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Modulation of angiogenesis by fibroblasts in tissue regeneration

Dissertação de candidatura ao grau de Doutor em Engenharia Biomédica, apresentada à Faculdade de Engenharia da Universidade do Porto

Faculdade de Engenharia
Universidade do Porto
Porto-2011

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The research described in this thesis was financed by:

Fundação para a Ciência e a Tecnologia: PhD grant, SFRH/BD/22090/2007;
Project ANGIO-POCTI/SAU-BMA/55556/2004; PTDC/SAU-OSM/102083/2008;
PEST-C/SAU/LA0002/2011and PEST-OE/SAU/UI0038/2011.

À minha mãe

Acknowledgments

Ao longo dos últimos quatro anos, com o seu esforço, tempo e saber, diferentes pessoas, de forma dedicada, contribuíram para a realização deste estudo. A todos quero deixar aqui o meu profundo reconhecimento.

Primeiramente, é ao Professor Doutor Mário Barbosa que devo agradecer por me ter acolhido no INEB. Assim, pude efectuar as pesquisas necessárias para a sua prossecução e participar em diversas actividades desenvolvidas pelo mesmo Instituto.

Agradeço ao Doutor Pedro Granja a oportunidade de levar a cabo este trabalho em colaboração com outras Instituições. E isto, indiscutivelmente, facilitou o meu crescimento intelectual e pessoal. Obrigada, pois, pelos oportunos conselhos científicos e amizade.

À Professora Doutora Raquel Soares que gentilmente me recebeu no Serviço de Biquímica da FMUP onde desenvolvi parte deste trabalho, devo igualmente úteis aconselhamentos de carácter científico e amizade.

To Prof. Dr. C. James Kirpatrick I would like to thank the possibility of working in his group and the way I have been received there. Thank you for all the support.
To Doctor Ronald Unger I must thank all the good lessons about endothelial cells and the scientific discussion.
To Doctor Christoph Brochhausen I would like to thank the collaboration and scientific discussion.

I would like to thank all my colleagues and friends at the RepairLab in Mainz for making my work and life an extraordinary experience: A special thank to Anne, Amparo, Roman, Eva, Kirsten and Marlin.

Por fim, quero agradecer aos membros do INEB pelas constantes provas de amizade, informações e debates, no âmbito de uma intensa e agradável atmosfera científica. Uma lembrança muito especial vai para Silvia Bidarra, Cristina Barrias, Keila Fonseca, Raquel Maia, Maria Oliveira, Raquel Gonçalves, Catarina Almeida, Joana Maciel, Daniela Sousa, Sidónio Freitas, Paula Parreira, Ana Lopes, Ana Paula Filipe e Virginia Fonseca.

Aos membros do Serviço de Bioquímica manifesto também, por idênticas razões, os meus sinceros agradecimentos. E muito em especial a Maria João, Rita Negrão, Rosário Monteiro, Nuno Alçada, Fátima Martel, Alejandro, Ana Faria, Ana Pirraco, Raquel, Sofia, Diogo e ao Pedro pela colaboração, boa disposição e amizade.

Dentre os meus próximos, à minha mãe, cujo incentivo persistente sempre me animou e sem a qual não disporia das condições mínimas para levar a cabo este projecto, quero significar a minha mais profunda gratidão.

Abstract

Angiogenesis, or the growing, expansion and remodeling of blood vessels in a developing vascular network, is considered of utmost importance in the development of new tissue. Vascularization is of relevance in tissue regeneration strategies by allowing cell survival through the supply of oxygen and nutrients for cells in tridimensional structures, such as tissues or artificial matrices. Synergetic strategies combining different biomaterials and components of the cellular microenvironment (cells, biomolecules and extracellular matrix components) have been proposed as a promising way to promote the regeneration of vascularized tissues.

Although endothelial cells, the cells lining blood vessels, seem to play the major role in new vessel formation, other cell types have been implicated in this phenomenon and their inter-relationships with endothelial cells have been explored. However, the interplay between fibroblasts and endothelial cells is far from understood, despite the fact that fibroblasts play key roles in wound healing and in vessel stabilization. Taking this into account, the present work is intended to investigate if angiogenesis and vasculogenesis can be modulated by fibroblasts in tissue regeneration.

In an initial approach the effect of growth factors (VEGF and bFGF) produced by fibroblasts were first observed to positively influence the viability of endothelial cells entrapped in RGD-alginate. Similarly, in angiogenesis assays, fibroblasts entrapped in RGD-alginate demonstrated the ability to improve the viability of endothelial cells and to support capillary-like structures assembly and increased length.

Indirect and direct cocultures studies were also performed to further elucidate the crosstalk between endothelial cells and fibroblasts. Results obtained showed that the two cells types interact with each other through exchange of growth factors, namely VEGF, bFGF, TGF-β1 and IL-8, which are known to influence angiogenesis. Fibroblasts promoted the formation and organization of capillary-like structures by endothelial cells, increased the amount of collagen in the cocultures and further determined the expression of alkaline phosphatase. High alkaline phosphatase expression could be co-localized with capillary-like structures and the interaction between the two cells types induced fibroblasts activation near microvessel-like structures.

Finally, the role of fibroblasts in the recruitment of endothelial cells was explored *in vivo*, to assess their influence on the formation of new blood vessels. Fibroblasts were entrapped in growth factor-reduced matrigel subcutaneously

implanted in mice. The vessels formed exhibited higher levels of hemoglobin, compared to the control matrix, implanted without fibroblasts, in which no vessel formation could be observed. The presence of vessels originating from the host vasculature suggested that host vascular response was involved, which constitutes a fundamental aspect in the process of neovascularization. Fibroblasts implanted within matrigel increased the presence of endothelial cells with positive staining for CD31 and for CD34, indicating the involvement of mature and also endothelial progenitor cells, and influenced the production of collagen, thus inducing the angiogenic process and promoting the formation of microvessels.

In conclusion, this work demonstrated the capacity of fibroblasts to modulate angiogenesis through the production of growth factors and extracellular matrix, thereby created a microenvironment capable of stimulating angiogenesis *in situ,* and can potentially be used in regenerative medicine or in delineation of new strategies in tissue engineering where vascularization is essential.

Resumo

A angiogénese, ou o crescimento, expansão e remodelação dos vasos sanguíneos sob a forma de uma rede vascular em desenvolvimento, é considerada de extrema importância no desenvolvimento de novos tecidos. A vascularização é importante nas estratégias de regeneração de tecidos por permitir a sobrevivência das células através do fornecimento de oxigénio e nutrientes às células em estruturas tridimensionais, tais como tecidos ou matrizes artificiais. Têm sido propostas estratégias sinérgicas, combinando diferentes biomateriais e componentes do microambiente celular (células, biomoléculas e componentes da matriz extracelular), como formas promissoras de promover a regeneração dos tecidos vascularizados.

Embora as células endoteliais, as células que revestem os vasos sanguíneos, pareçam desempenhar o papel preponderante na formação de novos vasos, têm sido implicados outros tipos de células neste processo e as suas inter-relações com as células endoteliais têm sido exploradas. No entanto, a interação entre os fibroblastos e as células endoteliais está longe de ser compreendida, apesar do papel fundamental dos fibroblastos na cicatrização de feridas e na estabilização de vasos. Tendo isto em conta, o presente trabalho pretende investigar se a angiogénese e a vasculogénese, outro processo de vascularização relevante na regeneração de tecidos, podem ser moduladas pelos fibroblastos na regeneração de tecidos.

Numa abordagem inicial, foi observado que fatores de crescimento (VEGF e bFGF) produzidos pelos fibroblastos influenciaram positivamente a viabilidade das células endoteliais encapsuladas em alginato-RGD. De forma similar, através de ensaios de angiogénese, fibroblastos encapsulados em alginato-RGD, demonstraram capacidade para melhorar a viabilidade das células endoteliais e de suportar a organização de estruturas capilares e de aumentar o seu comprimento.

Foram também realizados estudos em coculturas para elucidar a inter-comunicação entre as células endoteliais e os fibroblastos. Os resultados obtidos mostraram que os dois tipos de células interagem através da troca de fatores de crescimento, ou seja, VEGF, bFGF, TGF-β1 e IL-8, que são conhecidos por influenciar a angiogénese. Os fibroblastos promoveram a formação e organização de estruturas semelhantes a capilares pelas células endoteliais, o aumento da quantidade de colagénio nas coculturas e promoveram ainda a expressão de fosfatase alcalina. A elevada expressão de fosfatase alcalina pôde

ser co-localizada com estruturas semelhantes a capilares e a interação entre os dois tipos de células induziu a ativação dos fibroblastos em zonas adjacentes às estruturas microvasculares.

Finalmente, o papel dos fibroblastos no recrutamento de células endoteliais foi explorado *in vivo*, para avaliar a sua influência na formação de novos vasos sanguíneos. Os fibroblastos foram encapsulados em matrigel com fatores de crescimento reduzidos e implantados subcutaneamente em ratinhos. A presença de fibroblastos levou a um aumento da densidade vascular, avaliado pelo níveis superiores de hemoglobina, em comparação com a matriz do controlo, implantada na ausência de fibroblastos. A presença de vasos provenientes da vasculatura do hospedeiro sugeriu a envolvência de uma resposta vascular do hospedeiro, o que constitui um aspecto fundamental no processo de neovascularização. Os fibroblastos implantados em matrigel aumentaram a presença de células endoteliais com marcação positiva para CD31 e CD34, indicando o envolvimento de células endoteliais maduras bem como progenitoras, e influenciou a produção de colagénio, induzindo assim o processo angiogénico e promovendo a formação de microvasos.

Em conclusão, este trabalho demonstrou a capacidade dos fibroblastos para modular a angiogénese através da produção de fatores de crescimento e de matriz extracelular, criado assim um microambiente capaz de estimular a angiogénese *in situ*, o que pode potencialmente ser utilizado em medicina regenerativa ou na concepção de novas estratégias para engenharia de tecidos, onde a vascularização é essencial.

Abbreviations

ALP – Alkaline phosphatase

bFGF - Basic fibroblast growth factor (FGF-2)

CD31 – cluster of differentiation molecule 31 (=PECAM-1)

CD34 – cluster of differentiation molecule 34

EC - Endothelial cell

ECGF - Endothelial cell growth factor

ECM - Extracellular matrix

FB – Fibroblast

GFR – Growth factor reduced

HDF – Human dermal fibroblast

HDMEC - Human dermal microvascular endothelial cell

HFF-1 – Neonatal human dermal fibroblasts foreskin

HUVEC - Human umbilical vein endothelial cell;

IL-8 – Interleukin-8

MMP - matrix metalloproteinase

MSC – Mesenchymal stem cells

SMC - Smooth muscle cell

TGF-β1- Transforming growth factor beta-1

VEGF - vascular endothelial growth factor

vWF - von Willebrand factor

α-SMA – Alpha smooth muscle actin

Contents

Acknowledgements vii
Abstract xi
Resumo xv
Abbreviations xix
Contents xxiii
Preface xxvii

Chapter I
Introduction 1

1. Angiogenesis and vasculogenesis in tissue regeneration..... 3
1.1 Cells and tissues 6
1.1.1 Endothelial cells 7
1.1.2 Pericytes 7
1.1.3 Smooth muscle cells 8
1.1.4 Stromal cells 8
1.2 Growth factors 9
1.2.1 Vascular Endothelial Growth Factor 10
1.2.2 Basic Fibroblast Growth Factor 11
1.2.3 Transforming Growth Factor-beta1 12
1.2.4. Other factors that influence the angiogenic microenvironment 12
1.3. Extracellular matrix 13
1.3.1 Interactions between cells and ECM 13
1.3.2 Collagen 14
1.3.3 Glycoproteins 15
1.3.3.1 Alkaline phosphatase 15
2. Strategies to promote vascularization of bioengineered tissues 16
2.1 Growth factor delivery 17
2.2 Cell transplantation 18
2.3 Angiogenic artificial extracellular matrices 19
2.4 Combined strategies 21
3. Hypothesis and specific aims 25
4. References 26

Chapter II
Neonatal human dermal fibroblasts immobilized in RGD-alginate induce angiogenesis in tissue regeneration ……...45

Chapter III
Crosstalk between fibroblasts and endothelial cells promotes angiogenesis *in vitro*: putative role of alkaline phosphatase, growth factors and collagen ……..75

Chapter IV
Implanted neonatal dermal fibroblasts influence the recruitment of endothelial cells in mice ……103

Chapter V
Summarizing discussion and concluding remarks ……127

Chapter VI
Future Perspectives ……..145

APPENDIX
Pectin-based injectable biomaterials for bone tissue engineering……...……151

Preface

The present research work was developed as a contribution to overcome the difficulty in promoting vascularization in tissue regeneration. Cells, growth factors and the extracellular matrix play key roles in angiogenesis and vasculogenesis and their interplay, leading to successful vascularization, is far from understood. Based on previous work from research teams involved, on the crosstalk between endothelial cells and bone cells in the framework of bone tissue regeneration, the present work is intended to investigate if angiogenesis and vasculogenesis can be modulated by fibroblasts in tissue regeneration.

In this context, **chapter I** aims to introduce and summarize the central role of angiogenesis and vasculogenesis in tissue regeneration, as well as the different mechanisms, cells, biomolecules and extracellular matrix components that are known to contribute to this phenomena. This chapter is especially focused on the relevance, demonstrated over the last decade, of using synergetic strategies combining biomaterials and components of the cellular microenvironment to promote the regeneration of vascularized tissues.

In **chapter II,** the first experimental chapter of this work, the aim was to critically explore issues reported in previous studies of the research team, namely to improve the viability of cultured endothelial cells. The effect of entrapped fibroblasts in endothelial cells was explored, in terms of cell viability and ability to organize in tubular-like structures. An additional goal consisted in assessing the behavior of fibroblasts entrapped in alginate.

In **chapter III**, and following the assumptions outlined in the previous chapter, it was aimed to further assess how fibroblasts would influence the organization of endothelial cells in coculture systems, with a view to promoting vascularization. During this study different microenvironments were created (in direct or indirect contact) to investigate the interaction between endothelial cells and fibroblasts. Besides the influence of the cells themselves, the role of growth factors, the extracellular matrix and glicoproteins, namely alkaline phosphatase, were investigated in the development of the microvessel-like structures.

In **chapter IV** the role of fibroblasts in the recruitment of endothelial cells was explored *in vivo*, to assess their influence on the formation of new blood vessels. Fibroblasts were entrapped in growth factor-reduced matrigel, subcutaneously

implanted in mice and the ability of this system to influence vascularization of the implant was assessed biochemically and immunohistologically.

Finally, **chapter V** constitutes a critical discussion on all the issues previously raised, recurring to comparisons with the available literature on related matters, and where new hypotheses are put forward that had not been previously addressed regarding the modulation of angiogenesis by fibroblasts in tissue regeneration. Perspectives on future work are also discussed.

The **appendix** includes the publication entitled "Pectin-based injectable biomaterials for bone tissue engineering", in which the author of this thesis is a co-author, having contributed with know-how in several aspects. This publication relies in the same techniques used in this thesis for cell entrapment and assessment of their biological behavior. The development of such injectable cell entrapment systems is relevant in the framework of this thesis, as highlighted in the previous chapters.

Chapter I

Introduction

1. Angiogenesis and vasculogenesis in tissue regeneration

The term tissue regeneration became widely spread in the 1990s with the emergence of the field of tissue engineering. Tissue regeneration involves the capacity of the cells to renew and grow in order to generate new tissue for replacing diseased or damaged tissues or organs. Although coined only in the 1990s [1], the foundation of tissue engineering concepts, bridging biology and engineering knowledge, dates back from the 1930s, when Alexis Carrel with Charles Lindberg established the basic principles to grow viable and functional tissues and organs *in vitro* for use as replacement body parts [2]. However, only in the 1990s, Langer and Vancati [3] successfully regenerated cartilage through mouse subcutaneous implantation of synthetic biodegradable scaffolds seeded with chondrocytes. This interdisciplinary field uses principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [2]. Fig. 1 illustrates the challenge of using stem cells obtained from the bone marrow, in combination with a scaffold material that mimics the architecture of the tissue, in order to regenerate tissue function [4]. Vascularization plays a crucial role in regenerative medicine and is a phenomenon yet to be totally understood and mastered [4]. Through the restoration of the vascular system exchanges of oxygen, nutrients, growth factors and elimination of metabolites are possible, allowing survival of the new tissue [5]. The diffusion limit for oxygen is about 200 μm from blood vessels [6]. Thus, the recruitment of new blood vessels by activation of vasculogenesis and angiogenesis (the two main processes of vascularization), is still a great challenge in regenerative medicine.

In vasculogenesis blood vessel formation is directly orchestrated by endothelial precursor cells or angioblasts. In the past, vasculogenesis was exclusively associated with the concept of blood vessel formation during embryogenesis. However, recent data documented the presence of circulating endothelial progenitors cells in the adult leading to the assumption that vasculogenesis can also occur in adults [4, 6-9]. In angiogenesis new blood vessels are formed from the pre-existing vasculature [10, 11], which has proven to be essential for maintenance of tissue and organ regeneration [12, 13].

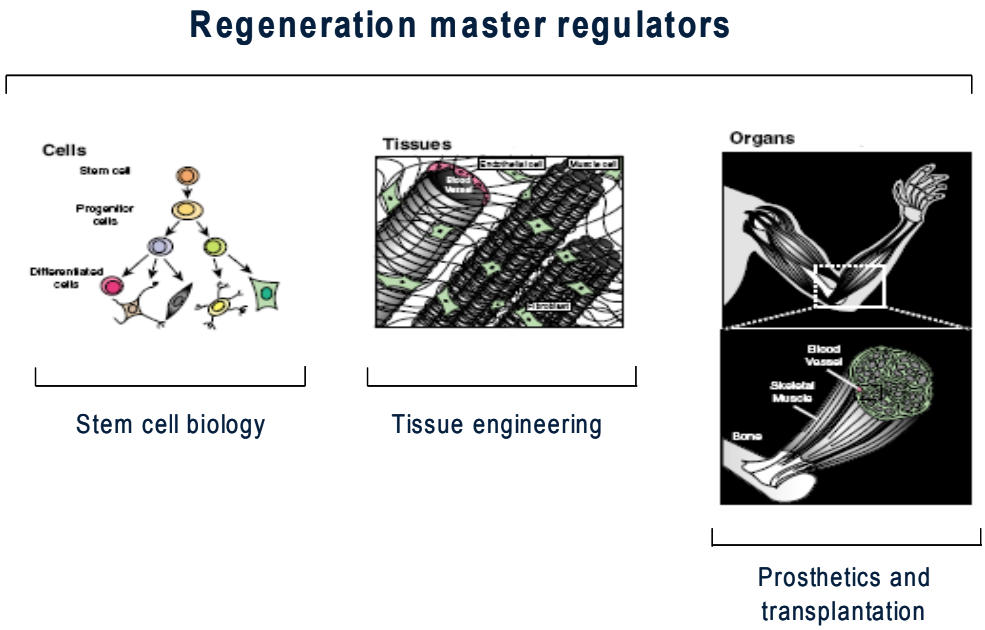

Figure 1. Tissue and organ regeneration recurring to *ex vivo* expansion of cells and their attachment to three-dimensional scaffolds. From Ingber et al. [4].

Arteriogenesis is the process of structural enlargement and remodeling of pre-existing small arterioles into larger collateral vessels [14]. Fig. 2 illustrates the development of the vascular system, from progenitor endothelial cells to tissue-specific specializations that determine the diversity of properties of endothelial cells.

In adults, angiogenesis is basically restricted to the female reproductive organs and wound healing on injured tissues [15]. For successful vascularization in regenerative medicine it is considered that the implanted construct needs to stimulate a quick and directional vascular growth from the host [16].

The body regulates angiogenesis by producing a precise balance of pro- and anti-angiogenic molecules that arise from different sources, such as endothelial, pericytes, smooth muscle, fibroblasts and blood cells and extracellular matrix components [18].

Angiogenesis is related with wound healing, inflammation as well as with pathological conditions such as rheumatoid arthritis, endometriosis, diabetic retinopathy, macular degeneration and tumor growth [19, 20]. On the other hand, angiogenesis is also related to growth repair, bone remodeling and tissue regeneration [21].

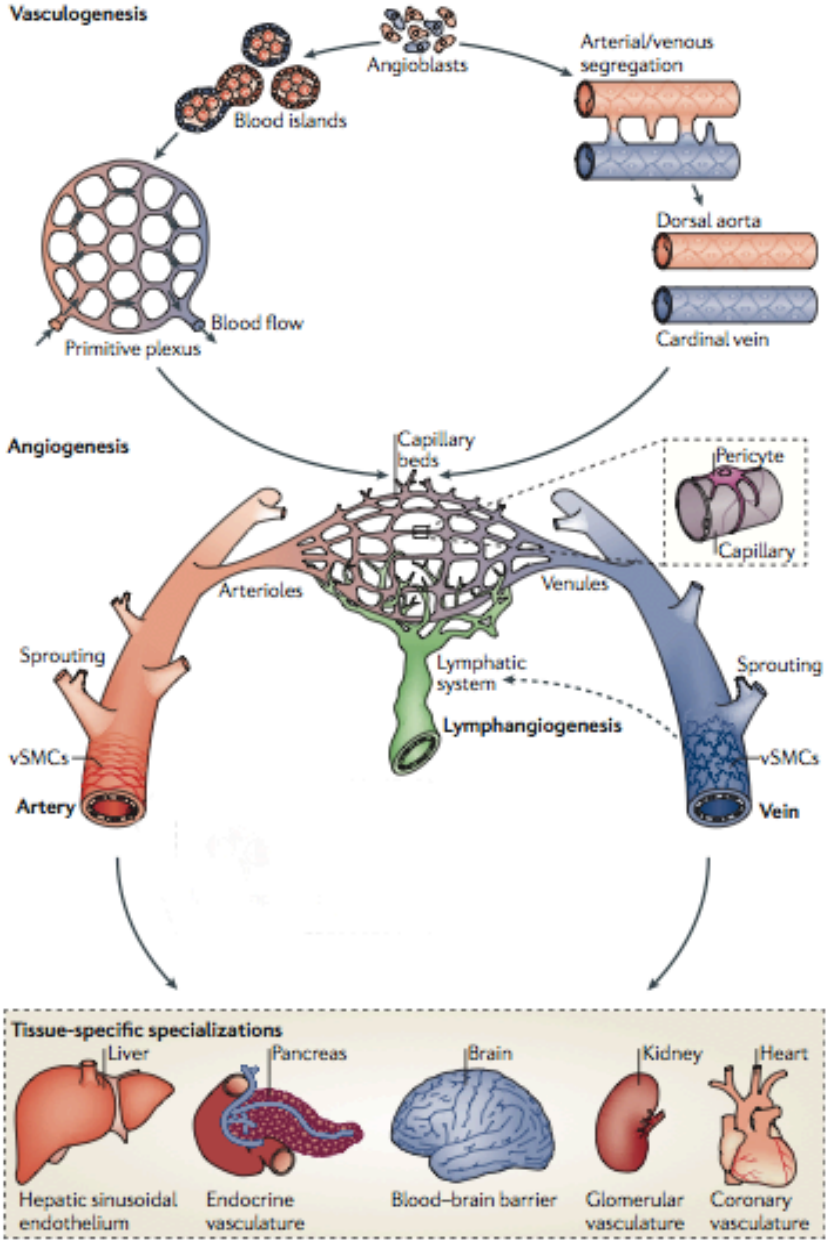

Figure 2. The road of the vascular system development, from progenitor endothelial cells to tissue-specific specializations. In vasculogenesis angioblasts may acquire arterial (red) or venous (blue) phenotype and shape into the dorsal aorta and cardinal vein, respectively, in the embryo. On the other hand, blood islands may be formed from angioblasts and create the primitive plexus. Then, the hierarchical network of arteries, arterioles and capillary beds may be organized by angiogenic remodeling. Mural cells are recruited to stabilize and to promote the maturation of the nascent vessels pericytes and smooth-muscle cells. In addition, the lymphatic system derived from venous vessels returns lymph fluid back to the vasculature. The tissue-specific specialization determines by new molecular cues the diversity of properties of endothelial cells. Adapted from Herbert et al. [17].

Taken into consideration the above-described process regarding vessels formation a detailed description of the cells, factors and the extracellular matrices involved is given below. Other variables are capable of influencing tissue vascularization, such as mechanical forces, electrical and chemical stimuli, which may considerably influence the new microenvironment. However, a review of these factors is out of the scope of the present manuscript and is available elsewhere [22-26].

1.1. Cells and tissues

The vascular system is responsible for blood transport to all tissues. Although there are different types of vessels (arteries, veins, venules and arterioles), vessels are formed by a layer of endothelial cells, which are covered by mural cells such as pericytes and smooth muscle cells (Fig.3).

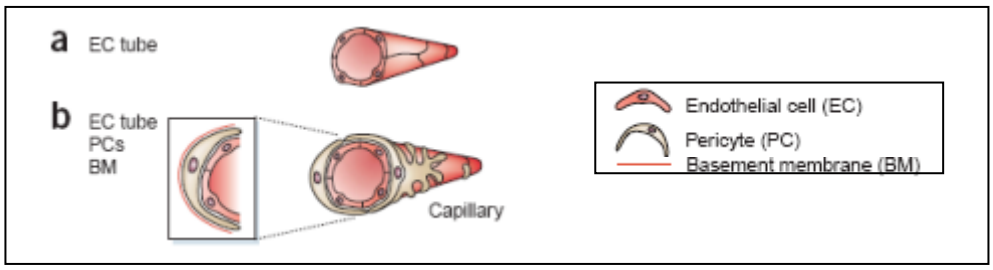

Figure 3. Scheme of vessels constitution. (a) Nascent vessels consist of a tube of endothelial cells. These mature into the specialized structures of capillaries, arteries and veins. (b) Capillaries, the most abundant vessels in our body, consist of endothelial cells surrounded by the basement membrane and a sparse layer of pericytes embedded within the endothelial cells basement membrane. Adapted from Jain [27].

Large blood vessels have a common basic structure, consisting of three different layers: the tunica intima, constituted by endothelium supported by a basement membrane and delicate collagenous tissue, an intermediate muscular layer, which is named the tunica media, and an outer supporting tissue layer called the tunica adventitia [27].

1.1.1. Endothelial cells

Endothelial cells play a pivotal role in angiogenesis. They form the endothelium, which is no longer considered an inert single layer of cells, that passively allows the passage of water and small molecules across the vessel wall, but an important player in the maintenance of tissues and its regeneration [28]. Endothelial cells are characterized by the expression of endothelial cell markers, such as von Willebrand factor (vWF), platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and vascular endothelial (VE)-cadherin. [29]. On the other hand, endothelial progenitor cells express other markers that characterize their immature stage of differentiation,
such as CD133 and CD34 [7, 30]. Vascular endothelial growth factor (VEGF) receptor-2 is a marker of active endothelial cells, present in nonquiescent endothelium as well as progenitor cells [29].
Depending on the tissues and organs the endothelium can acquire several features, namely continuous, fenestrated or discontinuous. In continuous capillaries endothelial cells line along all the surface of the vascular wall and can be found in the skin and muscles [31]. Fenestrated capillaries have small openings (80-100 nm in diameter) and can be found in the intestine, endocrine glands and kidney [31]. Finally, discontinuous capillaries or sinusoids have large gaps, many fenestrations and a discontinuous, or even absent, basal lamina and can be found in the liver, spleen and bone marrow [31]. Besides the assembly of vessel lumen, endothelial cells accomplish many other roles within our body. These cells are organized as a thin layer on the interior surface of all vessels and interactions with other cells and the extracellular matrix are crucial in endothelial cell function, such as in inflammation, angiogenesis cascade and transport of molecules through the vessel wall (either by active or passive mechanisms) [28].

1.1.2. Pericytes

Maturation of the nascent vessels involves the formation of a basement membrane and the support of new vessels by pericytes and smooth muscle cells. Pericytes have a prominent nucleous and a small cytoplasm and are associated with capillaries and post-capillary venules, providing structural support to endothelial cells [32]. Pericytes are plastic and have the capacity to differentiate

into other mesenchymal cell types, such as smooth muscle cells, fibroblasts or osteoblasts [28], although their ontogeny is not yet well understood. Gerhardt et al. report that pericytes have an intermediate phenotype between vascular smooth muscle cells and fibroblasts [33]. Some pericytes heterogeneity has been suggested based on the expression of α-smooth muscle actin (α-SMA) [34]. Pericytes can synthesize extracellular matrix proteins such as fibronectin, laminin [35], collagen [36] and glycosaminoglycans [37]. Hirschi et al. reported that the action of pericytes on the vasculature [32] seems to match the scope of ideal cells for tissue repair taken into account their recently documented multipotency [38].

1.1.3. Smooth muscle cells

Smooth muscle cells (SMC) secrete essential growth factors for vessel stabilization and preserve tissue integrity. SMC synthesize the connective tissue matrix of the vessel wall, which is composed by elastin, collagen and proteoglycans. Like endothelial cells, SMC have a very low proliferation rate in the normal artery but a high proliferate rate in response to vessel injury. SMC constitutes the vascular smooth muscle around the blood vessels whose function is to contract and to regulate blood vessel tone-diameter, blood pressure, and blood flow distribution [39]. Platelet-derived growth factor (PDGF) stimulates migration of smooth muscle precursors, during vascular development, which is necessary for the vessel wall formation [40].

1.1.4. Stromal cells

In general, the connective tissue can be formed by fibroblasts, adipocytes and persistent mesenchymal stem cells [41, 42]. In this context fibroblasts can be important regulators of angiogenesis.
Embryologically, fibroblasts arise from the relatively undifferentiated mesenchymal stem cells. Some of them remain in tissues such as the connective tissue. Fibroblasts are flat and irregular cells that synthesize the extracellular matrix of the connective tissue. Fibroblasts produce collagen, proteoglycans and elastin [43-45]. In addition, they secrete stimulatory growth factors, which provides as optimum environment to support epidermis formation and to facilitate skin wound healing [46]. Incorporation of fibroblasts in tissue

substitutes may be important as it acts as the key orchestrator in wound healing and enhances re-epithelialization [46]. Furthermore, there are no reports of hypertrophic scarring or keloid scarring following the intradermal injection of autologous fibroblasts, suggesting that fibroblast proliferation and collagen synthesis are naturally regulated by cell–cell and cell–ECM contact and by negative feedback [46]. Fibroblasts are activated when they express α-smooth muscle actin and some cytokines play a role in their activation, such as interleukin (IL)-1, tumor necrosis factor (TNF-α), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) [47]. These findings support the interest in studying the interactions between endothelial cells and fibroblasts, which may help to understand the crosstalk between these two types of cells and its implications in angiogenesis.

1.2. Growth factors

Growth factors are soluble peptides that are produced to induce cellular specific responses in order to restore the cellular homeostasis [48]. Depending of the distance of the site of synthesis (source) and site of action (target), the mechanisms can be designated as autocrine (effect on the producer cell itself), paracrine (effect on the neighboring cells) or endocrine (growth factors released directly into the circulation). Growth factors can affect cellular activities such as proliferation, differentiation, migration or gene expression [49]. When growth factors bind to the corresponding receptor, the signal is translated into the cell through signaling pathways that lead to gene expression induction or repression and specific biological response (Fig. 3); the response can differ from one cell type to another.

Experiments using conditioned media from cultured cells have proven to be an interesting and useful source of factors that may help to study the interactions between different cell types, although growth factors have short biological half-life periods. A variety of growth factors are involved in angiogenesis, such as VEGF, basic FGF, PDGF and TGF-β1 [6, 50].

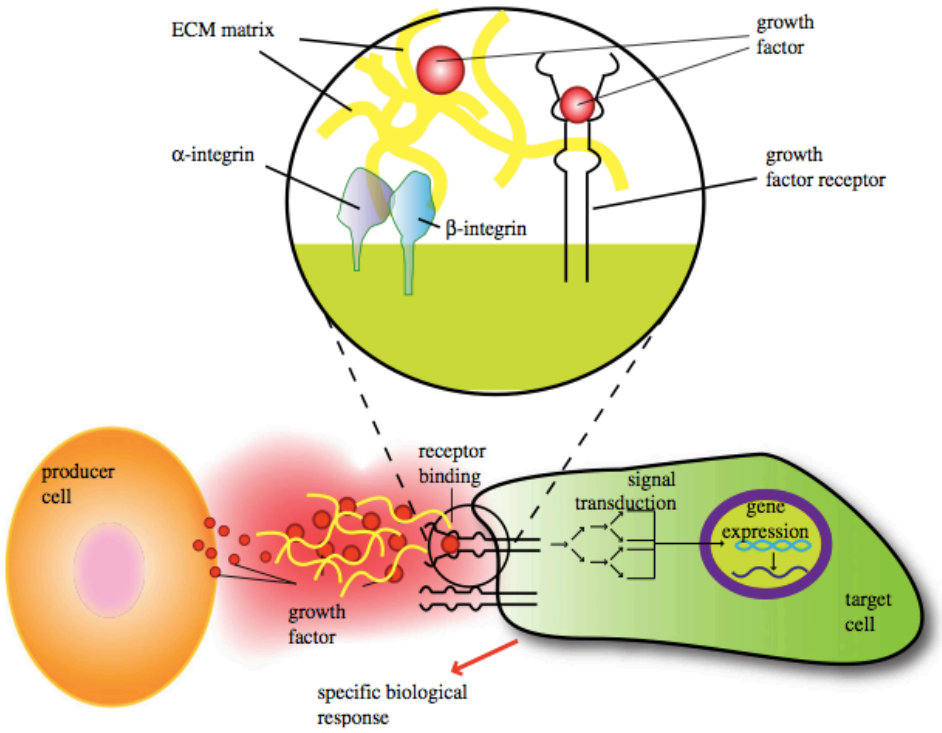

Figure 3. Illustration of growth factors presentation in a temporal and spatial fashion with cells and extracellular matrix. The producer cell secretes growth factors that create a gradient in the extracellular matrix. These growth factors will bind to target cell receptors and induce a specific biological response, or it will be stored in the extracellular matrix until its remodeling. From Lee et al. [51].

1.2.1. Vascular endothelial growth factor

VEGF is a homodimeric glycoprotein described as a vascular permeability factor with a heparin-binding domain [52, 53]. Four different major VEGF isoforms are generated through splicing: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ [54-56]. $VEGF_{121}$ is a free diffusible protein, unlike $VEGF_{165}$ that is secreted but linked to the cell surface and the extracellular matrix via its heparing-binding domain. These two isoforms induce mitogenic and permeability-enhancing activities on endothelial cells [54, 55, 57, 58]. *In vivo* VEGF has a complex and multifaceted role in angiogenesis and vasculogenesis. VEGF can bind to VEGF receptors 1 (VEGFR-1) and 2 (VEGFR-2), and in endothelial cells VEGFR-2 translates VEGF signals [6]. VEGF have been shown to promote permeability [59], to induce integrin expression [60], to enhance endothelial cell survival [61] and to induce matrix metalloproteinases (MMPs) secretion [62]. Bone marrow-derived mesenchymal progenitor cells secrete VEGF [63]. Additionally, VEGF is implicated in the

crosstalk between many types of cells, including neurons [64], smooth muscle cells [65], adipocytes [66] and fibroblasts [67].

1.2.2. Basic fibroblast growth factor

FGF has been associated with a wide range of cellular behaviors, including cell proliferation, differentiation, migration and survival [68, 69]. In humans 18 signaling FGFs have been found. FGFs are a family of small proteins containing a conserved core domain and two separate binding sites (one for FGF receptors and another one for heparin sulphate). The interaction of FGF with cell FGF receptor involves the formation of a FGF-heparin sulphate complex. FGF binds to heparin sulphate proteoglycans in the ECM, which protects FGF against degradation sequestering it [70]. FGF signaling is a complex and highly regulated process. Basic FGF (bFGF or FGF-2) influenced in an autocrine or paracrine manner the proliferation and differentiation of cells from epithelial, neuroectodermal and mesenchymal origin [71, 72]. bFGF has been shown to have a central role in fracture healing, bone remodeling and osteogenesis [68]. In addition, bFGF has been shown to be expressed by bone marrow stromal and peripheral blood cells [73].

Experimental evidence shows that bFGF can induce *in vitro* and *in vivo* a pro-angiogenic phenotype in endothelial cells, modulating endothelial cell proliferation [74] and migration [75, 76], protease production [76], and integrin and cadherin receptors expression [77-79].

As a consequence of its role in angiogenesis attempts of the use bFGF in tissue engineering are widely available [69, 80]. bFGF has been implicated in normal and pathological processes ranging from modulation of endocrine responses, embryogenesis, limb regeneration, and repair process as in neural, cartilage and soft tissues injury [81]. In wound healing, bFGF influences the proliferation of fibroblasts, endothelial cells, keratinocytes and smooth muscle cells [49]. bFGF is considered a potent angiogenic factor and its receptors can be found in almost all tissues in the body [49].

1.2.3. Transforming growth factor- β1

TGF-βs constitute a family of local mediators that regulate proliferation, differentiation and extracellular matrix metabolism [49]. In order to induce a biological response TGF-β needs to bind to cell surface receptors, consisting of multi-domain-transmembrane proteins with extracellular and intracellular domains with cinases activities [49]. TGF-β1 has been shown to possess pro- and anti-antiogenic effects in endothelial and other cells types: low doses seem to contribute to the angiogenic switch and high doses seem to led to inhibition of endothelial cells proliferation and migration [82, 83]. TGF-β1 stimulates the recruitment and proliferation of mesenchymal cells, and may modulate inflammation [69]. This growth factor has the capacity to bind to the extracellular matrix components, including type IV collagen, fibronectin, proteoglycans and heparin [49]. TGF-β has been proposed to be used in the clinical practice in the areas of soft and hard tissue healing, and control of chronic inflammatory diseases, associated with fibrosis and suppression of autoimmune diseases [84]. TGF-β is also involved in the process of wound healing. TGF-β1 levels show a transient increase after injury but in fibrosis TGF-β1 levels stay high for long periods. On the other hand, a decreased response to TGF-β1 by fibroblasts occurs in diabetic ulcers [85, 86].

1.2.4. Other factors that influence the angiogenic microenvironment

Other growth factors and cytokines may influence the process of angiogenesis and inflammation in order to orchestrate or to create an equilibrium leading to the formation of a new vascularized tissue. PDGF is mitogenic for fibroblasts, indirectly influences the production of collagen [87] and recruits the smooth muscle cells around nascent vessels to stabilize then into mature vessels [88]. Hepatocyte growth factor (HGF) induces angiogenesis in various animal models, stimulates the growth of endothelial cells and acts as a survival factor against endothelial cell death [89]. Cytokines, including interleukin (IL)-6 and IL-8, have been involved in the recruitment of endothelial cells and their organization due to the interplay between angiogenesis and inflammation [90, 91].

1.3. Extracellular matrix

The ECM plays fundamental roles during angiogenesis by providing the mechanical support and contributing to the maintenance of the tissue architecture, and also by providing the soluble and insoluble signaling required as described previously. The ECM can be described as a dynamic structure that regulates migration, proliferation and differentiation of endothelial cells due to intracellular signaling [92, 93]. The first step, in angiogenesis is the degradation of the sub-endothelial basement membrane and surrounding ECM. Therefore, several proteinase families are involved such as, plasminogen activators, matrix metalloproteinases (MMP) and their corresponding inhibitors, namely plasminogen activator inhibitor and tissue-type inhibitor of MMP (TIMP), respectively [94]. The fragmentation of the ECM leads to the release of matrix-bound angiogenic activators like bFGF, VEGF and TGF-β. In this regard, the remodeling of the ECM is intrinsically related with angiogenesis [82], and is implicated in vessel formation [95, 96] and vessel regression [97, 98].

The basement membrane consists of a network of molecules such as collagen IV, laminin and heparin sulphate proteoglycans [28], whereas typically components of the interstitial matrix are fibrillar collagens and glycoproteins, such as fibronectin [28]. The structured part consists of fibrous material, while the unstructured section can be described as an amorphous ground substance, that contains glycoproteins and electrolytes and other soluble substances of blood plasma.

After a skin injury, the development of a suitable ECM is necessary to allow cells to migrate. This matrix is formed by fibroblasts, monocytes, macrophages, lymphocytes and endothelial cells [99]. Collagen lead to the spreading of fibroblasts and endothelial cells, allowing the formation of capillaries [100].

1.3.1. Interactions between cells and ECM

Integrins are heterodimeric transmembrane proteins (each consisting of one α chain and one β chain) that mediate cell adhesion to the ECM and to the immunoglobulin superfamily. Integrins are important mediators in the regulation of intracellular signaling, due to the cell-ECM interactions [101]. The integrins pattern expressed in the cell surface dictates the cell adherence and the cell migration on different matrices. Integrins directly bind components of

the extracellular matrix such as fibronectin, collagen and laminin. Integrins also control the release of proteases that remodel the ECM, allowing cell motility. The sequence argine-glycine-aspartic acid (RGD) allows the interaction between the protein and the integrin receptors αVβ3 and α5β1 of the cell [102]. Arginine-glutamic acid-aspartic acid-valine (REDV) is an alternative splice from fibronectin, and it is recognized by α4β1 receptor in endothelial cells [103]. In the context of developmental and postnatal angiogenesis, vascular integrins appear as a target to improve or inhibit the neovascularization of the tissue [104].

Different combinations of integrin–growth factor receptors followed by distinct intracellular signaling pathways can avoid cell apoptosis. In the case of endothelial cells the crosstalks between αvβ3-bFGF and αvβ5-VEGFR-2 prevent apoptosis: the first preventing the intrinsic apoptosis pathway (is triggered by cellular stress) [105] and the second inhibiting extrinsic apoptosis (initiated through the stimulation of the transmembrane death receptors) [106].

For vessel stabilization endothelial cell-pericyte interactions are necessary and recent studies suggest the interaction of integrin α4β1on endothelial cells with vascular cell adhesion molecule 1 (VCAM1) on pericytes [107].

In tissue engineering the RGD sequence has been grafted to biomaterials to enhance adherence and viability of the cells [108, 109], unlike in cancer therapy where they are used to induce cell death [110, 111]. Regarding the soluble RGD-peptide, Aguzzi et al. reported a pro-apoptotic effect in endothelial cells [112, 113]. Interestingly, Kim et al. reported that in the case of endothelial progenitor cells the pro-apoptotic effect was not observed, but only an inhibition of the differentiation of the cells, maybe due to anti-adhesiveness of the RGD-peptide [114].

1.3.2. Collagen

Collagen is the most abundant protein in the ECM. All members of the collagen family are trimeric molecules composed of three polypeptide α chains resulting in a $(Glycine\text{-}X\text{-}Y)_n$ repeated structure (the X and Y position are often substituted by proline and hydroxyproline). In the endoplasmatic reticulum (ER) post-translational modifications occur in order to form the intramolecular hydrogens bonds, which will give stability to the structure. In connective tissues collagen types I, II, III, V and IX exist, being type I the most abundant in skin and bone.

Collagen plays an important role in organ development, wound healing and tissue repair, because of its interaction with growth factors and integrins of the cells, influencing adhesion, differentiation, growth and cellular reactivities [115]. Fibroblasts secrete and influence the alignment of collagen fibers in the ECM [116]. Collagen is found in high concentration in tendons, skin, bones, cartilages and ligaments, and these tissues are appropriate and abundant sources for isolation of this natural polymer [117].

Collagen can be readily processed into porous sponges, films and injectable cell carriers. Collagen has been used in cell encapsulation/entrapment due to its biocompatibility, biodegradability, abundance in nature and natural ability to bind cells [117, 118]. Challenges of using collagen as a material for cell delivery include the high purification cost, the natural variability of isolated collagens and the variation in enzymatic degradation (which depends on the location and state of the implant site) [118]. Collagen is a natural choice as artificial extracellular matrix in tissue engineering and has been used to engineer a large variety of tissues, including skin [119], bone [120, 121], heart valve [122], and ligaments [123], among many others.

1.3.3. Glycoproteins

Glycoproteins are carbohydrate-protein conjugates through a glycosidic link to the –OH of a serine or threonine residue (O-linked) or through an N-glycosyl link to the amide nitrogen of an aspargine residue (N-linked). The glycoproteins can be found in the blood, extracellular matrix, plasma membrane and inside some organelles of the cell. The length, variety and number of glycoconjugates allows many combinations that give proteins some tissue-specification and provides specific interactions between the cell membrane and the ECM [124]. The glycoproteins group represents 25% of non-collagenous proteins, which includes for instance alkaline phosphatase (ALP) [125, 126].

1.3.3.1. *Alkaline phosphatase*

In contrast to the large amount of evidence concerning the role of ALP in bone tissue, still very little is known about the clinical relevance of this protein in tissue regeneration. ALP is normally located either on the plasma membrane [127, 128] or in secretory vesicles [129] and, possibly, in the membrane

surrounding lipid droplets [130]. ALP is bound to cell membranes via a glycosylphosphatidyl-inositol (GPI) anchor [131, 132]. So, ALP is a glycoprotein that functions mainly as an ectoenzyme. Four isoenzymes have been described in humans: placental ALP (PALP), germ cell ALP (GCALP), intestinal ALP (IALP) and tissue-unspecific ALP (TNS-ALP). The endogenous substrates of ALP and its exact function are still not completely known [128, 130]. There is experimental evidence that the enzyme is involved in tissue development [133, 134], cellular differentiation [135, 136] and cell-cell interactions [137], although its precise role in these cellular events is also still not known. Hui et al. reported that collagen phagocytosis and apoptosis are induced by high levels of alkaline phosphatase expression in rat fibroblasts [138].

It has been hypothesized that ALPs may play a role in the processes of cell guidance and migration in a large variety of non-mammalian species. Several studies have demonstrated a potential involvement of ALP expression both in the migration of the pronephric duct during development in salamander [139, 140] and in growth cone guidance during axonal outgrowth from the distal limb in the developing grasshopper [141]. In addition, ALP expression is often restricted both temporally and spatially to cell populations that are involved in migration or morphogenesis [142]. Abnormal ALP isoenzymes expression has been found in malignant tissues, being often established as a useful prognostic indicator [137, 143].

2. Strategies to promote vascularization of bioengineered tissues

In tissue regeneration it seems fundamental to recreate a microenvironment capable of influencing cell guidance towards the regeneration process, instead of fibrosis or tumor development. Despite the same molecules and cells being involved in different processes, their proportions and half-life are different, resulting in microenvironment changes in different situations. Many strategies have been developed to promote vessel structure assembly and branching in order to maintain cellular functions and tissue integrity [144]. It seems important to identify a master regulator that leads to regeneration, due to the complexity of the process, in addition to understanding what happens in lower

organisms and in our embryology [4, 8, 145, 146]. Some approaches aiming at promoting vascularization will be presented and discussed, using growth factors, cells and artificial ECMs.

2.1. Growth factor delivery

Strategies involving the delivery of growth factors are aimed at using their signaling properties to stimulate the cells in the area to migrate, proliferate and differentiate. Regulation of angiogenesis involves the recruitment of endothelial cells to rapidly restore the vascularization of the new tissue formed and achieve homeostasis (oxygen and nutrients) of the tissue [147].

VEGF and bFGF have been often used to improve neovascularization by injecting these proteins in the circulation or into the target tissue [148, 149]. Growth factors delivered improved the chemotaxis of the cells and *in vivo* studies using their 'cocktails' [150] reported that recombinant human growth factors supplied additional blood flow to underperfused and incompletely revascularized areas [148, 151].

However, a number of studies reported that the subcutaneous injection of VEGF and bFGF presents several limitations related to their natural instability *in vivo* and to the possibility of uncontrolled effects at distant sites [152, 153]. Clinical trials reported that the administration of VEGF (Phase I) [148] and bFGF [154] was safe and feasible. However, VEGF (VIVA studies) in ischemia for vascular angiogenesis [155] and bFGF (FIRST study) in coronary artery disease [149] have not shown the expected benefit to the patients. Some disadvantages were appointed, such as their rapid clearance from the body, which lead to increasing the administered doses of these factors systemically into the body that, in turn, lead to unspecific and undesirable side effects, such as bleeding, tumor growth, atherosclerosis and restenosis [156-158]. Taken this in account it is important to understand the kinetics of the growth factors and their time-life in order to obtain the optimal dose and route of administration [8, 155]. Also, even though growth factors stimulate vessels formation, the newly formed vessels need to become mature vessels in order to maintain their stability and not regress. Taking this into account, the simple injection of angiogenic factors may be insufficient for stable vessel formation, and may even be dangerous [159]. Localized and sustained delivery of growth factors at specific sites instead of

free delivery in the circulation will probably allow a better and safer promotion of angiogenesis.

2.2. Cell transplantation

Several strategies designed to induce tissue vascularization rely on the transplantation of cells, mainly of the endothelial cell (EC) type. Several of these strategies have succeeded in promoting some vascularization although, generally, they were proven insufficient, with several limitations relying in the lost of cell viability after injection, the dispersion of the cells, their rapid clearance from the injection site and the lack of the right cues to develop their original role.

In general, the use of stem cells for transplantation, although promising, still involves several hurdles to be overpassed, namely the efficiency in the isolation process and the long culture time required to obtain the necessary amount of cells. Undifferentiated cells are more proliferative and present a higher regenerative potential compared to differentiated cells with endothelial phenotype [160]. In addition, mature ECs generally show less survival rates when injected in ischemic areas [161].

Cell transplantation in order to improve vascularization in the treatment of ischemic tissue in heart diseases is the basis of multiple animal studies and clinical trials [162]. In order to promote the regeneration of myocardial tissue, bone marrow-derived stem cells were injected in a mouse model and they differentiated in endothelial cells and smooth muscle cells that participated in the recovery of cardiac function [163].

Progenitor endothelial cells (EPC) are a source of endothelial cells, which are generated in bone marrow and circulate in the blood system participating in the neovascularization of new tissue [7, 164]. Transplanted endothelial progenitor cells have been shown to induce new vessel formation or restore the injured endothelial cell layers in the ischemic hindlimb [165]. In ischemic hindlimb EPCs showed better recovery of the limb when compared to simple injection of human microvascular vein ECs [165]. The EPC isolated from mononuclear cells from the peripheral blood express endothelial cells markers, but also monocytic markers and a delimited ability of expansion. On the other hand, outgrowth endothelial cells (OECs or late-EPCs) are another population of cells isolated from peripheral

blood showing high proliferative activity, when compared to EPC, and demonstrated a very stable phenotype during passages [29, 166]. These cells have demonstrated a capacity for use in cell-based therapies or in combination with biomaterials for improved angiogenesis [167].

Overall, the use of injected angiogenic cells as a strategy to promote vascularization has not been proved effective so far due to the limitations pointed above. Hence, the use of approaches involving cells combined with artificial extracellular matrices seems attractive in order to overcome several of the limitations described above.

2.3. Angiogenic artificial extracellular matrices

Different biomaterials are under investigation in order to promote vascularization of the new tissue formed in different organs such as in bone [168], heart [169], and skin [170]. The environment created by the scaffold should be designed to mimic the conditions of the natural tissue to be replaced, providing different soluble and insoluble signals, which will further influence cells in different ways, namely their migration, proliferation, and differentiation. Scaffolds have been designed with controlled biodegradation (matching tissue regeneration kinetics) and porosity to promote cellular colonization and ingrowth from the host, as well as with functionalities promoting cell adhesion [171-173]. Other requirements are biocompatibility and proper mechanical integrity of the scaffold [174].

In angiogenesis strategies using natural and synthetic matrices have been developed. Synthetic matrices, such as polyethylene glycol (PEG), poly-methacrylates, polylactides (PLA), due to lack of biological signaling ability, usually failed to induce vascularization [144]. Natural matrices, such as fibrin and collagen, are claimed to be more attractive for this purpose because of their natural biocompatibility and similarity with natural tissues [173, 175]. Callegari et al. reported that, after implantation in healthy or cryoinjured left ventricles of rats, a porous collagen scaffold was capable to induce a high angiogenic response [173].

In skin tissue engineering, clinically available acellular dermal substitutes, like Integra® (collagen) and Alloderm® (decellularized tissue matrix), are used for the treatment of burns and deep wounds and recommended as a temporary

barrier for skin grafts [170]. Although constituting useful therapeutic alternatives to traditional artificial skin products these products were shown to restore the epidermis but only a small part of the dermis, without a well-vascularized dermal wound bed for healing full thickness burns [170]. Usually, re-grafting is required and one of the main challenges is still a material able to rapidly become vascularized [170].

Overall the implantation of materials alone has resulted in poor vascularization *in vivo*. Hence, several strategies have been proposed and investigated to promote the adhesion and recruitment of endothelial cells, which seem to be essential to promote the vascularization of the scaffold in an environment where a variety of cell types are competing and assemble for tissue regeneration. One key aspect relies in the ability of the scaffold to allow the migration of endothelial cells to form vessels, and therefore increasing the porosity and the interconnectivity of the pores has been proposed as one viable strategy [176]. Another strategy relies is the grafting of peptides capable of enhancing the adherence of the cells, such as the RGD sequence (arginine-glycine-aspartic acid), present in many ECM proteins, such as fibronectin, vitronectin, and collagen [172, 177]. Hyaluronan hydrogels with open porous structure and viscoelastic properties, modified with laminin [178], poly-D-Lysine [179] or RGD peptides [180], were reported to support cell ingrowth and angiogenesis *in vivo* in the case of brain injury.

Other strategies have been developed in order to improve the selective recruitment of endothelial cells in the competitive microenvironment of the implant site, where smooth muscle cells and fibroblasts, among other cell types, are also present. One such strategy relies in grafting the arginine-glutamate-aspartate-valine (REDV) sequence, derived from fibronectin. Hubbell et al. demonstrated that REDV-coated surfaces in vascular grafts could improve the attachment of endothelial cells (HUVEC) but not fibroblasts or smooth muscle cells [181, 182]. This interactions between HUVECs and the REDV sequence was reported to occur through integrin $\alpha_4\beta_1$ [182]. Wei et al. also reported the selectivity of the REDV peptide for HUVECs compared to smooth muscle cells when immobilized onto a PEG structure [183].

Alternative approaches have been proposed to improve the selectivity between progenitor endothelial cells and the neighboring endothelial cells in the re-endothelialization of cardiovascular stents using an anti-CD34 antibody grafted to the surface of biomaterials for cardiovascular applications. A

polycaprolactone stent engrafted with an anti-CD34 antibody was shown to improve the adhesion of progenitor endothelial cells compared to HUVECs [184]. Lin et al. reported that an endothelialized stent coated with heparin/collagen multilayer coatings, and functionalized with an anti-CD34 antibody, was selective in promoting the attachment and growth of vascular endothelial cells and further demonstrated anti-restenosis effects [185].

Several alternative strategies have been proposed several of the components described previously, namely cells, growth factors and matrices, in order to improve cell-matrix interactions and to stimulate selective cell-signaling pathways leading to implant vascularization.

2.4. Combined strategies

In strategies to promote vascularization in regenerative medicine the extracellular matrices play fundamental roles by, on one hand, providing the support and the stability for cells to regenerate the injured area and, on the other hand, by additionally providing the insoluble and soluble signaling required to maintain cells viable, proliferating and differentiating.

As previously described, one of the potent facilitators of angiogenesis described in the literature is VEGF, which influences mature endothelial cells as well as progenitor endothelial cells, demonstrating effects on endothelial cell proliferation, motility, and vascular permeability [11, 186, 187]. Alginate gels incorporating VEFG or bFGF increased the number of blood vessels, although VEGF lead to an increased density of blood vessels [188]. Tanihara et al. reported that, *in vivo*, alginate with heparin and incorporating bFGF promoted angiogenesis, comparing to the control without bFGF, which showed no cellular infiltration nor angiogenesis, and further suggested that the release of the growth factor should be slow and directed to the target area, affecting only the adjacent tissues in order to minimize the local inflammatory response [189]. Iwakura et al. reported that bFGF incorporated into gelatin hydrogels improved the vascular density of the infarct zone after 4 weeks in a rat model [190]. Microbeads of alginate loaded with FGF implanted in the rat omental pouch model after 7 days increased vascular density compared with control rats implanted without growth factor, despite the recruitment of mural cells was not found to increase in the presence o FGF [191]. The delivery of TGF-β1 from

alginate hydrogels enhanced the natural dental pulp regeneration capacity [192]. These growth factors entrapped in alginate showed induction of neovascularization *in vivo* compared to subcutaneous injection of angiogenic factors not entrapped in alginate [51, 193, 194].

The combination more than one growth factors have also been investigated in order to obtain a stabilized vasculature instead of weaker blood vessels. Hao et al. reported that the sequential delivery of VEGF followed by PDGF from alginate hydrogels induced an angiogenic effect and functional improvement compared to the delivery of single factors after myocardial infarction [50]. Richardson et al. showed that co-delivery of PDGF and VEGF led to rapid formation of a mature vascular network after hind-limb ischemia induction compared to individual factors delivered alone [152]. Marui et al. incorporated, into collagen microspheres, bFGF and hepatocyte growth factor (HGF) and reported increased vessel formation and maturity [195]. Freeman et al., using alginate-sulfate hydrogels to deliver three angiogenic factors (VEGF, TGF-β1 and PDGF), showed an increase in vascularization compared to scaffolds delivering only bFGF [196]. Taken together, these examples indicate the importance of the action of multiple growth factor delivery for vascularization and hence better results in tissue regeneration. However, the optimal dose and spatial time delivery constitute aspects that clearly need to be further studied. In order to overcame these difficulties cells have been used, taking advantage of their capacity to secrete these pro-angiogenic growth factors and to provide feedback in reaction to the needs of the microenvironment.

Several different endothelial cells types have been used for promoting angiogenesis and vasculogenesis *in vitro* an *in vivo*. Human umbilical vein ECs (HUVEC) and human dermal microvascular ECs (HDMEC) are among the mature EC types most widely used [197, 198]. Alginate grafted with RGD peptides has been shown to improve cell attachment and formation of tubular-like structures using HUVECs [199] and progenitor endothelial cells [200]. In addition, covalent attachment of RGD peptidomimetics containing poligoethylene glycol spacers in poly(ethylene terephthalate) (PET), an inert polymer, demonstrated the capacity of improve the adhesion of the endothelial cells from the human saphenous vein [201]. Endothelial progenitor cell types are also becoming increasingly investigated for this purpose due to the advantages that they provide, as previously explained. The use of outgrowth endothelial cells within alginate have been shown to constitute a good strategy to improve the vascularization in

tissue damage, because alginate does not seem to adversely affect these cells and the microenvironment created does not seem to reduce the cells angiogenic potential [200]. Progenitor endothelial cells in combination with RGD-alginate as a delivery system were shown capable to create new vascular beds in ischemic tissues [202].

Vascularization strategies based in cocultures systems have shown that there are interactions between cells types with reciprocal beneficial effects in vascularization and tissue regeneration. In the case of endothelial cells different cells types showed ability to support the formation of pre-vascular structures, such as fibroblasts [203], smooth muscle cells [204], adipocytes [205], osteoblasts [198], as well as mesenchymal stem cells, in a general approach, due to their capacity to differentiate into different cell types [206]. The use of two or more cell types in coculture, creating and heterotypic microenvironment, also contributes to better understand cell behavior, in terms of spatial organization, proliferation and differentiation of the cells in two dimensional (2D) or three dimensional (3D) cultures. As a consequence, many cocultures studies are available using endothelial cells along with the major cell types of the tissue to regenerate, such as osteoblasts in bone, cardiomyocytes in heart and astrocytes in brain [206]. The crosstalk between endothelial cells and other cell types involve paracrine mechanisms (growth factors) and direct cell-cell communication that maintain the viability of the endothelial cells and stimulate the differentiation of the cells [207]. Villars et al. reported that connexin 43 is important in junctional communication between HUVECs and human osteoprogeniotor cells [208]. Connexin 43 expression in cardiac myocytes has also been promoted by microvascular endothelial cells [209]. Kaigler et al. reported improved vascularization in vivo using HUVECs and bone marrow stromal cells and further demonstrated the reciprocal beneficial interrelationship between the two cell types through exchange of VEGF, produced by the marrow cells, and bone morphogenetic protein (BMP)-2, produced by the ECs [210, 211]. Fuchs et al. reported that OECs cocultured with osteoblasts (MG63) or with progenitor osteoblasts formed a vascular-like network, and that this network was not formed using HUVECs [212]. Aguirre et al. highlighted the importance of the differentiation stage and the origin of mesenchymal stem cells in order to promote the formation of capillary-like structures when cocultured with EPCs [213].

The interaction between endothelial cells and fibroblasts are yet unclear, although the contribution of the latter cell type in tissue vascularization is considered important [42]. Cocultures of HUVECs and human periodontal ligament fibroblasts induced capillary formation [214]. Berthod et al. reported the importance of ECM produced by fibroblasts in the promotion of this capillary-like structures [203]. ECM and growth factors influenced the tubule formation, stability and differentiation. Conditioned media collected from alginate-bioactive glass beads cultured with fibroblasts showed an enhancement in endothelial cell proliferation due to the presence o VEFG secreted by fibroblasts [215].

Anastomosis of the newly formed vessels with the host vasculature is another major obstacle in the development of vascularization strategies. Ferreira et al, reported that endothelial-like and smooth muscle-like cells isolated from human embryonic stem cells, when subcutaneous implanted within Matrigel, formed microvessels structures and, I some cases, anastomosis with the host vasculature was observed [216]. An alternative study showed that spheroids of cocultures of HUVECs with mesenchymal stem cells subcutaneously inoculated in nude mice promoted anastomosis with the host vasculature to some extent [217]. 3D silk fibroin scaffolds colonized with outgrowth endothelial cells showed capacity to form microvessel-like structures important in the interconnection between the tissue engineered construct and host vasculature [218]. Complex biomaterials made of a combination of fibrin with synthetic poly(L-lactic acid) and polylactic-glycolic acid sponges, colonized with endothelial cells and fibroblasts, promoted an improvement in the neovascularization and perfusion of the implanted grafts [219].

The combination of matrices prevascularized with endothelial progenitor cells and hypoxia seems to be an alternative viable strategy to promote tissue vascularization favoring as well a link between the microvessels formed in the tissue construct and the existing vasculature from the host [220]. Tissue decellularization generated new insights in bioscaffold design [221]. Ott et al. described that a decellularized heart, when reseeded with endothelial and cardiac cells, was re-endothelialized and generated a pumping function [25]. Likewise, Baptista et al. reported the decellularization of a liver that maintained the vascular structure, which, when re-cellularized with endothelial and hepatic cells lead to right engraftment and re-vascularization, re-establishing basic liver functions [222]. The development of strategies to further elucidate these cell-

matrix interactions seem fundamental to advance the field of tissue regeneration and biomaterials design with a view to promote functional anastomosis between microvessels formed in tissue constructs with the existing vasculature from the host.

3. Hypothesis and specific aims

The hypothesis guiding the research reported in the present thesis is that a functional and viable vascular network may be promoted in tissue regeneration through the appropriate delivery of fibroblasts and endothelial cell, combined or not with angiogenic growth factors, implanted within biofunctionalyzed biodegradable hydrogel carriers.

In order to investigate this hypothesis different strategies were used with the following specific aims:

i) To assess if the survival and viability of endothelial cells can be improved and modulated by fibroblasts and/or angiogenic growth factors.

ii) To investigate if the functionality and ability of endothelial cells to form vessel-like structures can be influenced in indirect and direct coculture with fibroblasts.

iii) To study if entrapped fibroblasts can recruit endothelial cells and induce angiogenesis *in vivo*.

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CHAPTER II

Neonatal human dermal fibroblasts immobilized in RGD-alginate induce angiogenesis

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*Submitted (2011)

Abstract

Promoting angiogenesis in a damaged tissue is a major challenge for tissue regeneration. Recent findings in tissue engineering suggest that fibroblasts (FBs) play an important role in orchestrating the angiogenic process. Fibroblasts maintain the structural integrity of connective tissue by continuously secreting growth factors and extracellular matrix precursors, which are essential for endothelial cells (EC) adhesion and spreading, thus playing a crucial role in angiogenesis. We hypothesized that FBs immobilized in alginate gels grafted with the RGD peptidic sequence could influence the recruitment of ECs to improve vascularization. In this work the modulation of immobilized human FBs within the 3D synthetic extracellular matrix was assessed. Experiments using co-cultures of ECs and FBs in indirect contact as well as angiogenic assays were performed to assess the influence of FBs immobilized in RGD-alginate in ECs viability, stabilization, sprouting and assembly into capillary-like structures. This study demonstrates the ability of FBs immobilized within RGD-alginate microspheres to modulate and support capillary-like structures' assembly. These findings indicate that the microenvironment created by these stromal cells in the scaffold modulates capillary morphogenesis, thus stimulating angiogenesis *in situ*, and can potentially be used in regenerative medicine, in clinical scenarios where vascularization is essential.

Keywords: Alginate, angiogenesis, endothelial cells, fibroblasts, RGD peptide, injectable biomaterials.

1. Introduction

Strategies promoting tissue vascularization continue to represent a great challenge in regenerative medicine. A promising approach to address the problem of vascularization deficit of new tissue is the use of implantable three-dimensional (3D) biomaterials that can provide support and protection to cells transplanted for regeneration of skin (32), nerve (3), mammary gland (23), cardiovascular tissue (48), and bone (14,17,21,38). The 3D biomaterial structure is intended to mimic the extracellular matrix (ECM), thus creating an adequate microenvironment to allow the interaction between cells and the ECM. These interactions are fundamental in cell polarization and function and crucial for vascularization of the newly formed tissue (12). Angiogenesis, or the recruitment of new vessels to the tissue formed, is a process that plays a key role in various physiological conditions, including embryonic development, wound repair, inflammation, and regeneration (7).

In tissue engineering approaches, angiogenesis has been traditionally stimulated by paracrine signals, such as vascular endothelial growth factor (VEGF) (39) and basic fibroblast growth factor (bFGF) (13), using biomaterials like hydrogels for bioactive molecule delivery. However, in the framework of tissue vascularization, the uncontrolled release of angiogenic growth factors and/or alterations of the production of natural angiogenic inhibitors could deregulate the angiogenic balance (22). Immobilization of cells in 3D structures, instead of growth factors immobilization, constitutes an alternative strategy. The immobilization of cells inside alginate microspheres is here proposed as a minimally invasive (injectable) strategy for tissue regeneration (14,37,43). Alginate is an hydrogel that has been used as a biomaterial, both *in vitro* and *in vivo*, due to its several advantages, including biocompatibility and ability to be crosslinked under harmless mild conditions to cells (12). Coupling arginine-glycine-aspartic acid (RGD) peptidic sequence to alginate promotes cell attachment, leading to improvement of tissue formation (1). The present team previously demonstrated that immobilization of osteoprogenitor cells in RGD-alginate is a valuable strategy to maintain cells viable during the period of osteoblasts differentiation (5,14,21). It has also been previously shown that endothelial cells (EC) influence osteoprogenitor cells by releasing bone morphogenetic protein-2 (BMP-2) to modulate their osteogenic differentiation *in vitro* and *in vivo* (24). Reciprocally, bone cells produce VEGF that enhances EC

viability (25), thus generating a crosstalk that seems to stimulate both cell types. However, in this system, a significant decrease in ECs viability was revealed upon immobilization, even in the presence of osteoblasts (21).

Recent findings suggest that fibroblasts (FBs) also play an important role in orchestrating the angiogenic process (10,16,47). FBs are mesenchymal cells with many vital functions during development and in adult organisms (8). They are responsible for the synthesis of collagen, an ECM component that provides a structure for cell migration and release of angiogenic growth factors (8). However, the potential of FBs for improving vascularization in tissue regeneration strategies remains largely unexplored. Hence, in the present study it was hypothesized that FBs immobilized in RGD-alginate could influence ECs to improve vascularization. In this work the modulation of human ECs and FBs immobilized within a synthetic 3D extracellular matrix was assessed. Experiments using co-cultures in indirect contact and angiogenic assays were performed to assess the role of immobilized FBs in ECs viability, stabilization, sprouting and assembly into capillary-like structures.

2. Materials and Methods

2.1. Culture of endothelial cells and fibroblasts

Human umbilical vein endothelial cells (HUVECs, ScienceCell, herein called ECs) were cultured in M199 medium (Sigma), supplemented with 20% (v/v) inactivated fetal bovine serum (FBS; Gibco), 80 μg/mL heparin (Sigma), 30 μg/mL endothelial cell growth supplement (ECGS, BD Science), 100 U/mL penicillin G and 100 μg/mL streptomycin (Gibco). Cells were maintained in culture flasks coated with 0.2% gelatin (Sigma). Human dermal foreskin fibroblasts-1 (HFF-1, ATTC, herein called FBs) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with high glucose and with L-glutamine, supplemented with 15% (v/v) inactivated FBS (Gibco), 100 U/mL penicillin G and 100 μg/mL streptomycin (Gibco). The cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere until 90% confluence was reached. For each experiment, endothelial cells (ECs) and fibroblasts (FBs) were used at passages 6-8 and the culture medium was changed every 2 days.

2.2. Preparation of modified alginate

The alginate gel for cell immobilization was prepared according to the methods previously described by Evangelista et al. with minor modifications (14). Briefly, Protanal LF 20/40 sodium alginate (gift from FMC Biopolymers, Oslo, Norway) was used as high molecular weight (high content of guluronic acid units, high MW) component to prepare alginate microspheres. To obtain the low molecular weight (low MW) alginate γ-irradiation with a cobalt-60 source at a dose of 5 Mrad was used, as previously described by Kong et al. (27). Alginate was then purified through dialysis (MWCO 3500 membrane, Spectra/Por®) against distilled and deionized water for 3 days. After, sodium periodate was used for alginate oxidation, as previously described (6). Finally, oligopeptides with a sequence of $(Glycine)_4$-arginine-glycine-Aspartic acid-serine-proline (abbreviated as G_4-RGDSP; Peptides International, USA) were covalently grafted to the oxidized alginate (16.7 mg of peptide per gram of alginate) by using the aqueous carbodiimide chemistry (37). After reaction for 24 h the alginate was purified by dialysis (MWCO 3500 membrane, Spectra/Por®), and then filtered, lyophilized and stored at -20ºC until further use. In the present experiments, a 2 wt-% RGD-modified

alginate solution in 0.9 wt-% NaCl (Sigma) was used, corresponding to a 50:50 mixture of oxidized and RGD-modified alginate (25 wt-% high MW, 75 wt-% low MW) and unmodified high MW purified alginate (14).

2.3. Immobilization and culture of cells within alginate microspheres

Cells were tripsinized with 0.05% (w/v) trypsin/EDTA solution (Gibco), and the cell pellet was then resuspended in 0.9 wt-% NaCl (Sigma) solution and centrifuged in a 5 mL syringe. Afterwards, the cells were carefully homogenized with the 2 wt-% RGD-alginate in 0.9 wt-% NaCl solution using a dual-syringe system (5,14). A cell density of $20x10^6$ cells/mL of RGD-alginate solution was used (5). Subsequently, the mixture prepared was extruded under a coaxial nitrogen flow using a Var J1 encapsulation unit (Nisco, Switzerland) at a speed of 40 mL/h (Single-syringe infusion pump, Cole Parmer). The RGD-alginate microspheres were allowed to form in an isotonic 0.1 M $CaCl_2$ solution, and were kept therein for 10 min. The microspheres thus obtained were rinsed in Tris-buffered saline (TBS), followed by rinsing in culture medium. All microspheres produced were uniformly spherical (diameter of c.a. 800 μm). RGD-alginate microspheres containing cells were then cultured in dynamic culture conditions using 100 mL spinner flasks (Bellco Biotechnologies, Vineland, USA) maintained at 37ºC under a 5% v/v CO_2 humidified atmosphere. The culture medium was changed every three days. RGD-alginate microspheres without cells were also prepared as a control and maintained in the same conditions. After immobilization, RGD-alginate microspheres containing ECs were cultured in the presence of 5 ng/mL of recombinant vascular endothelial growth factor (VEGF, from Biomedical Technologies Inc., USA), or 5 ng/mL VEGF combined with 5 ng/mL recombinant basic fibroblast growth factor (bFGF, from Sigma, Portugal).

2.4. Diffusional study

The purpose of the diffusional study was to assess the diffusion of substances with distinct molecular weights into the RGD-alginate microspheres, namely albumin (Mw $6.9x10^4$) and lysozyme (Mw $14.3x10^3$). Microspheres were placed in a 15 mL centrifuge tube filled with a mixture of 7.5 mL of Tris-buffered saline (TBS, pH 7.4) and 0.25% of the diffusing solute (albumin or lysozyme, Sigma), as previously described by Orive et al. (33). At 15, 45 and 75 min, a 2 mL sample

was taken from the RGD-alginate microsphere suspension and analysed to measure the solute concentration. All diffusion experiments were performed under stirred conditions and in a 37ºC incubator. Albumin and lysozyme were measured by the micro BCA assay (41).

2.5. Uptake of Dil-ac-LDL by endothelial cells

Cells were incubated with fluorescent acetylated low-density lipoprotein (Dil-ac-LDL, from Biomedical Techologies Inc., USA), a specific marker for endothelial cells. After 4 h of incubation (10 µg/mL medium) at 37˚C, the medium containing Dil-Ac-LDL was removed and the cells were washed with probe-free medium. Immediately, the microspheres were visualized using a confocal laser scanning microscope (CLSM, Leica SP2 AOBS; Leica Microsystems) and LCS software (Leica Microsystems).

2.6. Metabolic activity by resazurin assay

The resazurin assay measures the innate metabolic activity of cells by assessing the fluorescence-intensity increase associated with the cellular reduction of resazurin to resorufin. Cell metabolic activity was assessed by culturing cells in 10% (v/v) resazurin solution (Sigma) for 4h at 37˚C. Afterwards, 100 µL/well were transferred to a 96-well black plate and fluorescence was measured at the excitation and emission wavelengths of 530 nm_{Ex} and 590 nm_{Em} in a Spectra Max Gemini XS (Molecular Devices). To allow recovery of cells immobilized in RGD-alginate, 50 mM ethylenediamine tetraacetic acid (EDTA) solution in phosphate buffered saline (PBS, pH 7.4) for 5 min, was used.

2.7. Cell viability by Flow Cytometry

Apoptosis was determined using Annexin V-FITC staining (BD Pharmingen™) according to the manufacturer's instructions. Propidium iodide (PI) is a standard flow cytometry viability probe and is used to distinguish viable from nonviable cells. Cells staining positive for annexin V-FITC and negative for PI are undergoing apoptosis. Samples of microspheres were transferred to a 24-well plate and incubated with 50 mM EDTA solution in PBS (pH 7.4) for 5 min to dissolve alginate and recover the cells, as previously described. Cells were then

washed in PBS and centrifuged at 1200 rpm for 5 min. Cells were then resuspended in 1x binding buffer at a concentration of $1x10^6$ cells/mL. After transferring 100 μL of the solution ($1x10^5$ cells) to a tube, 5 μL of annexin V-FITC and 5 μL of PI were added followed by incubation for 15 min at room temperature in the dark. Afterwards, 400 μL of 1x binding buffer were added to each tube and $1x10^4$ cells were used for this analysis. Data was collected with a flow cytometer (FACS Calibur, Becton Dickinson) and analyzed with FlowJo software.

2.8. Preparation of conditioned medium

Conditioned culture medium was obtained by culturing ECs (CMECs) or FBs (CMFBs) during 24h in M199 medium supplemented with 10% FBS, 80 μg/mL heparin, 30 μg/mL ECGS, penicillin G (100 U/mL), and streptomycin (100 μg/mL). The media were collected, centrifuged at 1200 rpm, during 5 min, and the supernatant frozen at -20ºC. Both cell types were separately plated at a density of $8x10^3$ cells/cm^2. After 24h incubation, media were changed: ECs were incubated in CMFBs and FBs were incubated in CMECs. Freshly prepared media were used as control for each cell culture. The metabolic activity of cells was then assessed by the Rezasurin assay upon a 24h incubation period.

2.9. Co-culture of endothelial cells and fibroblasts in indirect contact

FBs ($3x10^3$ cells) were seeded on the bottom of 24-well tissue culture plates and incubated for 24h. Afterwards, transwells with a membrane of 0.8 μm pores (BD Science) were coated with 50 μL of growth factor reduced-Matrigel® (GFR-Matrigel, BD Biosciences) and incubated for 30 min at 37ºC (cf. Fig. 5A). Then, ECs ($3x10^3$ cells) were seeded on top of the transwell insert, in indirect contact with the FBs in M199 medium supplemented with 10% FBS, 80 μg/mL heparin, 30 μg/mL ECGS, penicillin G (100 U/mL), and streptomycin (100 μg/mL). After 24h of incubation, cell metabolic activity was measured by the resazurin assay and the assembly of capillary-like structures was monitored by microscopic analysis (Axiovert 200M, Zeiss).

2.10. Assessment of capillary-like structures formation and stability

To assess the influence of FBs immobilized within RGD-alginate microspheres on capillary-like structures formation and development, a 3D assay was performed using growth factor reduced-Matrigel® (GFR-Matrigel, BD Biosciences)-coated plates, after adaptation from Soares et al. (42). Briefly, 200 μL of GFR-Matrigel® were added to a 24-well plate and incubated at 37º for 30 min to form a gel layer. Subsequently, RGD-alginate microspheres with or without (control) FBs were added to the well (approximately 54 ± 4 microspheres per well). Again, 200 μL of GFR-Matrigel® were added to the samples and incubated at 37º for 30 min. Afterwards, ECs ($5x10^3$ cells) were cultured on top of this gel in M199 medium. Under these conditions, ECs are known to assemble into capillary-like structures after 24h (42). A semi-quantitative measurement of the structures formed was carried out as previously described (5,42). The plates were photographed after 1 and 5 days post incubation using an inverted microscope (Axiovert 200M, Zeiss). The capillary-like structures formed were then counted in each well and the length was measured using imageJ64.

2.11. Ex-vivo assessment of sprouting using the aortic ring assay

The aortic ring assay was performed as a microvessel outgrowth analysis. Thoracic aortae were removed from normal Wistar rats and washed in PBS. After fibroadipose tissue removal, aortas were cross-sectioned (approximately 2 mm), washed in cell media and embedded on GFR-Matrigel® (BD Biosciences). First, 200 μL of GFR-Matrigel® were added to a 24-well plate and incubated at 37º for 30 min to form a gel layer. Afterwards, a single aorta section was placed in one side of the well. In the opposite side RGD-alginate microspheres with or without (control) FBs were added. Again, 200 μL of GFR-Matrigel® were added to the samples and incubated at 37º for 30 min. The aorta section and the microspheres were then cultured together in M199 supplemented with 10% FBS. Four days after incubation, vascular sprouting from aortic rings was then visualized on an inverted microscope (Axiovert 200M, Zeiss) and photographed. The composite images of aortic ring assays were created by using the photomerge tool (Photoshop CS4) to merge all the photos taken from the same well. The area of the sprouting in the microphotographs were identified using an image J64 and contrast was created to clearly identify the microvasculature.

Animal experiments were conducted according to accepted standards of animal care (Declaration of Helsinki, European Community guidelines 86/609/EEC) and Portuguese Act 129/92) for the use of experimental animals.

2.12. Statistical analyses

All experiments were performed in triplicate. Quantifications are expressed as mean ± standard deviation (SD). Statistical significance of difference between various groups was evaluated by analysis of variance (ANOVA) followed by Bonferroni test. For comparison between two groups the Student's *t*-test was used. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $p<0.05$.

3. Results

3.1. Immobilization of endothelial cells and fibroblasts inside RGD-alginate microspheres

After immobilization of ECs (Fig. 1A) or FBs (Fig. 1B) within RGD-alginate, homogeneous and spherical microspheres were obtained. In order to investigate if immobilized cells were having access to nutrients available in the medium, RGD-alginate microspheres without cells (Fig. 1C) were used. For that purpose, diffusional studies were carried out using two different solutes - albumin and lysozyme. The results evidence that both solutes investigated were able to diffuse into the microspheres and reach their equilibrium states at 15 min (Fig. 1D). This data indicates that low molecular weight compounds diffused into the microspheres.

3.2. Metabolic activity and viability of immobilized cells

The immobilization of ECs within RGD-alginate may represent an *in vitro* model to study the effect of growth factors in the metabolic activity of these cells in a 3D microenvironment. In our previous studies, ECs immobilized alone during one week within RGD-alginate microspheres presented decreased metabolic activity (21). In order to assess the effect of growth factors produced by FBs in the metabolic activity of immobilized ECs the resazurin assay was used as demonstrated in Fig. 2A. ECs immobilized in RGD-grafted alginate microspheres were cultured in medium supplemented with 5 ng/mL VEGF or with the same concentration of VEGF combined with 5 ng/mL bFGF. Additionally studies of dil-ac-LDL incorporation were performed in order to evaluate cell distribution. Results reveal that EC immobilized within RGD-alginate microspheres, in medium supplemented with VEGF, were homogenously distributed and that viable cells indeed incorporated the dil-ac-LDL dye, as illustrated at day 3 (Fig. 2B). For both conditions, the metabolic activity of immobilized ECs decreased during the period analyzed. However, the medium supplemented with the two growth factors provided a statistically significant improvement, which was higher at day 6, comparing to immobilized ECs cultured only in medium with VEGF ($p<0.05$ vs VEGF alone).

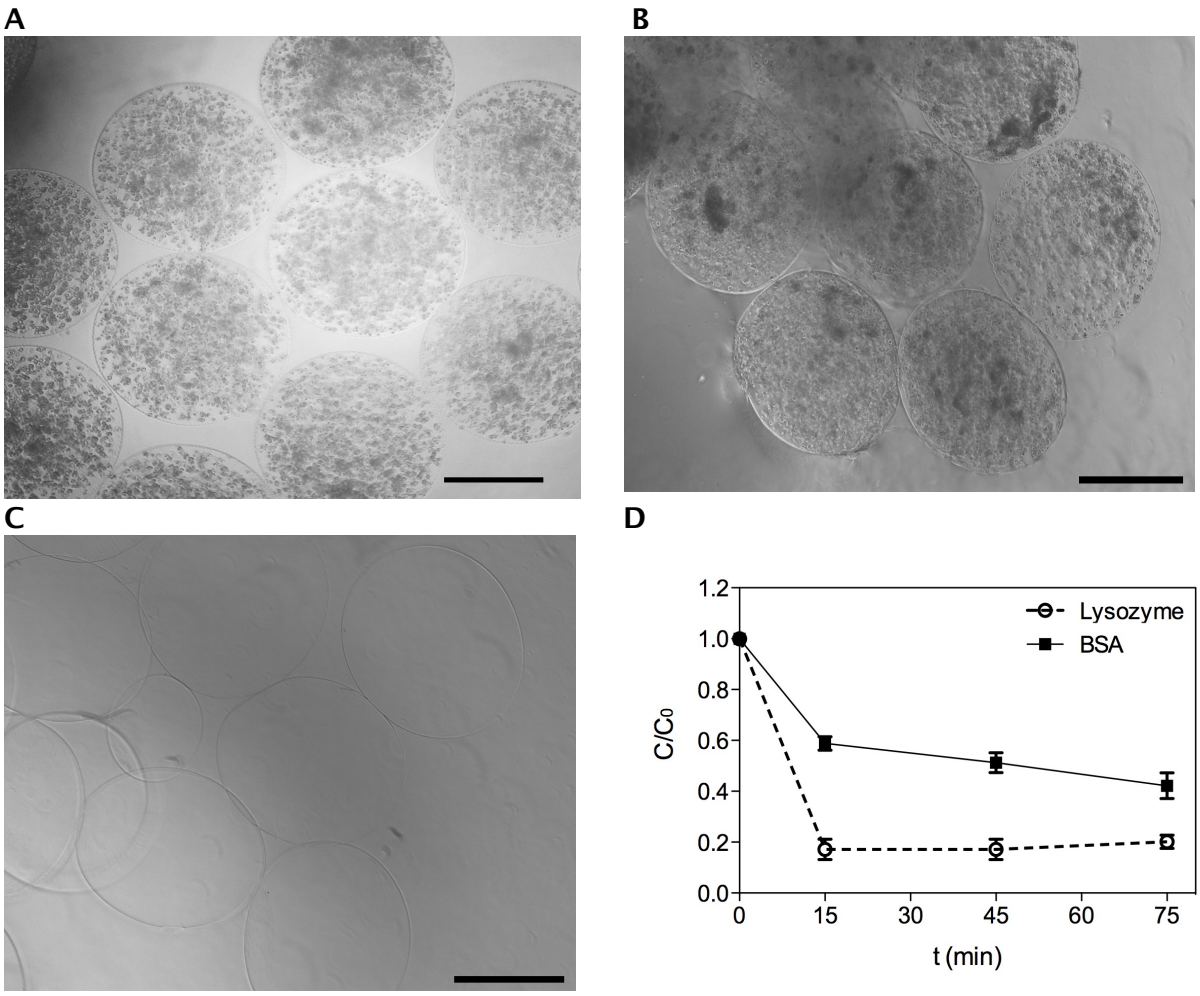

Figure 1. ECs and FBs immobilized in RGD-alginate microspheres. Photomicrographs of RGD-grafted alginate microspheres at day 4, illustrating the spherical and uniform shape of: (A) immobilized ECs; (B**)** immobilized FBs; and (C) without cells (control). Bar=500μm. (D) Quantification of albumin and lysozyme diffusion from the TBS solution into the RGD-alginate microspheres. Results are expressed as means±SD of at least three independent experiments.

The effect of cell apoptotic and necrotic activities was also assessed by flow cytometry analysis. Therefore, cells were labeled for annexin V FITC (apoptosis marker) and propidium iodide (PI, a necrosis marker). Cells that, during this period of time, were negative for both annexin V and PI corresponded to the population of viable cells. Apoptotic cells were positive for annexin V and negative for PI, while necrotic cells were positive for both annexin V and PI. Flow cytometry analysis of ECs immobilized in alginate microspheres and cultured, during 6 days, in medium supplemented with VEGF alone revealed only 12% of viability (Fig. 2C). Their viability considerably increased to 85%, in the same

conditions, when the medium was supplemented with VEGF in combination with bFGF (Fig. 2D).

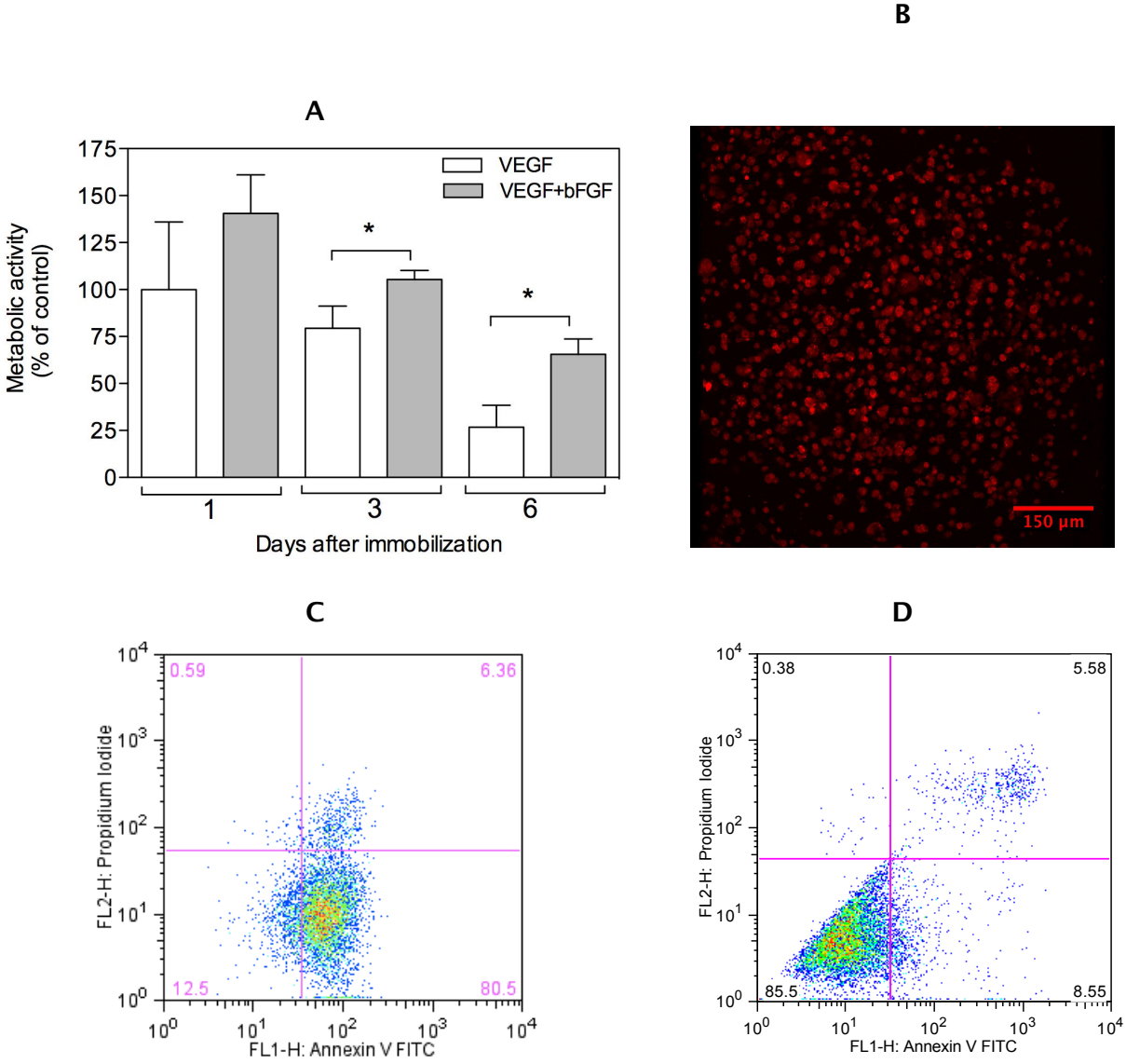

Figure 2. (A) Metabolic activity of ECs after immobilization in RGD-grafted alginate during 6 days, as assessed by the Resazurin assay. Immobilized ECs were cultured in the presence of 5 ng/mL of VEGF or 5 ng/mL of VEGF combined with 5 ng/mL bFGF. An increase in metabolic activity was observed, during 6 days in culture, upon combination of VEGF with bFGF. Bars correspond to the mean ± SD, in comparison to control (immobilized ECs at day 0), n=3, *p<0.05. (B) Dil-labeled low density lipoprotein (Dil-Ac-LDL) uptake by ECs, in medium supplemented with VEGF, at day 3, visualized using a confocal laser scanning microscope (bar=150 µm). (C) Flow cytometry data, at day 6, of ECs immobilized in RGD-grafted alginate and cultured in the presence of 5 ng/mL of VEGF. (D) Flow cytometry data, at day 6, of ECs immobilized in RGD-grafted alginate and cultured in the presence of 5 ng/mL of VEGF and 5 ng/mL of bFGF. The results were express in terms of percentage of cells expressing these markers.

When FBs were immobilized within RGD-alginate microspheres and cultured during 14 days their metabolic activity was also evaluated by the resazurin assay and the viability and apoptosis by flow cytometry analysis. Contrary to ECs, most of the immobilized FBs are autosufficient to maintain their metabolic activity during the whole experiment (Fig. 3A). Fig. 3B illustrates that immobilized fibroblasts present still approximately 69% viability at day 14 of culture.

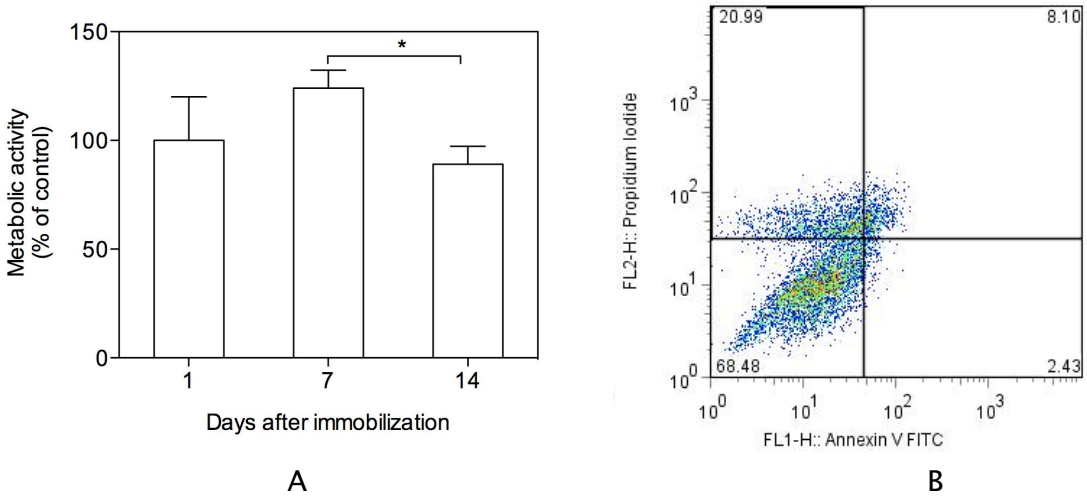

Figure 3. (A) Metabolic activity of fibroblasts after immobilization in RGD-grafted, as assessed by the Resazurin assay. These cells maintained their metabolic activity during 14 days of culture. Bars correspond to the mean ± SD, in comparison to control (immobilized fibroblasts at day 0), n=3, *p<0.05 (B) Flow cytometry data of fibroblasts immobilized and cultured in RGD-grafted alginate during 14 days. The cells were labeled for annexin V FITC and propidium iodide. The results were expressed in terms of percentage of cells expressing these markers.

3.3. Metabolic activity of cells using conditioned media

In order to examine whether ECs or FBs, in 2D culture, affect each others metabolic activity in a paracrine manner, conditioned medium of each cell culture was obtained and used in the other cell culture. This effect was compared with control ECs or FBs cultured in fresh medium. In comparison to the control, conditioned medium from FBs (CMFBs) increased ($p<0.05$ vs control) the metabolic activity of ECs (Fig. 4). An increase in metabolic activity can also be observed in Fig. 4, in which FBs were cultured in the presence of conditioned medium from ECs (CMECs) ($p<0.05$ vs. control).

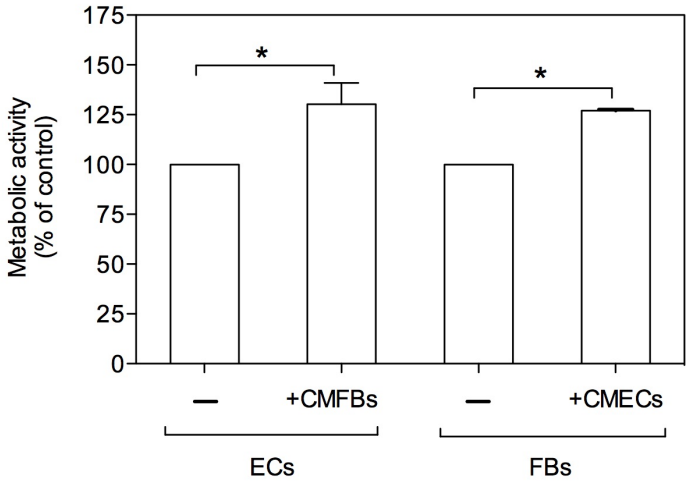

Figure 4. Metabolic activity of ECs or FBs in conditioned media. ECs were incubated during 24h in conditioned medium from fibroblasts (CMFBs) or in freshly prepared media (-, control). FBs were incubated during 24h in conditioned medium from EC (CMECs) or in freshly prepared media (-, control). Results are expressed as percentage of metabolic activity of cells in control media. Bars represent mean ± SD (n=3, *p<0.05 vs control).

3.4. Capillary-like structures formation in co-cultures in indirect contact

A transwell culture system was used to examine if FBs can influence the formation and organization of capillary-like-structures in indirect contact with ECs. For this purpose, FBs were cultured on the lower chamber of 24-well plates, and ECs were placed on a transwell insert coated with 50 µL of growth factor reduced (GFR)-Matrigel® (Fig. 5A). ECs co-cultured with FBs in indirect contact experienced a slight decrease in metabolic activity when compared to ECs in GFR-Matrigel® alone (Fig. 5B). However, the number of capillary-like-structures formed by ECs in GFR-Matrigel® when in co-culture with FBs was significantly higher than by ECs alone (Fig. 5C).

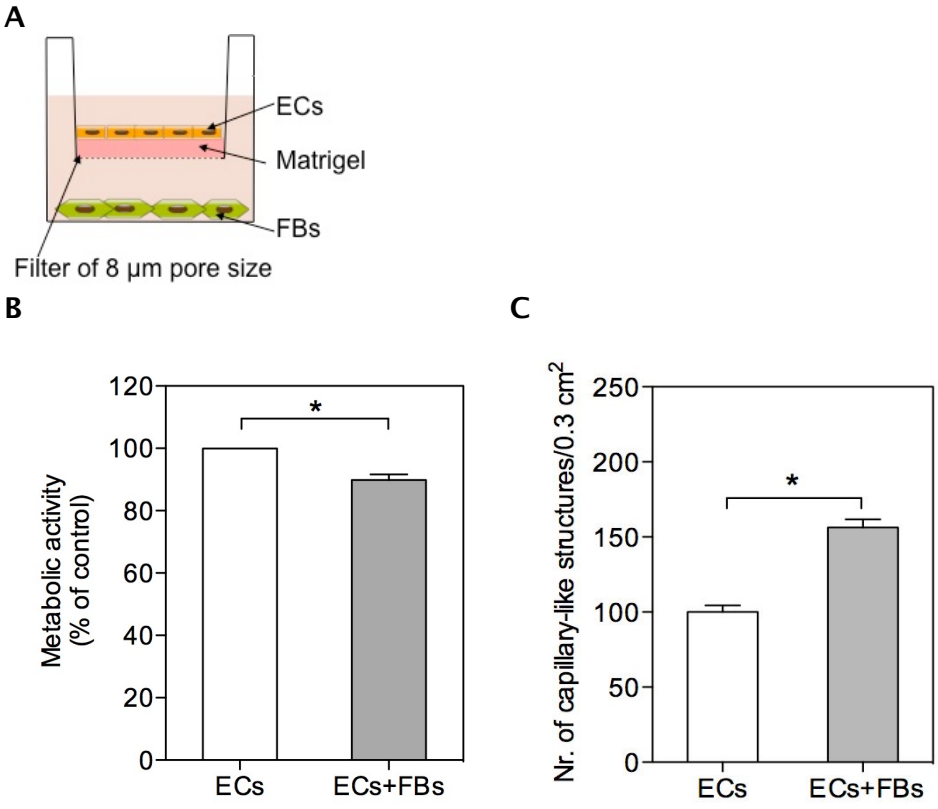

Figure 5. Co-cultures of ECs with FBs in transwells. (A) The FBs were cultured on the bottom and ECs were cultured on top of Matrigel-coated transwell inserts. (B) Metabolic activity of ECs cultured alone or in the presence of FBs (ECs+FBs, co-culture) during 24h; (C) Semi-quantification of the number of capillary-like structures formed during 24h. Bars correspond to the mean ± SD (n=3, *p<0.05).

3.5. Capillary-like structures formation by influence of immobilized FBs

The GFR-Matrigel® assay was used to assess if the ability of ECs to form capillary-like structures would be influenced by the presence of FBs. In Fig. 6A, the formation of capillary-like structures can be observed with ECs attaching and spreading to form typical polygonal forms after 24h. After 5 days of culture in GFR-Matrigel® the ability of ECs to form capillary-like-structures was not maintained (control ECs). On the contrary, the presence of fibroblasts immobilized within RGD-alginate (iFBs) maintained the assembly of ECs into capillary-like structures even after 5 days of culture (Fig. 6A). In the presence of iFBs a significant increase in the number of capillary-like structures formed could be observed, both at days 1 and 5, compared to the control (Fig. 6B).

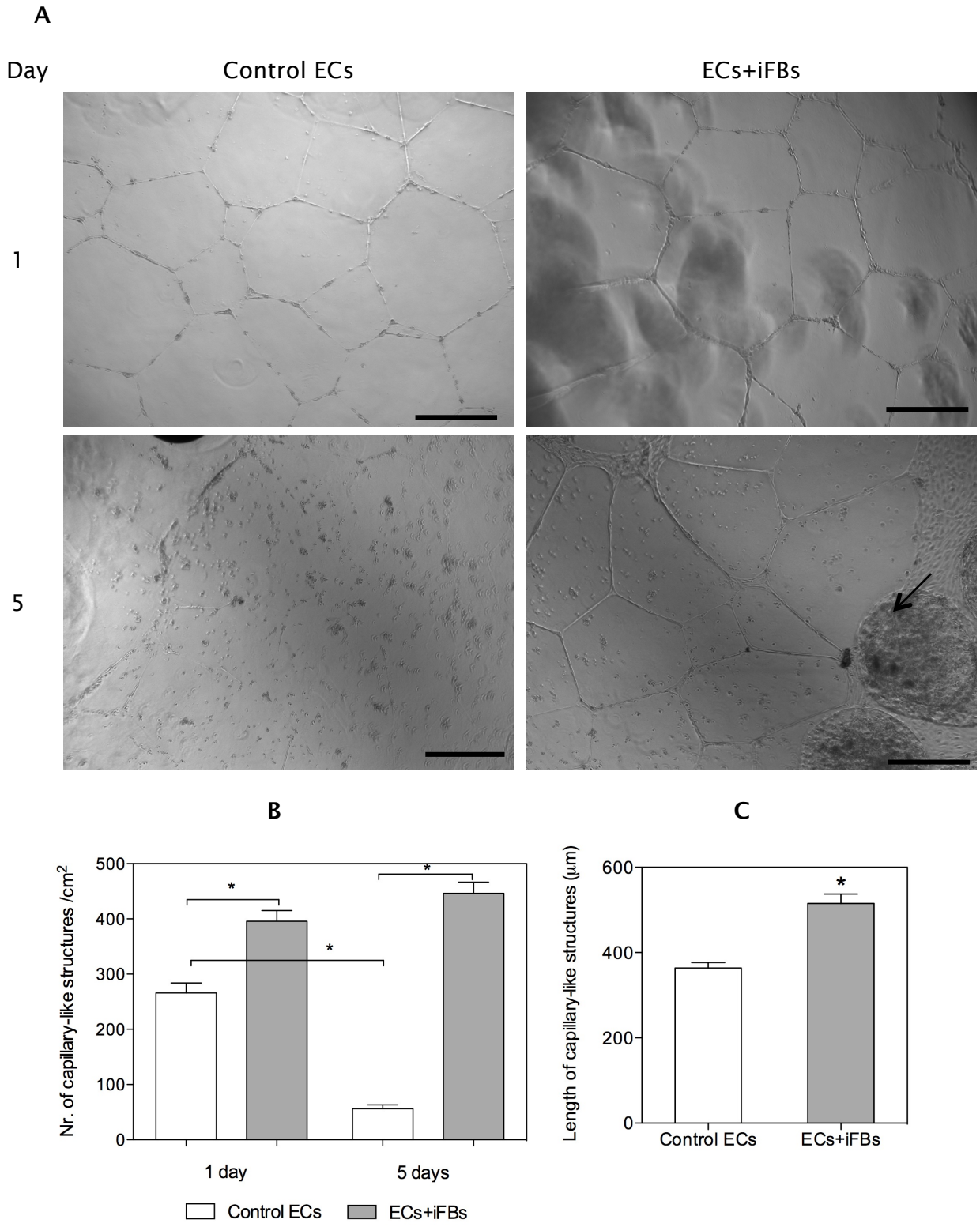

Figure. 6. Effect of fibroblasts immobilized within RGD-alginate on formation of capillary-like structures by neighboring ECs. (A) After 1 day of culture, ECs formed capillary-like structures and after 5 days of culture only ECs on matrigel in the presence of immobilized fibroblasts (ECS+iFBs, arrow) maintained the capillary-like structures formation (Bar=500μm). (B) Quantification of capillary-like structures formation. (C) Quantification of the length of capillary-like structures formed after one day. Results are means±SD of independent experiments (n=3, *p<0.05).

Furthermore, after 5 days a significant decrease in the number of capillary-like structures in the control condition could be quantified, compared to day 1. However, in the presence of iFBs, ECs maintained the capillary network even after 5 days of culture. The length of capillary-like structures formed was also quantified at day 1. When ECs were cultured in the presence of immobilized FBs, a significant increase in capillary-like structures length was observed ($p<0.05$), in comparison to the control (Fig. 6C). Taken together, these data suggest that immobilized fibroblasts support and preserve the capillary network.

3.6. Ex-vivo sprouting by influence of immobilized FBs

To further examine the effect of FBs in the angiogenic process, and considering that, *in vivo*, it also involves other cell types, such as smooth muscle cells, an approach with increased complexity was used, namely the aortic ring sprouting assay. Aortic rings were cultured in GFR-Matrigel® in the presence or absence of FBs immobilized in RGD-alginate (Fig. 7). The distance between the microspheres and the aortic ring was of approximately 6 mm (Fig. 7C). Sprouting of vessels was observed in both cases. However, in the absence of FBs (control), the sprouting scattered throughout the whole aortic ring (Fig. 7A). In the presence of immobilized FBs, sprouting of new capillaries was preferentially oriented towards the microspheres containing immobilized FBs (Fig. 7B). The overall area of sprouting was also quantified using image J, showing a decrease when FBs were present and compared to the control (Fig. 7D).

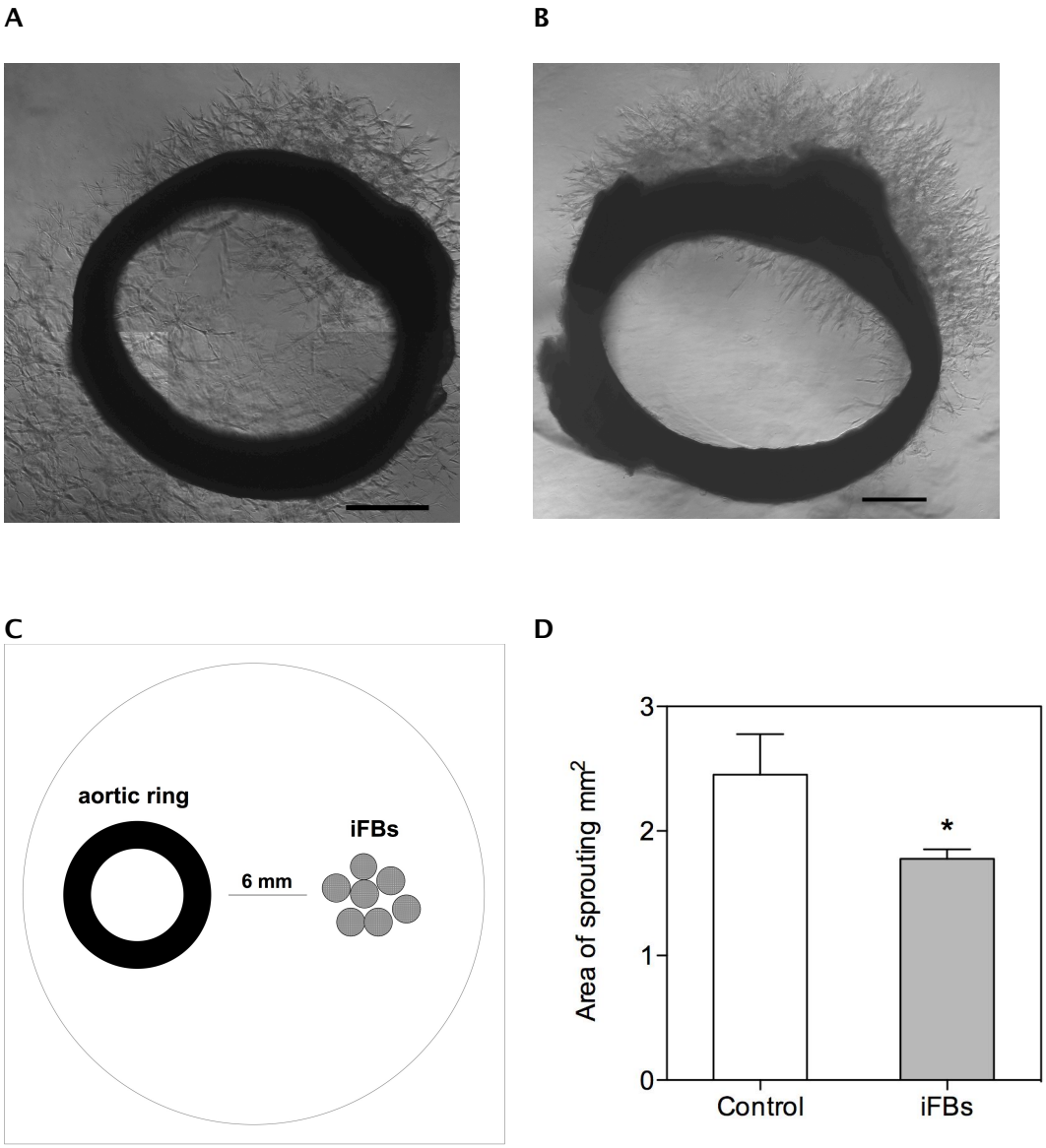

Figure 7. Effect of fibroblasts immobilized within RGD-alginate in capillary-like structures sprouting. Representative images of aortic ring at day 4 in the presence of microspheres of RGD-alginate: (A) without fibroblasts (control) and (B) with fibroblasts. (C) Scheme of well with aortic ring and RGD-alginate microspheres. (D) Quantification of the area of sprouting. Results are means±SD (n=3, *p<0.05). Bar=500μm.

4. Discussion

In tissue regeneration research, the use of models that mimic physiological conditions to test new strategies is of paramount importance since it depends on a number of intricate interactions between distinct cell types, growth factors and ECM components. One example is the formation of vessels, which is only possible in 3D assays of angiogenesis, a fundamental but yet unresolved problem in tissue regeneration. Interactions between mesenchymal and epithelial cells are known to play an important role in orchestrating the development and morphogenesis of tissues and organs (8). Bone cells of mesenchymal origin have been shown to exert a reciprocal influence on endothelial cells (4,18,21,25,45,46). Therefore, in the context of angiogenesis, it seems relevant to study how fibroblasts (FBs), which are committed mesenchymal cells, could influence the microenvironment of endothelial cells to trigger their assembly into capillary-like structures, and thus contributing to the regenerative process by promoting angiogenesis. Beyond the roles usually attributed to fibroblasts, namely contribution to pathological tumor development or fibrosis (26,29), these cells can also be advantageous in therapeutic applications since they are highly abundant, scattered throughout the organism and can be easily isolated in a noninvasive and patient-specific manner (40).

The present study investigated the modulation of endothelial cells (ECs) behaviour by fibroblasts (FBs) in terms of viability, metabolic activity and ability to form capillary-like structures in 3D models. In our study, the effect of supplementing the medium of ECs with a combination of VEGF and bFGF showed a synergistic effect of the two growth factors in improving metabolic activity of ECs immobilized in RGD-alginate microspheres. VEGF alone did not promote the same effect. FBs secrete a number of growth factors, such us bFGF and VEGF (30,36) and Presta et al. presented evidence suggesting the possibility that bFGF indirectly induces neovascularization by activation of the VEGF/VEGFR system (35). Our results are in agreement with Korff et al., who showed additive effects of VEGF and bFGF in decreasing apoptosis in spheroid endothelial cell clusters (28). The VEGF family has been described as assuming a pivotal role during embryonic development in vasculogenesis and angiogenesis (15). Soluble factors produced by FBs, such us VEGF and bFGF, are required for ECs survival, differentiation, and network formation (30), although their function decreases

during time in culture (9). Therefore, the transplantation of co-cultures is hereby appointed as a better strategy rather than growth factor delivery.

The use of co-cultures in tissue engineering is related with the fact that both cell types may support survival and growth of each other (11,21). In the present work, conditioned media from ECs and FBs demonstrated that both cell types produced soluble factors capable of influencing the metabolic activity of the other cell type, indicating the reciprocal inter-relationship between these two types of cells. Nevertheless, when ECs were cultured in GFR-Matrigel® and in the same chamber with FBs but in indirect contact, the metabolic activity of ECs did not change significantly, which can be explained by the fact that ECs were at different stages of development, namely proliferation or differentiation, thus exhibiting different levels of metabolic activity.

Proliferation or differentiation of ECs can be influenced by the 3D environment, presence of growth factors or presence of other cell types, such as FBs. According to Kunz et al. (30), in spheroid co-cultures of ECs and FBs low viability is not found in areas where ECs can migrate and form capillary-like-structures in the spheroids. Korff and Augusting (28) investigated a similar model, showing that cells at the surface monolayer of spheroids differentiated to a degree that rendered them survival factor-independent, although dependence on survival factors was observed concerning the unorganized cells in the spheroid center (28).

Having this in mind, co-cultures of ECs and FBs were used to study the influence of FBs immobilized within RGD-alginate in ECs viability and organization. Previous work from the present team showed that other cell types, namely preosteoblasts (MC3T3) (14) and human osteoprogenitor cells (5,21), can be stably immobilized in RGD-alginate, and maintained viable during long periods of time (29 days). These findings were confirmed in the present study, since fibroblasts immobilized in RGD-alginate microspheres during 14 days kept their high viability. The presence of the RGD sequence promoted cell adhesion to alginate and probably mediated survival signals that involve $\alpha_V\beta_3$ integrin signaling pathways (2,14,21,34). Integrins are known to play a central role in neovascularization, being $\alpha_V\beta_3$ integrin even considered a target for the development of anti-angiogenic therapies in cancer (35). In EC maturation and vessel wall assembly, ECM synthesis is a critical step. Previously, the production of collagen by MC3T3 cells, fundamental in the differentiation step, has been reported as significantly higher within RGD-alginate microspheres than in

unmodified alginate ones (14). Singh et al. found that FBs immobilized in alginate could produce collagen type I and, under adequate conditions, namely combination of hypoxia, BMP-2 supplementation, and long-term intermittent application of dynamic hydrostatic pressure, could be differentiated into the chondrogenic pathway (40). Additionally, Levenberg et al. suggested a close relationship between neosynthesis of ECM and increasing levels of VEGF secreted by FBs in the formation and maintenance of microvasculature (31).

In the present work the GFR-Matrigel® assay demonstrated that FBs immobilized within RGD-alginate were capable of influencing the formation of capillary-like structures by neighboring ECs, which is in agreement with our previous studies demonstrating similar results when using human mesenchymal stem cells (5). The length of capillary-like structures formed in the presence of immobilized FBs was higher than when ECs were cultured alone. Nevertheless, after 5 days of culture only the network formed in the presence of immobilized FBs was maintained. The preservation of the capillary like-structures with time is a fundamental issue in tissue regeneration. These results suggest that growth factors produced by FBs could act in a paracrine manner to induce and maintain capillary-like structures. Also, the ability of FBs to contribute to ECs survival and vessel assembly is probably closely related to their capacity to produce new ECM, thus creating a more adequate environment for capillary structure formation and maintenance. Grajar et al. reported that FBs stimulate ECs to form mature capillary networks and that this FBs-mediated capillary morphogenesis is dependent from matrix metalloproteinase production (20).

Given the complexity of the angiogenesis process, namely the existence of other structures and cells types besides ECs and FBs, such as pericytes and smooth muscle cells, the aortic ring assay was used to further assess the influence of immobilized FBs in the formation of capillary-like structures. The results suggest that the presence of immobilized FBs induced significant changes in the formation of capillary-like structures by ECs, namely a decrease in the overall area of sprouting of ECs when immobilized FBs were present, although, in the presence of FBs, the new capillaries formed showed a preferential orientation towards the microspheres containing FBs. Probably, a gradient of growth factors (e.g. bFGF) was produced by the immobilized FBs, directing sprouting. Soluble factors, like bFGF and other established angiogenic factors, can promote endothelial cell scattering during the first steps of the angiogenesis process and, along with the formation of cell-cell interactions, are required phenomena for

vessel maturation (35,44). For instance, bFGF has been reported to initially promote the disruption of the basal lamina by inducing protease production of various ECM components by ECs (19), thus contributing to the maturation of the vessels (35). The present study was able to show that FBs immobilized in RGD-alginate improved the heterotypic interactions within the microenvironment, resulting in a direct mechanism required for capillary formation.

5. Conclusions

The current study used different angiogenesis assays to test the influence of fibroblasts in the vascularization process. This study demonstrated the ability of FBs immobilized in RGD-alginate to modulate and support capillary-like structures assembly by endothelial cells. Taken together, these findings indicate that the microenvironment created by these stromal cells in the scaffold modulates capillary morphogenesis, thus stimulating angiogenesis i*n situ* and can potentially be used in tissue regeneration strategies, in clinical scenarios where vascularization is essential.

Acknowledgements

The work carried out at INEB was financed by FEDER funds through the *Programa Operacional Factores de Competitividade* – COMPETE and by Portuguese funds through FCT – *Fundação para a Ciência e a Tecnologia* in the framework of projects PEST-C/SAU/LA0002/2011, PEST-OE/SAU/UI0038/2011 and POCI/SAU-BMA/55556/2004 and the PhD scholarship SFRH/BD/40421/2007 for S.G.G.. The authors would like to express their gratitude to Raquel Gonçalves and Ana Rocha for the contribution in the FACS analyses and in the aortic ring assay, respectively.

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CHAPTER III

Crosstalk between fibroblasts and endothelial cells promotes angiogenesis *in vitro*: putative role of alkaline phosphatase, growth factors and collagen.

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*Submitted (2011)

Abstract

Interactions between the different cell-types, growth factors and extracellular matrix components involved in angiogenesis are crucial in the mechanisms of new vessel formation for tissue regeneration. The aim of the present study was to investigate if cocultured fibroblasts and endothelial cells (from macro- or microvasculature) could modulate growth factor production (VEGF, bFGF, TGF-β1 and IL-8), influence the formation of microcapillary-like structures by endothelial cells and affect fibroblast differentiation. Results obtained show that the two cells types interact with each other through exchange of growth factors (e.g., fibroblasts produce VEGF, which is known to influence angiogenesis). Fibroblasts promoted the formation and organization of capillary-like structures by endothelial cells, increased the amount of collagen in the cocultures and determined the expression of alkaline phosphatase. High alkaline phosphatase expression could be co-localized with capillary-like structures and the interaction between the two cells types induced fibroblast activation near microvessel-like structures.

Keywords: Alkaline phosphatase, angiogenesis, collagen, endothelial cells, fibroblasts, growth factors, regenerative medicine, tissue engineering.

1. Introduction

The formation of new blood vessels to support tissue growth and/or homeostasis in cancer or after injury and trauma is a critical issue in new therapeutic approaches in regenerative medicine. From the parallelism in the first steps of tissue regeneration, cancer and embryogenesis it is clear that vascularization is needed for tissue growth and development [1-3], as this allows the exchange of nutrients, gases and growth factors between blood and tissue. However, in tissue regeneration the establishment of a functional vascular system is a milestone yet to be achieved [4]. Stromal cells are of paramount importance in tissue regeneration in the adult, but their role in vascularization is still poorly understood [4-6].

Stromal cells, like fibroblasts, secrete growth factors, such as vascular endothelial cell growth factor (VEGF) [7, 8], basic fibroblast growth factor (bFGF) [9], transforming growth factor beta (TGF-β) [10] and interleukin 8 (IL-8) [11], that may act in a paracrine manner to modulate the healing process. Fibroblasts produce collagen, thereby creating a new matrix to fill a defect in the injured site [10] and to allow migration and sprouting of endothelial cells (ECs) to vascularize the newly formed tissue. The extracellular matrix (ECM) is a key factor for successful angiogenesis [12, 13]. Collagens form one of the major protein families in the ECM and can drive ECs migration by both chemotaxis and haptotaxis [13]. Recent findings in tissue engineering suggest that fibroblasts have important roles in orchestrating the angiogenic process [14-16] and in regulating the associated immune responses [17, 18]. Fibroblasts are connective tissue cells and have been shown to exhibit plasticity by differentiating into other members of the connective tissue family, including cartilage, bone, adipose tissue and smooth muscle cells [17]. So far, it is not clear if fibroblasts present within the connective tissue, besides maintaining the ECM and connective tissue homeostasis, also act as tissue-resident progenitors cells [19, 20].

The communication between different cell types also plays a paramount role in the regeneration process. Coculture systems constitute a promising instrument to mimic physiological processes in tissue regeneration and vascularization and may provide new insights into the underlying molecular mechanisms [21]. Cocultures of human mesenchymal stem cells/ECs [21-23], osteoblasts/ECs [12,

24], fibroblasts/ECs [4, 25], adipocytes/ECs [26, 27], smooth muscle cells/ECs [28], pericytes/ECs [29], fibroblasts/macrophages [5] and fibroblasts/ osteoblasts [30] show that the behavior of each cell type in terms of proliferation, migration and differentiation can be modulated in the presence of another cell type. It appears that fibroblasts are not just passive players in the angiogenesis process, and thus their coculture with ECs may further elucidate the contribution of fibroblasts to this important phenomenon.

The present study was aimed at investigating if fibroblasts in coculture with ECs, in indirect and direct contact, could modulate the vascularization process. ECs from macro- and microvasculature were used. The present findings show that fibroblasts influence the formation and organization of capillary-like structures by ECs, through the exchange of relevant growth factors, production of collagen and expression of alkaline phosphatase. Areas with co-localization of high expression of ALP and the presence of capillary-like structures are here described for the first time.

2. Materials and Methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from donors according to a previously described protocol [31] and cultured in M199 medium (Sigma-Aldrich, Germany), supplemented with 20% fetal calf serum (FCS, Invitrogen, Germany), 2 mM Glumatax I (Sigma-Aldrich, Germany), 25 μg/mL sodium heparin (Sigma-Aldrich, Germany) and 25 μg/mL endothelial growth factor supplement (ECGS; Becton Dickinson, USA), penicillin G (100 U/mL, Invitrogen, Germany) and streptomycin (100 μg/mL, Invitrogen, Germany). Human dermal microvascular endothelial cells (HDMECs) were isolated from juvenile foreskin and cultured in endothelial cell basal medium MV (PromoCell, Germany) supplemented with 15% FCS (Invitrogen, Germany), basic fibroblast growth factor (bFGF, 2.5 ng/mL, Sigma-Aldrich, Germany), sodium heparin (10 μg/mL, Sigma-Aldrich, Germany), penicillin G (100 U/mL, Invitrogen, Germany) and streptomycin (100 μg/mL, Invitrogen, Germany) [32]. HUVEC and HDMEC were cultured until confluence in culture flasks coated with 0.2% gelatin (Sigma-Aldrich, Germany), in a humidified atmosphere at 37ºC (5% CO_2), and used at passage 4.

Human dermal fibroblasts (HDFs) were isolated from juvenile foreskin and cultured in Dulbecco's modified eagle's medium (DMEM) with 45000 mg/L glucose (Sigma-Aldrich, Germany), 10% FCS (Invitrogen, Germany), penicillin G (100 U/mL, Invitrogen, Germany), streptomycin (100 μg/mL, Invitrogen, Germany) and 2 mM Glutamax I (Life Technologies, Germany). Cells were cultivated in a humidified atmosphere at 37ºC (5% CO_2) and used at passage 4.

2.2. Cocultures of endothelial cells and fibroblasts

Cocultures of HUVECs/HDFs and HDMECs/HDFs were prepared in endothelial cell basal medium MV (PromoCell, Germany) supplemented with 15% FCS (Invitrogen, Germany), bFGF (2.5 ng/mL, Sigma-Aldrich, Germany), sodium heparin (10 μg/mL, Sigma-Aldrich, Germany), penicillin G (100 U/mL, Invitrogen, Germany) and streptomycin (100 μg/mL, Invitrogen, Germany). Monocultures of HUVECs, HDMECs and HDFs were cultured in the same medium as a control for the different time periods studied.

2.2.1. Cocultures in indirect contact

To determine the effects of indirect contact, cocultures of HUVECs or HDMECs with HDFs and monocultures of HUVECs, HDMECs and HDFs were cultured in 24-well plate transwell with a pore size of 0.4 μm and with one individual cell-type seeded on each side of the membrane (HTS Transwell®, Corning, Germany). Firstly, HDFs ($5x10^3$ cells) were cultured on the bottom of the membrane. After 2h, the plate was the turned and 100 μL of collagen type I (0.03 mg/mL, BD Biosciences, Germany) were added to the top of the transwell. After 1h at 37ºC HUVECs or HMDECs ($15x10^3$ cells) were added to the top of the coated membrane. Finally, membranes were inverted and endothelial cell basal medium MV (PromoCell, Germany) supplemented with 15% FCS (Invitrogen, Germany), bFGF (2.5 ng/mL, Sigma-Aldrich, Germany), sodium heparin (10 μg/mL, Sigma-Aldrich, Germany), penicillin G (100 U/mL, Invitrogen, Germany) and streptomycin (100 μg/mL, Invitrogen, Germany) was added to the top and to the bottom chambers. After various incubation times the medium was collected and stored at -20ºC until further use.

2.2.2. Cocultures in direct contact

Cocultures of HUVECs or HDMECs with HDFs and monocultures of HUVECs, HDMECs and HDFs were cultured in 24 well-culture plates coated with 0.2% gelatin (Sigma, Germany). ECs and HDFs were seeded at the same time to the culture plate and cultured using the ratio of $2x10^4$ ECs:$1x10^4$ HDFs. For immunoflurescent staining, cocultures and monocultures were seeded onto Thermanox coverslips (13 mm, Nunc, Germany) and cultured in 24-well culture plates. After different time periods the medium was collected and kept at -20ºC until further analysis.

2.3. Protein concentration by enzyme-linked immunosorbent assay (ELISA)

As previously mentioned, culture supernatants collected from HUVECs, HDMECs and HDFs monocultures, as well as from HUVECs/HDFs and HDMECs/HDFs cocultures, were collected and stored at -20ºC. For cocultures in transwells (indirect coculture) the time points were 1 and 7 days. For cocultures in direct

contact the time points were 7, 14 and 21 days. The concentration of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8) and transforming growth factor-beta 1 (TGF-β1) was determined in triplicate using ELISA DuoSets® (R&D Systems), following the manufacturer's instructions [21]. The optical density of each well was measured using a microplate reader (GENios plus, TECAN, Germany) at 450 nm.

2.4. Immunofluorescent analysis of endothelial and fibroblastic markers

Cocultures seeded onto Thermanox coverslips (13 mm, Nunc, Germany) were prepared for immunoflorescent staining of endothelial markers PECAM-1 (CD31) and von Willebrand factor (vWF), of fibroblastic marker vimentin and of the activated fibroblast marker, α-smooth muscle actin (α-SMA). After fixation with 3.7% paraformaldehyde (Merck, Germany), cells were washed with PBS and permeabilized using 0.2% Triton-X 100/PBS for 10 min. After washing 4 times with PBS, cells were incubated for 45 min at room temperature with primary antibodies: mouse anti-human CD31 (1:50, Dako, Germany), rabbit anti-human vWF (1:8000, Dako, Germany), mouse anti-human α-SMA (1:100, Dako, Hamburg, Germany) and mouse anti-human vimentin (1:200, Progen, Germany) diluted in 1% bovine serum albumin/PBS. After washing 3 times with PBS, cells were incubated for 45 min at room temperature with the secondary antibodies: anti-mouse Alexa 488 (Molecular Probes, Germany) for CD31, vimentin and α-SMA staining and anti-rabbit Alexa 546 (Molecular Probes, Germany) for vWF, diluted 1:1000 in 1% bovine serum albumin/PBS. This incubation was followed by a brief rinse in PBS. Finally, cell nuclei were counterstaining with 1 μg/mL DAPI (Molecular Probes, Germany) in PBS for 5 min. After washing with PBS samples were mounted with Gel/Mount (Natutec, Germany) and examined by confocal laser scanning microscope (CLSM, LeicaTCS-NT, Leica Microsystems, Germany).

2.5. Alkaline phosphatase (ALP) staining

ALP staining was performed using a diagnostic phosphatase kit (Sigma-Aldrich, Germany) as described by the manufacturer. Briefly, the adherent cell cultures were washed with PBS, fixed with citrate-acetone-formaldehyde fixative solution for 30 sec, then stained with an alkaline-dye mixture at room temperature for 15 min. Naphthol AS-MS phosphate and Fast Red Violet B were used as substrate

and for enzyme activity detection, respectively. ALP staining was examined by CLSM at λ_{Ex} =633 nm.

2.6. Extracellular matrix (ECM) formation by collagen assay

The total collagen content of cell cultures was determined using the Sircol™ Collagen Assay Kit (Biocolor, UK), to assess ECM formation, according to the manufacturer's instructions. Sircol™ soluble collagen assay is a dye-binding method for the analysis of acid and pepsin-soluble collagens. The reagent binds to hydroxyproline, although it does not discriminate between collagen types I and V present in the ECM.

Briefly, the culture medium was removed and each well was washed with PBS. 500 µL of 0.1 mg/mL of pepsin solution in 0.5 M acetic acid (Sigma-Aldrich, Germany) was added to each well. The samples were incubated at 4ºC overnight. Then, 50 µL of acid neutralising reagent (Biocolor, UK), 100 µL of cold isolation and concentration reagent (Biocolor, UK) were added. The tube contents were mixed by tube inversion and incubated overnight at 4ºC. Afterwards, the tubes were centrifuged for 10 min at 12 000 rpm. Pellets were re-dissolved in 0.5 M acetic acid and 1 mL of Sircol™ dye reagent, which specifically binds to collagen. The reagents were mixed for 30 min. After centrifugation, pellets were suspended in 250 mL of the alkali compound included in the kit and optical density was evaluated using a microplate reader (GENios plus, TECAN, Germany) at the 540 nm. Each value of test samples was compared to the values obtained with collagen standard solutions provided by the manufacturer and used to construct a standard curve.

2.7. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism version 5.0 and statistical significances were evaluated using the Student's *t*-test. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $P<0.05$.

3. Results

3.1. Levels of VEGF, bFGF, IL-8 and TGF-ß1 in cocultures by indirect contact

The amounts of VEGF, bFGF, IL-8 and TGF-ß1 were determined in the supernatant of cocultures of ECs and fibroblasts in indirect contact, ECs and fibroblasts growing in close proximity since they were separated by a transwell coated with collagen, as well as in the controls (monocultures of HUVECs, HDMECs and HDFs) in one side of the transwell membrane. In monocultures of HUVECs and HDMECs the presence of VEGF could not be detected (Fig. 1). On the other hand, in HDF monocultures VEGF was detected and significantly increased from day 1 to 7. No VEGF could be detected in the supernatants collected from cocultures of HUVECs or HDMECs with HDFs.

The growth factor bFGF was included in the culture media, since it is necessary for ECs culture viability [24]. The presence of bFGF in HUVEC and HDMEC monocultures was detected both on day 1 and day 7 (Fig. 1). In monocultures of HDMECs a significant decrease in bFGF levels was observed on day 7 compared to day 1 (Fig. 1), that also being observed in monocultures of HDFs as well as in its cocultures with HUVECs or HDMECs. Thus, in cocultures, no production of bFGF occurred.

On the other hand, in all conditions tested with ECs a significant increase in IL-8 production on day 7 compared to day 1 was observed. In HDF monocultures no IL-8 was detected (Fig. 1).

The amount of TGF-ß1 present in the media increased after 7 days under all conditions tested, compared to day 1 (Fig. 1).

3.2. Expression of endothelial and fibroblastic markers in cocultures in direct contact

HUVECs and HDMECs in direct contact cocultures with HDFs resulted in different cell behaviour. HDMECs formed more colonies (open arrowheads) of cells compared to HUVECs (Figs. 2B and A, respectively) after 3 days. The HDFs, identified by nuclear staining (blue), showed some positive staining for α-SMA (Figs. 2A and B). In Fig. 2C, HDFs were stained positively for vimentin (green) surrounding a colony of HDMECs stained positively for vWF, although ECs also show some green fluorescence, as vimentin marks both fibroblastic and

endothelial phenotypes After 21 days in coculture with HDFs, HUVECs and HDMECs formed a well-defined network of microcapillary-like structures (Figs. 2D and E respectively), while in monoculture neither HUVECs nor HDMECs formed similar structures. All cell types in these cocultures were stained for nuclei (both HDFs and ECs, blue) and ECs for CD31 (endothelial cell-specific marker, green), allowing the observation of strings of microcapillary-like structures formed by HUVECs or HDMECs (Figs. 2D and E, respectively; stained green for CD31, arrows) amongst HDFs (blue nuclei and no CD31 staining, Figs. 2D and E, open arrows).

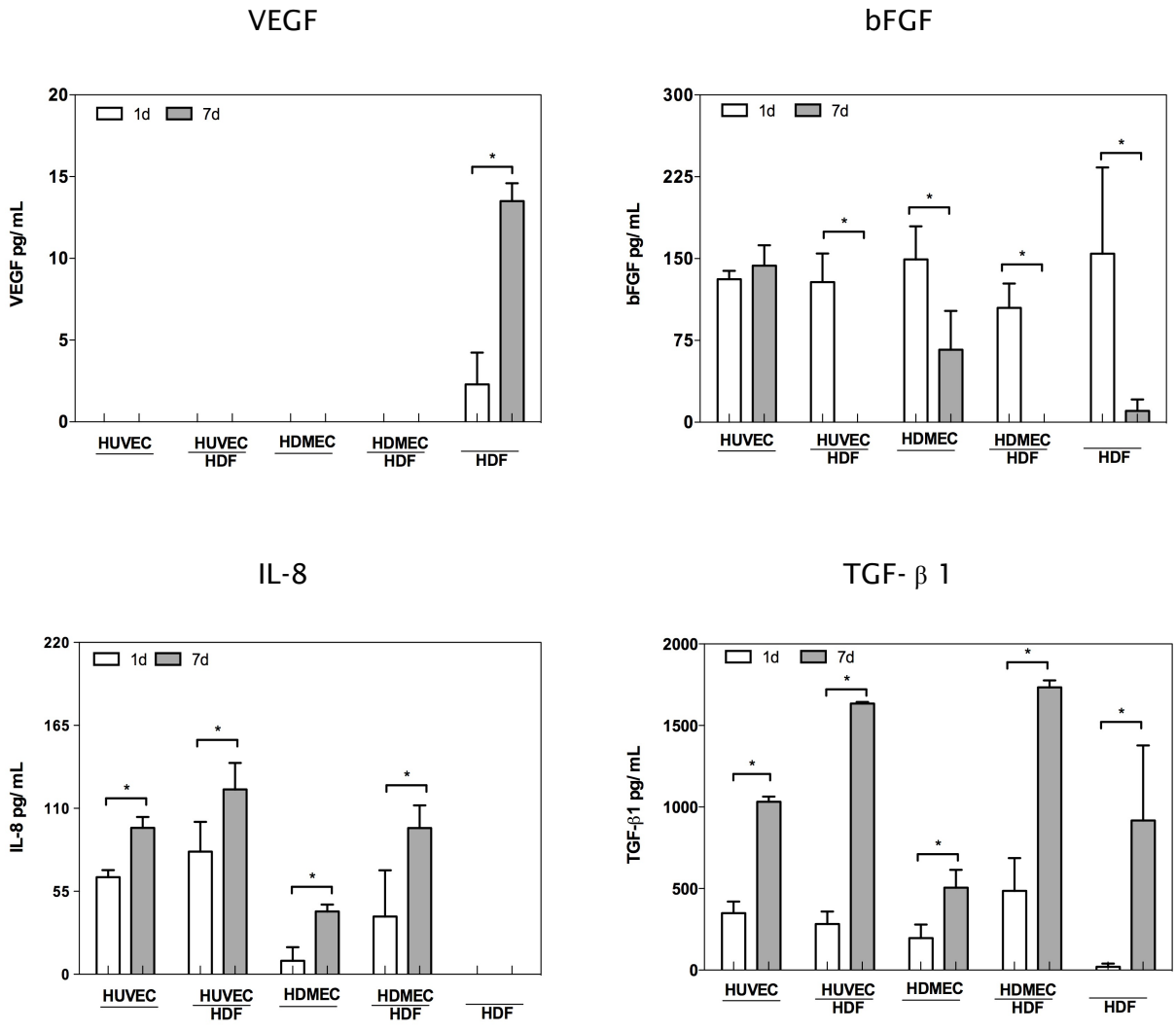

Figure 1. Concentration of VEGF, bFGF, IL-8, and TGF- β 1 in supernatants of monocultures of HUVECs, HDMECs or HDFs and of indirect contact cocultures of HUVEC or HDMEC with HDFs in tranwell systems after 1 and 7 days. Results are shown as mean ± SEM (n=3). * $p<0.05$.

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In Fig. 2G, HDFs stained for nuclei (blue) showed that cells began to grow in multilayers and most likely created a 3D environment that stimulated HDMECs (stained for CD31, arrows, green) to organize into microcapillary-like structures (arrowheads, Fig. 2G). Thus, direct cell contact between HDMECs or HUVECs with HDFs was required to observe the microcapillary-like formation by ECs.

3.3. Colocalization of microcapillary-like structures and expression of alkaline phosphatase

When HDFs were cocultured with HUVECs or HDMECs in direct contact for 7 days (Figs. 3A and B, respectively) areas with positive (red) and negative staining for ALP were observed. In both cocultures of HUVECs/HDFs and HDMECs/HDFs the presence of microcapillary-like structures was detected, which stained positively for CD31 (green) and nuclei (blue), while HDFs stained negatively for CD31 and positively for nuclei (blue). The presence of microcapillary-like structures could be colocalized with areas staining positively for ALP and thus further experiments were carried out to study the consistency of this phenomenon.

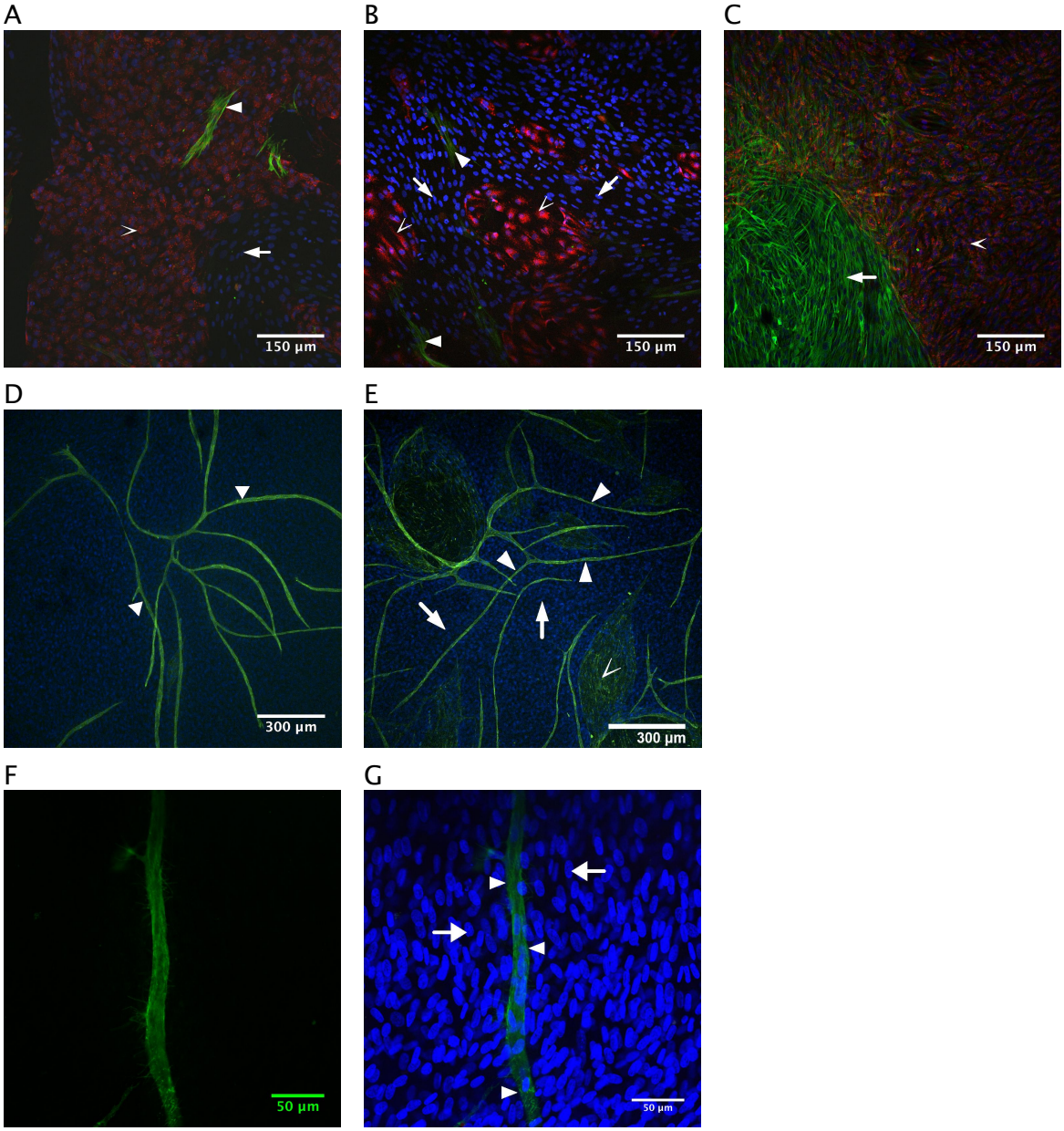


Figure 2. Microvessel-like structure formation in direct contact cocultures of endothelial cells with fibroblasts. HUVECs and HDMECs were cocultured with HDFs for 21 days. (A) HUVECs/HDFs at day 3, with HUVECs stained for vWF (open arrowhead, red), HDFs with DAPI-stained nuclei (arrow, blue) and negative for vWF, and active fibroblasts stained for α-smooth muscle actin (α-SMA; arrowhead, green); (B) HDMECs/HDFs at day 3, with HDMECs stained for vWF (open arrowheads, red), HDFs with DAPI-stained nuclei (arrows, blue) and negative for vWF, and active fibroblasts stained for α-SMA (arrowheads, green); (C) HUVECs/HDFs at day 3, with HUVECs stained with vWF (open arrowhead, red), HDFs stained more strongly for vimentin (arrow, green) than do ECs and nuclei for DAPI (blue). (D) HUVECs/HDFs at day 21, with microvessel-like structures (CD31, green, arrowheads) and nuclei (DAPI, blue); (E) HDMECs/HDFs at day 21, with microvessel-like structures (CD31, green, arrowheads) and nuclei (DAPI, blue). Not all HDMECs formed microvessel-like structures (open arrow heads); and HDFs with nuclei stained for DAPI (arrows, blue) and negative for CD31; (F, G) magnification of microvessel-like structure formed by HDMECs (CD31, green, arrowheads), surrounded by HDF (nuclei stained with DAPI, blue, arrows) in HDMECs/HDFs cocultures at day 21.

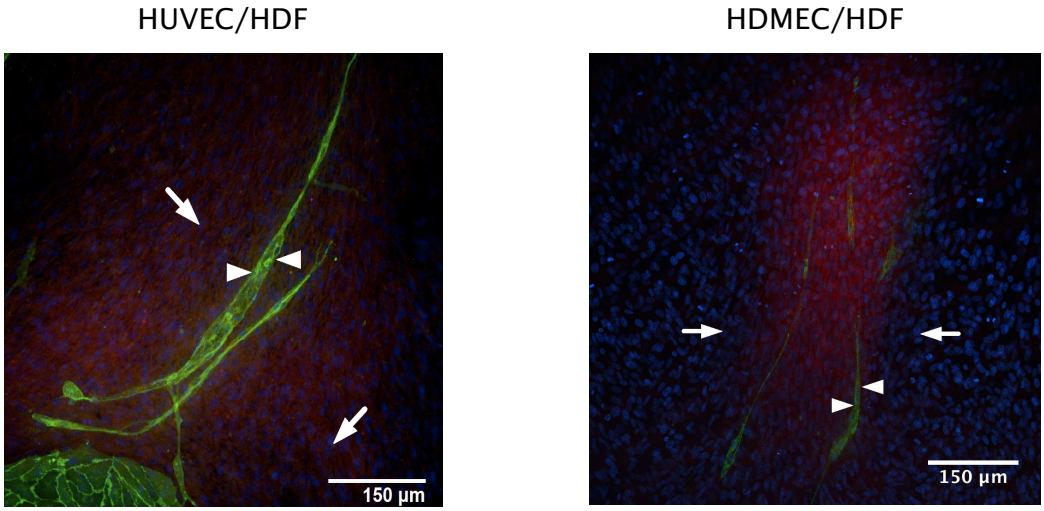
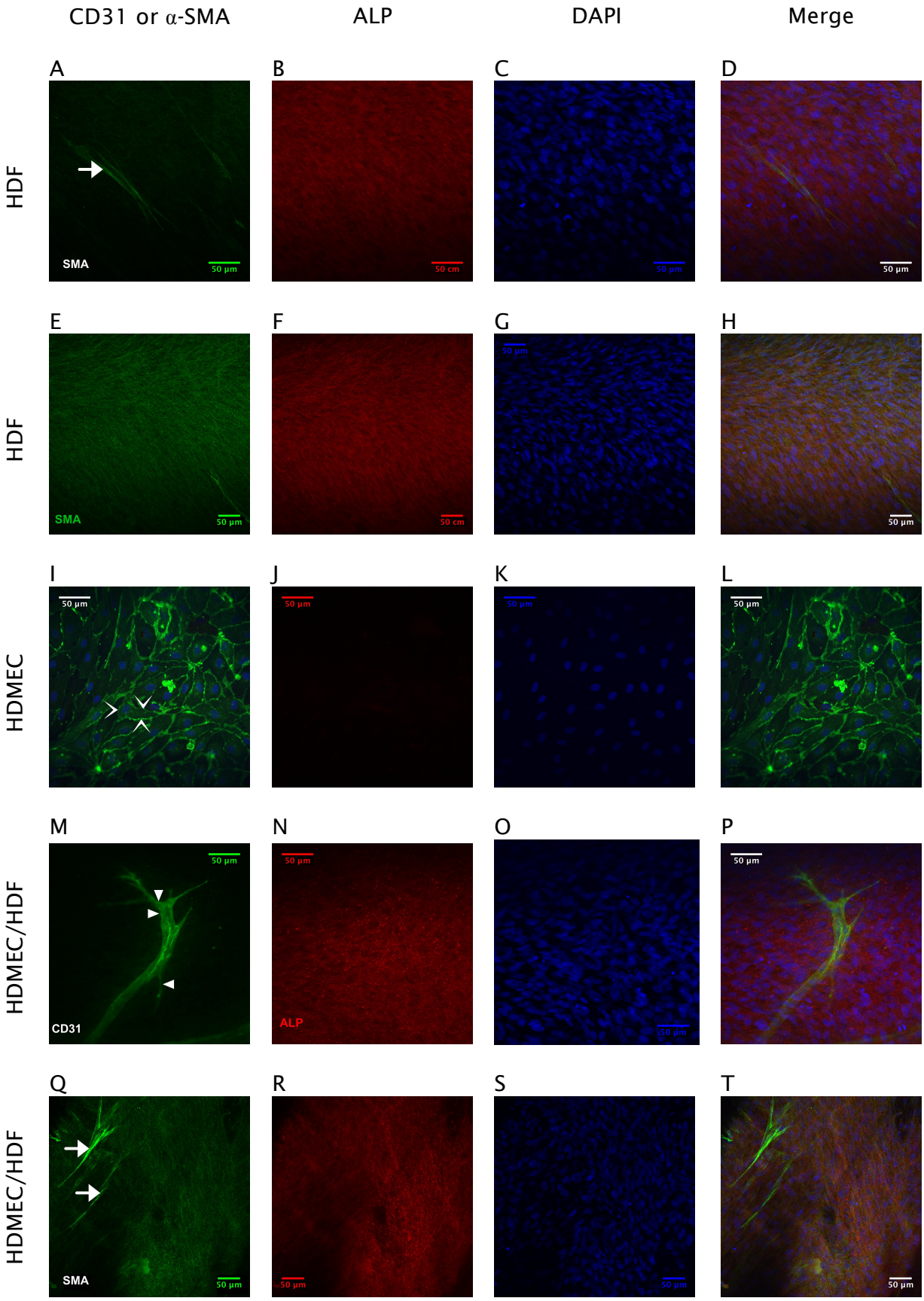


Figure 3. Cocultures of HUVECs or HDMECs with HDFs after 7 days in culture. Staining reactions for alkaline phosphatase (ALP) with Fast Red Violet B (red), HUVECs or HDMECs with CD31 (green, arrowheads) and nuclei with DAPI (blue). HDFs stained positively for DAPI (nuclei in blue, arrows) and negatively for CD31. Capillary-like structure formation was observed in areas stained positively for ALP.

Figure 4. Confocal laser scanning microscopy images of cocultures of HDMECs with HDFs after 14 days. (A, E) Monoculture of HDFs staining positively for α-smooth muscle actin (α-SMA, arrows) (green); (B, F) Monoculture of HDFs staining positively for ALP (red); (C, G) Monoculture of HDFs staining positively for DAPI (nuclei, blue); (I) Monoculture of HDMECs staining positively for CD31 (green, open arrowheads); (J) Monoculture of HDMECs staining negatively for ALP (red); (K) Monoculture of HDMECs staining positively for DAPI (nuclei, blue); (M) Cocultures of HDMECs/HDFs staining positively for CD31 and microcapillary-like structure formation (green, arrowheads); (N) Cocultures of HDMECs/HDFs staining positively for ALP (red); (O) Cocultures of HDMECs/HDFs staining positively for DAPI (nuclei, blue); (Q) Cocultures of HDMECs/HDFs staining positively for α-SMA (green, arrows); (R) Cocultures of HDMECs/HDFs staining positively for ALP (red); (S) Cocultures of HDMECs/HDFs staining positively for DAPI (nuclei, blue); (D, H, L, P, T**)** Merged images of each of the horizontal lines. Scale bars=50 μm.


CD31 or α-SMA
ALP
DAPI
Merge
HDF
HDF
HDMEC
HDMEC/HDF
HDMEC/HDF
A
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Q
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T
SMA
CD31
ALP
50 µm

To further characterize this behavior, the expression of ALP, CD31 and α-SMA was analyzed in cocultures of HUVECs or HDMECs with HDFs after 14 days in culture (monocultures were used as controls). HDF monocultures stained positively for ALP (red) throughout the culture (Figs. 4B and F), although the positive staining for α-SMA was irregular: some areas showed only 1 to 3 cells with positive staining for α-SMA (Fig. 4A) while others showed extensive positive staining (in the same monoculture, Fig. 4E). HDMEC monocultures stained negatively for ALP (Fig. 4J, red), and positively for CD31 (Fig. 4I, green), although no formation of microcapillary-like structures could be observed in these monocultures (Fig. 4I). However, in the cocultures of HDMECs with HDFs (HDMEC/HDF) areas with high expression of ALP (red) were observed (Figs. 4N and R). Furthermore, in ALP expression areas HDMECs in cocultures with HDFs organized into microcapillary-like structures, with positive staining for CD31 (Fig. 4M, green) and showing sprouting and cell tip structures (Fig. 4M, arrowheads). Also, in areas of high expression of ALP (Figs. 4R, red), cocultures of HDMECs/HDFs cells stained positively for α-SMA (Fig. 4Q). Positive staining for CD31 and α-SMA could not be observed in areas with low ALP expression in HDMECs/HDFs cocultures. Table 1 summarizes the data collected from this experiment. Similar results were obtained for cocultures of HUVECs/HDFs.

Table 1. Summary of the results from Fig. 4.

Cultured cells	ALP	CD31 (capillary-like structures)	α-SMA
HUVEC	-	-	-
HUVEC/HDF	+ (spots)	+	++
HDMEC	-	-	-
HDMEC/HDF	+ (spots)	+	++
HDF	+	-	+

(-) absent; (+) present (few); ++ present (abundant); ALP: alkaline phosphatase; α-SMA: α-smooth muscle actin; HDF: monoculture of human dermal fibroblasts; HDMEC: monoculture of human dermal microvascular endothelial cells; HUVEC: monoculture of human umbilical vein endothelial cells; HDMEC/HDF: coculture of HDMECs with HDFs; HUVEC/HDF: coculture of HUVECs with HDFs. Four different fields of the sample where used.

3.4 Influence of collagen production on microcapillary-like structure formation in cocultures of endothelial cells with fibroblasts

In order to investigate whether the formation of microcapillary-like structures could be related to collagen production, collagen levels in the extracellular matrix of cocultures of HUVECs or HDMECs with fibroblasts were quantified after 7, 14 and 21 days using the Sircol™ assay. Monocultures of HUVECs, HDMECs and HDFs were used as controls. Fig. 5 shows, at day 21, significant increases in the amount of collagen in all conditions tested compared to day 7. The amount of collagen significantly increased between day 14 and 21 in cocultures of HUVECs/HDFs and of HDMECs/HDFs, as well as in monocultures of HUVECs and of HDMECs. Thus, the production of collagen seems to have been influenced by the two cells types in the cocultures.

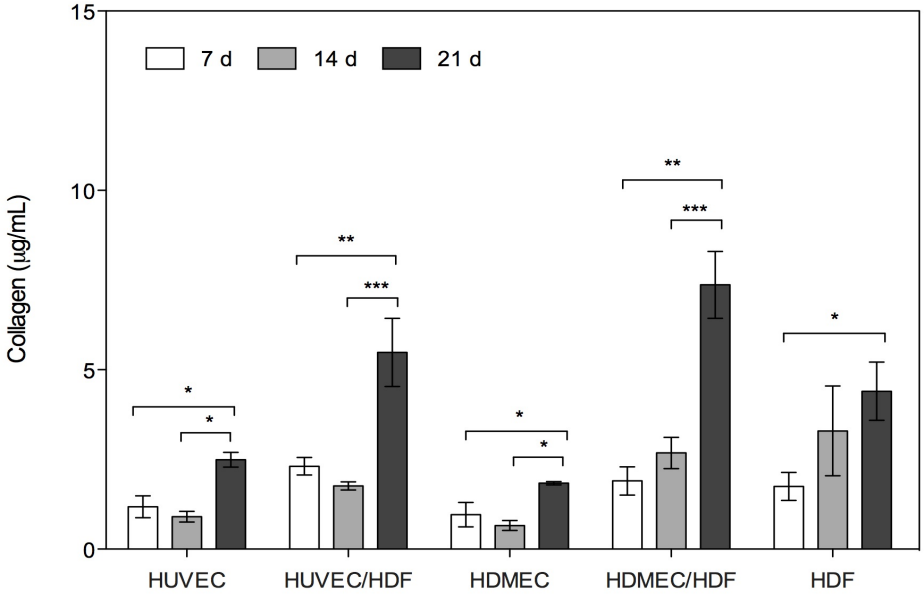

Figure 5. Collagen production after 7, 14 and 21 days of monocultures of HUVECs, HDMECS or HDFs, or of cocultures of HUVECs or HDMECs with HDFs. The results are presented as mean ± SEM (n=3). * $p<0.05$, ** $p<0.01$,*** $p<0.001$.

3.5 Levels of VEGF and TGF-β1 in cocultures by direct contact

In order to investigate if the formation of microcapillary-like structures and the increase in collagen levels in cocultures of HUVECs or HDMECs with HDFs would influence the media levels of VEGF and TGF-β1 supernatants of the

monocultures and cocultures were collected and analyzed by ELISA at days 7, 14 and 21. In agreement with the previous results from indirect coculture studies, VEGF was detected in HDF monocultures (Fig. 6A). VEGF levels at day 7 were significantly higher than at days 14 and 21. In both cocultures tested very low amounts of VEGF were measured at days 7 and 21. Contrary to results obtained with indirect contact cocultures, TGF-β1 levels were detected in HUVECs/HDFs (at days 7 and 14) and HDMECs/HDFs cocultures (at day 7), at low amounts (Fig. 6B). TFG-β1 was not observed in any of the monocultures tested.

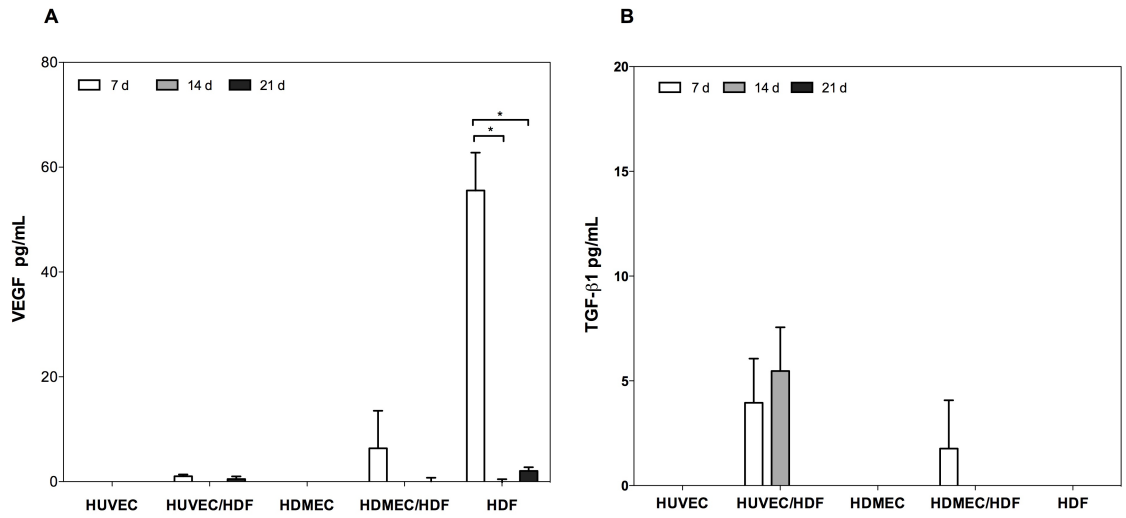

Figure 6. Cellular production of growth factors (VEGF and TGF-β1) by cells in monoculture and in direct contact cocultures, at 7, 14, and 21 days of culture. (A) VEGF and (B) TGF-β1 were quantified in the supernatants of HUVECs/HDFs and HDMECs/HDFs cocultures in direct contact, as well as in their monocultures (controls). Results are shown as mean ± SEM (n=3, *p<0.05).

4. Discussion

In vitro coculture systems have proven to be excellent models to analyse the cellular role of vascular-associated factors and to help elucidate their intracellular signalling pathways [33]. The *in vivo* microenvironment has diverse and complex interactions between multiple cell types and extracellular matrix (ECM) components that can be simulated in different combinations of coculture systems [33].

In order to investigate the interactions between endothelial cells (ECs) and fibroblasts and their effects on vascularization coculture systems of ECs/fibroblasts were established. Different cell types, such us pericytes and smooth muscle cells, are known to support the behaviour of ECs and thus play a prominent role in angiogenesis, but the role of fibroblasts in modulating vascularization is still poorly understood. Thus, an *in vitro* model was used here to assess the interaction between fibroblasts (human dermal fibroblasts, HDFs) and ECs from both the macrovascular (HUVECs) and the microvascular system (HDMECs).

The results obtained using indirect cocultures (transwells) showed that VEGF was not present in cocultures of HUVECs/HDFs or HDMECs/HDFs, although HDFs had the capacity to produce VEGF. It has been previously shown that stromal fibroblasts are an important source of the pro-angiogenic VEGF [7, 8]. A decrease in the amount of bFGF in time (from days 1 to 7) was observed, in HDF and HDMEC monocultures as well as in HUVEC/HDF and HDMEC/HDF cocultures. IL-8 was produced by HUVECs and HDMECs since no amount of IL-8 was observed in HDF monocultures. ECs are known to release IL-8 and this influences cell migration [34, 35]. In all cultures TGF-β1 was present and increased with time. TGF-β1 is known to influence the assembly and maturation of blood vessels [36], the production of collagen [37] and the activation of fibroblasts [38]. When fibroblasts are activated they stained positively for alpha-smooth muscle actin (a-SMA) [39].

In direct contact cocultures of HUVECs/HDFs and HDMECs/HDFs microvessel-like structures were observed. These microvessel-like structures were present in areas with high expression of alkaline phosphatase (ALP) and activated fibroblasts. Collagen production increased in all the cultures during an observation period of 21 days. On the other hand, VEGF production was high in HDF monocultures but observed only at much lower levels in cocultures of

HUVECs/HDFs and HDMECs/HDFs. Low levels of TGF-β1 were also observed in cocultures. These facts could indicate a consumption of VEGF by ECs, as required for their survival, migration and differentiation [40, 41]. Another possibility is the presence of ECM in direct contact cocultures, which could be responsible, to a certain degree, for the entrapment of growth factors produced by the cells [21].

It seems relevant to regenerative medicine to try and understand what biochemical cues influencing fibroblasts to contribute to regeneration instead of fibrosis are involved in this process. Fibroblast activation plays an important role in regulating immune responses [17, 18] and it is also known that small peptides released during remodeling of the extracellular matrix have profound effects on cellular immune responses [17, 42, 43]. Fibroblasts exhibit considerable intra-line plasticity that is dependent on the microenvironment. Recently, it has been reported that adult fibroblasts can be converted into pluripotent stem cells by transfection with only four transcription factors (oct3/4, Sox2, c-myc and Klf4) [20, 44]. Nanog, a transcription factor that regulates early development [45], has been found to be present in porcine [46] and human dermal fibroblasts [20] suggesting that nanog is important in maintaining the plasticity found in fibroblasts *in vivo* [20]. The present results further suggest that fibroblasts are not a passive cell-type in the complex signalling pathways of angiogenesis. Fibroblasts influenced the formation of microcapillary-like structures and collagen deposition, suggesting a dependency between the formation of microcapillary-like structures and the organization and thickness of the ECM, which is in agreement with previous studies [39].

Martins-Green *et al.* [20] have previously shown that peri-endothelial cells can arise by differentiation from fibroblasts and that they require interaction with ECs for that purpose. Faggin *et al.* [19] suggested that fibroblasts appear to serve as vascular cell progenitors in the injured rabbit carotid artery. In the present study, and in agreement with Nigau *et al.* [20], it was observed that, in the vicinity of the capillary-like structures, some fibroblasts became positive for α-SMA. Although the stimuli are not known, it is likely that cellular crosstalk between ECs of the capillary-like structures and fibroblasts results in the expression of such fibroblastic activation markers.

Alkaline phosphatase (ALP) is a widely used marker for the differentiation of pre-osteoblasts or human mesenchymal stem cells (hMSC) under osteogenic conditions [23, 47]. Previous studies have used ALP as a marker to assess the

differentiation of hMSCs into osteoblasts in coculture with ECs [48]. Here, the modulation of ALP produced by fibroblasts cocultured with HUVECs or HDMECs was investigated. Although the expression of ALP has been widely related to osteoblast differentiation and bone mineralization [49, 50], other perspectives and roles regarding ALP have been described. ALP has also been implicated in the regulation of cell growth and apoptosis [51]. In addition, ALP expression is often restricted both temporally and spatially to the population of cells involved in migration or morphogenesis [52], suggesting its involvement in cell guidance and migration. In the present study, microvessel-like structures were co-localized with areas with high levels of ALP expression in cocultures of ECs with fibroblasts, while this phenomenon was not observed in monocultures (controls). As far as we know, this is the first study showing the colocalization of microvessel-like structures with ALP. These findings support a possible role of ALP in cell-cell interaction (ECs-fibroblasts) [53] and cell migration [54-57]. Supporting this hypothesis, Friis *et al.* [58] reported an anti-angiogenic, dose-dependent, potential effect of levamisole on ECs propagated with fibroblasts, both *in vitro* and *in vivo* in a hetero-transplantation tumor model. Levamisole is a well-described and widely used tissue-non-specific alkaline phosphatase (TNS-ALP) inhibitor [59]. Levamisole-induced inhibition of microcapillary-like structures has been demonstrated although no clarification was provided about the roles of cell-cell interaction or cell migration in the ability of TNS-ALP to influence this process [58]. Furthermore, TNS-ALP has been reported to bind to collagen in the ECM [60, 61], which, together with our results, suggests an involvement of TNS-ALP in the angiogenesis process and particularly in the crosstalk between ECs and fibroblasts. Taken together, these results suggest a possible role of ALP in the modulation of angiogenesis and in cell adhesion that may be relevant for the development of new cell therapies in tissue regeneration. Collagen is one of the major proteins in the ECM and is produced by fibroblasts in wound healing. In cocultures of ECs and fibroblasts it was demonstrated that the presence of fibroblasts influenced the increase of collagen production with time. This observation suggests that the presence of collagen, and the fact that fibroblasts formed multilayers of cells in culture, induced ECs organization into microvessel-like structures, while these structures were absent in EC monocultures. Collagen type I is also known to drive EC migration by chemotaxis and haptotaxis [13]. In bone tissue engineering strategies the deposition of an extensive network of collagen by osteoblasts has been shown

to provide an essential 3D support for ECs to migrate and to organize into microcapillary-like structures by providing ECs with the chemical and physical cues for migration and proliferation [12]. Studies with cultured skin fibroblasts from a series of patients with osteogenesis imperfecta, a heritable defect that makes bones brittle, demonstrated that the mutations produced specific disruption in the biosynthesis of collagen type I, thus generating abnormal collagens fibrils [62]. These facts will influence matrix composition and the interaction with growth factors and cytokines important in cell behavior [63]. The coculture model presented here offers the possibility to delineate how specific proteins and growth factors are regulated as a result of interactions between fibroblasts and endothelial cells. Further studies are required to delineate the different subtypes of collagen produced in the cocultures compared with monocultures.

5. Conclusions

In the present study fibroblasts and endothelial cells (from macro or microvasculature) were cocultured and the production of growth factors was modulated by the crosstalk between these two cells types. The crosstalk occurring between ECs-fibroblasts appears to stimulate fibroblasts to produced collagen and growth factors that can positively influence the formation of microvessel-like structures. In addition, the interaction between these two cell types leads to the activation of fibroblasts (expressing α-smooth muscle actin) and to the expression of alkaline phosphatase near the microvessel-like structures. The latter raises the possibility that alkaline phosphatase could be involved in the process of vascularization.

Acknowledgments

The work carried out at INEB was financed by FEDER funds through the *Programa Operacional Factores de Competitividade* – COMPETE and by Portuguese funds through FCT – *Fundação para a Ciência e a Tecnologia* in the framework of projects PEST-C/SAU/LA0002/2011 and PEST-OE/SAU/UI0038/2011 and POCI/SAU-BMA/55556/2004 and the PhD scholarship SFRH/BD/40421/2007 for S.G.G.

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CHAPTER IV

Implanted neonatal human dermal fibroblasts influence the recruitment of endothelial cells in mice

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*To be submitted (2011)

Abstract

The vascularization of new tissue within a reasonable time is a crucial prerequisite for the success of different cell-and-material-based strategies. Considering that angiogenesis is a multi-step process involving humoral and cellular regulatory components, only *in vivo* assays provide the adequate information about vessel formation and the recruitment of endothelial cells. The present study aimed to investigate if neonatal human dermal fibroblasts could influence *in vivo* neovascularization. Results obtained showed that fibroblasts were able to recruit endothelial cells to vascularize the implanted matrix, which was further colonized by murine functional blood vessels after 1 week. The vessels exhibited higher levels of hemoglobin, compared to the control matrix, implanted without fibroblasts, in which no vessel formation could be observed. No significant differences were detected in systemic inflammation. The presence of vessels originated from the host vasculature suggested that host vascular response was involved, which constitutes a fundamental aspect in the process of neovascularization. Fibroblasts implanted within matrigel increased the presence of endothelial cells with positive staining for CD31 and for CD34 and the production of collagen influencing the angiogenic process and promoting the formation of microvessels.

New strategies in tissue engineering could be delineated with improved angiogenesis using neonatal fibroblasts.

Keywords: Angiogenesis, endothelial cells, fibroblasts, regenerative medicine, tissue engineering, vascular networks.

1.Introduction

Angiogenesis represents the process of formation of new blood vessels out of pre-existing ones, with significant value in development, reproduction and repair [1]. The basic vascular network is formed by endothelial cells, through which the exchanges of gases, growth factors, nutrients and elimination of metabolites between blood and tissue occur [2]. In fact, the diffusion distance of nutrients and oxygen out of blood vessels ranges from 100 to 200 μm [3]. In this context, the vascularization of new tissue within a reasonable time is a crucial prerequisite for the success of different cell-and-material-based strategies in the context of regenerative medicine [4]. One of the challenges in tissue engineering has become the development of artificial extracellular matrices that promote the ingrowth of microvessels in the injured site, for instance through the delivery of growth factors [5]. Alternatively, endothelial cell delivery systems, including their cocultures for instance with osteoblasts or mesenchymal stem cells in the framework of bone regeneration strategies, have also been shown to constitute adequate strategies to promote the formation of capillary networks, even without further addition of growth factors, indicating that the interaction between these cells types assures the release of the required growth factors [6, 7].

In tissue engineering strategies, where the vascularization of implanted cell-scaffold constructs is a requirement, with view to the use of a cell-scaffold construct, the interconnectivity between pores, in the case of porous scaffolds, or matrix timely degradation, are essential prerequisites for the success of both host cell invasion and proliferation and migration of implanted cells [8-11]. Additionally, the vascular networks formed need to be guided by the implanted artificial matrix to promote anastomosis into the host vasculature [12-14]. Matrigel has been extensively used as an artificial extracellular matrix to study angiogenesis *in vitro* and *in vivo* due to its unique characteristics. Matrigel is a mixture of extracellular matrix and basement membrane proteins extracted from the mouse Engelbreth-Holm-Swarm (EH) sarcoma that forms a hydrogel [15]. Considering that angiogenesis is a multi-step process, involving humoral and cellular regulatory components [16], *in vivo* angiogenesis assays constitute the most informative experiments in terms of vessel formation, endothelial cells recruitment and vessel functionality through analysis of anastomosis with host vasculature. Matrigel is especially suited for *in vivo* studies of angiogenesis due

to its similarity with the natural extracellular matrix, despite containing a number of growth factors believed to play key roles in the process. In the attempt to reduce the influence of the growth factors in angiogenesis experiments a reduced growth factor matrigel (GFR-matrigel) was developed. The subcutaneous implantation of matrigel with mesenchymal progenitor cells into mice showed the capacity of these cells to induce angiogenesis, and has been suggested as a model in development of approaches to improve neovascularization of engineered human tissues and organs [14].

The role of fibroblasts in tissue regeneration is probably underestimated in tissues other than the skin. Fibroblasts already constitute the dermis component of the commercial tissues engineered for dermal replacements used to improve the repair of chronic foot ulcers [17]. A cardiac patch using these cells has also been used to support the angiogenesis in infarcted heart tissue in animals models [18, 19]. Fibroblasts are known to maintain the structural integrity of connective tissues by continuously secreting growth factors and extracellular matrix precursors, which are essential for endothelial cells adhesion and spreading [20]. Furthermore, Kern et al. reported the lower immunogenicity of allogeneic fibroblasts grown in 3D comparing to fibroblasts grown in monoculture [21]. In the present work, it was hypothesized that neonatal human dermal fibroblasts may influence the neovascularization of the GFR-matrigel. In order to provide information about the morphology of fibroblasts and how they could influence the neovascularization an *in vivo* GFR-matrigel plug assay was used. Hemoglobin content and inflammatory levels were measured, and histological analyses were performed.

2. Materials and Methods

2.1. Culture of fibroblasts

Neonatal human dermal foreskin fibroblasts-1 (from American Type Culture Collection, ATCC, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with high glucose and with L-glutamine, supplemented with 15% v/v inactivated fetal bovine serum (FBS, from Gibco), 100 U/mL penicillin G and 100 μg/mL streptomycin (Gibco). The cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere and they were cultured until 90% confluence was

reached. Fibroblasts were used at passages 6-8 in every experiment and the culture medium was changed every 2 days.

2. 2. *In vivo* angiogenesis assay

Animal experiments were conducted according to accepted standards of human animal care, namely European Community guidelines (86/609/EEC) and Portuguese Act (129/92). *In vivo* matrigel plug assay was performed using a mixture of growth factor reduced Matrigel® (GFR-Matrigel®, BD Science) and heparin (25 U/mL, Sigma) with 10^6 fibroblasts. 500 μL of the GFR-Matrigel® mixture were subcutaneously inoculated into eight-weeks-old C57BL/6 male mice, from Charles River, (n=7). A control group was used, without cells (n=7). After 1 week mice were anesthetized by intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight) and euthanized, the matrigel plugs were removed, weighted, photographed and fixed. Mice blood was also collected for evaluation of inflammatory markers (N-acetylglucosaminidase activity and nitric oxide production). The formation of the functional vasculature in the matrigel plug was evaluated by determination of the hemoglobin concentration in the homogenized plug.

2.3 Determination of hemoglobin

Hemoglobin (Hb) content was determined in matrigel plugs collected 1 week after implantation, using a previously described procedure [22]. Matrigel plugs were homogenized in a water-heparin solution and centrifugated at 1500 *g* for 15 min at 20ºC. The Hb content was measured in the supernatant (100 μL) according to the Drabkin's method (Sigma) at 540 nm.

2.4 Determination of N-acetylglucosaminidase activity

Systemic inflammation can be ascertained measuring the activity of the lysosomal enzyme N-acetylglucosaminidase (NAG) in the serum, an enzyme present in high levels in activated macrophages. The blood was collected allowed to clot at room temperature for 30 min, followed by centrifugation at 1500 *g* for 15 min at 25ºC in order to obtain the serum. The serum was incubated during 10 min at 37ºC with 100 μL of p-nitrophenyl-N-acetyl-beta-D-

glucosaminide solution in a 96-well plate. The reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.6) and the substrate hydrolysis was measured at 405 nm [22].

2.5 Determination of nitric oxide

Nitric oxide (NO) production was measured indirectly using the Griess Reaction system, which uses 1% sulphanylamide and 0.1% *N*-1-napthylethylenediamine dihydrochloride under 2.5% phosphoric acid to measure nitrite (NO_2^-) in serum. 100 µL of serum and 100 µL of Griess Reagent were added to a 96-well plate. After 15 min at room temperature, protected from light, the azo product was measured at 550 nm.

2.6. Histological and immunohistological analyses

The explanted matrigel plugs were fixed in buffered formalin solution (10%), dehydrated using a graded ethanol series and automatically embedded in paraffin according to standard procedures. Paraffin sections of 4 µm thickness were cut, rehydrated and histochemically stained with hematoxylin and eosin staining according to standard procedures. To identify fibers built by fibroblasts the Elastica van Gieson (EvG) staining was performed according to laboratory standard methods.

The immunohistological detection of CD31 and CD34 was carried out with the peroxidase method by using DAB as chromogene. For this purpose monoclonal antibodies against CD31 (dilution 1:400, Santa Cruz, USA) and CD34 (dilution 1:200, Santa Cruz, USA) were used. The immunohistological stainings were automatically performed with help of an Autostainer plus (Dako, Germany) and the appropriate Dako-Staining kits. After the immunodetection the nuclei of cells were stained by hemalaun.

2.7 Transmission electron microscopy

For transmission electron microscopical analyses samples of 0.5 x 0.5 cm were cut out of the explants, fixed in buffered glutaraldehyde (2.5%) over night, contrasted with OsO_4 and then embedded in Agar 100 (Plano, Germany), which polymerized for at least 24 hours. After trimming first semithin sections were

performed to identify the region of interest, which was then cut ultrathin with the Ultracut E microtome (Leica, Germay). Transmission electron microscopical analyses were performed with a Phillips EM 410 (Phillips, Netherlands). Visualizations took place at 80 KV and were fotodocumented.

2.8 Statistical analyses

All experiments were performed at least in triplicate. Quantifications are expressed as mean ± standard error of the mean (SEM). For comparison between two groups the Student's *t*-test was used. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $p<0.05$.

3. Results

3.1. Hemoglobin content

To evaluate if the implanted human neonatal fibroblasts could modulate the angiogenic process *in vivo*, and the recruitment of endothelial cells from the host in particular, the matrigel plug angiogenesis assay was used. Upon removal the plugs were easily distinguishable from the surrounding tissues, and a well-defined plug between skin and muscles could be excised for the preparation of histological sections. The extension of functional neovascularisation was evaluated by measuring the amount of hemoglobin (Hb) present in the matrigel plugs implanted with or without fibroblasts after 1 week. Matrigel implants were inoculated with fibroblasts induced vascular development (Fig. 1B) contrary to what was observed in the control conditions, where no cells were implanted (Fig. 1A). The plugs implanted with fibroblasts showed an angiogenic response, highlighted by the red colour spread in the whole plug (Fig. 1C; arrows show vessels formed in the matrigel plug implanted with fibroblasts). As can be seen in Fig. 1D the Hb levels were significantly increased in the matrigel plugs implanted with fibroblasts compared to the control group (without fibroblasts).

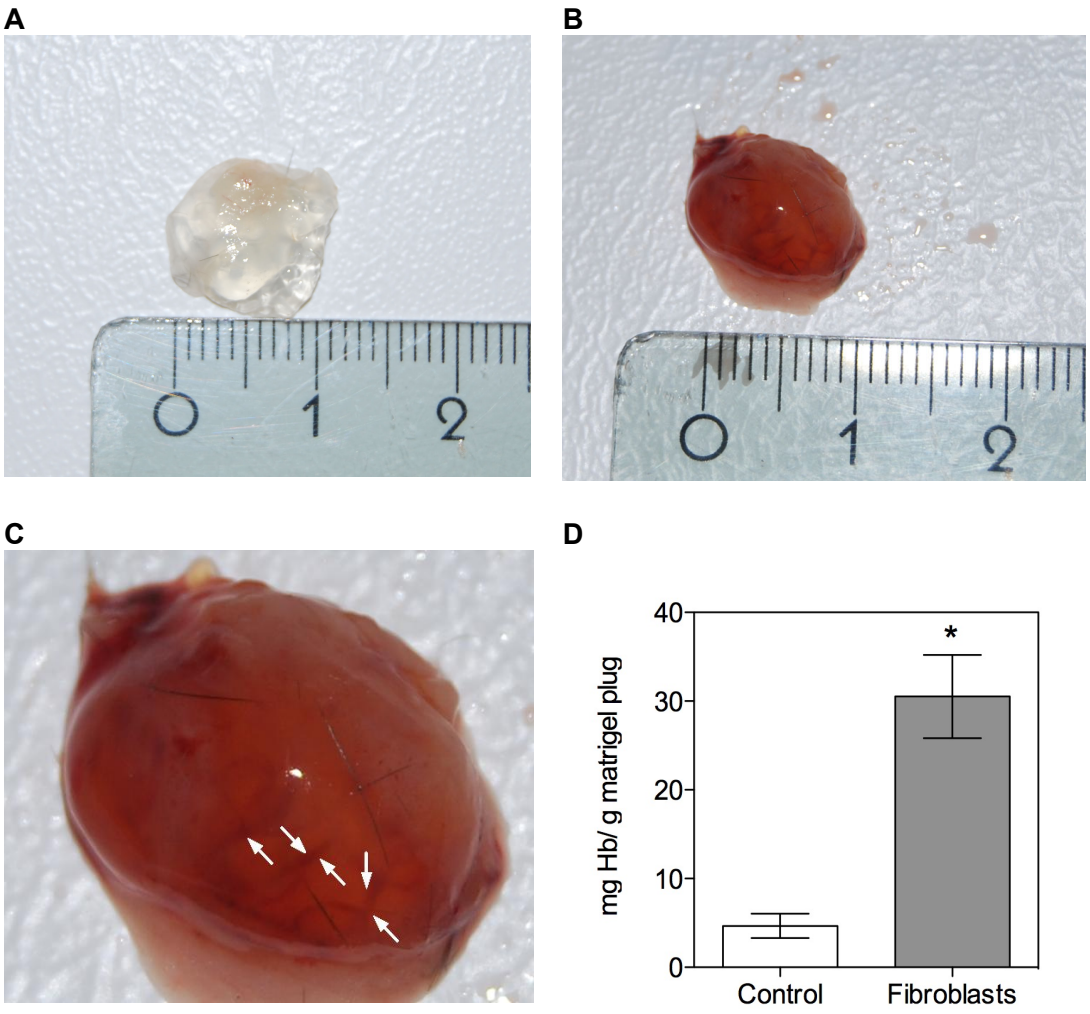

Figure 1. *In vivo* evaluation of angiogenesis using the matrigel plug assay. A mixture of matrigel and heparin, with or without fibroblasts (control), were implanted subcutaneously into C57BL/6 mice, and removed after 1 week. (A) Representative macroscopic visualization of matrigel plugs implanted without fibroblasts, which appear avascular; (B) macroscopic visualization of matrigel plugs implanted with fibroblasts; (C) magnification of image B, with arrows showing the vessels formed; (D) Quantification of haemoglobin (Hb) in the retrieved plugs, showing a significant increase in Hb levels upon fibroblasts implantation. The presence of fibroblasts increased vessel formation in the plug, significantly differing from the control. Results are means ± SEM of independent experiments (n=6). *$p<0.05$ vs. control.

3.2. Inflammation factors determination

To assess if implanted fibroblasts developed inflammation in mice during the experiment, NAG activity was evaluated in the serum of each mice. The results revealed no significant difference between the serum NAG activity levels of mice transplanted with fibroblasts compared to controls, indicating that no inflammatory response to fibroblasts implanted could be detected (Fig. 2A). Regarding the production of NO, no significant differences between the two test groups were detected either (Fig. 2B), confirming that no differences were obvious within these two groups.

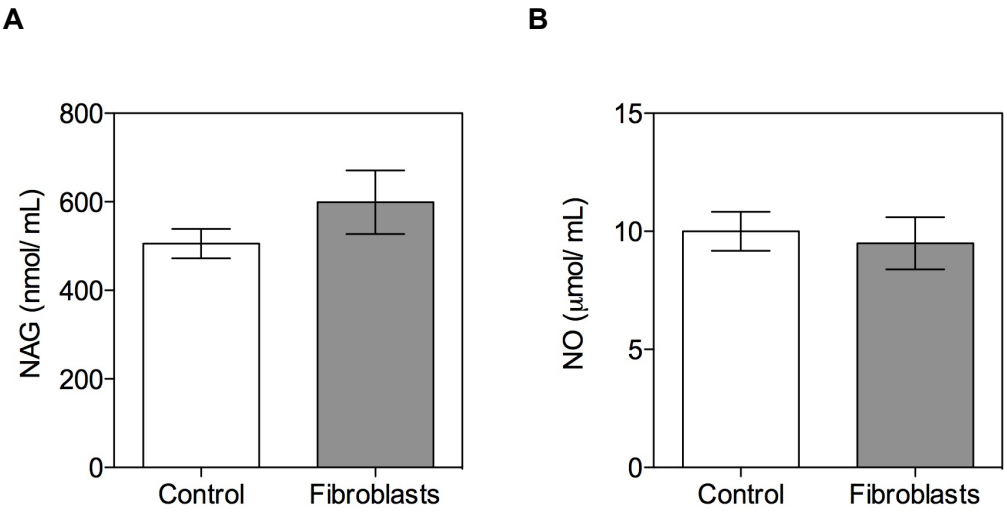

Figure 2. Determination of (A) N-acetylglucosaminidase (NAG) activity and (B) nitric oxide (NO) in mice serum. Results are means ± SEM of independent experiments (n=6).

3.3. Histological and immunohistological analyses

The histological analyses allow a microscopical view of the cell populations within the plugs in the two analyzed groups. All explanted plugs showed a slight inflammatory reaction with granulocytes and lymphocytes in the surrounding tissue after one week. Regarding the inflammatory reaction no differences could be found between the two analyzed groups. Furthermore, in all examined plugs an infiltration of cells could be found (Fig. 3). In matrigel with fibroblasts it was observed an accumulation of cells not only at the border but also in the periphery and the center of the plug (Fig. 3B). In this group, numerous groups of

circular arranged cells could be detected within the matrigel, which immunhistologically expressed CD31 (Fig. 3D, arrows). In the specimens without fibroblasts capillary-like tubes (black arrow) could be found solely in the periphery of the plugs but not in the center (Fig. 3C), which correspond to the small amount of hemoglobin detected in the control group.

In matrigel plugs implanted with fibroblasts the presence of some vessels inside (center) the implant was observed (Fig. 4A). Inside de matrigel, tube-like structures branching capillary arranged structures, aligned by endothelial cells, could also be identified, with erythrocytes and inflammatory cells within the lumen and near the periphery (Fig. 4B).

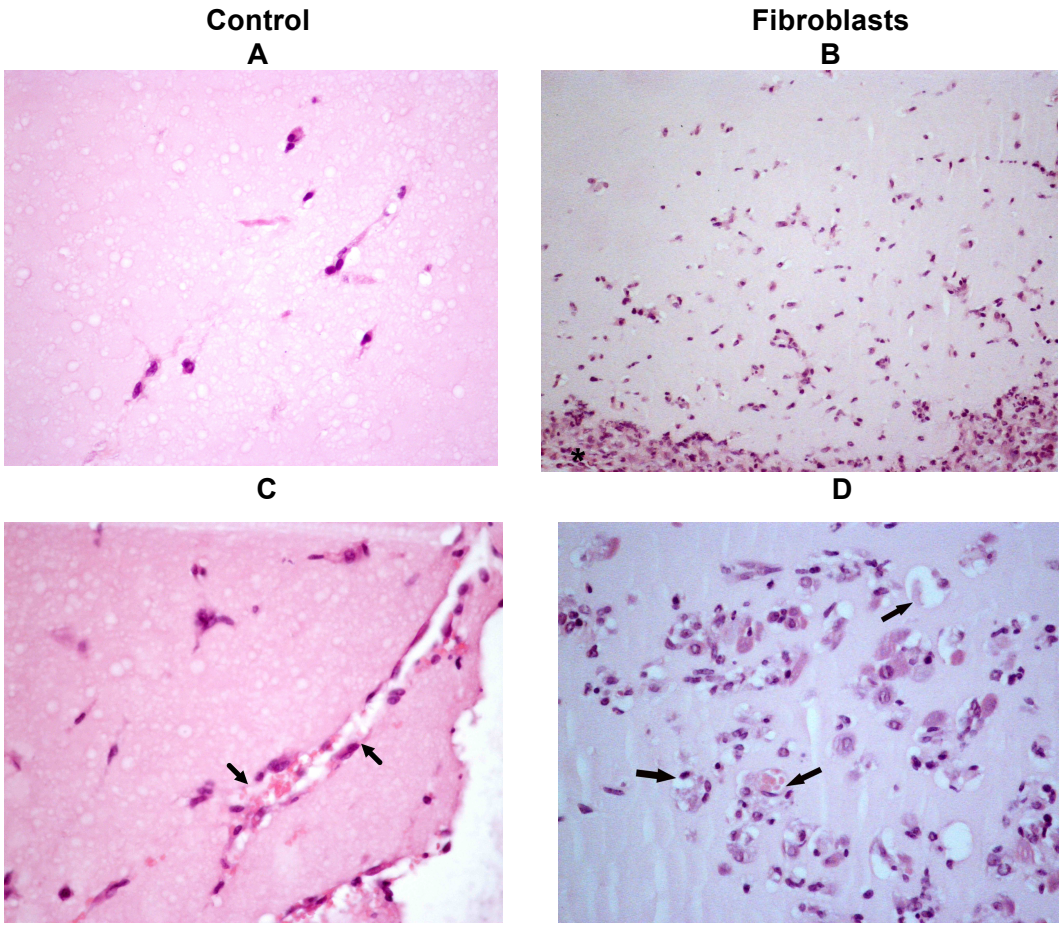

Figure 3. Representative images of hematoxylin & eosin staining of matrigel plugs implanted subcutaneously in C57BL/6 mice with (B and D) or without fibroblasts (control; A and C). (B) Asterisk pointing out population of cells in the border of the plug; (C) capillary-like tubes, arrow. (D) circular arranged cells, arrow. Original magnification: 400x (A, C and D) and 200x (B).

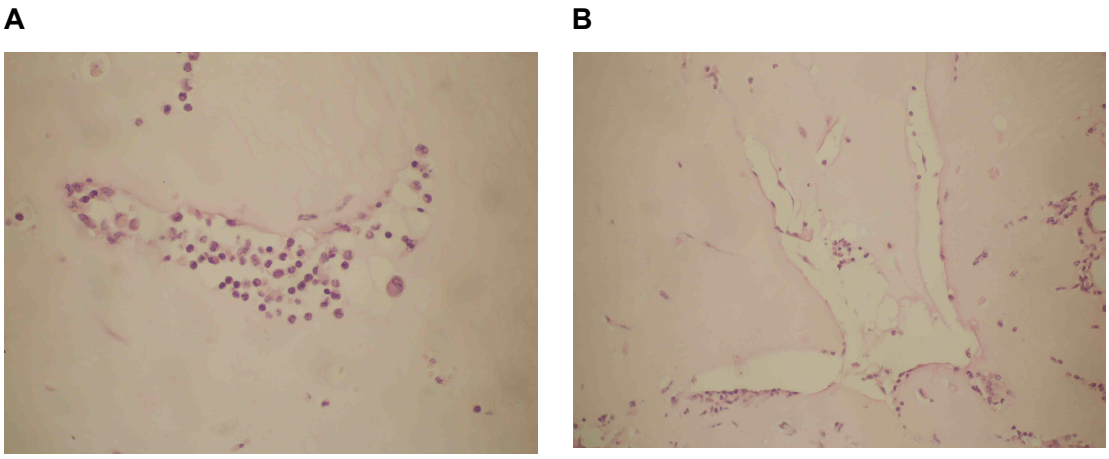

Figure 4. Branching network of vessels within the matrigel plus implanted with fibroblasts, with erythrocytes and inflammatory cells in the lumina: (A) vessel in the center of the matrigel; (B) interconnected vessels near the periphery of the matrigel plug. Original magnification 400x.

The *in vivo* recruitment of angiogenic network into the matrigel plugs implanted with or without fibroblasts was also evaluated. In order to visualize the formation of new blood vessels in the implanted plugs, histological sections were stained with antibodies against CD31 for the endothelial cells (mature vessels) and CD34 for progenitor endothelial cells that may also be present in initial steps of vessels formation. Neovascularization of the implanted matrigel plugs with fibroblasts was observed within 1 week after the injection. At this time it was possible to observe endothelial cells lining together and forming new blood vessels (Figs. 5B and 6A). The positive immunohistochemical staining for CD31, a marker for endothelial cells (black arrow, Fig. 5B), revealed the presence of microvessels (black circle, Fig. 5B). On the contrary, in the control group (Fig. 5A), only few endothelial cells were noticeable, marked positively for CD31, that were not organized in vessel structures.

In matrigel plugs implanted with fibroblasts (Fig. 6) it was also possible to observe endothelial progenitor cells, which collaborated in the process of formation of the blood vessels, and stained positive for CD34 (Fig. 6B). Near the periphery, it was observed vessel formation from the host vasculature staining positively for CD31 (Fig. 6C) and CD34 (Fig. 6D), indicating that both the mature and the progenitor endothelial cells participated in the process of vascularization.

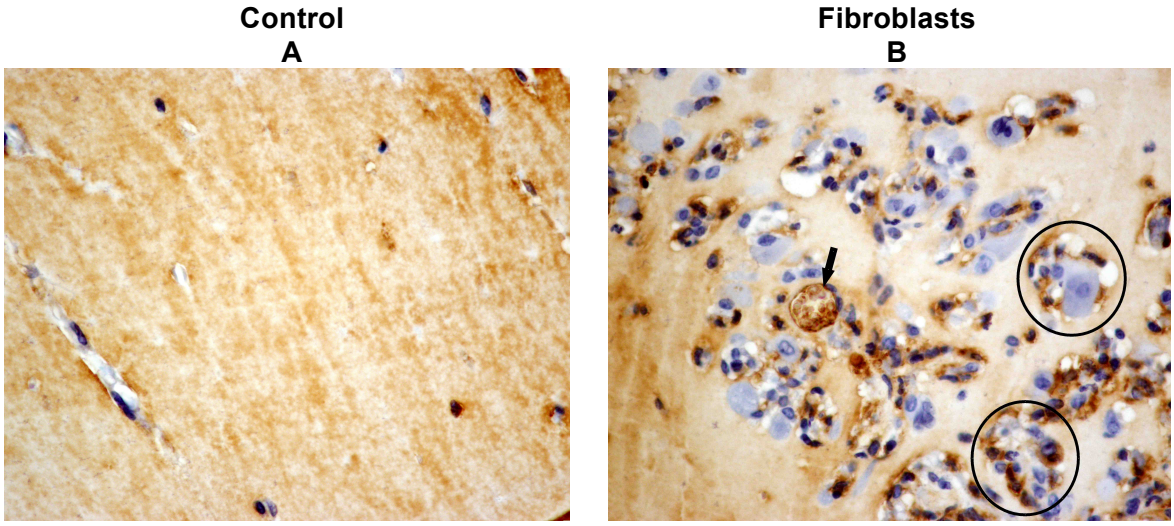

Figure 5. Cellular phenotypes colonizing matrigel plugs after 1 week of implantation. Representative images for CD31 staining: (A) matrigel implanted without fibroblasts (control) and (B) matrigel implanted with fibroblasts - arrow pointing out the CD31 positive cells and circular structures indicating the microvessels. Original magnification: 400x.

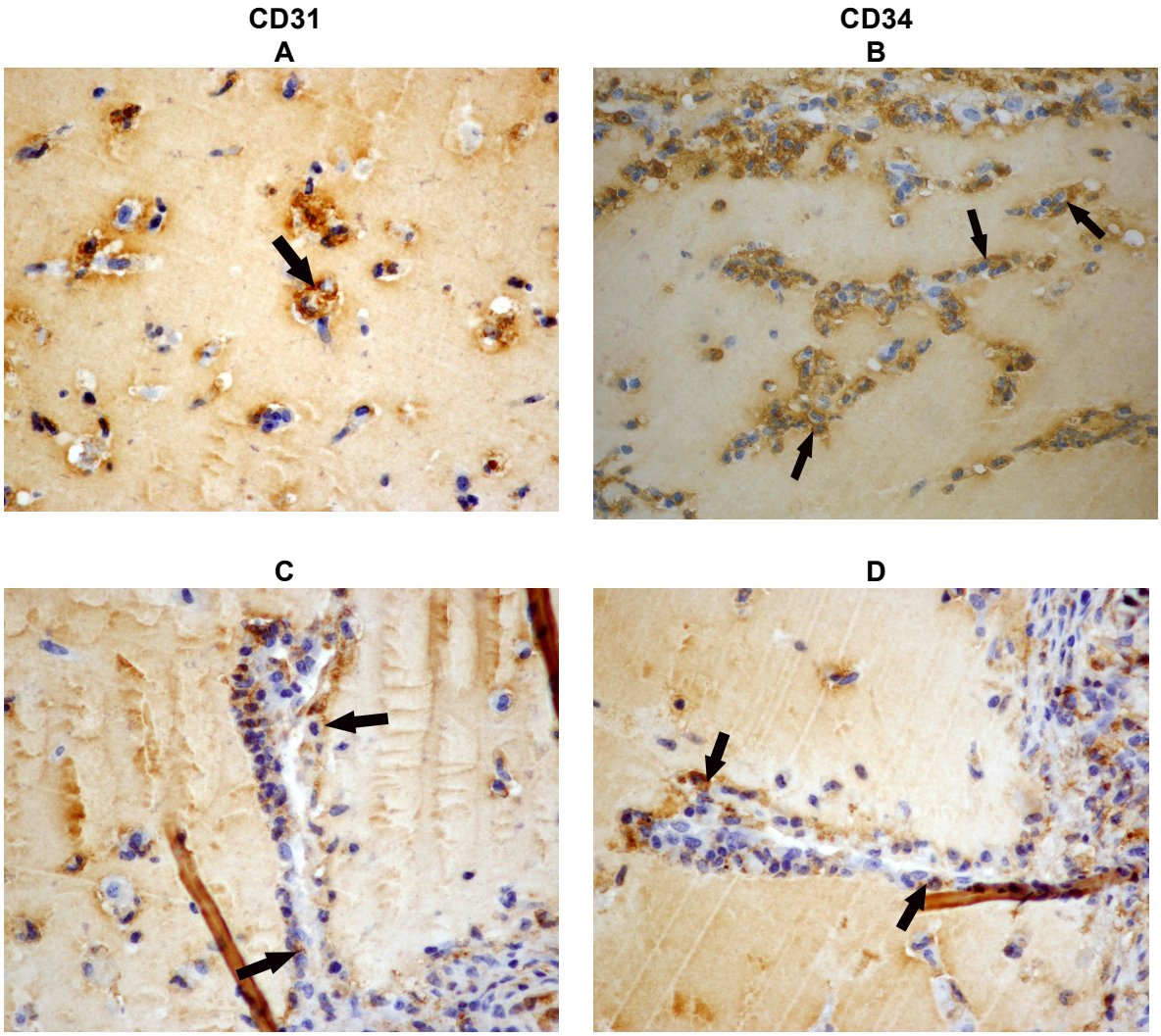

Figure 6. Cellular phenotypes colonizing matrigel plugs implanted with fibroblasts after 1 week of implantation. Representative images of stainings for CD31 and CD34: (A) microvessels formation, with arrows pointing CD31 staining; (B) microvessels formation, with arrows pointing CD34 staining; (C and D) consecutives images (C - CD31 staining; and D - CD34 staining), with arrows pointing the vessels formed from the periphery to inside the plug. Original magnification: 400x.

To access the presence of fibrillar collagen and to understand if the cells during the migration into matrigel leave a track of collagen that could guide the endothelial cells during angiogenesis, the Elastic van Gieson staining was used. Fig. 7A shows a track of collagen observed in the control samples (without fibroblasts). On the other hand, in the matrigel plugs implanted with fibroblasts (Fig. 7B) the collagen was observed in localized areas and around the microvessel (black arrow). This could be related with different time points of the vascularization of the matrigel.

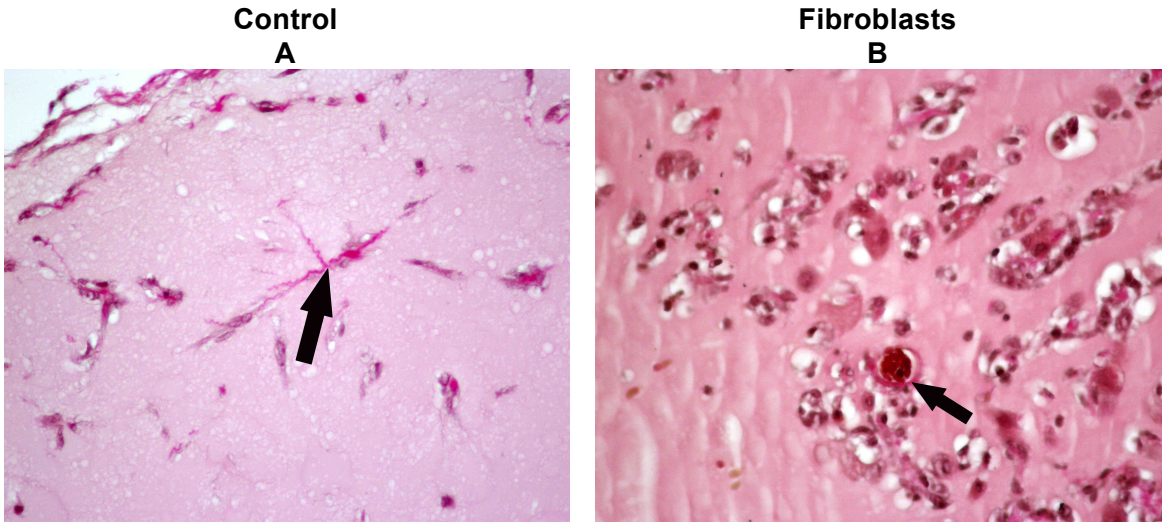

Figure 7. Elastic van Gieson staining identifying fibers such as collagen (pink color). (A) Matrigel implanted without fibroblasts (control), with arrows pointing the track of collagen. (B) Matrigel implanted with fibroblasts, with arrow pointing out the collagen in microvessels. Original magnifications: 400x.

Then, in order to access if fibroblasts produced collagen in the matrigel plug, ultrastructural studies (TEM) were performed in the explants after 1 week. In Fig. 8A fibroblasts are indicated by black arrows and the collagen produced by these cells are pointed out by black arrowheads. The high activity of protein synthesis that could be observed may be related with rough endoplasmic reticulum (RER, white arrow) presented over a large area of the cell (Fig. 8B). It was possible to observe the well-organized collagen bundles (black arrowhead) surrounded by the filopodia extensions of the fibroblast (white arrowhead, Fig. 8B).

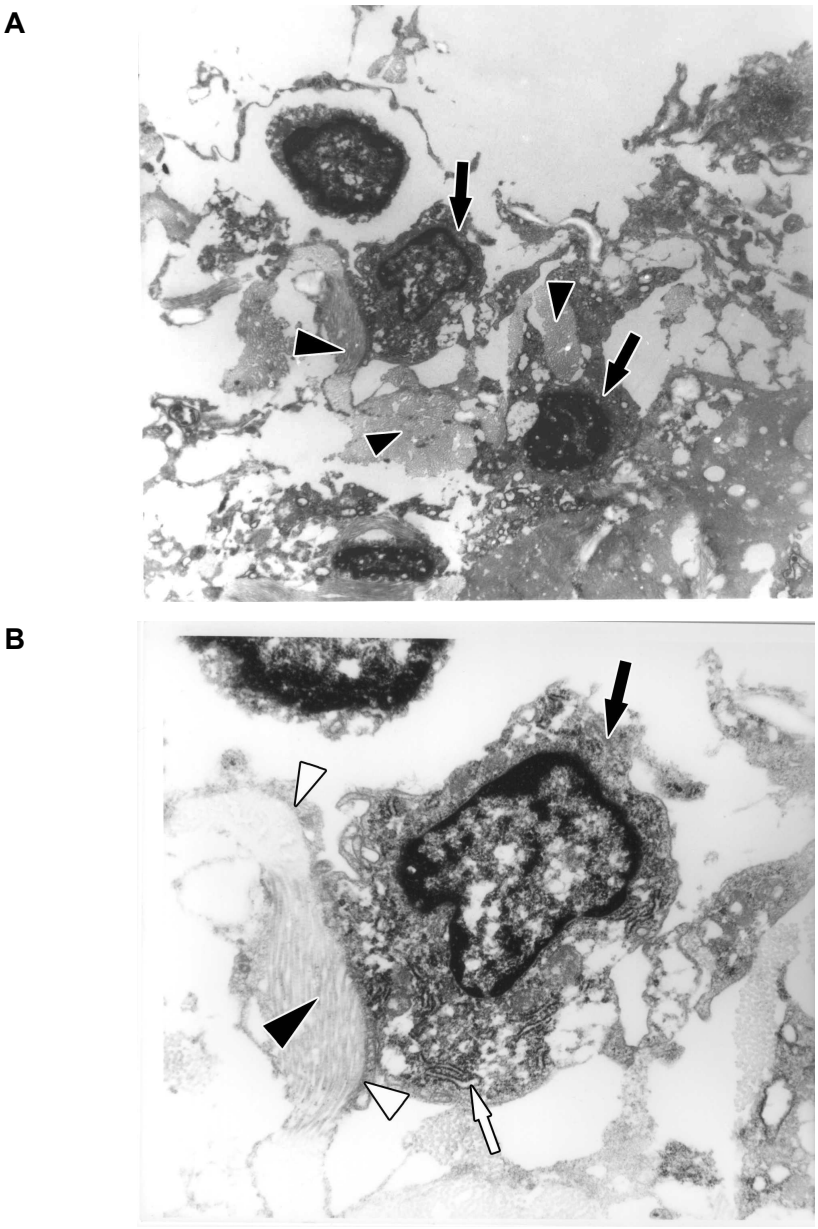

Figure 8. Ultrastructure of fibroblasts immobilized in a matrigel plug after 1 week of subcutaneous implantation by transmission electron microscopy. (A) The implanted fibroblasts produced collagen in the matrigel (black arrowhead indicates collagen; black arrows indicate fibroblasts); amplification: 6500x. (B) Magnified view of a fibroblast (black arrow), rough endoplasmic reticulum with high activity, high protein synthesis (white arrow), collagen (black arrowhead) surrounded by filopodia extensions of fibroblasts (white arrowhead); amplification: 17500X.

4. Discussion

Understanding how to promote angiogenesis in a damaged tissue, using cells embedded in an injectable biomaterial, continues to be a challenge in tissue regeneration. The need for proper vascularization, which involves the assembly of a microvascular network and its anastomosis to the host vasculature, remains one of the major hurdles for clinical success of complex tri-dimensional tissue engineered structures [23]. In order to address if neonatal human dermal fibroblasts could influence the formation of early microvascular structures the matrigel plug model was used. The matrigel plug assay has been described as a good model to study angiogenesis because it protects growth factors from degradation and dispersion [24] and additionally provides the embedded cells a viable environment to proliferate and differentiate without scattering [24].

The present *in vivo* study supports the hypothesis that implanted fibroblasts were able to recruit endothelial cells to vascularize an implanted artificial extracellular matrix with functional vessels after 1 week. As observed the same matrix implanted without fibroblasts did not vascularized, as confirmed by hemoglobin levels.

Soluble factors produced by fibroblasts, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), probably influenced the interactions with endothelial cells and their recruitment. VEGF is required for survival, differentiation, and network formation of endothelial cells [24]. bFGF promotes both endothelial cell scattering, that is required during the first step of the angiogenesis process, and the formation of the cell-cell interactions required for vessel maturation [24].

Angiogenesis and inflammation are intimately related since immune cells produce cytokines and growth factors that target endothelial cells and stimulated angiogenesis and, in turn, the new vessels formed transport oxygen and nutrients, as well as immune cells [25]. In tissue regeneration it seems important to modulate inflammation to an adequate level [26]. So, it was evaluated if the fibroblasts transplanted with matrigel changed the inflammatory profile, measuring inflammatory markers, namely NO and NAG. NAG is a lysosomal enzyme which activity is increased with monocytes and macrophages activation during inflammatory diseases [27]. NO is related with general inflammation and it is a vasodilator [28]. No significant differences were

detected in systemic inflammation between matrigel implanted with or without fibroblasts.

In matrigel plugs implanted with fibroblasts the presence of vessels with different endothelial cells, namely some with positive staining for CD31 and others for CD34, a marker for endothelial progenitor cells (EPC), outlines different stages of vascularization. The presence of vessels originating from the host vasculature is a remarkable observation, showing that host vascular response was involved, which is a key step for the clinical success of vascularization strategies. These findings indicate that the environment created by fibroblasts favors the recruitment of endothelial cells as well as the ingrowth of vessels from the host vasculature. EPCs are involved in promoting physiologic and pathologic neovascularization but questions remain about the magnitude of their contribution to newly forming blood vessels [29]. Melero-Martin et al. implanted EPCs in matrigel and reported no secretion of VEGF and no stimulation of murine vessel infiltration, although in cocultures of EPCs with mesenchymal stem cells better vascularization was observed [24].

Another key step in angiogenesis and vasculogenesis seems to be the production of new matrix. Mercier et al. reported that neurogenesis and vasculogenesis have implicated the participation of macrophages, fibroblasts and collagen [24]. Anghelina et al. reported that vascularization in a matrigel plug supplement with bFGF was influenced by initial cellular infiltration, with a predominant population of monocytes and macrophages [24]. The cells left a track forming cell columns after 1 week of implantation through secretion of angiogenic factors and enzymes able to degrade the matrix. A similar mechanism of cell invasion has been reported for leukocytes and tumor cells penetrating the matrix, or osteoclasts degrading and invading bone [24]. The amount of fibrillar collagen produced, apparently by fibroblasts, could derive from the circulating fibrocytes [30]. The present results showed the presence of collagen in matrigel plugs implanted with fibroblasts. By TEM, it was possible to observe the higher activity of the rough endoplasmic reticulum of fibroblasts, which is related with the production of collagen. Collagen acts as a potent pro-angiogenic milieu in itself [24], whereas the matrix-associated fibroblasts could lead the orientation of new blood vessels [31]. In addition, the microenvironment present in the implant is very important for cell fate. For instance, Yin et al. reported that the extracellular microenvironment (soluble factors, cell-matrix or cell-cell interactions) could influence the chondrogenic

differentiation of dermal fibroblasts [24], although Reddi et al. reported that the transformation of fibroblasts to chondroblasts was unstable when compared to osteoblasts [24]. Also, related with the ability of fibroblasts to synthesize collagen, Kellar et al. reported that dermal fibroblasts cultured in tridimensional structures could be used as a support or delivery system of cells, like stem cell populations in cardiac diseases [32]. Dermal fibroblasts have also been described as a population of cells with mesenchymal origin having a crucial role in communication with other cell types in response to contextual signals in skin physiology [33]. The present strategy revealed to provide an adequate environment stimulating collagen production by transplanted fibroblasts contributing to formation of new vessels as well as to the ingrowth of vessels from the host vasculature.

The matrigel plug assay is referred as an adequate model for *in vivo* studies of angiogenesis and vasculogenesis. Anghelina et al. suggested the use of matrigel plugs as experimental models of tissue regeneration, in which neovascularization is related with fibrosis and organogenesis and where inflammatory cells play a key structural role [24]. However, matrigel is not approved for use in humans. Taking this into account, new hydrogel systems need to be designed, providing an adequate microenvironment mimicking the natural extracellular matrix, with its capability to assure the signalling properties of soluble and insoluble components of the matrix. Alternative biofunctional hydrogels have been shown to provide adequate in vivo models of tumour angiogenesis [24]. New and complex biofunctional hydrogel matrices based on alginate have been recently developed, mimicking the extracellular matrix in its cell-specific adhesion and susceptibility to cell-driven proteolysis [24]. Other hydrogel-based systems have been reported as promising injective artificial extracellular matrices for cell transplantation and delivery [24]

5. Conclusions

The present *in vivo* study supports the hypothesis that fibroblasts embedded in matrigel plugs implanted in mice are able to recruit endothelial cells, including progenitor endothelial cells, to vascularize the matrix. The implanted matrigel with fibroblasts were further able to synthesize collagen and promote the ingrowth of vessels from the host vasculature. In the absence of an acute

inflammation the current strategy provides an adequate microenvironment for new strategies in tissue engineering, where neovascularization is essential.

Acknowledgements

The work carried out at INEB was financed by FEDER funds through the *Programa Operacional Factores de Competitividade* – COMPETE and by Portuguese funds through FCT – *Fundação para a Ciência e a Tecnologia* in the framework of projects PEST-C/SAU/LA0002/2011, PEST-OE/SAU/UI0038/2011 and POCI/SAU-BMA/55556/2004 and the PhD scholarship SFRH/BD/40421/2007 for S.G.G.

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CHAPTER V

Summarizing discussion and concluding remarks

Tissue engineering is a multidisciplinary field, which integrates knowledge from biomaterials science, molecular biology and biochemistry among others. The purpose of this field is to improve the quality of life of patients and increase their life expectancy. According to most of the data obtained on tissue engineering so far, one of the remaining limitations is the need of vascularization of the implant. However, many questions arise in order to understand what would the cues be in tissue regeneration to promote vascularization. Step by step, many improvements have been made in terms of biomaterial systems, in order to promote the delivery of endothelial cells and the recruitment of the host endothelial cells to the damaged area in different organs. The exact mechanism by which the extracellular matrix (ECM) acts to regulate migration, proliferation and differentiation of endothelial cells during the regenerative process needs to be elucidated, given the relevance of this phenomenon in tissue regeneration. The incorporation of growth factors and prevascularization of the implant could be a solution in order to create a microenvironment that allows the integration of the implant with host tissue in the right timing. Depending on the organ or tissue to regenerate, different strategies using heterotopic cell cultures were proposed. Nonetheless, the use of autologous bone marrow stromal cells as a source of different cell types related to the capacity of the cells to differentiate in different lineages opens a window in regenerative medicine. Stem cell properties of derived-bone marrow cells have been demonstrated [1]. Mesenchymal stem cells have been used in many studies to regenerate tissues. However, it has been demonstrated that mesenchymal stem cells and fibroblasts share much more features in common than it was established in the past [2]. In addition, fibroblasts were used to provide the appropriate niche for stem cell maintenance and differentiation *in vitro*. Nevertheless, fibroblasts have been shown to present different gene expression profiles depending on the specific tissue due to differential expression of homeobox (HOX) genes [3]. Beyond the roles usually attributed to fibroblasts in pathological conditions, namely contribution to pathological tumor development or fibrosis [4, 5], recent findings suggest that fibroblasts also play an important role in the angiogenesis process in tissue engineering [6-8]. Angiogenesis is a complex multistep process that requires the involvement of many cells types. Different cell types, such as pericytes and smooth muscle cells, are known to support the behaviour of ECs, thus playing a prominent role in

angiogenesis. However, the role of fibroblasts in modulating vascularization is poorly understood in the framework of tissue regeneration.

In this thesis (**chapter II**) alginate hydrogels were formulated to serve as implantable scaffolds to deliver fibroblasts and growth factors released by the cells. Previous reports showed that RGD-alginate microspheres support viability of pre-osteoblasts and mesenchymal stem cells [9, 10]. Although human umbilical ven endothelial cells (HUVEC) are a primary cell culture, not used for clinical application, our group has immobilized these cells in RGD-alginate as well and used this approach as an endothelial model to examine the interplay between endothelial cells (EC) and osteoprogenitor cells. This approach revealed that the presence of distinct types of cells (vascular wall cells and osteoprogenitor cells) enhanced each other's viability. Corroborating these findings, the use of growth factors such as VEGF and bFGF improved HUVECs viability in the 3D system, implying the relevance of interaction between distinct cell types in maintaining cells viability. In agreement, angiogenesis depends on major interactions between distinct cell types that release growth factors and ECM components [11]. Interactions between mesenchymal and epithelial cells are known to play an important role in orchestrating the development and morphogenesis of tissue and organs [3]. In order to create a microenvironment that could lead to the recruitment of host endothelial cells to the injury place, *in vitro* angiogenesis assays were performed in the current study. Fibroblasts entrapped in the RGD-alginate system were able to support the assembly of capillary-like structures by the HUVECs in matrigel-coated plates, and also influenced the area of sprouting in the aortic ring assay experiment. Both cell types produced soluble factors and this crosstalk probably activates signaling pathways that influenced sprouting and the organization of endothelial cells into a capillary-like structure in the extracellular matrix.

The presence of the RGD sequence in alginate promoted cell adhesion and probably mediated survival signals that involve $\alpha_v\beta_3$ integrin signaling pathways [9, 11-13], which will influence neovascularization in the implanted zone. Singh et al. found that fibroblasts immobilized in alginate produce collagen type I, leading to the assumption that these cells are capable of producing extracellular matrix inside the biomaterial, which can mediate growth factors release and cells differentiation [14]. Accordingly, another study highlighted the importance of osteoblasts producing collagen I that filled the spaces in the biomaterial and improved the formation of the microvessels structures when cocultured with

endothelial cells [15]. Levenberg et al. reported a close relationship between the neosynthesis of ECM and increasing levels of VEGF by fibroblasts in the formation and maintenance of the microvasculature [16]. Fibroblasts immobilized in the RGD-alginate increased the length of the capillary-like structures formed by endothelial cells, which is important for implant vascularization. These results suggest that growth factors produced by fibroblasts could act in a paracrine manner to induce and maintain capillary-like structures. The stimulation of production of metalloproteinases by the cells entrapped in alginate also seems to be important to neovascularization since it affects the degradation and remodeling of the extracellular matrix in the tissue [17]. The extracellular matrix is critical for blood vessel growth and maintenance by acting as scaffold support through which endothelial cells may migrate, and as a reservoir and modulator for growth factors. The present study was able to show that fibroblasts immobilized in RGD-alginate improved the heterotypic interactions within the microenviroment, resulting in a direct mechanism required for capillary formation.

Fibroblasts localize in peri-vascular regions of both small and large caliber vessels. To further investigate the interaction between ECs and fibroblasts, on **chapter III,** cocultures of endothelial cells from the macrovasculature (HUVECs) and microvasculature (HDMECs) with fibroblasts were used. Fibroblasts have been shown to exhibit plasticity by differentiating into other members of the connective tissue family, including cartilage, bone, adipose tissue and smooth muscle cells [18]. So far, it is not clear if fibroblasts present within the connective tissue, besides maintaining the ECM and connective tissue homeostasis, also act as tissue-resident progenitors cells [19, 20]. *In vitro* coculture systems have proven to be excellent models to analyze the cellular role of vascular-associated factors and to elucidate their intracellular signaling pathways [21]. In this study, fibroblasts were able to produce VEGF, explaining the effect on endothelial cell migration and microvessels formation in the literature [22, 23] and, in chapter II, when entrapped within alginate microspheres. ECs are known to release IL-8 that influences cell migration [24, 25]. In all cultures tested TGF-β1 was present and increased with time. TGF-β1 is known to influence the assembly and maturation of blood vessels [26], the production of collagen [27] and the activation of fibroblasts [4]. When fibroblasts are activated they became positive for alpha-smooth muscle actin (α-SMA) [28]. Cocultures of HUVECs or HDMECs established in direct contact with

human dermal fibroblasts (HDF) resulted in tubule-like structures formation. These vessel-like structures were present in areas with high expression of alkaline phosphatase (ALP) and activated fibroblasts. At the same time, collagen production increased in the cocultures along the experiment. On the other hand, VEGF production was high in HDF monocultures but observed only at much lower levels in cocultures of HUVECs/HDFs and HDMECs/HDFs. Low levels of TGF-β1 were also observed in cocultures in comparison to monocultures. These findings led us to hypothesize that VEGF is probably consumed upon activation of VEGFR2 in ECs cell membrane, resulting in survival, migration and differentiation of these cells [29, 30]. Another possibility is the presence of ECM in direct contact cocultures, which could be responsible, at least to a certain degree, for the entrapment of growth factors produced by the cells [31].

Fibroblasts activation plays an important role in regulating immune responses [18, 32] and it is also known that small peptides released during remodeling of the extracellular matrix have profound effects on cellular immune responses [18, 33, 34]. Fibroblasts exhibit considerable intra-line plasticity that is dependent on the microenvironment. Recently, it has been reported that adult fibroblasts can be converted into pluripotent stem cells by transfection with only four transcription factors (oct3/4, Sox2, c-myc and Klf4) [20, 35]. Nanog, a transcription factor that regulates early development [36], has been found to be present in porcine [37] and human dermal fibroblasts [20] suggesting that nanog is important in maintaining the plasticity found in fibroblasts *in vivo* [20]. The present results further suggest that fibroblasts are not a passive actor in the complex signaling pathways of angiogenesis. Rather, fibroblasts influence the formation of microcapillary-like structures and collagen deposition, suggesting a dependency between the formation of vascular structures and the assembly and thickness of the ECM, which is in agreement with previous studies [28]. Martins-Green *et al.* [20] have previously shown that peri-endothelial cells arise by differentiation from fibroblasts and that they require interaction with ECs for that purpose. Faggin *et al.* [19] suggested that fibroblasts appear to serve as vascular cell progenitors in the injured rabbit carotid artery. In the present study, and in agreement with Nigau *et al.* [20], it was observed that, in the vicinity of the capillary-like structures formed, some fibroblasts became positive for α-SMA. The extent of time of interaction between ECs of the capillary-like structures and fibroblasts could be a reason for these differences in terms of expression of activation markers.

Alkaline phosphatase (ALP) is a widely used marker for the differentiation of pre-osteoblasts or human mesenchymal stem cells (hMSC) under osteogenic conditions [10, 38]. Previous studies have used ALP as a marker to assess the differentiation of hMSCs into osteoblasts in coculture with ECs [11]. Although the expression of ALP has been widely related to osteoblasts differentiation and bone mineralization [39, 40], other perspectives and roles regarding ALP have been proposed. ALP has also been implicated in the regulation of cell growth and apoptosis [41]. In addition, ALP expression is often restricted both temporally and spatially to the population of cells involved in migration or morphogenesis [42], suggesting its involvement in cell guidance and migration. As far as we know, this is the first study showing the colocalization of microvessel like-structures with ALP. These findings support a possible role of ALP in cell-cell interaction (ECs-fibroblasts) [43] and cell migration [44-47]. Supporting this hypothesis, Friis *et al.* [48] reported an anti-angiogenic, dose-dependent, potential effect of levamisole on ECs propagated with fibroblasts, both *in vitro* and *in vivo* in a hetero-transplantation tumor model. Levamisole is a well-described and widely used tissue-non-specific alkaline phosphatase (TNS-ALP) inhibitor [49]. Levamisole-induced inhibition of microcapillary-like structures has been demonstrated although no clarification was provided about the roles of cell-cell interaction or cell migration in the ability of TNS-ALP to influence this process [48]. It was speculated that, in fibroblasts, ALP is a functional marker for a cellular subtype that is specialized in matrix degradation [50]. Furthermore, TNS-ALP has been reported to bind to collagen types I, II and X in the ECM [51, 52], which, together with our results, suggests an involvement of TNS-ALP in the angiogenesis process and particularly in the crosstalk between ECs and fibroblasts. Taken together, these results open the possibility of an important role of ALP in the modulation of angiogenesis and in cell adhesion that may be relevant for the development of new cell therapies in tissue regeneration.

Collagen is one of the major proteins in the ECM and is produced by fibroblasts in wound healing, and it is also known to drive ECs migration by chemotaxis and haptotaxis [53]. This observation suggests that the presence of collagen, and the fact that fibroblasts formed multilayers of cells in culture, induced ECs organization into microvessel-like structures, while these structures were absent in EC monocultures. Studies with cultured skin fibroblasts from a series of patients with osteogenesis *imperfecta*, a heritable defect that makes bones

brittle, demonstrated that the mutations produced specific disruption in the biosynthesis of collagen type I, thus generating abnormal collagens fibrils [54]. These facts will influence matrix composition and the interaction with growth factors and cytokines that is important in cell behavior [55]. The coculture model presented here offers the possibility to delineate how specific proteins and growth factors are regulated as a result of interactions between fibroblasts and endothelial cells.

Understanding how to promote angiogenesis in a damaged tissue, using cells embedded in an injectable biomaterial, continues to be a challenge in tissue regeneration. The need for proper vascularization, which involves the assembly of a microvascular network and its anastomosis to the host vasculature, remains one of the major hurdles for clinical success of complex tri-dimensional tissue engineered structures [56]. In **chapter IV,** in order to address how human dermal fibroblasts may influence the early microvascular structures formed on hydrogels and to observe the composition and fate of cellular infiltration, a Matrigel plug *in vivo* model was used. The present *in vivo* results support the hypothesis that fibroblasts can recruit endothelial cells from the host to vascularize the inoculated matrix. The Matrigel plug assay has been reported as a good model because it protects growth factors from degradation and dispersion [57, 58] and provides the embedded cells a viable environment to proliferate and differentiate without dispersion [59, 60].

Angiogenesis and inflammation seem to be interconnected processes, particularly in pathological situations. In fact, immune cells produce cytokines and growth factors, targeting endothelial cells to produce angiogenic factors. On the other hand, the newly formed vessels provide oxygen and nutrients for the immune cells [61]. Inflammation is a physiological response against harmful situations such as infection or tissue injury. In tissue regeneration, it seems important to prevent an exacerbated inflammation. For this reason, it was evaluated if the presence of fibroblasts in Matrigel would promote an increase in inflammatory markers (NO and NAG). No differences were observed in the levels of these markers in the presence and in the absence of fibroblasts inoculated within the Matrigel. Therefore, these findings suggest that the environment created by fibroblasts favors new vessels recruitment.

As previously mentioned, soluble factors produced by fibroblasts, such as VEGF and eventually others, required for the survival, differentiation, and network formation of ECs [29], could influence EC interactions in a paracrine manner.

Altogether, the previous findings led to the assumption that the transplantation of fibroblasts or other low differentiated cell types, results in the development of other cell phenotypes. Adipogenesis seems to be one of these responses [57]. Kawaguchi et al. [57] suggested the possibility that mesenchymal stem cells with fibroblasts morphology remain even in adulthood and differentiate into adipocytes, when invasion of fibroblast-like cells into a neovascularized Matrigel plug occurs. Therefore, neovascularization and adipogenesis seem to be parallel processes [57, 60]. On the other hand, Mercier et al. reported that neurogenesis and vasculogenesis involve the participation of macrophages, fibroblasts and collagen [62]. Endothelial progenitor cells (EPC) are implicated in promoting physiologic and pathologic neovascularization during wound healing and tumor growth, but questions remain about the magnitude of their contribution to newly forming blood vessels. Melero-Martin et al. implanted EPCs in Matrigel and reported no secretion of VEGF and no stimulation of murine vessel infiltration, although cocultures of EPCs with mesenchymal stem cells have shown a reciprocal and beneficial interrelationship between the two cell types, and relevant for vascularization strategies in regenerative medicine [59].

Anghelina et al. reported that vascularization in a Matrigel plug supplemented with bFGF is influenced by initial cellular infiltrate, predominantly composed by monocytes and macrophages [60]. The cells left a track forming cell columns after one week of implantation by secretion of angiogenic factors and enzymes capable of degrading the matrix. A similar mechanism of cell invasion has been reported for leukocytes and tumor cells penetrating the matrix, or osteoclasts degrading and invading bone [60]. The amount of fibrillar collagen, apparently produced by fibroblasts, could derive from the circulating fibrocytes [63]. The present results revealed the presence of fibroblasts secreting collagen by TEM analysis. The higher activity of rough endoplasmatic reticulum observed in fibroblasts was related with the production of collagen. Collagen acts as a potent pro-angiogenic milieu [64], whereas the matrix-associated fibroblasts guide new blood vessels [65]. From the standpoint of Kellar et al., dermal fibroblasts cultured in tridimensional structures could be used as a support or delivery system of cells like stem cell populations in cardiac diseases [66]. Dermal fibroblasts represent an essential population of cells with mesenchymal origin and have a crucial role in communication with other cell types in response to contextual signals in skin physiology [67].

Despite the relevance of the present findings, these studies were performed using Matrigel, a commercially available connective tissue, which is inadequate for implantation in humans. New and complex biofunctional hydrogel matrices based on alginate have been recently developed, mimicking the extracellular matrix in its cell-specific adhesion and susceptibility to cell-driven proteolysis [68]. These matrices, as well as other hydrogel-based systems, have been reported as promising injectable artificial extracellular matrices for cell transplantation and delivery and could be used instead of Matrigel [10, 68-73]. Nevertheless, these findings indicate that the microenvironment created by the stromal cells in the scaffold modulates angiogenesis through secretion and release of growth factors and collagen synthesis, which are likely to play key roles in the whole process of neovascularization.

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CHAPTER VI

Future perspectives

Although many interesting results were obtained in the present work, many other new questions arose and several issues should be further addressed in the future with the aim of generating a vascular network capable of rapidly anastomosing with the host vasculature in order to support the viability and integration of an implant.

The cell source used for cellular therapies aiming at promoting vascularization is a key issue. Fibroblasts are considered an important type of seed cell for tissue engineering and relevant in the crosstalk with endothelial cells and the immune system. However, the mechanisms behind these interactions are not yet clarified. The studies developed in this thesis were performed using HUVECs and HDMECs primary cell cultures, which are established and commercially available cell cultures. Since they cannot be used in the clinic, future work needs to be done using progenitor endothelial cells that have been reported to improve the development of vascular networks [1-3]. On the other hand, mesenchymal stem cells (MSCs) have been shown to serve as perivascular cells for *in vivo* vasculogenesis and could be used as a cell source for similar studies [4]. In addition, MSCs are one of the cell types under clinical trials for treatment of ischemic heart disease [6]. Moreover, MSCs may be isolated by minimally invasive procedures from a diversity of human tissues, including bone marrow [5], umbilical cord blood [6, 7], and adipose tissue [8]. It has also been reported that MSCs could differentiate into endothelial-like cells [9, 10], although the transplantation of MSCs alone promoted less vascularization than when MSC were accompanied with differentiated progenitor endothelial cells [4]. Dermal fibroblasts have been used in wound healing for chronic foot ulcers [11]. Nonetheless, for clinical use in ischemic situations other than in the skin, fibroblasts derived from other sources should be used. Taking into account that MSC and fibroblasts share some characteristics, it would be useful to understand if phenotypic alterations in mesenchymal stem cell-derived fibroblasts [12] could be influenced by soluble factors (VEGF, bFGF, TGF-β1, IL-8, among others) or extracellular matrix (ECM) components (collagen types I and IV, laminin and fibronectin). In addition, examining cell phenotypic alterations by immunocytochemistry and flow cytometry along the time (CD90, CD44, CD73, CD105, CD29, CD31, α-SMA, desmin, vimentin) would also enable to confirm the differentiating status of cells and their phenotypic profile.

Understanding the influence of bioactive molecules in cell differentiation is mandatory in order to achieve effective therapeutic strategies using

prevascularized biomaterials. The developmental status of tissues and different disease stages alter the microenvironmental regulation of stem cell behavior [13]. Endothelial-extracellular matrix crosstalk has been shown to require many biological factors, including VEGF, FGF and PDGF [14]. Cocultures of mesenchymal stem cells-derived fibroblasts with endothelial progenitor cells provide a means to examine how extracellular matrix components interfere with the organization of these cells and influence cell function. Technologies such as confocal laser microscopy, time-lapse microscopy using labeled cells and single-cell RT-PCR, single-cell microarrays and proteomics will be essential to identify the molecular mechanisms implicated. In addition, bioimaging analyses are an excellent tool to process this information.

Alkaline phosphatase (ALP) seems to play a part in angiogenesis as revealed by the present results, which indicated a crucial role in enhancing cell migration in the ECM. To further elucidate this phenomenon, gene and protein expression and colocalization studies, using fluorescent reported gene expression assays, as well as the inhibition of different ALP isoforms (such as tissue nonspecific, intestinal, and placental ALP), should be performed. These studies would enable the identification of the most relevant isoforms involved in this crosstalk between fibroblast-derived ECM components and angiogenesis, which can result in therapeutic benefits. Although Langer et al. reported that TNS-ALP represents the only ALP isoform present in the endothelium of blood vessels in brain [15], its role in angiogenesis and neurogenesis is still unclear.

The remodeling of the ECM plays a major role in controlling angiogenesis. The way in which biomaterials, as artificial ECMs, may enhance this role needs to be explored in cocultures of endothelial cells and fibroblasts. Taking this into account, it is important to evaluate the activity of metalloproteinases (MMP) and the ECM-derived proteins present in the supernatant of coculture assays (by zymography) since some of them could have pro- or anti-angiogenic effects. It is widely accepted that the pore size of scaffolds and their pore interconnectivity will influence the migration and organization of the pre-implanted cells. Instead, hydrogels susceptible to local proteolysis, such as RGD-alginate with a cleavable peptide by the action of MMPs [16], could be used to develop this prevascularized network. In addition, this biomaterial is free of proteins and growth factors, rendering this assay useful when compared to collagen gels or Matrigel. In addition, the presence of immune cells in cocultures should be investigated as well. Moreover, *in vivo* studies using these hydrogels would

provide valuable cues about the system's improvement. A valuable assay to assess vascularization of a therapeutically relevant clinical situation would consist in implanting, in a bone site, the this biofunctional alginate with cocultures of MSC-derived fibroblasts and EPCs to investigate promotion of vascularization (as well as anastomosis) and bone tissue regeneration *in vivo*. The sex-mismatch model, i.e., implanting male cells into female mice, allowing to examine the presence of colocalization for Y-chromosomes in the new vessels, seems to be a good model for *in vivo* studies and fate mapping.

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APPENDIX

Pectin-based injectable biomaterials for bone tissue engineering

BioMACROMOLECULES

ARTICLE

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Pectin-Based Injectable Biomaterials for Bone Tissue Engineering

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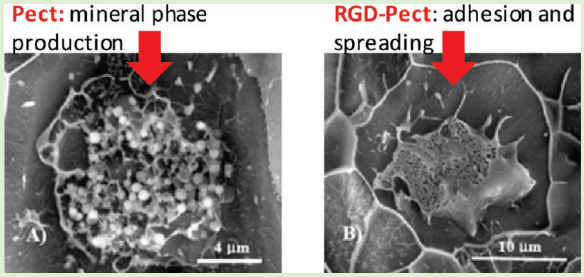
ABSTRACT: A variety of natural polymers and proteins are considered to be 3D cell culture structures able to mimic the extracellular matrix (ECM) to promote bone tissue regeneration. Pectin, a natural polysaccharide extracted from the plant cell walls and having a chemical structure similar to alginate, provides interesting properties as artificial ECM. In this work, for the first time, pectin, modified with an RGD-containing oligopeptide or not, is used as an ECM alternative to immobilize cells for bone tissue regeneration. The viability, metabolic activity, morphology, and osteogenic differentiation of immobilized MC3T3-E1 preosteoblats demonstrate the potential of this polysaccharide to keep immobilized cells viable and differentiating. Preosteoblasts immobilized in both types of pectin microspheres maintained a constant viability up to 29 days and were able to differentiate. The grafting of the RGD peptide on pectin backbone induced improved cell adhesion and proliferation within the microspheres. Furthermore, not only did cells grow inside but also they were able to spread out from the microspheres and to organize themselves in 3D structures producing a mineralized extracellular matrix. These promising results suggest that pectin can be proposed as an injectable cell vehicle for bone tissue regeneration.



1. INTRODUCTION

Natural polymers secreted by plants and bacteria are involved in the growth of organisms and in the intercellular signaling by regulating the ionic exchange at the cell surface.[1,2] They also protect the living organism by creating a molecular architecture acting as a barrier against external threats.

In tissue engineering, an ideal biomaterial would be able to reproduce these complex functionalities by mimicking the natural extracellular matrix (ECM), thus providing biospecific cell adhesion and the subsequent control of cellular functions. With this aim, the extracellular natural polymers from different sources can be considered to be potential candidates for substituting the ECM because of their typical gel-forming capability.

Among these polymers, alginates and pectins are polyuronates physiologically subjected to calcium-induced gelation,[3] resulting from specific and strong interactions between calcium ions and carboxyl groups of guluronate and galacturonate blocks in alginate and pectin, respectively.[4] The resulting egg-box structure can be reproduced in vitro[5] by interaction of the polymer with divalent cations, therefore allowing the immobilization of bioactive components or cells inside the gel structure. However, as other natural polymers, alginates and pectins resist protein adsorption and cell adhesion because of their hydrophilic nature.[6]

Cell adhesion to the matrix is required for the survival of many cell types. In particular, integrin-dependent cell adhesion, which consists of the interaction between integrin receptors and ECM, plays critical roles in many cellular functions, including migration, proliferation, differentiation, and apoptosis.[7,8] The RGD (Arg-Gly-Asp) sequence, present in adhesive ECM proteins, was identified by Pierschbacher et al.[9] as the minimal peptide sequence required for the adhesion of integrins to the ECM components.[10−12] Rowley et al.[6] first described the modification of alginate with an RGD-containing oligopeptide using the carbodiimide chemistry to graft covalently the oligopeptide to the carboxyl groups of alginate. Further studies showed that the immobilization of preosteoblasts or osteoprogenitor cells within RGD-modified alginate promoted cell viability, metabolic activity, adhesion, and differentiation.[13,14]

In this work, we propose to use pectin as a cell carrier able to stimulate bone tissue formation. Pectin was modified with RGD-containing oligopeptides through the carbodiimide chemistry (Figure 1) and compared with unmodified pectin in promoting cell adhesion. Pectin and RGD-pectin microspheres are here proposed as a cell vehicle for bone tissue regeneration.

Pectins are mainly used as thickeners or jellifying agents in the food industry, and they have also been recently considered for

Received: September 17, 2010
Revised: January 14, 2011
Published: February 08, 2011

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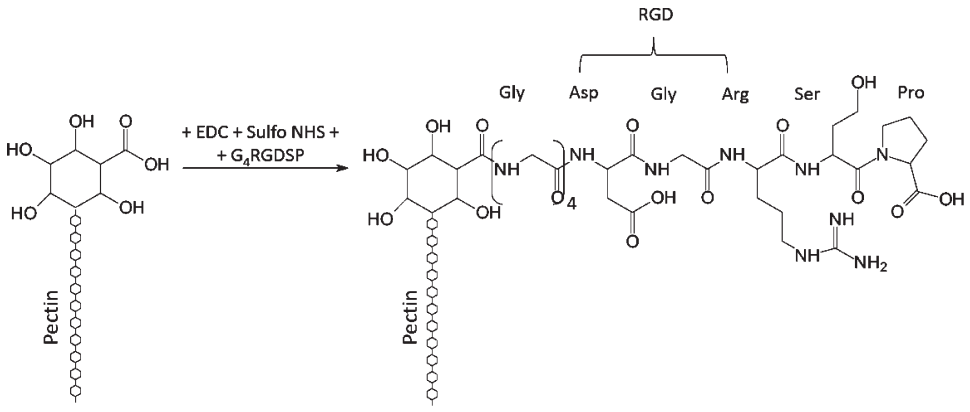

Figure 1. Functionalization of pectin with the RGD-containing oligopeptide.

drug delivery. In particular, pectin gels or tablets are used for targeted drug release in the colon because of pectin's ability to remain intact throughout the gastrointestinal tract and to be degraded by the colon's microflora.[15,16] In recent works, pectin has been proposed for cancer treatments because of its intrinsic properties[17−19] and for gene therapy.[20,21] However, only a few studies have investigated the use of pectin as a biomaterial for tissue regeneration.[22,23]

In a bone tissue engineering context, pectin was found to have a great potential. Previous studies demonstrated that it is possible to trigger the growth and differentiation of mammalian bone cells by enzymatically modifying the branched regions of pectin (rhamnogalacturonan I and rhamnogalacturonan II). Kokkonen et al.[24] used two enzymes to obtain different pectin branched regions from apple tissues. It was reported that local bone growth and differentiation can be stimulated by rhamnogalacturonans with shorter side chains.

Pectin gels are attractive cell carriers also because their solubility can be more easily modulated compared with other natural polymers, such as alginates. Calcium ions can be quickly displaced from pectin egg-box structure by other counterions (such as Na^+), leading to the formation of soluble pectin from the insoluble gel. Therefore, pectin gels may be considered as externally tunable systems for the release of immobilized cells or therapeutic agents. Furthermore, the gels can promote the nucleation of a mineral phase on pectin microspheres,[25] with the formation of biomimetic constructs, better mimicking the natural architecture of the bone.

2. MATERIALS AND METHODS

2.1. Pectin Purification and Coupling with G_4RGDSP Oligopeptides. Low methoxyl (LM) pectin, kindly provided by Herbstreit & Fox Neuenbuerg (Germany), was purified by washing pectin (10 g) in ethanol (100 mL, 50% (v/v)) for 30 min, followed by separation through filtration. Then, pectin was rinsed twice with ethanol (75 mL 50% (v/v) and 50 mL 95% (v/v)) and finally dried until constant weight.

Pectin was covalently modified (Figure 1) with the oligopeptide $(glycine)_4$-arginine-glycine-aspartic acid-serine-proline (Commonwealth Biotechnologies, Richmond, VA), using aqueous carbodiimide chemistry[6] as follows: Pectin solution (1% (w/v)) was prepared by dissolving pectin overnight in 2-(*N*-morpholino) ethanesulfonic (MES) acid buffer (0.1 M MES buffering salt, Sigma, and 0.3 M NaCl, Merck), and the pH was adjusted to 6.5 with 1 M NaOH. Then, sulfo-NHS (Pierce Chemical, 27.40 mg), EDC (Sigma, 48.42 mg), and the oligopeptide (16.70 mg) were added per gram of pectin, and the solution was allowed to react for 20 h under constant stirring. The reaction was quenched with hydroxyl amine (Sigma, 18 mg), dialyzed against distilled water and NaCl for 3 days, then treated with activated charcoal (Sigma, 0.5 g) for 30 min, filtered, and lyophilized.

2.2. Cell Immobilization Inside Microspheres. MC3T3-E1 preosteoblasts (purchased from ECACC - European Collection of Cell Culture, Porton Down, Salisbury Wiltshire, U.K.; from mouse calvaria) below passage 15 were cultured in 300 cm^2 flasks and grown in α-minimum essential medium (α-MEM, Gibco), supplemented with fetal bovine serum (FBS 10% (v/v), Gibco) and penicillin/streptomycin (P/S 1% (v/v), Gibco). Cells were kept at 37 °C with 5% CO_2 and 95% humidity, and the medium was changed every 2 days.

Microspheres of pectin and RGD-coupled pectin (RGD-pectin) were prepared from aqueous solutions (2.4% (w/v)) filtered with 0.2 μm filters. The cells were harvested by trypsin treatment after reaching 90% confluence and were carefully mixed with pectin or RGD-pectin solutions (at a density of 20×10^6 cells/mL) using a dual-syringe system to obtain homogeneous mixtures, as previously described.[13,14] Microspheres without cells were also generated as control. Microspheres were obtained using an electrostatic bead generator system (VarV1 encapsulation unit, from Nisco) at a speed of 20 mL/h and 5 kV electrostatic potential using an isotonic $CaCl_2$ solution (0.1 M) as gelling agent. After gelation, the microspheres were washed twice with α-MEM and incubated in complete α-MEM under dynamic conditions in spinner-flasks up to 29 days. The culture medium of pectin and RGD-pectin microspheres carrying cells was supplemented with ascorbic acid (Merck, 50 μg/mL) and β-glycerophosphate (Sigma, 10 μg/mL) to induce osteogenic differentiation. The experiment was repeated twice to check for the reproducibility of the results.

2.3. Microsphere Dissolution Test. Pectin and RGD-pectin microspheres (1 g/mL) were incubated in phosphate-buffered saline (PBS, pH 7.4) at 37 °C after preparation. In each time stage, the incubation solution was removed, and the microspheres were dried at 37 °C until constant weight. The dried mass variation was determined with the following eq 1

$$\Delta w = \frac{(w_t - w_0)\cdot 100}{w_0} \quad (1)$$

where w_t is the dry weight at time point t and w_0 is the initial dry weight of the microspheres. Analyses were performed using six samples per time point, and the error was calculated with standard deviation.

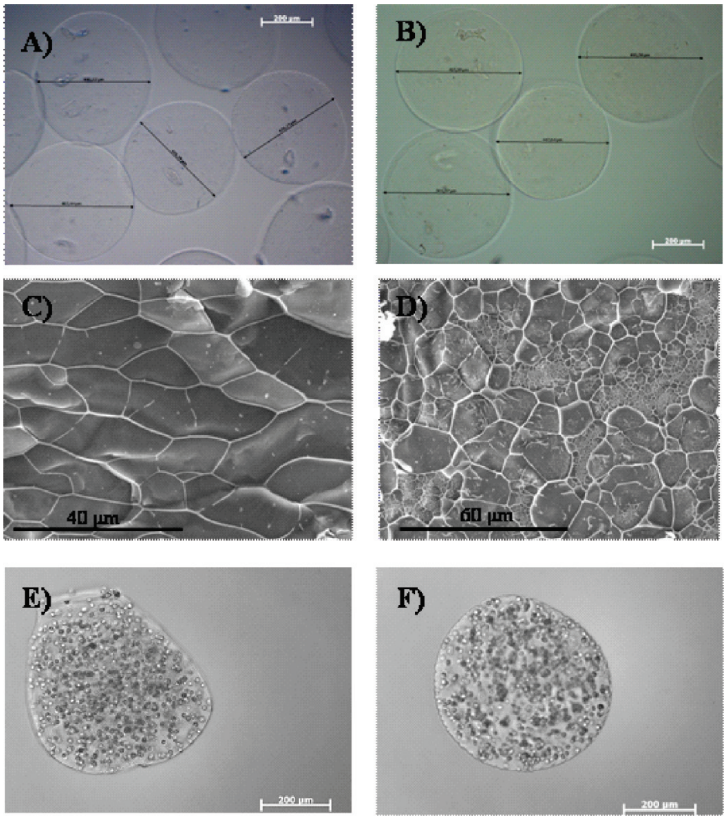

Figure 2. Diameters and spherical morphology of (A) pectin and (B) RGD-pectin microspheres acquired by optical microscopy. Structure and porosity of microspheres, acquired by CryoSEM of (A) pectin and (D) pectin coupled with a peptide containing the RGD sequence after cross-linking with calcium ions. Optical microscopy images of MC3T3-E1 cells distribution inside (E) pectin and (F) RGD-pectin microspheres. Scale bars of optical microscopy images = 200 μm.

Table 1. Dissolution of Pectin Microspheres at High Concentration in PBS (1g/1 mL)[a]

time	weight variation [%]	
	pectin	RGD-pectin
0	0 ± 0	0 ± 0
10 min	+60 ± 9	+1 ± 11
30 min	+33 ± 14	+3 ± 12
1 h	−20 ± 11	+1 ± 11
4 h	−62 ± 37	−51 ± 13
24 h	−85 ± 17	−98 ± 7

[a] Positive or negative values indicate an increase or decrease in weight of microspheres, respectively, in relation to the reference (weight of microspheres at time 0). After 24 h, pectin and RGD-coupled pectin microspheres were completely dissolved. Values indicated are mean ± standard deviation.

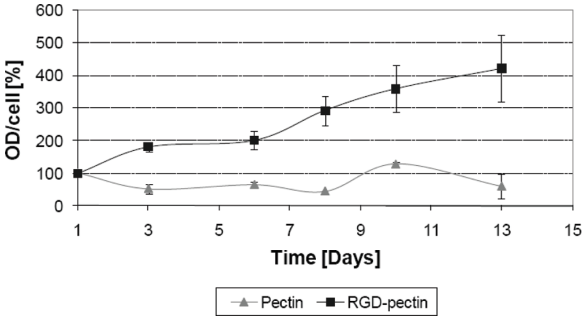

Figure 3. Metabolic activity of MC3T3 cells immobilized in pectin and RGD-pectin microspheres measured after 1, 3, 6, 8, 10, and 13 days of dynamic culture. The metabolic activity of the first day after cell immobilization was taken as the reference (100%).

2.4. Cell Characterization. *2.4.1. Cell Metabolic Activity.* The resazurin reduction assay was performed on the cells within microspheres after 1, 3, 6, 8, 10, and 13 days of dynamic culture to measure cell metabolic activity. This assay depends on the ability of metabolically active cells to reduce the amount of oxidized resazurin (blue) to resorufin (red), which exhibits strong emission at wavelengths >550 nm. Pectin and RGD-pectin microspheres, with or without cells, were recovered from the spinner flasks and incubated with resazurin solution (200 μL, 10% in complete α-MEM) in a 96-well culture plate for 4 h. The absorbance of 100 μL of each well was measured with a KC4 Synergy HT (Biotek Instruments) plate reader (absorbance read at 570 nm with reference to 600 nm). Microspheres containing cells were subsequently dissolved in each well plate using ethylenediaminetetraacetic acid (EDTA 50 mM, Sigma), and the cells were counted. Cell

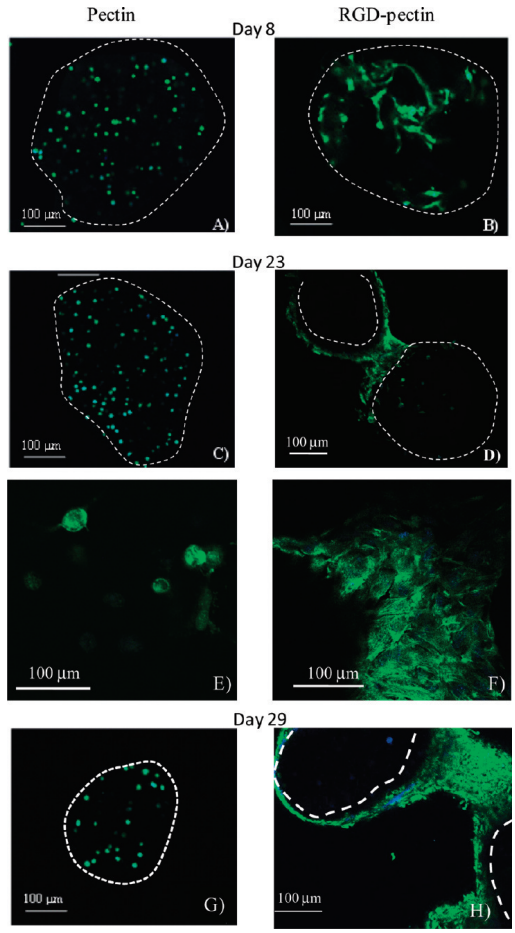

Figure 4. Confocal microscopy images of F-actin staining of cells immobilized on pectin and RGD-pectin microspheres: phalloidin marks the actin of the cytoskeleton (green) and DAPI labels the nuclei (blue) of MC3T3 cells. From day 8 of incubation onward, cells immobilized in RGD-pectin microspheres exhibited spreading and adhesion to the matrix (B) and created 3D connections among different microspheres (D and H). Cells immobilized in pectin microspheres, instead, maintained the round shaped morphology (A,C,G) during the 29 days of dynamic culture. The dashed lines indicate the borders of the microspheres or of the aggregates of microspheres. Images of single cells were acquired at higher magnification (40×) at day 23 to show round cells immobilized in pectin microspheres (4E) and spread cells growing in the space between adjacent microspheres (4F).

metabolic activity was quantified for each well using the following eq 2

$$(\mathrm{Abs_{cell}} - \mathrm{Abs}) / n \qquad (2)$$

where $\mathrm{Abs_{cell}}$ is the absorbance of microspheres with cells, Abs is the absorbance values of microspheres without cells, and n is the number of counted cells.

2.4.2. Cell Structure. At days 1, 3, 8, 16, 23, and 29 postimmobilization, samples of microspheres were washed in $CaCl_2$ (0.1 M) and fixed in formaldehyde (3.5% (v/v)). The microspheres were incubated with Alexa Fluor 488 phalloidin (6.6 μM in 0.1 M $CaCl_2$, Molecular Probes, Ex 495/Em 518 nm) at 37 °C for 20 min to stain actin filaments (f-actin) and then with DAPI (1.5 μM) for 10 min at room temperature to

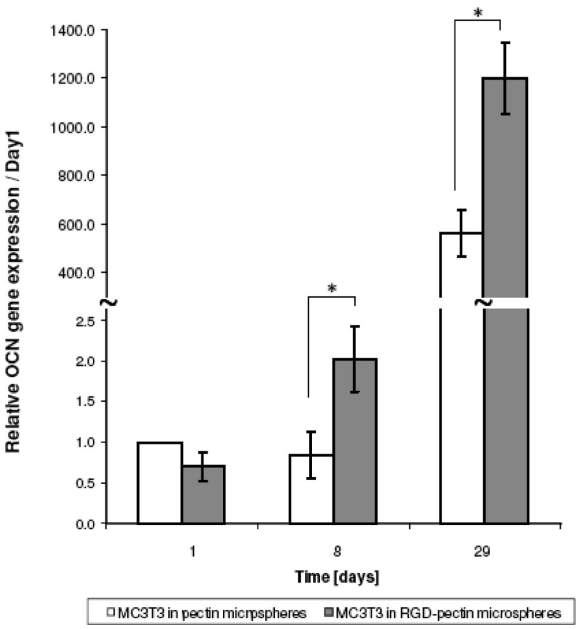

Figure 5. Osteocalcin (OCN) gene expression of immobilized cells. Expressed data are relative to day 1. The amount of the OCN gene expressed by cells increased with time. When MC3T3-E1 cells were immobilized in RGD-pectin microspheres, gene expression of OCN increased significantly, compared with pectin microspheres.

visualize the cell nuclei. Images were acquired by laser scanning confocal microscopy (Leica SP2 AOBS SE, Leica Microsystems, Germany) through the objective HC PL APO Lbl. Blue 20×/0.70 IMM/CORR (used with water).

2.4.3. Osteocalcin Expression. After 1, 8, and 29 days of in vitro culture, samples of microspheres were collected and dissolved in EDTA (50 mM). Cells were washed in cold PBS by centrifugation. Total RNA was extracted from the cells and purified using RNeasy Mini Kit (Qiagen); RNA (500 ng) was used as template for single-strand cDNA synthesis with the Superscript II reverse transcriptase kit (Invitrogen) in a final volume (20 μL) containing oligo(dT) 12−18 (0.5 μg/μL), dNTPs (10 mM), RT buffer (10×), $MgCl_2$ (25 mM), DTT (0.1 M), and RNaseOUTribonucleaseRNase inhibitor. Real-time semiquantitative PCR was performed in a reaction (final volume 20 μL) containing cDNA (1 μL), the primers sequence (200 nM), SYBR Green (10 μL, Invitrogen), and sterile distilled water. Primers for osteocalcin (OCN) (forward 5′-CTCTGTCTCTCTGACCTCACAG-3′, reverse 5′-CAGGTCCTAAATAGTGATACCG-3′) and -actin (forward 5′-GTGGGCCGCTCTAGGCACCAA-3′, and reverse 5′-GTCTTTGATGTCACGCACGATTTC-3′) were used at the annealing temperature of 56 °C.

2.4.4. Histochemical Staining. The osteogenic differentiation of the immobilized cells was evaluated through Von Kossa, Alizarin red, and Masson's Trichrome stainings after 1, 3, 9, 16, 23, and 29 days of dynamic culture. The microspheres were collected, fixed in formaldehyde (3.5% (v/v)), dehydrated with a graded series of ethanol, and embedded in paraffin blocks. Sections (8 μm of thickness) were prepared, dewaxed in xylene (Merck), and rehydrated in a decreasing graded series of ethanol followed by pure water.

Von Kossa and Alizarin red stainings allow the detection of matrix mineralization induced by differentiated osteoblastic cells. The Von Kossa staining is based on the combination of silver with insoluble salts like phosphates and carbonate bound to calcium, whereas Alizarin red complexes with calcium in a chelative process. For Von Kossa staining,

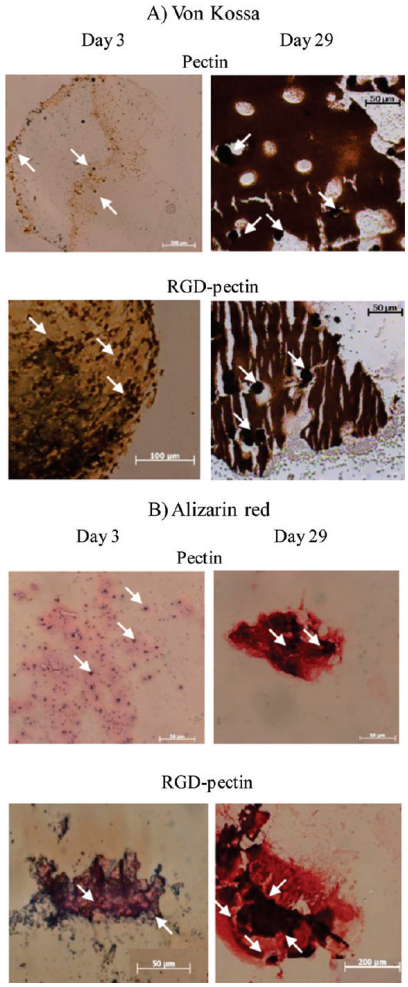

Figure 6. (A) Von Kossa and (B) Alizarin red staining of MC3T3-E1 cells immobilized in pectin and RGD-pectin microspheres after 3 and 29 days of dynamic culture. Phosphates and carbonates bound to calcium are stained in brown (A), and calcium deposits in red (B). The arrows indicate some of the immobilized cells.

the sections were incubated in silver nitrate (Merck, 2.5% (w/v)) for 30 min, followed by incubation in sodium thiosulfate (Sigma, 5% (w/v)) for 3 min. In the case of Alizarin red staining, the sections were incubated in Alizarin red solution (2% (w/v), pH 4.3) for 10 min. The slides were kept at 4 °C until observation by optical microscopy.

The Masson's trichrome staining was performed as indicated by the manufacturer (Sigma kit HT15-1KT), allowing us to investigate the formation of collagen fibers in the ECM.

2.5. CryoSEM and EDS Analyses. Images of the microspheres were acquired by cryoSEM (cryo scanning electron microscopy) at days 1, 8, and 29 postimmobilization. The microspheres were rapidly immersed in liquid nitrogen ($T = -210$ °C) for 3 min and vacuum-transferred to an Alto 2500 (Gatan) cryo preparation chamber coupled to the JEOL JSM 6301F scanning electron microscope. Frozen samples were mechanically fractured, and the temperature was set at −95 °C for 2.5 min to sublimate the water from the microspheres. Finally, the fractured microspheres were coated with Au/Pd and observed at −130 °C using a primary accelerating voltage (15 kV). Microanalysis was performed with an energy dispersive spectrometer (EDS) to identify and label the atoms of interest.

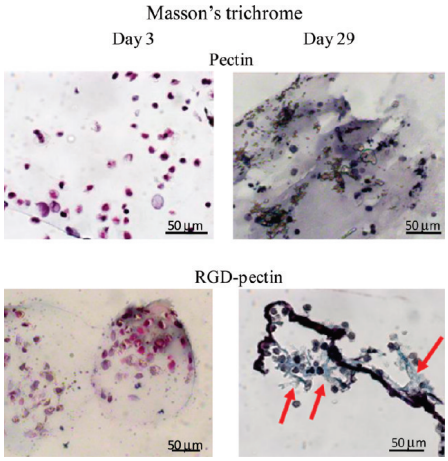

Figure 7. Masson's trichrome staining, which stains collagen in blue, cytoplasm in red and nuclei in black, performed on MC3T3-E1 cells immobilized in pectin and RGD-pectin microspheres after 3 and 29 days of dynamic culture. The arrows show the presence of collagen in RGD-pectin microspheres after 29 days of incubation. Scale bars = 50 μm.

2.6. Statistical Analyses. All measurements regarding cell viability, metabolic activity, and gene expression were tested in triplicate. Student's t test was used to make comparisons among the data, and the results were considered statistically different when $p < 0.05$.

3. RESULTS

3.1. Preparation of Pectin Microspheres. Homogeneous and spherical pectin and RGD-pectin microspheres were obtained using an electrostatic bead generator system. The diameter of the microspheres varied from 300 to 530 μm (Figures 2A,B,E,F), and their internal structure was found to be dependent on the presence, or not, of the peptide containing the RGD sequence. By using the cryoSEM technique, it was observed that RGD-pectin microspheres (Figure 2D) showed a different texture in comparison with pectin microspheres (Figure 2C), namely, an apparently tighter pore structure, which is probably related to the RGD grafting. Nevertheless, although the RGD grafting modified pectin structure, thus affecting hydrophilicity and the ability to form cross-links, these structural changes did not produce noticeable effects on the swelling tests performed (data not shown), where no differences between pectin and RGD-pectin microspheres were measured.

3.2. Microsphere Dissolution Tests. Pectin microspheres, modified or not, resulted highly susceptible to ion exchange: when suspended at low concentration in phosphate-buffered saline (PBS), tris-buffered saline (TBS), or other solutions containing Na^+ ions, they rapidly dissolved. Using higher amounts of microspheres, they dissolved as well, but the solubilization was found to be slower (Table 1). For this reason, the protocols originally used to prepare and characterize alginate microspheres[13] were slightly modified, using an isotonic $CaCl_2$ solution (0.1 M) for washing the microspheres instead of TBS.

3.3. Viability and Metabolic Activity of Immobilized Cells. MC3T3-E1 preosteoblasts were used as a model cell line because of their high growth rate and because of the fact that they can be easily differentiated into the osteogenic pathway in vitro.

The immobilized cells were homogeneously distributed within the microspheres (Figure 2E,F), and the majority of

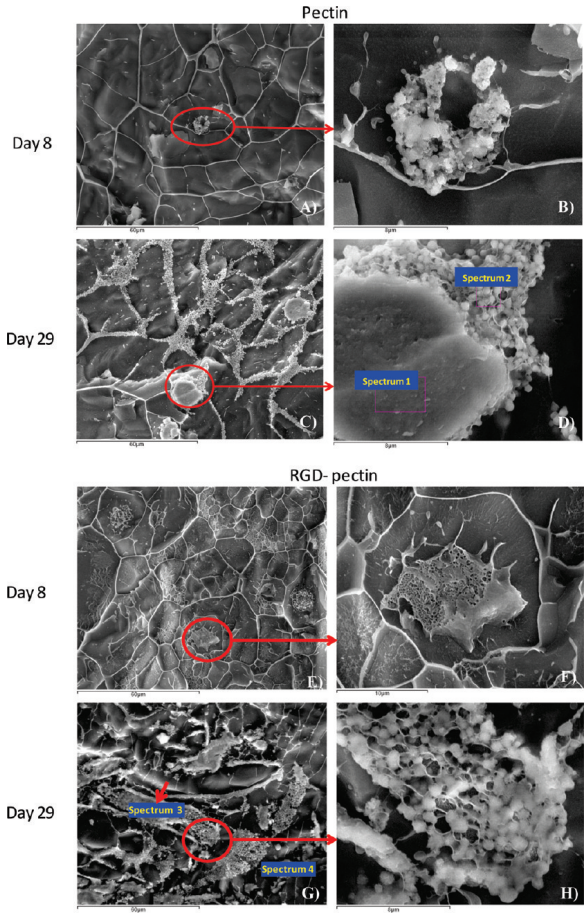

Figure 8. CryoSEM images of immobilized MC3T3-E1 cells after 8 and 29 days of culture inside unmodified and RGD-grafted pectin microspheres. (A−D) Cells immobilized in pectin microspheres were kept round and produced mineralized nodules. Cells immobilized in RGD-pectin microspheres adhered and spread (E−H) with observable cytoplasmic extensions (F). After 29 days of incubation, mineralization could be observed both on the cells and on the internal structure of the microspheres (C,D and G,H). The circles identify the regions where images at higher magnification were acquired. The blue labels indicate the spots where the EDS analyses were performed.

the cells remained viable throughout the 29 days of the experiment.

MC3T3-E1 cells immobilized in pectin microspheres exhibited a constant metabolic activity, which was significantly lower than the metabolic activity measured for cells immobilized in RGD-pectin microspheres at any time point analyzed (Figure 3). A significant increase in the metabolic activity of cells loaded in RGD-pectin microspheres was observed after the sixth day of dynamic culture (Figure 3). The metabolic activity could not be measured from the 13th day of incubation onward because of the consistent mineralization of pectin and RGD-pectin microspheres promoted by cells, which hindered the dissolution of the microspheres.

3.4. Cell Structure. MC3T3-E1 cells immobilized within pectin microspheres maintained a round morphology during the 29 days of the experiment, as shown by the fluorescence staining of F-actin (Figure 4A,C,E,G). On the opposite, cells immobilized within RGD-pectin microspheres were already adhered and spreading to the RGD-pectin matrix after 8 days of dynamic culture (Figure 4B). Furthermore, it was possible to observe connections between cells from adjacent microspheres, constituting a 3D bridge-like structure. The mineralized ECM matrix produced by these cells embedded several adjacent RGD-pectin microspheres in insoluble aggregates and did not allow staining the core of these microspheres (Figures 4D,F,H).

3.5. Cell Differentiation. OCN gene expression was assessed to evaluate the osteogenic differentiation. After 29 days of dynamic culture, MC3T3-E1 immobilized in pectin or RGD-pectin microspheres highly expressed the OCN gene (Figure 5). Moreover, at days 8 and 29 postimmobilization, the cells immobilized in pectin grafted with the RGD-containing oligopeptide showed a significantly higher OCN gene expression, as compared with nonmodified pectin.

MC3T3-E1 cells loaded in pectin and RGD-pectin microspheres produced a mineralized matrix, as shown by Von Kossa (brown deposits) and Alizarin red (red deposits) stainings of the microspheres (Figure 6). The mineralization appeared to be produced earlier from day 3 onward, by cells immobilized in RGD-pectin microspheres, although after 29 days of culture, it was comparable to that produced by cells immobilized in pectin microspheres (Figure 6). The Masson's trichrome staining showed the formation of a collagen-containing ECM after 29 days of incubation (Figure 7).

The EDS analyses confirmed these results: the presence of calcium phosphates (calcium and phosphorus peaks at 3.7 and 2.1 keV, respectively) was detected on the surface of cells and on the internal structure of the microspheres (Figures 8 and 9).

Calcium phosphates were found on pectin structure after 29 days of incubation in a-MEM (Figure 10). When in the presence of cells, this deposition appeared to start earlier and to be promoted in the surroundings of cells after 8 days of incubation. In this stage, mineralization in acellular microspheres (Figure 10A) did not occur. In addition, after 29 days of immobilization, the more consistent calcium phosphate deposition was observed over cells or in their vicinity rather than in areas in which no cells were present (Figure 8C,D and G,H) for either pectin or RGD-pectin microspheres.

The Ca/P ratio of calcium phosphates formed by preosteoblasts immobilized in RGD-pectin (Ca/P = 1.61; spectrum 3 of Figure 9) and in pectin microspheres (Ca/P = 1.48; spectrum 1 of Figure 9) was closer to Ca/P ratio of hydroxyapatite (Ca/P close to 1.7) than in the case of acellular microspheres (Ca/P = 2.62; spectrum 1 of Figure 10).

4. DISCUSSION

In this work, the use of pectin microspheres as cell carriers for bone tissue regeneration was investigated. According to the literature, size and porosity of microspheres are two important conditions to take into account because they deeply affect cell viability.[26] In this study, the parameters of electrostatic bead generation were optimized to obtain microspheres with diameters in the range of 300−500 μm. These dimensions are considered to be suitable to convey the incoming flow of oxygen and the outgoing of catabolites according to literature, where the maximum diameter permitting convenient oxygenation was suggested to be 1 mm.[27] Pectin and RGD-pectin microspheres presented similar dimensions and showed comparable homogeneous distribution of

dx.doi.org/10.1021/bm101110x |*Biomacromolecules* 2011, 12, 568–577

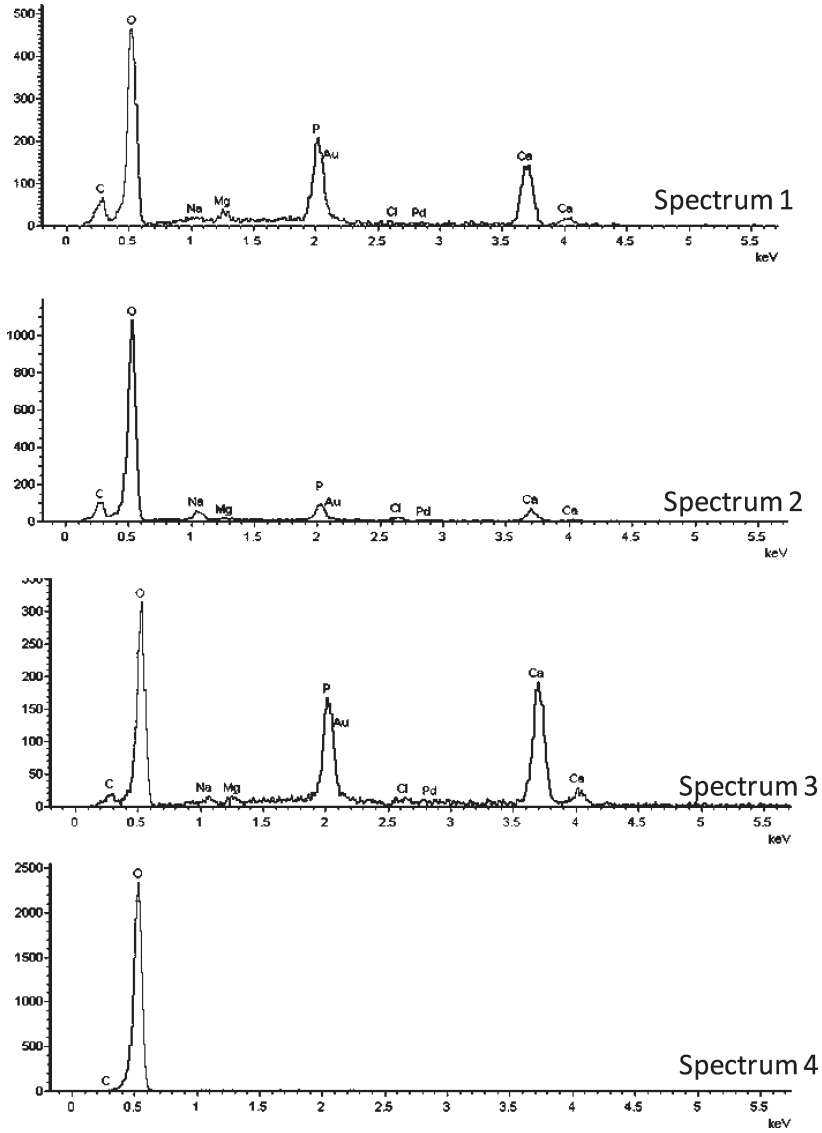

Figure 9. EDS analysis of the regions labeled in Figure 8. Calcium (peak at 3.7 keV) and phosphorus (peak at 2.1 keV) are revealed both on the surface of MC3T3-E1 cells (spectra 1 and 3) and on pectin structure (spectrum 2), where nodules of mineralization were observed. A spot of an RGD-pectin microsphere where no cells were present was also analyzed (spectrum 4) and did not show evidence of mineralization nodules.

immobilized cells. MC3T3-E1 cells maintained their viability during the 29 days of the experiment both in pectin and in RGD-pectin microspheres, thus emphasizing the potential of pectin microspheres as a cell vehicle for bone tissue regeneration.

The possibility of using sodium-containing compounds to dissolve easily pectin microspheres is here proposed as an interesting way to trigger their in vivo dissolution: the controlled release of cells from the microspheres possibly placed in a bone defect could be stimulated by injecting small volumes of physiological solution. Alternatively, an additional thin coating with controllable fast degradation could provide the release of sodium.

Bone tissue engineering requires the ability to induce cell proliferation and differentiation by regulating the cellular response through the physicochemical environment. Accordingly, the mimicking of the physiological environment can be obtained with the design of new functionalized biomaterials. Some bone ECM proteins, such as fibronectin, osteopontin, bone sialoprotein, thrombospondin, type I collagen, and vitronectin, have chemotactic or adhesive properties because they contain an RGD sequence that specifically binds to integrin transmembrane receptors.[7]

Osteoblasts adhesion is essential for signal transduction and for the regulation of gene expression because the ECM-mediated cell spreading can stimulate the nuclear matrix and therefore modify gene expression, inducing cell proliferation and differentiation.[28] The biomimetic modification of biomaterials with RGD-containing peptides thus constitutes a promising approach for bone tissue regeneration by promoting cell adhesion.

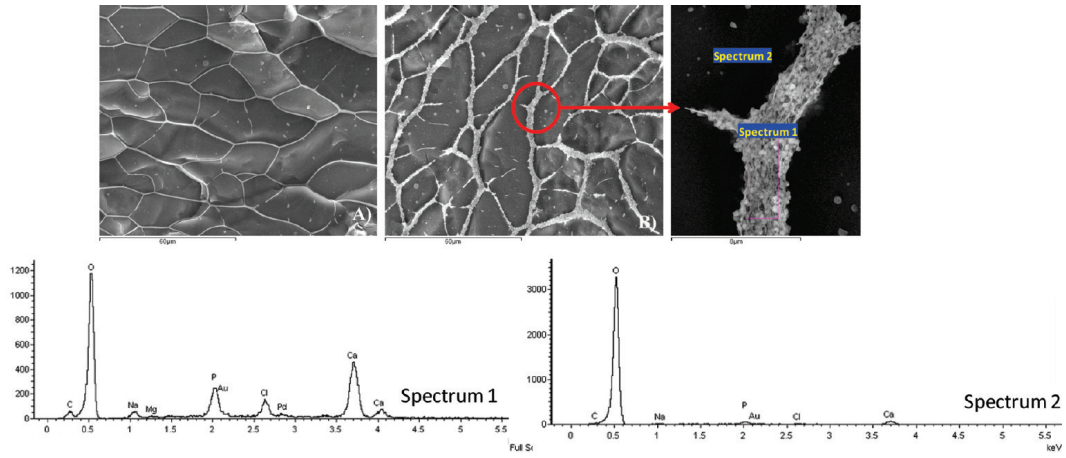

Figure 10. CryoSEM images of pectin microspheres without cells after (A) 8 and (B) 29 days of incubation and EDS analyses of the labeled regions. Calcium (peak at 3.7 keV) and phosphorus (peak at 2.1 keV) are revealed on pectin structure (spectrum 1), where nodules of mineralization were observed.

In this work, remarkable differences were observed in the structure of microspheres prepared from pectin or from pectin modified with the oligopeptide RGD (RGD-pectin) (Figure 2C, D). Also, and based on previous findings,[13] cells were immobilized at a high density (20×10^6 cells/mL) to facilitate cell−cell interactions inside the microspheres. The present work shows that MC3T3-E1 preosteoblasts were able to spread and to establish contacts between cells of the same or from adjacent microspheres when pectin was modified with a peptide containing the RGD sequence (Figure 4B,D,F,H). The process of integrin-mediated cell adhesion in RGD-pectin microspheres comprised a cascade of different events:[29,30] at first, cells established contact with the RGD-coupled material, and they began to flatten. This led to actin filaments elongation (Figure 4B), which linked the ECM to the molecules of actin cytoskeleton. Cytoplasmatic extensions were visible from 8 days of incubation onward (Figures 4B,D,F and 8F-H). On the contrary, MC3T3-E1 cells immobilized in unmodified pectin microspheres did not adhere to the material and retained a round morphology over the experimental time period (Figures 4A,C,E,G and 8A−D). Although the round morphology of preosteoblasts may be related to cell death, in this case, the up-regulation of OCN and the production of a calcium- and phosphorus-containing mineral phase suggest that cells were alive up to the end of the experiment (29 days), as confirmed also by live/dead assay performed (data not shown).

As expected, the metabolic activity of MC3T3-E1 cells resulted enhanced for RGD-pectin microspheres when compared with that in pectin microspheres (Figure 3).

Preosteoblasts were found only inside the core of the pectin microspheres, whereas interestingly, they were able to spread out from the RGD-pectin microspheres and establish 3D contacts connecting adjacent microspheres (Figure 4D,F,H). Cell growth outside the microspheres was not observed in a previous study performed with microspheres made of alginate coupled to RGD.[13]

Some hypotheses may explain the different behavior of cells immobilized in the two different polysaccharides. First, the cell behavior may be related to the molecular structure of pectin, characterized by the long side chains, rhamnogalacturonan I and rhamnogalacturonan II, which are not present in the linear unbranched structure of alginate. Also, the anchorage of cells to the favorable conformation of pectin modified with the RGD motif may have stimulated cell growth on the surface of the microspheres, with a consequent enhancement of viability and metabolic activity. In this case, a better exchange with the culture medium and with oxygen is established, thus resulting in improved proliferation and secretion of new ECM components, connecting cells on adjacent microspheres. It is not clear how cells could reach the external surface of the microspheres. A migration from the core of the microsphere can be considered, coupled to a possible partial dissolution of pectin, exposing the previously immobilized cells to the surrounding environment.

Finally, this work shows that preosteoblastic cells immobilized inside pectin microspheres, and particularly inside RGD-pectin, are able to differentiate into the osteoblastic phenotype. OCN, a specific marker of the bone differentiation, was expressed by MC3T3-E1 immobilized in both pectin types but in higher amounts in pectin modified with the RGD sequence (Figure 5). Even though the significant upregulation of OCN is suggestive of osteogenic differentiation, the expression of other osteogenic markers (osteopontin, alkaline phosphatase, or RUNX2, for instance) should be further investigated.

Pectin, as other natural polymers, is known to mineralize in the presence of media having specific ionic species in adequate concentrations (e.g., SBF).[25,31] However, the autocalcification of pectin microspheres prepared without cells as control appeared not before 29 days of incubation (Figure 10B), whereas cells immobilized in pectin microspheres were found to produce mineralization nodules already after 8 days of culture (Figure 8A,B), whose Ca/P ratios suggest an atomic composition similar to the one of hydroxyapatite. Hence, it is possible to conclude that the calcium phosphate phase produced by cells appeared earlier and seemed to induce an intense mineralization on pectin and RGD-pectin structures.

Preosteoblasts immobilized in RGD-pectin microspheres seem to induce additionally the synthesis of a new ECM with collagen fibers (Figure 7), which eventually results in a more complex and natural new tissue. Also, when the cells were immobilized in RGD-pectin microspheres, mineralization appeared to occur earlier than in pectin microspheres.

Previously, only enzymatically modified high-methoxyl pectin was tested as a coating for tissue engineering,[22,24] showing interesting properties to improve the biocompatibility of implant materials. Here we demonstrated that even unmodified LM pectin itself is suitable for tissue engineering. Indeed, pectin microspheres can be used to immobilize cells under conditions compatible with high cell viability and metabolic activity. Pectin, especially if grafted with the RGD sequence, can promote the osteoblastic differentiation of MC3T3-E1, as shown by histochemical analyses and OCN expression.

The use of pectin microspheres as injectable scaffolds for bone tissue regeneration may avoid the dispersion of cells after injection and provide a complete adaptation to the bone defect. Biodegradation is a fundamental requirement for tissue regeneration: biodegradable microspheres can serve as carriers to provide initial mechanical support and a replaceable matrix for the ingrowth of new tissue. As new bone tissue forms inside and on the surface of microspheres, pectin seems capable of gradually degrading, thus allowing sufficient time for a physiological regeneration of damaged tissues. The degradation products are eliminated through the natural metabolic pathways of the organism. The possibility of inducing pectin dissolution through an adequate amount of sodium-containing solutions may be useful to obtain controlled and adjustable biodegradation.

This work provides a model of injectable microspheres loaded with preosteoblasts aimed at bone tissue regeneration, although other cell types (especially stem cells) or even cocultures may be in principle successfully immobilized in pectin microspheres for soft or hard tissue engineering. Furthermore, pectin microspheres can be employed for the in situ controlled release of growth factors, drugs, enzymes, or proteins, in combination with cells, to treat different pathologies.

5. CONCLUSIONS

In this work, a model for cell delivery and tissue regeneration has been developed, and encouraging results have been obtained. The present findings confirmed the possibility of using unmodified and modified LM pectin as an innovative biomaterial, showing interesting properties for bone tissue regeneration. For the first time, the possibility of using pectin microspheres not only as drug delivery systems but also as injectable tissue regeneration scaffolds has been assessed. The chemical grafting of the peptide containing the RGD sequence on pectin structure improved preosteoblasts adhesion, stimulating their metabolic activity and differentiation. Furthermore, pectin and RGD-pectin microspheres incubated in the culture medium induced the deposition of calcium phosphates, thus behaving as biomimetic scaffolds. When in the presence of preosteoblasts, mineralization occurred earlier, and it was more consistent in the cell surroundings, thus providing interesting properties as scaffolds for bone repair.

In vitro and in vivo studies with human cells are now needed to better explore the potential of this system to promote bone tissue regeneration.

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ACKNOWLEDGMENT

This work was partially supported by grants from the Italian Institute of Technology (IIT, Istituto Italiano di Tecnologie) within the project "Nanobiotechnology - research line 1: biosensors and artificial bio-systems".

We would like to acknowledge Herbstreit & Fox Neuenbuerg (Germany) for kindly providing the LM pectin used in this study. We thank Daniela Silva and Rui Fernandes for their technical support on cryoSEM and histological analyses, respectively.

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