

Universidade do Porto
Faculdade de Engenharia



**The influence of cell density and culture
conditions within 3D hydrogel matrices on
mesenchymal stem cells behavior**

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ABSTRACT

This study addressed the effect of culture conditions, namely the initial cell density, on hMSC behavior upon 3D-culture within RGD-alginate matrices. Low-to-high densities in the range of 2-to-15 million cells per mL of hydrogel were tested. Structural electron microscopy analysis showed that the polymer network surrounded entrapped cells, and that cell-matrix interactions were promoted to some extent, as suggested by the presence of cytoplasmatic extensions around cells. Entrapment at the highest density promoted a more interesting cell response, with significant differences in relation to the other tested densities. Cell viability was better maintained and more cell-cell interactions were elicited. Along one week of culture, the total cell number of cells and DNA content on the 3D matrices remained constant, showing that the entrapped cells were not proliferating. This was consistent with the observed steady-state in terms of metabolic activity, and was most probably related to the spatial restrictions imposed by the alginate network, as both metabolic and mitotic activity could be recovered when cells were moved from 3D to 2D. After 2 weeks, MSCs in high-density 3D-culture under osteogenic conditions, which accelerated matrix degradation, were shown to adopt a more spread shape, forming cellular networks inside the hydrogels. Under those specific conditions, the formation of multicellular aggregates was also detected. Those structures stained negative for the proliferation marker Ki67, and possibly resulted from the migration and intercellular adhesion of previously isolated cells. Cells within large aggregates produced endogenous ECM, as demonstrated by the presence of collagen and fibronectin, and stained intensively for ALP, a commonly used bone phenotypic marker. Overall, these observations highlight the potential of RGD-alginate hydrogel as injectable hMSC-delivery systems for bone regeneration applications, and put in evidence the importance of selecting the most appropriate cell entrapping density for a given application.

RESUMO

Este estudo abordou o efeito das condições de cultura, nomeadamente a densidade celular inicial, sob o comportamento de hMSC em culturas 3D em matrizes de alginato-RGD. Várias densidades, de 2 a 15 milhões de células por mL de hidrogel, foram testadas. Análise estrutural com microscopia eletrónica demonstrou que a rede de polímero rodeia as células aprisionadas, e que as interações célula-matriz foram promovidas em certa medida, como sugerido pela presença de extensões citoplasmáticas ao redor das células. O confinamento na maior densidade promoveu uma resposta celular mais interessante, com diferenças significativas em relação a outras densidades testadas. A viabilidade celular foi melhor mantida e mais interações célula-célula foram conseguidas. Ao longo de uma semana de cultura, o número total de células de células e conteúdo de DNA nas matrizes 3D permaneceu constante, mostrando que as células confinadas não proliferaram. Este resultado foi consistente com o estado estacionário observado em termos de atividade metabólica, que estará, muito provavelmente, relacionado com as restrições espaciais impostas pela rede de alginato, já que a atividade metabólica e mitótica poderam ser recuperadas quando as células foram transferidas de 3D para 2D. Após 2 semanas, MSCs em culturas 3D de alta densidade, sob condições osteogénicas, que aceleraram a degradação da matriz, adotaram uma forma mais esticada, formando redes de células no interior do hidrogel. Sob essas condições específicas, a formação de agregados multicelulares também foi detectada. Estas estruturas não expresaram o marcador de proliferação Ki67 e, possivelmente, resultaram da migração e adesão intercelular das células previamente isoladas. Células dentro de grandes agregados produziram ECM endógena, como demonstrado pela presença de colágeno e fibronectina, e coraram intensamente para ALP, um marcador fenotípico do osso comumente utilizado. Em geral, estas observações evidenciam o potencial do hidrogel de alginato-RGD como sistema injetável de entrega de hMSC para aplicações de regeneração óssea, e coloca em evidência a importância de selecionar a densidade celular de confinamento mais apropriada para uma dada aplicação.

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
a. u.	Arbitrary units
ALP	Alkaline phosphatase
ATP	adenosine triphosphate
BCA	Bicinchronic Acid
BM	Basal Medium
BSA	Bovine serum albumin
Calcein AM	Calcein acetoxymethyl ester
cryo-SEM	Cryo-scanning electron microscopy
CSLM	Confocal Scanning Laser Microscopy
DAPI	4',6-diamino-2 phenylindole
DMEM	Dulbecco's Modified Eagle Medium
dsDNA	Double-stranded DNA
ECM	Extracellular Matrix
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
EDTA	Ethylenediaminetetracetic acid
EMT	epithelial-mesenchymal transition
EthD-1	Ethidium Homodimer-1
Ex/Em	Excitation spectrum/Emission spectrum
FAK	focal adhesion kinase
FBS	Fetal bovine serum
FN	fibronectin
G4RGDSP	(glycine)4-arginine-glycine-aspartic acid-serine-proline
G6-P-DH	glucose-6-phosphate dehydrogenase
GAGs	Glycosaminoglycans
GDL	Glucone delta-lactone
h	Hours
H&E	Hematoxylin-Eosin
HK	hexokinase
hMSC	Human mesenchymal stem cell
HMW	High Molecular Weight
LMW	Low Molecular Weight
MES buffer	2-(N-morpholino)ethanesulfonic acid

min	minutes
MT	Masson's Trichrome
Mw	Molecular Weight
MWCO	Molecular Weight cut-off
NADPH	nicotinamide adenine dinucleotide phosphate
OD	Optical density
OM	Osteogenic medium
ON	Overnight
PBS	Phosphate buffered saline
Pen/Strep	penicillin streptomycin
PFA	Paraformaldehyde
pHEMA	Poly(2-hydroxyethyl methacrylate)
PKC	protein kinase C
RFU	Relative fluorescence unit
RGD	arginine-glycine-aspartic acid
rpm	Rotation per minute
RT	Room Temperature
s	seconds
SD	Standard deviation
Sulfo-NHS	Sulfo- (N-hydroxysulfosuccinimide)
TBS	Tris-buffered saline
TBS/Ca	TBS with 7.5 mM CaCl ₂
TCPS	tissue culture polystyrene
TEM	Transmission electron microscopy
UV	Ultra Violet
wt	Weight

I. INTRODUCTION

I. Introduction

1. The extracellular matrix: structure, composition and cell-matrix interactions

The architecture and organization of multicellular organisms is governed by cohesion mechanisms, being cells held together by cell junctions, cell adhesions and the extracellular matrix (ECM) [1-3]. The ECM provides structural support to the cells, and profoundly influences many aspects of cellular behavior such as survival, proliferation, migration and differentiation. The ECM is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them (Fig. 1) [2].

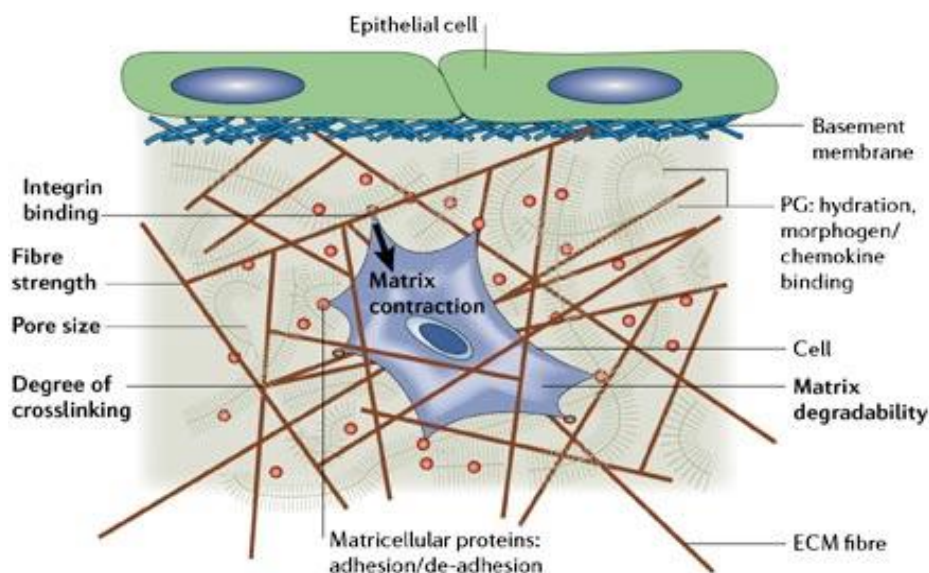


Figure 1. Connective tissue with a cell surrounded by extracellular matrix, underlying an epithelium ([4])

1.1. ECM structure and composition

Variations in the relative amounts of the different types of matrix macromolecules and the way in which they are organized in the ECM give rise to a diversity of forms adapted to the functional requirements of the particular tissue [2]. In connective tissues, such as bone or tendons, the ECM is abundant and rich in collagen, whereas at the interface between an epithelium and connective tissue, named basal lamina or

basement membrane, the ECM is thin and sparse and serves as a barrier with an important role in controlling cell behaviour [2]. Histological images of distinct tissues with loose and dense ECMs are presented in Fig. 2 as an illustrative example.

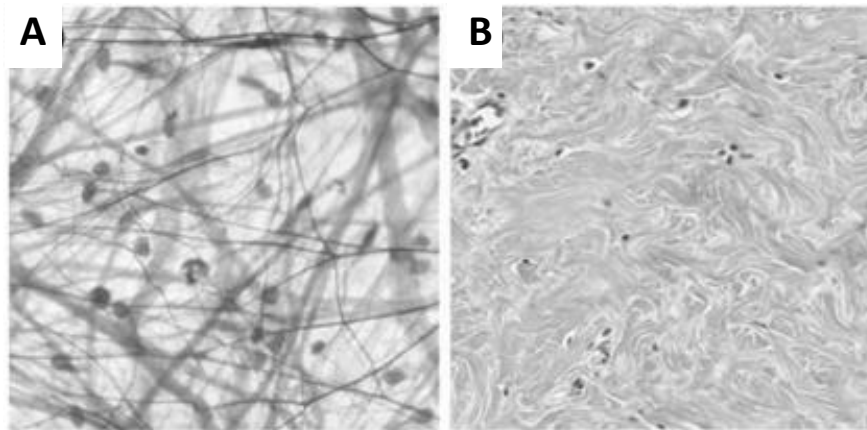


Figure 2. Images of different ECM architectures: **(A)** loose connective tissue showing fibroblasts embedded in protein fibers with an irregular orientation and large spaces in between (40x); and **(B)** dense connective tissue with cells collagen fibers irregularly oriented but densely packed (20x). Adapted from: ([5]).

The macromolecules that compose the connective tissue's ECM of most mammals are essentially glycosaminoglycan (GAGs), which are polysaccharide chains usually found covalently linked to proteins in the form of proteoglycans; fibrous proteins, including collagen and elastin; and other specialized proteins such as fibronectin, and laminin, which have both structural and adhesive functions [2, 6-8]. Differences between the ECM's composition in different tissues dictates their specific physical properties [6]. Cells in different tissues secrete their own ECM: mostly fibroblasts in connective tissues, and osteoblasts or chondroblasts in specialized connective tissues such as bone and cartilage, respectively [2]. The constituents of the ECM with major relevance for the present work are briefly described in the next sections.

1.1.1. GAGs and proteoglycans

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains composed of repeating disaccharide units, an amino sugar such as *N*-acetylglucosamine or *N*-acetylgalactosamine, which usually is sulfated, and an uronic acid (glucuronic or iduronic) [2]. Four main groups of GAGs are distinguished according to their sugars,

the type of linkage between the sugars, and the number and location of sulfate groups: hyaluronan, chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate [2, 8]. The GAG chains are covalently attached to proteins as proteoglycans, except for the case of hyaluronan.

Because there are sulfate or carboxyl groups on most of their sugars, GAGs are highly negatively charged, which attracts osmotically active cations, such as Na^+ , creating a swelling pressure. Furthermore, the polysaccharide chains are stiff, not able to form globular structures, and are strongly hydrophilic. GAGs tend to adopt extended conformations, occupying large volume relating to their mass, therefore forming highly hydrated gels even at low concentration. These characteristics enable the matrix to withstand compressive forces [1, 2]. As already referred, hyaluronan is an exception, as it has no backbone proteins linked to the main chain. Hyaluronan is composed of identical repeating disaccharide sequences and no sulfated sugars, and due to its higher molecular weight, has important space filling functions, creating free-spaces in embryogenesis and wound healing where cells can migrate to, also acting as a lubricant in the joint fluid [2]. Syndecan-1 also differs from other proteoglycans, since it is an integral transmembrane protein [8]. GAGs and proteoglycans can assemble and associate to form larger polymeric structures, even with other matrix components such as collagens [2].

1.1.2. Collagens

Different types of collagen, a fibrous protein, exist in the ECM exhibiting distinct and unique properties, such as tensile strength and fibril formation. An essential structural feature of these molecules is the classical triple helix, in which three collagen polypeptide α chains, extremely rich in glycine and proline, form a rope-like structure [1, 2, 8]. In fibrillar collagens, as type I, II, III, V and XI, the triple helix represents the major part of the molecule, in which the collagen molecules assemble forming collagen fibrils which in turn aggregate to form collagen fibers. Type I collagen, the principal collagen molecule in skin and bone, is the most common. This fibril assembly will only occur after secretion since collagen is synthesised in a pro-collagen form, only achieving its activated state after pro-peptides cleavage. This strategy guides the intracellular formation of the triple stranded collagen molecule and prevents the intracellular formation of the collagen fibrils [2, 8].

Collagen types IX and XII decorate the surface of collagen fibrils, being therefore called fibril-associated collagens. Collagen type IV, a network-forming type of collagen, is the major constituent of basal membranes, with more than half of its molecular weight represented by non-triple helicoidal, glycoprotein-like structure, and type VII collagen is an anchorin fibril, helping attach the basal lamina to connective tissue underneath it [8].

1.1.3. Fibronectin

Different types of glycoproteins appear to play a crucial role in cell-matrix interactions. Special focus has been given to fibronectin (FN), the first and perhaps the better studied example [8-10]. FN serves as substrate for different adhesion molecules, including integrins that are heterodimeric cell surface receptors [11]. Fibronectin is secreted by cells in the form of non-functional building units, which are subsequently assembled in the extracellular space into functional supramolecular structures, in a highly regulated manner [11].

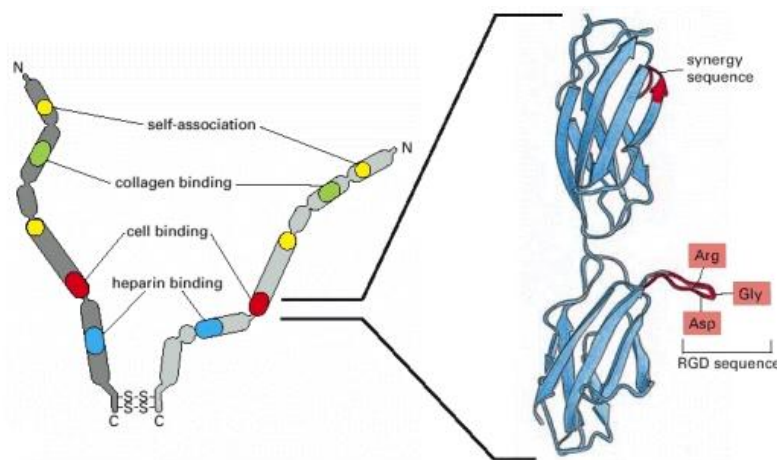


Figure 3. Fibronectin molecule highlighting the RGD sequence for cellular adhesion ([2]).

The disulfide-bonded FN dimers possess specific binding sites for multiple ECM components (Fig. 3), and dimerization seems to be required for FN assembly into a fibrillar matrix. FN has been shown to interact with integrins through a small sequence of aminoacids, Arginine-Glycine-Aspartate or RGD, modulated by the neighboring “synergy site” [7, 8].

The FN molecule exists in two forms, a cellular FN which assembles into fibrillar matrix in the tissues, and the plasma FN, a soluble, non-fibrillar form. The assembly of FN fibrils is a cell-driven process in which integrins play a central role. Integrins shift between low and high affinity conformations, so called inactive and active forms, respectively. Therefore, tissues expressing activated integrins, immediately binds cellular FN, which is followed by generation of mechanical tension through cells cytoskeleton, leading to the stretching of the FN molecule, revealing the cryptic “self-association” sites and binding other FN molecules. The final result is fibril formation via an assembly chain-reaction [11].

1.2. Dynamics of cell-ECM interactions

The highly dynamic interactions between cells and the ECM along with their key importance are nowadays well recognized, and the ECM is no longer thought to be only a structural support to maintain tissue and organ configuration [7, 8, 12-15]. It is widely accepted that there is an informational feedback loop between cells and ECM, where the differentiation status of the cell, along with space and time, are known to play a role. The ECM connects with cells by means of receptors such as integrins, creating outside-in signaling, and in turn the cell itself can modify its own ECM through inside-out signaling. This vicious cycle makes both ECM and cells inseparable in terms of tissue functionality and modifications, and derangements of the ECM may be the basis of diseases [8].

1.2.1. Cell-Matrix adhesion

Cell–matrix adhesions are essential for many key cellular functions, and as a result they play central roles in embryonic development, remodeling, and tissues homeostasis. Matrix adhesion signals cooperate with other pathways to regulate biological processes such as cell survival, cell proliferation, wound healing, and tumorigenesis [12].

Anchorage-dependent cells require a support matrix in order to survive [16], and undergo apoptosis in the absence of cell–matrix interactions. This type of apoptosis,

which occurs as a result of cell detachment from the ECM, is specifically named anoikis [17, 18]. For example, studies from Meredith and colleagues indicate that ECM is required for cell survival, since endothelial cells incubated in suspension were induced to undergo anoikis, which was reverted when cells were plated on an integrin $\beta 1$ monoclonal antibody, and observed a similar result with neuronal cells [19].

Cell adhesion to the ECM induces discrete cell surface structures tightly associated with the matrix termed cell–matrix adhesions, which mediate direct interactions of the cell with its extracellular environment [12].

These cells-ECM contact regions contain integrin receptors as their major transmembrane proteins [6, 7, 12]. There are 24 distinct integrin receptors that bind various ECM ligands with different affinities [20]. Some integrin subunits are ubiquitously expressed, while other subunits are expressed in a tissue- or stage-restricted manner [12]. Each integrin consists of a heterodimer made from one α and one β subunit. Both subunits are type I transmembrane glycoproteins with relatively large extracellular domains and, with the exception of the vertebrate $\beta 4$ subunit, short cytoplasmic tails [21]. With their extracellular domains, distinct integrin heterodimers (e.g. $\alpha 5 \beta 1$) bind to specific ECM components (e.g. fibronectin), whereas their cytoplasmic tails firmly interact with adaptor proteins, such as vinculin, paxillin, and α -actinin, that link them to the actin cytoskeleton (Fig. 4) [6].

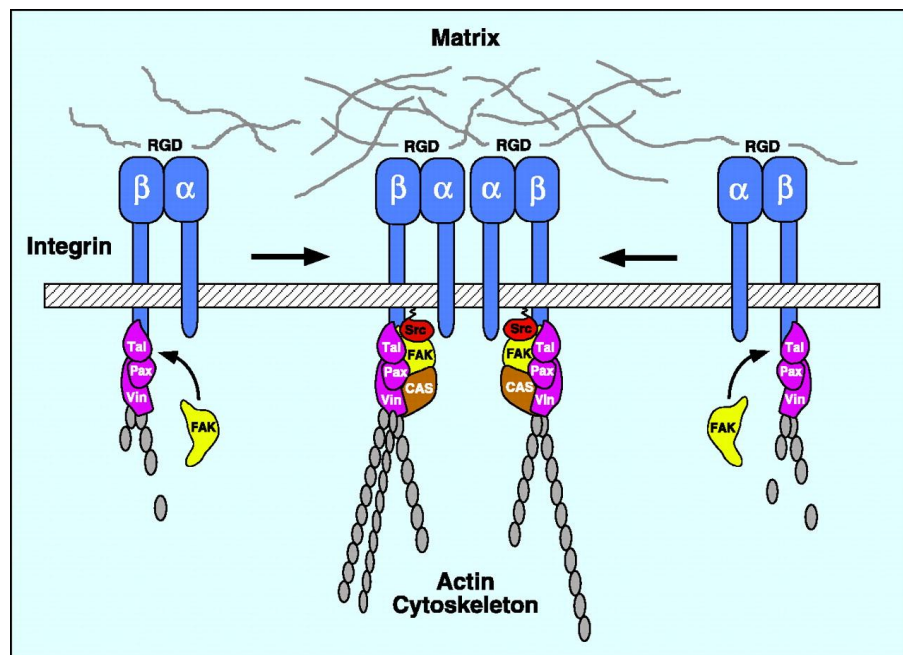


Figure 4. Matrix binding promotes integrin clustering and association with the cytoskeleton ([22]).

The extracellular domains of integrin receptors bind ECM ligands and divalent cations, but they can also associate laterally with other proteins at the cell surface, such as tetraspanins, growth factor receptors, ECM proteins, and matrix proteases or their receptors [12, 22].

Therefore, integrins provide a major mechanism for connecting the intracellular and extracellular compartments, transmitting mechanochemical information. Cell adhesion to the ECM transmits information via integrin receptors that regulates intracellular signalling via outside-in signalling, while conversely, intracellular signals can induce changes in integrin conformation and activation that alter its ligand-binding activity in an inside-out signalling. Integrin engagement with matrix can also affect integrin activation, providing bidirectional crosstalk between inside-out and outside-in signalling [12].

Integrins specify protein recruitment to adhesion complexes and thereby selectively link matrix adhesions to various downstream signalling cascades that control cytoskeletal organization, gene regulation, and diverse cellular processes and functions [12, 21]. The clustering of integrins activates non-receptor tyrosine kinases such as focal adhesion kinase (FAK) and Src, and regulates members of the protein kinase C (PKC) family, PI3-kinase and RPTP α [12, 21]. They can influence the organization of the cytoskeleton by regulating the activity of the Rho family of small GTPases [23]. Cell-ECM adhesions and the actin cytoskeleton are involved in mechanotransduction [24].

1.2.2. Cell migration in the ECM

Cell migration is crucial in diverse biological processes as embryonic development, inflammatory response, wound repair, and tumour formation and metastasis, occurring both in normal and pathological states [25-27]. Cells generally migrated in response to a gradient of a specific cue [27]. The nature of that cue often defines the type of directed migration. By chemotaxis, cells move toward a positive gradient of soluble stimulants (chemokines and growth factors); while by haptotaxis, cells respond to a gradient of substrate-bound molecules [28]. Chemokinesis is another type of cell movement, also induced by soluble molecules of the surrounding environment, but the elicited response is a random, non-oriented taxis that provides more scanning of the environment than migration between two distinct points.

Cells can undergo specific migration patterns as single or collective migrating cells. Single migrating cells can employ different migration mechanisms, namely mesenchymal or ameboid modes, where different integrin profiles are expressed. Different cell morphologies, migration modes, and transitions between those are illustrated in Fig. 5 [5, 29].

The mesenchymal migration mode is the most well studied [24, 30, 31], where responding to mechanical or chemical cues, cells migrate following the complete five-step sequence proposed by different authors, which encompasses: 1) protrusion of the leading edge, 2) cell-matrix interaction and formation of focal contacts, 3) recruitment of surface proteases to ECM contacts and focalized proteolysis; 4) cell contraction, and finally 5) detachment of the trailing edge [5, 24, 27, 32]. Ameboid migration, typically observed in leukocytes, hematopoietic stem cells and some tumour cells, does not involve integrins but other weaker adhesions instead. Since cells are extremely deformable and can squeeze through the ECM, this mechanism allows them to travel rapidly through different tissues environments [24]. This ameboid migration mode includes cell shape change and amoeboid propulsion, formation of lateral footholds or cytoplasmic streaming [33, 34]. Furthermore, cells can undergo a phenomenon designated by epithelial-mesenchymal transition (EMT) and its reverse MET, transiting between mesenchymal and ameboid migration modes, which is a strategy to maintain tissue homeostasis being also involved in tumour cell migration [35, 36].

There are different mechanisms by which cells overcome the physical barriers imposed by the ECM, such as shape change, contact-dependent matrix remodeling, and proteolysis by secreted proteases. The mechanism of shape change induces reversible alterations of the ECM structure, by traction and bending of fibers, and cells follow the path of least resistance, adapting morphologically to the ECM environment [32], whereas the two latter processes cause irreversible changes of both matrix protein structure and overall matrix architecture [5].

As proposed by different authors [5, 29], differences in matrix architecture, as well as stiffness, govern the ability of cells to migrate through the ECM, with or without the assistance of proteolytic degradation, as illustrated in Fig. 6.

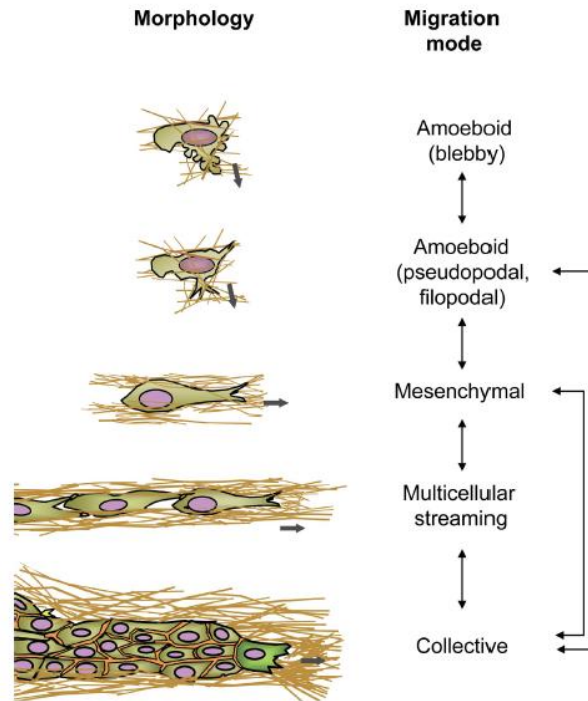


Figure 5. Cell morphologies, migration modes, and transitions. Cells may adopt rounded or spindle-shaped morphologies and migrate through individual, loosely connected, or collective patterns. Each migration mode is governed by a set of specific molecular mechanisms ([29]).

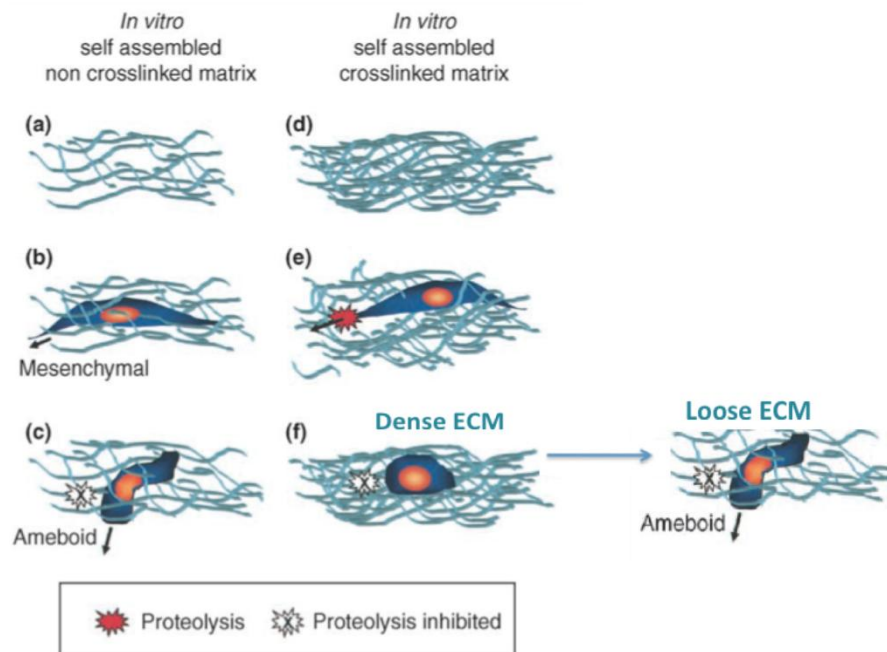


Figure 6. Schematic representation of cell movement through collagen-based matrices as a function of matrix architecture (adapted from [5]).

Based on the use of collagen-based ECM models, the authors discuss some mechanisms of cell movement through the ECM [5]. In non-crosslinked matrices (Fig. 6a) cells show typical mesenchymal migration along the fibers (Fig. 6b). However, in the presence of protease inhibitors (Fig. 6c), cells switch to an ameboid migration mode squeezing through the interstitial spaces. Highly cross-linked dense ECMs (Fig. 6d), limit cell movement as a result of the small spaces between fibers. In this case, ECM-degradation is required for cell migration (Fig. 6e). Protease inhibition causes cell rounding, and if the interstitial spaces are too small, migration cannot proceed (Fig. 6f). However, if there are some gaps within the matrix or it becomes less dense, those voids are detected by cells and may eventually permit non-proteolytic migration within the matrix.

Through the mechanism of proteolysis, the ECM can be remodeled through enzymatic cleavage of its components by several families of proteases, including matrix metalloproteinases (MMPs) [7]. MMPs modulate both cell-cell and cell-ECM interactions by direct proteolytic degradation or activation of cell surface proteases and ECM proteins [37]. MMP substrates include peptide growth factors, tyrosine kinase receptors, cell-adhesion molecules, cytokines and chemokines, as well as other MMPs and unrelated proteases.

2. Hydrogels that mimic the natural ECM

2.1. Types of ECM-like hydrogels

Hydrogels are an important class of biomaterials that capture many characteristics of the natural ECM. These features include a high content of water and a good permeability, the latter allowing easy transport of oxygen, nutrients, waste or even soluble factors [38]. Hydrogels are crosslinked hydrophilic polymeric chains forming reticulated structures [39], resembled in many aspects the GAGs present in the natural ECMs. Some types of hydrogels are of particular interest as they can be formed under mild conditions and can be easily modified. There is also the possibility to design permissive hydrogels, which provide a three-dimensional (3D) microenvironment but lack the interactivity with cells, or promoting hydrogels, that intend to have a bioactive role and direct interaction with cells through their surface receptors [38, 40]. Permissive

hydrogels are frequently used as “inert” backgrounds that are converted into promoting hydrogels through chemical derivatization (e.g., with peptide epitopes or adhesion proteins), which affords a means to control cell–matrix interactions in a highly controlled manner [13, 41-64].

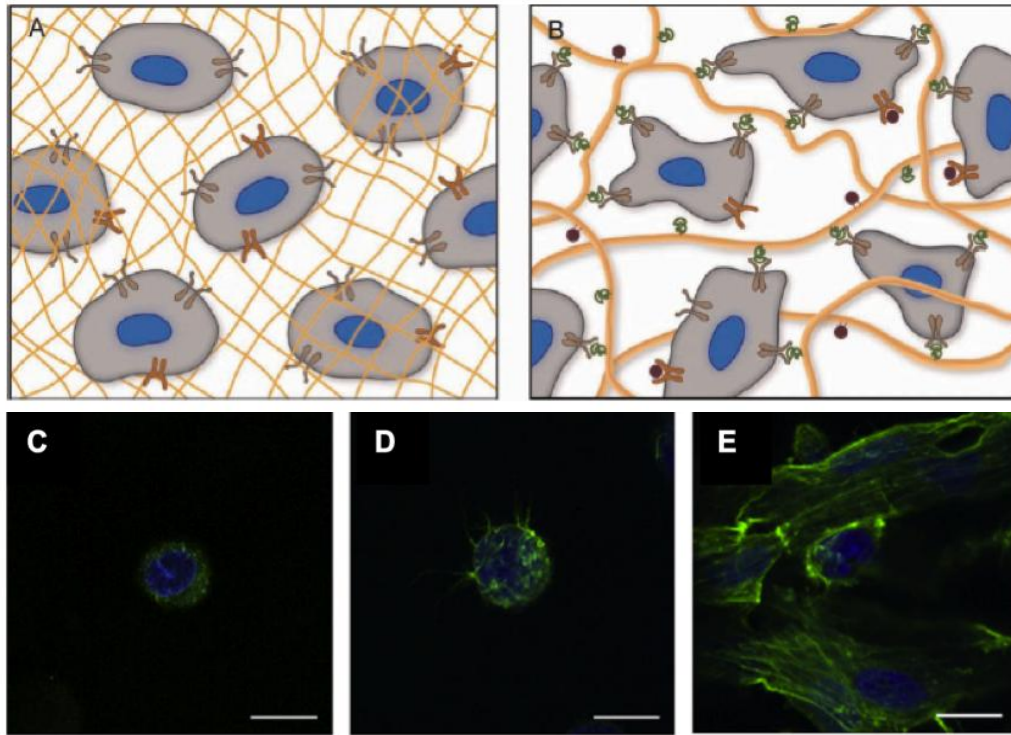


Figure 7. Schematic representation of **(A)** permissive and **(B)** promoting hydrogels. Mesenchymal stem cells encapsulated in **(C)** unmodified alginate, a permissive hydrogel, and **(D)** RGD-alginate or **(E)** a RGD/PVGLIG-alginate, two promoting hydrogels (adapted from [38] and [48]).

As illustrated in Fig.7A, permissive hydrogels provide a 3D environment for culturing cells while maintaining their viability, but don't promote specific cell-matrix interactions. On the other hand, promoting hydrogels (Fig.7B) present specific signals, for example integrin-binding sites (green) and ECM (yellow fibers) bounded growth factors, and are able to direct cell behavior [38]. Fig. 7C-E, represent images of mesenchymal stem cells (MSCs) cultured under 3D conditions in (C) unmodified alginate, (D) alginate modified with a cell-adhesive peptide (RGD), and (E) alginate modified with both RGD and a protease-sensitive peptide (PVGLIG). These images illustrate how a permissive hydrogel (unmodified alginate) can be converted by chemical modification with peptides in cell-instructive (promoting) hydrogels (RGD- and RGD/PVGLIG-alginate) [48].

Hydrogels can be both of synthetic or natural origin. Some common examples of synthetic hydrogels, include poly(ethylene glycol) (PEG), Poly (glycolic acid) (PGA), Poly (lactic acid) (PLA), Poly (lactic-co-glycolic acid) (PLGA), poly (ε-caprolactone) (PCL) and poly (propylene fumarate) (PPF) [38, 53, 65]. PEG hydrogels are amongst the most extensively used 3D matrices, and are perhaps one of the best-studied examples of specifically designed cell-instructive hydrogels. Their non-cytotoxicity, high versatility and ease of modification have become major advantages in ECM mimicking strategies. Different modifications have been tested, including chemical grafting of cell-adhesion ligands, use of cross-linking moieties sensitive to specific classes of proteases, use of PEG macromers with various molecular weights and number of arms, and chemical immobilization of ECM growth factors [66-69]. For example, Raeber and co-workers designed preteolytically degradable PEG-based hydrogels, modified with RGD and protease-sensitive peptides to promote cell-matrix adhesion and cell-driven matrix degradation [67]. West and co-workers showed that changes on the size and amino-acid sequence of protease-sensitive oligopeptide domains, accounts for controlled protease selectivity [66]. Seliktar and colleagues, modified PEG hydrogels with an engineered variant of vascular endothelial growth factor (VEGF), sensitive to MMP-2 proteolysis, that can be activated by migrating endothelial cells, upon addition of TGF-β1, similar to what occurs in the natural ECM [69]. Many other very interesting examples on the use of synthetic hydrogels as ECM-analogues were found in the literature, but a detailed analysis of these materials was out of the scope of this study.

Natural hydrogels are made of different ECM counterparts, and some examples include collagen, fibrin, hyaluronic acid, and also solubilised ECMs such as Matrigel. Other hydrogels from natural sources include alginate, chitosan or silk fibrils [38].

Alginates, the hydrogel-forming class of polymers selected for the present study, are extremely versatile biomaterials likely to find increasing utility in a wide range of medical applications, namely as vehicles for cell/drug delivery and as matrices for TE [70]. They are naturally derived polysaccharides, composed of (1-4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers, varying in amount and sequential distribution along the polymer chain. The alginate molecule is a block copolymer of GG, GM and MM sequences. Divalent cations such as Ca²⁺ bind the GG regions of adjacent alginate chains, resulting in ionic crosslinking, and the formation of a hydrogel from aqueous alginate solutions. Crosslinking may be promoted *in situ*, under mild conditions, and a variety of techniques can be used to control gelling and mechanical properties of the resulting hydrogels [70]. When used for

3D cell culture, unmodified alginate formulations present an environment completely devoided of cell-matrix interactions. As such, cell viability in unmodified alginate hydrogels is generally very low, depending on the cell type. This can be easily overcome through chemical modification with cell-adhesion peptides, such as Arg-Gly-Asp (RGD) [47, 48, 59, 71, 72]. Additional covalent modifications of alginates provide further control over receptor-mediated cell response. In a recent study [48], alginate has been modified not only with RGD but also with MMP-sensitive peptides, to mimic two key features of the natural ECM: cell adhesion and proteolytic degradation. A schematic representation of bifunctional alginate hydrogels and their interaction with entrapped cells is depicted in Fig. 8. Soluble Na-alginate chains (Fig. 8A) are chemically modified with cell-adhesion (RGD) and MMP-sensitive (PVGLIG) peptides (Fig. 8B). The latter are expected to establish links between alginate chains. Calcium is added as the main crosslinking agent (Fig. 8C), promoting the formation of a 3-D network via interchain ionic binding. The mild crosslinking process can be performed in the presence of cells that become entrapped within the 3D matrix (Fig. 8D). The RGD motifs present in the matrix provide for integrin-mediated cell adhesion, while PVGLIG residues render the matrix partially degradable by cell-driven local proteolysis (Fig. 8E). Cellular proteases cleave the MMP-sensitive peptide, overcoming biophysical hydrogel resistance (Fig. 8F). Entrapped cells are able to dynamically interact with the hydrogel matrix and neighbouring cells (Fig. 8G).

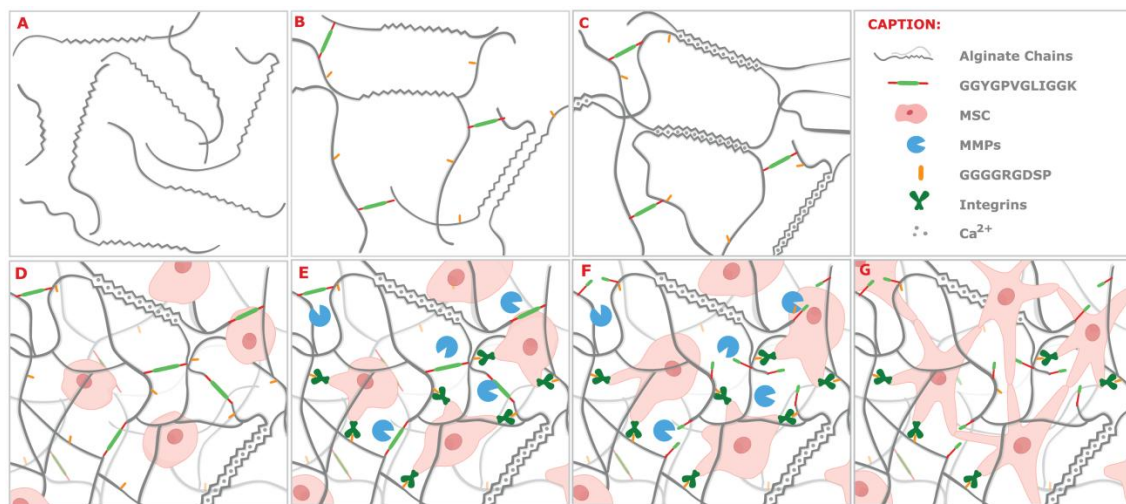


Figure 8. Schematic representation of the strategy used to prepare bifunctional-alginate, and establishment of cell-hydrogel interactions after cell entrapment within the modified-alginate matrices ([48]).

Moreover, other studies have highlighted the usage of alginate hydrogels as ECM-like matrices and its versatility. Studies of Evangelista and colleagues with MC3T3-E1 pre-

osteoblast cultured within RGD-alginate microspheres have shown that cells maintained high viability for up to 24 days in culture, and underwent terminal differentiation into osteoblasts [47]. Using the same alginate encapsulation scheme, the work of Bidarra et al showed that this system was able to maintain a hMSC viability of 90% for 21 days, and was also efficient in promoting osteogenic differentiation upon induction, with ALP activity at day 21 being considerably higher in 3D cultures compared with monolayer cultures [42]. Studies from Alsberg et al indicate that the degradation rate of alginate hydrogel beads used for transplanting bone-forming cells dramatically influences subsequent bone formation [73]. Furthermore, HUVECs cultured in 1wt% RGD-alginate matrices were able to maintain high viability (80%) after 48h, whereas viability in unmodified alginate decreased to 40%, enabling cells to spread and form a cellular network and also to migrate from the matrices [41].

2.2. Applications of ECM-like hydrogels

An emerging area of interest is the design of hydrogels for regenerative therapeutic applications, namely as injectable cell-delivery vehicles [41, 51, 61, 64, 74-77]. Because of their very interesting properties, numerous groups are also exploring the development of hydrogel-like matrices as advanced 3D-cell culture models for in vitro studies [38, 52, 53, 59-61, 78] Fig 9.

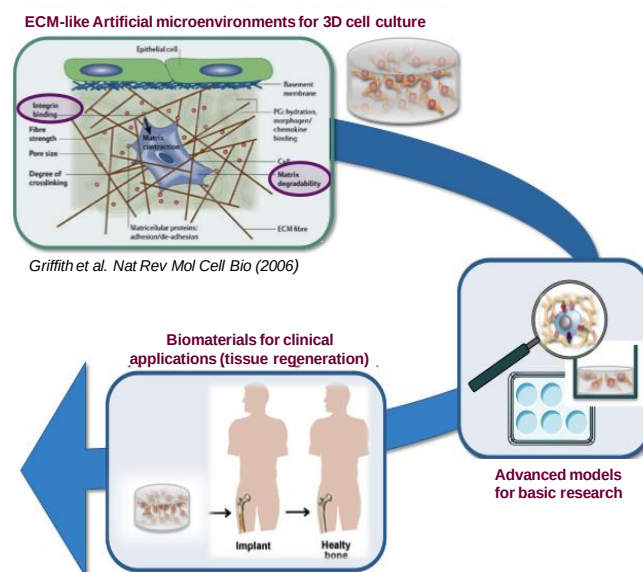


Figure 9. Hydrogels are being increasingly applied for the design of ECM-like artificial microenvironments for cell entrapment. These systems may find applicability both as advanced 3D models for basic research, or as (injectable) biomaterials for regenerative medicine applications.

2.2.1. Applications in regenerative medicine

Cell transplantation via direct injection in injured sites is a promising regenerative therapeutic strategy. However, retention of the transplanted cells at the injection sites remains a central issue following injection of dissociated cells. In fact, cell death; leakage and migration may occur when cells are delivered as a single cell suspension, resulting in poor survival and tissue integration. In alternative, by delivering cells in biomaterial scaffolds some of these limitations may be overcome [79, 80]. Injectable biomaterials, commonly consisting of hydrogels, present additional advantages as they provide a means to fill tissue-defects of different shapes and sizes using minimally invasive surgical techniques, which provide less discomfort to patients [61, 64].

In ideal circumstances such material should be: (1) easy to manipulate and implant through minimally invasive surgery; (2) crosslink under mild conditions to allow matrix formation in the presence of cells without affecting their viability; (3) form highly hydrated networks with adequate stiffness mimicking the natural ECM; (4) enable cell migration and matrix remodelling; (5) modulate cell response by incorporating biochemical cues; (6) act as a vehicle to deliver cells into the implant site; and (7) allow the easy combination of the desired functionalities to attain tailored biospecificity [81] [51, 61, 64, 74-77]. The design of these artificial, yet biologically meaningful, ECM-analogues represents thus a significant engineering challenge.

2.2.2. Application of ECM-like hydrogels as substrates for 3D cell culture

Traditionally, investigation of tissues physiology and pathology has been performed in vitro using two-dimensional (2D) flat plastic dishes (eg. culture plates and flasks) or substrates, where cells grow in monolayers and are in direct contact with the culture medium. These experiments brought the basis of all our understanding of such phenomena. However, a three-dimensional and more realistic environment should give more reliable results, since, as previously seen, the natural substrate for cells is the ECM, a 3D microenvironment [7, 82-84].

Petersen, Tanaka and respective co-workers' demonstrated that cells do not behave in a natural fashion when they are excised from the 3D native tissue and cultured in a 2D

substrate, resulting in considerable discrepant behaviours between in vitro and in vivo [38]. Regarding those two environments, differences can also be observed in many cellular processes, since a cell cultured in a 2D substrate does not encounter the same restrictions nor the same connections and interactions than within a 3D environment [38].

Fig. 10 illustrates how cells may in fact experience drastically different environments between 2D and 3D culture. As depicted, neural cells cultured in monolayer (Fig. 10A) are constrained to extend processes in the plane. However, when cultured within hydrolytically PEG based hydrogels (Fig. 10B) the same cells form neurospheres and extend processes isotropically in three dimensions. In contrast, (Fig. 10C) mesenchymal stem cells are able to spread in 2D RGD-alginate films, (Fig. 10D) but adopt a round-shape in 3D due to the physical barrier imposed by the matrix.

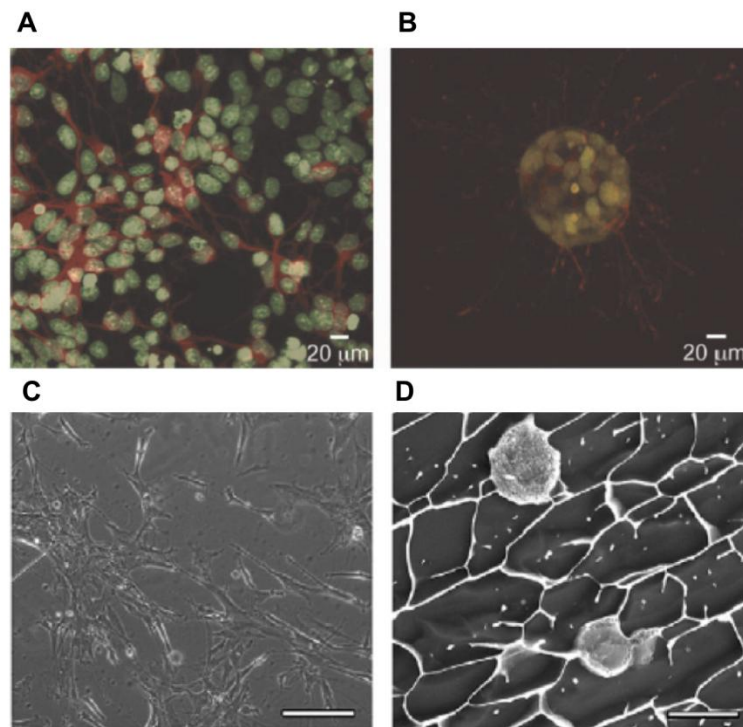


Figure 10. 2D and 3D substrates present radically different environments to cultured cells. Neural cells are constrained to extend processes in 2D (A), but form neurospheres and extend processes in 3D. MSCs attach and spread on 2D RGD-alginate substrates (C), but adopt a round-shape in 3D matrices (D).

In another example, studies of Gruber and colleagues comparing cells cultured in a monolayer versus 3D cultured disc cells when grown in 3D culture, human disc cells form multi-celled colonies instead of the flattened, spindle shape phenotype 2D cultured cells [85].

As a consequence of the remarkable different environments presented to cells in 2D vs 3D-cultures, other cellular activities such as migration, proliferation and differentiation are also affected [86, 87].

In conclusion, although 2D systems provide a useful tool to access cellular behaviour, they are too simplistic models that do not embrace the complexity of the natural ECM and tissues, and often fail to reproduce the *in vivo* situation [88]. To overcome these limitations, 3D matrices are emerging as more suitable models, which in some situations may eventually bridge the gap between *in vivo* and traditional monolayer cultures [89].

2.2.3. Relevance of dynamic vs static culture conditions in 3D cultures

The paradigm shift from 2D to 3D settings is underway and progressing rapidly. Implementation of effective 3D approaches will however require the development of improved cell culture systems. Traditional static culture techniques using flat plastic dishes (eg. culture plates and flasks) are generally adequate for working under 2D conditions, as cells in monolayers are in direct contact with the culture medium. Yet, these are often inadequate for culturing cells under 3D conditions, where there are mass transfer limitations and simple diffusion is not sufficient to ensure an efficient oxygenation, nutrition and waste removal [82, 90]. This becomes increasingly important as samples become thicker, affecting mostly cells at the core. Within a 3D matrix, cells at different locations and depths frequently become heterogeneously distributed and/or exhibit non-comparable growth kinetics, viability and functional characteristics, which introduces significant and potentially confounding variables to the model. Moreover, the metabolic requirements of the cells or tissues may ultimately be unsustainable for long culture periods. To overcome such limitations, 3D cell-material constructs should ideally be cultured under dynamic conditions [90-92].

Bioreactors can be designed in order to establish spatially uniform cell distributions on 3D scaffolds, maintain desired concentrations of gases and nutrients in the culture medium, enhance diffusion of biochemical factors to the construct interior, as well as promote continuous removal of by-products of cellular metabolism [46, 93-96]. Different types of dynamic systems have been proposed, from simple stirred systems, as spinner-flasks, to perfusion chambers and to more complex bioreactors with oxygen and nutrient consumption/metabolites production control. Diverse variations include the

fixed-bed bioreactor, making use of a basket in a stirred vessel, the use of non-mechanical agitation as air-lift or fluidized bed bioreactors and diverse configurations to support samples of different shapes and sizes [97].

Spinner flasks, which were used in this work, are the simplest stirred systems that can be directly scaled-up from static cultures. They have long been used as *in vitro* systems for cell culture, particularly for stem cells expansion, being appropriate for the efficient growth of suspension cultures as well as for the growth of attachment-dependent cells seeded on microcarriers or other types of scaffolds. Spinner flasks generate convective forces through a magnetic stirrer bar that allows continuous mixing of the culture media around 3D constructs. They thus provide high mass and hydrodynamic shear transfer levels and minimize metabolites and oxygen concentration gradients [96]. However, the turbulent flow conditions created by the impeller may render these systems inappropriate for the culture of cells in fragile, free-floating samples, such as hydrogels, which can be mechanically damaged. To overcome this limitation, a novel sample holding unit, consisting of a multi-compartment chamber adaptable to standard spinner-flasks, was designed and used in a set of studies of the present work for the 3D-culture of hMSC under dynamic conditions.

3. The use of MSC in regenerative applications

Tissue engineering (TE) is an interdisciplinary field that involves cell biology, materials science, bioreactor engineering, and clinical research. The aiming of TE strategies is to regenerate damaged tissues, instead of replacing them, by developing biological substitutes that restore, maintain or improve tissue function [58, 98]. The integrated usage of cells, chemical or physical signals and the scaffold is a template for tissue formation by allowing cells to migrate, adhere, and produce tissue [58].

The usage of mesenchymal stem cells (MSC) due to its potential of self-renewal, differentiation into multiple lineages such as adipogenic, chondrogenic and osteogenic (Fig. 11), their homing capacity and immune-modulatory properties [99], has been of rising interest as versatile and a promising source for injured tissues [56]. Adult MSC obtained from bone marrow contribute for the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma [100]. These are fibroblastic, adherent cells and the possibility to expand these cells in order to

obtain sufficient amount for the demands of clinical use, as well as the possibility to induce specific cell differentiation is of high interest.

Their commitment and efficient differentiation is dependent of several factors, such as basal nutrients, cell density, spatial organization, mechanical forces and growth factors and cytokines [100]. In physiological environment, cells reside in a niche where its stemness is kept and upon injury, they are able to proliferate, migrate to the location and differentiate into the specific cell lineage [101, 102]. Within the skeletal tissues, MSC contribute to the normal remodeling and repair processes by providing the pool of osteoblasts necessary to form the mineralized matrix of bone. In vivo, it was already demonstrated that MSC transplantation in small and large animal models increased bone production and tissue stiffness [103].

In vitro, MSCs are able to differentiate into multiple lineages upon induction. For example, MSCs differentiate into osteoblasts in the presence of β -glycerophosphate, 2-phospho-L-ascorbic acid and dexametasone [104], adopting a typical morphology and expressing several bone phenotypic markers, including a mineralized matrix [104, 105].

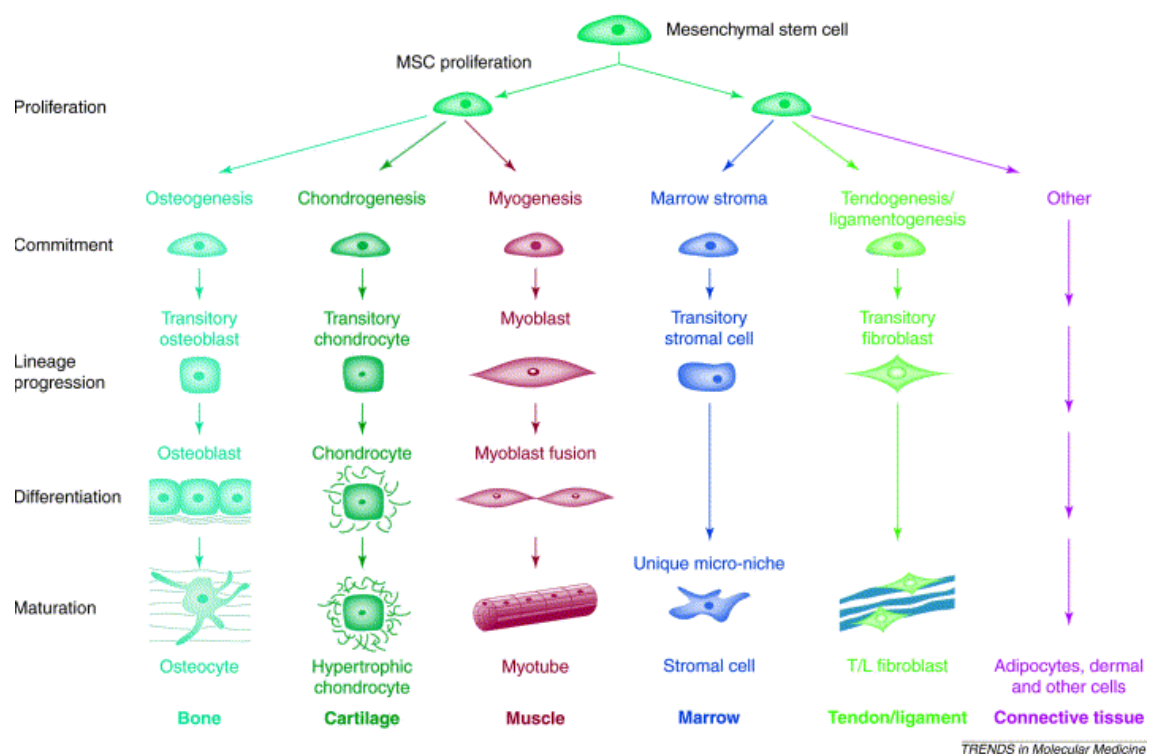


Figure 11. Differentiation of MSC into mesodermal lineages: bone, cartilage, muscle, stromal cells of bone marrow, tendon/ligament, adipocytes and connective tissue ([99]).

MSCs have a considerable therapeutic capacity in the repair of osteochondrogenic defects, diseases/disorders including: osteogenesis imperfecta, rheumatoid arthritis, osteoarthritis, bone fracture, craniosynostosis in pre-clinical and some clinical settings [103]. Furthermore, the therapeutic potential of MSCs is extended to other applications: ongoing clinical trials using MSCs include ischemic stroke, multiple sclerosis, acute leukemia, graft-versus-host disease, critical limb ischemia and articular cartilage among others [106]. However, despite the significant potential, some challenges in MSC's clinical applications include low survival of transplanted cells, limited targeting capabilities, and low grafting efficiency. Thus, basic research plays an important role in uncovering the dynamic interplay between stem cells and their microenvironments.

The final and ultimate goal of the clinical applicability of stem cell-based therapeutics and tissue engineering requires, therefore the fundamental understanding of their behavior under 3D biomimetic conditions, fully exploiting this systems as *in vitro* cell culture systems and the development of efficient technologies for high-throughput production of progenitors and therapeutic cells suitable for on-demand transplantation [107].

4. Aim and structure of the thesis

Cell-instructive hydrogels, including peptide-modified alginates, have been finding increasing applications in the Regenerative Medicine and Cell Research fields. They are conceptually attractive, as they mimic many key properties of natural ECMs and provide adequate microenvironments for embedding cells under real 3D conditions.

A wide range of factors modulates cells response upon 3D-culture within hydrogel-like artificial ECMs. These include several matrix-related features, such as the type and density of matrix-bound biochemical cues, the structural and mechanical properties (e.g. porosity and stiffness) of the matrix, and also its biodegradability. A number of other factors, such as cell type and density, culture media, culture conditions (dynamic vs. static), etc., also have an important influence. Therefore, studying and understanding the behavior of cells cultured within such complex, yet biologically meaningful, microenvironments is far from being simple.

The aim of this work was to characterize the behavior of MSCs cultured under 3D conditions within alginate matrices, previously developed by our group for bone regeneration applications, looking in particular to the effect of cell density (low-to-high) and culture conditions. Different techniques were used to provide complementary information on cell behavior under different perspectives, namely in terms of cell viability, metabolic activity, morphology and distribution within the matrix, proliferation, production of endogenous ECM and ability to undergo osteogenic differentiation. Some of the methodologies were optimized and for the first time implemented.

Alginate hydrogels incorporating RGD peptides (to enhance specific matrix–integrin interactions), and with the ability to crosslink *in situ* in the presence of calcium, were selected as the 3D matrices. These hydrogels do not contain protease-sensitive moieties and for that reason cannot be enzymatically degraded by cells. MSC's response to such microenvironments is not yet fully understood.

This introductory chapter provides an overview of some concepts with relevance for the present investigation. In the next chapter, the main results are described and discussed. Some complementary analyses are provided in Annexes I and II. Finally, the last chapter presents the general conclusions and possible directions for future research. These future studies will be very important to address several new and important questions that arose along this work.

II. MATERIALS AND METHODS

II. Materials and Methods

1. Preparation and characterization of RGD-alginate hydrogels

1.1. Preparation of peptide-grafted alginate

Ultra pure alginate with high content of guluronic vs mannuronic acid units (>60%, NovaMatrix, FMC Biopolymers) and different molecular weights were used. High Molecular Weight Alginate (HMW; M_w in the range of 75 to 220 kDa) was oxidized and modified as described bellow, whereas Low Molecular Weight alginate (LMW; M_w <75 kDa) was used as received.

For the oxidation process, sodium periodate (Sigma-Aldrich) was added to a 1 wt.% HMW alginate solution in deionized water, to yield a 1% oxidation per mol of alginate [44], and stirred for 19 h in the dark, followed by the addition of an equimolar amount of ethylene glycol (Merck) to stop the reaction. The solution was stirred for 1 h and 30 min at room temperature (RT), and then dialyzed against deionized water for 3 days (MWCO 1000 membrane, Spectrum Lab). The oxidized HMW alginate was lyophilized and stored at -20°C until further use.

The cell-adhesion peptide sequence (glycine)₄-arginine-glycine-aspartic acid-serine-proline (G4RGDSP, GenScript), hereafter abbreviated as RGD, was coupled to oxidized HMW alginate using carbodiimide chemistry, as described in detail in previous studies [47]. Briefly, a 1 wt.% alginate solution in 0.1 M MES buffer (Sigma) was prepared and stirred overnight (ON). Sulfo-NHS (Thermo Scientific) and EDC (Sigma) at 1:2 molar ratio were then sequentially added and stirred for 15 min, to activate polymer COOH- groups before addition of the RGD peptide (17 mg/g alginate). After stirring for 24 h, the reaction was quenched with Hydroxylamine (NH₂OH, Sigma). Non-reacted species were separated by dialysis (MWCO 3500 membrane, Spectrum Lab) against solutions of decreasing concentration of Sodium Chloride (NaCl, Merck) and finally in deionized water. The recovered solution was lyophilized and the RGD-alginate was stored at -20°C until further use.

1.2. Quantification of RGD peptides grafted to alginate

UV absorbance spectra of soluble RGD peptide (1 mg/mL) and RGD-alginate samples (1 wt.%) were acquired in the 200–300 nm region, using a 384-well UV transparent

microplate (Greiner) in a UV spectrophotometer (PowerWave XS, BioTek). The same solutions were also assayed using the BCA Protein Quantification Kit (Pierce), according to the manufacturer's instructions. A set of soluble RGD solutions at concentrations ranging from 0 to 1 mg/mL prepared in 1 wt.% unmodified alginate were used as standards. The efficiency of the coupling process was calculated as: efficiency (%) = (grafted peptide/input peptide) x 100. All measurements were made using at least n = 3 replicates.

1.3. Bioactivity of immobilized RGD: hMSC 2D-culture on alginate films

To probe the cell-adhesive properties of the immobilized RGD peptide, a 2D study was carried out using alginate hydrogel films. Solutions of unmodified and RGD-grafted alginate at 2 wt.% were spin-coated (Spin-Coater, SCS G3P-8) on plastic coverslips (Sarstedt) previously coated with poly-D-lysine (Sigma). Immediately after alginate deposition (120 μ L, 5000 rpm, 30 s), crosslinking was promoted by immersion in a 0.1 M CaCl₂ (Sigma) solution. After being thoroughly rinsed with sterile tris-buffered saline (TBS, 50 mM Tris in 150 mM NaCl, pH 7.4, Sigma), films were seeded with human mesenchymal stem cells (hMSCs) at 2×10^4 cells/cm² and incubated at 37 °C. More details on the hMSCs used in this study are provided in section 2.1. After incubation ON, cells on the top of unmodified and RGD-modified alginate films were imaged by optical microscopy.

1.4. Preparation of RGD-alginate hydrogels by in situ crosslinking

To prepare in situ crosslinking hydrogel matrices an internal gelation strategy adapted from Kuo *et al.* was used [51, 108]. The alginate gel precursor solution was a 50:50 v/v binary mixture of RGD-grafted oxidized HMW alginate and unmodified LMW alginate, prepared at a total alginate concentration of 2 wt.% in 0.9 wt.% NaCl. This formulation (hereafter designated RGD-alginate) has been previously optimized by our team [42, 47]. The solution was sterile-filtered (0.22 μ m) and thoroughly mixed with an aqueous suspension of CaCO₃ (Fluka) at a CaCO₃/COOH molar ratio of 1.6. To trigger gel formation, a fresh solution of glucone delta-lactone (GDL, Sigma) was added. The CaCO₃/GDL molar ratio was set at 0.125. The gelation time was 30 min. The contour-adaptability of alginate hydrogels prepared using this method, was first evaluated by casting the hydrogels in different molds. For the subsequent cell culture studies hydrogels were prepared as described in detail below.

2. hMSC 3D-culture within alginate hydrogels

2.1. Routine culture of human mesenchymal stem cells (hMSC)

hMSC were purchased from Lonza (PT-2501, Lot. No. 6F4392, obtained from 19 years-old male donor). Cells were routinely cultured in a basal medium (hereafter designated as BM) composed of low-glucose Dulbecco's Modified Eagle Medium with glutamax (DMEM, Gibco) supplemented with 10% v/v Fetal Bovine Serum (FBS, MSC-qualified, Gibco) and 1% v/v Penicillin/Streptomycin (Pen/Strep, Gibco). Cultures were maintained at 37°C under a humidified atmosphere of 5% v/v CO₂ in air, with culture medium being changed twice a week, and were trypsinized when reaching 70% confluence. In the different studies hMSC in passage 7 to 9 were used, except for differentiation studies, in which all experiments were performed with cells at passage 7.

2.2. Preparation of hMSC-loaded 3D alginate matrices

hMSCs at different densities (2, 5, 7.5 and 15x10⁶ cells/mL gel, designated as D1, D2, D3 and D4 respectively), were mixed with sterile alginate gel precursor solutions and crosslinking agents and carefully homogenized. *In situ* crosslinking was promoted directly in a culture plate for suspension cells (Greiner) by placing 20 µL of this mixture into each well. For the set of “static vs dynamic-culture” studies, the mixture was loaded into a 1mL syringe (4 mm inner diameter, Terumo) to form a cylindrical hydrogel that was afterwards sliced with a razor blade into small discs of 3 mm high. In both cases, gelation was allowed to proceed for 30 min at 37°C. For the majority of studies, hMSC-loaded matrices (20 µL) were then transferred to pHEMA-treated [109] 24-well suspension-culture plates and the appropriate medium (described in section 2.3) was added. In “static vs dynamic-culture” studies (Annex I), the 3D matrices (4 mm diameter x 3 mm high, approx. 40 µL) were cultured under dynamic conditions as described in section 2.4. During culture, cells were maintained at 37°C under a humidified atmosphere of 5% v/v CO₂ in air.

2.3. hMSC 3D-culture under basal vs osteogenic conditions

Cell-loaded hydrogel 3D matrices were cultured in BM unless otherwise stated. For different studies, cells cultured in 2D tissue-culture treated polystyrene (TCPS) culture plates in BM were used as a control. In an individual set of studies (Annex II) the use of high (4.5 g/L) vs low-glucose (1g/L) DMEM (HGluc or LGluc) and different feeding

regimes were tested. In other studies, osteogenic differentiation was induced by culturing cells in an osteogenic medium (OM) composed by low-glucose DMEM with 10%v/v FBS (pre-selected batch from PAA), 1%v/v Pen/Strep, 100 nM dexamethasone (Sigma), 10mM β -glycerophosphate (Sigma) and 0.05 mM 2-phospho-L-ascorbic acid (Fluka) [104], for up to 3 weeks. In these differentiation studies, cells cultured in BM were used as a control.

2.4. hMSC 3D-culture under static vs dynamic conditions

Cell-loaded hydrogels discs were cultured under dynamic culture conditions in a sample holding device (under patent application) adapted to a spinner flask (V=25mL, Bellco Biotechnologies). Different stirring velocities (15 to 100 rpm) were tested. As a control, similar samples were cultured in pHEMA-treated 6-well TCPS plates (hereafter designated as static conditions). The ratio of medium volume per disc was maintained at 4 mL/disc in both, static and dynamic conditions.

3. Analysis of hMSC behaviour in 3D-culture

At various time points, different techniques were used to assess hMSCs behavior in 3D alginate matrices. The assays were either performed *in situ* (i.e. in cells within the hydrogel 3D matrix) or after releasing cells from the matrices. In the latter, cell recovery was performed by dissolving the hydrogels in a 0.25 wt.% Trypsin (Sigma)/50mM Ethylenediaminetetracetic acid (EDTA, Sigma) solution for 5-10 min, which yielded a homogenous cell suspension. The different assays are described in detail below.

3.1. Structural analysis of hMSC-loaded alginate hydrogels

One hour after cell entrapment, structural information concerning the arrangement of cells within the hydrogels was screened using cryo-scanning electron microscopy (cryo-SEM). Samples were placed between miniature rivets on a vacuum transfer rod, slam-frozen until -210 °C in N₂ slush and transferred to the cryostat chamber (-30 °C). The top rivet was flicked off to produce a fractured surface, which was sublimed at -95 °C and coated with gold/palladium. Samples were finally transferred to the cryo-SEM microscope chamber (JEOL JSM 6301F/Oxford INCA Energy 350/ Gatan Alto 2500) for analysis.

Ultra-structural analysis of entrapped hMSC was analyzed under transmission electron microscopy (TEM) at day 7. Matrices were washed in TBS-Ca (TBS with 7.5 mM CaCl_2) and fixed in 2 wt.% glutaraldehyde/ 0.1 M sodium cacodylate (pH 7.4) for 1 h. After washing twice in 0.1 M sodium cacodylate buffer for 10 min the discs were fixed in 2% v/v osmium tetroxide in 0.1 M sodium cacodylate for 1 h followed by 2 times washing step in 0.1 M sodium cacodylate buffer for 10 min. Samples were dehydrated in a gradient series of ethanol solutions for 10 min each (70%, 80%, 90% and 100% v/v) and finally incubated for 10 min in a propylene oxide solution. Inclusion in EPON resin was performed by immersion of alginate matrices in a gradually increasing series of propylene oxide to EPON as follows: 2:1, 1:1, 1:2 and 0:1 for 30 min each. At the end, inclusion of alginate discs in EPON resin was performed in a silicon mold. EPON polymerization took place at 60 °C for 24 h. Sections with 70 nm thickness were prepared using a diamond knife (Diatome) and were recovered to 200 mesh Cu-grids. Staining of sections with 2 wt.% uranyl acetate and saturated lead citrate solution, for 7 min each, was performed before observation. Visualization was carried out at 50 kV in a Zeiss EM 902A microscope. For each time point and condition, four different microspheres were analyzed and two different grids with two sections each were prepared and observed.

3.2. Cell quantification and viability analysis

Cell viability was assessed using the Trypan Blue exclusion assay in recovered cells or the Live/Dead Assay (Invitrogen) in whole-mounts to provide information about cell distribution in 3D.

After hydrogel dissolution, 10 μL of cell suspension were mixed with 10 μL of 0.4% v/v Trypan Blue staining solution (TB, Sigma), and live/total cells were counted with a Neubauer chamber. Cell Viability was quantified as the percentage of cells excluding trypan blue in relation to the total number of cells. Three replicates per condition were analyzed.

Live/Dead assay was performed according to the manufacturer's instructions. Briefly, cell-loaded matrices were washed three times with serum-free DMEM without phenol red (Gibco), to remove/dilute esterases, and then incubated with 1 μM Calcein AM and 2.5 μM Ethidium Homodimer-1 (EthD-1) for 45 min at 37°C, protected from light. Afterwards, the supernatant was removed and fresh serum-free DMEM without phenol red was added. Calcein AM (Ex 485 nm/ Em 530 nm) stains live cells green and EthD-1 (Ex 530 nm/Em 645 nm) stains dead cells red. Samples were imaged with Confocal

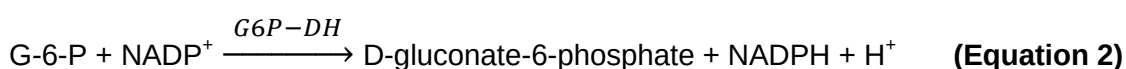
Scanning Laser Microscopy (CSLM, Leica SP2 AOBS) using LCS Software (Leica). Further image treatment was performed using ImageJ 1.43u software (Wayne Rasband).

3.3. Cell metabolic activity

Cell metabolic activity was assessed using the resazurin assay. Resazurin is a non-fluorescent compound that can be reduced by reductases in living cells, yielding the highly fluorescent compound resofurin (Ex 530/Em 590). The assay is non-destructive to cells, enabling continuous monitoring of the culture. 3D hMSCs matrices were incubated with 20% v/v of the stock resazurin solution (0.1mg/mL resazurin (Sigma) in TBS) in medium for 2 h at 37°C. The supernatant was then transferred to a 96-well plate black with clear bottom (Greiner) and fluorescence measurements were carried out using a spectrophotometer microplate reader (Biotek Synergy MX).

3.4. D-glucose consumption

For glucose quantification, spent media from hMSC cultures were collected at different intervals of time and centrifuged at high-speed. At each time point, small-volume samples were taken and exchanged for new media, yielding a final 1% dilution factor. The supernatants obtained were stored at -20°C until further analysis. The D-glucose UV-method kit (Boehringer Mannheim/R-Biopharm, Roche) was used according to manufacturer's instructions but adapted to a microplate format. This assay is based on the stoichiometric conversion of D-glucose to NADPH that can be measured by means of light absorbance at 340 nm. The 2-step reaction is catalyzed by the enzymes hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6-P-DH), as represented in equations 1 and 2.



Briefly, solution 1 (ATP, NADP) was added to samples diluted in deionized water, and after 5 min absorbance was measured at 340 nm using a 96-well UV-Star Half Area microplate (Greiner) in a microplate reader (PowerWave XS, Biotek). The obtained values were set as a blank (A1). Solution 2 (HK, G6-P-DH) was then added, and after 45 min absorbance at 340 nm was measured again. The amount of NADPH, and consequently of D-glucose, was obtained by subtracting A2 to A1 and converting into

mg/mL by using a standard curve of D-glucose in the range of 0.5 – 0.05 mg/mL. For all measurements, n=3 replicates were used. D-glucose specific consumption was calculated using the consumed glucose between days of culture and divided by total number of cells and days in culture.

3.5. Cell morphology

The F-actin cytoskeleton of hMSCs in alginate 3D matrices was observed by CSLM. Alginate matrices were washed with TBS-Ca and fixed for 20 min with 4% v/v paraformaldehyde (PFA, Sigma) in TBS-Ca at RT. After washing in TBS-Ca twice, cell membrane of cells immobilized within the matrices were permeabilized using 0.1% Triton X-100 (Sigma) in TBS-Ca for 5 min and further washed twice again with TBS-Ca. Samples were incubated for 30 min with 1 wt.% bovine serum albumin (BSA, Merck) in TBS-Ca for blocking, and then incubated for 1h in a 6.6 μ M Alexa Fluor 488 Phalloidin (Ex 495 nm/Em 518 nm) (Molecular Probes, Invitrogen) and 5 μ M EthD-1 in a 1-wt% solution of BSA in TBS-Ca. Alexa Fluor 488 Phalloidin labels F-actin present in cell cytoskeleton and counterstaining is obtained with EthD-1 labeling cell nuclei. After a final washing step, cells were observed with CSLM.

3.6. Total dsDNA quantification

After dissolution of cell-loaded hydrogels, cells were recovered by centrifugation at 10,000 rpm for 5 min, washed with PBS, centrifuged again and stored at -20°C until further use. Cells were lysed by incubation in 1% v/v Triton X-100 for 1 h under agitation at 4°C. Samples were then diluted 10 $\times$ in PBS and used for double-stranded DNA (dsDNA) quantification using the Quant-iT PicoGreen dsDNA kit (Molecular Probes, Invitrogen), according to manufacturer's instructions. Briefly, samples were transferred to a 96-well plate black with clear bottom (Greiner) and diluted in TE buffer (200mM Tris-HCl, 20 mM EDTA, pH7.5). After adding the Quant-iT PicoGreen dsDNA reagent, samples were incubated for 5 min at RT protected from light, and then fluorescence measurements were performed in a spectrofluorometer (Biotek Synergy MX) with excitation at 480 nm and emission at 520 nm. Fluorescence values were converted into ng/mL using a standard curve of lambda DNA in the range of 1-1000 ng/mL. For each condition, n=3 replicates were analyzed.

3.7. Histological analysis

3.7.1. *Hematoxylin-Eosin (H&E) and Masson's Trichrome (MT) stainings*

After day 14 of culture under basal or osteogenic conditions, cell-loaded alginate matrices with different cell densities were recovered and washed with TBS-Ca and fixed with 4% v/v PFA in TBS-Ca for 20 min. Dehydration in a gradient series of ethanol was performed and the matrices were embedded in paraffin blocks. Sections of 3 μ m thickness were sequentially recovered. After diafanization in xylene and rehydration in decreasing gradient series of ethanol and finally water, samples were stained as follows: for H&E staining, sections were incubated in Gill's Hematoxylin for 5 min and, after a washing step in water, counterstained with Eosyn Y for 1 min. For MT staining, a modified MT kit (Sigma-Aldrich) was used. Sections were incubated with Celestine Blue for 5 min, washed in water, stained with Gill's Hematoxylin for 5 min and washed again in water for 2 min. Afterwards, sections were incubated for 1h with Bouin solution, washed in water, and stained in Biebrich Scarlet-Acid Fuchsin solution for 5 min. After another washing step with water, sections were mordant in fresh phosphomolybdic acid/phosphotungstic acid solution for 5 min and stained with aniline blue for 5 min. After staining, sections were dehydrated, diafanized in xylene and mounted using histofluid mounting medium (Marienfeld). Finally, samples were visualized under a light microscope and imaged.

3.7.2. *Immunohistochemistry: Ki67 and fibronectin stainings*

After 14 days of culture, paraffin sections of the different samples were obtained as already described in the previous section, diafanized and rehydrated. Sections were washed three times in TBS for 5 min, and heat-induced epitope retrieval in 10 mM sodium citrate (Sigma) buffer at pH 6 was conducted for 10 min. After washing three times for 5 min in PBS, cells were permeabilized by incubating sections 5 min in 1% v/v Triton X-100, and washed three times for 5 min in PBS. To quench auto-fluorescence, sections were incubated for 5 min in very fresh 0.1% wt.% sodium borohydride (Sigma) in Tris/EDTA solution at pH 9, and washed once again in PBS. Blocking non-specific binding was performed by incubating with 4% v/v FBS, 1% v/v BSA solution in PBS for 1h at RT. After another washing step, sections were incubated ON at 4°C with primary antibodies in blocking solution. The following dilutions of antibodies were used: rabbit polyclonal anti-Ki67 (Abcam) at 1:100, and mouse anti-human monoclonal HFN 7.1 (Developmental studies hybridoma bank) at 1:1000000. Afterwards, sections were

washed three times in PBS for 5 min each, and incubated for 1 h with secondary antibodies at the following conditions: Alexa Fluor 594 F(ab')₂ fragment of goat anti-rabbit (1:1000), Alexa Fluor 488 goat anti-mouse IgG (H+L) (1:1000), both from Molecular Probes (Invitrogen) and counterstained with DAPI (1:1000), in blocking solution in the dark. Sections were washed three times in PBS and sections mounted with Vectashield (Vector). Samples were imaged with an inverted fluorescence microscope (Axiovert 200M, Zeiss).

3.7.3. Whole-mounts cytochemistry: Alkaline phosphatase (ALP) staining

Cell-loaded alginate matrices were washed three times with TBS-Ca and fixed with 4% v/v PFA in TBS-Ca for 20 min at RT, followed by another washing step, and incubated for 30 min in Naphtol AS-MX phosphate/Fast Violet B salt (Sigma) at 37°C, protected from light. After washing, pictures were taken using a stereoscope (SZX10, Olympus) with a digital camera (DP21, Olympus).

4. Statistical analysis

All results are shown as Mean±SD. SPSS Statistics 17.0 software was used to apply statistical tests and results were considered statistically significant when $p < 0.05$. Cell viability, total cell number and cell metabolic activity data were analyzed using one-way ANOVA, complemented with Tukey post-hoc test whereas data regarding total DNA and glucose concentration were analyzed using non-parametric Mann-Whitney test.

III. RESULTS AND DISCUSSION

III. Results and Discussion

1. Preparation and Characterization of RGD-alginate

In this study, RGD-functionalized alginate was synthesized, characterized and further used to prepare hydrogel matrices for culturing hMSCs under 3D conditions.

Alginate is a non cell-adhesive polymer but provides a good background for chemical modifications. Since matrix adhesiveness is known to play a key role in cell survival, proliferation, migration and differentiation [61, 110], alginate was grafted with a peptide sequence containing RGD, an integrin-mediated cell adhesion motif present in natural ECM proteins such as fibronectin [22]. This peptide was grafted to alginate through standard aqueous carbodiimide chemistry, and the success of the immobilization process was confirmed via UV spectra analysis of both RGD-alginate and soluble peptide. The UV spectra of RGD-alginate (normalized against the blank – unmodified alginate), is depicted in Fig.12 showing the presence of a characteristic peak around 230 nm, with a similar pattern as the one shown for the soluble RGD, which indicates that the peptide was effectively grafted to alginate.

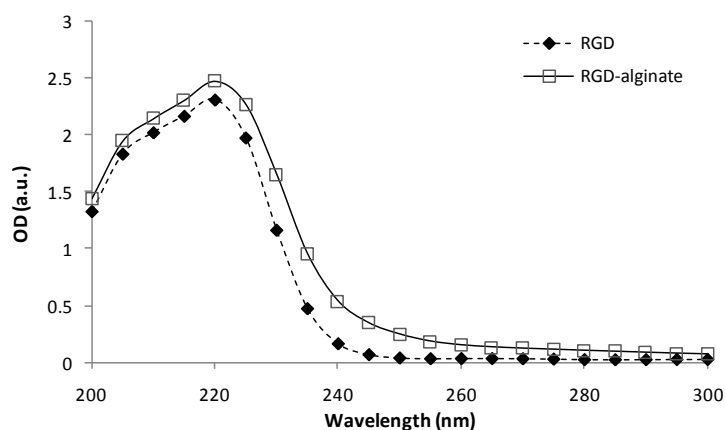


Figure 12. UV spectra of RGD-alginate (plotted against the blank – unmodified alginate) and soluble RGD peptide. Both spectra reveal similar peak profile around 230 nm.

The amount of covalently immobilized peptide was estimated using the BCA assay and a reaction yield of approximately 60% was obtained. Therefore, the RGD-grafted alginate used in this study had a content of approximately 10 mg of immobilized peptide per gram of alginate.

For the cell culture studies an hydrogel formulation previously optimized by our group, was used [41, 42, 47]. The gel precursor solution consisted on a 50:50 v/v binary mixture of RGD-grafted oxidized HMW alginate and unmodified LMW alginate, prepared at a total alginate concentration of 2 wt.% in 0.9 wt.% NaCl. Therefore, the RGD concentration in the final hydrogel matrices was around 110 μ M. This density has an order of magnitude comparable to the RGD density of commonly used 3D matrices, both of artificial [41, 42, 47, 59, 72] and natural origin, the later including tumour-derived ECM that has an RGD density of 140 μ M [111].

The bioactivity of the immobilized RGD peptide, i.e. its cell-adhesion properties, was probed by performing preliminary cell adhesion studies in a 2D set-up. As depicted in Fig. 13, hMSCs seeded onto RGD-alginate hydrogel films were able to attach and were already spread after 24h, as expected, while on control surfaces (unmodified alginate) cells were unable to adhere and formed cell aggregates that were easily displaced and washed out.

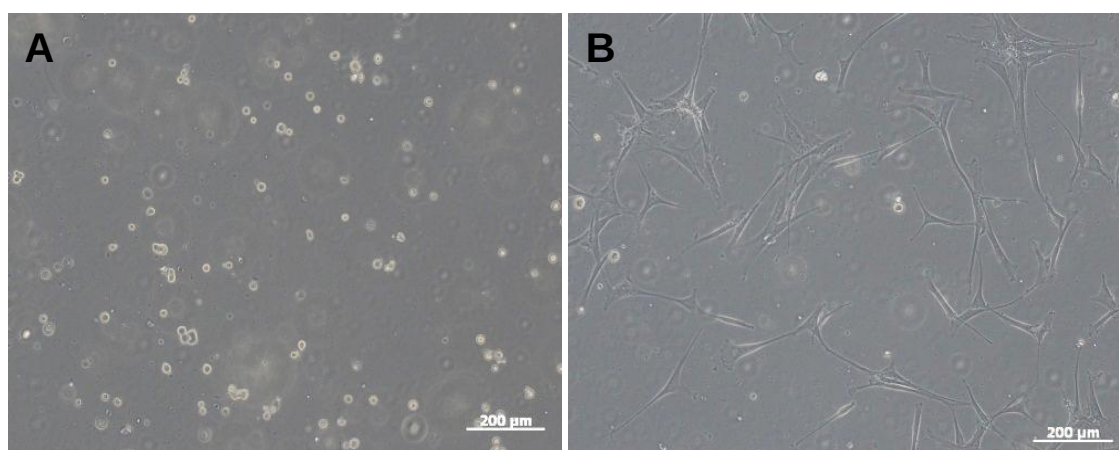


Figure 13. Optical microscopy images of hMSCs on the top of alginate films prepared by spin-coating at 24 hours post-seeding (without medium change) on (A) unmodified alginate and (B) RGD-modified alginate. Scale bars: 200 μ m.

2. Establishment of 3D hMSC cultures within alginate hydrogels

The in situ crosslinking properties and contour-adaptability of the RGD-alginate hydrogels prepared by an internal gelation strategy was firstly demonstrated by casting the hydrogels using different molds as illustrated in Fig 14A.

To promote the crosslinking of the alginate chains, a combination of CaCO_3 and GDL was used as previously described [48, 51, 108]. CaCO_3 , a calcium salt with limited solubility, was used as a source of calcium ions. The release of Ca^{2+} into the solution was promoted by the generation of an acidic pH with GDL, a slowly dissociating acid. Once released, Ca^{2+} ions can participate in the interchain ionic binding between carboxyl groups (COO^-) of guluronic acids blocks in the polymer chain, giving rise to a hydrogel network. Representative images of the 3D alginate hydrogel matrices used in this study, and of their method of preparation, are also depicted (Fig 14B-D). For the preparation of cell-loaded 3D matrices, cells were directly mixed with gel precursor solutions before crosslinking, becoming physically entrapped within the final hydrogel matrix.

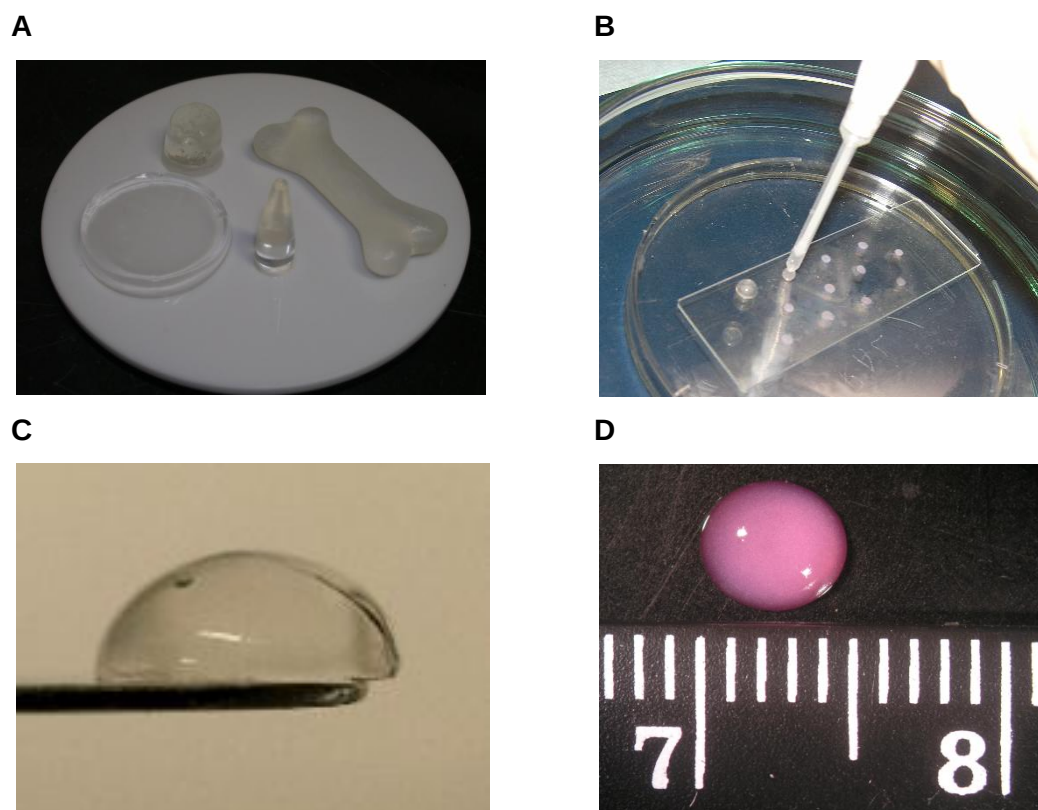


Figure 14. **A.** Different shapes of alginate gels obtained by internal gelation method. The alginate was gelled *in situ* by the liberation of calcium ions from an insoluble compound (CaCO_3) through pH decrease that accompanies GDL hydrolysis in solution. **B.** Formation of alginate discs with immobilized cells obtained from a 20 μL alginate drop using a positive-displacement micropipette. **C.** and **D.** Representative images of the alginate discs used in this work.

The microstructure of the hMSC-loaded alginate matrices immediately after entrapment was imaged using cryo-SEM. This technique was very interesting for studying the detailed morphology of the 3D matrices, since it allows the analysis of samples containing high amounts of water like hydrogels [112]. After a freezing step, samples

were fractured and the water present within the structure was partially removed in a controlled manner by sublimation with no creation of ice crystals. Therefore, contrary to conventional SEM, the cryo-SEM technique provided direct observation of the samples without any dehydrating step, thus avoiding distortion of the matrix architecture. In Fig. 15A, a representative image of one cell and the surrounding alginate network is presented. The alginate network appears homogenous and cells were restrictively confined and individually isolated within the matrix, which provided a real 3D microenvironment to cells. This structural information was confirmed by histological analysis of H&E-stained cryo-sections of the matrices (Fig. 15B). The distribution of cells as individual entities within the matrix, as well as the alginate network can be observed. Both images clearly show that the mean pore size of the hydrogel network is smaller than the maximal diameter of the cell body. As a result, the matrix barriers forced the entrapped cells to adapt their morphology and adapt a round shape.

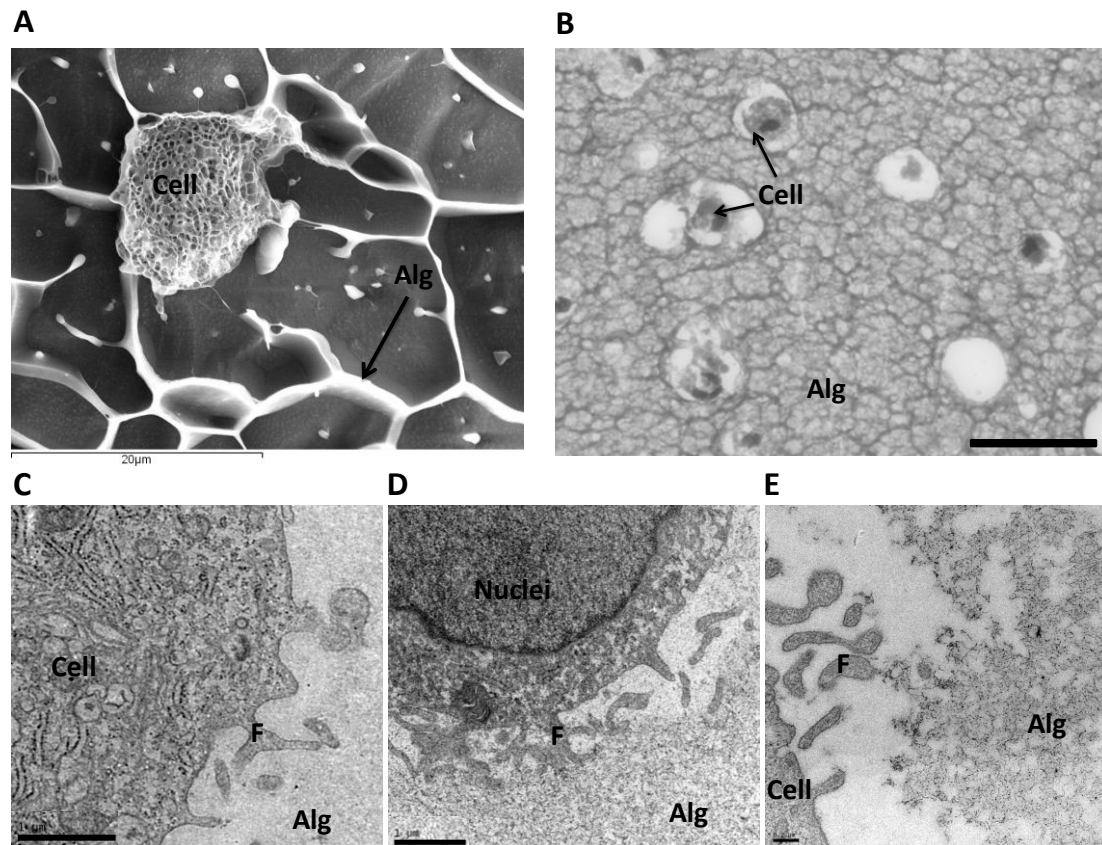


Figure 15. A, B: Cryo-SEM image (scale bar, 20µm) and H&E-stained cryo-sections (scale bar, 50 µm) Original of hMSCs-loaded alginate matrices 1h post-entrappment. C-E: TEM image of the samples after 1 week of culture (scale bar, C 1µm, D 1µm, E 0.2µm) Alg – alginate; F- filopodia.

At day 7, ultra-thin sections of cell-loaded alginate matrices were imaged by TEM, and cell-matrix interactions were observed in more detail (Fig. 15 C-E). The presence of cell

filopodia-like membrane protrusions extending to the extracellular space puts in evidence the establishment of a physical connection between the entrapped cells and the surrounding alginate matrix, most probably mediated by the RGD adhesion sites, which is in accordance with previously reported data [47]. In some cases (D, E) these connections were disrupting during sample processing.

3. Analysis of hMSC behavior in 3D culture

Mesenchymal stem cells (MSCs) have become a key tool in regenerative therapies. However, most *in vitro* research has focused on their behavior in 2D-culture, and relatively little is known about cell behavior in 3D-culture. Moreover, there is an increasing interest in using 3D-cultures as more reliable and sophisticated models for mechanistic *in vitro* studies, as it is now widely recognized that there are vast differences between conventional 2D and 3D-cultures. This has been prompted the need to better evaluate the performance of 3D-cultured and thoroughly characterize the behavior of cells when cultured under such conditions.

To address this, the present study focused on the effect of different culture conditions, namely the initial cell density, on hMSC cultured under 3D conditions within alginate hydrogel matrices. The 3D hMSC culture was monitored according to cell viability, metabolic activity, glucose consumption, proliferation, production of endogenous ECM and osteogenic differentiation.

3.1. Effect of cell density on cell's viability and metabolic activity

To evaluate the effect of cell density of hMSC behavior in 3D, cells were entrapped within alginate matrices at the following concentrations: 2×10^6 cells/mL (D1), 5×10^6 cells/mL (D2), 7.5×10^6 cells/mL (D3) and 15×10^6 cells/mL (D4). Most studies in the literature were performed at lower densities, in the range of 1 to 5×10^6 cells/mL [43, 113-116], while only a few were performed using high-density cultures, in the range of 5 to 20×10^6 cells/mL [47, 48, 117].

Cell viability was evaluated at days 0, 1, 4 and 7 by trypan blue exclusion assay and results are depicted in Fig. 16. Immediately after entrapment within the alginate matrices (day 0), a high cell viability ranging from $91.2 \pm 4.8\%$ (D1) to $82.6 \pm 3.3\%$ (D4) was observed. The slightly lower initial cell viability obtained for the highest cell density (D4) may in part be related to the hydrogel dissolution procedure, which required multiple pipetting to suspend the hydrogel matrix into a single-cell suspension and that may have lead to some shear-induced damage. Other authors have already reported a similar circumstance [118].

Along the time of culture, a slightly decrease in cell viability was observed for all cell densities tested, and viabilities ranging from $56.4 \pm 5.8\%$ (D1) to $77.4 \pm 5.3\%$ (D4) were observed after 7 days of culture. Differences in cell viability between days 0 and 7 were statistically different ($p < 0.05$) for the range of densities D1 to D3. For the highest cell density (D4), no significant differences in cell viability were observed after one week of culture, and approximately 80% of the entrapped cells were still viable. Comparable values were found in literature for 3D hMSCs cultured within alginate microspheres at a density of 20×10^6 cells/mL [42].

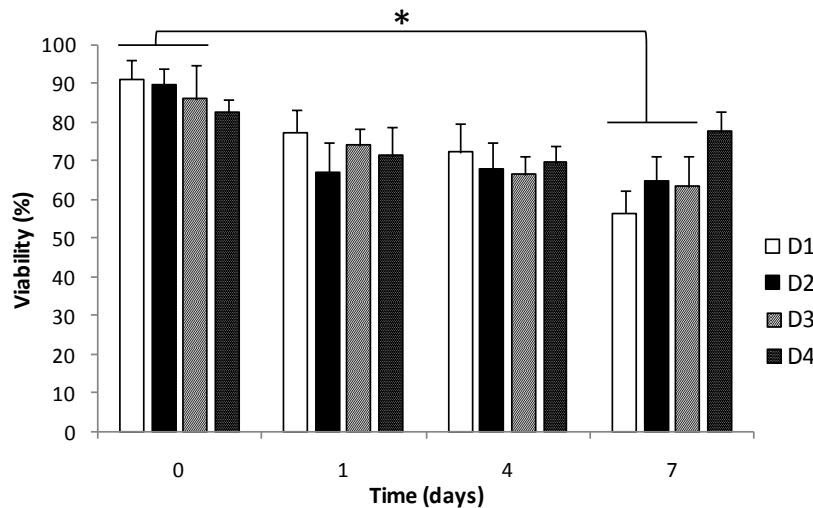


Figure 16. Viability of hMSCs in 3D alginate matrices at different cell densities (2×10^6 cells/mL (D1) – 15×10^6 cells/mL (D4)) along 1 week of culture. At the highest cell density, the viability is maintained constant and high along the time. Data is shown as Mean $\pm$ SD (n=3). (*) represents statistical differences ($p < 0.05$).

The better maintenance of cell viability observed for the highest density cultures may have resulted from a more efficient intercellular signaling, and the eventual establishment of some cell-cell interactions, more prone to occur under this density.

The metabolic activity of hMSCs immediately after entrapment within 3D alginate matrices at low-to-high cell densities was analyzed through the Resazurin assay. The metabolic activity of the same total amounts of hMSCs maintained under standard 2D conditions was also analyzed for comparison. This enabled not only to assess the immediate consequences of cell entrapment, but also to evaluate the effectiveness of using this type of colorimetric metabolic assays, which do not necessarily correlate linearly with increasing cell densities or between 2D and 3D-cultures, as already suggested in the literature [119].

As presented in Fig.17, in 2D-cultured hMSCs, a clear rise in metabolic activity was recorded as cell density increased, with a linear response ($R^2=0.9977$) from D1 to D3 (equivalent to 40 000 – 150 000 cells), but a loss of linearity at D4.

Compared with the 2D-culture, 3D-cultured cells produced a significantly lower fluorescent signal, as depicted in Fig. 17., but a linear relationship between fluorescence and cell numbers was still maintained for the all range of densities tested ($R^2=0.9735$). RFU values ranged from of 2,000 to 12,000 for cell densities of 2×10^6 cells/mL (D1) and 15×10^6 cells/mL (D4), respectively.

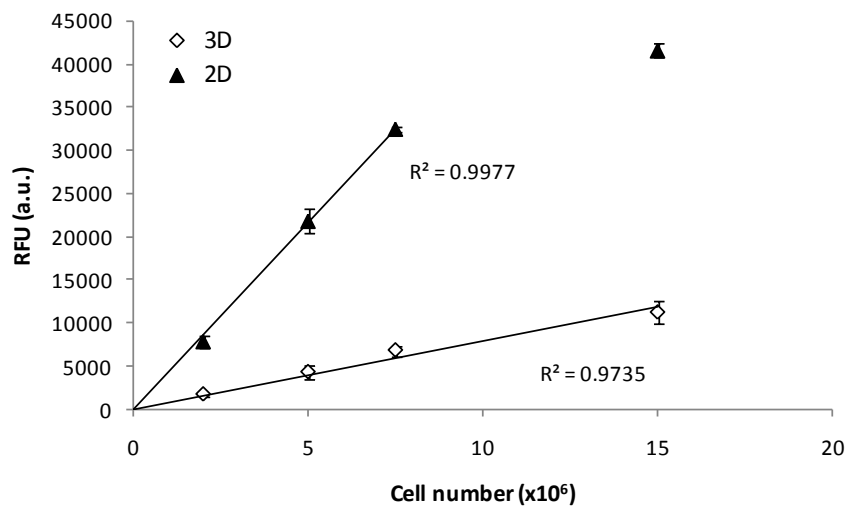


Figure 17. Metabolic activity of hMSCs in 2D standard cultures (TCPS) and in 3D alginate matrices, at day 0, with different cell densities (D1 = 2×10^6 cells/mL – D4 = 15×10^6 cells/mL). Metabolic Activity of 2D cells was up to 5-fold increased compared to 3D. Data is shown as Mean $\pm$ SD (n=3).

To guarantee that these differences between 2D and 3D were not a consequence of limited substrate/product diffusion into and out of the 3D construct during the resazurin assay incubation period, a set of additional experiments were performed to investigate if the metabolic activity of 3D-cultured cells was being underestimated. However, the metabolic activity of 3D-cultured cells assayed immediately after being released from

the matrices, at the different time points, was still significantly lower than those of 2D-cultured cells (data not shown).

The metabolic activity of 3D-hMSC was subsequently followed in time along 1 wk of culture (Fig. 18). For all the densities tested, the metabolic activity gradually decreased along the period of culture, and for D4 attained a nearly steady-state profile from day 4. Similar results were reported, where in alginate microspheres, hMSCs were seen to slightly decrease metabolic activity during the first 2 weeks of culture [42].

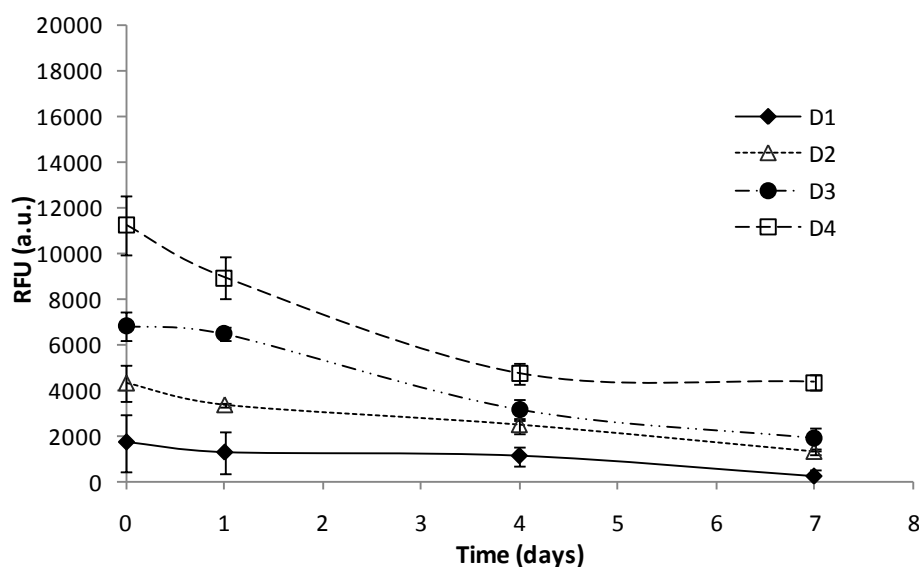


Figure 18. Metabolic activity of hMSCs within 3D alginate matrices along one week of culture, at different cell densities (2×10^6 cells/mL (D1) – 15×10^6 cells/mL (D4)). Data is shown as Mean $\pm$ SD (n=3).

To evaluate the reversibility of the observed metabolic inhibition, 3D-hMSCs cultured at the highest cell density (D4) were recovered from alginate matrices after 1week of 3D culture and re-plated under standard 2D conditions. Cells were then cultured for one additional week and their metabolic activity was followed as depicted in Fig 19.

The results showed that the metabolic activity of the recovered cells gradually increased along the time, increasing up to 5-fold by day 15 (Fig 19A), and that cells were able to proliferate as detected by routine optical microscopy observations (data not shown). At the end of the culture, a Live/Dead assay was performed and cells in 2D were shown to be mainly viable as observed in Fig. 19B. The cells were well spread and formed a monolayer.

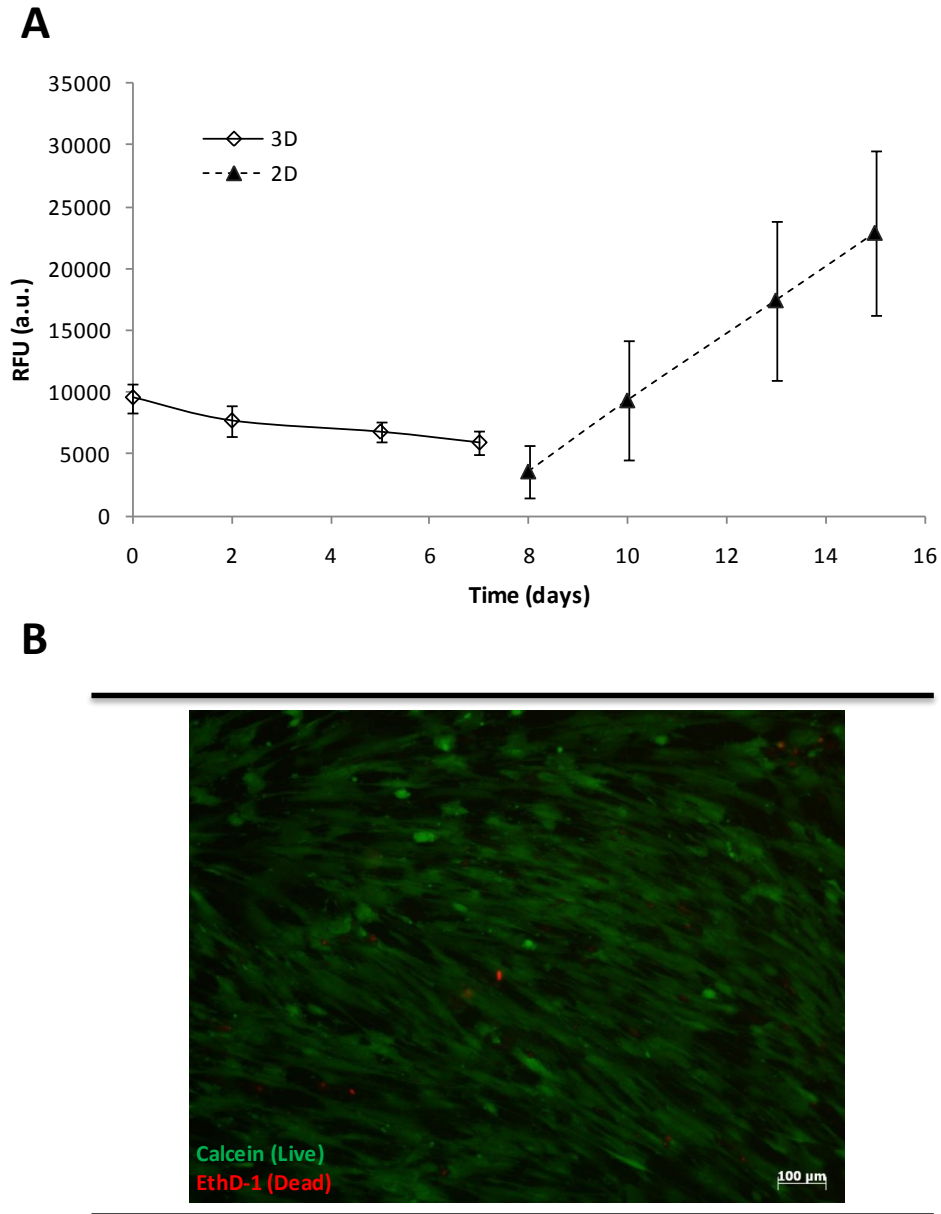


Figure 19. hMSC cultured in 3D conditions (3D) for one week and after recovery from alginate matrices, cultured for an additional week in standard 2D conditions (2D). **A.** Metabolic activity follow-up for 2 weeks under different conditions (3D or 2D). Data is shown as Mean $\pm$ SD from 1 representative experiment (n=3). **B.** Live/Dead Cytotoxicity/Viability assay performed at day 15 in 2D cultured cells, showing mainly viable cells. Live cells are green and dead cells are red (Scale bar, 100 μ m).

These results are in agreement with findings from Hunt *et al*, where fibroblasts were seen to significantly decrease their metabolic activity within 3D alginate constructs, but subsequently recovered a normal mitotic activity upon retrieval [117].

To rule out the possibility that the observed decrease in metabolic followed by a steady-state in 3D was not a consequence of nutrient and O₂ transport limitations within the hydrogel matrices, as suggested by other authors [118], some control

experiment were performed under dynamic conditions. These are presented in Annex 1. Results showed however that there were no significant differences between dynamic and static conditions, suggesting that the decrease in metabolic activity observed under 3D conditions was not an outcome of diffusional limitations.

To gain further insights into 3D cell metabolism, the glucose levels in the medium were monitored along the time (Fig 20A). hMSCs were cultured at different densities within 3D alginate matrices for 7 days, without any medium exchange, and the spent medium was collected at different time points (days 0, 1, 4, 7). A reduction in glucose concentration in the medium along the period of culture was observed for all cell densities, indicating glucose consumption, which is an indicator of cellular metabolic activity. The glucose depletion in the media was proportional to cell density, as expected. For the highest cell densities ($D3 = 7.5 \times 10^6$ cells/mL and $D4 = 15 \times 10^6$ cells/mL) it could be observed that after 7 days the medium was completely glucose-depleted. For the lowest cell densities, a decrease of approximately 20% and 40% of glucose was observed at day 7, for D1 and D2, respectively.

Since this experiment showed that in high-density cultures the medium become completely glucose-depleted after 4 days of culture, a set of experiments were performed to establish the most adequate feeding regimens. These are presented in Annex II, and showed that exchanging the medium every 2 days is sufficient to prevent periods of glucose starvation in 3D cultures, which was adopted in subsequent studies. Differences in glucose consumption between 2D and 3D hMSC cultures were analyzed at day 1. This time point was selected because it was the only one where glucose was still available in all the cultures, and where the total amount of cells at 2D and 3D was still comparable. As depicted in Fig 20A, the glucose depletion in the media was proportional to cell density and, interestingly, differences between 2D and 3D were small (Fig. 20B).

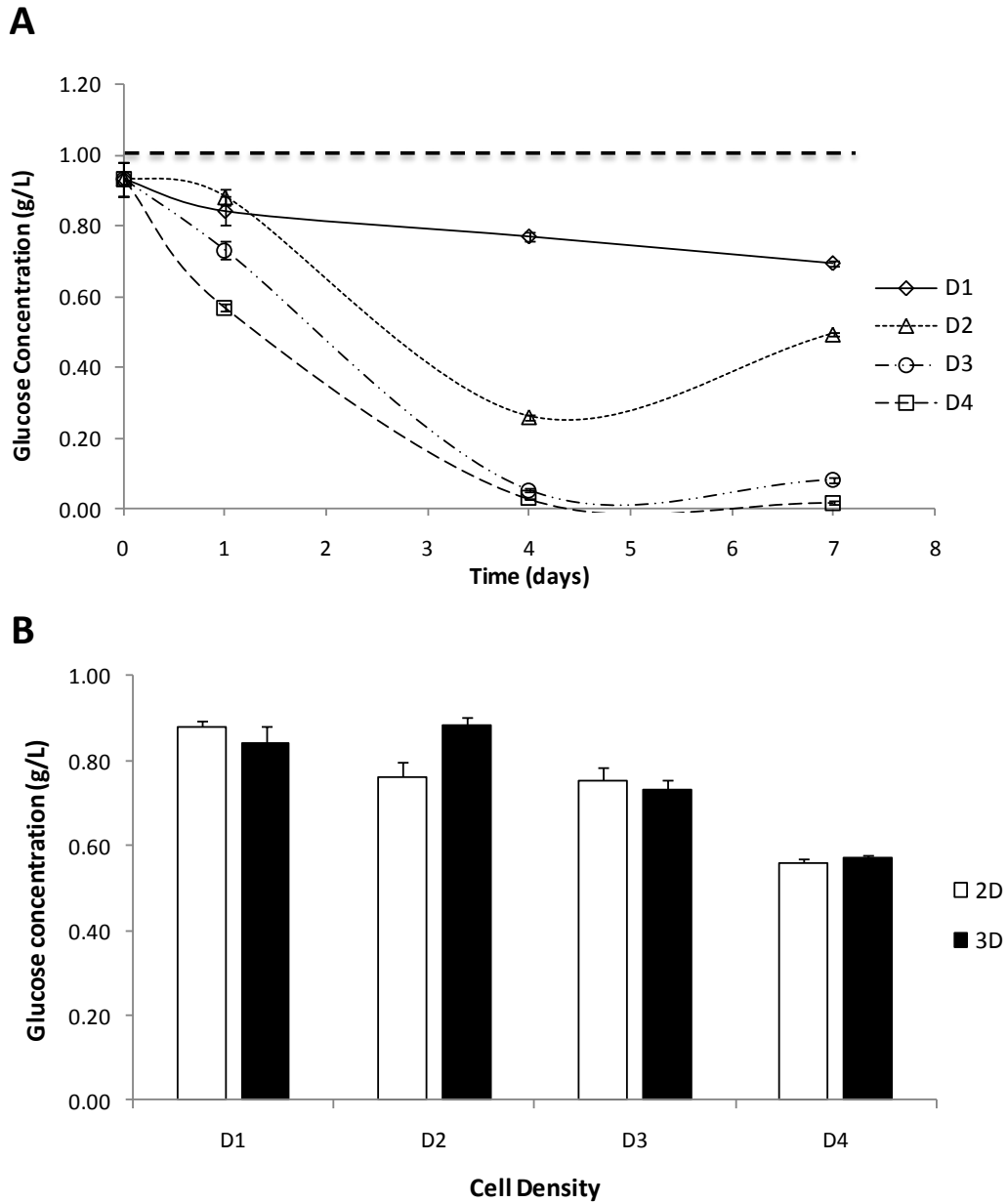


Figure 20. A) Glucose concentration on cell culture medium of 3D hMSC maintained in culture for 1 week at different cell densities (D1 (2×10^6 cells/mL) - D4 (15×10^6 cells/mL)). Dashed line represents the maximum glucose concentration on the medium (1g/L). Data is shown as Mean $\pm$ SD. **B)** Glucose concentration on cell culture medium of 2D vs 3D hMSC cultures of different densities after 1 day in culture. Data is shown as Mean $\pm$ SD (n=3).

Anaerobic glycolysis and the aerobic citric acid cycle are the two primary means for cells to obtain energy [120]. In fact, it is widely known that MSCs exhibit high levels of glycolytic metabolism and a recent study has suggested that MSCs have a mixed metabolism utilizing both glycolysis and citric acid cycle (approximately 30% of total ATP generation) [121]. To gain further insights into the relative metabolic state of MSCs in 2D vs 3D cultures, whether aerobic or anaerobic, it will be important in future

studies to look at the fate of the glucose consumed by the cells.

This could be performed by quantification of lactate production in the presence and absence of sodium azide (an enzyme inhibitor in the aerobic oxidative phosphorylation). The higher the amount of lactate, the more likely the anaerobic metabolic state [120]. Some preliminary lactate quantification assays were conducted in the present study but further optimization was needed.

3.2. Effect of cell density on 3D hMSC proliferation

The effect of cell density on 3D cell proliferation was analyzed by counting the total number of cells per matrix after 0, 1, 4 and 7 days of culture. The results obtained for the different densities are presented in Fig. 21. At day 0, immediately after entrapment and for all cell densities, the average total number of cells present in each matrix was in accordance with the range of expected theoretical value, respectively from D1 to D4. However, those values remained essentially constant throughout the period of culture, suggesting that the entrapped cells were not able to proliferate, independently of the original cell density.

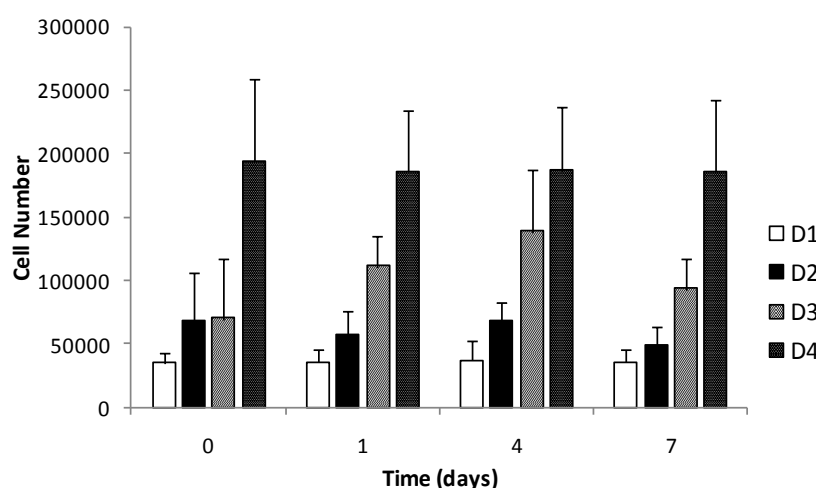


Figure 21. Total cell number of 3D hMSC upon retrieval from alginate matrices, at different time points and cell densities (D1(2 x 10⁶ cells/mL) – D4 (15 x 10⁶ cells/mL)) along one week of culture. Data is shown as Mean±SD.

DNA quantification was also performed using the same samples as shown in Fig. 22.

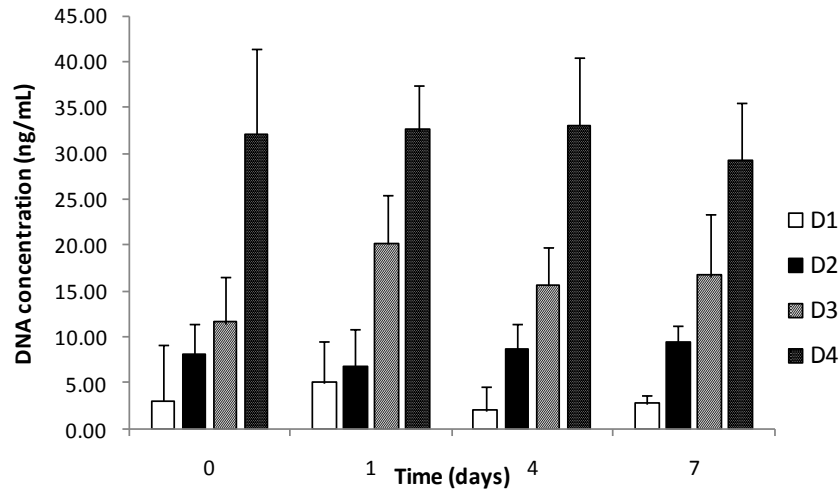


Figure 22. DNA concentration (ng/mL) of 3D hMSCs within alginate matrices along 1 week of culture, at different cell densities (D1 (2×10^6 cells/mL) – D4 (15×10^6 cells/mL)). Data is shown as Mean $\pm$ SD.

At day 0, the dsDNA content of the different samples was proportional to their respective original cell density, as expected. In agreement with the previous observations, no increase on the dsDNA content was observed along the time, further indicating that cells were in a non-proliferating state.

These results are consistent with the steady-state metabolic activity reported in the previous section. Altogether these data point out to an altered metabolism of 3D-cultured hMSCs as compared with traditional 2D-cultures.

Growth arrest and metabolic inhibition following entrapment in alginate hydrogel matrices has been previously reported by other authors. For example, Markusen and coworkers did not observe proliferation of hMSC in alginate beads after 2 weeks of culture [118], also seen by Duggal *et al* in similar matrices[122]. Studies from Lan and coworkers using HepG2 cells grown on 2D (collagen coated well plate) and 3D alginate matrices, shown that the number of cells cultured on the collagen coated well plate (2D culture) increased dramatically (4.5 times higher than day 0) after 2 weeks of culture, while 3D cell number was maintained and no significant differences were seen [123].

Altogether, results obtained by us and other authors suggest that when cultured under real 3D-conditions (i.e. within hydrogel-like matrices), cells may become spatially constrained by a polymeric network that commonly has a pore size significantly smaller than cellular dimensions. This was demonstrated in the present case by cryo-SEM analysis. Therefore, in order to be able to proliferate and migrate, cells need to overcome physical impediments posed by the surrounding environment. If unable to do it, cells possibly become locked into a steady state that does not allow cell division.

Although future studies will need to be carried out to draw a valid conclusion, it can be speculated that upon entrapment, cells somehow enter in a quiescent-like state. In fact, the cardinal feature of quiescence, which distinguishes it from other non-dividing cell states, such as senescence, apoptosis, and terminal differentiation, is that quiescence is reversible [124]. This was demonstrated in this study, by showing that cells recovered by 3D-cultures re-entered the cell cycle and began to proliferate with a concomitant increase in metabolic activity. Moreover, as here demonstrated especially for the high-density cultures, cells remained with a high viability along the entire period of culture.

3.3. Effect of cell density on the osteogenic differentiation of 3D-cultured hMSC

As multipotent cells, hMSCs are able to differentiate into the osteogenic lineage opening a wide field of opportunities in bone regeneration area. As a regenerative medicine strategy, the use of injectable hydrogels such as alginate may be of great interest. Therefore, it is important to evaluate the behavior of this system upon osteogenic induction, and in particular look at the effect of cell density on that process.

To induce osteogenic differentiation of 3D-cultured hMSCs, medium was supplemented with a specific batch of FBS, previously selected for this purpose; L-ascorbic acid, to induce collagen synthesis and deposition; β -glycerophosphate, to provide a source of inorganic phosphate for mineralization; and dexamethasone, which promotes bone nodules formation [125].

hMSC-loaded alginate matrices were cultured under basal and osteogenic conditions and monitored along 21 days of culture. For some of the tested conditions, significant alterations on the constructs shape and size were detected along the time, even at the macroscopic level. Images of the samples at days 7 and 14, at the lowest (D1) and highest (D4) cell densities tested, and in basal versus osteogenic conditions are presented in Fig. 23. Along the first week of culture under osteogenic conditions, the alginate discs diameter decreased while cell density increased (Fig. 23A,B). This effect was also detected under basal conditions but a very low extent (Fig 23 E,F). The reduction on matrix diameter on high-density cultures may be a consequence of cell-mediated hydrogel contraction, as cells probably pulled the polymeric matrix to a certain extent as they formed interconnected multicellular networks.

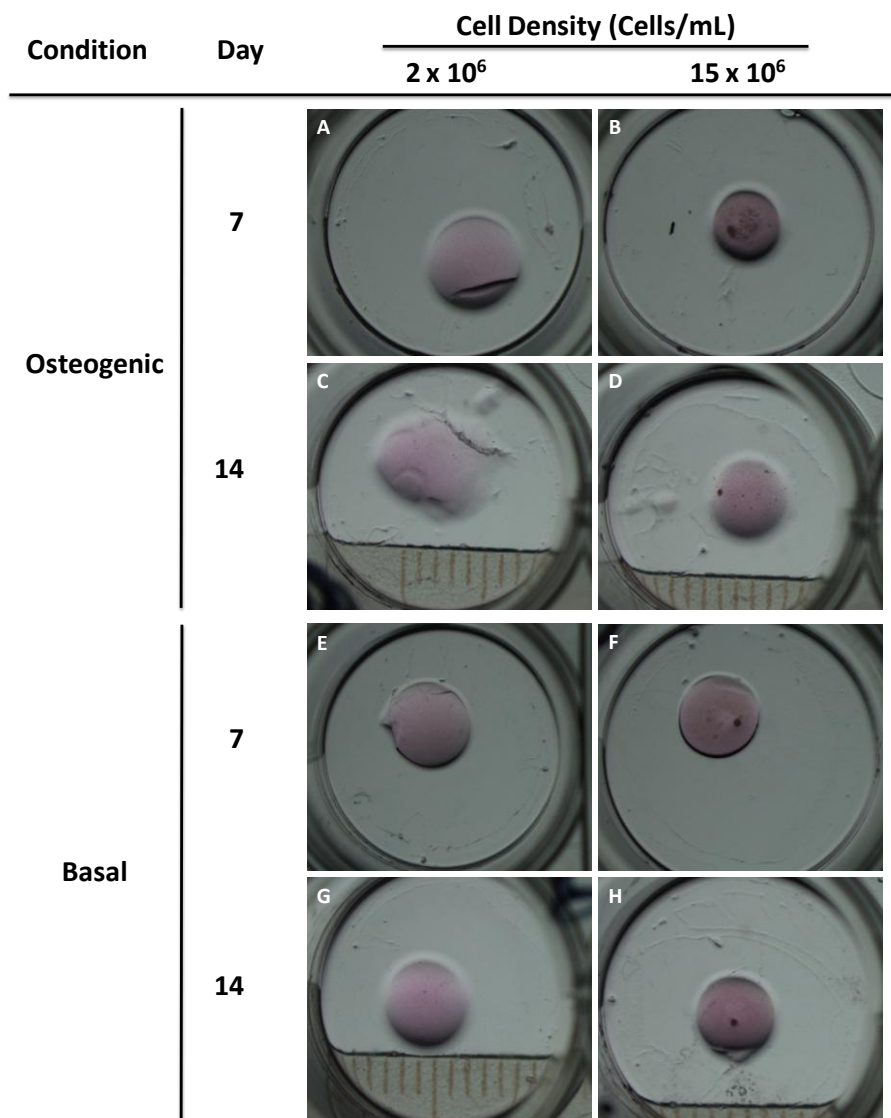


Figure 23. Macroscopic view of cell-loaded hydrogels at different cell densities (2×10^6 cells/mL – 15×10^6 cells/mL) at days 7 (A - D) and 14 (E - L), cultured under osteogenic (A – H) and basal conditions (I-L). Stereoscope images, original magnification 10x.

Along the remaining weeks, a loss of integrity in the discs was seen under osteogenic conditions (Fig. 23C,D), suggesting that prolonged times of culture lead to an expansion and softening of the matrices and ultimately to physical breakdown, which was more pronounced in low-density cultures. These effects were not seen under basal conditions (Fig. 23G,H). In ionically crosslinked alginate matrices, the stability of the crosslinks and the physical properties of the hydrogel are greatly affected by the ionic environment of the medium. In typical OM formulations, beta-glycerophosphate is exogenously added to release inorganic phosphate that will initiate the mineralization of

osteoid nodules in vitro [126]. In this case, the increased concentration of phosphate ions in the medium probably promoted the sequestering of some alginate-bound calcium, contributing to the gradual disassembling of the hydrogel matrices. By days 17-19, all the matrices were already disintegrated, releasing the cells, and for that reason studies could not be more prolonged in time.

Nevertheless, at the highest cell density (D4), the integrity of the matrices was better retained in time, compared with the others. Again, this was possibly due to the higher extent of cell-cell interactions in high-density cultures, which probably conferred additional cohesion to the matrices. Moreover, it is already well established that migrating cells in a 3D culture may create profound changes in matrix architecture, ultimately leading to contraction of the lattice [127], something that possibly also occurred in the present case.

Several studies in the literature have shown that scaffold degradation may directly impact the metabolism and osteogenic differentiation of hMSCs [73]. Studies of Park and colleagues, for example, have shown that a scaffold with higher degradability enhances osteogenesis [128], by providing more space for new tissue formation.

Cell morphology and re-arrangement within the matrices was analyzed by phase contrast imaging of hMSCs within 3D alginate matrices at day 14, under different culture conditions, as depicted in Fig. 24. Differences among the various cell densities were observed.

Under basal conditions (Fig. 24A, C, E, G), cells remained essentially round and individually dispersed within the matrix, except for the higher density of 15×10^6 cells/mL (Fig. 24G) where a few cells with a slightly spread shape were observed. Under osteogenic conditions, at low cell densities ($2 - 5 \times 10^6$ cells/mL, Fig. 24 B, D) cells were also shown to maintain a round shape. However, for the higher cell densities (Fig. 24 F, H), especially for D4, cells presented a more spread shape. Moreover, for the D4 density, some multicellular aggregates were found, as seen in Fig. 24 H.

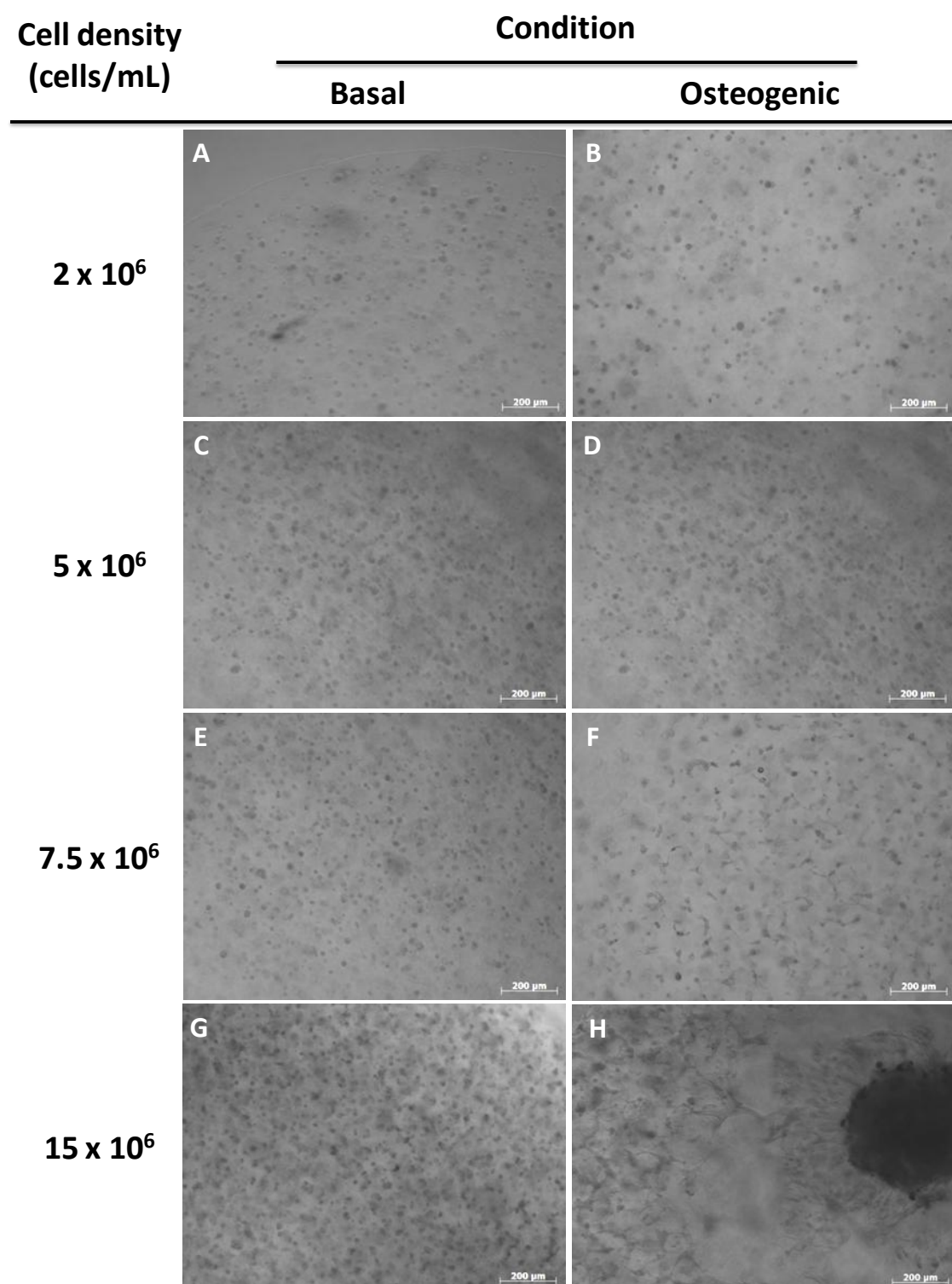


Figure 24. Phase contrast images of 3D hMSCs at different cell densities (2×10^6 cells/mL, 5×10^6 cells/mL, 7.5×10^6 cells/mL and 15×10^6 cells/mL), cultured under Basal (A, C, E, G) and Osteogenic (B, D, F, H) conditions, at day 14.

The previously described opening of the lattice structure along the time, and reduction

in matrix mechanical stiffness most likely facilitated cell spreading and the establishment of cell-cell interactions, accounting for the differences in cell morphology observed between basal and osteogenic conditions.

To better analyze the morphological alterations that hMSCs experienced when cultured under 3D conditions at different densities, under basal or osteogenic conditions, some histological analyses were performed. Representative images of H&E-stained sections at day 14 are depicted in Fig. 25. As depicted, cells under basal conditions remained essentially round and homogeneously distributed within the matrix, as single entities, independently of the cell density (Fig. 25 A, C, E). A similar behavior was observed for cells cultured at the lowest density under osteogenic conditions (D1, Fig. 25B). However, at the intermediate and highest density (D3 and D4, Fig. 25D, F), cells cultured under osteogenic conditions exhibited a more spread shape (D4, Fig. 25F). Cell morphology and rearrangement within the matrix were particularly distinct at the highest cell density. Cells presented a high degree of spreading and multiple cell aggregates were detected within the matrix, which was an unexpected observation. The cell shape has long been linked to ECM-dependent changes, essentially via integrin signaling pathways as already demonstrated in 3D tissues, and a more spread shape has been generally associated with osteoblastic commitment [73].

To evaluate if the 3D-cultured hMSCs were able to produce their own ECM, a very important feature for regenerative medicine applications, the deposition of endogenous collagen was analysed. Contrary to monolayer cultures where ECM components may be released into the media, in 3D microenvironment many of the secreted endogenous ECM may become retained between and around the cells which produced it, enhancing our ability to morphologically detect them [85]. Even so, depending on their molecular sizes, some secreted proteins can still readily diffuse through the hydrogel and into the media, and be hardly detectable in the matrix.

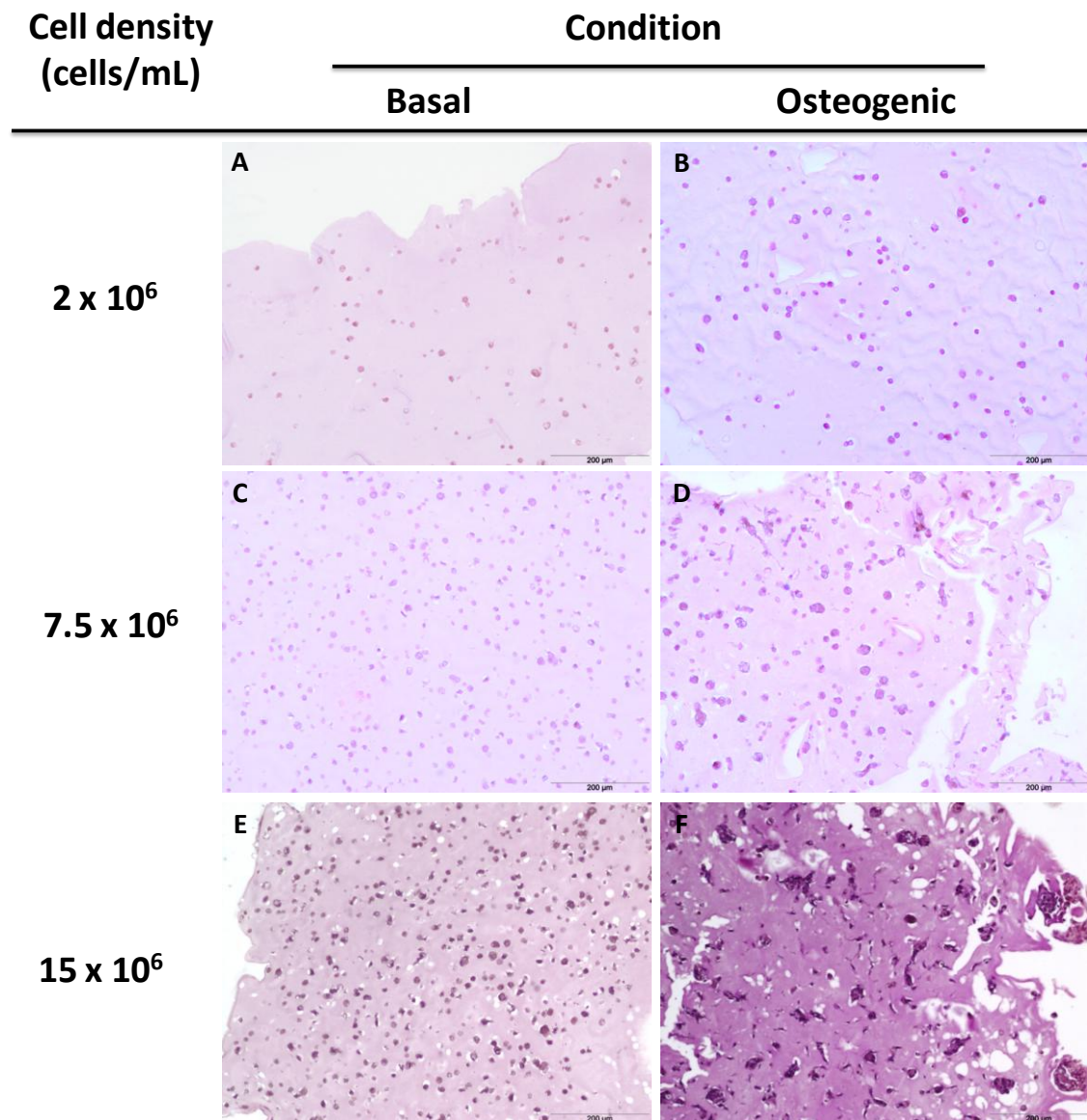


Figure 25. H&E-stained of paraffin sections (3 μ m) of 3D hMSC cultured at different cell densities (D1= 2×10^6 cells/mL, D3= 7.5×10^6 cells/mL and D4= 15×10^6 cells/mL) under basal (A, C, E) or osteogenic (B, D, F) conditions, for 14 days Scale bars 200 μ m.

The presence of collagen on day 14 samples was analyzed in paraffin sections stained with Masson's trichrome (MT), which stains collagen in blue, nuclei in dark red/purple and cytoplasm in red/pink. The differences in collagen deposition in basal versus osteogenic conditions at different cell densities were compared (Fig. 26). In basal conditions, collagen deposition was not detected at none of the cell densities tested (Fig. 26A, C, E). The same was observed for cells cultured at the lower cell densities under osteogenic conditions (Fig. 26B, D). However, in samples of high-density cultures (D4) under osteogenic conditions (Fig. 26F) some collagen deposition was detected (blue), mainly within large cell aggregates.

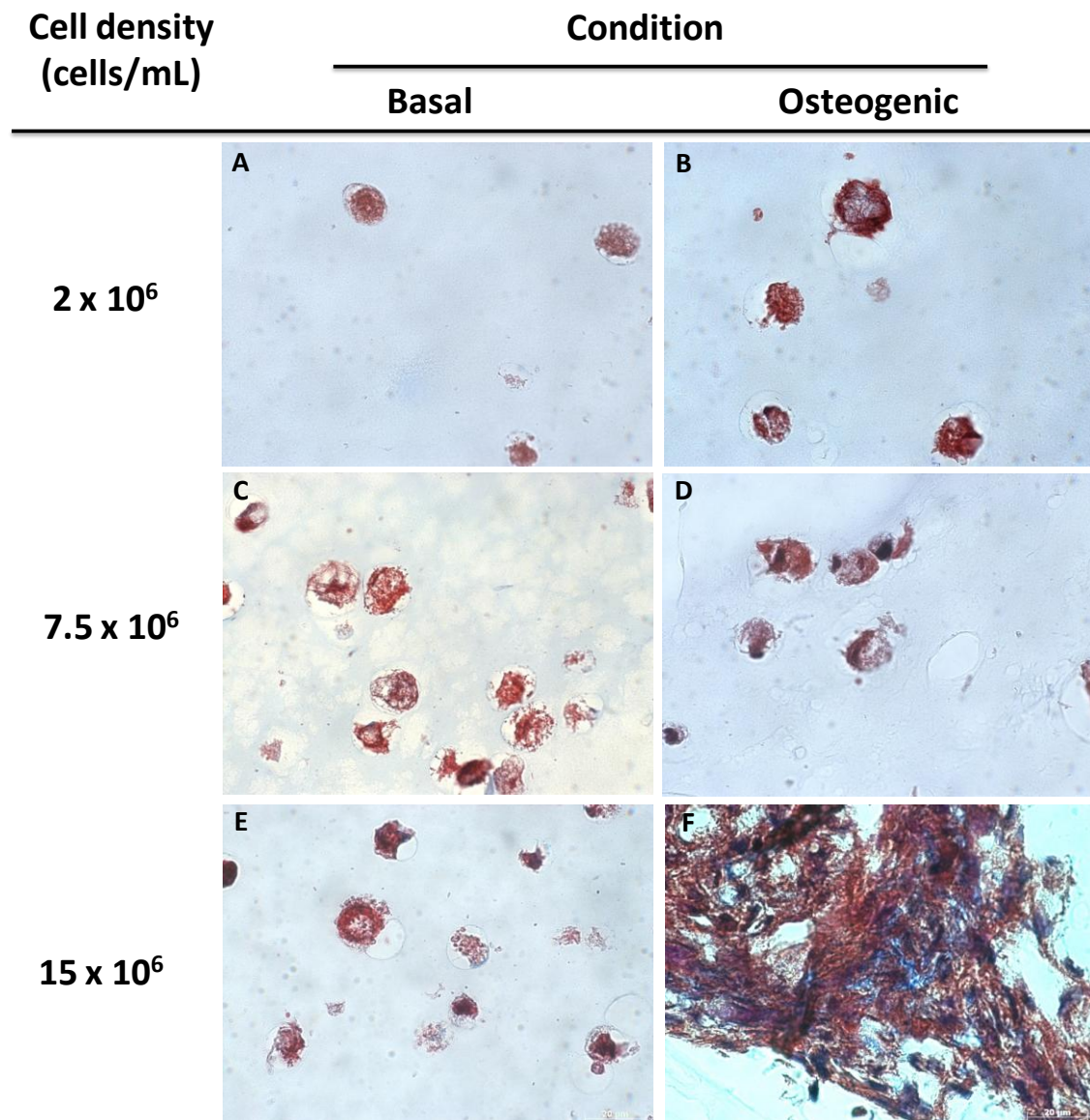


Figure 26. MT-stained paraffin sections (3 μ m) of 3D hMSC cultured at different cell densities (D1= 2×10^6 cells/mL, D3= 7.5×10^6 cells/mL and D4= 15×10^6 cells/mL) under basal (A, C, E) or osteogenic (B, D, F) conditions, for 14 days. The collagen appears in blue, nuclei in dark red/purple and cytoplasm in red/pink. Scale bars 20 μ m.

Cell-secreted ECM, which is known to stabilize scaffolds against degradation [63], may have also contributed to the previously described enhanced integrity of the high cell density matrices upon osteogenic culture. In some studies the higher amount of endogeneous ECM deposition from fetal chondrocytes was related with a high cell density culture [129].

Since the more promising results were obtained at the highest cell density used (15×10^6 cells/mL), subsequent studies were performed using only this density and the

behavior of 3D hMSCs upon osteogenic induction conditions was further investigated. Results are presented in the following section.

3.3.1. Osteogenic differentiation of hMSC cultured at high density in 3D alginate matrices

Despite the more interesting results obtained in this study, and the greater potential for tissue engineering applications, high-density cell cultures frequently exacerbate oxygen and nutrients limitations causing a reduction in cell viability. Therefore, the viability of high-density 3D hMSC cultures under basal and osteogenic conditions was assessed through the Live/Dead assay by confocal microscopy along 2 weeks of culture. The Z-projection of CSLM images are shown in Fig. 27. Live cells are stained in green while dead cells are stained in red. No significant differences on cell viability were detected along the time, either under basal or osteogenic conditions. Importantly, no apparent differences in cell viability were observed between the core and the periphery of the matrices, suggesting that the hydrogel network posed no limitations to the diffusion of nutrients and metabolites into and out of the constructs. To characterize cell morphology and cytoskeleton re-organization upon 3D-culture under basal and osteogenic conditions, F-actin of cells within alginate matrices was labeled in green with phalloidin-conjugated AlexaFluor 488, while nuclei were counterstained in red with EthD-1 (Fig. 28). At the onset of the culture, hMSCs were homogeneously distributed as individual entities all over the alginate matrix. Under basal conditions, in accordance with the previous observations by histological analysis of H&E-stained sections, cells within the matrices remained isolated and maintained a round shape throughout the culture. In opposition, cell morphology was markedly different under osteogenic conditions. Cell spreading was observed from day 7 with the formation of extended F-actin stress fibers, as can be seen in more detail in Fig. 29A,B. Cells were able to form an intricate cell network, and also multi-cellular aggregates as previously observed.

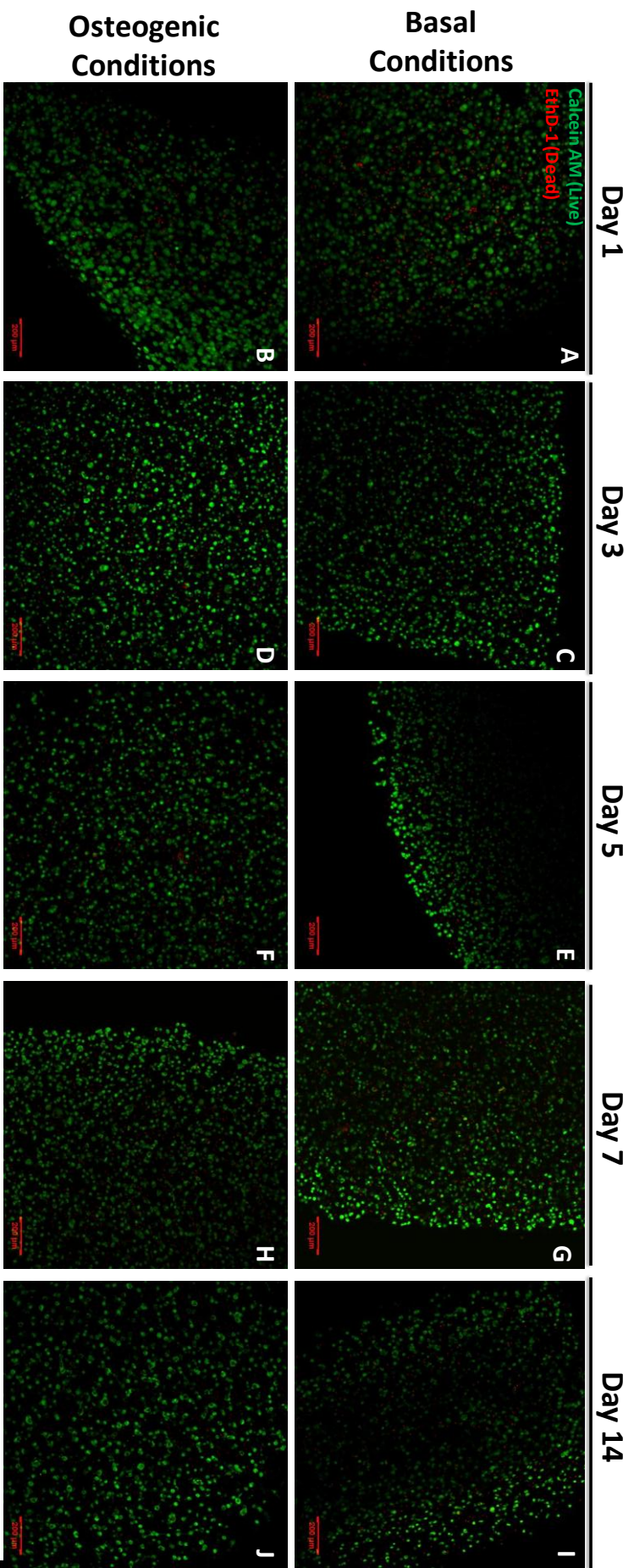


Figure 27. Live/Dead Cytotoxicity/Viability Assay CSLM images (Z-projection) of 3D hMSCs, at a density of 15×10^6 cells/mL, cultured under basal (A, C, E, G, I) or osteogenic conditions (B, D, F, H, J) for 2 weeks. Live cells are green and dead cells are labeled red. Scale bars 200 μm .

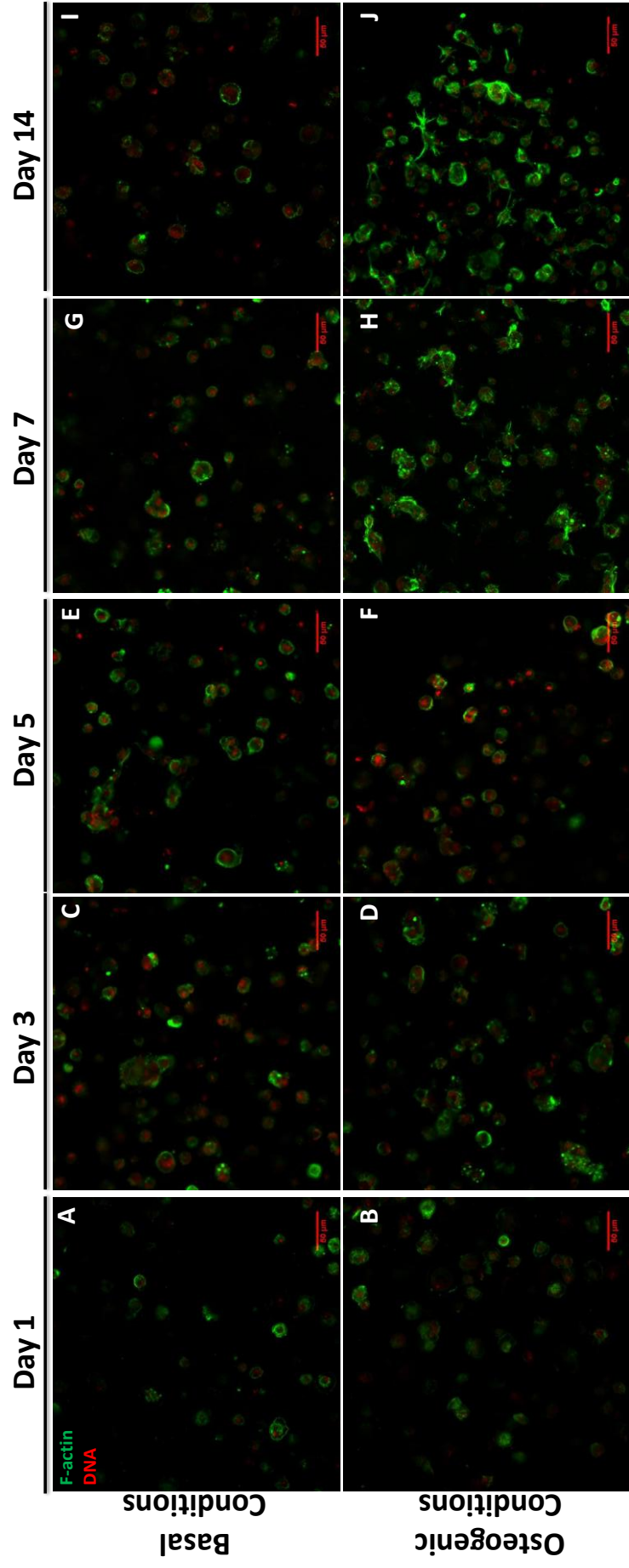


Figure 28. F-actin Staining CSLM images (Z-projections) of 3D hMSC, at a density of 15×10^6 cells/mL, cultured under basal (A, C, E, G, I) or osteogenic (B, D, F, H, J) conditions, followed for 2 weeks. F-actin is green and nuclei are counterstained red. Scale bars 50 μ m.

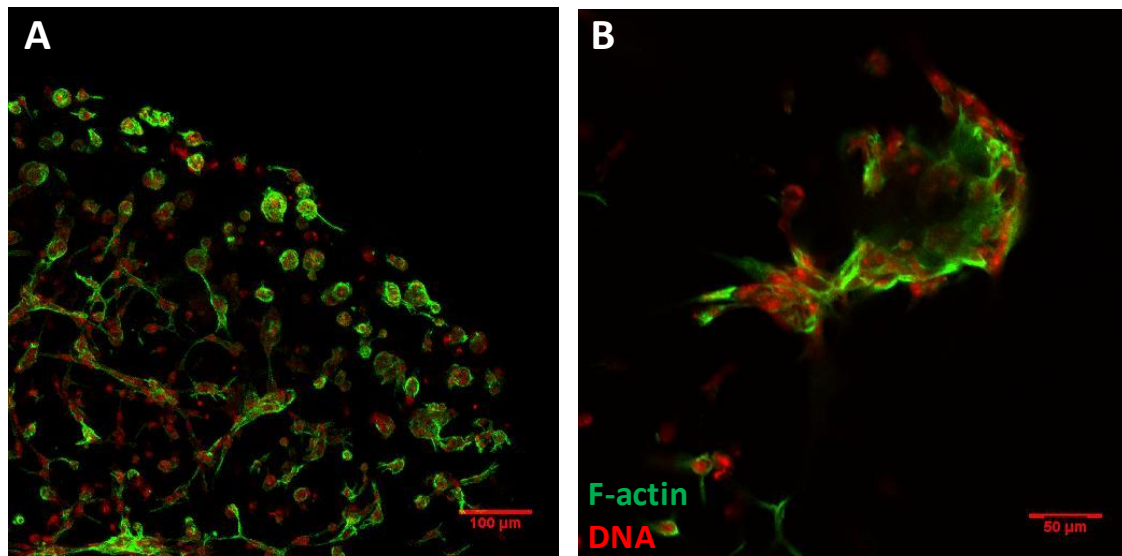


Figure 29. F-actin CSLM image (Z projection) of 3D hMSC at a cell density of 15×10^6 cells/mL cultured under osteogenic conditions at day 15, **(A)** showing an intricate cell network along the thickness of the alginate disc and **(B)** aggregated cells. Scale bars: A - 100µm; B - 50µm.

These cellular aggregates were further investigated under different aspects. They were easily detectable, even at a macroscopic level (Fig. 30A). In Fig. 30B, obtained under phase contrast microscopy at day 14, one cell cluster is shown to be formed by a core of multiple aggregated cells surrounded by well spread cells, bordered by cell-free hollow space with occasional connections with cells distant from the aggregate. These aggregates were found distributed within the matrix both at the core and at the periphery of the disc, as illustrated in Fig. 30C, D, where several round-shape smaller aggregates and one large cluster are depicted. In these larger cell aggregates (Fig. 30E, F), the deposition of an endogenous ECM was detected (MT-stained sections showing collagen in blue) (Fig. 30F, G). In Fig. 30F it is possible to see the considerable size of the cell aggregate and the distribution of the surrounding cells. These cells are spread and disposed radially relative to the aggregate, leading to the hypothesis of migration of these surrounding cells towards the aggregate.

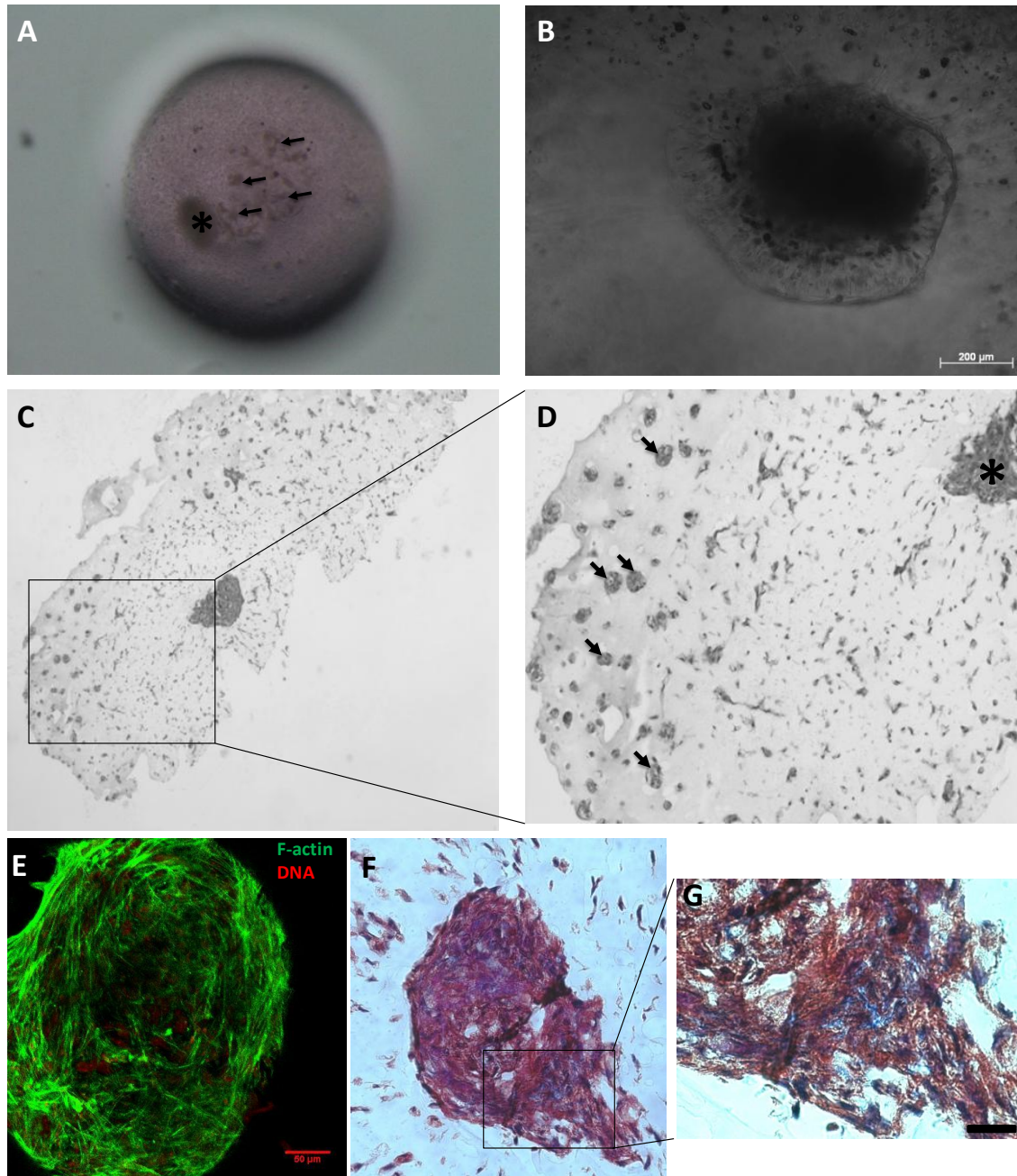


Figure 30. Cell aggregates seen in 3D hMSC cultured at high cell density (15×10^6 cells/mL) under osteogenic conditions. (A) Macroscopic view at day 7 (Stereoscope image, original magnification 6.3x). **(B)** Phase contrast images at day 14. Scale bar 200 μ m. **(C)** H&E-stained section at day 14 (Original magnification 4x) and **(D)** detailed view from a specific area (Original magnification 10x). **(E)** F-actin CSLM image (Z projection) at day 15. **(F)** Masson's trichrome staining of paraffin sections (3 μ m) at day 14. Series of overlapping tiled images acquired over a defined area (MozaiX application for AxioVision Software) of a cell aggregate and **(G)** a detail of an original 100x magnification image. (*) and arrow represent different multicellular aggregates with different morphological characteristics.

To better understand the origin of these aggregates, whether cell clusters arose essentially from cellular migration and subsequent aggregation as opposed to mitosis, an immunostaining for the cell cycle-related proliferation marker Ki67 in paraffin sections was performed. The Ki67 antigen is present during all active phases of the cell

cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). As depicted in Fig. 31, no Ki67 positive cells were observed within the larger cell aggregates, except for occasional situations, as shown in detail of Fig. 31B. Therefore, the hypothesis of cell migration followed by cell–cell adhesion of neighboring cells with subsequent aggregate formation within the matrix seems convincing. This suggests that the higher cell-cell signaling combined with the formation of some voids in the matrix along the culture may have enabled cells to migrate to some extent. This possibility will be further investigated in future studies. This result further supports the previous hypothesis that 3D hMSCs were in a non-proliferating state.

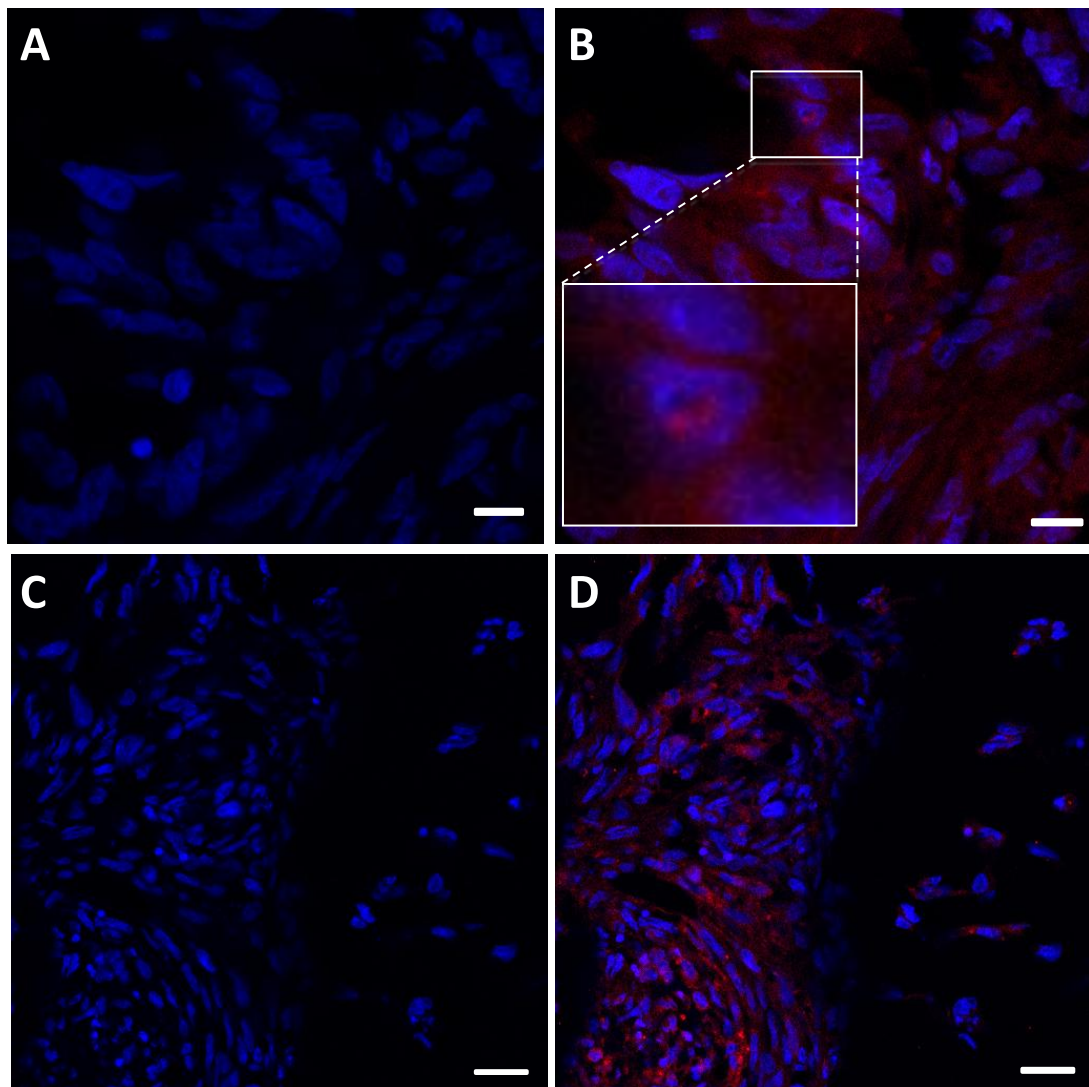


Figure 31. Immunostaining for Ki67 proliferation marker on paraffin sections (3μm) of cell aggregates observed in osteogenic conditions for 15×10^6 cells/mL density, at day 14. **(A, C)** Nuclei were stained blue with Dapi, **(B, D)** Ki67 is red. No Ki67 positive cells (stained nuclei) were detected, only non-specific staining in the cytoplasm. A punctual situation of Ki67 positive staining is seen in detail of B. Scale bars: A, B: 10μm; C, D: 30μm

The specific reasons underlying the formation of large cell aggregates in high-density 3D cultures under osteogenic conditions is not completely clear. It probably arose from a combination of factors, namely the higher degree of cell-cell interactions and the gradual degradation of the hydrogels under osteogenic conditions. Studies of Ryan and colleagues have shown that there is a competition between cell-substrate adhesion and cell-cell adhesion, which leads to the formation of cell aggregates when those cell-cell contacts are predominant [130]. The growth of cell clusters, presumably arising from the migration and aggregation of neighboring cells, was presumably facilitated by the formation of voids along the bulk hydrogel due to a gradual loss of the ionic crosslinks.

Similar phenomena of cell-aggregation within 3D hydrogel matrices have already been reported by other authors [63, 131-134]. For example, Maguire et al. described the aggregation of hepatocytes within alginate beads [134], and concluded that the aggregation process is a dominant mechanism underlying cell differentiation within the hydrogel bead microenvironment. Moreover, they suggest that changes in alginate concentration and cell seeding density could be used to modulate differentiation by altering the aggregation process.

In a very recent study, Zhang C et al. showed that chondrocytes encapsulated in PEG hydrogels containing oligo(trimethylene carbonate) (OTMC) moieties underwent spontaneous aggregation during *in vitro* culture [63]. The aggregation of cells was found to be dependent on the initial cell density, as well as the mesh size of the hydrogels. A higher cell density resulted in an earlier onset of cluster formation, as well as larger clusters. In addition, the same study demonstrates that the aggregation of cells in some of the tested hydrogel formulations resulted in enhanced endogenous matrix production, which is in accordance with our results.

Studies of Boland *et al.* using cell printing techniques have also seen this cellular aggregation driven by cell-cell contact, when aggregates are placed closely within collagen gels resulted in the fusion of adjacent cell aggregates, with a sequential formation of elongated rod-like tissue constructs [132], highlighting the high cell density effect.

In Lin and coworkers' studies on the formation of multicellular spheroids, the steps leading to the formation of such structures were proposed as being initiated as an early aggregation by cell surface integrins promoted by RGD motifs of ECM fibers, followed by up-regulated cadherin expression in a delayed phase and finally, cell adhesion through homophilic cadherin-cadherin binding between cells conferring strong cell adhesion, obtaining these spheroids [135]. The same study safeguards the usage of

these aggregates for tissue engineering strategies, since they may function as building blocks for tissue reconstruction [133].

In an attempt to better characterize the production of endogenous ECM by entrapped hMSCs, some preliminary immunostainings for fibronectin were also performed as shown in Fig. 32. As observed for collagen, an higher fibronectin amount was detected in osteogenic conditions, especially around cell within large cell aggregates.

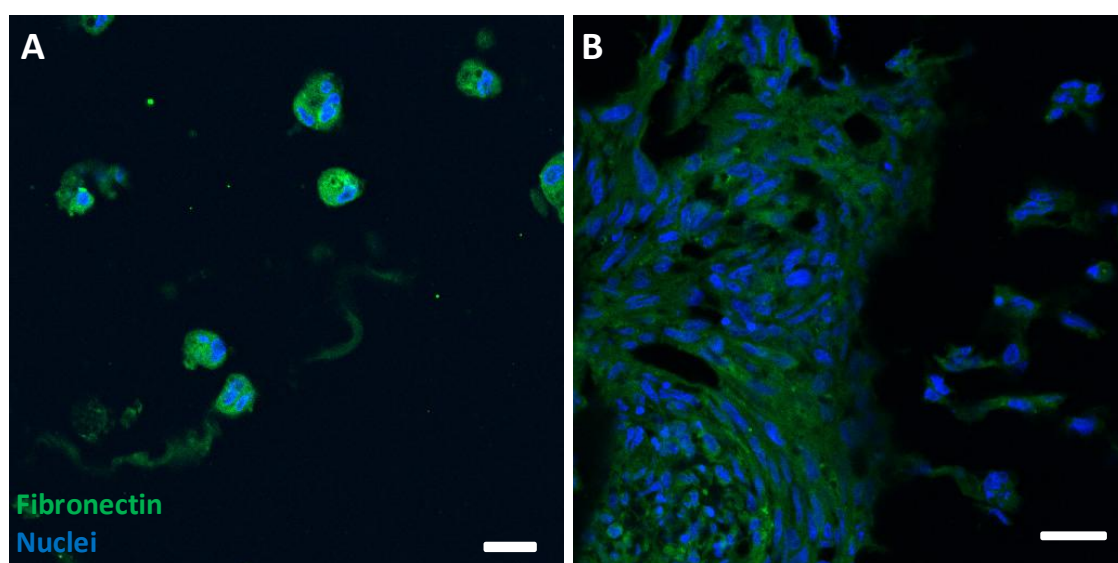


Figure 32. Immunostaining for Fibronectin of paraffin section (3 μ m) of 3D hMSCs within alginate matrices at a cell density of 15×10^6 cells/mL at day 14, cultured under **(A)** basal conditions and **(B)** osteogenic induction conditions. Scale bars: A -10 μ m; B-30 μ m.

As already described, the presence of collagen, a common bone phenotypic marker, was detected in high-density 3D cell cultures. The expression of alkaline phosphatase (ALP), another early marker for osteoblastic differentiation was also analyzed and the results are depicted in Fig. 33. At day 14, samples of 3D-cultured hMSCs under basal conditions (Fig. 33A) were negative for ALP staining, while cells cultured under osteogenic conditions, stained positive for this marker (Fig. 33B, pink color), which was particularly intense within multi-cellular aggregates. Altogether, results regarding collagen and ALP expression in high-density 3D cell cultures, suggest that hMSCs remained responsive to external stimuli upon entrapment within alginate hydrogels, and able to differentiate along the osteoblastic lineage upon induction. In future studies, the expression of other bone phenotypic markers will have to be analyzed and quantified.

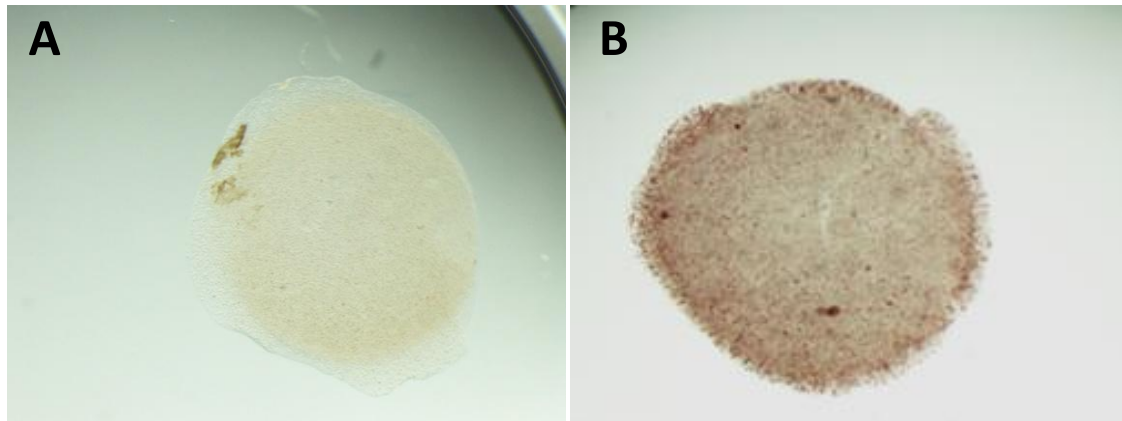


Figure 33. ALP staining of high-density 3D hMSCs within alginate matrices after 14 days of culture under **(A)** basal conditions and **(B)** osteogenic conditions. A significantly high ALP expression was only detected in cells cultured under osteogenic conditions. Images were obtained with a stereoscope (original magnification 20x).

IV. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

IV. Concluding Remarks and Future Perspectives

The focus of the present study was to evaluate the effect of different culture conditions, namely the initial cell density, on hMSC behavior under 3D conditions. For that, RGD-functionalized alginate hydrogels were used as 3D matrices, and the response of hMSC after entrapment was analyzed at different levels.

RGD-alginate hydrogels have been extensively used for the 3D culture of different types of cells and numerous studies can be found in the literature [41-43, 47, 48, 72, 117, 118, 136]. Different parameters are now recognized to influence cell response in alginate 3D cultures, including several matrix-related factors such as composition, mechanical properties (stiffness), and degree of crosslinking. The influence of the initial cell entrapment density has however been poorly investigated until now.

In this study, MSC entrapment densities in the range of 2 to 15 million cells/mL of gel were tested. In the literature, most studies in 3D used lower cell densities (1 to 5 million cells/mL [43, 113-116]). However, increasing cell-densities are likely to favor the establishment of cell-to-cell contacts and to promote intercellular communication, consequently triggering a wide number of important signaling mechanisms. In fact, contrary to what is generally observed in traditional 2D-cultures, cell-cell interactions may be hindered in 3D, if cells are entrapped at low cell densities, which turns crucial the optimization of the initial cell density [83].

While in 2D the effect of initial cell density in cell behavior has been widely studied [137, 138], in 3D that parameter has only recently been explored. One example of muscle-derived cells cultured in 3D collagen gels shows that different higher cell densities (8 million cells/ml) promote myoblast fusion but had only a little effect on matrix remodeling [139]. Another study shows that higher hMSC density (30 millions/ml) in 3D agarose hydrogels increase glycosaminoglycans (GAG) and collagen content when compared with a lower cell density (10 million/ml) [140]. This was also shown for mature chondrocytes, for which higher cell densities were related with higher tensile moduli and greater biochemical content [140]. However, if cell density is sufficiently high, an effect may not be seen among different cell densities, as demonstrated by the maintenance of GAG content when equine MSCs initial density increases from 25 to 50 million cells/ml in alginate hydrogels [141].

In the present study with RGD-alginate hydrogels, the highest cell density tested (15 million/mL) was the one that promoted a more interesting cell response, with significant

differences in relation to the other tested densities. At this density, cell viability was better maintained (remaining at ~80% for up to 2 weeks in culture), and cell-cell interactions as well as ECM deposition were promoted to a greater extent.

The levels of (mitochondrial) metabolic activity observed during all the experiments in 3D (independently of cell density or glucose availability) were much lower than in standard 2D conditions. However, cell viability remained high, and metabolic activity levels reverted when 3D cells were re-cultured in monolayer. MSCs metabolism is highly flexible, occurring either by aerobic oxidative phosphorylation (in the mitochondria) or anaerobic glycolysis, which is responsible for about 30% of total ATP generation [121]. The results obtained in this study demonstrated “normal” glucose consumption levels coupled with low (mitochondrial) metabolic activity in 3D hMSC cultures, which suggests a predominance of the anaerobic metabolism, in opposition to 2D, in which high mitochondrial metabolic activity is observed. However, this is only a hypothesis and the underlying reasons are not clear. Since cell metabolism can be affected by different factors (e.g., glucose, glutamine or oxygen) it would be important to evaluate them in order to better understand the observed behavior.

Although glucose starvation after day 4 was observed in our first studies, viability in 3D alginate hydrogels remained relatively high throughout one week of culture. It is known that MSCs metabolism is also highly adaptable under stress conditions (low oxygen or low glucose), which is a key factor for the maintenance of high survival rates [142]. For example, MSCs have been shown to survive better to ischemic environments (low glucose, low oxygen, high lactate) than cardiomyocytes, being also able to maintain its multi-differentiation capacity [142]. Nevertheless, since it has also been described that MSCs survival and function can be dramatically reduced under certain situations of severe glucose depletion [143], different strategies to overcome glucose (and possibly other nutrients) starvation were attempted (Annex I and II). In the end, low-glucose medium was used, as recommended for maintaining hMSCs in 2D culture [144], and glucose availability has been improved by increasing the frequency of medium exchange.

Proliferation of 3D-cultured MSCs was also evaluated. The maintenance of the total cell number and DNA content along 1 wk of culture suggested that entrapped cells entered in a non-dividing state. This may be related with space restrictions imposed by the alginate network, leading to changes in the cell cycle, an hypothesis that has already been raised by other authors, using alginate and other protease-insensitive polymers such as RGD-PEG [43, 123]. Those studies highlighted that proliferation in

3D, compared to 2D cells require strategies to overcome the physical impediment posed by the matrix. These restrictions may induce growth-arrest, stimulating cell senescence or favoring quiescent-like steady-states. The second hypothesis seems more plausible, since cells maintained high viability throughout the culture period, and re-gain the ability to proliferate after being transferred from 3D to 2D cultures, pointing out to the reversibility of the observed mitotic inhibition, already described [117]. These hypotheses must however be confirmed in futures studies, namely through an exhaustive analysis of the cell-cycle in 2D vs 3D by flow cytometry, and by looking at different cell bioactivity markers by Western Blot.

At the highest cell density, and when cultured under osteogenic conditions, the behavior of 3D-cultured hMSCs differed significantly from that observed for the other densities. In fact, after 2 weeks of culture, MSCs were shown to adopt a more spread shape, forming cellular networks inside the hydrogels and even multicellular aggregates.

The formation of multicellular aggregates under those specific conditions (high density osteogenic cultures), presumably resulting from the migration and intercellular adhesion of previously isolated cells, was an unexpected observation in this study. Of notice was the fact that such aggregation was apparently related with the production of endogenous ECM. This phenomenon showed that the nature of network structures and crosslinks influenced the mechanism cells employed to overcome the physical barrier imposed by the hydrogel. In fact, cells remained dispersed as individual entities during the first days of culture, but as the matrix degraded and voids were created, cells increased the ability to move inside the matrix and to migrate, possibly using an “ameboid-like” mechanism [5].

The apparent relationship between hMSCs aggregation and osteogenic differentiation potential remains unclear. From classical 2D studies, there is some evidence that a 3D microenvironment is important for bone formation in vitro. For example, the formation of bone nodules associated with the expression of a mineralized ECM in MSC cultures [145], indicates the importance of 3D cellular interactions for osteogenesis. However, information about osteogenic differentiation of hMSCs aggregates is still scarce. There has been a series of publications on aggregation of MSCs either as a procedure for enhancing chondrogenic differentiation of the cells or to increase their therapeutic potential [131, 146, 147]. Only a few studies evaluated osteogenic differentiation on 3D hMSC aggregates, and those were more specifically carried out using spheroids. For example, a 3D spheroid culture system on micropatterned substrates has been shown

to significantly improve the osteogenic differentiation of MSCs, as compared with 2D [148]. In another study [149], a technique for creating homogeneous hMSC spheroids has been optimized, and the resultant aggregates were successfully induced to undergo osteogenic differentiation. The authors propose that those 3D systems better mimic the *in vivo* physiological conditions, where cells are surrounded by other cells or fibrous layers, bridging the gap between conventional monolayer cultures and tissues.

In this study, aggregated hMSCs were shown to be able to produce endogenous ECM, as demonstrated by collagen deposition and fibronectin production, and stained intensively for ALP, a commonly used bone phenotypic markers. To our knowledge, there are no other studies in the literature showing the deposition of endogenous ECM in osteogenic MSC cultures under real (hydrogel-like) 3D conditions. To consistently demonstrate that cells effectively differentiate along the osteoblastic lineage, other specific bone phenotypic markers should be analysed and quantified in future experiments.

As a conclusion, this study demonstrated that, when cultured at high-density within RGD-alginate hydrogels, hMSC remained viable, produced their own ECM and could be successfully induced to differentiate along the osteoblastic lineage. The 3D matrices could be formed *in situ*, and degraded over time under osteogenic conditions, eventually releasing the entrapped cells. Overall, these observations highlight the potential of these hydrogels as injectable hMSC-delivery systems for bone regeneration applications, and put in evidence the importance of selecting the most appropriate cell entrapping density for a given application. Moreover, this study emphasizes the use of these hydrogels as *in vitro* platform for cell behavior studies, which better mimic 3D architecture of living tissues than standard 2D culture plates. Several important questions remained however unanswered and further studies should be carried out to address them.

V. REFERENCES

V. References

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ANNEXES

Annex I. Culture of hMSC in 3D alginate matrices under dynamic conditions

To evaluate if hMSC within 3D alginate matrices become subjected to diffusional limitations (nutrients, O₂, metabolites), some studies were performed under dynamic conditions. With this purpose, a standard spinner-flask (Bellco, 25 ml) was modified with a sample holding device, which was specifically designed at INEB for 3D cell within hydrogel-like matrices (currently under patent application). This device has multiple compartments that can accommodate several discs in independent chambers, protecting these fragile cell-loaded matrices from the shear stress induced by mechanical stirring. The metabolic activity of hMSC cultured in 3D hydrogels under static vs. dynamic conditions was compared. To guarantee similar glucose availability, the ratio $V_{\text{medium}}/V_{\text{disc}}$ was maintained between the two conditions: 1 disc/4ml of culture medium in static and 6 discs/24 ml of culture medium in the spinner flask. Two different initial cell densities ($D3=7.5 \times 10^6$ cells/mL and $D4=1.5 \times 10^6$ cells/mL) and different stirring speeds (15, 50 and 100 rpm) were tested. Cells (mitochondrial) metabolic activity was evaluated at day 7. The results are summarized in Fig 34.

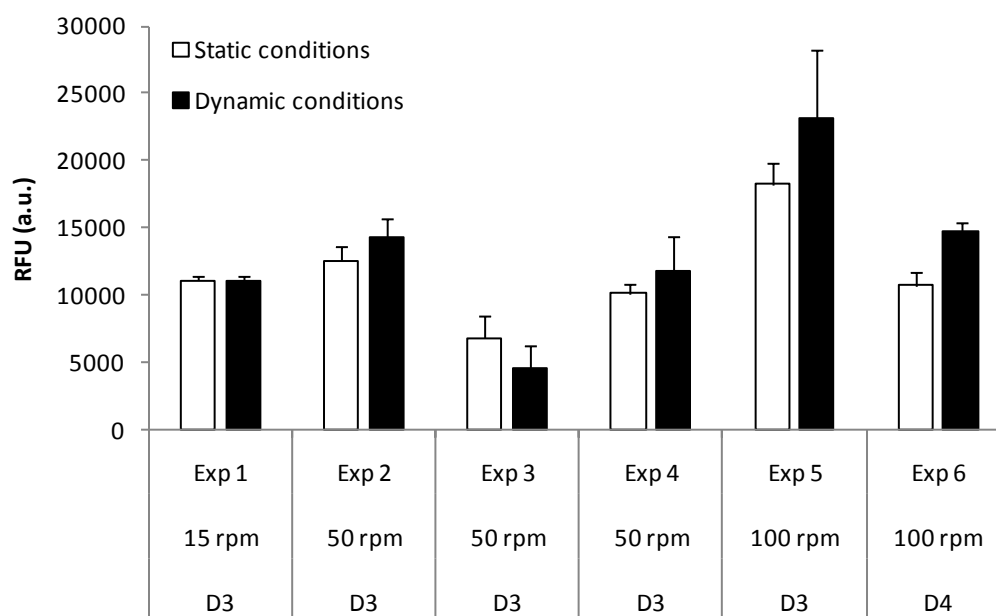


Figure 34. Metabolic activity of 3D hMSCs cultured for 7 days at different cell densities ($D3=7.5 \times 10^6$ cells/mL and $D4=1.5 \times 10^6$ cells/mL), under static or dynamic conditions (stirring speed: 15, 50 or 100 rpm). Results shown concern to day 7 of culture, and different experiments are shown (Exp 1 – 6).

In all the experiments no significant or consistent differences were observed between cells cultured under static vs dynamic conditions, and the metabolic levels remained lower than the ones typically observed for an equivalent density of cells cultured under 2D conditions (data presented on the Results section). It could be concluded that the lower metabolic activity on 3D vs. 2D cultures were not a consequence of diffusion limitations within the 3D matrices. Therefore, and for practical reasons, all the subsequent studies were carried out under static conditions.

Annex II. Effect of glucose concentration and feeding regimen on high-density 3D hMSC cultures in alginate matrices

Ideal medium formulations for culturing hMSCs in traditional 2D (tissue-culture polystyrene) monolayers cultures have been widely described in the literature. In particular, low cell seeding densities, low glucose concentration, presence of Glutamax and the appropriate fetal calf serum revealed to be effective for the isolation and expansion of multipotent hMSCs [144, 150]. Our team has been routinely using low-glucose DMEM medium (with the adequate supplements) in 2D hMSC cultures, which has demonstrated to be efficient not only for MSC expansion but also to induce osteogenic differentiation. For this reason, the first studies of this work were performed with this glucose concentration (1g/L). In a first approach, the same medium was maintained along 1 week of culture. However, since in 3D hMSCs cultures, glucose-depletion (only at the higher densities, D3 and D4) and low mitochondrial metabolic activity (independently of the cell density) were observed along one week of culture (even if the viability remained high), the effect of glucose concentration and the feeding regimen were addressed.

High-density (D4: 15×10^6 cells/mL) hMSCs-loaded alginate matrices were cultured with low-glucose DMEM (LGluc, 1g/L) and high-glucose DMEM (HGluc, 4.5 g/L) along one week of culture. In both conditions, the medium was exchanged at days 3 and 5. The glucose concentration patterns for the different conditions are depicted in Fig. 35.

In low-glucose medium, glucose-depletion was already observed after 3 days of culture, but the subsequent medium exchanges at each 2 days proved to be sufficient in preventing further glucose starvation. When high-glucose medium was used; glucose was consumed at similar rates, but never falling below 2.8 g/L.

The effect of glucose concentration and feeding regimen in both cell viability and metabolic activity of hMSC in 3D matrices was then analyzed. At day 1, a viability ranging from $80.8 \pm 1.5\%$ (HGluc) to $86.2 \pm 1.3\%$ (LGluc) was observed (Fig. 36A), and was fairly maintained during the 7 days in culture for the two conditions tested. Concerning the mitochondrial metabolic activity, it can be observed from (Fig 36B) that immediately after entrapment (day 0), the fluorescence signal was within the same range of values obtained for this cell density in the previous studies (Fig 18). During the following 7 days, the metabolic activity decreases to approximately 70% of its initial value, which was slightly better than the previously obtained results. However no significant differences were observed between low- and high-glucose media.

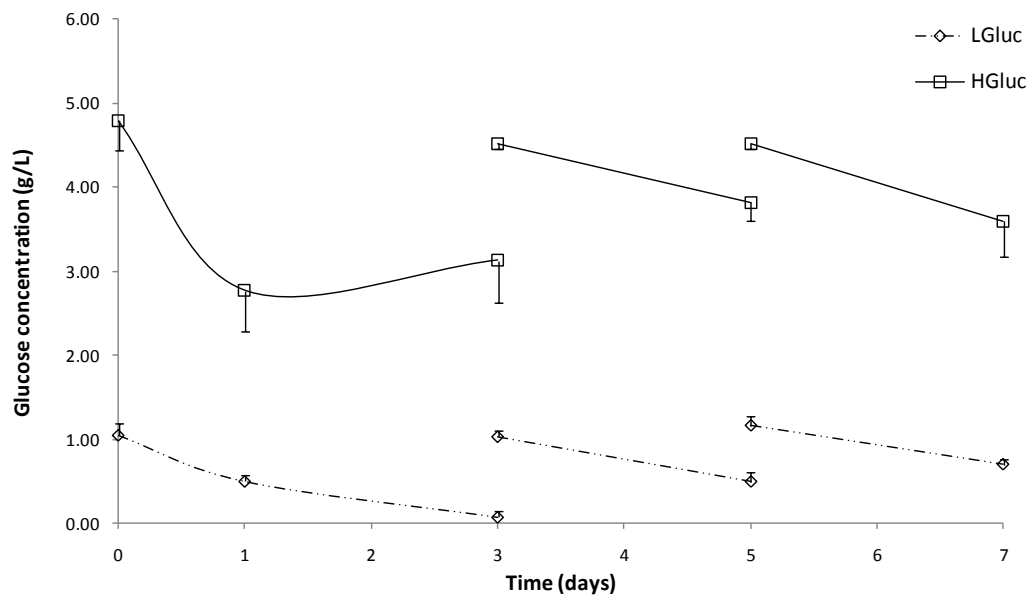


Figure 35: Glucose concentration in culture media of hMSC within alginate 3D matrices (15×10^6 cells/mL), kept in culture for 7 days. Cells were cultured in low-glucose media (LGluc) and high-glucose (HGluc) media with medium exchange at days 3 and 5.

From these results we concluded that the concentration of glucose in the medium does not significantly affect cell viability nor mitochondria metabolic activity in 3D-cultures. Studies from Weil and coworkers conclude that hMSCs may be resistant to the short-term effects of high glucose since proliferation and growth factor production in response to a chosen stimuli are unaffected by glucose concentration of the culture medium [151]. These results are in agreement with ours, where no differences in cellular behavior were observed.

Therefore, in subsequent studies, we opted to maintain the hMSCs in low-glucose medium, and exchanged the medium every 2 days to avoid periods of glucose starvation in 3D cultures.

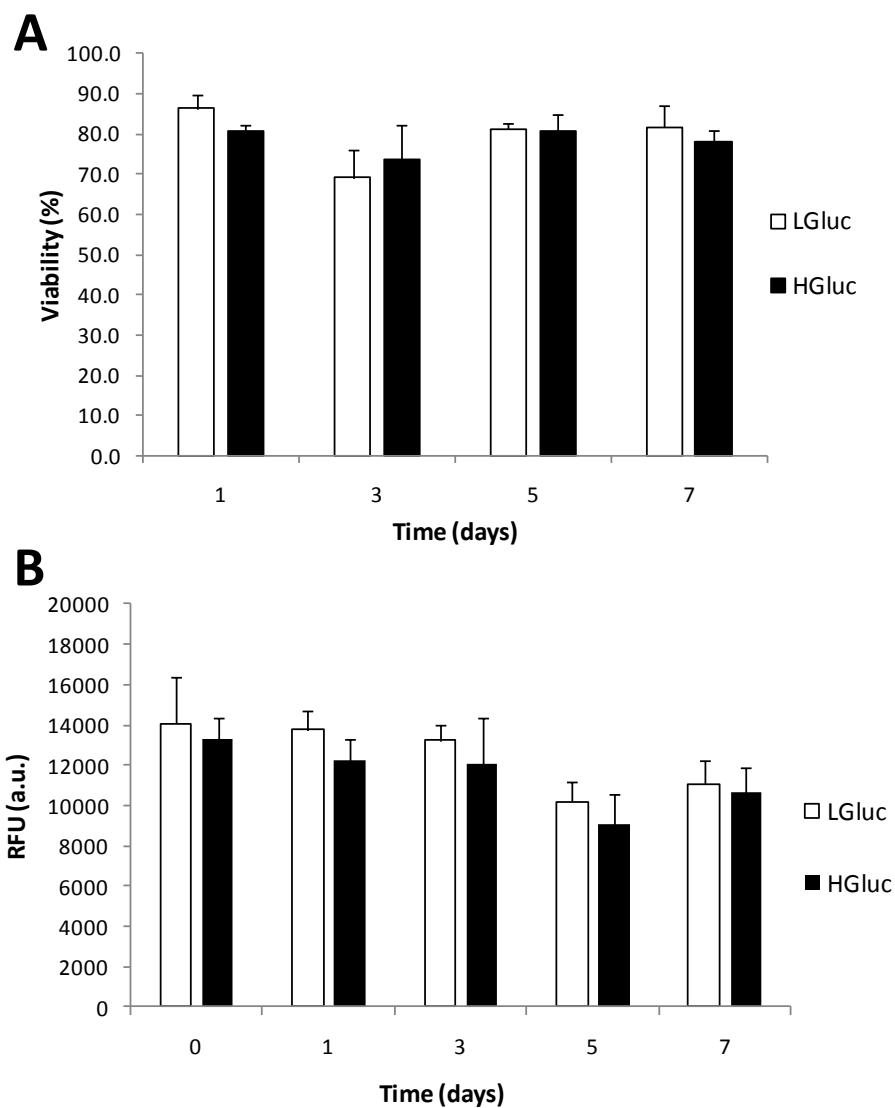


Figure 36. Viability (**A**) and metabolic activity (**B**) of high-density hMSCs cultured in 3D alginate matrices along 7 days of culture in low-glucose (LGluc) or high-glucose (HGluc) DMEM. The medium was exchanged at days 3 and 5. Results are presented as Mean $\pm$ SD.

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