different cell ratios. The percentage of HUVECs in the cocultures was estimated using anti-human CD31 (PECAM-1) antibody labeled with FITC (BD Biosciences). Cell suspensions were stained for 15 min, and for each day and ratio, 10,000 events were analyzed in triplicate on a FACSCalibur flow cytometer (Becton Dickenson). Data processing was performed using FlowJo software 8.7.

Alkaline phosphatase (ALP) activity

For ALP cytochemistry cells were incubated in the dark for 1h at 37ºC in Naphtol AS-MX phosphate/Fast Violet B salt (Sigma), as recommended by the manufacturer. After being rinsed with deionized water and air-dried samples were observed under an inverted microscope (Axiovert 200M, Zeiss).

For quantitative ALP analyses, cell lysates were obtained by treatment with 1 % v/v Triton X-100 under brief sonication in ice, and then incubated with the chromogenic substrate 2 mM ρ–nitrophenol phosphate in 0.2 M bicarbonate buffer (pH 10), 0.05 %

v/v Triton X-100, and 4 mM $MgCl_2$ (Sigma) for 1h at 37 ºC. The reaction was stopped by adding 1 M NaOH and absorbance was read at 405 nm in a microplate reader (SLT Spectra). The amount of product was obtained from a p-nitrophenol standard curve. The enzymatic activity was normalized to total protein concentration, obtained using the bicinchoninic acid protein assay (BCA, Pierce), and expressed as nmol/min/mg protein.

Mineralization

For assessment of mineralization by von Kossa staining cells were fixed with 3.7% v/v formaldehyde in PBS during 15 min. Then, cells were incubated in 2.5 wt-% silver nitrate (Sigma) for 30 min under UV light, followed by incubation in 5 wt-% sodium thiosulfate (Aldrich) for 3 min. Finally, wells were rinsed with deionized water and air-dried prior to observation under an optical microscope.

Phenotype analyses through mRNA expression

Total RNA was extracted from MSCs in mono- and cocultures, at weekly time points, using the Total RNA isolation Kit (Macherey-Nagel), as recommended by the manufacturer. Subsequently, 1 µg of the total RNA was used as template for single strand cDNA synthesis with the Superscript pre-amplification system (Invitrogen) in a 20 µL final volume containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 5 mM MgCl2, 10 mM DTT, 0.5 mM of each dATP, dCTP, dGTP and dTTP, 0.5 µg oligo(dT)12-18, and 50 U of reverse transcriptase. After incubation at 42ºC for 50 min, the reaction was stopped at 70ºC for 15 min. After the cDNA synthesis reaction, qReal-Time PCR was carried out in a total volume of 25 µL of a mixture containing 5 µL of cDNA diluted at a 1:80 ratio (corresponding to 3.125 ng of total RNA), 200 nM of each forward and reverse primers and 1x Mesa Green qPCR MaterMix Plus for Sybr Assay (Eurogentec). Primers of ubiquitary ribosomic protein P0 forward: 5 ´ ATGCCCAGGGAAGACAGGGC 3´; reverse: 5’ CCATCAGCACCACAGCCTTC 3´; type I collagen (COL I) forward: 5’ GGA ATG AGG AGA CTG GCA ACC 3´ and reverse 5´ TCA GCA CCA CCG ATG TCC AAA 3´; alkaline phosphatase (ALP) forward 5´ AGC CCT TCA CTG CCA TCC TGT 3´; reverse 5´ ATT CTC TCG TTC ACC GCC CAC 3´; transcription factor Runx2 forward 5’ GTGCCTAGGCGCATTTCA 3’ and reverse 5’ GCTCTTCTTACTGAGAGTGGAAGG 3’; osteocalcin (OCN) forward 5’ TGAGAGCCCTCACACTCCTC 3’ and reverse 5’ ACCTTTGCTGGACTCTGCAC 3’; bone sialoprotein (BSP) forward 5’ CGATTTCCAGTTCAGGGCAGT 3’ and reverse 5’

CAACACTGGG CTATGGAGAG G 3'. qRT-PCR experiments were run using an iQ5 (Bio-Rad) and analyzed with the iCycler IQ™ software. Relative quantification of gene amplification by qRT-PCR was performed using the cycle threshold (C_t) values and relative expression levels were calculated as follows: 2^(C_t P0gene - C_t gene of interest). The expression value for each target gene was normalized to the P0 value at each time point. Afterwards, the gene expression at each day was normalized to day 0. For each PCR, samples were analyzed in duplicate and three independent experiments were performed.

Statistical analyses

Statistical analyses were performed using SigmaStat (Statistical Software, STAT32 MFC Application). Statistical significance was assessed by using two way analysis of variance (ANOVA) in conjunction with Tukey's test, pairwase multiple comparison procedures. Differences were considerer statistically significant when *p* values were lower than 0.05. All data are presented as mean values ± standard deviation.

RESULTS

Selection of base medium

HUVECs and MSCs behavior was evaluated in several commercial media, including Iscove Modified Dulbecco Medium (IMDM), Medium 199 (M199), Dulbecco's Modified Eagle's medium (DMEM) and a mixture 1:1 of the last two media. The three media present some major differences in terms of composition, (composition provided by the manufacturer). For instance, IMDM and M199 have a higher number of amino acids than DMEM. IMDM has the highest glutamine concentration, followed by DMEM and then M199. Additionally, M199 has the lowest concentrations on vitamins and the other two have a similar composition. In the inorganic salts content, such as calcium, chlorine, iron, potassium, no major differences were found. Regarding other components, IMDM is the only with HEPES (5958 mg/L) and presents a D-Glucose concentration (4500 mg/L) higher than the others (1000 mg/L). Moreover, M199 does not have sodium pyruvate in its composition.

To evaluate the effect of the different media on cell behavior, metabolic activity and protein quantification assays were carried out (Fig. 1). Overall, there was a

continuous increase in terms of the metabolic activity and protein content along the time, for both HUVECs and MSCs, in all the media tested. However, the medium that showed the best results was different in both cell types. Considering both assays, for HUVECs the following profile could be outlined: IMDM > M199 = M199+DMEM > DMEM. For MSCs the following sequence was obtained: M199+DMEM > DMEM > IMDM = M199. In a coculture approach, the selected medium should take into consideration the behavior of both cell types. Collectively, the described data pointed towards the selection of the combined medium M199+DMEM, which provided a performance close to the ideal in both situations.

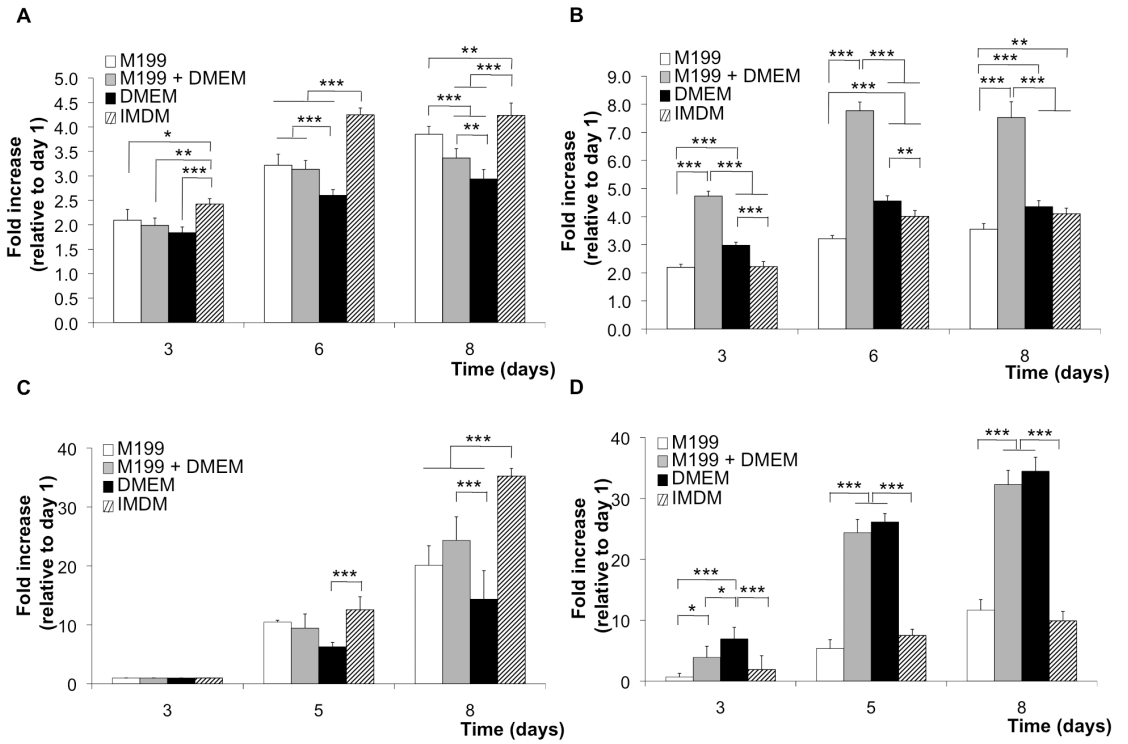


Figure 1. (A,B) Metabolic activity and (C,D) protein concentration of (A, C) human umbilical vein endothelial cells and (B,D) mesenchymal stem cells cultured under four different media: M199, M199 with DMEM, DMEM and IMDM. The initial cell seeding density was the same in all the conditions ($3x10^3$ cells/cm^2). Data are expressed as mean ± standard deviation (n=3). (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

Coculture study: cell quantification, morphology and differentiation

Cocultures were established by seeding the two different cell types in direct contact at a cell ratio of 1:1. Both cell types were cultured (in mono- and coculture) in the selected medium, and the evolution on the number of total cells along the time was

analyzed, as depicted in Fig. 2. Cells in coculture exhibited, as a whole, a significant increase in number throughout the culture, reaching from day 14 values significantly higher than any of the monocultures, and continued to proliferate along the last week (Fig. 2A).

When both cell types were analyzed in separate, it could be seen that while the number of MSCs in monoculture significantly increased until day 14th and then remained practically unchanged, the MSCs in coculture (CoMSCs) increased in number throughout the 21 days (Fig. 2C). Moreover, CoMSCs presented higher proliferation rate than MSC in monoculture (at 1.5 x10^3 or 3 x10^3 cells/cm^2) in the last two weeks of culture. MSCs in coculture increased rapidly, reaching approximately 80% of the cells in coculture after 1 week, and then cells continued to proliferate until the end of the culture, reaching a percentage of 94% (Fig. 2B).

The HUVECs in monoculture showed higher proliferation rate than the MSCs on the 1st week of culture (Fig. 2A). However, the number of cells significantly decreased thereafter. Although the percentage of HUVECs in coculture (CoHUVECs) progressively declined, cells continued to proliferate during the 2nd week (Fig. 2D).

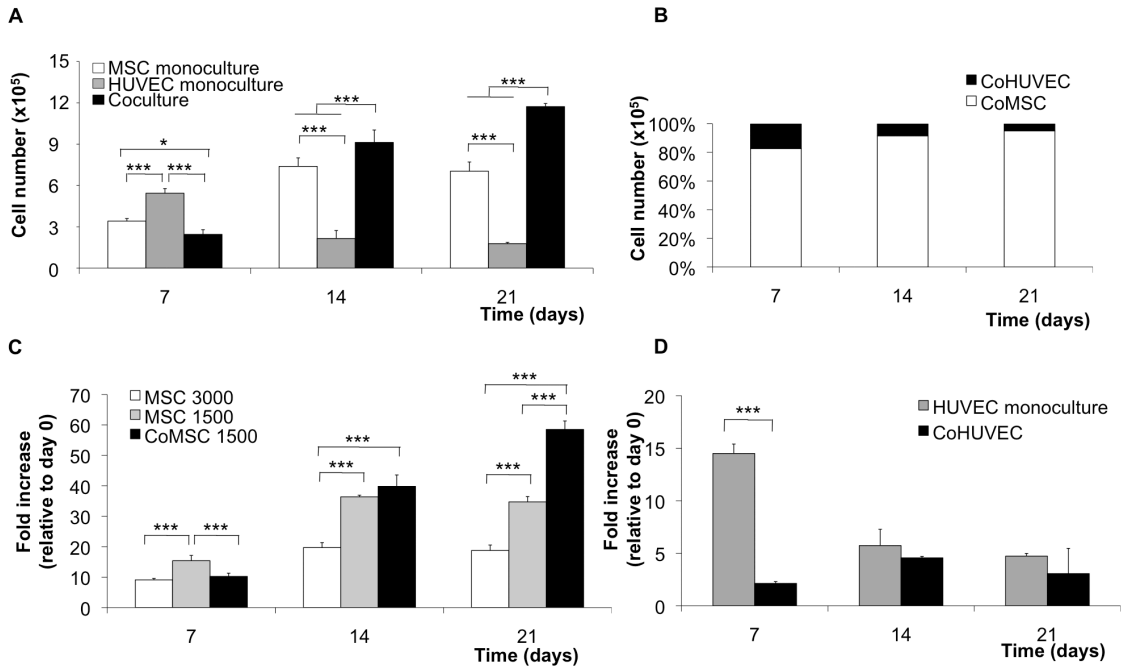


Figure 2. Mesenchymal stem cells (MSC) and human umbilical vein endothelial cells (HUVEC) cultured in mono- and coculture during 21 days. (A) Total cell number of MSC, HUVEC and coculture of both cell types; (B) Relative number of MSC (CoMSC) and HUVEC (CoHUVEC) in the coculture; (C) Fold increase in cell number relative to day 0 of MSC in the coculture (CoMSC 1500; 1500 cells/cm^2 of MSCs with 1500 cells/cm^2 of HUVECs), MSC in monoculture with a cell density of 3000 cells/cm^2 (MSC 3000), and MSC in monoculture with a cell density of

1500 cells/cm^2 (MSC 1500); (D) Fold increase in cell number relative to day 0 of HUVEC in mono- and co-culture during 21 days. Cocultures were established by seeding the two cell types in direct contact at a cell ratio of 1:1 with a final cell seeding density of 3x10^3 cells/cm^2 and the medium used (mixture 1:1 of DMEM and M199) was the same in all the conditions tested. For monocultures cell density was of 3x10^3 cells/cm^2 with the exception of MSC 1500. Data are expressed as mean ± standard deviation (n=3). (* $p \leq 0.05$; *** $p \leq 0.001$)

The cell morphology study, revealed evident differences between cells organization in MSCs mono- and cocultures, as can be depicted in Fig. 3A. MSCs in monoculture appeared randomly distributed at the cell culture surface along the first 2 weeks, revealing, at day 21, a fibroblastic-like shape with stress fibers running in parallel according to cell orientation. In coculture, cells were rearranged in a significantly different manner, forming a kind of tubular-like cellular network. This type of structure becomes less evident along the time, as the cell density rose. At day 21, it was possible to visualize a crisscrossed pattern of actin cytoskeleton with thick stress fibers.

Differences in cells morphology were also evident on the ALP cytochemical analysis (Fig. 3B). However, in cocultures it was possible to detect a much higher amount of ALP positive cells in all time points. For instance, at day 14, the wells from the coculture revealed an almost monochromatic (pink) surface. These results were further corroborated by the ALP colorimetric assay (Fig. 3C). ALP activity, normalized to total protein concentration, reached significantly higher levels in coculture than in monoculture. In monoculture the levels of ALP remained low and constant throughout the culture and always below the values reached in coculture.

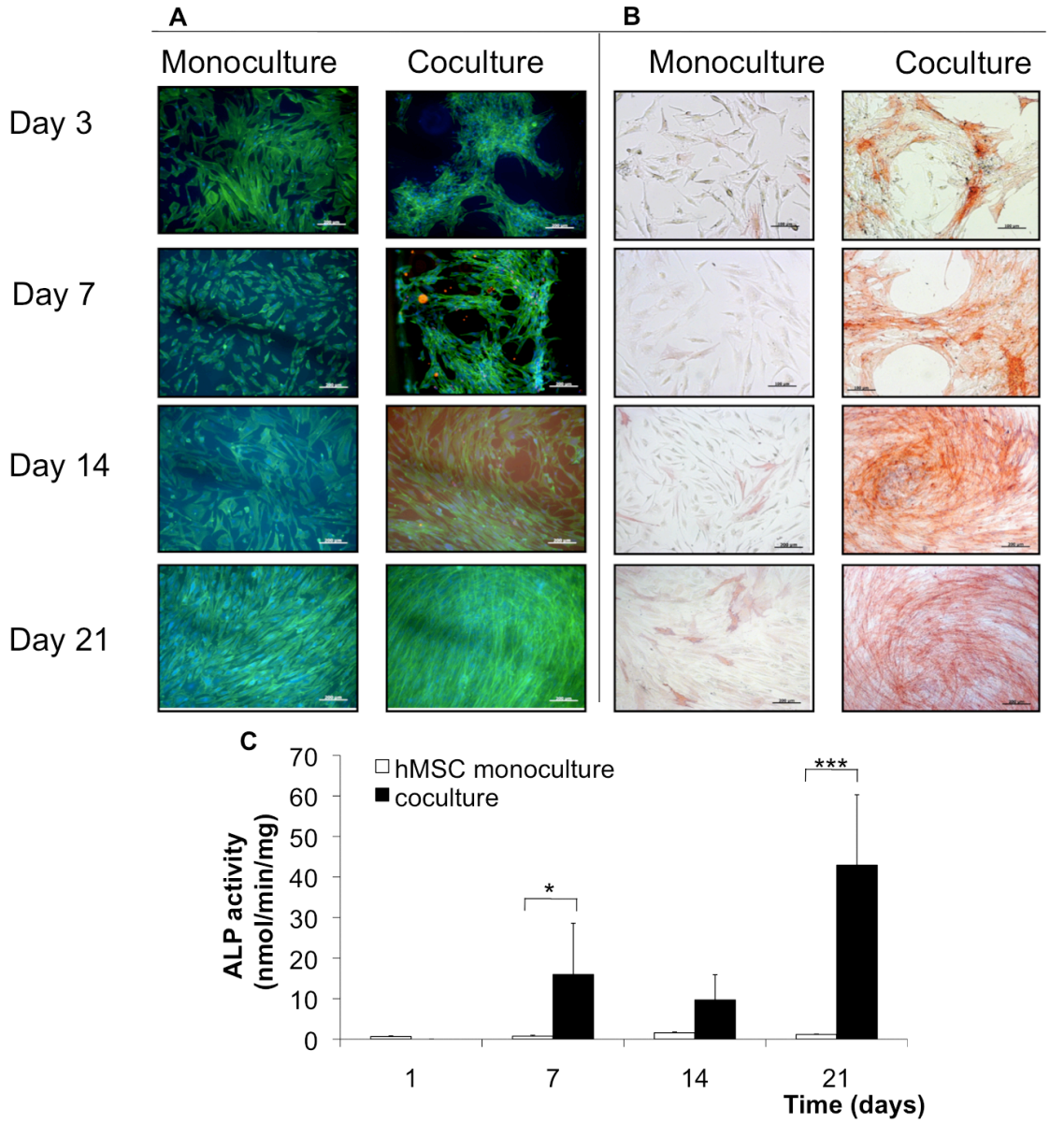


Figure 3. (A) Immunocytochemistry of actin filaments (green) and nuclei (blue) and (B) ALP histochemistry and (C) ALP activity in MSCs cultured alone or cocultured with HUVECs at different time points under basal conditions. Cocultures were established by seeding the two different cell types together at a cell ratio of 1:1 with a final cell seeding density of $3x10^3$ cells/cm^2. Bars correspond to 200 μm. Data are expressed as mean ± standard deviation (n=3). (* $p \leq 0.05$; *** $p \leq 0.001$)

As previously described, the two types of cells in coculture were separated and additional differentiation studies were performed using only the MSC fraction. The gene expression profile of several osteogenic markers was analyzed (Fig. 4). The gene profile for ALP and COL I revealed a significant difference between MSC in monoculture and cocultured MSC (CoMSC) (for ALP $p < 0.05$ and for type I collagen $p = 0.01$). Runx 2 and BSP presented a significant increase after one week of culture in

CoMSC. The late-stage osteogenic differentiation marker, OCN, even after 21 days of culture did not show significant differences between MSC and CoMSC.

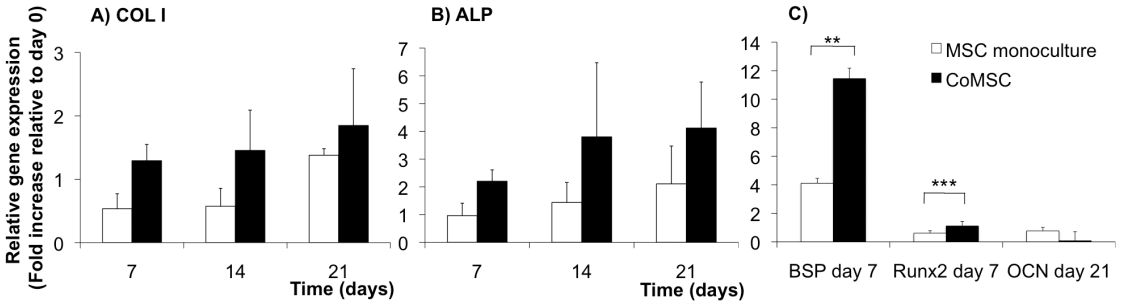


Figure 4. Gene expression levels of osteogenic markers of mesenchymal stem cells (MSC) growing in monoculture and in coculture with endothelial cells (CoMSC) by qPCR. (A) type I collagen (COL I), (B) alkaline phosphatase (ALP) and (C) bone sialoprotein (BSP) and transcription factor Runx2 at day 7 and osteocalcin (OCN) at day 21. To perform gene analysis on CoMSC, cells were separated by magnetic cell sorting using magnetic beads coupled with an antibody against CD31. CoMSC presented significantly higher expression levels for COL I and ALP than MSCs in monoculture (collagen type I $p = 0.01$ and ALP $p < 0.05$). The experiment was performed three times, each in triplicate. (** $p \leq 0.01$; *** $p \leq 0.001$)

Effect of different cell ratios on MSC behavior

In order to evaluate the effect of MSCs and HUVECs relative ratios on MSCs behavior, the following combinations were tested: 25%HUVEC-75%MSC, 50%HUVEC-50%MSC and 75%HUVEC-25%MSC. MSCs monocultures were used as a control. The influence of endothelial cells on the metabolic activity, proliferation and osteogenic differentiation of MSCs was analyzed.

Concerning metabolic activity (Fig. 5A), the three different ratios presented a significant increase along the first 2 weeks of culture, followed by a plateau, although the MSC monoculture showed the highest fold increase after 1 week. During the 2nd and 3rd weeks this behavior changed, and cells in coculture reached metabolic activity values significantly higher than the monoculture, at all the ratios tested. Among the different ratios, the combination 50%HUVEC-50%MSC was the one that presented the highest increase, although not significantly different from the others.

The evolution of the number of cells along the time is depicted in Fig. 5B, showing that in all the three ratios tested, the percentage of MSCs increased, while the relative amount of HUVECs decreased. In fact, MSCs were able to proliferate throughout the 21 days of culture and the ratio that showed the highest MSCs proliferation was the one that started with a lower percentage of MSCs (75%HUVEC-

25%MSC). Moreover, in this ratio, HUVECs presented the lowest decline.

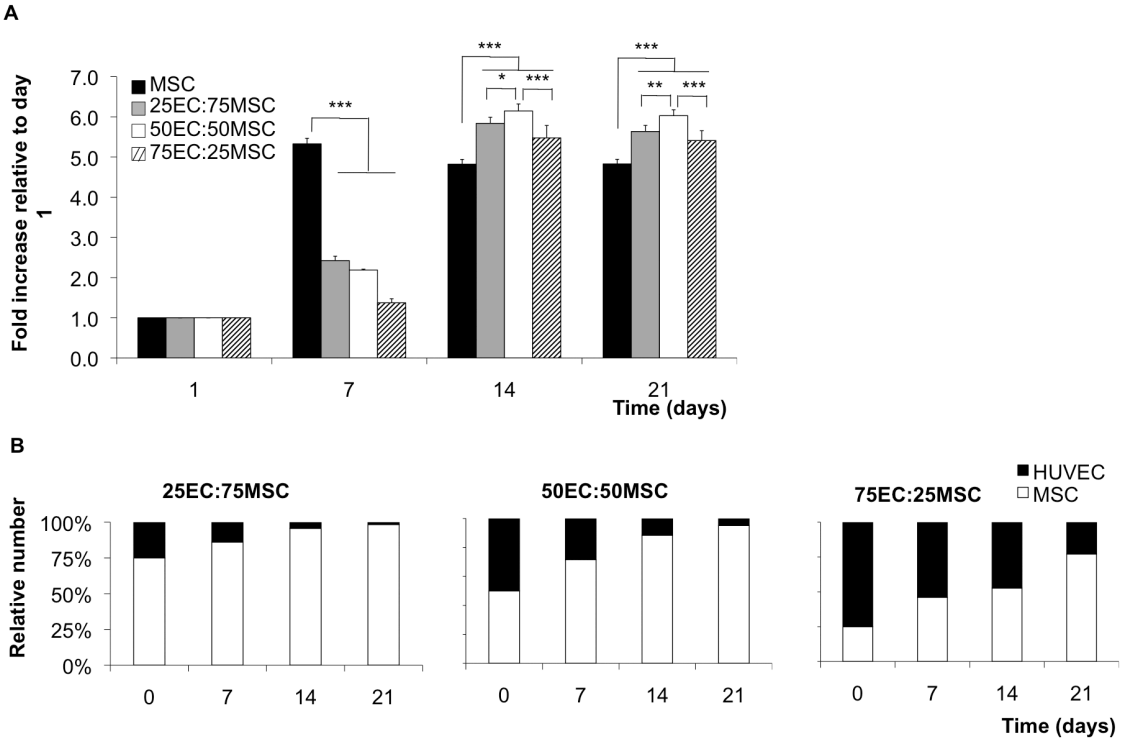


Figure 5. (A) Metabolic activity, relative to day 1, of mesenchymal stem cells (MSC) in monoculture or in coculture with human umbilical vein endothelial cells (HUVEC) and (B) the percentage of both cell types in the coculture during 21 days. Cocultures were tested using different relative cell ratios: 25% HUVEC with 75% MSC (25EC:75MSC), 50%HUVEC with 50% MSC (50EC:50MSC) and 75% HUVEC with 25% MSC (75EC:25MSC). Independently of the cell ratio, cocultures have also the same final density of $3x10^3$ cells/cm^2. Thus, the used percentage for each cell type in the coculture was related to this final value of $3x10^3$ cells/cm^2. Metabolic activity was assessed using the resazurin-based assay and relative quantification was obtained by flow cytometry. Data are expressed as mean ± standard deviation (n=3).

To analyze MSCs differentiation in the coculture systems, ALP activity was measured weekly for a 3 weeks period, as illustrated in Fig. 6. Under basal conditions (Fig. 6A), MSCs in monoculture kept a similar low value (practically null) throughout the time, without significant variations. However, in the presence of HUVECs, there was an increase on the ALP activity. Although the cocultures showed the highest values, only in the last week they reached values significantly higher than those obtained in monoculture. The ratios 25%HUVEC-75%MSC and 50%EC-50%MSC were the ones resulting in the highest ALP activity values. Under osteogenic conditions, MSCs monoculture showed a significant increase on ALP activity during the 1st week reaching then a plateau (Fig. 6B). In general, in the first 2 weeks of culture, the three

relative ratios tested did not present significant ALP differences between them. In the last week there was a significant upregulation in ALP activity in all the cocultures, with values significantly higher than the MSCs monoculture.

When comparing osteogenic with basal conditions, ALP activity values were not very different but were in general slightly higher in the former. Additionally, all the ratios tested achieved significantly higher ALP activity values than the monoculture.

Cytochemical stainings of ALP activity and mineralization nodules are presented in Fig. 7, and provide additional qualitative evidence that cells differentiated along the osteoblastic lineages at all ratios tested.

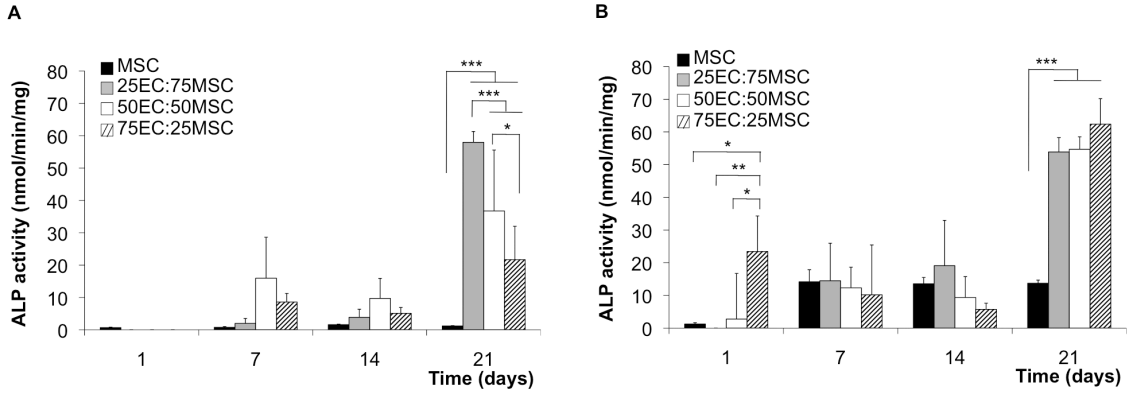


Figure 6. Alkaline phosphatase activity of mesenchymal stem cells (MSC) cultured alone or cocultured with human umbilical vein endothelial cells (HUVEC) at different relative cell ratios under (A) basal conditions and (B) osteogenic conditions. Results are expressed in nmol per min per mg of protein. Cocultures were tested at different relative cell ratios: 25% HUVEC with 75% MSC (25EC:75MSC), 50% HUVEC with 50% MSC (50EC:50MSC) and 75% HUVEC with 25% MSC (75EC:25MSC). Independently of the cell ratio cocultures were also prepared at a total density of $3x10^3$ cells/cm^2, and the percentage for each cell type was calculated with reference to that density. Data are expressed as mean ± standard deviation (n=3). (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

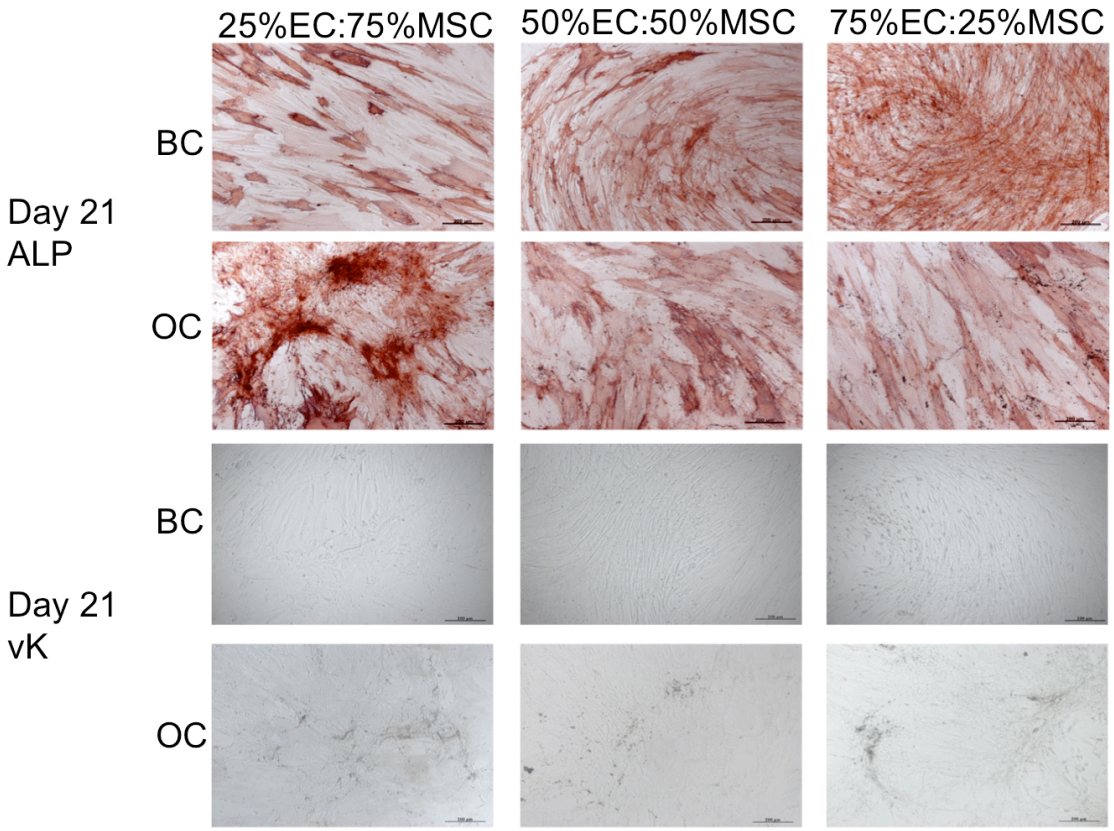


Figure 7. Alkaline phosphatase activity (ALP) and von Kossa (vK) histochemistry in cocultures after 21 days, under basal conditions (BC) and osteogenic conditions (OC). Cocultures were tested at different relative cell ratios: 25% HUVEC with 75% MSC (25EC:75MSC), 50% HUVEC with 50% MSC (50EC:50MSC) and 75% HUVEC with 25% MSC (75EC:25MSC). Independently of the cell ratio cocultures were also prepared at a total density of $3x10^3$ cells/cm^2, and the percentage for each cell type was calculated with reference to that density. Bars correspond to 200 μm.

DISCUSSION

A large number of MSCs committed to the osteogenic lineage is necessary for creating a tissue-engineered implant able to fill bone defects. It is generally accepted that MSCs play a critical role in bone tissue regeneration due to their differentiation capacity into distinct end-stage cell types, immunosuppressive properties and ability to produce a broad spectrum of bioactive macromolecules that are capable of establishing a regenerative microenvironment [26]. In bone marrow, MSCs represent a very small fraction of all nucleated cells: less than 0.01% [27, 28].

As previously pointed out, this study focused on the use of a MSCs-endothelial cells coculture system as a strategy to modulate MSCs expansion and osteogenic differentiation *in vitro*. Our team is specifically interested in addressing this subject in

the context of a minimally invasive bone regeneration strategy using injectable 3D matrices [29-33]. As reviewed by Grellier and colleagues (2009) there are some studies on the reciprocal regulation and functional relationship between bone and endothelial cells which, in turn, may be greatly influenced by the culture conditions [13].

In a preliminary phase of the present study, and due to its impact in future cell studies, the appropriate medium for the coculture system was established. It is worth mentioning that, due to the dramatic influence that the batch of serum may have on MSCs behavior at different levels (proliferation, differentiation and gene expression) [6], a previous serum batch-selection was made and it was used to supplement all the tested media. Media selection was based on cell metabolic activity, total protein content and optical microscopy analyzes (Fig. 1). The tested media included those routinely used to culture the two cell types in monoculture (DMEM and M199 for MSCs and HUVECs, respectively) and also IMDM, a medium reported in several works for expansion and further coculture of both cell types [10, 12, 20, 24, 34, 35]. In several other studies, the medium selected for the cocultures was the same one used for ECs monocultures [21-23, 36-42]. Here, the medium selected was a DMEM+M199 mixture (1:1). After one week of culture, the cell performance in the presence of this medium was closer to the one obtained with DMEM and M199 for MSCs and HUVECs, respectively. Moreover, by choosing this mixed medium, cells in coculture will continue to be in contact with their expansion medium.

Several published studies on this topic provide insights into the relationship between the two cell types but there is still a limited knowledge concerning the proliferation behavior of cells in coculture. For a detailed analysis on cell proliferation in coculture, immunomagnetic beads coupled with an anti-CD31 antibody were used to separate both cell types. This technique allowed confirming that both cell types were proliferating, although with significantly different growth rates, being it higher for MSCs. This difference in cell proliferation could explain the different percentage of both cell types in coculture (Fig. 2). Moreover, MSCs growing in coculture (CoMSC) showed a clearly higher cell growth rate as compared with MSCs in monolayer. On one hand, this result points towards the potential of HUVECs to stimulate MSCs proliferation. On the other hand, MSCs could curtail ECs capability for expansion due to their high proliferative capacity in coculture. Notwithstanding that HUVECs in monoculture presented a higher growth rate than in coculture, CoHUVECs proliferated for a longer period than HUVECs alone. This ruled out the possibility that CoHUVECs may be dying since, in fact, there was an increases in cell number. Noteworthy, in monoculture

when endothelial cells are maintained at confluence for an extended period of time, they become tightly packed but show no tendency to overlap or overgrow. Then, when cell density becomes to high, cells start to detach and die [43].

Another parameter that could influence cell proliferation was the extracellular matrix produced, since an increase on one of its major components was detected (upregulation of collagen type I). In parallel, a study was perform to validate that the significant increase in CoMSC proliferation was indeed related with HUVECs presence, and not with differences in terms of cell seeding densities (CoMSCs cell seeding density was 1500 cells/cm^2 instead of 3000 cells/cm^2 as the MSC monoculture). For that purpose MSCs were seeded alone at 1500 cells/cm^2, which allow comparing with CoMSC of the same real "age" (having gone through the same cell divisions) during the 21 days of culture (Fig. 2C). Although MSCs at lower cell density presented a higher proliferation rate in the first weak, in the following two weeks CoMSCs continued to exhibit the highest proliferation (Fig. 2C), corroborating the potential of HUVECs to stimulate MSCs proliferation.

It is well established that the cytoskeleton plays important roles in cell morphology, adhesion, growth and signaling. The actin network, one of the three components of the cytoskeleton, is of critical importance in the determination of the mechanical properties of living cells. In this study, it was shown that the actin cytoskeleton changes from an apparently well-organized structure, with long fine fibers running in parallel along the cell axis in MSCs monocultures, to a more random arrangement of the actin cytoskeleton in cocultures (Fig. 3A). Yourek and colleagues observed this type of cytoskeleton changes upon osteogenic differentiation [44]. They further stated that this reorganization could be related with the natural reaction of the actin cytoskeleton in bone cells to the shear stress that occurs *in vivo* during bone modeling/remodeling. Grellier *et al.* (2009) also observed a similar cell rearrangement in osteoprogenitor cells (HOPs) cocultured with ECs [12]. They further observed HUVECs migration along HOPs and suggested that the direct contact between the two cell types could stimulate the release of chemotactic factors that, in turn, would stimulate HUVEC migration.

To support the hypothesis that cytoskeleton changes of CoMSC could be related with their osteogenic differentiation in the presence of endothelial cells, further studies were made. Consequently, ALP cytochemistry and activity, and also gene expression analyzes of osteogenic markers, were performed. ALP activity is considered an early osteogenic marker since its expression increases from the beginning of cell

differentiation and increases throughout extracellular matrix maturation [45]. As shown in Fig. 3B there was an increased in the number of ALP stained cells in coculture comparing to monoculture even after only 3 days, suggesting a commitment of CoMSCs into the osteogenic lineage. The ALP activity profiles and gene expression were assessed and both support the previous data (Figs. 3C and 4B). The gene profile of type I collagen, a protein of the extracellular matrix and the main organic component of bone tissue, was also higher in CoMSC (Fig. 4A). In order to further analyze osteogenic commitment of CoMSC, gene expression of two other early markers of osteogenic differentiation, Runx2 and bone sialoprotein (BSP), was evaluated by real time quantitative RT-PCR assay. Runx2, is an earlier transcription factor proven essential for commitment to osteoblatogenesis. BSP is a major non-collagenous extracellular matrix protein in bone and promotes the initial formation of mineral crystals [46, 47]. Both genes were significantly upregulated, which further confirms the differentiation of CoMSC along the osteoblastic lineage (Fig. 4C). Other gene that was investigated was osteocalcin (OCN) that is a noncollagenous calcium-binding bone protein and is considered a late-stage osteogenic differentiation marker [48]. OCN expression did not increase and, even after 21 days, there was no upregulation. Overall, the genetic profile obtained points towards an osteogenic commitment of MSCs culture in the presence of ECs, but cells were not fully mature.

The effect of the relative HUVECs-MSCs ratio was also investigated. This aspect can be controversial since many different combinations were found in the literature: 4:1 [39], 2:1 [10, 12, 20, 30, 35, 49], 1:1 [4, 9, 21-23, 36, 38, 50, 51], 1:1.5 [37], 6.5:1 [41], 3:1 [24], 1.5:1 [52] of endothelial and osteoblast-like cells, respectively. The majority of these coculture studies have used higher amounts of endothelial cells than bone cells, although the medium composition was often different. Moreover, among these works only one mentions a preliminary cell ratio study to justify the selected ratio used [41]. Although authors did not show any results, they stated that a ratio of EC:osteoblasts between 5:1 an 10:1 resulted in the presence of both cell types after 1 week of culture, whereas ratios of 1:1, 1:5 and 1:10 resulted in substantial reduction of EC number after the same period. In the present work, a relative cell ratio study was also performed, with an increasing on the relative amount of HUVECs: 25%HUVEC-75%MSC, 50%HUVEC-50%MSC and 75%HUVEC-25%MSC, to investigate how the different ratios could influence cell behavior. Regarding metabolic activity (Fig. 5A), monoculture reaches the maximum at day 7, which is probably related to a higher MSC proliferation. In the coculture, in the following 2 weeks there was a significant increase in metabolic activity,

mostly related to a higher CoMSC proliferation, as observed earlier. This one-week difference may be related to the decrease in cell number of ECs in the coculture that was further compensated with CoMSC proliferation (Fig. 5B). The cell quantification assay provided additional information on the behavior of the two cell types at each cell ratio throughout the time (Fig. 5B). As expected, higher initial amounts of HUVECs led to a higher percentage of HUVECs in the coculture at day 21. Additionally, HUVECs relative number decreased throughout time, while MSCs increased using all the three relative cell ratios tested. In short time studies the initial cell seeding may not be essential, although for longer periods the cell ratio in the coculture needs to be carefully evaluated. Concerning ALP activity (Fig. 6A) no major differences were found between the different cell ratios until the last week, where the ratio with higher relative amounts of MSC presented higher ALP activity.

Additionally, cell studies were also performed in the presence of osteogenic media. This allowed assessing the *in vitro* mineralization of MSC in contact with ECs due to the presence of an inorganic phosphate source (beta-glycerophosphate). It was possible to observe mineral deposits in the cell culture of all the ratios (Fig. 7). Moreover, cells in osteogenic conditions showed some changes in cell morphology comparing to cells in basal conditions (Fig. 7). However, regarding ALP activity, no major differences were found between basal and osteogenic conditions in all ratios (Fig. 6).

Collectively, the present findings demonstrate that, by coculturing MSCs with HUVECs, there was not only an enhancement of osteogenic differentiation, but also a significant increase on MSCs proliferation. This augmentation in cell proliferation occurred in the presence of ECs, independently of relative cell ratios, but was favored by higher relative amounts of HUVECs. The main conclusions drawn from this work may be helpful for the development of a strategy for large-scale production of mesenchymal stem cells committed into the osteogenic lineage for tissue engineering applications, where a high amount of cells is required. Further studies are being carried out to assess the relevance of the present findings in an *in vivo* scenario. Previous results from our group provided a clear indication that ECs are also able to induce the commitment of MSCs into the osteogenic lineage *in vivo*. In fact, when both cell types were cocultured within a 3D matrix and then implanted in a rat femoral critical-sized defect, there was a significant increase in mineralization compared with the implantation of MSC alone [30]. Additional studies are being carried out to get further insight into cocultured cell behavior *in vivo*, under different experimental conditions. In

case of success, that knowledge would be useful for developing minimally invasive regenerative therapies using injectable 3D matrices as (multi)cell delivery systems [29-32].

This work constitutes a step forward in the pursuit for a better understanding of the interaction between these two cell lines, which can easily find application in several other tissue engineering approaches where vascularization is of prime importance. To our knowledge, the current paper showed for the first time that coculturing MSC with HUVECs significantly increased MSCs proliferation, independently of the relative cell ratio.

ACKNOWLEDGEMENTS

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Chapter III

Immobilization of human mesenchymal stem cells within RGD-grafted alginate microspheres and assessment of their angiogenic potential *

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ABSTRACT

In this work, human mesenchymal stem cells (hMSC) immobilized in RGD-coupled alginate microspheres, with a binary composition of high and low molecular weight alginate, were investigated. Cells immobilized within RGD-alginate microspheres (during 21 days) showed metabolic activity with an overall viability higher than 90%, short cell extensions and, when induced, they were able to differentiate into the osteogenic lineage. In osteogenic conditions (comparing to basal conditions) immobilized cells presented alkaline phosphatase (ALP) activity and an upregulation of ALP, collagen type I and Runx 2 expression. Moreover, mineralization was also detected in immobilized cells under osteogenic stimulus. In addition, it was demonstrated for the first time that MSCs immobilized in this 3D matrix were able to enhance the ability of neighboring endothelial cells to form tube-like structures. Overall, these findings represent a step forward in the development of injectable stem cell carriers for bone tissue engineering.

Keywords: Biomaterials, Angiogenesis, Bone Tissue Engineering, Regeneration, Cell Encapsulation, Mesenchymal Stem Cells, RGD peptide.

INTRODUCTION

The development of injectable hydrogels for bone tissue engineering, allowing the localized and controlled delivery of cells with a high viability, offers an attractive strategy to fill-in bone defects. These polymeric systems can be implanted in the body using non-invasive surgical procedures, which reduce patient discomfort and promote faster recoveries at lower costs [1]. For this type of strategy, calcium-crosslinked sodium alginate hydrogels have several advantageous features over other gel matrices used for cell immobilization, namely the ability to: a) crosslink *in situ* under mild conditions; b) encapsulate living cells; c) form 3D constructs; and d) be easily sterilized and stored [2, 3]. As a consequence, alginate, a natural polysaccharide extracted from seaweed, has been extensively used in the form of microspheres for cell immobilization [4-6]. Additionally, microspheres can be prepared in a controlled way, resulting in beads with a narrow size distribution. These regular spherical particles with a controlled size, combined with a vehicle with *in situ* gel-forming ability, can be used as an injectable bone filler system [7].

Alginate, an originally non-cell interactive polymer, can additionally be covalently modified with peptides containing the RGD sequence to promote cell adhesion [8, 9]. Moreover, its molecular weight can be modified through irradiation and oxidation to modulate its viscoelastic properties, as well as its degradation rate [9]. The lower viscosity of the irradiated polymer results in reduced shear forces during the cell-polymer mixing process and, consequently, in higher cell viability [10]. Previous studies using RGD-alginate microspheres with a binary composition of high and low molecular weight already demonstrated the usefulness of this 3D system for the immobilization of MC3T3-E1 osteoblast-like cells [8, 11].

In the context of bone regeneration, it seems relevant to investigate the use of alginate microspheres as carriers for more clinically relevant cell types, such as human mesenchymal stem cells (hMSCs). hMSCs have been widely investigated for bone tissue engineering strategies due to their high proliferative potential, their default osteogenic differentiation pathway, and their paramount role in endochondral and intramembranous bone formation *in vivo* [12-15]. The combination of these cells with alginate hydrogels has already shown some encouraging results. Markusen *et al.* demonstrated that MSCs could be encapsulated in RGD-alginate beads maintaining a viability higher than 80% during two weeks [16]. Duggal *et al*. provided some insights on cell behavior changes when moving from a 2D to a 3D system, using alginate as the

culture substrate [17]. However, none of these studies examined the ability of MSCs immobilized in this 3D matrix to influence the formation of tube-like structures by endothelial cells.

Angiogenesis is the process by which the budding of pre-existing vessels extends the vascular network, which involves, in an initial phase, recruitment of endothelial cells and, later on, pericytes and smooth muscle cells to the development of capillaries and large vessels, respectively [18, 19]. Angiogenesis is closely associated with bone resorption and remodeling, which makes it a key process in the success of bone regeneration strategies [20, 21].

Besides being a natural source for bone cells, MSCs may also play a fundamental role in the angiogenesis process [22]. These cells are capable of influencing endothelial cells, by promoting their migration and tube-like structures formation, as demonstrated by Gruber *et al*. [23]. Potapova and co-workers additionally showed that conditioned medium from hMSCs culture supported migration, extracellular matrix invasion and survival of endothelial cells [24].

In the present work, the viability, metabolic activity and osteogenic potential of hMSCs immobilized within RGD-alginate microspheres and cultured under basal and osteogenic induction conditions were characterized in detail. The ability of this cell-material 3D construct to enhance the sprouting of vessels, under influence of neighboring endothelial cells, was also investigated. The development of an injectable system, able to simultaneously provide a source of viable bone cells and to stimulate an angiogenesis-like response in the host, may represent a step forward in bone regeneration strategies.

MATERIALS AND METHODS

Alginate preparation

Alginate for cell immobilization was prepared as a binary composition of high and low molecular weight alginate, as previously described by Evangelista *et al.* [8]. Sodium alginate powder with a high content of guluronic units (MVG Protonal LF 20/40, a gift from FMC Biopolymers, Oslo, Norway) was used as the high molecular weight component (HMW). The low molecular weight (LMW) alginate was obtained by gamma irradiating HMW with a cobalt-60 source, as previously described by Kong *et al.* [10].

The alginate preparation consisted in a three-steps procedure, as described by Evangelista *et al.* [8]. Firstly, alginates with both molecular weights were purified through dialysis against distilled and deionized (DD) water for 3 days, followed by oxidation with sodium periodate. Then, covalent grafting of the oligopeptidic sequence of (Glycine)4-Arginine-Glycine-Aspartic acid-Serine-Proline (abbreviated as G_4-RGDSP; Peptide International, USA) was carried out using aqueous carbodiimide chemistry [9]. For this latter step, 22 µmol of the peptide were used per gram of alginate polymer. Alginate gel for cell immobilization was prepared in 0.9 % sodium chloride (NaCl, Sigma) at a final concentration of 2 wt-%. It was composed of a 50:50 mixture of oxidized and RGD-modified alginate (25 wt-% HMW, 75 wt-% LMW) and unmodified HMW purified alginate.

Cell cultures

Human mesenchymal stem cells (hMSC, Lonza) were cultured at 37ºC under a 5% CO_2 humidified atmosphere and expanded in MSCGM (MSC growth medium, Lonza). For the experiments, cells were used at passage 8 and cultured in basal medium: Dulbecco's modified Eagle medium with low glucose (DMEM, Gibco), supplemented with 10% v/v inactivated fetal bovine serum (FBS, Gibco) and 1% v/v penicillin/streptomycin (P/S, Gibco). To induce hMSCs differentiation along the osteoblastic lineage, cells were cultured with osteogenic medium: basal medium supplemented with 10 mM β-glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma) and 50 µg/mL ascorbic acid (Fluka). The culture medium was changed every other day.

Human umbilical vein endothelial cells (HUVECs, ScienCell) were cultured at 37ºC under a 5% CO_2 humidified atmosphere, in M199 medium (Sigma) supplemented with 10% v/v inactivated FBS, 1% v/v P/S, 0.1 mg/mL of heparin and 0.03 mg/mL of endothelial cell growth supplement (Sigma) in 0.1% w/v collagen coated flasks.

Immobilization and dynamic culture of hMSCs within alginate microspheres

For immobilization within alginate microspheres cells were harvested before reaching confluence, using 0.05% w/v trypsin/ethylenediamine tetraacetic acid (EDTA) solution, then resuspended in 0.9 wt-% NaCl and loaded in a 5 mL syringe. After centrifugation and discarding of the supernatant, cells were homogeneously mixed with 2 wt-% RGD-modified alginate solution, using a dual-syringe system, with a final concentration of $20x10^6$ cell/mL of alginate solution. Subsequently, the alginate-cell

suspension was extruded under a coaxial nitrogen flow using a Var J1 encapsulation unit (Nisco, Switzerland) at a speed of 40 mL/h (Single-syringe infusion pump, Cole Parmer), to an isotonic 0.1 M $CaCl_2$ solution to crosslink the alginate molecules. The microspheres were kept therein for 10 min, then rinsed in Tris-buffered saline (TBS) and finally rinsed in culture medium. All microspheres produced were uniformly spherical with an average diameter of 820 ± 30 µm (Fig. 1).

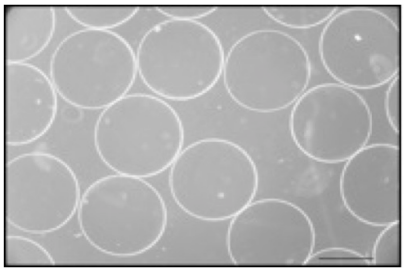


Figure 1. Photomicrograph of RGD-grafted alginate microspheres, illustrating their spherical and uniform shape. The average diameter of microspheres measured was 820 ± 30 µm (n = 100) (Bar = 500 µm).

Cells immobilized within RGD-alginate microspheres were cultured under dynamic culture conditions for up to 21 days, using 100 mL spinner flasks (Bellco Biotechnologies, Vineland, NJ, USA). Half of the microspheres were cultured in basal medium and the other half in osteogenic medium. The microspheres were maintained at 37ºC under a 5% v/v CO_2 humidified atmosphere. For control purposes, monolayer (2D) studies were carried out using hMSC suspensions seeded directly in 24 well plates with a final concentration of $3x10^3$ cells/cm^2.

Cell quantification, viability and metabolic activity

At days 1, 7, 14 and 21, for each condition, three samples containing microspheres were transferred from the spinner flask to a 24 well plate. Then, the number of microspheres per well was counted. Finally, cells were released from the microspheres through alginate dissolution using a 50 mM EDTA solution in PBS (pH 7.4) for 5 min. Cells were quantified and their viability was determined using the trypan blue exclusion test. For that purpose, an aliquot of cell suspension was mixed with 0.4 wt-% trypan blue (Sigma) and then transferred to a Neubauer chamber for counting. Cell viability was determined by scoring the percentage of unstained cells in the total cell number.

To assess metabolic activity three samples of microspheres for each condition were removed from the spinner flasks at days 1, 7, 14 and 21 and transferred to a 24 well plate. A resazurin solution (Sigma) was added to fresh medium at a final concentration of 10% (v/v). Cells were incubated in this solution at 37ºC for 4 h, after which 100 µL/well were transferred to a 96 well black plate and fluorescence was measured (530 nm_{Ex}/590 nm_{Em}) in a Spectra Max Gemini XS (Molecular Devices). The fluorescence was normalized to the number of microspheres per well.

Microscopic analyses of immobilized cells

After fixation in 4% paraformaldehyde/TBS for 20 min and further permeabilization with 0.1 % TritonX-100 (Sigma) for 5 min, the cell cytoskeleton was stained for F-actin with Alexa Fluor 488-phalloidin (Molecular Probes) and the cell nuclei with propidium iodide (Sigma). The morphology of immobilized cells was analyzed using confocal laser scanning microscopy (CLSM) (Leica SP2AOBS; Leica Microsystems).

For scanning electron microscopy (SEM) observation, microspheres were washed twice in TBS and fixed in 1.5% glutaraldehyde in sodium cacodylate (0.14 M) for 1 h at room temperature, followed by dehydration with a graded ethanol series (60%, 70%, 80%, 90% and 99%) for 10 min in each solution. Microspheres were maintained in 99% ethanol until critically point dried (CPD 7501, Quorum Technologies). After sectioning the microspheres with a blade, they were sputter-coated with gold using a JEOL JFC-100 Fine Coat Ion Sputter device and observed using a JEOL JSM-6301F scanning electron microscope (SEM).

Quantification of alkaline phosphatase (ALP) activity

For quantitative ALP analyses, three samples of cell lysates for each condition, obtained by brief sonication in ice with 1% v/v Triton X-100, were incubated with the chromogenic substrate 2 mM ρ–nitrophenol phosphate in 0.2 M bicarbonate buffer (pH 10), 0.05% v/v Triton X-100, and 4 mM $MgCl_2$ (Sigma) for 1 h at 37ºC. The reaction was stopped by adding 1 M NaOH and absorbance was read at 405 nm in a microplate reader (SLT Spectra). The amount of product was obtained from a p-nitrophenol standard curve. The enzymatic activity was normalized to total protein concentration, obtained using the bicinchoninic acid protein assay (BCA, Pierce), and expressed as nmol/min/mg protein.

Assessment of mineralization by Von Kossa staining

At day 21 microspheres were recovered from the spinner flasks and washed with TBS. After fixation in 4% paraformaldehyde/TBS for 10 min, samples were dehydrated in a gradient series of ethanol and then embedded in paraffin blocks. Later, sections of 6 µm thickness were prepared. Prior to staining, sections were dewaxed in xylene and rehydrated in a decreasing gradient series of ethanol followed by water. Sections were then incubated in 2.5 wt-% silver nitrate (Sigma) for 30 min under UV light, followed by incubation in 5 wt-% sodium thiosulphate (Aldrich) for 3 min. Finally, sections were washed and imaged under an inverted microscope (Axiovert 200M, Zeiss).

Phenotype analyses through mRNA expression

At days 1, 7, 14 and 21, total RNA was extracted from three samples of cells entrapped in microspheres using the RNeasy Mini Kit (Qiagen) protocol, as recommend by the manufacturer. Subsequently, 0.25 µg of the total RNA were used for random hexamers first strand synthesis to generate single stranded cDNA using the SuperScript^{TM} First-strand synthesis system for RT-PCR (Invitrogen). RNA quantification was performed by using a NanoDrop 1000 spectrophotometer.

After the cDNA synthesis reaction, qReal-Time PCR was carried out in a total volume of 20 µL of a mixture containing 1 µL of cDNA (corresponding to 12.5 ng of total RNA), 0.25 µM of each forward and reverse primers and 1x iQ SYBR Green Supermix (Bio-Rad). qRT-PCR experiments were run using an iQ5 (Bio-Rad) under the following conditions: 95°C for 3 min, followed by 40 cycles at 94°C for 30 s, 60°C for 45 s and 72°C for 30 s, and the last step at 55º for 10 s. All reactions were performed in duplicate. After completion of the PCR cycling, melting curves, obtained by increasing the temperature from 60°C to 96°C in increments of 0.5°C, were examined to ascertain specificity of the PCR products. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous assay control. Relative quantification of gene amplification by qRT-PCR was performed using the cycle threshold (C_t) values and relative expression levels were calculated as follows: 2^(C_t GAPDHgene - C_t gene of interest). The expression value for each target gene was normalized to the GAPDH value at each time point. Afterwards, the gene expression at each day was normalized to day 1, which was the same sample for basal and osteogenic conditions. The sequence and length of the primer pairs used is indicated in Table 1.

Table 1. Sequence of primer pairs used for qRT-PCR.

Gene name	Ref. sequence	Sequence of primers (5'-3')	Length (bp)
GAPDH	NM_002046	AGCCACATCGCTCAGACAC GCCCAATACGACCAAATCC	66
ALP	BC021289	AGAACCCCAAAGGCTTCTTC CTTGGCTTTTCCTTCATGGT	74
COL1A1	NM_000088	GGGATTCCCTGGACCTAAAG GGAACACCTCGCTCTCCA	63
Runx2	NM_001024630	GTGCCTAGGCGCATTTCA GCTCTTCTTACTGAGAGTGGAAGG	78

Induction of tube-like structures formation

The effect of immobilized hMSCs on tube-like structures formation and development was investigated using HUVECs cultured in the commercially available 3D matrix Matrigel (Becton Dickinson, USA). After 21 days of culture, microspheres containing the hMSCs, kept in basal and osteogenic conditions, were transferred to a 48 well plate. The medium was removed and then 150 µL/well of matrigel basement membrane was added and allowed to stand for 30 min at 37ºC to form a gel layer. After gel polymerization, HUVECs ($5x10^3$ cells) were added to each well. The medium used in each condition consisted on an equal mixture of M199 (culture medium for HUVECs) and DMEM (expansion medium for MSCs). Matrigel without microspheres was used as control.

A quantitative measurement of tube-like structure formation in Matrigel was carried out. Comparisons among HUVEC cultures plated simultaneously for the three different conditions were performed after 24 hours (three wells for each condition: matrigel alone, matrigel with microspheres kept in basal and osteogenic conditions). The number of tube-like structures formed (vessels) was then counted in each well. Each tubular structure observed between the ramifications was considered one vessel. Mean values were obtained by evaluating the three wells under the same treatment. The experiment was repeated three times.

Detection of vascular endothelial growth factor (VEGF)

After 21 days, samples of conditioned media from microsphere cultures maintained in basal and osteogenic conditions were collected from the spinner flasks and centrifuged to remove cell debris. Levels of VEGF released by the embedded hMSCs into the surrounding media were assessed using a commercial VEGF ELISA Kit (R&D Systems), as recommended by the manufacturer.

Statistical analyses

Statistical analyses were performed using SigmaStat (Statistical Software, STAT32 MFC Application). Statistical significance was assessed by using two way analysis of variance (ANOVA) in conjugation with Tukey's test, pairwase multiple comparison procedures. The comparison of results from the tube-like structures formation assays was conducted by one way ANOVA in conjugation with Tukey's test. Differences were considerer statistically significant when *p* values were lower than 0.05. All data are presented as mean values ± standard deviation.

RESULTS

Cell quantification, viability and metabolic activity

hMSCs were recovered from the microspheres at days 1 and 21 and counted. Overall, the total number of cells per microsphere was not significantly different between the two conditions (basal and osteogenic) and between the two days evaluated (Fig. 2).

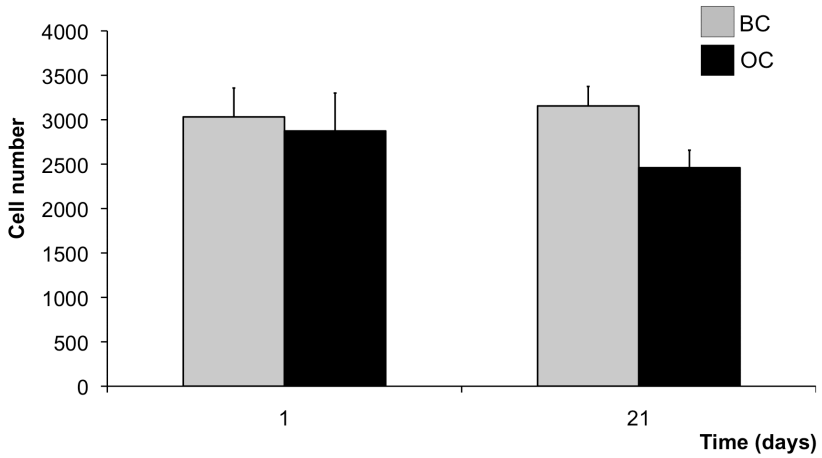


Figure 2. Number of immobilized hMSCs per microsphere after 1 and 21 days in culture, in basal (BC) and osteogenic medium conditions (OC). Data are expressed as mean ± standard deviation (n=3).

At days 1, 7, 14 and 21 post-immobilization, cell viability was assessed (Fig. 3). At day 1 it was approximately 90% in both conditions, and it did not markedly changed until day 21. Moreover, the fraction of viable cells was similar in the two media

conditions tested and was generally constant for 2 weeks (no significant differences were observed until day 14).

These findings were confirmed by determining metabolic activity using the resazurin assay. As can be seen in Fig. 4, the metabolic activity of cells cultured under basal and osteogenic conditions was similar for each time point. Under both conditions, the cells metabolic activity slightly decreased during the first two weeks of culture, but recovered completely by the end of the culture period tested (21 days).

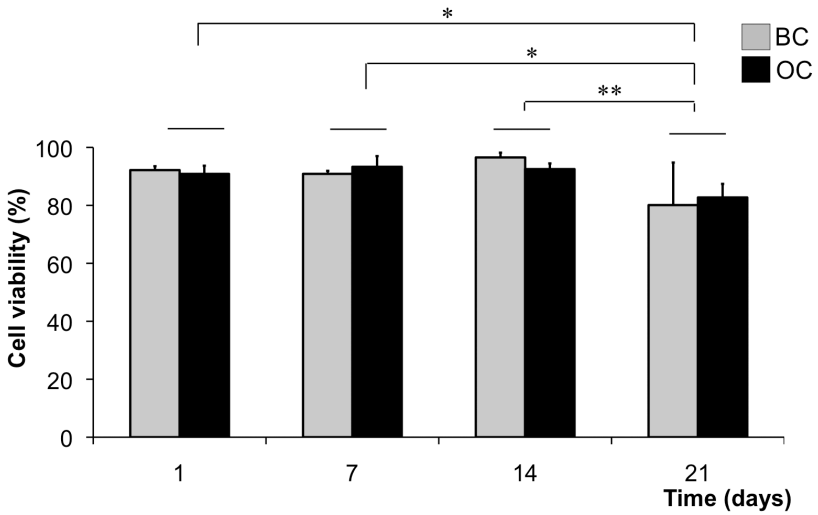


Figure 3. Viability of hMSCs immobilized in RGD-grafted alginate microspheres in basal (BC) and osteogenic medium conditions (OC), after 1, 7, 14 and 21 days of culture. (* $p \leq 0.05$; ** $p \leq 0.01$) Data are expressed as mean ± standard deviation (n=3).

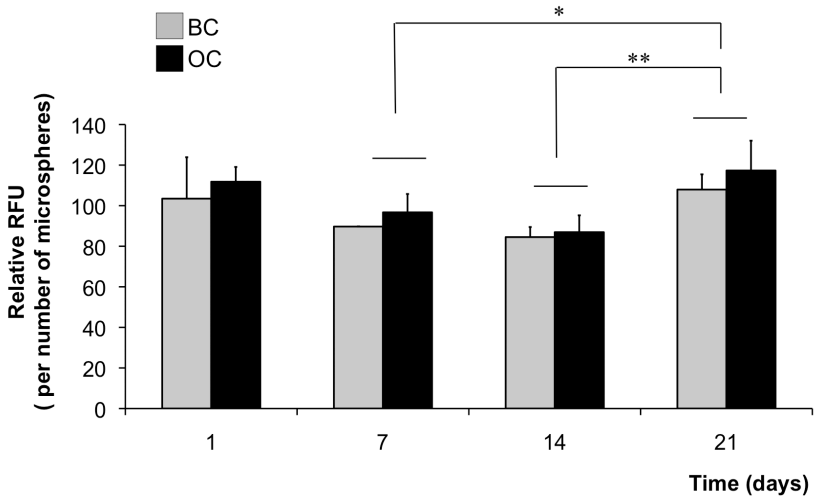


Figure 4. Metabolic activity of human mesenchymal stem cells immobilized within RGD-grafted alginate microspheres, using the resazurin based assay after 1, 7, 14, and 21 days of culture under basal (BC) and osteogenic medium conditions (OC). RFU: relative fluorescence units. (* $p \leq 0.05$; ** $p \leq 0.01$) Data are expressed as mean ± standard deviation (n=3).

Cell morphology

hMSCs morphology inside alginate microspheres was assessed by CLSM by staining the F-actin filaments, as shown in Fig. 5. Cells interacted to some extent with the hydrogel matrix, as suggested by the presence of short cytoplasm extensions around the cells, but remained typically with a round shape.

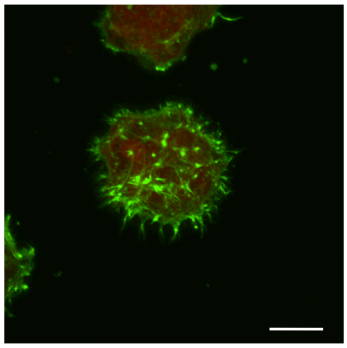


Figure 5. Cytoskeleton organization of human mesenchymal stem cells inside alginate microspheres stained with phalloidin Alexa Fluor 488 for F-actin (green) and propidium iodide (red) for nucleus (Bar = 10 μm).

SEM analyses allowed the observation of the inner part of microspheres, including the hydrogel structure embedding the cells. Fig. 6 shows SEM micrographs of the internal part of a microsphere with hMSCs, after 21 days in culture, confirming the presence of cells and their interaction with the hydrogel matrix.

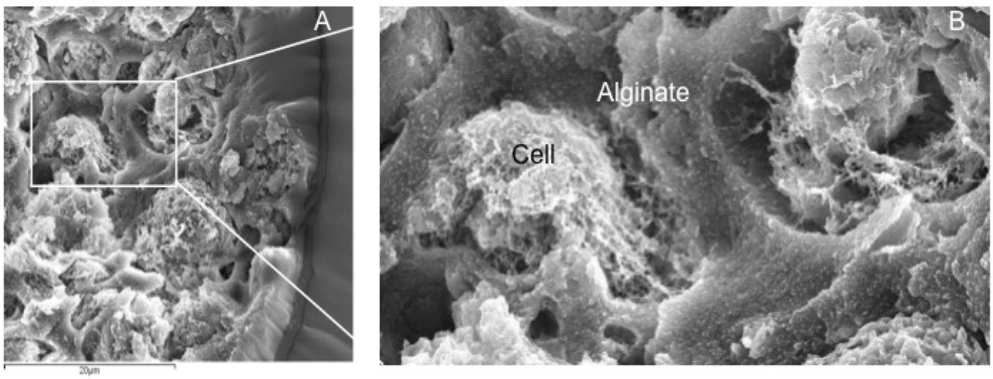


Figure 6. Scanning electron microscopy (SEM) images of hMSCs immobilized within alginate microspheres, cultured for 21 days. (A) Cells inside the microsphere; and (B) a 3.6 magnification of the cell-alginate interface.

Cell differentiation

Alkaline phosphatase activity

As illustrated in Fig. 7, the alkaline phosphatase (ALP) activity of cells cultured under basal conditions remained relatively low and constant throughout the experiment, both in 2D (monolayer) and 3D (microsphere) cultures, indicating almost no hMSC differentiation under these conditions. There were no significant differences during the 21 days for each type of culture under basal conditions. However, when comparing 2D and 3D cultures, the latter ones showed significantly higher ALP activity values at days 1 and 21, while at day 14 2D cultures exhibited a significantly higher value.

Monolayer cultures (2D) treated with osteogenic media exhibited a significant increase ($p<0.001$) of ALP activity from day 1 to day 7, when a plateau was reached. On the other hand, cells within microspheres (3D) exhibited ALP values that gradually increased over time. Comparing 2D with 3D cultures in osteogenic media, on the first day there were no significant differences. ALP activity levels were significantly higher in 2D comparing to 3D cultures at day 7 ($p<0.001$) but no differences were observed at day 14. The ALP activity considerably increased in 3D cultures at day 21, becoming significantly higher than in 2D cultures ($p<0.001$).

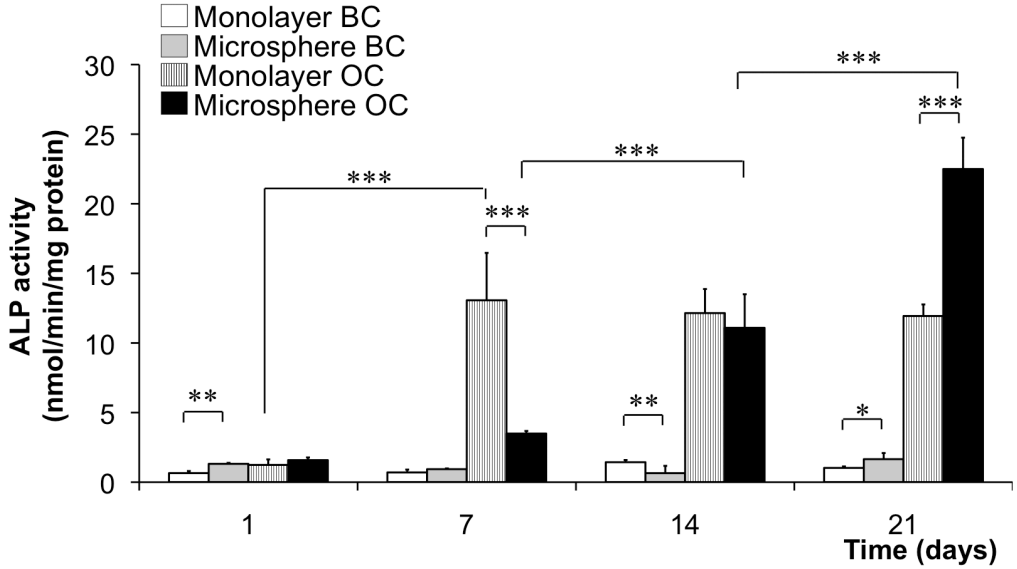


Figure 7. Expression of ALP activity by hMSCs cultured in monolayer (2D) and inside microspheres (3D), after 1, 7, 14, and 21 days, under basal (BC) and osteogenic medium conditions (OC). (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) Data are expressed as mean ± standard deviation (n=3).

Von Kossa staining

One of the hallmarks of osteogenic differentiation is the formation of extracellular mineralized deposits. To assess the presence of these deposits the von Kossa staining was performed. hMSCs immobilized in alginate microspheres under osteogenic conditions showed large areas of mineralization (dark), with mineral nodules after 21 days of incubation, as illustrated in Fig. 8. Mineralization was not detected in immobilized cells cultured under basal conditions nor in microspheres without cells, in agreement with previous work [8].

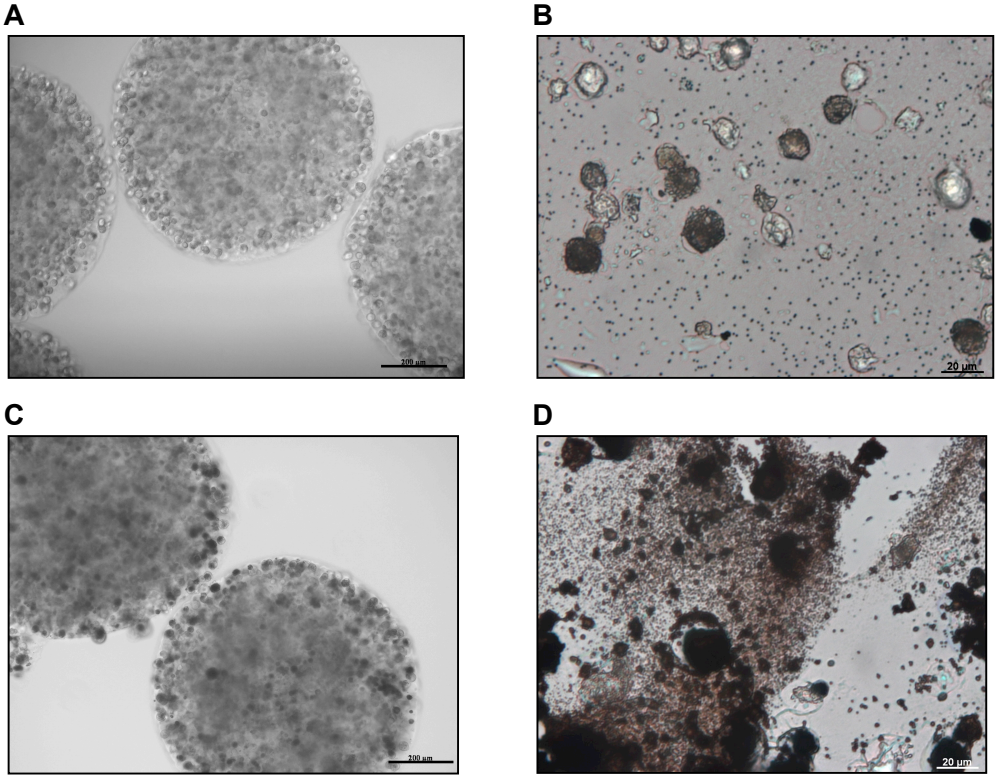


Figure 8. Optical microscopy observation of hMSCs immobilized and cultured for 21 days inside RGD-alginate microspheres, in basal conditions (A, B) and in osteogenic conditions (C, D). A and C microsphere outline with cells; B and D a microsphere detail at a higher magnification. Von Kossa staining was performed in 5 µm thick paraffin sections.

Quantitative real time PCR analyses of osteogenic markers

In order to study the gene expression profile of immobilized cells cultured in basal and osteogenic conditions, real time PCR analyses of osteoblastic differentiation markers were carried out at defined time points (1, 7, 14 and 21 days) (Fig. 9). One of the genes studied was the runt homology domain transcript factor Runx2, which is a key osteoblastic modulator during bone formation [25]. Both in basal and osteogenic

conditions, Runx2 was significantly upregulated from day 1 to day 7 ($p<0.001$), reaching a maximum at the end of the first week of immobilization, in the case of osteogenic culture conditions (Fig. 9A). Runx2 expression levels were significantly higher in osteogenic conditions at day 7. Afterwards, a decrease was observed, although not significant. Finally, on day 21, the Runx2 expression significantly decreased comparing to day 7 ($p<0.001$). Overall, Runx2 expression was significantly higher in osteogenic conditions compared to basal conditions ($p=0.001$).

ALP expression (Fig. 9B) was significantly higher ($p<0.001$) in cells cultured in osteogenic medium comparing to basal one, at all time points analyzed. In osteogenic conditions, a ten-fold increase was observed at the end of the first week and the ALP expression level remained unchanged on the second week. A small decrease occurred at the third week. The expression levels in basic medium conditions were kept low for all time points tested.

Collagen type I, the major component of the extracellular matrix in bone tissue, was significantly upregulated ($p<0.001$) in osteogenic conditions during the first 2 weeks of culture (Fig. 9C). In both media tested, this gene exhibited significantly higher mRNA levels ($p<0.001$) at day 14 comparing to days 7 and 21.

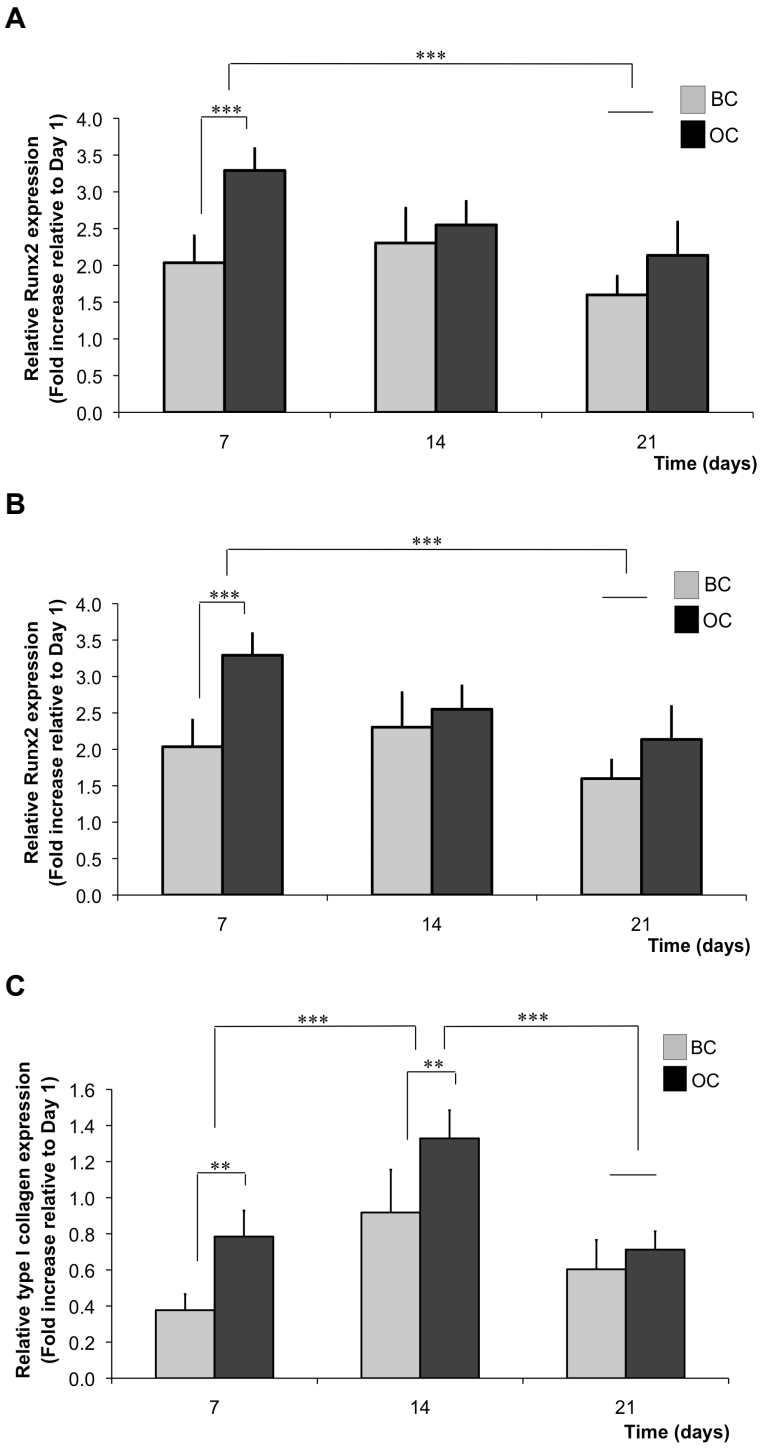


Figure 9. Human mesenchymal stem cells gene expression levels of three osteogenic markers: A) transcript factor Runx2; B) alkaline phosphatase; and C) collagen type I. These expression levels were obtained recurring to real-time PCR, after cell immobilization in RGD-grafted alginate microspheres at different time points (BC - basal conditions; OC - osteogenic conditions). (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) Data are expressed as mean ± standard deviation (n=3).

Induction of tube-like structures formation by HUVECs: an *in vitro* model of angiogenesis

Matrigel matrix serves as a substrate for *in vitro* endothelial cell invasion and tube-like structures formation assays, and can also be used to assess *in vivo* angiogenic activity. In this study, Matrigel was used to evaluate if immobilized hMSCs could influence the ability of endothelial cells (HUVECs) to form tube-like structures. In matrigel alone (control) HUVECs adhered to the surface within 18 h and formed networks of branching and anastomosing cells, with multicentric junctions that lasted 24 to 48 h (Figs. 10 and 11), in agreement with previous reports [26, 27]. In the presence of microspheres containing immobilized hMSCs, the amount of tube-like structures formed significantly increased, independently of the culture conditions used (Figs. 10 and 11). Although microspheres cultured under osteogenic conditions promoted the highest amount of tube-like structures formation, it was not significantly different from the results obtained under basal conditions.

VEGF was present in the conditioned medium collected from microspheres-containing hMSCs maintained in basal and osteogenic conditions for 21 days, as assessed by ELISA analysis. This assay confirmed that the VEGF produced by the cells was able to diffuse into the medium.

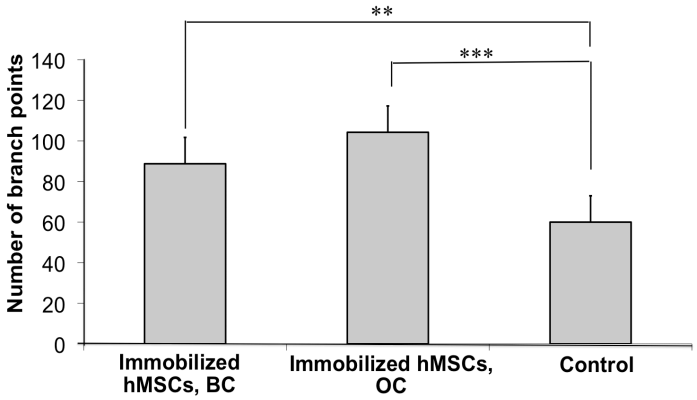


Figure 10. Quantification of tube-like structures formed by HUVECs cultured for 24 h alone on matrigel or in the presence of hMSCs immobilized inside RGD-alginate microspheres. Cultures were carried out in basal (BC) and in osteogenic medium conditions (OC). The control was matrigel alone. Measurements were taken from a representative experiment repeated three times. (** $p \leq 0.01$; *** $p \leq 0.001$)

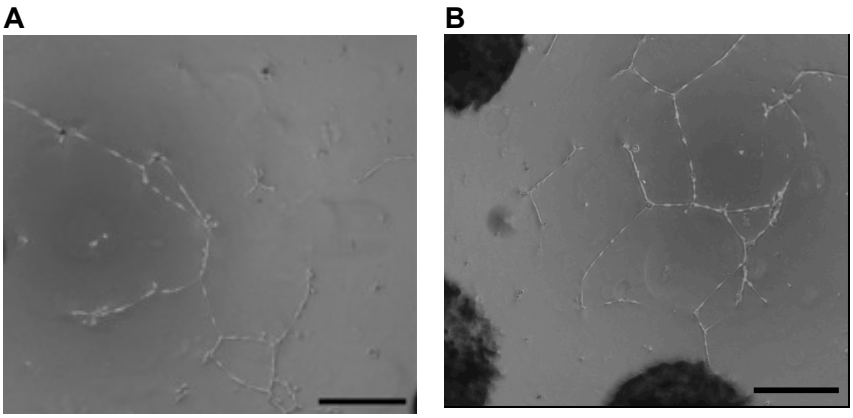


Figure 11. Tube-like structures formed by HUVECs after 24 hours on matrigel. (A) HUVECs cultured alone on matrigel; and (B) HUVECs cultured in the presence of hMSCs immobilized inside RGD-alginate microspheres (Bar = 500 μm).

DISCUSSION

To construct a functional engineered tissue, relevant native cell types, or their precursors, have to be either included or recruited into the scaffold along with vascular cell types [19]. Basically, this means that to accomplish this daunting task of mimicking functionality and complexity of natural tissues, encouraging infiltration of the host vessels is mandatory. This generally recognized demand has inspired the development of several distinct therapeutic angiogenesis approaches, based in the use of scaffolds combined with cells and/or growth factors [11, 28-30].

As an organ, bone is not an exception in this need for vascular development. The clear synergy between bone formation and angiogenesis uplifted the development of strategies that incorporate neovascularization approaches [19, 31]. In the present work, a system based on the combination of RGD-modified alginate, as a synthetic extracellular matrix, and hMSCs is proposed and analyzed in detail.

The present work was designed to aim for the preparation of an injectable bone regeneration system. For that purpose, alginate microspheres with an average size of 800 μm (Fig. 1) were prepared. This size range was selected mainly to facilitate handling during the several assays performed, although these 3D matrices can be easily reduced in size down to 200 μm by simply varying the type of the encapsulation unit used. Additionally, as previously pointed out, these microspheres can be combined with an hydrogel vehicle to assist injection, especially using the smaller sizes [7]. The

reduction in size can further contribute to improve the exchanges between cells and medium, thus facilitating cells access to nutrients and oxygen.

Markusen *et al.* previously encapsulated MSCs in alginate-GRGDY beads and investigated their viability and proliferation [16]. Abbah *et al.* studied the osteogenic behavior of rabbit bone marrow cells encapsulated in alginate microbeads, with and without a poly-L-lysine coating, and reported an increase in cell number until day 14 [32]. More recently, Duggal *et al.* investigated the phenotype and gene expression of hMSC in a 3D alginate system as compared to a 2D culture [17]. The present team already demonstrated that the present RGD-modified alginate hydrogel, in contrast with unmodified alginate, was able to promote a much higher level of adhesion and differentiation of MC3T3-E1 pre-osteoblastic cells [8]. Also, Grellier *et al*. explored the ability of this matrix to enhance mineralization in a bone defect after immobilization of human osteoprogenitors combined with endothelial cells [11]. Although in all these studies the authors showed the interest of using RGD-grafted alginate as a 3D matrix for bone tissue engineering applications, little information still exists about cytoskeleton organization, cell viability, cell-matrix interactions and cell differentiation. More importantly, the effect of the entrapped cells in the regulation of new vessel growth has never been assessed. The present work, besides successfully demonstrating that RGD-alginate microspheres promoted hMSCs adhesion, maintained their metabolic activity and supported their osteogenic differentiation, also showed the capacity of this 3D cell culture system to enhance *in vitro* endothelial cell invasion and tube-like structures formation.

Similarly to previous reports [16, 17], the present work demonstrated that hMSCs could be entrapped in RGD-alginate maintaining a high viability, and be kept metabolically active during 3 weeks under 3D culture. Moreover, hMSCs immobilized in this 3D scaffold were also able to respond to environmental biochemical cues from the media by differentiating when cultured in osteogenic media [32].

Furthermore, the proliferation of immobilized cells in basal and osteogenic conditions was also assessed, during 21 days of dynamic culture conditions, by the resazurin-based assay and through cell quantification (Figs. 2 and 3). The results obtained using both assays showed no significant differences between basal and osteogenic conditions for every time point analyzed. Regarding metabolic activity there was a significant increase over the last week of culture. However, the total cell number did not significantly change from the beginning until the end of culture. This suggests that there was no proliferation inside the microspheres over the time course of the

experiment. Cell viability was also evaluated, revealing that the fraction of viable cells was similar in both environments and was kept higher than 90% until day 14 (Fig. 3). Although the total cell number was kept unchanged, the metabolic activity of immobilized cells increased (Fig. 4) which points to cell activities other than proliferation, such as cell differentiation by synthesis of extracellular matrix. The lack of proliferation could be due to minimal space for growth of entrapped cells, which are probably spatially constrained by the polymeric network. In another related independent work, Ma and co-workers [3] also observed proliferation inhibition of cells entrapped in alginate microspheres, which provide suspension conditions and arrest cells on G0G1 phase.

In the current study, analyses of cytoskeleton organization demonstrated that cells were able to establish sites of interaction with the polymeric matrix (Figs. 5 and 6). As hypothesized by Evangelista *et al.* [8] the covalently coupled RGD peptides promote interaction with integrins of the cell membrane, thus leading to assembly of actin filaments. Cells immobilized in the matrix without RGD peptides remained round and were unable to establish interactions with the polymer [8].

The ability of hMSCs to generate distinct lineages is now a well-established concept and their osteogenic differentiation potential has become one of the most widely investigated topics in bone regeneration [32]. Promising results have been obtained when using osteoblasts differentiated from MSCs in bone regenerative processes [33, 34]. In view of these facts, and aiming to develop a bone regeneration strategy, the differentiation of immobilized hMSCs towards the osteogenic lineage was explored by analyzing several cell differentiation markers. Firstly, the expression of ALP, an early osteoblastic marker, that reflects not only the stage of cell differentiation but also represents the percentage of committed osteoprogenitor cells, was evaluated. The activity of this enzyme increases during synthesis of the extracellular matrix, which corresponds to the beginning of cell differentiation [35]. In this study, ALP activity was analyzed in immobilized cells and compared with cells growing in monolayer. In basal conditions, ALP values were higher in immobilized cells, with the exception of day 14. In osteogenic conditions, only at day 21 was ALP activity significantly higher in immobilized cells than in monolayer culture (Fig. 7). In osteogenic conditions, since cells were entrapped within the alginate matrix, contact with osteogenic factors may be slower, due to diffusion limitations, which could lead to a delay in osteogenic differentiation. That could explain for at least one-week retardation on the osteogenic process, when comparing to monolayer culture in the same conditions. As previously

pointed out, microspheres with a high diameter were used to facilitate handling, although their size can be considerably reduced using the present technology, down to 200 mm. This size reduction would certainly contribute to facilitate exogenous stimulation by soluble factors. Nevertheless, after 3 weeks in osteogenic culture conditions, immobilized cells expressed ALP values two times higher than the monolayer culture. These findings seem to indicate that, despite the slower diffusion, the 3D structure was able to stimulate osteogenic differentiation. An additional evaluation of ALP expression from immobilized cells was carried out by real time RT-PCR. Also, Von Kossa staining of immobilized cells cultured under osteogenic conditions showed formation of mineral deposits, which further confirms osteogenic differentiation (Fig. 8). Real time quantitative RT-PCR assays were performed for transcripts that code for osteoblast-related proteins, including a major transcription factor for regulating osteogenesis, Runx2 (Fig. 9A). Results showed that Runx2 expression was affected during cell differentiation, with its level increasing during the osteogenic induction, which is in agreement with its function as a key transcription factor in the regulation of bone differentiation markers [25]. In addition, since its activity may be modulated through cell contact with the extracellular matrix, the 3D alginate matrix could have had an important role in its regulation and have been responsible for its upregulation in basal conditions. In conformity with previous results, ALP expression was significantly upregulated under osteogenic conditions (Fig. 9B). Finally, the expression of collagen type I also increased during osteogenic differentiation, which is consistent with its role in osteoblast maturation (Fig. 9C) [36]. In fact, ALP and type I collagen are crucial during extracellular matrix production, which is necessary for mineralization.

As stressed before, a decisive factor that needs to be taken into account for bone regeneration strategies is angiogenesis, which is a process closely allied to osteogenesis. It plays a critical role in the initiation of fracture healing and in the promotion of endochondral and intramembranous ossification during bone growth [21, 37, 38]. During the angiogenesis process, by which the budding of pre-existing vessels extends the vascular network, endothelial cells are intensively recruited [18, 19]. The Matrigel assay, a reconstituted basement membrane that promotes morphological differentiation of human endothelial cells, is a well-known system for studying angiogenesis induction [27]. In the present work, the Matrigel assay demonstrated that hMSCs immobilized inside RGD-grafted alginate were capable of influencing angiogenesis by significantly enhancing the ability of neighboring endothelial cells to

form tube-like structures (Figs. 10 and 11). Since the two cell types were not in direct contact, these results suggest that growth factors produced by the hMSCs, like the vascular endothelial growth factor (VEGF) [39-41], may have influenced endothelial cells behavior. The presence of VEGF was detected in the culture medium, showing not only that hMSC were able to produce this signaling protein, but also that it was able to diffuse through the hydrogel network into the surrounding medium.

Yu et al. recently provided further insights into the effect of alginate biopolymers as synthetic extracellular matrix and RGD peptides as endothelial cell-matrix mediators [42]. In their study they showed that that RGD peptides conjugated to alginate promoted an enhancement of angiogenesis when studying the regeneration of cardiac tissue by improving myocardial function.

Overall, in the present study, it was demonstrated that RGD-grafted alginate microspheres not only promoted hMSCs adhesion and supported their osteogenic differentiation, but also provided the adequate environment for these cells to stimulate neighboring endothelial cells to form tube-like structures.

CONCLUSIONS

This study showed that RGD-alginate hydrogels were able to support hMSCs differentiation along the osteogenic lineage when cells were immobilized within this 3D matrix. Moreover, it was demonstrated that this system enhanced the ability of neighboring endothelial cells to form tube-like structures. Collectively, these findings demonstrate the potential of this approach as a therapeutic strategy for bone regeneration.

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Chapter IV

Evaluation of injectable *in situ* crosslinkable alginate matrix for endothelial cells delivery*

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ABSTRACT

Cell-based therapies offer an attractive approach for revascularization and regeneration of tissues. However, and despite the pressing clinical needs for effective revascularization strategies, the successful immobilization of viable vascular cells within 3D matrices has been difficult to achieve. In this paper the *in vitro* potential of a natural, injectable RGD-alginate hydrogel as an *in situ* forming matrix to deliver endothelial cells was evaluated. Several techniques were employed to investigate how these microenvironments could influence the behavior of vascular cells, namely their ability to promote the outward migration of viable, proliferative cells, retaining the ability to form a 3D arrangement. Cells within RGD-grafted alginate hydrogel were able to proliferate and maintained 80% of viability for at least 48h post-immobilization. Additionally, entrapped cells created a 3D organization into cellular networks and, when put in contact with matrigel, cells migrated out of the RGD-matrix. Overall, the obtained results support the idea that the RGD peptides conjugated to alginate provide a 3D environment for endothelial cells adhesion, survival, migration and organization.

Keywords: Alginate; Cell adhesion; Endothelial cell; RGD peptide; Scaffold.

INTRODUCTION

The field of regenerative medicine is based on the patient's own competence to induce the repair of injured tissues, and consequently restore their natural functions *in situ*. This natural healing capacity, coupled with the use of an adequate scaffold providing a synthetic extracellular matrix (ECM) to organize cells into a three-dimensional (3D) architecture, guides the formation and growth of the new tissue.

Paramount to a good performance of a biomaterial scaffold is its capacity to allow the proper vascularization and therefore, vascular cell survival and organization. Attempts to mimic vascularization in engineered tissues will lead to more clinically relevant cell-based therapies. Aside from strategies based on promoting angiogenesis from the host, in the past few years a growing interest in biomaterial *in vitro* prevascularization has emerged into the limelight [1-4]. Although vascularization remains an unsolved challenge in tissue engineering, encouraging developments have been made to grow and maintain, *in vitro*, these self-assembled biological vascular networks [5, 6]. Polymers with the ability of forming 3D hydrogel networks offer an exciting possibility for the *in situ* delivery of vascular cells, allowing their organization into a 3D architecture and assisting cells in repopulating the surrounding tissue after transplantation [7].

Hydrogels have been proposed with different functions in the field of tissue engineering, namely as space filling agents, drug and cell delivery vehicles and 3D scaffolds [7]. Hydrogel matrices are water-swollen crosslinked polymeric structures that are mechanically flexible and simulate to a certain extent the natural ECM [8]. Highly hydrated hydrogels are advantageous for tissue regeneration applications because of their high permeability to oxygen, nutrients and water-soluble metabolites [9]. Additionally, some hydrogel matrices can be prepared from soluble precursors solutions that crosslink *in situ*, which allows their application at the injury site by minimal invasive methods [8]. One example is alginate, a hydrogel-forming natural polysaccharide that has been intensively investigated for a variety of biomedical applications, namely for cell delivery in tissue regeneration therapies [10]. Although being originally a non cell-interactive polymer, alginate can be covalently modified with peptides containing the amino acid sequence arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD) to promote cell adhesion [11, 12]. Moreover, its molecular weight can be modified through irradiation and oxidation to modulate its viscoelastic properties, as well as its degradation rate [12]. The lower viscosity of the irradiated polymer results in

reduced shear forces during the cell-polymer mixing process and, consequently, in higher cell viability [13]. Previous studies from our group, using RGD-alginate microspheres with a binary composition of high and low molecular weight, already demonstrated the usefulness of this 3D system for the immobilization of osteoblast-like cells [11, 14, 15]. Alginate, coupled with vascular endothelial growth factor (VEGF), has also been proposed as macroporous scaffolds for endothelial progenitor cells (EPCs) delivery [16]. However, only a limited number of reports exist so far regarding the use of injectable *in situ* crosslinking systems for vascular cell delivery [15, 17-20]. For example, Kraehenbuehl and co-authors incorporated a Tß4 peptide in a synthetic hydrogel, to stimulate vascular organization [21]. In a previous work with alginate, immobilized endothelial cells showed a fast decreased in metabolic activity throughout time, due to the high alginate concentration that impeded cell-cell contact [15].

The aim of this study was to evaluate the applicability of injectable RGD-alginate hydrogels as vehicles for the minimally invasive delivery of endothelial cells. Several techniques were employed to investigate the outcome of these microenvironments on vascular cell behavior, namely on their ability to promote the outward migration of viable, proliferative cells, retaining the ability to form tubular-like structures.

MATERIALS AND METHODS

Alginate preparation

Alginate for cell immobilization was prepared as a binary composition of high and low molecular weight alginate, as previously described [11, 14]. Sodium alginate powder with a high content of guluronic units (M VG Protonal LF 20/40, a gift from FMC Biopolymers, Oslo, Norway) was used as the high molecular weight component (HMW). The low molecular weight (LMW) alginate was obtained by gamma irradiating HMW with a cobalt-60 source, as previously described by Kong *et al.* [13].

The alginate preparation consisted in a three-steps procedure, as explained by Evangelista *et al.* [11]. Firstly, alginates with both molecular weights were purified through dialysis against distilled and deionized water for 3 days, followed by oxidation with sodium periodate. Then, covalent grafting of the oligopeptidic sequence of (Glycine)4-Arginine-Glycine-Aspartic acid-Serine-Proline (abbreviated as G_4-RGDSP; Peptide International, USA) was carried out using aqueous carbodiimide chemistry [12]. For this latter step, 22 µmol of peptide were used per gram of alginate polymer. Alginate gel for

cell immobilization was prepared in 0.9 wt-% sodium chloride (NaCl, Sigma) at a final concentration of 1 wt-%. It was composed of a 50:50 mixture of HMW and LMW, both alginates oxidized and RGD-modified. For control purposes the same mixture of HMW and LMW was used but without RGD.

Formation of alginate hydrogel

Hydrogel formation was carried out under simulated physiological conditions using an internal gelation strategy as previously described [22, 23]. Briefly, alginate gel precursor solutions at 1 wt% in 0.9 wt% NaCl were mixed with an aqueous suspension of calcium carbonate ($CaCO_3$, Fluka) until homogenization. The gelling process was triggered by adding a fresh solution of glucone delta-lactone (GDL, Sigma). The molar ratio of $CaCO_3$/GDL was set at 0.125. Gel formation continued at standard incubator conditions (37ºC, 5% CO_2) for approximately 1h. The gelation time can be easily adjusted by changing the molar ratio of $CaCO_3$/GDL, as previously shown [24].

Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cells (HUVECs, ScienCell) were routinely grown at 37ºC under a 5% CO_2 humidified atmosphere, in M199 medium (Sigma) supplemented with 10% v/v inactivated FBS, 1% v/v Penicillin/Streptomicin, 0.1 mg/mL of heparin and 0.03 mg/mL of endothelial cell growth supplement (Sigma) in 0.1% w/v collagen coated flasks. For the experiments cells were used until passage 8 and cultured in complete endothelial cell growth medium-2 (EGM-2, Lonza).

Cell attachment on alginate films

Solutions of unmodified and RGD-grafted alginate at 1 wt-% were spin-coated (Spin Coater, SCS G3P-8) on plastic coverslips (Starstedt) previously coated with poly-lysine (Sigma). Immediately after alginate deposition (100 μL, 9000 rpm, 1 min), crosslinking was initiated by immersion in 0.1 M $CaCl_2$ solution. Then, films were transferred to a 24 well plate and carefully rinsed with sterile water and culture medium. A total of 40,000 cells were seeded on the top of the alginate films and cultured at 37ºC under 5% CO_2 humidified atmosphere. After 24h, films were imaged under an inverted microscope (Axiovert 200M, Zeiss).

3D cell culture within alginate hydrogel

For immobilization within alginate hydrogel, cells were harvested before reaching confluence, using 0.05% w/v trypsin/ethylenediamine tetraacetic acid (EDTA) solution (Sigma). After centrifugation and discarding of the supernatant, HUVECs were homogeneously mixed at a final concentration of $20x10^6$ cell/mL with 1 wt-% of alginate gel precursor and crosslinking agents as described on section 2.2. Crosslinking was promoted directly in the culture plates by placing 20 µL of the solution into each well of a 24 well suspension culture plate, which allowed obtaining small and uniform hydrogel matrices. Samples were placed in the incubator at 37ºC under 5% CO_2 humidified atmosphere during 1h, until gelling was completed. Finally, complete EGM-2 was added do each well. The cell-loaded alginate hydrogels were cultured during 72h and medium was changed after 24h. As a control, cells were entrapped within alginate without RGD and kept under the same conditions. For the different cell studies three hydrogel matrices were analyzed for each condition.

Cell viability

At different time points (0, 24 and 48h) three hydrogel matrices for each condition were dissolved using a 50 mM EDTA solution in PBS (pH 7.4) for 5 min to allow cell recovery. Cell viability was determined using the trypan blue dye exclusion test. For that purpose, an aliquot of cell suspension was mixed with 0.4 wt-% trypan blue (Sigma) and then transferred to a Neubauer chamber for counting. Cell viability was determined by scoring the percentage of unstained cells in the total cell number.

CFSE-based cell-division tracking

HUVECs were incubated with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) for 15 min at 37°C at 10 µM CFSE in PBS, washed twice with PBS with 20% FBS and once in medium. CFSE-labeled cells were entrapped in alginate with and without RGD peptides as previously described. To monitor cell proliferation rates, cells were recovered by dissolving the alginate matrices with EDTA and the levels of CFSE in cells were analyzed in triplicate on a FACSCalibur flow cytometer (Becton Dickenson). Data processing was performed using FlowJo software 8.7.flow cytometry. As cells divide, the CFSE fluorescence intensity is split roughly evenly between the two daughter cells. This intensity provides an indirect measurement of the number of divisions a cell has undergone.

Cell morphology

After 24 and 48h, HUVECs morphology was evaluated through staining of their cytoskeleton. For that purpose, cells were fixed in 4% w/v paraformaldehyde/tris buffered saline (TBS) for 20 min, permeabilized with 0.1% v/v TritonX-100 (Sigma) for 5 min, and finally the cell cytoskeleton was stained for F-actin with Alexa Fluor 488-phalloidin (Molecular Probes) and the cell nuclei with Hoechst 33342 at 1 µg/mL. The morphology of entrapped cells was analyzed using confocal laser scanning microscopy (CLSM; Leica SP2AOBS, Leica Microsystems).

Immunoflurescent analysis of von Willebrand expression

After 24h, HUVECs immobilized within alginate hydrogels were fixed in 4% w/v paraformaldehyde/TBS for 20 min, and permeabilized with 0.2% v/v TritonX-100 (Sigma) for 10 min. After washing, the primary antibody (1:200, vWF, DakoCytomation) was added and incubated overnight. Then, samples were washed and the secondary antibody (1:1000, anti-rabbit Alexa 594, Molecular Probes) was added and incubated for 1h at room temperature. Finally the nuclei were stained with Hoechst 33342 at 1 µg/mL and samples were examined by CLSM.

Phenotype analyses through mRNA expression of Angiopoetin-2

Total RNA was extracted from HUVECs using the total RNA isolation kit (Macherey-Nagel), as recommended by the manufacturer. Subsequently, 1 µg of the total RNA was used as template for single strand cDNA synthesis with the Superscript pre-amplification system (Invitrogen) in a 20 µL final volume containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 5 mM MgCl2, 10 mM DTT, 0.5 mM of each dATP, dCTP, dGTP and dTTP, 0.5 µg oligo(dT)12-18, and 50 U of reverse transcriptase. After incubation at 42ºC for 50 min, the reaction was stopped at 70ºC for 15 min. After the cDNA synthesis reaction, qReal-Time PCR was carried out in a total volume of 25 µL of a mixture containing 1 µL of cDNA diluted at a 1:10 ratio (corresponding to 100 ng of total RNA), 200 nM of each forward and reverse primers and 1x Mesa Green qPCR MaterMix Plus for Sybr Assay (Eurogentec). Primers of angiopoietin 2 (Ang-2) forward: 5´ GGATGGAGACAACGACAAATG 3´; reverse: 5’ GGACCACATGCATCAAACC 3´; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5’ AGCCACATCGCTCAGACAC 3´ and reverse 5´ GCCCAATACGACCAAATCC 3´. qRT-PCR experiments were run using an iQ5 (Bio-Rad) and analyzed with the iCycler IQ^{TM} software. Relative quantification of gene amplification by qRT-PCR was

performed using the cycle threshold (C_t) values and relative expression levels were calculated using the 2^(-$\Delta\Delta C_T$) method. The expression value for each target gene was normalized to the GAPDH value at each time point. Afterwards, the fold change in the Ang-2 gene normalized to GAPDH and relative expression at time zero was calculated for day 2. For each PCR, samples were analyzed in duplicate and three independent experiments were performed.

***In vitro* cell migration assay**

The cell migration assay was carried out using FluoroBlok™ inserts with an 8 μm pore size (Becton Dickenson) in a 24-well tissue culture insert carrier plate (Becton Dickenson). Cells were pre-labeled with the fluorescent probe CellTracker Green CMFDA (Molecular Probes) at 15 μM for 30 min, and then entrapped within RGD-modified and unmodified alginate matrices, as previously described. Afterwards, the hydrogel matrices were transferred to the apical side of the FluoroBlok inserts, and EGM-2 medium with (lower chamber) or without (upper chamber) growth factors was added. Fresh VEGF (Sigma) and fibroblast growth factor (FGF, Sigma) were added to the lower chamber every 24h to act as chemoattractants. At different time points (0, 1.5, 5, 18, 24, 48 and 72h), fluorescence intensity from the basal side was measured in bottom-reading mode using a microplate spectrofluorimeter (BioTek), at wavelengths of 492/517 nm (Ex/Em). To convert fluorescence readings into cell numbers, a standard curve was established for each experiment. Fluorescence images of migrating cells were collected using an inverted fluorescent microscope. Fluorescence-labeled cells within the alginate matrices were imaged by confocal microscopy. Samples were assayed in triplicate and the experiment was repeated three times.

***In vitro* Matrigel sprouting assay**

The ability of outward migrating cells to form tubular-like structures was analyzed by placing the cell-loaded alginate hydrogel matrices in contact with a matrigel basement membrane layer (Becton Dickinson). Samples were cultured with complete EGM-2 and placed in the incubator at 37ºC under 5% CO_2 humidified atmosphere. After 24h, cell sprouts were visualized at different magnifications under an inverted microscope (Axiovert 200M, Zeiss).

Activity of matrix metalloproteinases (MMPs) by zymography

To detect MMP enzymatic activity from entrapped HUVECs, cell culture supernatants were analyzed by gelatin-zymography. After 24 and 48h supernatants were collected and centrifuged to remove cell debris. Conditioned media were loaded into gelatin-SDS polyacrylamide gels. Sample volumes were adjusted to yield equivalent total cell protein contents, which were quantified by the Dc Protein assay kit (BioRad) in lysates of cells recovered from the hydrogels. The gel was run in 1x Tris-Glycine SDS running buffer at 60 V (Mini Protean Tetra Cell system, BioRad). After electrophoresis, gels were washed twice and subsequently incubated in MMP substrate buffer for 16h at 37ºC. Afterwards, gels were washed and then stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich). MMPs proteolytic activity was visualized as the presence of clear bands against a blue background of Coomassie Blue-stained of gelatin substrate.

Statistical analyses

Statistical analyses were performed using SigmaStat (Statistical Software, STAT32 MFC Application). Statistical significance was assessed by using two-way analysis of variance (ANOVA) in conjunction with Tukey's test, pairwase multiple comparison procedures. The comparison of results from the CFSE-based assay was conducted by one-way ANOVA in conjugation with Tukey's test. The t-test was adopted for statistical comparison between the groups in the Ang-2 profile analysis. Differences were considered statistically significant when p values were lower than 0.05. All data are presented as mean values ± standard deviation.

RESULTS

Cell behavior on RGD and unmodified alginate films

Preliminary cell adhesion studies were performed in a 2D environment to better illustrate the biological interaction between HUVECs and the covalently modified alginate. HUVECs seeded onto RGD-coupled alginate hydrogels were able to attach, and were already well spread after 24h, while on control surfaces (unmodified alginate) cells were unable to adhere and formed cell clusters. This phenomenon was observed

under light microscopy and is well illustrated in Fig. 1. After medium change the cell aggregates formed on unmodified alginate were easily displaced and washed out.

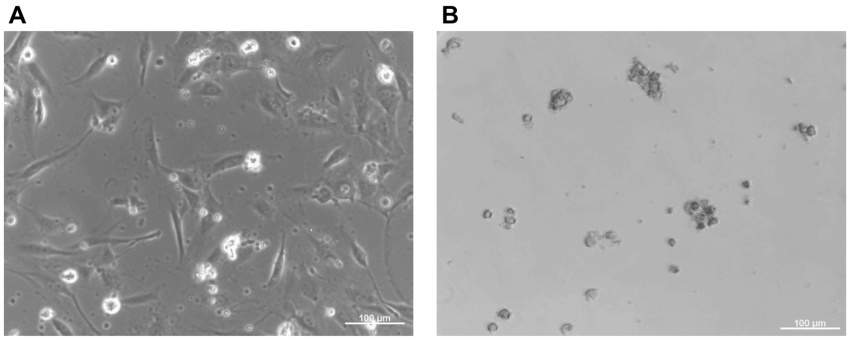


Figure 1. Photomicrographs of human umbilical vein endothelial cells (HUVECs) cultured on the top of alginate hydrogel films at 24h post-seeding without medium change: (A) RGD-modified alginate, (B) unmodified alginate (control).

In situ crosslinking of alginate solutions

Injectable *in situ*-crosslinkable hydrogels need to be able to adjust to complex forms at the site of injury. To confirm that alginate was able to conform to different shapes, gel precursor and crosslinking agents were mixed and hydrogels were casted in different molds as can be depicted in Fig. 2A. For the subsequent cell culture studies, hydrogels were prepared from 20 µL drops of alginate gel precursor solution mixed with cells (Figs. 2B, C and D).

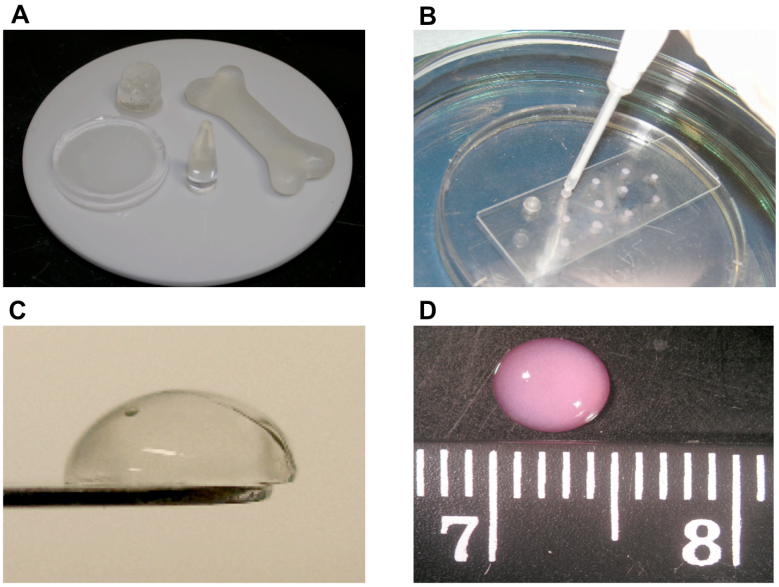


Figure 2. A) Different shapes of alginate hydrogels obtained by an internal gelation method. The alginate was gelled *in situ* by the liberation of calcium ions from an insoluble compound ($CaCO_3$) through pH decrease that accompanies glucone delta-lactone hydrolysis in solution. B) Formation of alginate matrices with immobilized cells obtained from a 20µL alginate drop by using a positive-displacement micropipette. C) and D) Representative images of alginate matrices used in this work.

Effect of RGD peptides on HUVECs survival: Viability and angiopoietin-2 expression

Cell viability, morphology and proliferation studies were performed to analyze cellular behavior under 3D conditions, within adhesive and non-adhesive alginate hydrogels. HUVECs recovered from both types of hydrogel matrices immediately after entrapment (day 0) did not present significant differences in terms of viability (Fig. 3A). After 24h, cells within RGD-alginate matrices already presented a higher viability (80%) compared to cells within unmodified hydrogels (63%). After 48h, this difference became more pronounced, with cellular viability decreasing to nearly 40% within unmodified alginate, and remaining at near 80% within RGD-alginate.

Angiopoietin-2 (Ang-2) belongs to a family of angiogenic modulators that is almost exclusively produced by endothelial cells [25]. To examine the Ang-2 expression in response to the presence or absence of RGD as cell-matrix mediator, a qPCR assay was carried out. As illustrated in Fig. 3B there were differences between the two conditions, with cells within unmodified alginate presenting a significantly higher Ang-2 expression.

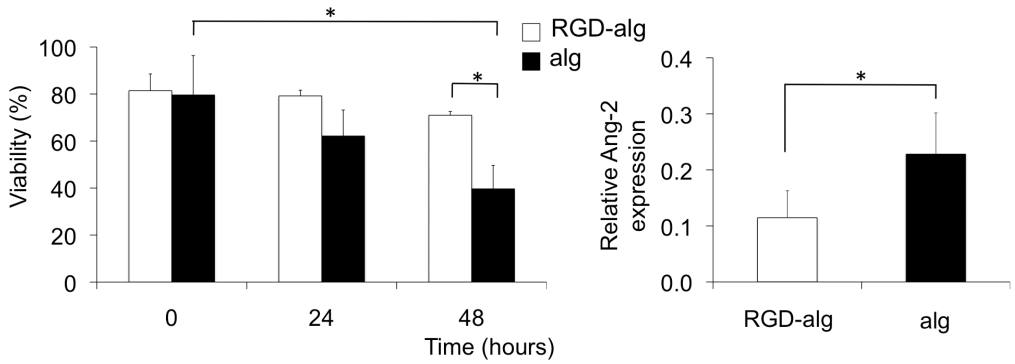


Figure 3. A) Viability of human umbilical vein endothelial cells (HUVECs) cultured under 3D conditions within RGD-modified (RGD-alg) and unmodified alginate (alg), estimated using the trypan blue exclusion assay. B) Gene expression levels of angiopoietin-2 (Ang-2) of HUVECs within RGD-modified and unmodified alginate by qPCR 48h pos-immobilization. Each experiment was performed three times, each in triplicate. Data are expressed as mean ± standard deviation (n=3). (* $p \leq 0.05$)

Cell morphology

The morphology of immobilized HUVECs was assessed by CLSM by staining the F-actin filaments, as shown in Fig. 4. In RGD-alginate, after 24h, HUVECs were able to spread and build a cellular network, which was maintained at least for 48h post-immobilization. Additionally, cells stained positively for the endothelial cell marker vWF. This network design was not attained within unmodified alginate, where cells remained with a round shape and presented no detectable cytoplasm extensions.

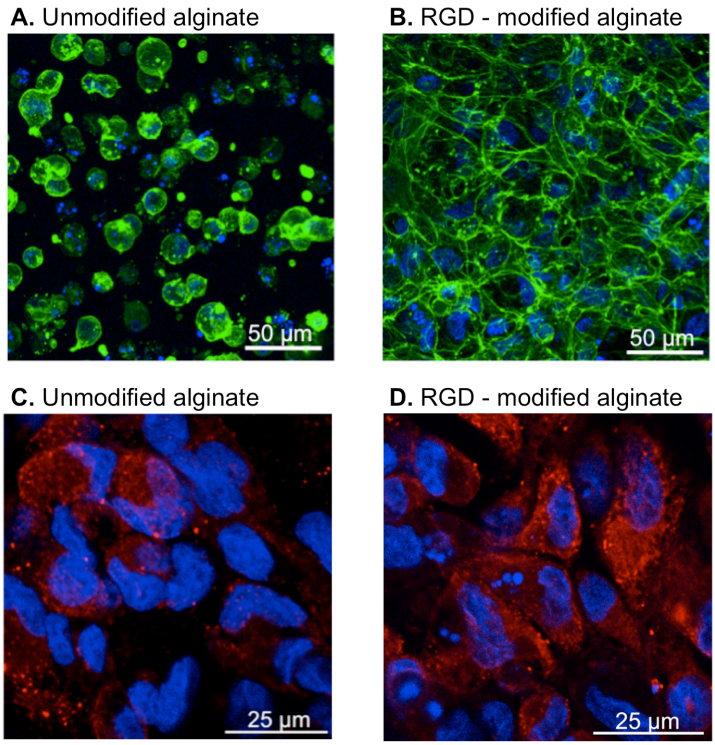


Figure 4. Human umbilical vein endothelial cells (HUVECs) cultured under 3D conditions within alginate hydrogels with or without RGD peptides 48h pos-immobilization. A and B) Cells were staining with Alexa Fluor 488 phalloidin (in green) and Hoechst 33342 (nuclei in blue). C and D) Cells were analyzed for the endothelial marker von Willebrand factor (vWF) (red) and Hoechst 33342 (nuclei in blue). Constructs were visualized under confocal fluorescence microscope. A and C) Representative images of HUVECs immobilized within unmodified alginate (without RGD); B and D) HUVECs immobilized within RGD-modified alginate.

Proliferation of ECs-loaded hydrogels

Cell proliferation on modified and unmodified alginate hydrogels was analyzed using the CFSE labeling assay. A representative histogram of flow cytometry data is shown in Fig. 5A. The extent of HUVECs proliferation is related with the decrease on CFSE staining intensity, demonstrating different rates of cell division for the two assayed conditions. Looking at the geometric mean of CFSE intensity represented in Fig. 5B it can be concluded that cells within RGD-alginate matrix presented a higher rate of cell division (lower CFSE intensity) than cells within the unmodified alginate.

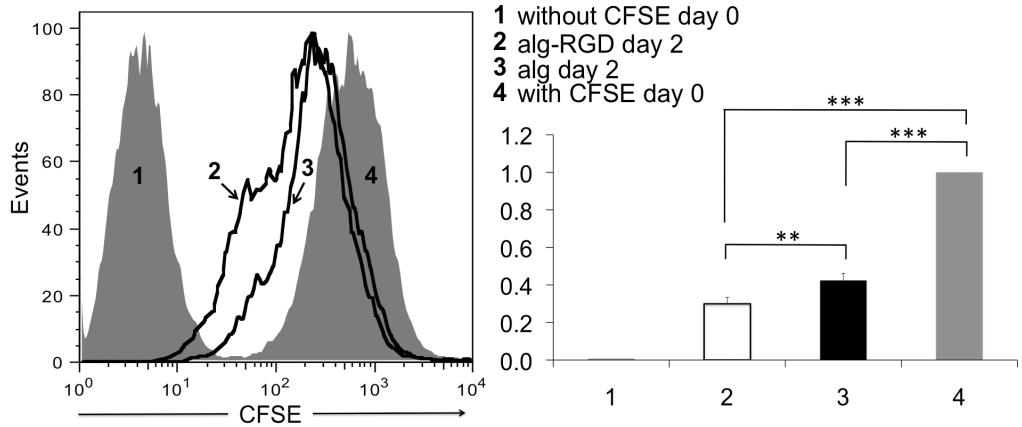


Figure 5. A) CFSE histogram data obtained by flow cytometry for human umbilical vein endothelial cells proliferation within alginate hydrogels (curve 1 – cells without CFSE, curve 4 – cells with CFSE at day 0; line 2 – cells with CFSE after 48h within RGD-modified alginate (RGD-alg); line 3 – cells with CFSE after 48h within unmodified alginate (alg). B) Geometric mean of CFSE intensity relative to cells with CFSE at day 0 (corresponds to maximum of intensity) of three different experiments. Data are expressed as mean ± standard deviation (n=3). (** $p \leq 0.01$; *** $p \leq 0.001$)

Cell migration and MMPs activity

Outward migration of HUVECs from alginate hydrogels was investigated using a transwell system. Fluorescence-labeled cells were entrapped within alginate matrices (Fig. 6A), and those that were able to escape from the hydrogel matrices were induced to migrate through the fluorescence-blocking membrane of the insert by chemotaxis (Fig. 6B). A time course of the cell migration up to 72h is illustrated by a representative experiment in Fig. 6C. Transwells with RGD-modified alginate matrices showed a significantly higher fluorescence throughout time, which corresponds to a higher number of cells that were able to cross the membrane of the fluoroblok.

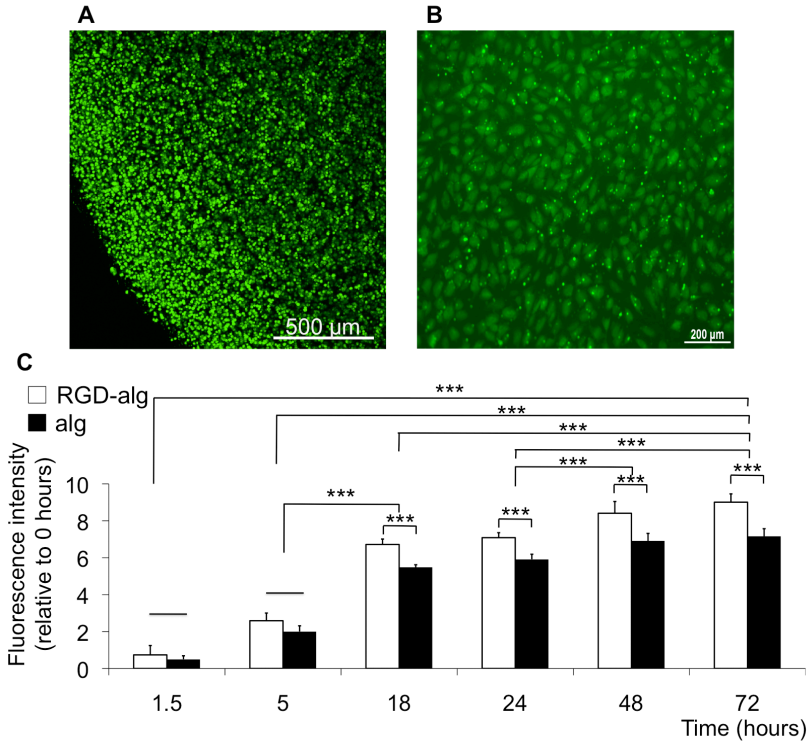


Figure 6. (A) Human umbilical vein endothelial cells (HUVECs) labeled with CellTracker^{TM}green fluorescent marker immobilized within RGD-modified alginate matrix. (B) Fluorescently labeled migrating HUVECs at the basal side of the insert membrane. (C) Fluorescence intensity (relative to 0 hours) of fluorescently labeled HUVECs that evade the alginate matrix and migrate through the FluoroBlok membrane along a period of 72h. The experiment was performed three times, each in triplicate. Data are expressed as mean ± standard deviation (n=3). (*** $p \leq 0.001$)

The next step consisted in evaluating if the entrapped HUVECs that exited the alginate matrix retained the ability to form tube-like structures. In other words, the ability of RGD-alginate hydrogels to support outward migration of HUVECs from the matrix in an organized and functional way was assessed. Hydrogel matrices containing cells were embedded in matrigel (Fig. 7), to mimic implantation in tissues. After 24 and 48h, no cellular sprouts were observed in the vicinity of control alginate matrices, but only a few dispersed cells. On the other hand, already after 24h, several cellular branches were protruding from the RGD-alginate hydrogel matrices, forming tubular-like structures that invaded the surrounding matrigel layer.

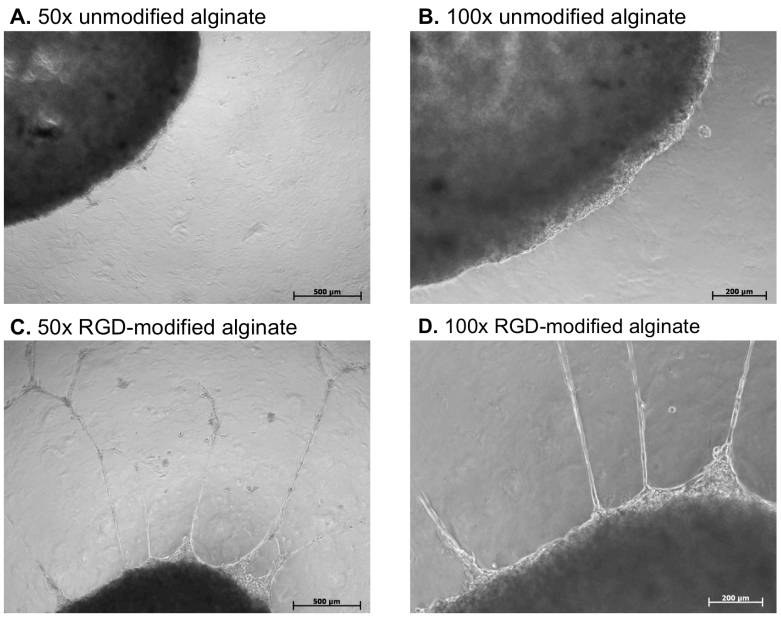


Figure 7. Representative phase-contrast micrographs of human umbilical vein endothelial cells that have migrated out from the alginate matrix into the surrounding tissue mimic (Matrigel), after 24h, at different magnifications: A) a B) Unmodified alginate; C) and D) RGD-modified alginate.

Endothelial cell migration through extracellular matrices is known to be dependent and controlled by MMP-mediated matrix degradation [26]. High levels of protease expression are required for cells to survive and migrate. Zymography results (Fig. 8) show that HUVECs secreted the two active MMP2 species (62 and 64 kDa), which increased after 48h and were detected using unmodified and RGD-modified alginate. However, in the case of cells immobilized with alginate grafted with RGD peptides, a higher activity of both active MMP2 species was observed. Although the latent form of MMP9 (92kDa) was detected in the medium, its active species were not observed.

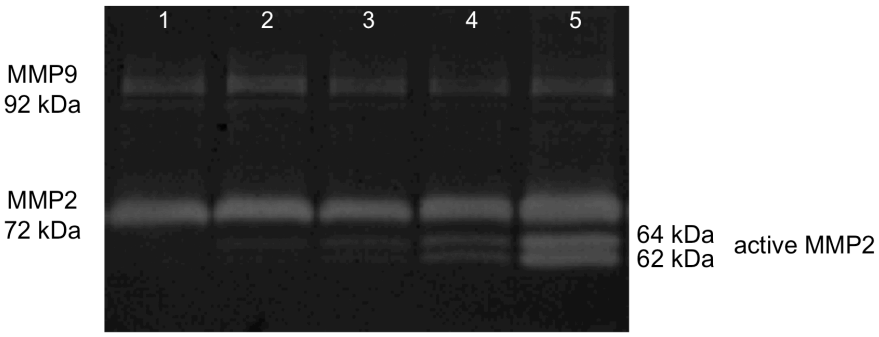


Figure 8. Enzymatic activity of MMP-2 and MMP-9 obtained from human umbilical vein endothelial cells (HUVECs) supernatants analyzed by gelatin zymography. HUVECs entrapped within unmodified alginate for 24h (lane 2) and 48h (lane 3) and entrapped within RGD-modified alginate for 24h (lane 4) and 48h (lane 5). In lane 1 fresh medium was run as a control. These results are representative of three separate experiments.

DISCUSSION

Three-dimensional (3D) scaffolds offer an exciting possibility to mimic functionality and complexity of native tissues. However, to construct a functional tissue, the formation of a vascular network is required. The vascularization of engineered tissues *in vitro* may be seen as an alternative to improve cell survival after transplantation, since functional blood vessels can facilitate transport of oxygen and nutrients. Moreover, considering the fact that several attempts to accomplish revascularization solely through delivery of growth factors have largely failed in clinical trials, endothelial cells transplantation stands out as a promising approach [27].

Polymers with the ability to form 3D hydrogel networks, such as alginate, have been used in a variety of medical applications including cell encapsulation. In particular, this anionic polysaccharide has, as additional advantage, the capacity to be transformed from liquid to solid *in situ* under mild conditions. The idea behind this work was to analyze this injectable biomaterial as a vehicle for vascular cell-based therapies, providing a microenvironment that enhances cell survival and that could be used for repopulation of surrounding tissue after injection. From an application standpoint, these injectable matrices would also help to tackle problems of surgical invasiveness.

Alginates are non-fouling and non-adhesive to cells, which can be overcome through chemical modification with cell-adhesion peptides, such as the amino acid sequence arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD) found in many ECM proteins including fibronectin, laminin, vitronectin and collagen [7]. The RGD modification alone was sufficient to transform alginate from a relative bioinert polysaccharide into a substrate capable of supporting endothelial cell anchorage and consequently, allowing cell interaction. HUVECs adhesion to the hydrogel films was possible due to the presence of these RGD peptides, as is shown by the limited cell adhesion and spreading observed on the unmodified surface (Fig. 1). The integrins that bind to the ECM provide not only anchorage for endothelial cells, but also information concerning their microenvironment, contributing to cell's decision to proliferate, migrate or die [28]. More precisely, the integrin $\alpha_v\beta_3$, that binds to RGD, plays an important function mediating cells survival and have been associated to cell migration and invasion [28, 29]. Yu and co-workers used RGD peptides conjugated with alginate to improve heart functionality by regulating endothelial cell function in a chronically injured heart tissue [29]. They were able to demonstrate that this hydrogel can be used as an effective

acellular strategy due to the fact that integrin-ligand interaction may induce host tissue regeneration. Although they demonstrated an improvement in a specific disorder, for other situations the application of the biomaterial *per se* may be insufficient, and the co-transplantation of cells may be additionally required.

In regenerative medicine, the use of injectable biomaterials, such as alginate hydrogels, with the capacity to adjust to complex shapes at the site of injury (Fig. 2) represents an important advantage [9]. Moreover, the hydrogel network acts as an artificial ECM that organizes cells into an adequate 3D architecture, offering structural support and preserving cells phenotype and function, which may not be accomplished in 2D cultures. From 2D to 3D studies, a key parameter to examine was the capacity of alginate hydrogels to support HUVECs viability, considering that most biomaterials tested for that purpose poorly supported vascular cells survival [15, 27]. In point of fact, in a prior work with 2 wt-% modified alginate, endothelial cells showed an abrupt loss of viability after immobilization [15]. To overcome this drawback, several studies with lower alginate concentrations were performed until 1 wt-%. With this concentration HUVECs were able to maintain their viability and spread within the hydrogel matrix. As illustrated in Fig. 3, HUVECs retained their viability for at least 48h post-immobilization in the presence of RGD peptides. On the other hand, within unmodified alginate cells viability decreased rapidly throughout time. This phenomenon could be explained by the fact that endothelial cells are cell-anchorage dependent, and the lack of proper integrin-ligand interactions, resulting from the unavailability of RGD peptides, leads to cell death [30]. Some authors associate Ang-2 as an originator of endothelial cell apoptosis, or in some unfavorable circumstances (hypoxia, serum starvation, absence of cell/matrix interaction) capable of activating signaling pathways that promote cell survival [31]. A higher Ang-2 expression was observed in cells immobilized within unmodified alginate, which could be interpreted as a sign of endothelial cells stress (unfavorable condition), due to the absence of cell anchorage points [32].

A successful matrix for tissue regeneration should ideally promote the development of a vascular network. In the present work, the angiogenic potential of HUVECs immobilized within an hydrogel matrix grafted or not with RGD peptides, under a pro-angiogenic environment, was analyzed. HUVECs were able to organize into 3D cellular networks within RGD-alginate hydrogels after 24h (Fig. 4) but remained round and isolated within unmodified alginate. Moreover, they continued to express endothelial specific markers, such as vWF, a glycoprotein that mediates platelet adhesion at sites of vascular injury [33]. The expression of this marker, which was

observed by confocal microscopy, indicated that HUVECs within the RGD-alginate matrix have been able to preserve functionality (Fig. 4D). Additionally, CFSE staining analyses confirmed HUVECs proliferation after 48h post-immobilization in both alginates, comparing to day 0 (Fig. 5). However, cells within RGD-alginate showed a higher rate of cell division comparing to cell within unmodified alginate.

The dynamics of cell-ECM interactions contribute not only to cell-matrix adhesion but also to cell activities such as cell proliferation, invasion and migration. Cell migration is vital for many physiological processes including tissue vascularization. The injectable matrix used here for immobilizing vascular cells based on 1 wt-% modified alginate allowed this process, promoting a significantly higher cell migration out of the alginate matrix in the presence of RGD peptides (Fig. 6). In contact with a basement membrane extract rich in ECM proteins, only cells within RGD-alginate matrix were able to migrate out from the alginate in an organized way, producing several branches that protruded from the hydrogel matrices and invaded a Matrigel layer as tubular-like structures (Fig. 7). A possible explanation may rely on the higher proteolytic ability (expressed by higher enzymatic activity of MMP2) of cells entrapped within RGD-alginate hydrogel, which was demonstrated by zymography. This enhanced capacity may assist cells in degrading the surrounding Matrigel layer, and thus facilitate ECM invasion by the tubular-like structures. The gelatin zymogram of the supernatants revealed that HUVECs in the presence of RGD, a molecular recognition motif of some ECM proteins, secreted higher amounts of both latent and active MMP-2, which increased from 24 to 48h (Fig. 8). This is in agreement with the literature, which demonstrated that proMMP-2 activation is modulated by the number of bindings of the cell surface integrin receptors to the ECM [34]. In point of fact, integrin binding can regulate MMP activity and ECM proteolysis. Moreover, the MMP-2 activation represents a critical level of regulation [26, 35].

Among the enzymes that play a critical role in extracellular matrix turnover, MMP-2 and MMP-9 are the most important in the angiogenesis process, due to their collageneolytic activity that enables endothelial cells to invade the basement membrane of vascular structures [35]. Regarding MMP-9, this enzyme also participates in the degradation of basement membranes, but the mechanisms that trigger its activity are not well understood so far. In the present study, it was not possible to detect active MMP-9, probably due to its low concentration in the supernatant.

In summary, whether vascular cells are delivered from an implanted hydrogel or invade from the host tissues, rapid stimulation of vascular organization is critical for cell

survival within the implant. In this report several fundamental problems associated with an *in situ* gel forming system for vascular cells were taken into consideration – loss of viability, lack of vascular-like organization and absence of cell migration. The results obtained support the potential of using injectable alginate hydrogels as adequate carriers for the minimally invasive delivery of vascular cells.

CONCLUSIONS

In this work the *in vitro* potential of a natural hydrogel for the delivery of vascular cells was explored. The obtained results support the idea that an RGD-alginate matrix can recreate an adequate 3D microenvironment for HUVECs adhesion, survival, migration and 3D organization. To our knowledge, this was the first study showing the potential of injectable alginate hydrogels as an *in situ* gel forming delivery system for vascular cells, which supports the *in vitro* formation of cellular networks by migrating cells.

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Chapter V

Concluding remarks and future perspectives

Bone is one of the few organs that retains the potential for regeneration in adult life, as it possesses the ability to heal and to remodel [1]. In some clinical settings, however, bone is not able to heal by itself and further treatment is needed: fusion of bones, reconstruction of shattered bones, filling of bone defects caused by cysts or tumors, and stimulation of bone growth to help anchor an artificial joint or other implant [2, 3]. For most of these injuries, available treatments are still inadequate. Therefore, in the present thesis we propose a new strategy that might constitute a solution for some for some of these problems.

The main objective of this thesis was the development of a bone tissue engineering strategy based on an injectable system in combination with mesenchymal stem cells (MSCs) and endothelial cells (ECs). The proposed system would potentially act as a filler to stimulate bone repair and to enhance bone function.

Modern tissue engineering is increasingly using three-dimensional structured biomaterials as artificial extracellular matrices in combination with stem cells as cell source and/or biologically active molecules [4]. A bio-inspired material was herein investigated, not only to serve as cell carrier by providing the adequate physical and mechanical support, but also to actively influence cell behavior, such as cell adhesion, proliferation, differentiation and function [5].

MSCs have been widely explored for bone tissue engineering strategies due to their differentiation capacity into distinct end-stage cell types, their paramount role in endochondral and intramembranous bone formation *in vivo*, immunosuppressive properties and ability to produce a broad spectrum of bioactive macromolecules capable of establishing a regenerative microenvironment [6-14].

The crosstalk between MSCs and ECs has already been identified: a paracrine effect mediated by vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP)-2 and insulin-like growth factor (IGF) production, and a juxtacrine mechanism by gap junctional activity (connexin 43) [15-18]. Although the proficiency of this dialogue had been evaluated in the behavior of both cells, it has not been considered as a key parameter for large-scale production of MSCs. Taking into account that a large number of MSCs committed to the osteogenic lineage is required for creating a tissue-engineered implant able to fill bone defects, we investigated whether a coculture system of both cell types could modulate the phenotype and proliferation of harvested MSCs. We were able to demonstrate that, by coculturing MSCs with HUVECs, there was not only an enhancement of osteogenic differentiation, but also a significant increase on MSCs proliferation. This augmentation in cell

proliferation occurred independently of the relative cell ratios used, but was favored by higher relative amounts of HUVECs. Hence, the obtained data suggest that HUVECs not only modulate MSC phenotype but also their proliferation rate. Such finding, constituting a step forward in understanding the interaction between these two cell types, can easily find application in several tissue engineering approaches, where a high amount of cells is required and where vascularization has prime importance.

As the field of tissue engineering progresses, improvements in the development of more refined and tailored cell-based therapies to replace, repair or enhance the function of damaged tissues or organs are emerging. The possibility of combining cells with biomaterials to create “living cell medicines” has open new doors. For clinical applications, the delivery of cells through minimally invasive approaches may be very attractive , since these systems offer specific advantages, such as ease of application, confined delivery and improved patient compliance and comfort [19].

In the context of bone regeneration, and considering previous work from our group, [20, 21] it seemed relevant to us to investigate the use of alginate microspheres as carriers for MSCs. Calcium-crosslinked sodium alginate hydrogels present several advantageous features over other gel matrices used for cell immobilization, namely the ability to crosslink under mild conditions, to immobilize living cells and to form 3D constructs. In addition, they can be easily sterilized and stored, and have been extensively used in the form of microspheres for cell immobilization [22-26]. In this work, alginate microspheres with an average size of 800 μm were prepared. It is worth mentioning that this size range was selected mainly to facilitate handling during the several assays performed, although these 3D matrices can be easily reduced in size down to 200 μm by simply varying the encapsulation settings used.

Another parameter taken into account was the fact that alginate, although originally a non-cell interactive polymer, can be covalently modified with peptides containing the RGD sequence to promote cell adhesion [20, 27]. Moreover, its molecular weight was modified through irradiation and oxidation to modulate its viscoelastic properties, as well as its degradation rate [28]. The lower viscosity of the irradiated polymer results in reduced shear forces during the cell-polymer mixing process and, consequently, in higher cell viability [29]. Previous studies using RGD-alginate microspheres with a binary composition of high and low molecular weight alginate already demonstrated the usefulness of this 3D system for the immobilization of MC3T3-E1 osteoblast-like cells [20, 21]. As highlighted above, MSCs represent a more clinically relevant cell source. Accordingly, we characterized in detail the viability,

metabolic activity and osteogenic potential of MSCs immobilized within RGD-alginate microspheres cultured under basal and osteogenic induction conditions. We were able to demonstrate that MSCs could be entrapped in RGD-alginate maintaining a high viability, and be kept metabolically active during 3 weeks under 3D culture. Moreover, MSCs immobilized in this 3D alginate matrix were also able to respond to environmental biochemical cues from the media by differentiating into osteoblasts when cultured in osteogenic media.

It is now widely accepted that the success of a bone tissue engineering implant largely relies on its capacity to influence the surrounding environment and, eventually, on triggering new vessel formation. For that reason, we also studied the ability of a MSCs-alginate 3D construct to enhance the sprouting of vessels by neighboring endothelial cells. By applying a well-known methodology, the Matrigel assay, we observed that MSCs immobilized inside RGD-grafted alginate microspheres significantly enhanced the ability of neighboring endothelial cells to form tube-like structures. In addition, the presence of VEGF was monitored in the culture medium, showing not only that hMSC were able to produce this signaling protein, but also that it was able to diffuse through the hydrogel network into the surrounding medium.

In future works, the *in vivo* performance of MSCs-loaded microspheres upon implantation on critically sized bone-defects created in suitable animal models would provide further insights into the usefulness of this system under physiological conditions. Nevertheless, to be applied via minimally invasive surgical strategies (for instance, through injection), the system described above would require a vehicle with *in situ* gel-forming ability to deliver the MSC-loaded microspheres into the defect and to keep them in place. Some hydrogel matrices, like alginate, can be prepared from soluble precursor solutions that crosslink *in situ*, which would allow the application of the microspheres at the injury site by minimally invasive methods [30, 31]. As a future perspective, the injectability of these microspheres deserves investigation. An ideal vehicle, with adequate rheological properties allowing the implantation of microspheres through a minimally invasive approach, needs to be developed. Although alginate stands up as a good candidate, a meticulous study must be carried out.

Of vital importance to a good performance of a biomaterial scaffold is its capacity to allow the proper vascularization, and therefore, vascular cell survival and organization. In our experimental conditions, immobilized MSCs were able to stimulate ECs in a reconstituted basement membrane (Matrigel). The next step consisted in evaluating the survival of ECs within the alginate matrix, which was proven to be low.

In point of fact, in a prior work with 2 wt-% modified alginate, endothelial cells showed an abrupt loss of viability after immobilization [21]. To overcome this drawback, several studies with lower alginate concentrations were performed until 1 wt-%. Using this concentration we were able to develop a hydrogel matrix where ECs retained their viability at least 48h post-immobilization. Moreover, such cells spread, formed a cellular network and were able to migrate out from the alginate hydrogels. In light of a clinical application, the vascular cell source needs to be carefully addressed. Although in this work we used a very well characterized endothelial cell line to demonstrate the alginate potential, from a clinical perspective, mature ECs present some disadvantages, such as reduced availability and proliferation rate. As an alternative, autologous cells like outgrowth endothelial cells (a subpopulation of endothelial progenitor cells) appear as a powerful candidate for regenerative medicine, as more knowledge about their biological properties is being revealed [32-38]. In spite of some controversy, these cells have been successfully applied in 3D matrices and there is no doubt regarding their ability to enhance angiogenesis and vascular repair [37, 39-42]. Although ECs have an inherent capacity to form tubular structures, the formation of long-lasting vessels within a 3D matrix using a coculture system with pericytes or smooth muscle cells should be evaluated as well [11, 43, 44].

Overall, we were able to demonstrate that MSCs could be expanded *ex vivo* in the presence of ECs and further delivered in a RGD-modified alginate. Finally, we studied the potential of this biomaterial-based system as an *in situ* gel forming delivery system able to promote the outward migration of viable, functional and proliferative endothelial cells. As future perspectives, although RGD-modified alginate successfully immobilized MSCs and ECs, further modifications should be considered to improve the 3D alginate microenvironment. For instance, modification of alginate with a protease-labile double-end grafted crosslinking peptide, cleavable by matrix metalloproteinases, would provide a more dynamic 3D environment, allowing cells to remodel the matrix and create pathways [31].

The development of a potential injectable system, able to simultaneously provide a source of viable bone and vascular cells and to stimulate an angiogenesis-like response in the host, may represent a breakthrough in bone regeneration strategies.

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