



Bioengineering a 3D heterotypic tumor model to study the dynamics of endothelial cells recruitment and growth

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Dissertation for the Degree of Master of Science in Bioengineering at the Faculty of Engineering of the University of Porto and Abel Salazar Biomedical Sciences Institute of the University of Porto

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*“Nós nunca nos realizamos.
Somos dois abismos - um poço fitando o céu.”*

Bernardo Soares, Livro do Desassossego

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Abstract

In breast cancer, the influence of the microenvironment, namely extracellular matrix (ECM) mechanical properties and composition, and tumor angiogenesis, appear to have a key role in tumor progression and metastatic spread. The development of three dimensional (3D) *in vitro* models that, apart from the parenchymal compartment, also mimic both the tumor stroma and angiogenic processes, will certainly help clarifying signaling pathways and molecular patterns involved in tumor progression, including the role of vascular cells in all the process.

The arise of new approaches to study cancer biology with 3D systems using different materials is becoming the basis of the next generation of cancer research platforms. A 3D system makes possible to study tumor cells behavior in a more biologically relevant setting and can be used for more specific drugs screening to define treatments more accurately and personalized. In this context, the present work had the main objective of studying the dynamics of endothelial cells recruitment and growth in a bioengineered 3D heterotypic tumor model of human breast tissue.

In the first part we wanted to clarify the impact of tumor stiffness and low oxygen levels in endothelial cell mobility and recruitment. For this, a tumor-like 3D construct based on breast fibroblasts (hMFs) and cancer epithelial cells (MDA-MB-231) cultured within soft RGD-modified alginate matrices was established. Cell morphology and metabolic activity were monitored throughout time and compared to 3D monocultures of both cells. We also monitored the expression of carbonic anhydrase (CA9, a hypoxia sensor) and matrix metalloproteinases (MMP) production in these 3D tumor-like constructs, in response to altered stiffness and hypoxic conditions, during 2 weeks of culture. For each condition and time point, medium was collected and further used to evaluate paracrine effects on endothelial cells proliferation and migration. Overall, our data support the association between hypoxia or increased stiffness on endothelial cell recruitment.

Lastly, to allow a more reliable screen of the impact of soluble factors on vascularization, we optimized a vascular construct by establishing a co-culture of outgrowth endothelial cells (OECs) and hMFs on porous alginate scaffolds. We were able to observe organization of OECs in 3D capillary-like structures throughout the scaffold, as well as cellular outgrowth when constructs were embedded in both collagen type I and fibrin matrices.

In the future, a 3D tri-culture platform can be developed by combining the vascularized porous scaffold with hydrogel-embedded primary patient-derived cells, providing a platform for anti-angiogenic drug screening, in a precision medicine context.

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Table of Contents

Abstract.....	iii
Table of Contents	v
List of Figures	vii
List of Tables.....	xii
List of Abbreviations	xiii
1. Introduction	xvi
1.1. Triple Negative Breast Cancer: an overview	2
1.2. Tumor Microenvironment	3
1.3. Metastatic Process	5
1.4. Tumor angiogenesis/vascularization	6
1.5. 2D versus 3D	8
1.6. Bioengineering matrices for in vitro 3D breast tumor angiogenesis.....	8
1.7. Natural-based scaffolds	9
1.8. Synthetic-based matrices	10
2. Hypothesis, Strategy and Aims	13
3. Materials and Methods.....	15
3.1. Cell Maintenance.....	15
3.1.1. Primary cultures	15
3.1.2. Cell lines.....	15
3.2. Synthesis of RGD-grafted alginate	16
3.3. 3D culture of epithelial cells and hMFs in RGD-alginate matrices.....	16
3.4. Stiffness increase of Co-cultured-RGD-alginate matrices	17
3.5. Rheological measurements.....	17
3.6. Hypoxic culture of MDA-MB-231-hMFs 3D cultured in RGD-alginate matrices	18
3.7. Evaluation of alginate embedded cells metabolic activity	18
3.8. Isolation of cells from 3D RGD-alginate matrices.....	18
3.9. RNA expression quantification	19
3.10. Scratch/wound healing assay	19
3.11. Directed migration assay.....	20
3.12. Matrix metalloproteinases (MMPs) secretion by zymography	20
3.13. Preparation of RGD-grafted alginate scaffolds	21
3.13.1. Freeze-drying technique	21

3.13.2.	Freeze-drying associated with Particle leaching technique	21
3.14.	Characterization of RGD-grafted alginate scaffolds	22
3.15.	Seeding of human mammary fibroblasts and endothelial cells on RGD-alginate scaffolds	23
3.15.1.	Scaffold preparation	23
3.15.2.	Seeding approach.....	23
3.15.2.1.	Cell seeding on the top of the scaffold.....	23
3.15.2.2.	Cell seeding on the top of the scaffold followed by centrifugation	23
3.16.	Outward cell migration assay in fibrin or collagen I gel.....	24
3.16.1.	Collagen type I.....	24
3.16.1.1.	Collagen type I matrices	24
3.16.1.2.	Collagen type I sandwich assay	25
3.16.2.	Fibrin sandwich assay	25
3.17.	Immunostaining	26
3.17.1.	3D culture of epithelial cells and hMFs	26
3.17.2.	Transwell inserts.....	26
3.17.3.	Co-culture RGD-alginate scaffolds	26
3.17.4.	3D in vitro Sprouting Assay	27
3.18.	Statistical analysis	27
4.	Results and Discussion	29
4.1.	Synthesis of RGD-grafted alginate	29
4.2.	3D culture of epithelial cells and hMFs in RGD-alginate matrices.....	30
4.3.	Effect of increasing the stiffness of RGD-alginate matrices.....	33
4.4.	Mimicking hypoxic tumor core	35
4.5.	Evidences of ECM remodeling.....	39
4.6.	Paracrine effect of tumor-like constructs in endothelial cell recruitment	40
4.7.	Preparation and characterization of RGD-grafted alginate scaffolds	44
4.8.	Seeding of human mammary fibroblasts and endothelial cells on RGD-alginate scaffolds	47
4.9.	Cells seeded on RGD-alginate scaffolds outgrowth	51
5.	Concluding Remarks and Future Perspectives	55
6.	References.....	57
	Supplementary Information	67

List of Figures

Figure 1. Overview of relevant molecular alterations in triple-negative breast cancer (TNBC): The increased immunological infiltrate, a high proliferation rate, and homologous recombination deficiency are common themes in TNBC classification. Based on molecular results, important therapy strategies for TNBC are emerging and currently being tested in clinical studies: Immune therapy, Platinum-based chemotherapy, Poly(ADPribose) polymerase (PARP) inhibitor therapy. 3

Figure 2. Schematic illustration of a typical tumor microenvironment. Cancer cells reside in a complex microenvironment containing various supporting cells, extracellular matrix and a suite of signaling molecules..... 5

Figure 3. The sequential process of Cancer metastasis. Tumor cells undergo epithelial-to-mesenchymal transition (EMT) empowering cell migration and infiltration. Cancer cells intravasate into the vascular (A) and circulate throughout the body as circulating tumor cells (B). These cells survive the circulation, extravasate and invade distant tissues by reestablishing characteristics of their corresponding primary tumor, through the mesenchymal-to-epithelial transition (MET) (C). Cells that successfully adapt to their microenvironment and resume proliferation successfully form overt secondary tumors (D). Adapted from [60]..... 6

Figure 4. Simplified scheme of the interactions between tumor cells and endothelial system in the hypoxic tumor core. Tumor cells produce pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and pro-inflammatory cytokines stimulating endothelial cells to proliferate. Adapted from [66]. 7

Figure 5. Proposed 3D heterotypic tumor model to study the dynamics of cell recruitment and growth. Co-cultures of MDA-MB-231 and hMF are entrapped within RGD-modified alginate matrices and exposed to different culture conditions. Conditioned medium is collected from this tumor-like construct and its paracrine effect is evaluated in EC proliferation and migration in 2D. And later on, it will be assessed the impact of these soluble factors using a more biologically relevant vascular construct. 14

Figure 6. Experimental procedure for the preparation of the 3D cell-laden matrices. Small drops of cell-laden alginate were applied on top of a Teflon plate between two spacers (black

arrows) (A) and then another Teflon plate was placed on top of those spacers (B). After gelation, 3D matrices were obtained (C, D)..... 17

Figure 7. Schematic representation of the transwell directed migration assay..... 20

Figure 8. Schematic representation of the freeze-drying association with particle leaching technique. NaCl particles were fractioned and sieved to a particle size of 150-250 μm , and subsequently incorporated into the alginate precursor solution. After the first lyophilization, obtained cylindric scaffolds were placed in distilled water for 24h with agitation to leach out the salt particles, changing the water 3 times. Then leached samples were frozen at -20°C and freeze-dried again for 48h. 22

Figure 9. Experimental design for co-culturing OECs and hMFs in RGD-alginate scaffolds. Two strategies were tested: total cell seeding on the top of the scaffold (A), followed by centrifugation (B). 24

Figure 10. Experimental design to analyze cell capacity to outgrow from the alginate scaffold. Colonized scaffolds were incorporated within collagen type I (A, B) or fibrin gel (C). 25

Figure 11. Morphological and functional features of MDA-MB-231 cells, hMFs and co-cultures within 3D RGD-alginate matrix. (A) Representative CLSM images of spheroids formed after 7 days by MDA-MB-231 cells (a), hMFs (b), and co-cultures (c). (B) Representative CLSM images of spheroids formed after 14 days by MDA-MB-231 cells (d), hMFs (e), and co-culture (f). Spheroids were stained with DAPI (scale bar: 100 μm). (C) The metabolic activity of the different cultures showed an increase until day 7, followed by stabilization. Data is presented as mean $\pm$ stdev (n=3) (*p=0.0185, **, *** p=0.0004, and **** p<0.0001). (D) Fold increase of metabolic activity relative to day 1. 32

Figure 12. Experimental and theoretical prediction for the metabolic activity of cells within 3D matrix. It is assumed that as total cell density is the same (5×10^6 cells/mL), each cell type is responsible for half of the metabolic activity registered when in monoculture. Data are presented as mean $\pm$ stdev (n=3). 33

Figure 13. Rheological measurements. Viscoelastic properties (elastic, G' and viscous, G'' components of the shear moduli, and phase angle, δ) of 1 wt.% RGD-alginate with MDA-MB-

231-hMFs (A). To provide physiologically-relevant biomechanical cues, alginate hydrogels properties were tailored to attain viscoelastic properties ($G' \approx 200$ Pa) comparable to that of normal breast tissue and mammary gland, and alternatively ($G' \approx 3000$ Pa) close to mammary tumor stiffness. For that it was investigated which ionic crosslinker (Ca^{2+} , Sr^{2+} , Ba^{2+}) would drive to the desired stiffness increasing. Fold increase of matrix stiffness relative to control (B). 34

Figure 14. Metabolic activity of monocultures of metastatic breast cancer cell line (MDA-MB-231) and human mammary fibroblast (hMFs) and co-coculture of both cell types during 2 weeks of culture. Cell-laden hydrogels were stiffened at the first day of culture (light bars, stiff) or maintained in culture without changes (dark bars, soft). The pattern of metabolic activity observed in both stiffnesses is similar. Cells seems to be proliferating until day 7, then a slightly decreased occurs, remaining approximately stable until day 14. 35

Figure 15. Metabolic activity of monocultures of metastatic breast cancer cell line (MDA-MB-231) and human mammary fibroblasts (hMFs) and co-coculture of both cell types at (A) day 7 and at (B) day 14. Cell-laden hydrogels were stiffened at the first day of culture (light bars, stiff) or unchanged (dark bars, soft). Both matrices were maintained in normal culture conditions (21% v/v O_2 , 5% v/v CO_2 , Normoxia) or under hypoxia (1% v/v O_2 , 5% v/v CO_2). 37

Figure 16. qRT-PCR quantification of relative mRNA expression of CA9 (carbonic anhydrase 9, hypoxic marker). Data normalized to cells cultured for 7 days under normoxia conditions (Control). 38

Figure 17. Activity of MMP2 and MMP9 secreted by 3D cocultures of MDA-MB-231 and hMFs embedded within soft or stiff alginate matrices under normoxia or hypoxia conditions for 7 days. (A) Quantification of MMP2 and MMP9 secretion from 3 different experiments. (B) Gelatin zymography for the different conditions tested in 1 experiment. MMP9 band appears at around 92 kDa and MMP2 at 72 kD. (C) Fold increase of MMP2 and (D) MMP9 secretion relative to the total amount detected in soft matrices of 1 experiment. 40

Figure 18. Analysis of OECs migration/proliferation under different conditioned media by in vitro scratch/wound assay. The conditioned media was collected from co-cultures of MDA-MB-231 and fibroblasts culture in 3D alginate matrices for 7 days (D7) or 14 days (D14) in soft or in stiff matrices, under normoxia or hypoxia conditions. Percentage of wound closure percentage in all the conditioned media tested (A). Red dot line corresponds to the

percentage of wound closure when cells were culture only with DMEM (negative control). Green dot line corresponds to the percentage of wound closure when cells were culture with EGM-2MV (positive control). Wound appearance in the moment of the scratch (0h) and after 24h for the controls (B) and for the tested conditions, soft (C) and stiff (D) matrices. The white dot lines define the area lacking cells..... 42

Figure 19. Transwell migration assay towards conditioned medium. Transwell membranes were used to quantify OECs direct migration and analyzed under CLSM. Representative images were selected for each condition, positive control (EGM-2MV) and negative control (DMEM) (scale bar: 200µm). 43

Figure 20. SEM images of RGD-grafted alginate scaffolds horizontal cross-section. Two techniques were considered: freeze-drying (A), particle leaching (B). Pore size differences between freeze-drying (C) and particle leaching (D). Green arrows indicate interconnected pores. Two magnifications are shown, 35x (A, B), and 100x (C, D). 46

Figure 21. Cylindric scaffolds obtained after freeze drying. Using a scalpel blade, the obtained cylindric scaffolds were then cut into small ones with an average height of 1.5 mm. 46

Figure 22. Immunostaining of hMFs and ECs seeded on top of RGD-grafted alginate scaffolds after 14 days of culture. 2% wt. RGD-grafted alginate scaffolds were prepared by freeze drying technique. Two final cell densities were used: 50×10^4 cells/scaffold (A) and 25×10^4 cells/scaffold (B). Cytoskeletal organization of hMFs were observed in both sides of the scaffold (i, ii), under a CLSM and demonstrated by F-actin (magenta), laminin (green), CD31 (red) and DAPI (blue). (Scale bar: 100 µm). 48

Figure 23. Immunostaining of hMFs and OECs seeded on RGD-grafted alginate scaffolds at a cell density of was 50×10^4 cells/scaffold. 2% wt. RGD-grafted alginate scaffolds were prepared by freeze-drying technique with particle leaching (A, B) and without particle leaching (C, D). Cells were seeded on top of the scaffold (B, D), followed by centrifugation (A, C). Cytoskeletal organization of hMFs and OECs after 14 days of culture were observed under a CLSM and demonstrated by laminin, DAPI and CD31. CD31 and laminin markers were not visible in the scaffold prepared by freeze drying technique without particle leaching and cell-seeded with centrifugation (C). The dashed white line illustrates the limits of cross-

section of the scaffold. (Scale bar: 500 μm). Diagram illustrating the cross-section of the scaffold (E). Gray arrow points the plan from which CLSM images were taken. 50

Figure 24. Immunostaining of hMFs and OECs seeded on 2% wt. RGD-grafted alginate scaffolds prepared by particle leaching after 14 days of culture (A). After seeding 50×10^4 cells on top of the scaffold, it was performed a 5 minutes-centrifugation at 1000 rpm. Cytoskeletal organization of both cells were observed under a CLSM and demonstrated by laminin (green) and CD31 (red). The dashed white line illustrates the limits of cross-section of the scaffold. (Scale bar: 500 μm). Zooming 2x zone confined by dashed yellow line, it is possible to observe endothelial cells organization into tubular-like structures, near to scaffold pores (a, white arrow). 51

Figure 25. Cellular outgrow in collagen type I gels during 20 days of culture. hMFs-OECs-seeded alginate scaffolds cultured for 10 days were transferred to a collagen layer. Two different collagen type I concentrations were tested (2mg/mL, A-C and 3mg/mL, D-F). Images were obtained from inverted microscope (A, B, D, E), inverted fluorescence microscope (C), and CLSM (F).10x 52

Figure 26. Immunostaining of cell outgrowth of the scaffolds. hMFs-OECs-seeded alginate scaffolds, cultured for 7 days, were embedded between two layers of collagen type I (A), or fibrin gel (B). Cytoskeletal organization of the cells after 3 days of culture were observed in the scaffold (i) and in matrix (ii), under a CLSM and demonstrated by F-actin (green), and DAPI (blue). (Scale bar: 200 μm). 53

List of Tables

Table1. Bioengineered matrices for in vitro 3D breast tumor angiogenesis	12
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List of Abbreviations

3D - Three Dimensional

2D - Two Dimensional

ATP - Adenosine triphosphate

BCA - Bicinchoninic Acid

bFGF - basic Fibroblast Growth Factor

BRCA - Breast Cancer gene

BSA - Bovine Serum Albumin

CA9 - Carbonic anhydrase 9

CAF - Cancer Associated Fibroblast

CLSM - Confocal Laser Scanning Microscopy

CXCL12 - C-X-C Motif Chemokine Ligand 12

DMEM - Dulbecco's Modified Eagle Medium

EBM - Endothelial Basal Medium

EC - Endothelial Cell

ECM - Extracellular matrix

EDC - ethyl-(dimethylaminopropyl)-carbodiimide

EDTA - ethylenediaminetetraacetic acid

EGF - Endothelial Cell Growth Factor

EGM - Endothelial Cell Growth Medium

EHS - Englebreth Holm Swarm

EMT - Epithelial to Mesenchymal Transition

ER - Estrogen Receptor

FGF-2 - Fibroblast Growth Factor 2

FBS - Fetal Bovine Serum

FGS - Fibroblast Growth Supplement

FM - Fibroblast Medium

G4RGDSP - (glycine)4-arginine-glycine-aspartic acid-serine-proline

GA - GentamicinAmphotericin

GDL - Glucose delta-lactone

GLUT2 - Glucose transporter 2

HBSS - Hank's Balanced Salt Solution

hEGF - human Epidermal Growth Factor

HER-2 - Human Epithelial Growth Factor Receptor 2

HIF-1 α - Hypoxia-inducible factor 1-alpha

hMFs - Human mammary fibroblasts

HUVEC - Human Umbilical Vein Endothelial Cell

IARC - International Agency for Research on Cancer

IGF - 1 - insulin growth factor-1

LDH A - Lactate dehydrogenase A

LVR - Linear viscoelastic region

MET - Mesenchymal to Epithelial Transition

MMP - Matrix Metalloproteinase

MES - -(N-morpholino)ethanesulfonic acid

OECs - Outgrowth Endothelial Cell

ON - Overnight

PARP - Poly(ADPribose) polymerase

PDGF - Platelet derived growth factor

PEG - Polyethylene glycol

PFA - Paraformaldehyde

PR - Progesterone Receptor

qRT-PCR - Quantitative Real-Time PCR

RGD - Arginine-Glycine-Aspartic acid

RT - Room Temperature

SDF-1 - Stromal Cell-Derived Factor 1

SDS - Sodium dodecyl sulfate

SEM - Scanning Electron Microscopy

A-SMA - alpha Smooth Muscle Actin

Sulfo-NHS - N-Hydroxy-sulfosuccinimide

TBS - Tris Buffered Saline

TGF- β - Tumor Growth factor beta

TNBC - Triple Negative Breast Cancer

uPA - urokinase Plasminogen Activator

VEGF - Vascular Endothelial Growth Factor

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1. Introduction

Breast cancer is the leading cause of cancer death in women worldwide, leading to 626 679 out of 9.6 million of cancer deaths in 2018, according to the International Agency for Research on Cancer (IACR, 2019). In particular, the most aggressive subtype of breast cancer is Triple Negative Breast Cancer (TNBC). TNBC is characterized for the absence of receptors for the hormones estrogen (ER) and progesterone (PR) or the human epidermal growth factor receptor (HER)-2, making hormone therapy or HER-2-targeting drugs not eligible treatments. The main drug treatment used is chemotherapy, which carries the problem of chemotherapy resistance development within a few months by most patients.

TNBC affects mainly younger women, and despite all the progress in breast cancer diagnosis and treatment, most deaths are associated with the spread of malignant cells to distant sites of the body. This is a process denominated metastasis which leads to the growth of new tumors in secondary organs, being extremely difficult to cure [1]. Alarmingly, once the disease becomes metastatic, the median survival is around 12 to 15 months.

This subtype represents a minority (10-20%) of all breast cancer cases [2], however due to its poor prognostic, the need of cancer research directed to metastatic triple-negative disease, namely the angiogenesis role in this complex process, is crucial to better identify effective treatment options and improve patients outcome [2, 3].

Most of the cancer research performed in laboratory is based in two-dimensional (2D) cell culture systems, like plastic or glass surfaces, which is very limited since it does not represent human biology [4]. The arise of new approaches to study cancer biology based on three dimensional (3D) systems using different materials is becoming the basis of the next generation of cancer research platforms [5]. A 3D system makes possible to observe tumor cells behavior and can be used to more specifically screen for drugs in a way to make drug treatments more accurate and more personable [5]. It has been shown that most of the drugs tested in 2D exhibiting positive outcomes fail once they reach the clinic [6, 7]. 3D culture of tumor cells offers the opportunity of following their response to drug stimulation and the interactions that tumor cells establish with the surrounding environment [7]. This is not possible to address in 2D culture systems as the lack of a third dimension makes them unable to represent the whole surrounding tissue environment [7, 8].

Next generation breast cancer treatments could be designed based on the information about patient's cancer retrieved from 3D models. Hopefully, the ideal 3D breast cancer model will provide data regarding the aggressiveness level and the metastatic potential, and ultimately it will provide guidance for a more suited treatment to the patient.

1.1. Triple Negative Breast Cancer: an overview

Triple-negative breast cancer represents 10% to 20% of all breast cancers [2, 9, 10], being more prevalent in non-white African women, particularly in premenopausal women under the age of 50 years [9-12].

TNBC is characterized by a lack of expression of the main breast cancer biomarkers, namely estrogen receptor [13-16], progesterone receptor [8, 16], or lack of Human Epidermal Growth Factor Receptor 2 gene overexpression and/or amplification [17-19]. Subsequently, the disease presents a clinical challenge since this breast cancer subtype does not respond to endocrine or anti-HER2 therapy [20, 21]. For that reason, chemotherapy is the only conventional therapeutic approach currently available [13, 16, 22]. The therapeutic strategies are moving towards neoadjuvant studies [23] involving the administration of chemotherapy drugs before surgery [24]. This approach seems to be very effective in a restricted minority of women with TNBC, but for the majority of TNBC patients the outcome is unlucky [16, 23].

Despite of TNBC being a highly heterogenous disease [25], the study of gene expression profile, histopathology, and genomic alterations brings some common and recurrent features (Figure 1) [16, 19, 26]. Regarding clinical and pathological aspects, TNBC is widely associated with a high proliferation rate, an increased immunological infiltrate [13, 26, 27], a basal-like and a mesenchymal phenotype [13, 28, 29], and a deficiency in homologous recombination [30], which is associated with the loss of BRCA1 or BRCA2 function [13, 31, 32]. Concerning the metastatic potential in triple-negative breast cancer, this phenomenon tends to be more aggressive and prone to visceral tissues, including brain and lungs, rather than other breast cancer subtypes [33].

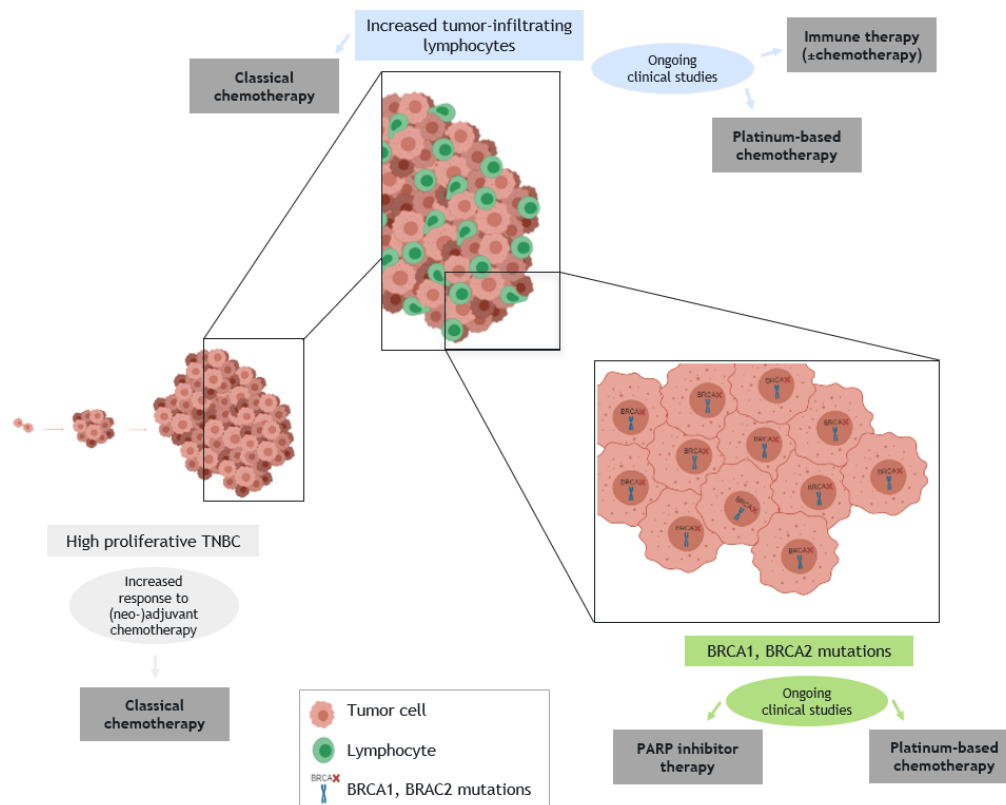


Figure 1. Overview of relevant molecular alterations in triple-negative breast cancer (TNBC): The increased immunological infiltrate, a high proliferation rate, and homologous recombination deficiency are common themes in TNBC classification. Based on molecular results, important therapy strategies for TNBC are emerging and currently being tested in clinical studies: Immune therapy, Platinum-based chemotherapy, Poly(ADPribose) polymerase (PARP) inhibitor therapy.

1.2. Tumor Microenvironment

Cancer cells reside in a complex microenvironment containing extracellular matrix (ECM), adipocytes, stromal cells, blood vessels, and a suite of signaling molecules [34, 35]. These environmental components highly organized in a 3D structure (Figure 2) collectively contribute to the tumor-stromal interaction and tumor progression through extensive inputs to cells from cell-cell, cell-matrix, and soluble signals [35-38].

The stromal component of tumor microenvironment is formed by the acellular part (ECM), stromal cells, and peptide factors such as growth factors, chemokines, cytokines and antibodies. ECM is mainly constituted by collagen, fibronectin, laminin and proteoglycan [39], complemented with specific components according to each tissue [40]. Stromal cells include fibroblasts [13, 41, 42], adipocytes (mesenchymal supporting cells) [43], cells of the vascular system [44] and immune

cells [45-47], which have a pronounced influence in tumor progression [34]. Fibroblasts synthesize and secrete mostly collagen and fibronectin to the ECM and become activated by tissue injury. In tumor microenvironment, these activated fibroblasts in close proximity with tumor cells are termed cancer-associated fibroblasts (CAFs) and are an essential component in tumor progression, namely cancer cell growth, inflammation, angiogenesis, and invasion [41, 42, 48-50]. One pathway that is thought to lead to tumor progression is related with the communication between CAFs and endothelial cells (EC) through growth factors and chemokines (CXCL12/SDF-1 and FGF-2) secretion [50, 51].

During tumor progression, the composition of ECM suffers some changes partly due to CAFs activity [50, 52]. Frequently, there are an upregulation of ECM synthesis, a post-translational modification and an extensive remodeling of ECM by proteins known as matrix metalloproteinases (MMPs), leading to an increase of cellular migration and survival. These alterations are translated in signals which promote tumor progression [36, 37, 49]. For example, in mammary tissue, the basement membrane - a specialized ECM produced by myoepithelial and stromal cells, where the basal surface of epithelial cells is attached - is breached when epithelial tumors proceed to invasive malignancy [36, 37, 49]. Regarding the interstitial matrix, which consists of fibrillar collagens, fibronectin, and proteoglycans and provides strength and structure to the tissue, its remodulation increases in tumor, being a crucial process for angiogenesis [13, 52, 53].

The referred alterations in ECM are ultimately reflected in the increase of stiffness [34], mainly due to collagen crosslinking [54], leading to the formation of hard nodules which separates tumor tissue from normal surrounding tissue. The increased rigidity enhances the disorganization of nonmalignant epithelial cells and its invasive phenotype [54-56].

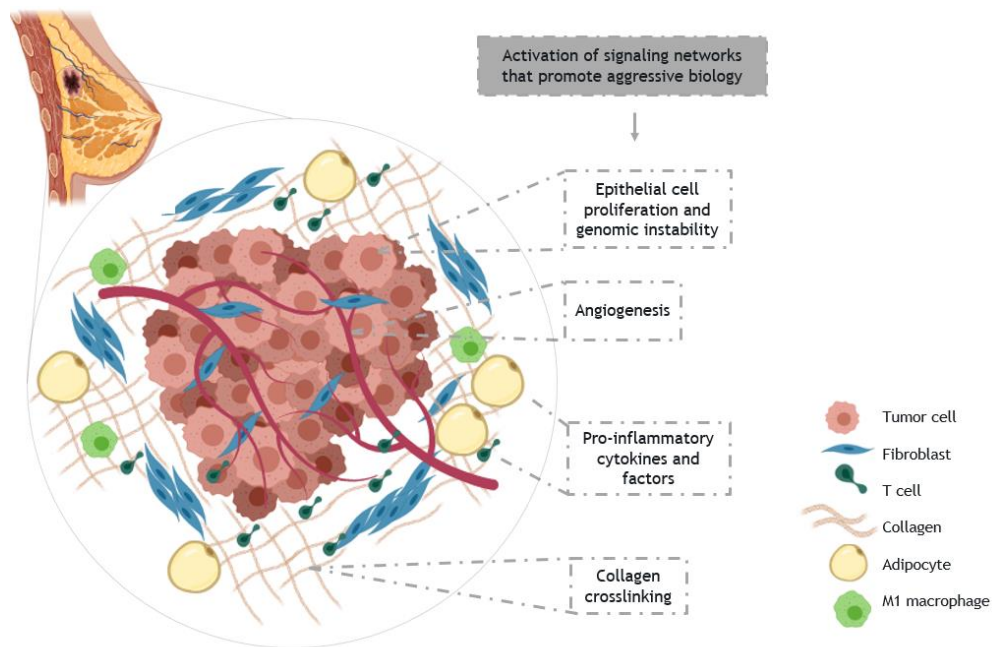


Figure 2. Schematic illustration of a typical tumor microenvironment. Cancer cells reside in a complex microenvironment containing various supporting cells, extracellular matrix and a suite of signaling molecules.

1.3. Metastatic Process

Once tumor cells acquire the invasive phenotype, they gain the ability of penetrating the surrounding tissues and the metastatic process is unleashed.

The metastatic process can be divided in the following sequential steps: invasion of the tumor cells, intravasation (Figure 3A) and dissemination through lymphatic or blood vessels (Figure 3B), extravasation and colonization [3, 57]. Preceding invasion, tumor cells detach from cell-extracellular matrix (cell-ECM) and cell-cell-adhesion bindings, and an active degradation of the ECM occurs through MMPs, the urokinase plasminogen activator (uPA) system, and heparanase [57, 58]. Having an invasive phenotype, tumor cells can migrate from the confined primary site singly (a protease-dependent mesenchymal movement involving cell phenotypic changes: epithelial-to-mesenchymal transition (EMT) (Figure 3C), or protease-independent ameboid movement) or collectively (cells connected via intercellular junctions circulate as emboli in the vessels and create tube-like microtracks to ensure mass migration) [58, 59]. Once in circulation, tumor cells must escape from the immune system until adhere to capillary beds within the target organ and extravasate [58, 59] (Figure 3D).

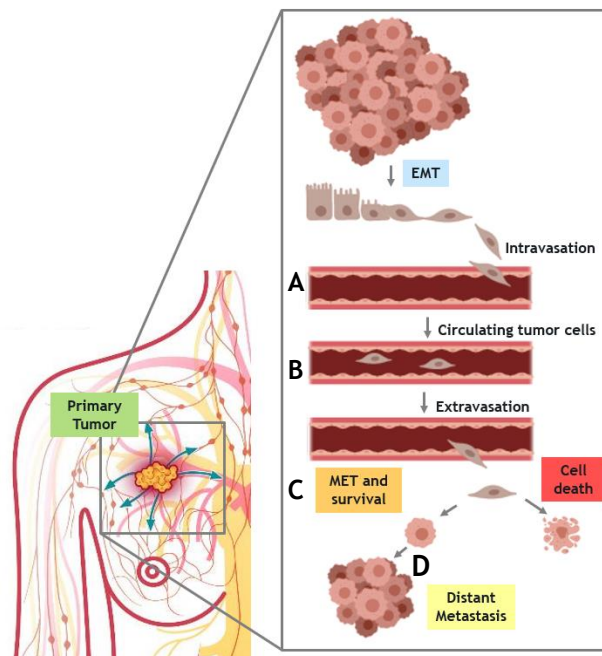


Figure 3. The sequential process of Cancer metastasis. Tumor cells undergo epithelial-to-mesenchymal transition (EMT) empowering cell migration and infiltration. Cancer cells intravasate into the capillary (A) and circulate throughout the body as circulating tumor cells (B). These cells survive the circulation, extravasate and invade distant tissues by reestablishing characteristics of their corresponding primary tumor, through the mesenchymal-to-epithelial transition (MET) (C). Cells that successfully adapt to their microenvironment and resume proliferation successfully form overt secondary tumors (D). Adapted from [60].

1.4. Tumor angiogenesis/vascularization

Whether in the primary tumor or after metastasization, it is known that tumors recruit blood vessels to ensure their growth. For that, supporting cells from the tumor microenvironment and the hypoxic nature and nutrient poor tumor core are involved in the production of signaling cascades promoting angiogenesis [61-63]. If the tumor is not sufficiently close to the vascular system, nutrient and oxygen levels are not reestablished, and tumor becomes dormant [61, 63, 64]. To avoid this phenomenon, it occurs the ‘angiogenic switch’ that is characterized by the release of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF/FGF-2) leading to new endothelial cells attraction to the local [63, 65, 66] (Figure 4). The endothelial cell migration involves ECM degradation, and once cells reach the tumor site, they start to form capillary structures to reestablish oxygen and nutrient levels

[58, 62]. In contrast to the normal vasculature, these capillary structures are irregularly shaped, tortuous, leaky, and immature, and provide a route of dissemination [62]. It is known that a high density of micro vessels is generally associated to higher tumor metastatic potential [61], however, no conclusions are yet established regarding the correlation between angiogenesis patterns and metastatic dissemination [67]. That possible relation is a challenge for many researchers who are immersed in developing alternative systems to improve therapeutic efficacies against tumors [63, 64, 68, 69].

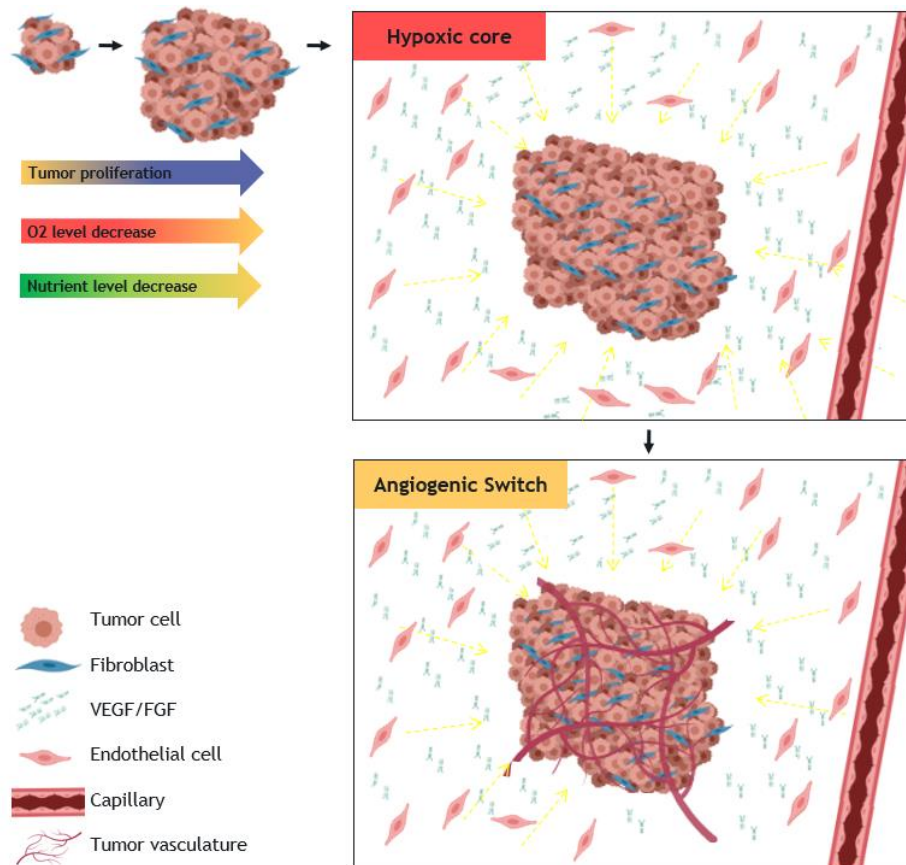


Figure 4. Simplified scheme of the interactions between tumor cells and endothelial system in the hypoxic tumor core. Tumor cells produce pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and pro-inflammatory cytokines stimulating endothelial cells to proliferate. Adapted from [66].

1.5. 2D versus 3D

The role of tumor's microenvironment and angiogenesis in the metastatic process is not completely understood. Over the past decades, most elucidations in the process of understanding cancer had resulted from fundamental biology research and translational and clinical studies with the objective of transforming a deadly disease into one with ever evolving therapeutic options and increasing survival rates [35-37, 45]. To this progress in knowledge have contributed studies with *in vitro* cancer models, mainly those which better balanced the setup simplicity and the clinical transfer of results to human patients [69]. First *in vitro* studies were performed in two-dimensional (2D) cell cultures. However, having in mind the importance of cellular microenvironment, the change of paradigm was eminent, and culture cells within a three-dimensional (3D) system became the gold standard for the *in vitro* cancer studies [70]. In 2D cell cultures the physiological cell organization, polarization and function is difficult to achieve. In its turn, 3D culture systems can combine the relative simplicity of *in vitro* cell cultures and the acquisition of more relevant results to an *in vivo* environment, as tissues and tumors are 3D structures of extracellular matrix and multiple cell types that interact in a complex manner rather than being a simple cellular monolayer [69, 70]. Moreover, several methodologies have been developed to monitorize 3D cell culture systems, namely regarding cell proliferation, gene expression, and signaling pathway activation [5].

1.6. Bioengineering matrices for in vitro 3D breast tumor angiogenesis

As mentioned above, the importance of the tumor angiogenesis and microenvironment as a key factor to determine the tumor response to an external pressure, such as drug treatment, is already recognized but it is not completely understood. Moreover, most of the knowledge on this field came from *in vitro* studies comprising the conjugation of tumor and vascular models [64, 71]. Biomaterials to be applied on vascularized models development have to combine several characteristics. Therefore, there are some key factors to be taken into account when choosing the biomaterial for that purpose. Firstly, its origin, which can be either classified as natural (materials that appear naturally in the world) or synthetic (materials created by chemical synthesis) [72, 73]. In addition, important features to take into consideration include biocompatibility, controlled biodegradability, surface properties, mechanical properties, and last but not least, pore density and structure [64, 71].

When in contact with the biomaterial, cells have to keep the capacity to survive, migrate and proliferate (biocompatibility). The material itself has to present a degradation rate compatible with simultaneous production and renovation of new ECM proteins, and one strategy for that can be the crosslinking of enzyme-sensitive peptides to the scaffold (for example, to attach peptides sensitive to MMPs activity to a hydrogel) [74]. To promote the interaction between cells and the biomaterial, a surface modification must be considered, namely with ligands (binding sites and signaling peptides), such as Arg-Gly-Asp (Arginine - Glycine - Aspartic Acid, RGD) ligands [75]. In terms of mechanical properties, the biomaterial should match the mechanical environment of the target anatomical site [76]. To facilitate vessel growth, nutrient and waste transport, biomaterials need to have interconnected pores [64, 71]. Porosity is defined as the volume fraction of the material that is pore and interconnectivity of the pore architecture. These properties are highly tissue-specific, since different cell types prefer different pore sizes, which can be controlled by conditions such the freezing time of the biomaterial [64, 71].

1.7. Natural-based scaffolds

The great majority of 3D approaches for breast tumor angiogenesis engineering is directed to the use of natural-based scaffolds [77]. Natural materials have several distinct advantages. Their intrinsic structure provides both chemical and physical cues to promote cancer morphogenesis [77], endothelial cells capillary morphogenesis [78, 79], and sprouting [80, 81]. They are biocompatible, biologically active, and degradable. Examples of natural materials that have been used extensively in the vascularization of tumor constructs include collagen type I and Matrigel® [82-85].

Matrigel® is a basement membrane extracted from Englebreth-Holm-Swarm (EHS) tumor. Several studies where endothelial cells were co-cultured with breast epithelial cells on top of Matrigel showed the remarkable influence of endothelial cells in tumor angiogenesis and breast cancer morphology, although using 2D models and lacking the ability to mimic the extracellular matrix structure [86].

The relevance of endothelial cells in breast cancer biology was further explored by Ingthorsson and co-workers, who isolated breast luminal, myoepithelial and endothelial cells from reduction mammoplasties, and embedded them in reconstituted basement membrane in direct co-culture with endothelial cells and separated by Transwell filters [82]. Besides having stimulated proliferation of both luminal and myoepithelial cells in co-culture, endothelial cells induced

luminal epithelial cells to form large acini/ducts with a large and clear lumen, and also stimulated growth and cloning efficiency of normal and malignant breast epithelial cell lines [82].

Besides Matrigel®, collagen type I has also been used as a natural matrix for tumor angiogenesis engineering. Szot and colleagues bioengineered a 3D *in vitro* system based on collagen I hydrogels, where cells were grown on 3D polymeric scaffolds inducing upregulation of characteristic genetic markers, such HIF-1 α and VEGF-A, to promote an *in vivo* phenotype [83]. Later, that system evolved into a bilayer-bioengineered tumor angiogenesis model, in which breast cancer cells, MCF7 or MDA-MB-231 cells, were cultured in bulk collagen I hydrogel, and telomerase-immortalized human microvascular endothelial cells were cultured on the surface of an acellular layer of collagen I hydrogel separating the two cell types [84]. This model represents the first demonstration of invasive angiogenic sprouting into a 3D matrix induced by tumor-endothelial cell paracrine signaling. It was indeed shown that endothelial cells invaded the collagen layer and assembled into capillary-like tubule network with lumen formation and anastomosing branches in the presence of MDA-MB-231 but not MCF7 cells, due to the more aggressive invasion phenotype of MDA-MB-231 cells than MCF7 cells [84].

Using the minitumor model, a 3D spheroid-based system incubated in collagen type I that was designed by Correa de Sampaio and colleagues, endothelial cells and fibroblasts in coculture with the breast cancer cell line MDA-MB-231 presented a quantifiable capillary-like sprouts formation typical of early stages of tumor angiogenesis [85]. The main conclusion was that endothelial cell precapillary sprouts are supported by the fibroblasts, which act as mural cells, and their growth is increased by the presence of cancer cells [85].

1.8. Synthetic-based matrices

Early in the history of *in vitro* tumor angiogenesis models, and to overcome the main limitations of natural materials-based models, Werner and co-workers presented an *in situ*-forming starPEG-heparin hydrogels scaffold, composed of multi-armed PEG and heparin crosslinked via cytocompatible Michael type addition that enabled cells to be embedded in 3D [87]. Polyethylene glycol (PEG) is a synthetic, non-biodegradable, and biocompatible polymer, whose repeating alkane-ether motif makes it extremely hydrophilic and biochemically inert [72]. This was the first report of tumor-endothelial paracrine signaling-induced invasive angiogenic sprouting into a 3D matrix, and a big evidence of fibroblasts importance in cancer since it showed that the addition of fibroblasts abrogated the apoptosis process triggered when endothelial cells contact cancer cells [87]. Bray and colleagues also projected 3D *in vitro* model of tumor

angiogenesis based on glycosaminoglycan hydrogel [88]. They established different co-cultures, breast or prostate epithelial carcinoma cells (MCF-7, MDA-MB-231, LNCaP, PC3) with HUVECs and mesenchymal stromal cells in matrices fabricated from synthetic starPEG and maleimide functionalized heparin, which presented less sensitive to chemotherapy than 2D cultures, and similar tumor regression to that displayed *in vivo* [88].

Furthermore, to investigate the effect of the microenvironment in cancer progression, specially the effect of different ECM ligands on tumor cell invasion and angiogenesis, Taubenberger and colleagues decorated MMP-degradable biohybrid polyethylene glycol-heparin hydrogels with the peptide motifs RGD, GFOGER (collagen I), or IKVAV (laminin-111), and analyzed their influence on 3D breast (MCF-7) and prostate (PC-3, LNCaP) cancer cell cultures, and co-cultures with endothelial and mesenchymal stromal cells [89]. While the morphology of poorly invasive MCF-7 and LNCaP cells was not modulated by any of the peptide motifs, the aggressive PC-3 cells exhibited an invasive morphology when cultured in hydrogels comprising IKVAV and GFOGER motifs compared to RGD motifs or non-functionalized controls [89].

The table (Table 1) below summarizes current established approaches for modeling tumor angiogenesis in *in vitro* 3D systems as it was described above.

Table1. Bioengineered matrices for in vitro 3D breast tumor angiogenesis

Material	Example	Model	Cells
Natural	Matrigel	Breast luminal and myoepithelial cells embedded laminin rich in direct co-culture with endothelial cells and by separation of transwell filters [82]	BRENCs, MCF10A, D382, MCF7, T47-D, MDA-MB-231
	Collagen type I	3D spheroid-based system incubated in collagen type I leads to the formation of capillary-like sprouts typical of early stages of tumor angiogenesis [85]	HUVECs, NHDF, MDA-MB-231
		Cells grown on 3D polymeric scaffolds presented upregulation of characteristic genetic markers to promote an <i>in vivo</i> phenotype [83]	MDA-MB-231
		Breast cancer cells cultured in a bulk collagen type I hydrogel, and microvascular endothelial cells cultured on the surface of an acellular layer of collagen type I hydrogel that separated the two cell types [84]	MDA-MB-231, MCF7, TIMEs
Synthetic	PEG-heparin	Multiparametric hydrogels support 3D <i>in vitro</i> tumor angiogenesis [87]	MCF-7, MDA-MB-231, HUVECs, MSCs
		<i>In situ</i> -forming starPEG-heparin hydrogels - scaffolds that are composed of multi-armed PEG and heparin crosslinked via cytocompatible Michael type addition that enabled cells to be embedded in 3D [88]	ECs, SCs, SMCs, HDFs
		Matrix metalloproteinase-degradable biohybrid PEG-heparin hydrogels [89]	HUVECs, SMCs, HDFs, MSCs, HepG2

2. Hypothesis, Strategy and Aims

During breast cancer development/progression, stromal components, as well as tumor tissue stiffness and low oxygen levels, may have an important role in endothelial cells (EC) recruitment.

To try to clarify this, we decided to use RGD-modified alginate hydrogel, which can mimic the native ECM, to culture both human mammary fibroblast (hMF) and cancer epithelial cells (MDA-MB-231) in a 3D setting. With these tumor-like 3D constructs, we intended to monitor MMP production and CA9 expression (a well-known tumor-hypoxic marker) in response to altered stiffness and hypoxic conditions. We further aimed to understand the crosstalk between our tumor-like construct and EC. For that we intend to study the effect of conditioned medium obtained from these 3D co-cultures in EC proliferation and migration by performing both scratch and a transwell migration-based assays.

However, since this is a quite reductionist approach, we intend to further understand the impact of these soluble factors using a more biologically relevant vascular construct. To achieve this, we intend to establish vascularized construct by co-culturing outgrowth EC (OEC) and hMF on alginate porous scaffolds. OECs were selected as EC source since they have high proliferation rate and no tissue specificity. Ideally, scaffold should be porous to facilitate cellular infiltration and 3D capillary-like organization throughout the structure, and later allow for cellular outgrowth.

Furthermore, in the future, this bioengineered platform could be explored with primary patient-derived cells, in a precision medicine context. Due to platform compartmentalization, the 3D microenvironments can be optimized to each cell type to ensure optimal conditions for cell growth and maintenance.

The major aim of this thesis was to bioengineer 3D heterotypic tumor models to study the dynamics of endothelial cells recruitment and growth (Figure 5). Specific goals were:

- a. To develop tumor-like constructs, through the establishment of 3D co-cultures of human breast cancer epithelial cells (MDA-MB-231) with hMF within alginate-RGD hydrogel, with tunable mechanical properties;
- b. To analyze the paracrine effect of tumor-like constructs on EC recruitment and growth;

- c. To develop a **vascularized construct**, through the establishment of co-culture of OEC and fibroblasts on alginate porous scaffolds.

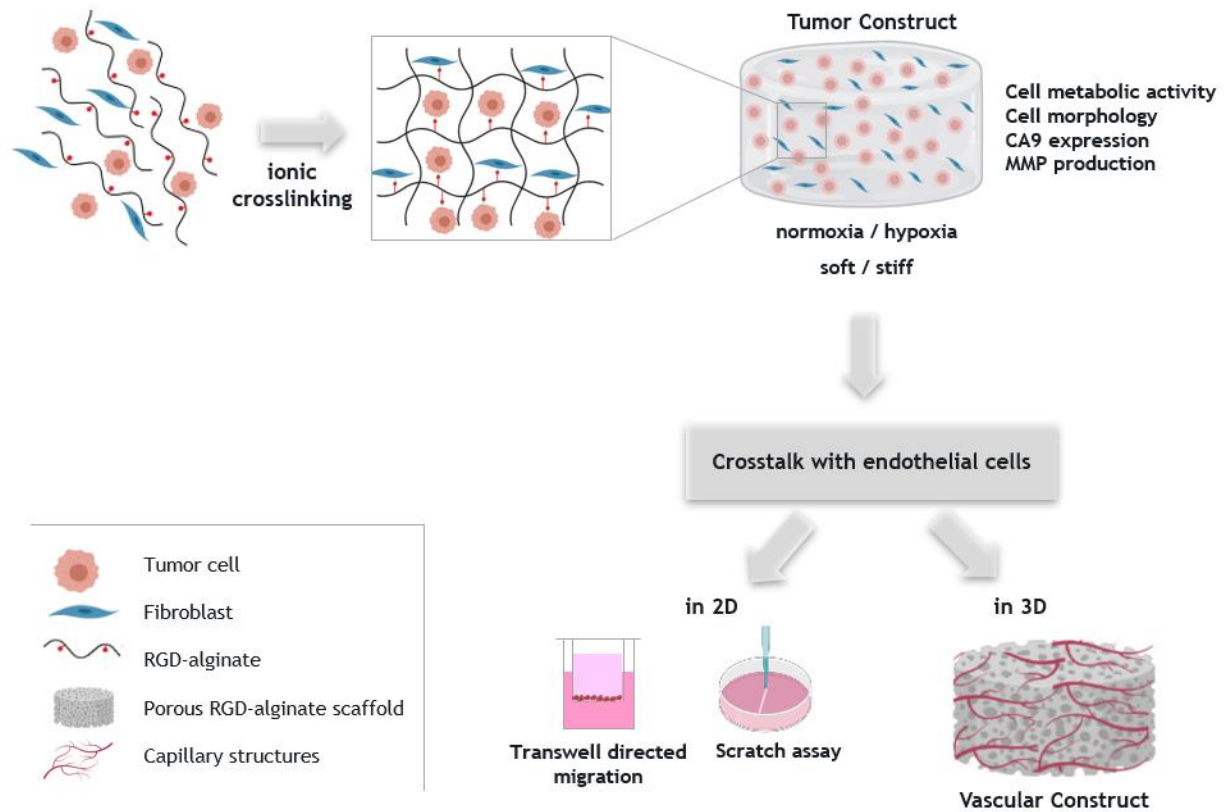


Figure 5. Proposed 3D heterotypic tumor model to study the dynamics of cell recruitment and growth. Co-cultures of MDA-MB-231 and hMF are entrapped within RGD-modified alginate matrices and exposed to different culture conditions. Conditioned medium is collected from this tumor-like construct and its paracrine effect is evaluated in EC proliferation and migration in 2D. And later on, it will be assessed the impact of these soluble factors using a more biologically relevant vascular construct.

3. Materials and Methods

3.1. Cell Maintenance

3.1.1. Primary cultures

Outgrowth endothelial cells (OECs) were isolated from human umbilical cord, as previously described by Torres and colleagues [90]. To culture these cells, endothelial cell growth medium (EGM-2MV, Lonza) supplemented with 5% v/v fetal bovine serum (FBS) was used. EGM-2 MV medium was prepared by supplementing EBM-2 with ascorbic acid, hydrocortisone, gentamicin-amphotericin (GA)-1000 and human GF cocktail, which included epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor- β (hFGF- β) and insulin growth factor-1 (IGF-1). Media was changed every two days, to remove non-adherent cells, and when cells reached approximately 80% confluency, they were trypsinized and used. OECs were used between passage 5 and passage 8.

Human mammary fibroblasts (hMFs, Innoprot) were maintained in fibroblast culture medium (FM, Innoprot) composed of 2% v/v of FBS, 1% of Fibroblast Growth Supplement (FGS) and 1% of penicillin/streptomycin (Pen/Strep), until the first trypsinization. Thenceforward, hMFs were cultured in Dulbecco's Modified Eagle Medium with Glutamax (DMEM GlutaMAXTM, Gibco) supplemented with 10% v/v FBS and 1% v/v Pen/Strep. hMFs were used between P6 and P12.

All the cells were maintained in a 5% v/v CO₂ humidified incubator at 37°C in order to maintain physiological conditions.

3.1.2. Cell lines

Basal aggressive metastatic breast cancer cell line, MDA-MB-231, was maintained in DMEM with Glutamax (Gibco) supplemented with 10% v/v FBS and 1% v/v Pen/Strep.

Cells were maintained in a 5% v/v CO₂ humidified incubator at 37°C in order to maintain physiological conditions.

3.2. Synthesis of RGD-grafted alginate

High molecular alginate (PRONOVA) was covalently grafted with cell-adhesion peptides (glycine)4-arginine-glycine-aspartic acid-serine-proline (G4RGDSP, GenScript) using aqueous carbodiimide chemistry. Alginate solutions (1 wt.%) in MES buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) were prepared and stirred overnight (ON) at room temperature (RT). N-Hydroxy-sulfosuccinimide (Sulfo-NHS, Pierce) and 1-ethyl-(dimethylaminopropyl)-carbodiimide (EDC, Sigma 27.4 mg per g of alginate) were sequentially added at a molar ratio of 1:2, followed by 100 mg of RGD peptide (Genscript) per g of alginate. Control samples were prepared without the addition of peptide. After stirring for 20 h at RT, the reaction was quenched with hydroxylamine (NH₂OH, Sigma) and the solution was dialyzed against deionized water for 3 days (MWCO 3500, Spectrum Labs). After purification with activated charcoal (0.5 g per g of alginate, Sigma), RGD-alginate was lyophilized and stored at -20°C until further use. The amount of grafted RGD was quantified using the BCA Protein Assay (Pierce). Briefly, samples (1 wt.% RGD-grafted alginate) were incubated in BCA reagent for 30 min at 37°C in the dark and the absorbance was read at 560 nm in a microplate reader (Biotek Synergy MX). A set of RGD solutions (0 to 1 mg/mL in 1 wt.% HMW0) was used as standards to produce a calibration curve. Typically, the reaction yield was determined by BCA total protein assay, as described in [91, 92].

3.3. 3D culture of epithelial cells and hMFs in RGD-alginate matrices

In situ forming alginate hydrogel matrices were prepared by internal gelation, using calcium as ionic crosslinker as previously described [91-93]. For that, RGD-alginate precursor solutions were sterile-filtered (0.22 µm) with a sterile suspension of CaCO₃ in 0.9% wt.% NaCl at a Ca²⁺/COO⁻ molar ratio of 0.288, and the gelling process was triggered through the addition of a filtered (0.22 µm) fresh GDL in 0.9% wt.% NaCl at a CaCO₃/GDL molar ratio of 0.125. A final RGD-modified alginate concentration of 1 wt.% with 200 µM of RGD is obtained by adding the previous solution to the cell suspension (5 × 10⁶ cells/mL). Cells were trypsinized and, centrifugated (1100 rpm, 5 min) and after remove the supernatant, cells were resuspended in 0.9% wt.% NaCl. Four different cultures were established: hMFs monocultures, MDA-MB-231 monocultures, and coculture of hMFs and MDA-MB-231 (cells ratio 1:1) Finally, the cell suspension was homogeneously mixed with the RGD-alginate solution and crosslinking agents. The 3D matrices were obtained by quickly applying 20 µL drops of the mixture between Teflon plates separated by 750µm-thickness spacers

(Figure 6). Then the Teflon plates with the 3D matrices were kept in a petri dish, at 37°C in a 5% v/v CO₂ incubator and removed 20 to 30 minutes after triggering the gelling process, which is after adding the GDL [94]. The 3D matrices were transferred to a 24-well plate and incubated with culture medium at 37°C and after 30 min the medium was removed.

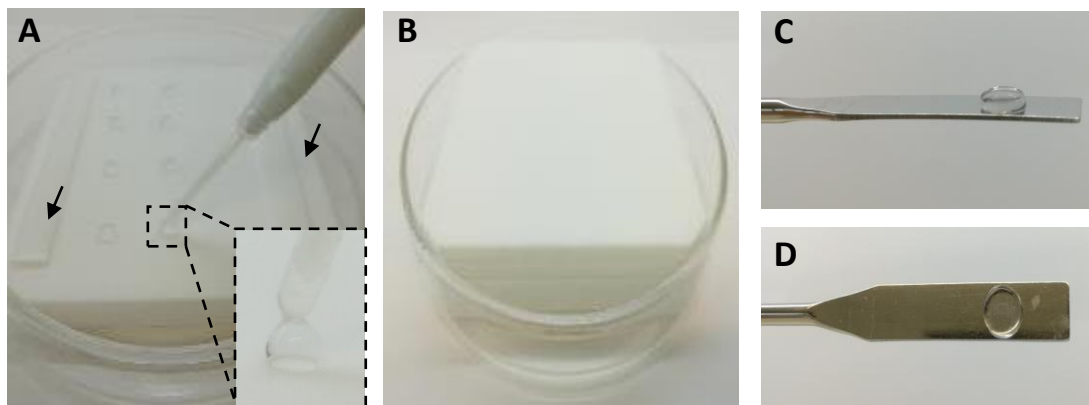


Figure 6. Experimental procedure for the preparation of the 3D cell-laden matrices. Small drops of cell-laden alginate were applied on top of a Teflon plate between two spacers (black arrows) (A) and then another Teflon plate was placed on top of those spacers (B). After gelation, 3D matrices were obtained (C, D).

3.4. Stiffness increase of Co-cultured-RGD-alginate matrices

The 3D matrices were transferred to a 24-well plate and incubated with culture medium at a 5% v/v CO₂ humidified incubator at 37°C for 24 h. After that, culture medium was changed to BaCl₂ solution (50 mM) (E.Merck, Darmstadt) for 15 min in order to increase the matrix stiffness. Then 3D matrices were transferred to a 24-well plate and incubated with culture medium at 37°C, and after 30 min the medium was changed.

3.5. Rheological measurements

Rheological measurements were carried out using a Kinexus Pro rheometer (Malvern). Hydrogel discs were analyzed at day 7 with cells under standard culture conditions and 4 mm cylinders were punched from the original ones immediately before analysis. All samples were assayed using a plate-and-plate geometry (4 mm diameter, sandblasted surfaces) and were compressed to 20% of their original thickness to avoid slippage. A solvent trap was used to minimize sample drying. All measurements were performed at 37 °C (Peltier system). Stress sweeps (0.05 Hz) were first performed to determine the LVR. Frequency sweeps (0.01-2 Hz) were

then performed within the LVR. The values of the shear moduli (G' and G'') and phase angle, were obtained at a frequency of 0.05 Hz. Samples were analyzed in triplicate.

3.6. Hypoxic culture of MDA-MB-231-hMFs 3D cultured in RGD-alginate matrices

After 4 and 11 days of culture under normoxic conditions (21% v/v O_2 , 5% v/v CO_2 humidified incubator at 37°C), the 3D matrices were transferred to a 24-well plate and incubated with culture medium at hypoxic conditions (1% v/v O_2 , 5% v/v CO_2 humidified incubator at 37°C) for 3 days. On the 3rd day, conditioned medium was collected, and cells were retrieved from alginate and stored at -20°C.

3.7. Evaluation of alginate embedded cells metabolic activity

The metabolic activity of entrapped cells in alginate matrices was determined through the a resazurin-based assay. To this end, 3D matrices were incubated with 20% v/v of stock resazurin solution (0.1mg/mL, Sigma) in Dulbecco's Modified Eagle Medium with Glutamax (DMEM GlutaMAX™, Gibco) for 2h at 37°C protected from light. Then, fluorescence measurements were performed using a microplate reader (Biotek Synergy MX) with excitation/emission at 530/590nm.

3.8. Isolation of cells from 3D RGD-alginate matrices

3D RGD-alginate matrices were degraded with alginate lyase (10mg/mL, Sigma) by incubating for 30 minutes at 37°C. Cells and degraded alginate were recovered by centrifugation (1500 rpm, 5 min), then incubated with 0.05% w/v trypsin/50 mM EDTA solution for 15 min. Cells were recovered by centrifugation (2000 rpm, 5 min), washed with PBS and stored at -20°C.

3.9. RNA expression quantification

RNA was extracted from cells entrapped in alginate matrices (hMFs and MDA-MB-231 cells) at day 7 and day 14 under normoxic and hypoxic conditions using the Quick-RNA MiniPrep (Zymo Research), as recommended by the manufacturer. Subsequently, 500 ng of total RNA were reversed transcribed to single stranded cDNA using Superscript First-strand synthesis and random hexamer primers (Invitrogen). Quantitative Real-Time PCR (qRT-PCR) was carried out using source RNA from 2 biological replicas for the target gene *CA9* and for endogenous control *GAPDH* using as probe sets Mm00486909, Mm.PT.47.16166845, (Integrated DNA Technologies), respectively. Samples were run in triplicates in the ABI Prism 7000 Sequence Detection System under the following conditions: 95 °C for 20 sec, followed by 40 cycles at 95 °C for 3 sec and 60 °C for 30 sec. Data was analyzed by the comparative $2(-\Delta\Delta CT)$ method.

3.10. Scratch/wound healing assay

OECs (P5) were seeded in a 48-well plate (10,000 cells/well) and cultured in EGM-2MV medium until reaching ~80% confluency. A scratch was made manually down the center of the well with a p1000 micropipette tip in one swift motion as previously described [95]. Conditioned medium from 3D matrices cultured under hypoxia and normoxia was applied and pictures were taken at 3 different locations along the scratch wound immediately after scratching. Conditioned medium volumes were adjusted to yield equivalent total cell protein contents and DNA content, which were quantified by the BCA Protein Assay (Pierce) and Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) in lysates of cells recovered from the hydrogels, respectively [96]. EGM-2 MV medium was used as positive control, and complete DMEM GlutaMAX™ as negative control. The cells were incubated in a 5% v/v CO₂ humidified incubator at 37°C, and after 24 h pictures were then taken at the same locations along the scratched wound that were imaged immediately after scratching. Wound closure was quantified as the difference in scratch width between the initial and final images at 5X using 3 pictures per well.

3.11. Directed migration assay

To assay directed migration of OECs *in vitro*, OECs (P5) were seeded into Transwell inserts (8 μm pores; Corning Inc.) at a cell density of 20,000/transwell in 200 μL of EBM-2MV (5% FBS) (Figure 7). Transwell inserts were then placed within the well, containing the 700 μL of conditioned medium from 3D matrices cultured under hypoxia and normoxia. Sample volumes were adjusted to yield equivalent total cell protein and DNA contents, as previously described. The plates were incubated in a 5% v/v CO_2 humidified incubator at 37°C, and after 18 h inserts were then removed using sterile tweezers, fixed with 4% formaldehyde in PBS and stained with DAPI. Mosaic images were obtained using a confocal laser scanning microscopy (CLSM, Leica) and processed using Fiji Imaging Software.

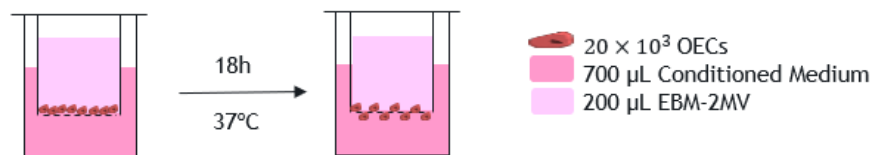


Figure 7. Schematic representation of the transwell directed migration assay.

3.12. Matrix metalloproteinases (MMPs) secretion by zymography

To detect expression of MMPs (MMP2 and MMP9) by 3D cultured cells, cell culture supernatants were analyzed by gelatin-zymography. After 72 h in normoxic conditions (21% v/v O_2 , 5% v/v CO_2 humidified incubator at 37°C) and hypoxic conditions (1% v/v O_2 , 5% v/v CO_2 humidified incubator at 37°C), conditioned media were collected, centrifuged to remove cell debris and loaded into gelatin-SDS polyacrylamide gels. Sample volumes were adjusted to yield equivalent total cell protein and DNA contents, quantified in lysates of cells recovered from the hydrogels, as previously described [96]. The gel was run in 1 $\times$ Tris-Glycine SDS running buffer at 60 V (Mini Protean Tetra Cell system, BioRad). After electrophoresis, gels were washed twice and subsequently incubated in MMP substrate buffer for 16 h at 37 °C. Afterwards, gels were washed and stained with Coomassie Brilliant Blue R-250 (Sigma). MMPs proteolytic activity was visualized as the presence of clear bands against a blue background of Coomassie Blue-stained gelatin substrate. Thereafter, the gel was washed with distilled water, scanned using the GS-800 Calibrated Densitometer (Bio-Rad) and MMPs activity was estimated by densitometric analysis (Quantity One, Bio-rad).

3.13. Preparation of RGD-grafted alginate scaffolds

3.13.1. Freeze-drying technique

Scaffolds were prepared by internal gelation, using calcium as ionic crosslinker as previously described by [90, 92]. RGD-alginate precursor solutions were homogeneously mixed with a suspension of calcium carbonate (CaCO_3 , Fluka) in water at a $\text{CaCO}_3/\text{COOH}$ molar ratio of 0.288, and the gelling process was triggered through the addition of fresh gluconic solution of delta-lactone (GDL, Sigma) in water at a CaCO_3/GDL molar ratio of 0.125. A final RGD-modified alginate concentration of 2 wt.% with 600 μM of RGD was obtained and quickly added in 96-well plate (250 μL /well), where it gelled. Three-dimensional scaffolds were generated by freeze-drying technique. After the gelation time (approximately 45 minutes, at RT) the sample was frozen at -20°C , and then lyophilized at 0.008 mBar and a temperature of -80°C for 48h. Using a scalpel blade, the obtained cylindric scaffolds were then cut into small ones with an average height of 1.5 mm.

3.13.2. Freeze-drying associated with Particle leaching technique

Alginate scaffolds were also prepared using the particle leaching method, in which sodium chloride (NaCl) was used as a porogen to form the porous structures (Figure 8). NaCl particles were fractioned and sieved to a particle size of 150-250 μm , and subsequently incorporated into the alginate precursor solution (5 mg/mL, corresponding to 0.5-1 wt% of the solute, were added into the solution). After homogenization, this solution was mixed with a suspension of calcium carbonate in MilliQ water at $\text{Ca}^{2+}/\text{COO}^-$ molar ratio of 0.288, and the gelling process was triggered through the addition of GDL in MilliQ water at a CaCO_3/GDL molar ratio of 0.125. A final concentration of 2 wt.% was obtained and this alginate solution was finally mixed and quickly added in a 96-well plate (250 μL /well), where it gelled. After the gelation time (approximately 45 minutes, at RT) the sample was frozen at -20°C , and then lyophilized at 0.008 mBar and a temperature of -80°C . The obtained cylindric scaffolds were then placed in distilled water for 24h with agitation to leach out the salt particles, changing the water 3 times. The leached samples were frozen at -20°C , and then freeze-dried again for 48h. Using a scalpel blade, the obtained cylindric scaffolds were then cut into small ones with an average height of 1.5 mm.

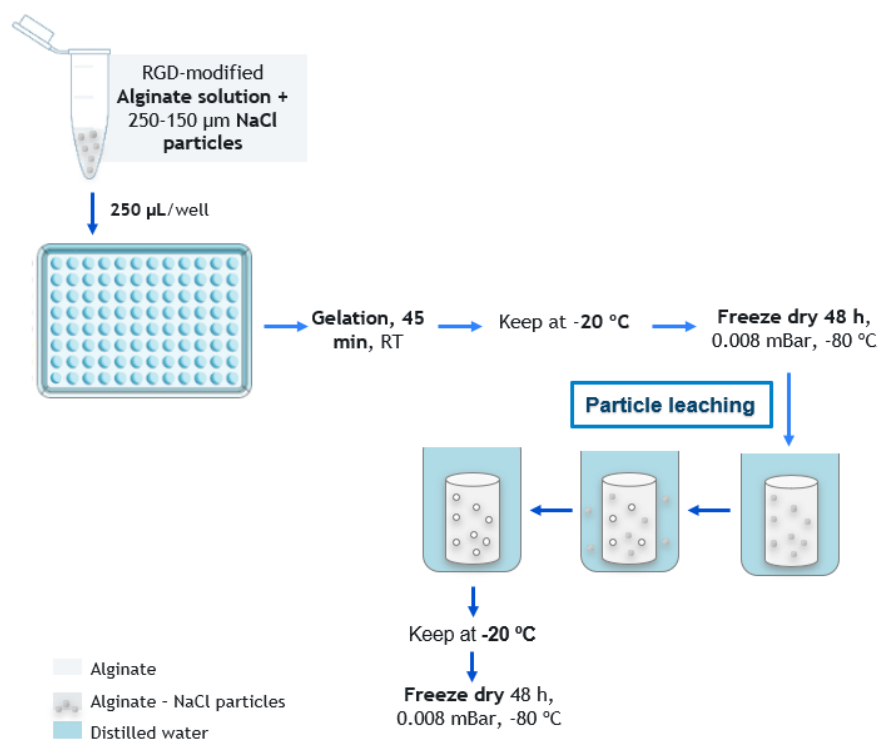


Figure 8. Schematic representation of the freeze-drying association with particle leaching technique. NaCl particles were fractioned and sieved to a particle size of 150-250 µm, and subsequently incorporated into the alginate precursor solution. After the first lyophilization, obtained cylindric scaffolds were placed in distilled water for 24h with agitation to leach out the salt particles, changing the water 3 times. Then leached samples were frozen at -20°C and freeze-dried again for 48h.

3.14. Characterization of RGD-grafted alginate scaffolds

The scaffolds characterization was performed by Scanning Electron Microscopy (SEM) analysis of the surfaces and cross-sections, using a High-resolution Scanning Electron Microscope with X-Ray Microanalysis (JEOL JSM 6301F/Oxford INCA Energy 350) at an accelerating voltage of 15 kV. Samples were coated with Au/Pd thin film, by sputtering for 10 seconds and with a 15 mA current, using the SPI Module Sputter Coater equipment. The average pore diameter was also analyzed by manual measurement of SEM images, using Fiji Imaging software.

3.15. Seeding of human mammary fibroblasts and endothelial cells on RGD-alginate scaffolds

3.15.1. Scaffold preparation

RGD-alginate scaffolds were sterilized by wash with ethanol 70% for 20 minutes, following by rinsing 3 times in sterile TBS/CaCl₂. Scaffolds were then transferred to a 48-well plate and incubated with cell culture medium (DMEM), at 37°C. 10 minutes before the seeding, cell culture medium was removed.

3.15.2. Seeding approach

3.15.2.1. Cell seeding on the top of the scaffold

hMFs and OECs were seeded simultaneously at two different final cell densities, 25×10^4 cells/scaffold and 50×10^4 cells/scaffold, in a total volume of 30 μ L that was added to the top of the scaffold (cell ratio 1:1) (Figure 9A).

After allowing cell adhesion for 2h, EGM-2MV, Lonza was added, and the seeded scaffolds were incubated.

3.15.2.2. Cell seeding on the top of the scaffold followed by centrifugation

hMFs and OECs were seeded simultaneously at a final cell density of 50×10^4 cells/scaffold in a total volume of 30 μ L that was added to the top of the scaffold (cell ratio 1:1) and the well plate was placed in a plate centrifuge (Centrifuge 5810 R, Eppendorf AG, 2231, Germany) and centrifuged for 5 minutes (1000 rpm, 21 °C) (Figure 9B) [97]. Scaffolds were then transferred to a 48-well plate and after allowing cell adhesion for 2h, EGM-2MV was added, and the seeded scaffolds were incubated.

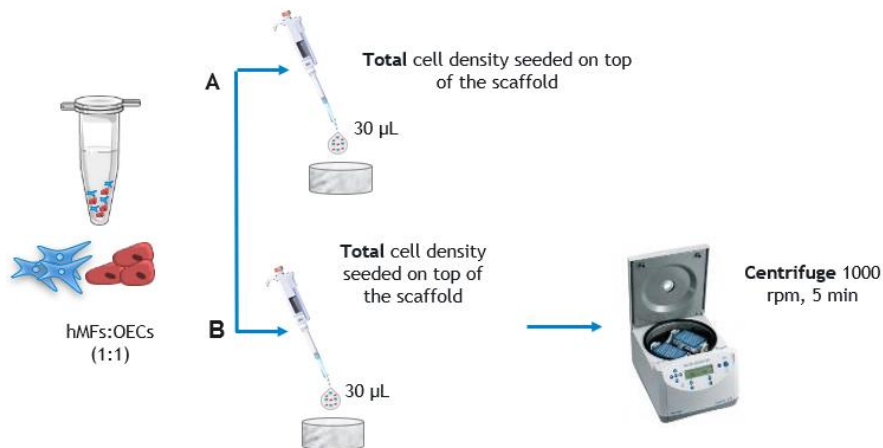


Figure 9. Experimental design for co-culturing OECs and hMFs in RGD-alginate scaffolds. Two strategies were tested: total cell seeding on the top of the scaffold (A), followed by centrifugation (B).

3.16. Outward cell migration assay in fibrin or collagen I gel

To determine cells outgrow from the alginate scaffold, colonized scaffolds were incorporated within collagen type I or fibrin gel.

3.16.1. Collagen type I

3.16.1.1. Collagen type I matrices

hMFs-OECs cultured on alginate scaffolds for 10 and days were transferred to a thin layer of collagen type I (Figure 10A). Collagen type I matrices were prepared by adding 25 µL of 10x PBS, 1.34 µL of NaOH (1mol/L), 331 µL of DMEM with Glutamax (DMEM GlutaMAX™, Gibco) and 58.4 µL of collagen type I (8.56 mg/mL) (Corning), as recommended by the fabricant (Merck KGaA, 2019). This solution was added at a 24-well plate (250 µL/well) and incubated at 37°C for 15 minutes to allow for gel formation (not completely before the addiction of the scaffold). After that, hMFs-OECs-seeded alginate scaffolds were transferred to the collagen type I matrices. A volume of 500µL of EGM-2MV fully supplemented media was added to maintain the cells alive while keeping the scaffold in direct contact with the collagen type I gel and changed every day for 20 days, during which representative pictures were taken.

3.16.1.2. Collagen type I sandwich assay

hMFs-OECs cultured on alginate scaffolds for 7 days were embedded between two layers of collagen type I gel (Figure 10B) that were prepared as previously described. A volume of 500 μ L of EGM-2MV fully supplemented media was added to maintain the cells alive while keeping the scaffold in direct contact with the fibrin gel and changed every day for three days, during which representative pictures were taken.

3.16.2. Fibrin sandwich assay

hMFs-OECs cultured on alginate scaffolds for 7 days were embedded between two layers of fibrin gel (Figure 10C). Fibrin gel was formed with a volume of 61.2 μ L of fully supplemented EGM-2MV media added to 183.5 μ L of fibrinogen (4mg/mL in 0.9% NaCl, Sigma) and 24.5 μ L of aprotinin (500 μ g/mL in PBS, Sigma). This solution was mixed with 19.2 μ L of thrombin (2.08U/mL in PBS, Sigma) and 211.5 of 1x PBS and was incubated at 37°C for 30 minutes to allow for gel formation. A volume of 500 μ L of EGM-2MV fully supplemented media was added to maintain the cells alive while keeping the scaffold in direct contact with the fibrin gel and changed every day for three days, during which representative pictures were taken.

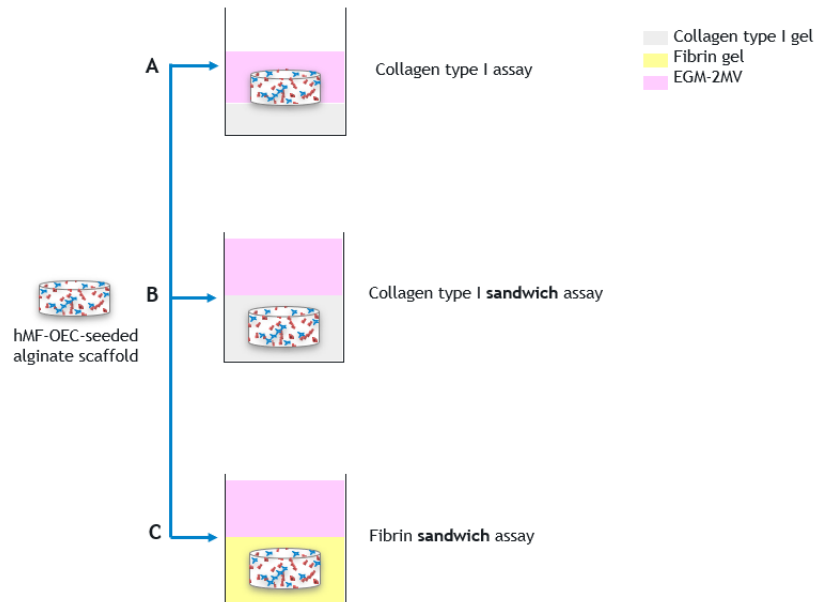


Figure 10. Experimental design to analyze cell capacity to outgrow from the alginate scaffold. Colonized scaffolds were incorporated within collagen type I (A, B) or fibrin gel (C).

3.17. Immunostaining

3.17.1. 3D culture of epithelial cells and hMFs

3D matrices were washed twice with HBSS (1x), fixed with 4% v/v PFA in HBSS (20 min at RT) and washed again with HBSS (1x). Then, 3D matrices were permeabilized with 0.2% v/v Triton X-100 for 10 min and blocked for 1h with wt.% BSA solution in HBSS. After that, 3D matrices were incubated ON at 4°C with primary antibodies in 1.5 wt.% BSA in HBSS: rabbit anti-E-cadherin (1:100, Cell Signaling 31955), mouse anti-vimentin (1:50, Santa Cruz sc-6260) and phalloidin 647 (1:100, Flash Phalloidin Red 647, Biolegend 424205). After washing, 3D matrices were incubated in the dark for 1 h at RT with the secondary antibodies Alexa Fluor 594 goat anti-rabbit (1:500, Invitrogen A11020) and Alexa Fluor 488 donkey anti-mouse (1:500, Invitrogen A21202).

Finally, the scaffolds were washed 3 times with HBSS, the nuclei were counterstained with DAPI. 3D imaging of the samples was accomplished by CLSM (SP5, Leica) and data analysis was performed using Fiji Imaging software.

3.17.2. Transwell inserts

After removing culture medium, transwell inserts were washed carefully with PBS (1x), fixed with 4% v/v PFA in PBS (20 min at RT) and washed again with PBS (1x). A cotton-tipped applicator was used to carefully remove the media and remaining cells that have not migrated from the top of the membrane without damaging it. Then, membranes were removed from the inserts using a scalpel and mounted in a glass slide with Vectashield with DAPI (Sigma). Images of the samples were obtained using CLSM (SP5, Leica).

3.17.3. Co-culture RGD-alginate scaffolds

Co-cultured scaffolds were washed twice with HBSS (1x), fixed with 4% v/v PFA in HBSS (20 min at RT) and washed again with HBSS (1x). Then, scaffolds were permeabilized with 0.2% v/v Triton X-100 for 10 min and blocked for 1h with 1.5 wt.% BSA solution in HBSS. The co-cultured scaffolds of OECs and hMFs were incubated with phalloidin 647 (1:100, Flash Phalloidin Red 647, Biolegend 424205), with mouse anti-CD31 (1:50, DAKO M0823) combined with rabbit anti-laminin (1:100, Sigma L-9393) ON at 4°C. After washing, scaffolds were incubated for 1 h at RT with the secondary antibodies Alexa Fluor 594 goat anti-mouse (1:500, Invitrogen A11020) and Alexa Fluor 488 goat anti-rabbit (1:500, Invitrogen A11008). Alternatively, avoiding the permeabilization step,

the co-cultured scaffolds of OECs and hMFs were incubated with phalloidin 647 (1:100, Flash Phalloidin Red 647, Biolegend 424205), with rabbit anti-fibronectin (1:200, Sigma F3648) combined with mouse anti-collagen type IV (1:100, DAKO M0785) ON at 4°C. After washing, scaffolds were incubated in the dark for 1 h at RT with the secondary antibodies Alexa Fluor 594 goat anti-rabbit (1:500, Invitrogen A11072) and Alexa Fluor 488 donkey anti-mouse (1:500, Invitrogen A21202).

Finally, the scaffolds were washed 3 times with HBSS, the nuclei were counterstained with DAPI. 3D imaging of the samples was accomplished by CLSM (SP5, Leica) and data analysis was performed using Fiji Imaging software.

3.17.4. 3D in vitro Sprouting Assay

Samples were washed twice with HBSS (1x), fixed with 4% v/v PFA in HBSS (20 min at RT) and washed again with HBSS (1x). After, collagen I matrices and fibrin gels were permeabilized with 0.2% v/v Triton X-100 for 20 min and blocked for 1h with 1.5 wt.% BSA solution in HBSS. Afterwards, samples were incubated with phalloidin 647 (1:100, Flash Phalloidin Red 647, Biolegend 424205), with mouse anti-CD31 (1:50, DAKO M0823) combined with rabbit anti-laminin (1:100, Sigma L-9393) stirring for 2h at RT, and after that, stored ON at 4°C. After washing, scaffolds were incubated for 4 h at RT with the secondary antibodies Alexa Fluor 594 goat anti-mouse (1:500, Invitrogen A11020) and Alexa Fluor 488 goat anti-rabbit (1:500, Invitrogen A11008). Alternatively, it was also used phalloidin 488 (1:100, Flash Phalloidin Green 488, Biolegend 424201), with rabbit anti-fibronectin (1:200, Sigma F3648) combined with mouse anti-CD31 (1:50, DAKO M0823) as primary antibodies. In that case, the secondary antibodies used were Alexa Fluor 647 donkey anti-rabbit (1:500, Invitrogen A31573) and Alexa Fluor 594 goat anti-mouse (1:500, Invitrogen A11020).

Lastly, samples were washed 3 times with HBSS, the nuclei were counterstained with DAPI. 3D imaging of the samples was accomplished by CLSM (SP5, Leica) and data analysis was performed using Fiji Imaging software.

3.18. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software version. One-way ANOVA test was used for the analysis of the metabolic activity. Results for all analysis with 'p' value less than 0.05 were considered to indicate statically significant differences.

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4. Results and Discussion

4.1. Synthesis of RGD-grafted alginate

Regarding the development of *in vitro* tumor models or other tissue engineering applications, the choice of the biomaterial must comply with several key requisites, related with biocompatibility, controlled biodegradability, surface properties, mechanical properties, architecture and pore density, among others. A common requisite when selecting the biomaterial is its capability of providing structural support and biological signaling to cells, in a similar way as the native ECM does *in vivo* [75], which has a great impact in modulating cell response [40].

Alginate meets the aforementioned requirements, being widely used as ECM analogue in 3D models. The low toxicity and high biocompatibility of this natural polymer is already well established [72, 94]. Moreover, alginate allows the formation of soft hydrogels under physiological conditions, forming a porous mesh that allows nutrients, oxygen and growth factors to easily diffuse into the hydrogel, reaching the cells that are entrapped within the polymer network [72, 94].

However, alginate is bio-inert, meaning that it is not able to interact with cells, unless it undergoes biofunctionalization [94]. In order to promote alginate-cell interaction, bioactive peptide sequences that are known to bind to integrin receptors on the cell surface can be covalently linked to alginate [98, 99]. The most common peptides used to mimic adhesive properties of ECM proteins and, subsequently, to stimulate cell differentiation and proliferation, are Arg-Gly-Asp (RGD) ligands [99, 100]. These peptides are very abundant in adhesion proteins, such as fibronectin, laminin and fibrinogen [101].

Herein, to promote cell attachment, high molecular alginate was covalently grafted with the cell-adhesion peptides (glycine)⁴-arginine-glycine-aspartic acid-serine-proline, using aqueous carbodiimide chemistry as previously described [93]. The use of carbodiimides is reported as a popular method for binding peptide amine groups to polymer carboxylic groups, resulting in amide formation [102]. Water-soluble EDC is a widely used carbodiimide for aqueous crosslinking [102]. It has been described that carbodiimide-based chemical modification of alginate typically yields 0.1-1.0% mol peptide per mol of alginate monomer [98, 100]. Here, the amount of grafted RGD was quantified using the BCA Protein Assay, being the coupling efficiency obtained 21%, which is equivalent to 5.48 mM of RGD per mol of alginate. This concentration was found to be similar to that which is reported for ECM-derived biological matrices [103], and proper to promote endothelial cell attachment [93].

4.2. 3D culture of epithelial cells and hMFs in RGD-alginate matrices

Cancer cells reside in a complex microenvironment containing various supporting cells, ECM and a cocktail of signaling molecules. These environmental components collectively contribute to tumor-stromal interactions and tumor progression [104]. Cells reside in highly structured 3D environment and receive extensive inputs from cell-cell, cell-matrix, and soluble signals. The stromal component of tumor microenvironment has an acellular portion, the ECM, and stromal cells, in great part fibroblasts [13, 41, 42, 104], which synthesize and secrete different ECM components, particularly collagens and fibronectin [51].

The establishment of the proposed tumor like constructs was based on previous work, where soft alginate hydrogel functionalized with RGD peptides was used to mimic the 3D microenvironment of normal murine mammary epithelial EpH4 cells [105]. That study reported that the use of 1 wt.% RGD-alginate hydrogels and a cell density of 5×10^6 cells/mL allowed to create a 3D microenvironment where epithelial EpH4 cells formed large spheroids with high cell viability [105]. Epithelial morphogenesis was also supported under similar conditions when using human breast epithelial cells, MCF10A (Figure S1). Thereby, herein, the same conditions were selected to establish 3D cultures of human mammary fibroblasts and highly invasive human breast cancer MDA-MB-231 cells.

In order to validate the proposed tumor like 3D constructs and understand better the influence of fibroblasts presence, we assessed the metabolic activity and morphology of MDA-MB-231 cells and hMFs, either in monoculture or in co-culture, within 3D RGD-alginate matrix (Figure 11).

Regarding metabolic activity, MDA-MB-231 cell monoculture and MDA-MB-231-hMFs co-culture presented higher metabolic activity than hMFs monoculture (Figure 11C), although, all the conditions presented a gradual increase in metabolic activity until day 7 (Figure 11D). This increase suggests that MDA-MB-231 were actively proliferating, both in monoculture and co-culture. Accordingly, in both cultures, we were able to observe the formation of multicellular spheroids after 7 days in culture (Figure 11A-a and 11A-c) that increased in size throughout time (Figure 11B-a and 11B-c). Importantly, in both MDA-MB-231 monoculture and coculture we could detect some spheroids with lumenization and peripheral nuclear alignment (Figure 11A and B, green and yellow arrows). As time progressed, metabolic activity levels slightly decreased from day 7 to day 12, and from that point on they stabilized until day 14 (Figure 11C and 11D). Despite this, from day 7 to day 14, the number of spheroids seemed to increase in number and in size. For instance, it was observed an increase in terms of diameter of MDA-MB-231 spheroids, from an average of 32 μm at day 7 (Figure 11A-a) to 38 μm at day 14 (Figure 11B-d). In coculture spheroids

the diameter duplicated, reaching an average diameter of around 53 μm by day 14 (Figure 11B-f). Still, it cannot be assured that hMFs contributed physically to the increase on spheroids size, or if it was just through any paracrine interaction, since no specific hMFs marker was used herein to allow for specific fibroblast localization. MDA-MB-231 is an epithelial cell line that lacks the expression of typical epithelial markers such as E-cadherin and β -catenin at the cell membrane [106] and are positive for mesenchymal markers, like fibronectin and vimentin, which posed some technical limitations for cell identification [107]. One possible approach to explore in future studies could be labelling cells with differently colored cell trackers, before embedding both cells in alginate, and following cell distribution throughout time.

Concerning hMFs monoculture, these cells do not organize in a spheroid conformation (Figure 11A-b and 11B-b), but tended to aggregate forming small clusters or remained individually distributed within the matrix with essentially round shape. These findings were not totally unexpected, as the alginate used is not degradable, thus physically restricting the typical spreading of fibroblasts [108]. Nevertheless, at higher magnification it was possible to observe some cytoplasmic protrusions into the matrix (data not shown). However, hMFs mitochondrial metabolic activity slightly increased along time (Figure 11C and 11D), showing that entrapped hMFs remained viable.

Even if hMFs in monoculture showed a slower increase in metabolic activity over time (Figure 11C and 11D) than in coculture, the presence of these cells seems to have positively influenced the overall proliferative capacity of co-culture cells, as well as epithelial spheroids formation, as mentioned before. Comparing to a theoretical estimative, in which half of the total metabolic activity may be assumed to relate to each cell type, experimental results from hMFs-MDA-MB-231 co-culture were very similar in the first week of culture (Figure 12). However, during the second week the experimental values were slightly higher than the theoretical ones, suggesting a positive synergy between both cell types.

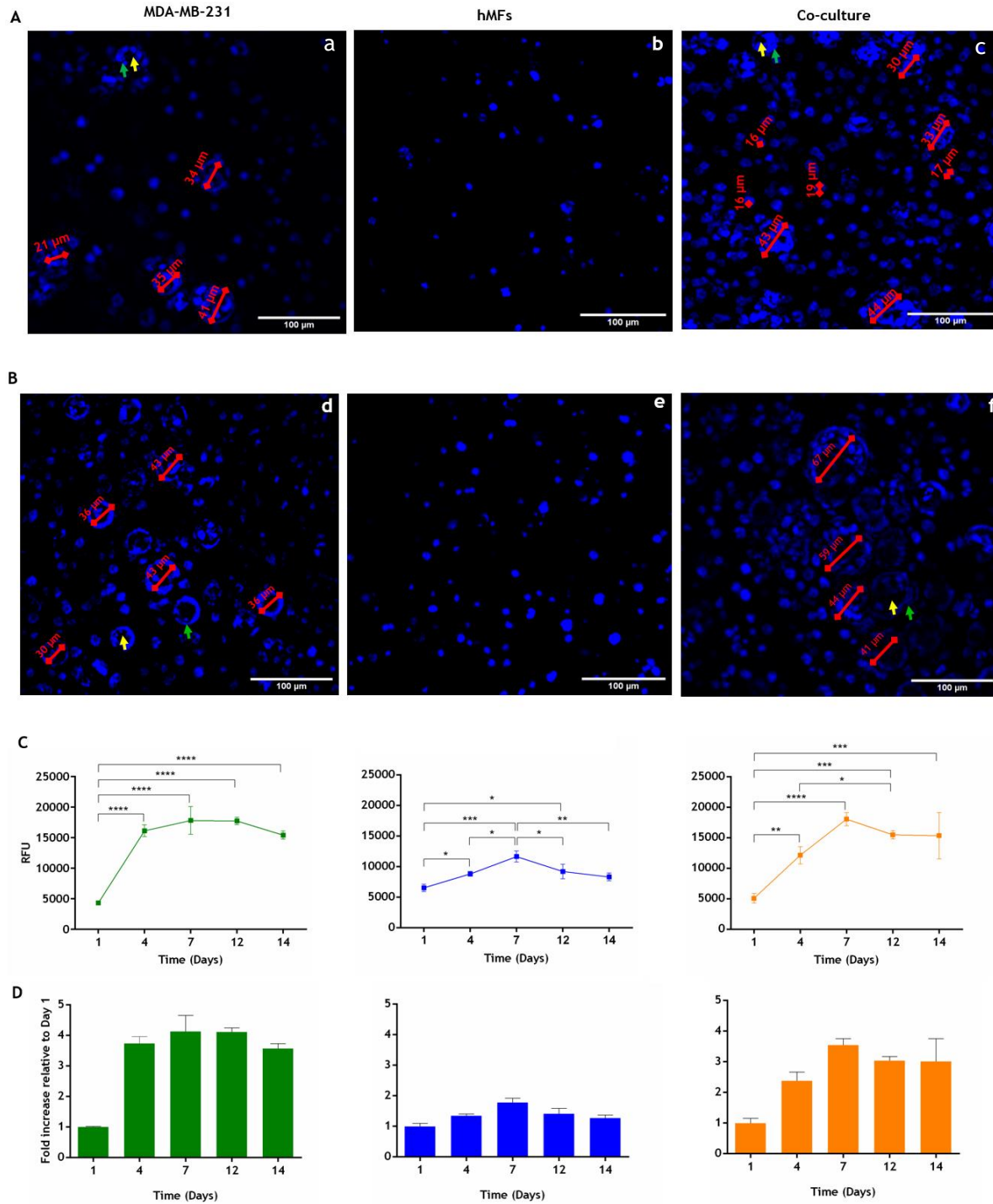


Figure 11. Morphological and functional features of MDA-MB-231 cells, hMFs and co-cultures within 3D RGD-alginate matrix. (A) Representative CLSM images of spheroids formed after 7 days by MDA-MB-231 cells (a), hMFs (b), and co-cultures (c). (B) Representative CLSM images of spheroids formed after 14 days by MDA-MB-231 cells (d), hMFs (e), and co-culture (f). Spheroids were stained with DAPI (scale bar: 100 μ m). (C) The metabolic activity of the different cultures showed an increase until day 7, followed by stabilization. Data is presented as mean $\pm$ stdev (n=3) (*p=0.0185, **, *** p=0.0004, and **** p<0.0001). (D) Fold increase of metabolic activity relative to day 1.

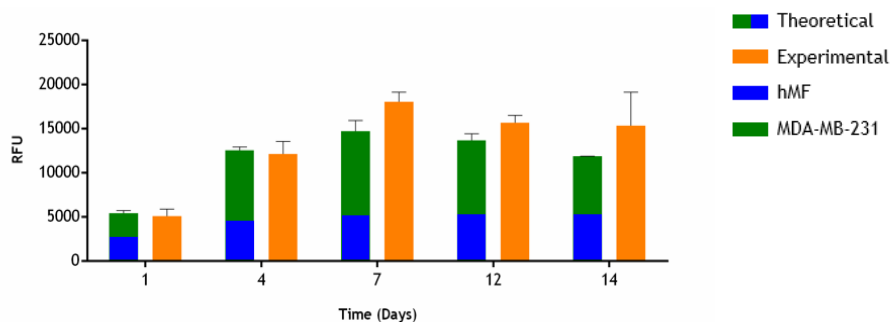


Figure 12. Experimental and theoretical prediction for the metabolic activity of cells within 3D matrix. It is assumed that as total cell density is the same (5×10^6 cells/mL), each cell type is responsible for half of the metabolic activity registered when in monoculture. Data are presented as mean $\pm$ stdev ($n=3$).

4.3. Effect of increasing the stiffness of RGD-alginate matrices

As mentioned before, the ECM plays an undeniably important role in cancer progression, undergoing dynamic transformations in terms of structure and composition. Such alterations significantly influence cellular behavior, affecting migration, proliferation, morphology, as well as differentiation. Changes in matrix stiffness also occur during tumor progression [56, 104], being reported that healthy mammary gland tissue has an elastic modulus of 100-200 Pa, whereas in mammary tumors stiffness increases to 1000-4000 Pa. The increase of tumor ECM stiffness can result from tumor cells interaction with surrounding matrix, as reported for MDA-MB-231 cells [109].

In order to provide physiologically-relevant biomechanical cues, alginate hydrogels properties were tailored to attain viscoelastic properties comparable to that of normal breast tissue and mammary gland ($G' \approx 200$ Pa), and alternatively, to that of breast tumors ($G' \approx 3000$ Pa).

We successfully obtained softer hydrogels with stiffness comparable to that of normal mammary tissue, as demonstrated by rheological analysis of cell-laden 3D matrices (Figure 13A - Ctrl). These matrices were subsequently treated with ionic crosslinkers to promote stiffening. Although CaCl_2 has been used as stiffening agent in previous works [110], calcium ions are implicated in several cellular functions and variations in calcium concentration might thus interfere with key cell signaling or cell adhesion mechanisms. Therefore, different ionic crosslinkers: calcium chloride (CaCl_2), strontium chloride (SrCl_2) and barium chloride (BaCl_2), were

tested. Comparing the effect of the three stiffening agents, the elastic modulus (G') of the hydrogels decreased in the order $\text{BaCl}_2 > \text{SrCl}_2 > \text{CaCl}_2$ (Figure 13B). The desired effect, in terms of stiffness fold increase, was obtained with BaCl_2 , with values reaching around 2257 Pa, as demonstrated by rheometry analysis of cell-laden 3D matrices

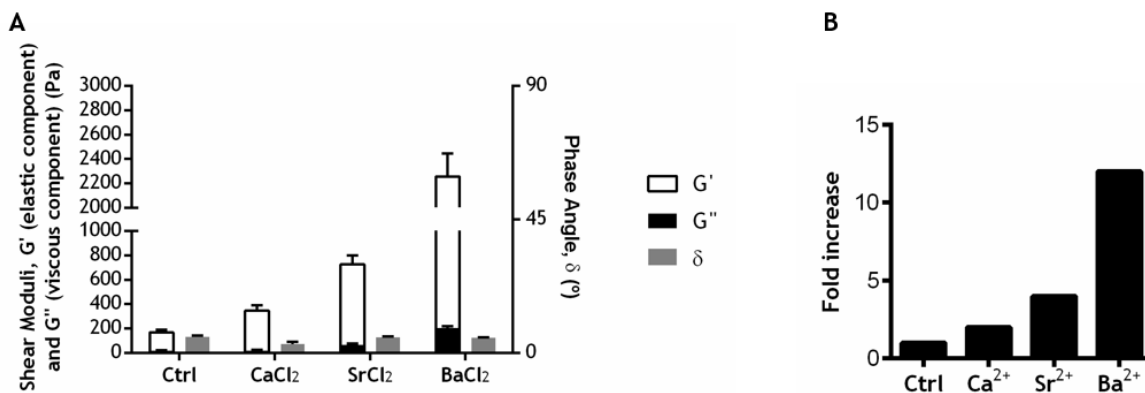


Figure 13. Rheological measurements. Viscoelastic properties (elastic, G' and viscous, G'' components of the shear moduli, and phase angle, δ) of 1 wt.% RGD-alginate with MDA-MB-231-hMFs (A). To provide physiologically-relevant biomechanical cues, alginate hydrogels properties were tailored to attain viscoelastic properties ($G' \approx 200$ Pa) comparable to that of normal breast tissue and mammary gland, and alternatively ($G' \approx 3000$ Pa) close to mammary tumor stiffness. For that it was investigated which ionic crosslinker (Ca^{2+} , Sr^{2+} , Ba^{2+}) would drive to the desired stiffness increasing. Fold increase of matrix stiffness relative to control (B).

The existing literature shows that mechanosensing and metabolic pathways are interrelated and can be modulated through ECM mechanical properties. Cancer cells normally upregulate their metabolism in order to cope with the high-energy needs. This adaptation is known as the Warburg Effect [111], which is characterized by a shift of aerobic respiration towards aerobic glycolysis, and allows faster ATP obtainment [112]. Interestingly, there are evidences for a positive response on MDA-MB-231 metabolism to matrix stiffness increase [112, 113].

Our results showed a decreased in metabolic activity of MDA-MD-231 cells and hMFs, either in monoculture or co-culture (Figure 14). This decrease was more pronounced in MDA-MB-231 monoculture over the first days (until day 7) (Figure 14, light green bars), which then become less evident, following the stabilization tendency until day 14, hMFs also decreased their mitochondrial activity, however differences between cell behavior in soft and stiff matrix were more significant from day 7 onwards (Figure 14, light blue bars). In fact, this cellular response seemed to be reproduced in co-culture, where the same tendency of metabolic activity diminution from day 7 was observed (Figure 14, light orange bars). An explanation for the observed results is still

unclear. On one hand, matrix stiffness in itself may be interfering with normal cell metabolism and/or inducing some phenotypic alterations. On the other hand, differences may be related with a lower cell number due to a more restrictive network on stiffened matrices, which may provide a physical barrier for cell growth (Figure S2) [114]. Finally, results might be affected by artifacts related with the resazurin-based assay that was used to detect metabolic activity. This assay is based in the conversion of the blue nonfluorescent dye resazurin to the pink fluorescent resorufin by mitochondrial enzymes, thus evaluating the mitochondrial metabolic activity. It has been described that MDA-MB-231 cells exhibit some metabolic dysfunction, resorting to glycolysis instead of mitochondrial respiration to manage metabolic requirements [115]. Perhaps this might explain the decrease in mitochondrial metabolic activity detected by the resazurin-based assay. To evaluate such possibility, an assay based in ATP quantification could be performed in the future, to get more reliable results in terms of cellular metabolic activity.

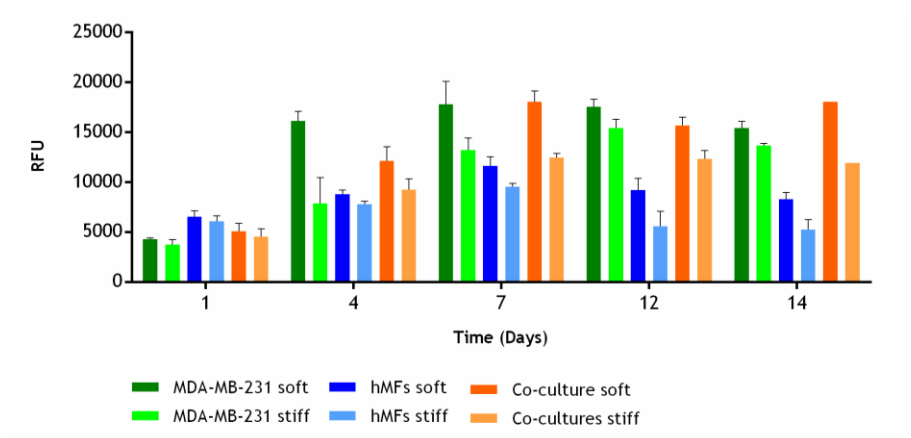


Figure 14. Metabolic activity of monocultures of metastatic breast cancer cell line (MDA-MB-231) and human mammary fibroblast (hMFs) and co-culture of both cell types during 2 weeks of culture. Cell-laden hydrogels were stiffened at the first day of culture (light bars, stiff) or maintained in culture without changes (dark bars, soft). The pattern of metabolic activity observed in both stiffnesses is similar. Cells seem to be proliferating until day 7, then a slightly decrease occurs, remaining approximately stable until day 14.

4.4. Mimicking hypoxic tumor core

Environmental stresses in tumor core, characterized by hypoxia, nutrient deprivation and increased levels of metabolic wastes and carbon dioxide are well established. Hypoxia leads to the expression of a number of gene products that are involved in tumor progression, invasion and metastasis formation. At molecular level, the adaptation of tumor cells to hypoxic conditions is

largely regulated by hypoxia-inducible factor-1 α (HIF-1 α), a transcription factor that accumulates in response to decreased cellular oxygen levels [116]. Carbonic anhydrase 9 (CA9) is considered one of the most sensitive endogenous sensors of HIF-1 activity [116].

MDA-MB-231 cells and hMFs cultured within RGD-alginate matrices for 4 and 11 days of culture under normoxic conditions, were exposed to hypoxic conditions for 3 days, in order to closely mimic the *in vivo* situation. The impact of this hypoxic stage was then investigated in cellular metabolic activity and in CA9 expression.

The mitochondrial metabolic activity of cells cultured under hypoxic conditions for 3 days, showed a decrease at day 7, comparing to cells cultured under normoxic conditions (Figure 15A). At day 14, this difference was less pronounced (Figure 15B). Besides that, the pattern previously observed for metabolic activity of cells on stiff and soft matrices seemed to be maintained, being the levels registered on stiffer matrices (light bars) slightly lower than those of cells cultured in soft matrices (dark bars). In fact, this is true for all conditions, except for hMFs monoculture at day 14, under hypoxic conditions (Figure 15B). Since this was against what had been previously observed under normoxic conditions, and only occurred for that specific sample, this result was not considered significant. From what is reported in 2D cultures, the inhibitory effect of hypoxia in MDA-MB-231 cells growth is not significant [117], and the same is true for fibroblasts [118, 119]. In fact, preservation of metabolic activity levels was expected, since MDA-MB-231 cells present lower basal oxygen consumption rate [120], regardless of decreasing in stiff environments [120]. The variation in oxygen tension due to the exposition to a hypoxic environment should not be significant enough to induce a decrease in metabolic activity. One possible explanation could be related to the fact that in hypoxic conditions some cells decrease their mitochondrial activity [121]. Since resazurin is converted in mitochondria and mitochondria activity is reduced, this could explain the observed reduced conversion of resazurin in resorufin.

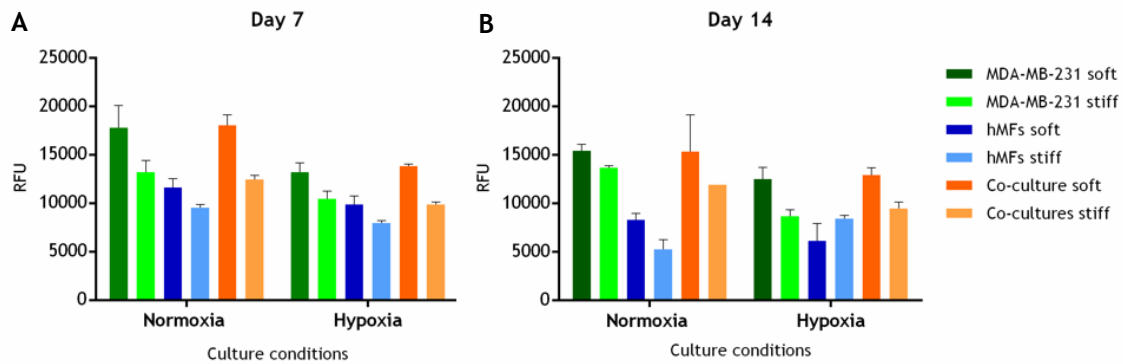


Figure 15. Metabolic activity of monocultures of metastatic breast cancer cell line (MDA-MB-231) and human mammary fibroblasts (hMFs) and co-culture of both cell types at (A) day 7 and at (B) day 14. Cell-laden hydrogels were stiffened at the first day of culture (light bars, stiff) or unchanged (dark bars, soft). Both matrices were maintained in normal culture conditions (21% v/v O₂, 5% v/v CO₂, Normoxia) or under hypoxia (1% v/v O₂, 5% v/v CO₂).

CA9 is a well-known tumor-hypoxic marker, which some studies have shown to be present in tumor cells, such as MDA-MB-231 [122]. Considering what had been demonstrated in MDA-MB-231 cells 2D cultures, it was expecting an increase in CA9 expression in response to hypoxia [122, 123].

Results presented herein are preliminary, corresponding to one experiment, which unfortunately could not be repeated. Cells cultured in soft matrices for 7 days were used as a control. It was possible to observe an upregulation in CA9 expression in soft matrices from day 7 to 14 (Figure 16). This could be related to the observed increased in spheroids size that could lead to a hypoxic center with proliferative cells in the periphery. To confirm this hypothesis, an immunostaining of ki-67 (proliferation marker) combined with a caspase 3 (apoptotic marker) could be performed in future studies. Another interesting result was obtained with stiff matrices under normoxic conditions at day 7 (Figure 16, Normoxia). Comparing both, it was possible to observe an increase in CA9 expression when cells were cultured in stiffer matrices. This result corroborates what we have previous highlighted, which is that the matrix stiffness in itself may interfere with oxygen diffusion. However, further analysis needs to be considered.

As expected, when soft matrices were cultured for 3 days under hypoxic conditions, we were able to observe an increased in CA9 expression, indicating a cell response to the low levels of oxygen

Overall, the combination of stiff matrices and hypoxic conditions resulted in a decreased in CA9 expression (Figure 16). An explanation for the observed results is still unclear. Further analysis on other pathways that are upregulated during hypoxic conditions need to be studied, such as, glut2, LDH A and lox.

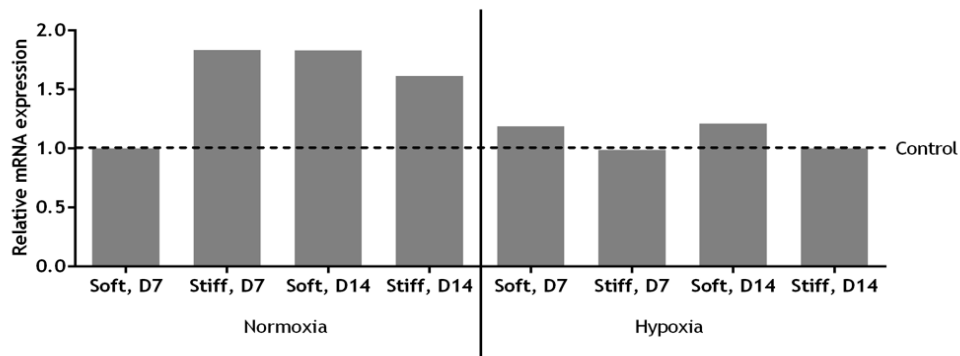


Figure 16. qRT-PCR quantification of relative mRNA expression of CA9 (carbonic anhydrase 9, hypoxic marker). Data normalized to cells cultured for 7 days under normoxia conditions (Control).

4.5. Evidences of ECM remodeling

Migration and invasion are extremely important processes on tumor progression. These two processes are often correlated with increased levels of matrix metalloproteinases (MMP) activity [124], namely MMP2 and MMP9, which have a key role in ECM degradation [125]. MMPs are produced both by tumor cells and stromal cells present in tumor microenvironment. This production occurs in response to reciprocal paracrine stimulatory interactions [126].

In order to understand possible alterations in the invasive capacity of cells under the effect of increased stiffness and hypoxic conditions, activity of MMP2 and MMP9 in conditioned media from 3D co-cultures was quantified. Since inconsistent results were obtained for day 14 (initially, two time points were chosen, days 7 and 14), the presented results correspond only to the first time point (7 days).

Globally, we observed increased expression of MMP2 comparatively to MMP9 (Figure 17A and 17B). Under normoxic conditions, stiffened matrices presented higher production of both MMPs comparing to soft (Figure 17C and 17D). Since increasing matrix stiffness will constrict the space where MDA-MB-231 cells and hMFs can be organized, it would be expected that cells could react to the tension exerted by surrounding matrix, increasing secretion of MMPs to allow ECM degradation and allow cell mobility [127]. This could explain why MMPs production is increased in stiff conditions. When comparing normoxia vs. hypoxia, it was observed an increase on both MMPs production linked to low oxygen levels (Figure 17C and 17D). These findings were expected, since it has been reported that hypoxia activates several pathways related to ECM degradation and remodeling [128].

Considering that a synergistic effect could result from a combination between stiffness and hypoxia, an increased MMP production would be expected. However, that increase was only detected for MMP9 (Figure 17D), while for MMP2 a slight decrease was observed (Figure 17C). Hypoxia and tumor-associated stiffness are triggers for a wide range of pathways, whose participants can interact and perhaps have mutual inhibitor effect. Yet, no conclusions can be taken based in the obtained results, since the experience should be repeated and further supported by the study of markers secreted by tumor cells, which are already known to be implicated in ECM remodeling by MMPs (one example is LOX gene) [128].

It would be newsworthy to understand if the alginate matrix stiffening combined with MDA-MB-231 produced factors could induce hMFs activation (by investigating the presence of α -SMA marker, for example), since hMFs activation is related to MMP-9 enhanced production [129].

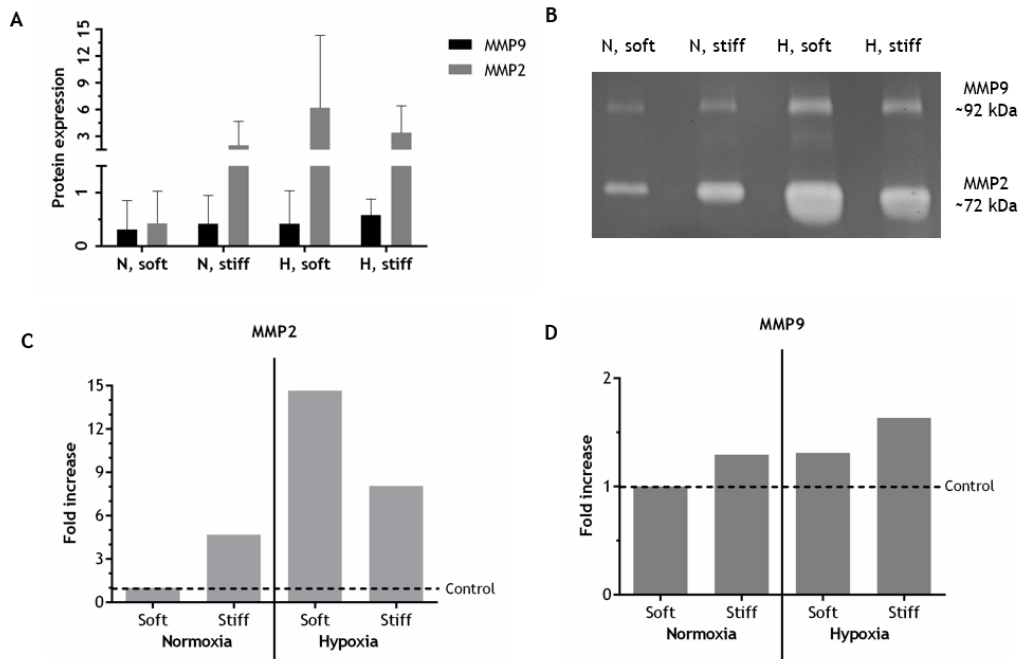


Figure 17. Activity of MMP2 and MMP9 secreted by 3D cocultures of MDA-MB-231 and hMFs embedded within soft or stiff alginate matrices under normoxia or hypoxia conditions for 7 days. (A) Quantification of MMP2 and MMP9 secretion from 3 different experiments. (B) Gelatin zymography for the different conditions tested in 1 experiment. MMP9 band appears at around 92 kDa and MMP2 at 72 kDa. (C) Fold increase of MMP2 and (D) MMP9 secretion relative to the total amount detected in soft matrices of 1 experiment.

4.6. Paracrine effect of tumor-like constructs in endothelial cell recruitment

Cells present in tumor microenvironment are responsible for producing factors that stimulate tumor angiogenesis, such as vascular endothelial growth factor (VEGF) or basic fibroblast factor (bFGF/FGF-2) [63]. VEGF is directly stimulated by low oxygen levels, and tumor-associated stiffness is also described as a tumor angiogenesis inducer, by directly increasing expression of VEGF, or indirectly by blocking the existing vasculature surrounding the tumor mass to promote hypoxia and VEGF secretion [63, 65].

In order to evaluate the paracrine effect of co-cultures in endothelial cell recruitment, the effect of conditioned medium in endothelial cell proliferation and migration was assessed by a scratch assay and a direct migration assay, respectively. Progenitor outgrowth endothelial cells, OECs, were chosen for these assays since they are described as being able to respond to angiogenic stimuli via factors present in the extracellular environment [65].

Globally, a positive effect of different conditioned media in OECs proliferation was observed. Comparing to the negative control (only DMEM), all the wound closure percentages were superior, and even higher than the positive control (EGM-2MV) (Figure 18A). Since EGM-2MV contains a cocktail of endothelial cell growth factors, these results could indicate the presence of additional factors and/or at different concentrations in conditioned media, which potentiated OECs migration/growth.

On soft matrices under normoxia, wound closure was superior in samples at day 14 comparing to day 7. Comparing normoxia vs. hypoxia in soft matrices we also observed a higher EC proliferation under hypoxia conditions. Both results support our previous CA9 and MMP results, suggesting that CA9 upregulation, higher MMP secretion and EC recruitment are correlated with tumor-hypoxia conditions [130]. In opposite to that observed on CA9 expression and MMP secretion, at day 7 no striking difference were detected between stiff vs. soft matrices under normoxia conditions.

Regarding the combination of both stimuli, hypoxia and stiffness, there was a decrease in OECs proliferation as compared to control soft matrices (Figure 18A). As previously mentioned, the combination of both conditions could have a mutual inhibitor effect. Still, to validate this hypothesis further studies are mandatory

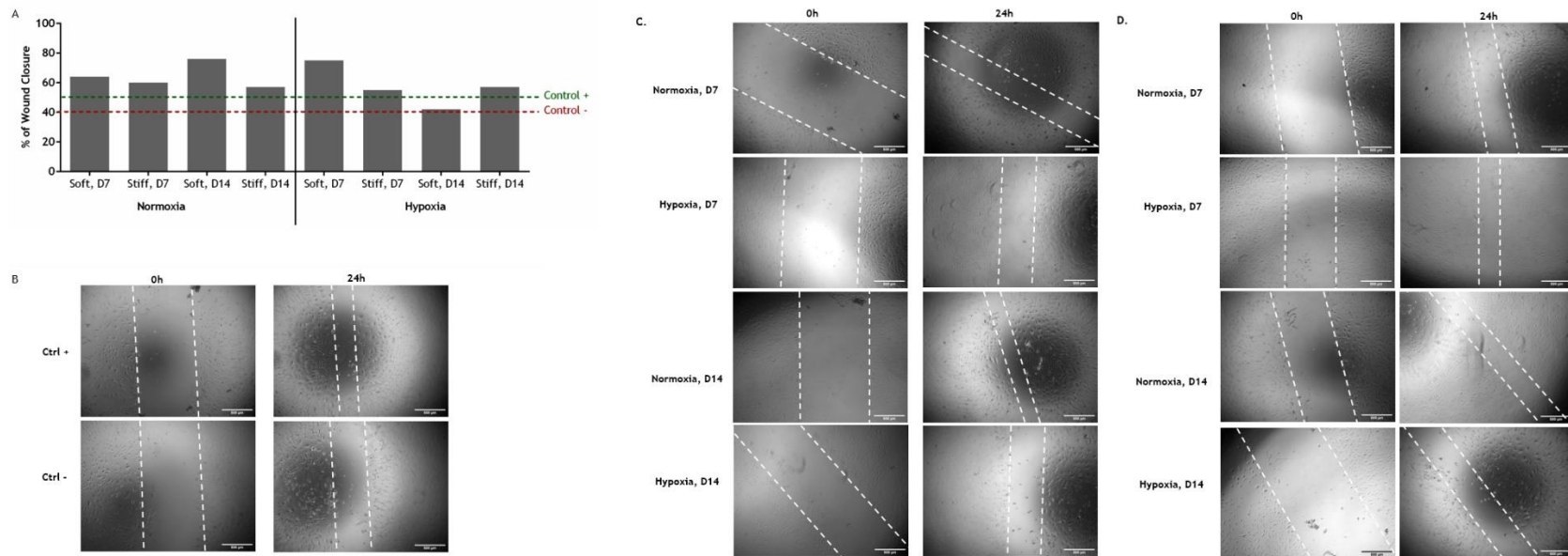


Figure 18. Analysis of OECs migration/proliferation under different conditioned media by in vitro scratch/wound assay. The conditioned media was collected from co-cultures of MDA-MB-231 and fibroblasts culture in 3D alginate matrices for 7 days (D7) or 14 days (D14) in soft or in stiff matrices, under normoxia or hypoxia conditions. Percentage of wound closure percentage in all the conditioned media tested (A). Red dot line corresponds to the percentage of wound closure when cells were culture only with DMEM (negative control). Green dot line corresponds to the percentage of wound closure when cells were culture with EGM-2MV (positive control). Wound appearance in the moment of the scratch (0h) and after 24h for the controls (B) and for the tested conditions, soft (C) and stiff (D) matrices. The white dot lines define the area lacking cells.

Although transwell migration-based experiments should to be repeated and a quantitative analyze performed, preliminary results are in agreement to those obtained with the scratch assay, showing higher EC migration in all conditioned media, comparing to negative control (Figure 19A). Under normoxia (Figure 19C), at day 7, enhanced EC migration in conditioned medium from stiff matrices was observed in comparison to soft matrices. In soft matrices under normoxia, EC migration appears to be dependent of culture time, since higher number of EC were observed in samples exposed to conditioned medium from day 14, comparing to those from day 7 (Figure 19C). Once again, the combination of both hypoxia and increased stiffness seemed to have a non-stimulatory effect in ECs migration, comparing to results from soft matrices (Figure 19D). Moreover, it seems that hypoxia did not significantly affect ECs migration, since the number of nuclei observed in the lower part of the transwell membrane appeared to be inferior to the one presented under normoxia (Figure 19C and 19D).

In some samples there are almost no differences, which may be related to the FBS percentage (10%) used in the conditioned media. This amount of serum could be masking the real effect of factors secreted by cells entrapped in alginate matrices. In the future, this assay could be repeated with lower percentage of FBS. Also, to complement these studies on ECs recruitment, a 3D sprouting assay could be performed.

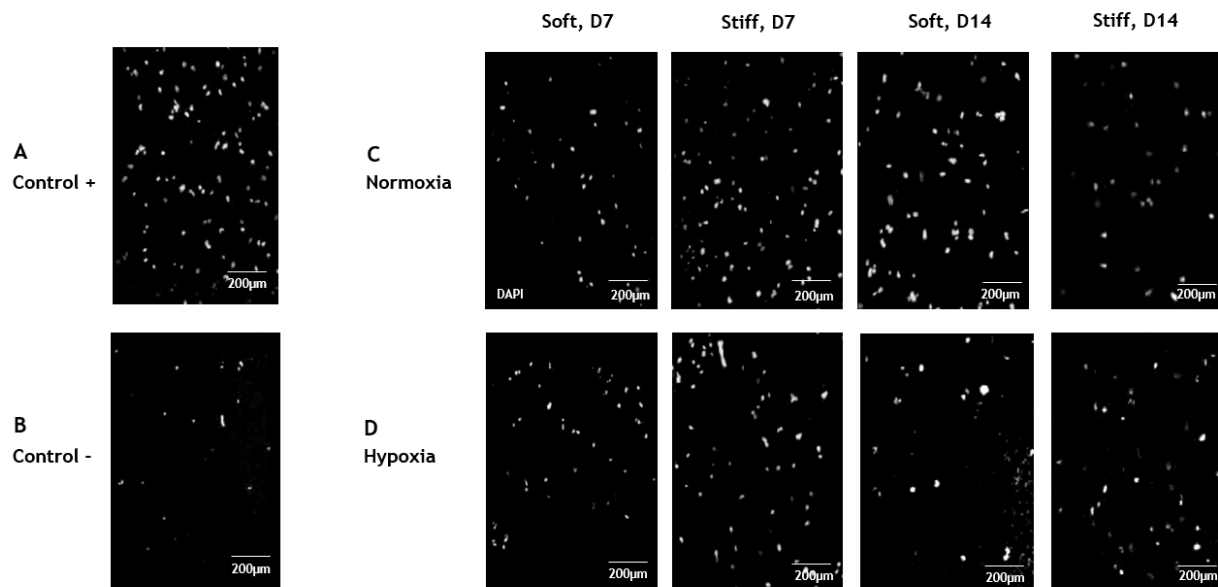


Figure 19. Transwell migration assay towards conditioned medium. Transwell membranes were used to quantify OECs direct migration and analyzed under CLSM. Representative images were selected for each condition, positive control (EGM-2MV) and negative control (DMEM) (scale bar: 200µm).

4.7. Preparation and characterization of RGD-grafted alginate scaffolds

Cell behavior in porous 3D scaffolds, as well as nutrients/oxygen/metabolites transport, is highly affected by pore size, pore shape, and pore volume fraction, since these variables affect total surface area and distribution of cell ligands [131]. It is reported that pore size essentially affects cell migration and adhesion, while pores interconnectivity contributes to cell growth and diffusional mechanisms within the scaffold [132, 133].

Aware of the importance of having a biomaterial with pores large enough to promote cell migration and, at the same time, small enough to retain a critical total surface area for appropriate cell interactions, scaffolds were prepared from a 2 wt.% RGD-modified alginate hydrogel, which was frozen at -20°C after being crosslinked at RT. Two different techniques were used to obtain porous 3D scaffolds (Figure 20A and 20B). The first one was the freeze-drying technique, which is based on direct sublimation of the ice present in the material due to the low pressure (near to vacuum), followed by the removal of the unfrozen water by desorption. This method allows the achievement of a highly porous sponge, in which pore shape results from that of ice crystals [131, 134]. Images obtained by SEM show scaffolds with sponge-like morphology and an average pore size between 96.64-552.5 μm (Figure 20C). Accordingly, it was already reported that the freeze drying procedure allows the production of scaffolds with pore diameter of 55-243 μm and porosity ranging from 33% to 68% depending on the freeze-drying pressure applied [135].

The freeze-drying technique presents advantages regarding simplicity, quickness and, importantly, the fact that is used water and ice crystals instead of organic solvents [131, 134]. Furthermore, it allows the formation of pores with sizes that can support the generation of blood vessels since EC migration and metabolites exchanges are guaranteed within pores with a minimum size of 30-40 μm [131, 136]. However, it was also showed that larger pores, 160 to 270 μm facilitate new vessels formation throughout a scaffold [131]. Thus, to address the challenge of creating structures with bigger pores and homogeneous size another approach was tested: the particle leaching technique associated with freeze drying.

The process is similar to freeze-drying technique, but in this case sodium chloride (NaCl) particles with size between 150 and 250 μm are incorporated into the alginate precursor solution [137]. Images obtained by SEM show the production of larger pores, with less variable size (average pore size between 175.1 and 450.9 μm) (Figure 20D) than the ones previously obtained with the freeze-drying technique (Figure 20C). Given that the freeze-drying process is less controllable, being strongly influenced by the water ratio of the polymer solution [135], it tends result in bigger difference in terms of pore size within the scaffold. On the contrary, in

combination with particle leaching technique, it leads to more uniform pore size, since theoretically, this is essentially controlled by the size of the porogen used [131, 134].

Observing SEM images from particle leaching-produced scaffolds, the internal structure seems to have lost integrity (Figure 20B). This can be due to the fact that increasing the pore size, affects the stability of the scaffold, as well as its capacity to provide physical support [138]. It is easily conceived to assume that as the obtained structure exhibits larger pores, in average, their architecture can be more easily destroyed when the initial cylindric 3D structures are cut into smaller discs (Figure 21). Another explanation is that NaCl particles may aggregate, due to their high hygroscopic nature, which compromises their homogenous distribution in the alginate solution. The possible formation of NaCl clusters could compromise the porous architecture. To avoid that, NaCl particles were sieved again and new scaffolds have been prepared (data not shown).

Regarding the interconnectivity of the pores (Figure 20, green arrows), no relevant differences were seen between the two techniques, as it was expected, since these techniques do not adequately allow to control pore shape and inter-pore distance [131].

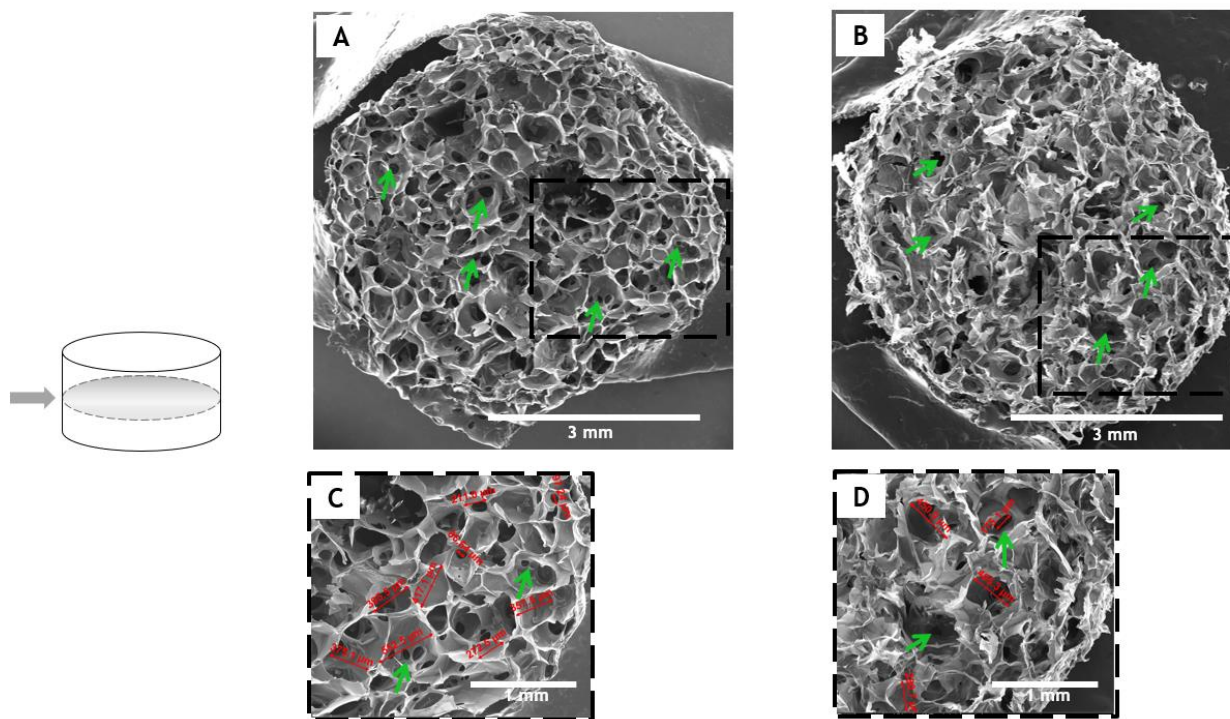


Figure 20. SEM images of RGD-grafted alginate scaffolds horizontal cross-section. Two techniques were considered: freeze-drying (A), particle leaching (B). Pore size differences between freeze-drying (C) and particle leaching (D). Green arrows indicate interconnected pores. Two magnifications are shown, 35x (A, B), and 100x (C, D).

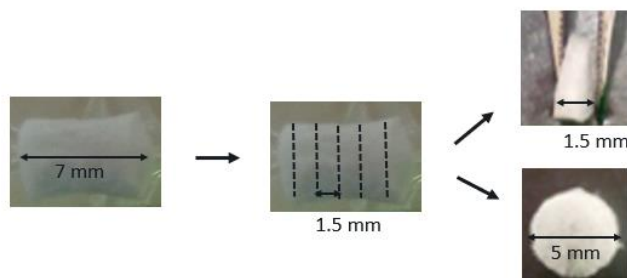


Figure 21. Cylindric scaffolds obtained after freeze drying. Using a scalpel blade, the obtained cylindric scaffolds were then cut into small ones with an average height of 1.5 mm.

4.8. Seeding of human mammary fibroblasts and endothelial cells on RGD-alginate scaffolds

The establishment of endothelial cell network is enhanced by the presence of stromal cells, such as fibroblasts, which produce soluble factors and ECM components, stabilizing vascular structures through cell-cell and cell-matrix interactions [48, 49]. OECs present high proliferative capacity in long term monolayer cultures [139]. When seeded on Matrigel matrix, these cells are able to form interconnected cord-like structures, and co-culture with stromal cells favors the formation of micro vessel-like structures [140]. In the present work, porous RGD-grafted alginate scaffolds were seeded with hMFs and OECs. When establishing co-cultures, the cell ratio is an important factor to take into account. Some works report that a higher ratio of endothelial cells would guarantee the formation of tubular-like structures [141-143]. On the other hand, a superior ratio of stromal cells may be desirable and, in cocultures of endothelial cells and fibroblasts, it has been showed that matrix proteins produced by fibroblasts support endothelial cell migration and the formation of a capillary-like tubular structure [144, 145]. In the light of previous work done in the group (data not published), no significant improvements resulted from the use of different ratios of the two cell types on similar alginate scaffolds. Therefore, herein the cellular ratio was set to 1:1.

Two seeding approaches were tested for their potential to provide better cell distribution throughout the scaffold (Figure 9). Endothelial cell organization within scaffolds was analyzed by CD31 (endothelial marker) and laminin staining. As the alginate used for the scaffold's preparation was previously grafted with RGD peptides, cells were able to adhere and spread, producing ECM proteins [146], such as laminin, which is determinant to the basement membrane assembly [146].

In both strategies, the total volume of cell suspension was directly added on top of the scaffold. In one of the conditions, after dropping 30 μ L cell suspension on the top of the scaffold, a centrifugation step was implemented (5 min, 1000 rpm) [97].

In the first strategy, two different final cell densities, 50×10^4 cells/scaffold (Figure 22A), and 25×10^4 cells/scaffold (Figure 22B), were tested. The best outcome, both in terms of cell spreading throughout the scaffold, and OECs organization into microvessel-like structures, was obtained with the highest seeding cell density (Figure 22A). Figure 22 shows cellular distribution along the scaffold, at each side of the scaffold (Figure 22i and 22ii), after 14 days of culture in EGM-2MV. In both seeding approaches, a higher cell density was observed on-top of the scaffold, where seeding was performed (Figure 22i). However, the presence of cells in both sides (Figure 22i, 22ii) suggests that cells can migrate through the porous scaffolds, even in scaffolds prepared by the freezing-drying technique. These results also showed that, after 14 days of culture, hMFs

tend to fill spaces within the scaffold and produce ECM (Figure 22A-c, 22A-d, 22B-j and 22B-k) (Figure S3), whereas OECs form tubular architectures, preferentially aligned along pores' walls (Figure 22A-a, 22A-b, 22B-g and 22B-h).

As mentioned before, since optimal cell distribution throughout the scaffolds was obtained with the highest seeding cell density tested (50×10^4 cells/scaffold) (Figure 22A), this density was the one selected for subsequent experiments

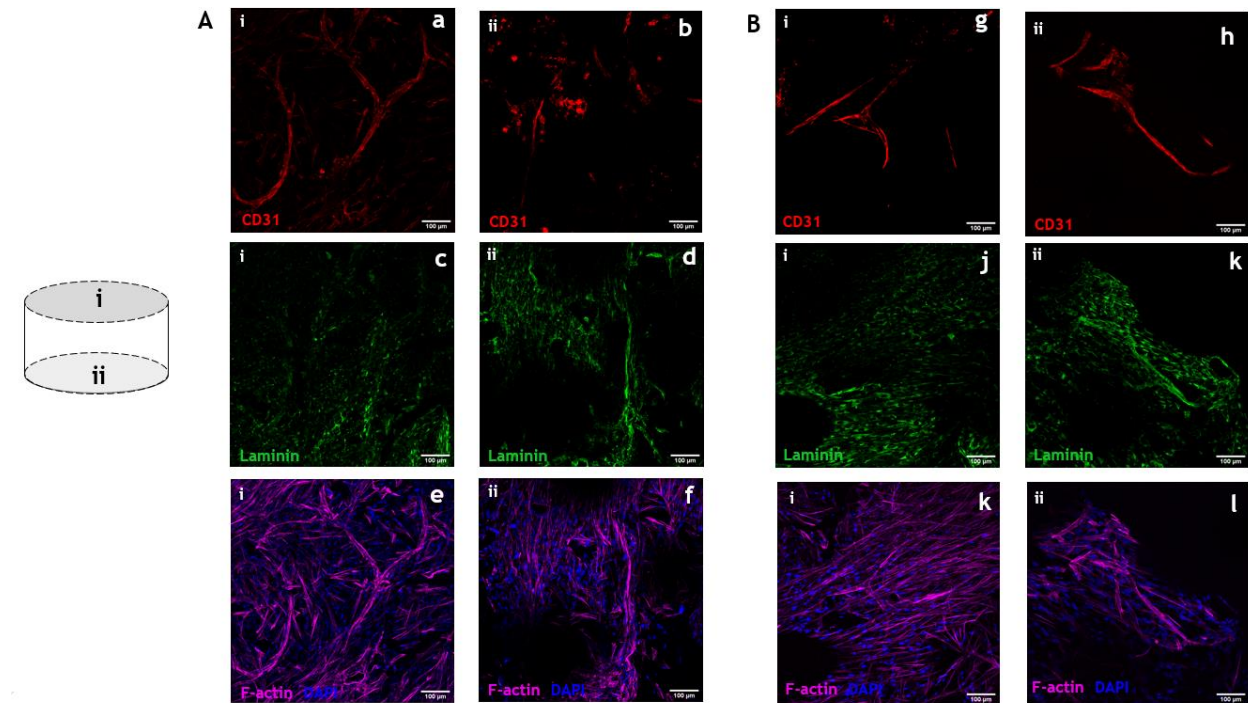


Figure 22. Immunostaining of hMFs and ECs seeded on top of RGD-grafted alginate scaffolds after 14 days of culture. 2% wt. RGD-grafted alginate scaffolds were prepared by freeze drying technique. Two final cell densities were used: 50×10^4 cells/scaffold (A) and 25×10^4 cells/scaffold (B). Cytoskeletal organization of hMFs were observed in both sides of the scaffold (i, ii), under a CLSM and demonstrated by F-actin (magenta), laminin (green), CD31 (red) and DAPI (blue). (Scale bar: 100 μ m).

To compare, simultaneously, how the scaffold preparation approach and the seeding strategy influence cellular organization within the scaffolds, different cross sections of 14-cultured scaffolds were analyzed under CLSM (Figure 23). Overall cellular organization within the scaffolds was demonstrated by the presence of F-actin and DAPI. Endothelial cell organization scaffolds was analyzed by CD31 (endothelial marker) staining, and fibroblasts ability to produce ECM proteins was analyzed by laminin staining.

The combination of particle leaching with cell centrifugation had clearly the best outcome in terms of cell infiltration and organization within the scaffold (Figure 23A-c). Comparing to the

scaffold produced without centrifugation during seeding (Figure 23B), it was observed a lower density and organization of both cells types (Figure 23B-a and 23B-b).

Regarding the scaffold prepared by freeze-drying technique without particle leaching (Figure 23C and 23D), the influence of centrifugation was not as significant as in the previous condition. Between centrifuged (Figure 23C) and non-centrifuged scaffolds (Figure 23D), there seems to be a slight difference in terms of cellular spread next to the side where the seeding was performed (Figure 23C, red arrow).

Comparing freeze-drying technique, with and without particle leaching, and without centrifugation (Figure 23B and 23D), there were no significant differences appeared in terms of cellular spread and organization, being the F-actin and DAPI distribution similar between the two sample (Figure 23B-c and 23D-c). One explanation could be that pores were not large enough to improve cell infiltration and eventually OECs organization in tubular-like architecture within the scaffold. Therefore, to improve scaffold colonization a centrifugation step may effectively be necessary to facilitate cell infiltration. These results can also be interpreted in light of the pores interconnectivity. Comparing scaffolds from the two different approaches, the interconnected pore area is higher in scaffolds with particle leaching, or at least, it is better defined. This could be clarified by additional optimization of NaCl particles concentration used for scaffold preparation. It would also be interesting to analyze the influence of freezing rate and pressure during the lyophilization process. In parallel, along all steps, SEM analysis could be performed to confirm the real influence of the referred parameters in the final 3D porous structure.

Overall, the RGD-grafted alginate scaffold produced by freeze drying technique with particle leaching, in combination with the centrifugation-based seeding strategy, clearly resulted in the best outcome. We clearly observed OECs organization into tubular-like structures in the scaffold core and near the pores (Figure 24-a, white arrow), as depicted in Figure 24. Therefore, this combinatory approach was the one chosen for the subsequent experiments

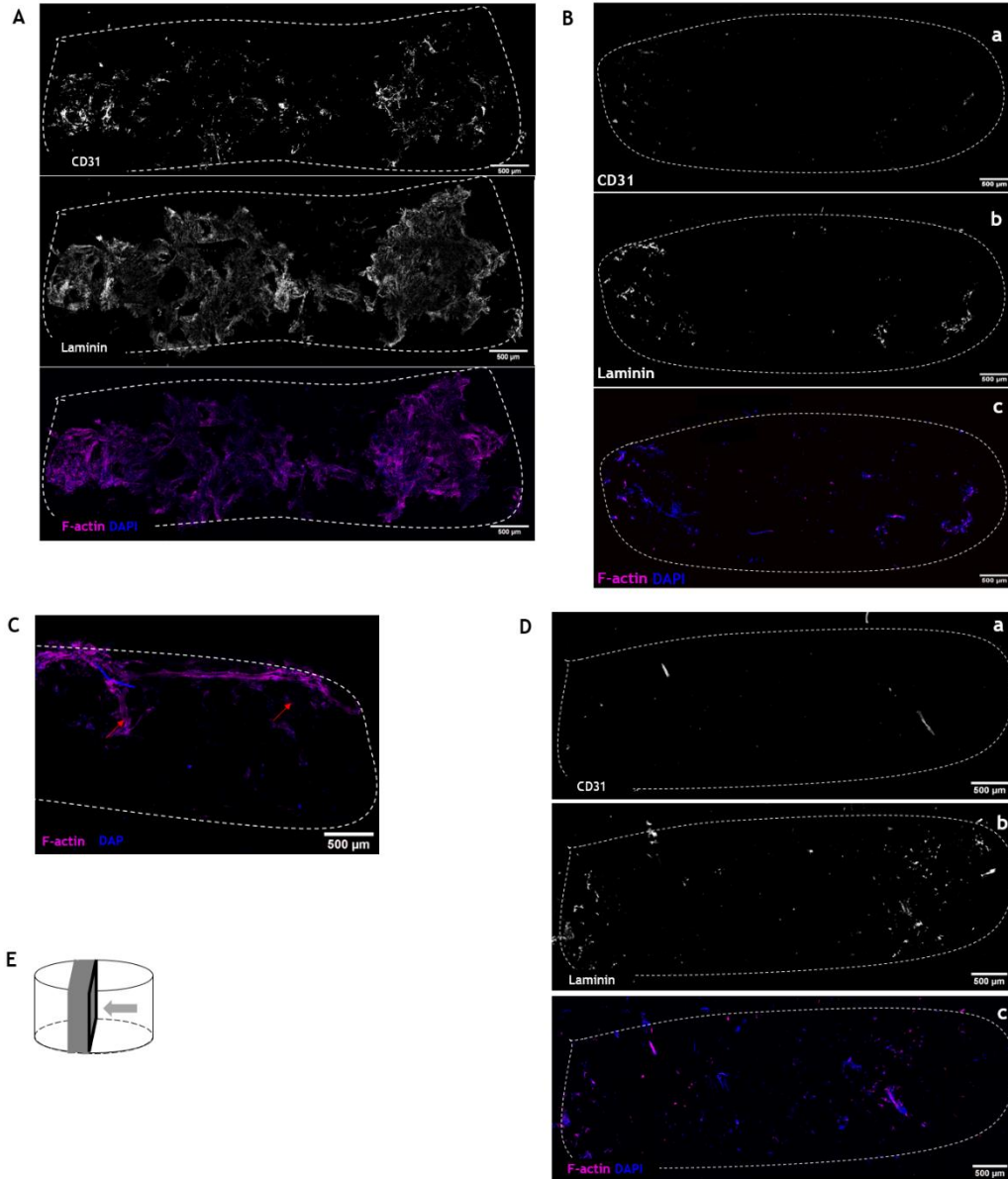


Figure 23. Immunostaining of hMFs and OECs seeded on RGD-grafted alginate scaffolds at a cell density of was 50×10^4 cells/scaffold. 2% wt. RGD-grafted alginate scaffolds were prepared by freeze-drying technique with particle leaching (A, B) and without particle leaching (C, D). Cells were seeded on top of the scaffold (B, D), followed by centrifugation (A, C). Cytoskeletal organization of hMFs and OECs after 14 days of culture were observed under a CLSM and demonstrated by laminin, DAPI and CD31. CD31 and laminin markers were not visible in the scaffold prepared by freeze drying technique without particle leaching and cell-seeded with centrifugation (C). The dashed white line illustrates the limits of cross-section of the scaffold. (Scale bar: 500 μm). Diagram illustrating the cross-section of the scaffold (E). Gray arrow points the plan from which CLSM images were taken.

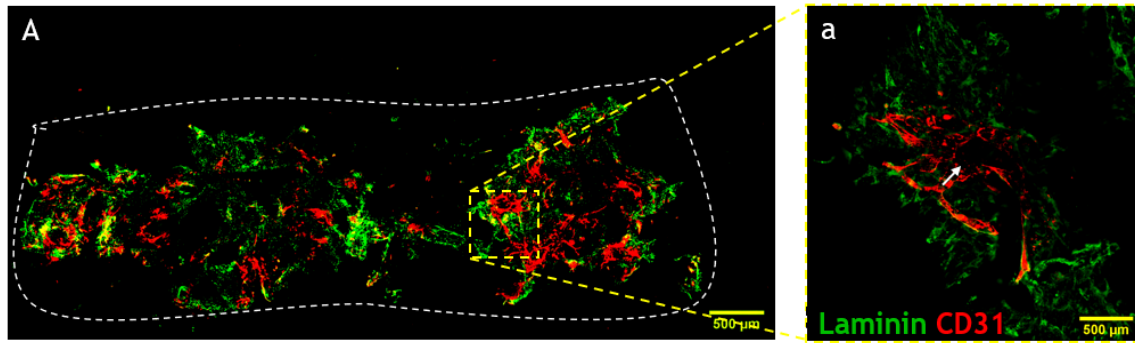


Figure 24. Immunostaining of hMFs and OECs seeded on 2% wt. RGD-grafted alginate scaffolds prepared by particle leaching after 14 days of culture (A). After seeding 50×10^4 cells on top of the scaffold, it was performed a 5 minutes-centrifugation at 1000 rpm. Cytoskeletal organization of both cells were observed under a CLSM and demonstrated by laminin (green) and CD31 (red). The dashed white line illustrates the limits of cross-section of the scaffold. (Scale bar: 500 μm). Zooming 2x zone confined by dashed yellow line, it is possible to observe endothelial cells organization into tubular-like structures, near to scaffold pores (a, white arrow).

4.9. Cells seeded on RGD-alginate scaffolds outgrowth

Whether in the primary tumor or after metastization, it is known that tumors recruit blood vessels to ensure their growth and avoid dormancy. Tumor vascularization, known as “angiogenic switch”, occurs mainly via sprouting angiogenesis as a result of imbalanced expression and function of pro-angiogenic factors [61, 63, 64]. Angiogenic switch is a complex, multistep and dynamic process, which involves ECM degradation as well as EC migration and proliferation [58, 62]. The use of 3D matrices in order to study the dynamics of EC recruitment and growth of new vessels toward the tumor mass can greatly improve our understanding of tumor-driven angiogenesis, leading to the development of better therapeutic strategies to selectively fight cancer. 3D approaches have been developed utilizing protein-based natural materials such as collagen type I, fibrin or laminin-rich basement membrane extract known as Matrigel, mainly due to their broad availability and ease of handling. Additionally, such ECM-derived scaffolds carry a variety of intrinsic signals, which ensure excellent cell survival rate and efficiently guide morphogenetic processes such as EC morphogenesis and sprouting.

In the first part of this work, we evaluated the paracrine effect of tumor-like constructs on the behavior of EC in 2D. In the second part, we intended to further understand EC dynamics in a more physiologic environment, by exploring a vascularized 3D construct. As a proof of concept, we started by analyzing if these vascularized 3D scaffolds would allow cell sprouting/outgrowth when embedded in two different natural matrices, collagen type I and fibrin. Both proteins are found in ECM microenvironment and are considered to be optimal substrates for angiogenesis [147]. Fibrin

deposition is commonly observed in angiogenesis associated with wound healing and tumor growth [148]. Type I collagen is a major component of tumor ECM and several in vitro studies demonstrate that type I collagen gel supports angiogenesis as well as fibrin gel.

Scaffolds seeded with hMFs and OECs were cultured for 10 days and then transferred to a thin layer of collagen type I (2 or 3 mg/mL) for 20 days. Noteworthy, in a preliminary test using 1.5 mg/mL collagen type I, early detachment of the scaffolds from this collagen layer was observed, which made us increase the concentration. In the less dense 2 mg/mL collagen type I matrix, outward cell migration started earlier (at day 2) (Figure 25A), as compared to denser 3 mg/mL matrices (Figure 25D). On the 6th day of culture, approximately the same average migration was observed at both concentrations (Figure 25B and 25E), and migration seemed to have stabilized. At the 20th day, the number of migrating cells was similar to the one observed at day 6, as observed either by confocal microscope (Figure 25F) or inverted fluorescence microscope (Figure 25C). Since we were able to observe cell sprouting already after 2 days in 2 mg/mL matrices, we selected this concentration for subsequent studies.

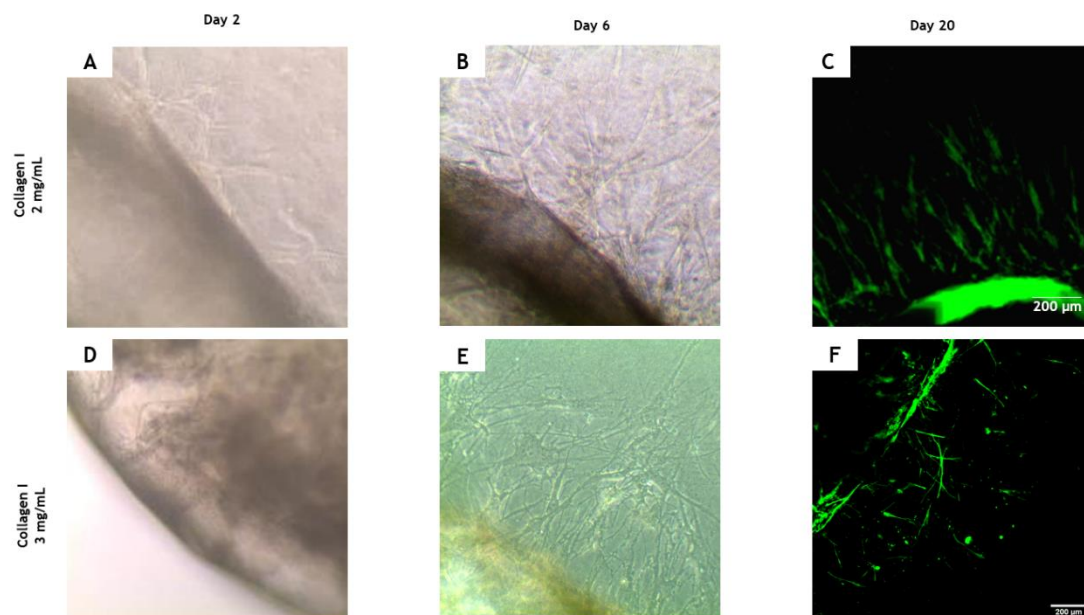


Figure 25. Cellular outgrow in collagen type I gels during 20 days of culture. hMFs-OECs-seeded alginate scaffolds cultured for 10 days were transferred to a collagen layer. Two different collagen type I concentrations were tested (2mg/mL, A-C and 3mg/mL, D-F). Images were obtained from inverted microscope (A, B, D, E), inverted fluorescence microscope (C), and CLSM (F).10x

Since after 7 days in culture it was already possible to observe EC organization in 3D alginate porous scaffolds (data not shown), for the following studies this time point was selected. Thus, hMFs-OECs seeded on alginate scaffolds for 7 days were embedded between two layers of 2 mg/mL collagen type I gel, or as alternatively, scaffolds were embedded between two layers of fibrin gel. After 3 days, cytoskeletal organization (F-actin) was analyzed in collagen I (Figure 26A) and fibrin gel (Figure 26B), by CLSM. A shorter time of culture in these matrices were used (as comparison with the first approach, 20 days), due to the lower stability of the fibrin gel [149]. Interestingly, higher cellular outgrowth was observed in fibrin gels (Figure 26B) than in collagen (Figure 26A). Despite collagen type I being the main component of the ECM in breast tissue, promoting epithelial cell branching during the mammary gland development [150], fibrin is naturally angiogenic. Fibrin stimulates endothelial assembly [81, 149, 151] and promotes production of ECM proteins, including basement membrane proteins such as laminin [152]. Therefore, fibrin gel is considered as reference (positive control). However, expression of the endothelial cell marker CD31 was not detected, even when the antibody concentration was increased (from a dilution of 1:100 to 1:50) in both matrices. The presence of sprouting OECs outside the scaffolds remains unclear, and the assay should be repeated, but it would be expected, taking into account the remarkable formation of endothelial tubular-like structures within the scaffold (Figure 24). Furthermore, other question arising is related to the long-term stability of endothelial cells architecture [149, 153]. In this regard, it is extensively reported that the use of support cells, such as fibroblasts, tend to improve capillary-like structures formation and stability, avoiding retraction and de-stabilization [153].

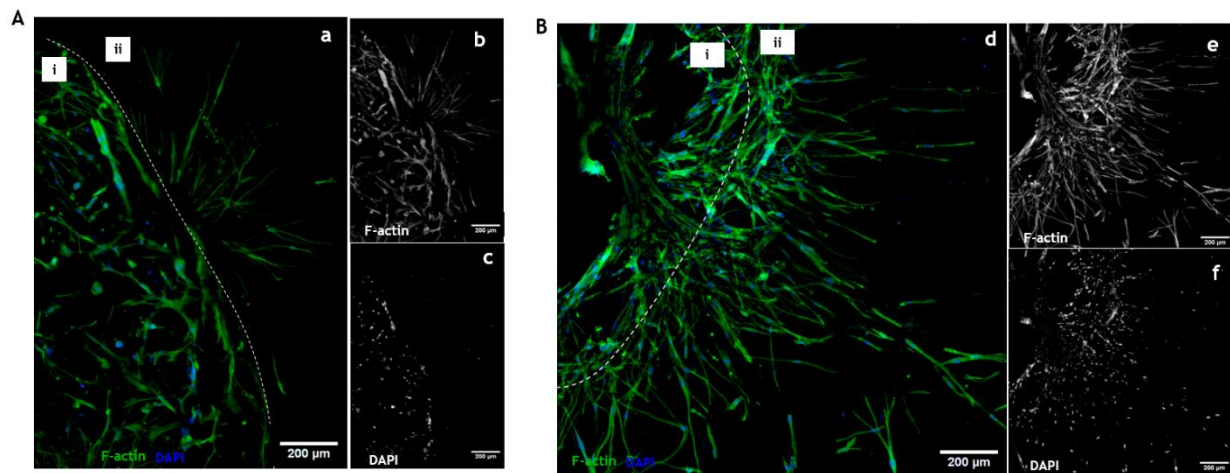


Figure 26. Immunostaining of cell outgrowth of the scaffolds. hMFs-OECs-seeded alginate scaffolds, cultured for 7 days, were embedded between two layers of collagen type I (A), or fibrin gel (B). Cytoskeletal organization of the cells after 3 days of culture were observed in the scaffold (i) and in matrix (ii), under a CLSM and demonstrated by F-actin (green), and DAPI (blue). (Scale bar: 200 μ m).

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5. Concluding Remarks and Future Perspectives

Triple-Negative Breast Cancer represents 10% to 20% of all breast cancer cases, exhibiting an aggressive clinical course and final outcome [1]. Its poor prognostic is generally related to early and higher rates of distant recurrences, associated with metastatic processes, typically in visceral organs [10]. Once TNBC is negative for the receptors that potentially could respond to hormones, eligible treatments for this subtype of breast cancer are restricted [2]. The main drug treatment used is chemotherapy, which carries the problem of resistance development within a few months by most patients. There is an evident need to identify effective treatment options for improved outcomes [2, 3]. Research directed to metastatic triple-negative disease should attempt to clarify the role of different players and mechanisms, which contribute to the complex process of tumor development/progression, such as angiogenesis.

The arise of new approaches to study tumor angiogenesis based on 3D systems, which include biomaterial-based matrices, is becoming the basis of the next generation of cancer research platforms [5]. In particular, exploring the role of ECM composition and mechanical properties in cancer cells behavior has been earning increasing interest.

In the present work, a 3D *in vitro* model of breast tissue was developed by co-culturing mammary fibroblast and cancer epithelial cells (MDA-MB-231) within RGD-modified alginate hydrogel, used as an ECM-analogue [105]. Comparing to monocultures, it was observed an increase in MDA-MB-231 spheroid size when these cells where co-cultured with hMFs, suggesting a positive effect of hMFs on spheroids formation. However, it remains unclear if this contribution is resultant from direct cell culture and/or paracrine interaction.

To provide physiologically-relevant biomechanical cues, alginate hydrogels properties were fine-tuned to attain viscoelastic properties (stiffness) comparable to that of normal breast tissue ($G' \approx 200$ Pa), as well as tumor tissue ($G' \approx 3000$ Pa). The metabolic activity of cells cultured in stiff matrices decreased, which could be due to physical space constrains, as these matrices are denser. In order to closely mimic the *in vivo* situation, the effect of induced hypoxia was also evaluated, [61-63], by investigating metabolic activity and CA9 expression. There was a decrease in the metabolic activity of cells exposed to hypoxia, but results were unclear, and the lack of specificity of the assay used to measure this parameter may have led to artifacts. In agreement with what was expected, when soft matrices were cultured for 3 days under hypoxic conditions, increased CA9 expression was observed, indicating that 3D-cultured cells were able to sense and response to the lower oxygen levels. Throughout time, in soft matrices, CA9 was upregulated, which could be correlated with the increase in spheroids size and the formation of a hypoxic center with proliferative cells at the periphery. To confirm this hypothesis, it would be interesting

to perform an immunostaining of ki-67 (proliferation marker) combined with a caspase 3 (apoptotic marker). However, the combination of stiffer matrices and hypoxic conditions resulted in a decreased in CA9 expression, which need to be clarified through further analysis on other pathways that are upregulated during hypoxic conditions, such as, glut2, LDH A and/or lox, among others.

Co-cultures of hMFs and OECs entrapped in 3D alginate matrices, registered higher levels of MMP2 and MMP9 production when exposed to hypoxia, as detected in conditioned media. This increase was also observed in conditioned media from stiffer matrices, as compared to softer ones. It would be interesting to evaluate if stiffening of the alginate matrix combined with MDA-MB-231 may induce hMFs activation, since hMFs activation is related to enhanced MMP-9 production [129].

The impact of the factors produced by MDA-MB-231 cells cultured with hMFs in a 3D matrix in tumor angiogenesis was assessed by exposing EC to conditioned medium obtained from this co-culture. In terms of endothelial proliferation and migration, results from both the scratch and directed transwell migration assays seemed to correlate with what was observed in CA9 mRNA expression. This supports the association between hypoxia and EC recruitment to the tumor site, in order to reestablish local oxygen levels. These studies on the paracrine effect of epithelial and stromal cells in our tumor-like construct were performed on OECs cultured in traditional 2D monocultures. However, since this is a quite reductionist approach, we intended to proceed studies using a more biologically-relevant setting, so we decided to develop a vascularized 3D construct that could be used in the future.

To this end, a co-culture of OECs and hMFs was established on porous alginate 3D scaffolds. The freeze-drying technique associated with particle leaching allowed to obtain scaffolds with high porosity. The seeding of both cell types on one side of the scaffold, followed by a centrifugation step, facilitated cell penetration through the pores and, consequently a better cell distribution and 3D capillary-like organization. Moreover, the vascularized 3D scaffold allowed cell outward migration, when in embedded in two different natural matrices, collagen type I and fibrin.

In the future, a 3D tri-culture platform can be developed by combining the vascularized porous scaffold with hydrogel-embedded primary patient-derived cells, in a precision medicine context. Also, the possibility of using a proteolytically-degradable alginate hydrogel, such as MMP-sensitive alginate, should be considered in order to allow cell migration directed by soluble factors produced by cancer cells and/or fibroblast. Other interesting approach would be to induce gradual stiffening of the alginate matrix over the time and evaluate the influence in cellular behavior.

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Supplementary Information

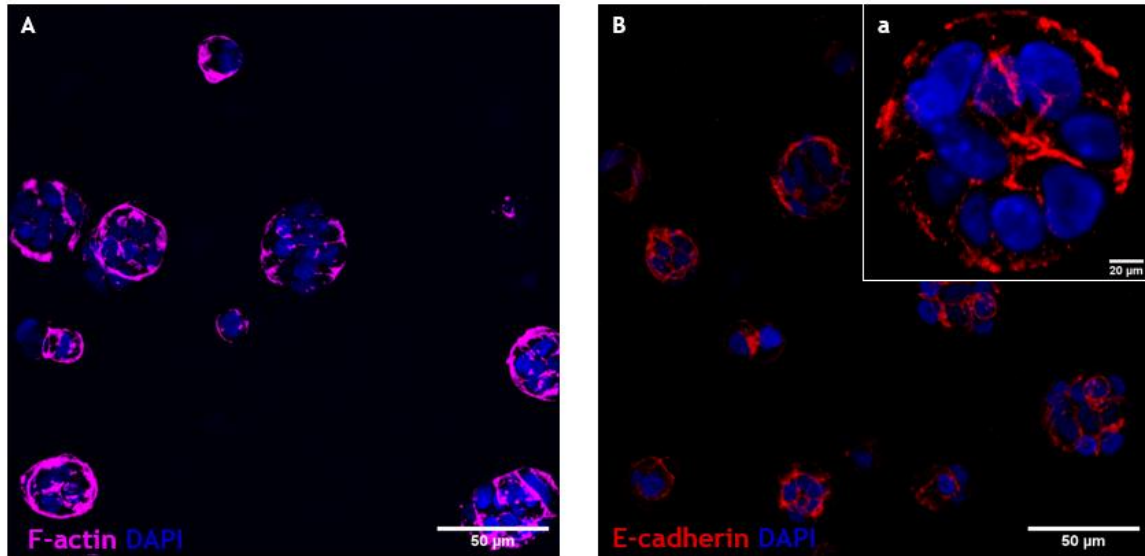


Figure S1. Culture in RGD-alginate 3D matrix supports epithelial morphogenesis. CLSM images of 3D-cultured MCF10A cells at day 14, showing: spheroids formation (A), and expression of the classical epithelial marker E-cadherin (B). Scale bars (A and B: 50 µm; a: 20 µm).

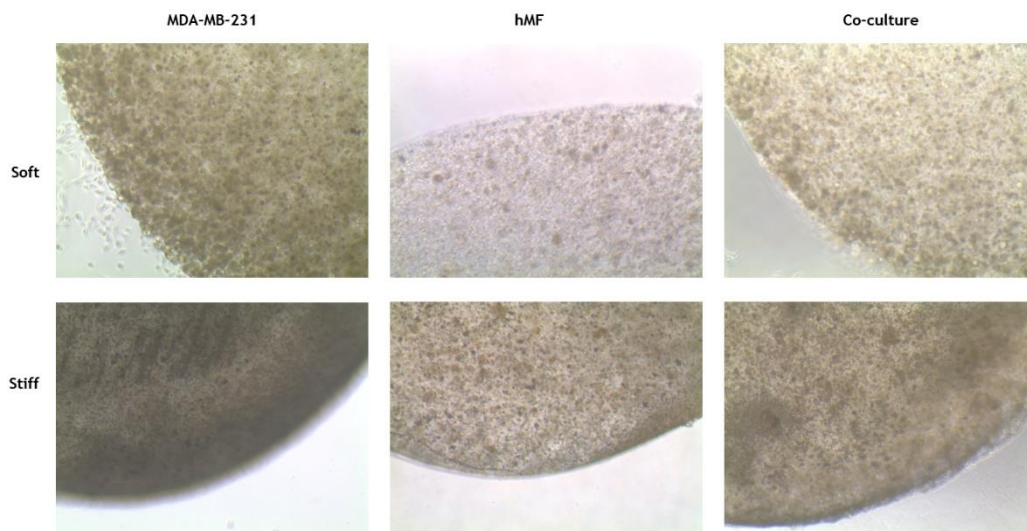


Figure S2. MDA-MB-231 and hMF monocultures, and co-culture of MDA-MB-231 and hMFs, at day 7 in RGD-alginate soft and stiff matrices. It is observed a constriction in alginate matrix, which can limit cell growth and proliferation. Microscopic observation (4x magnification)

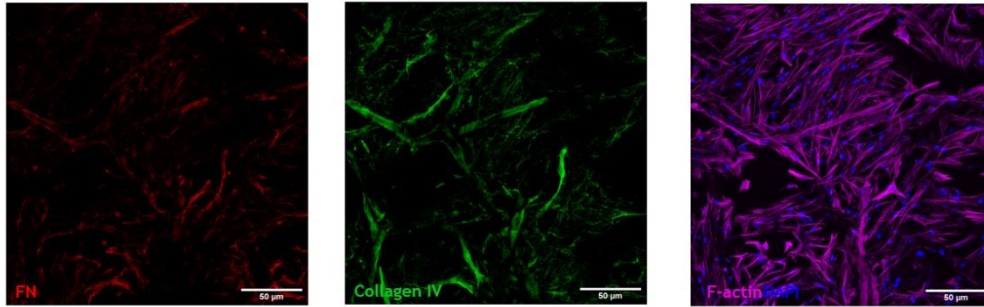


Figure S3. Immunostaining of hMFs and ECs seeded on top of RGD-grafted alginate scaffolds (50×10^4 cells/scaffold) after 14 days of culture. 2% wt. RGD-grafted alginate scaffolds were prepared by freeze drying technique. Scaffolds were staining for fibronectin (FN, red), collagen IV (green), F-actin (magenta) and DAPI (blue) and observed CLSM. (Scale bar: 50 μ m)