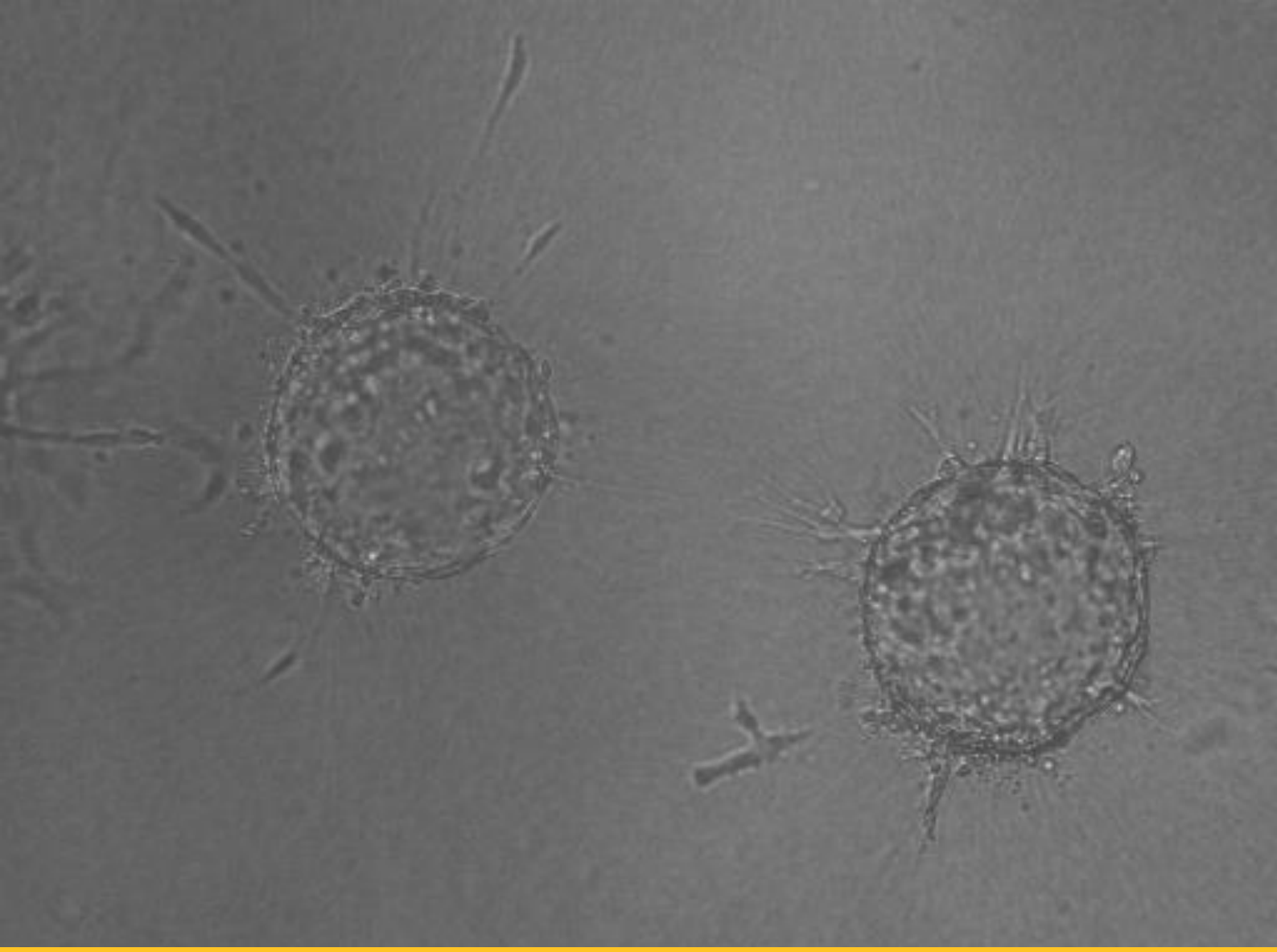

Teresa Margarida Ribeiro Rodrigues

QUANTITATIVE CHARACTERIZATION OF VASCULAR FORMATIONS *IN VITRO* AND *IN VIVO*

Mestrado em Investigação Biomédica

2013

· U C ·

UNIVERSIDADE DE COIMBRA

Teresa Margarida Ribeiro Rodrigues

QUANTITATIVE CHARACTERIZATION OF VASCULAR FORMATIONS *IN VITRO* AND *IN VIVO*

Dissertação de Mestrado em Investigação Biomédica, na especialidade de Oncobiologia, apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre.

Orientadores: Doutor Rui Travasso e Doutor Henrique Girão

Coimbra, 2013

· U C ·

UNIVERSIDADE DE COIMBRA

O trabalho apresentado nesta dissertação foi realizado no Centro de Oftalmologia e Ciências da Visão, Instituto de Imagem Biomédica e Ciências da Vida (IBILI) da Faculdade de Medicina da Universidade de Coimbra, sob a orientação do Doutor Rui Travasso e do Doutor Henrique Girão, ao abrigo do projecto de investigação PTDC/SAU-ENB/110354/2009 (Fundação para a Ciência e a Tecnologia - FCT), no âmbito do Mestrado de Investigação Biomédica (MIB) da Faculdade de Medicina da Universidade de Coimbra.

Agradecimentos

Esta tese representa para mim o culminar de um percurso, o final de uma etapa! Amadureci, cresci, não em altura, eu sei! Mas em conhecimento. Foi-me dada oportunidade de viver, de partilhar momentos e experiências que certamente me moldaram. Como tal não posso deixar de agradecer àqueles que estiveram presentes e tiveram um papel importante nesta fase.

Ao Rui, pelo apoio, orientação e discussão de ideias, e principalmente pela oportunidade que me deu para integrar este projecto de investigação, obrigada!

Aos colegas e amigos, Ana Soares, Inês Mahú, João e Tânia, companhia em dias de laboratório (e por vezes também noites, fins de semana e afins…), de gabinete, banhos de sol pós-almoço, brincadeiras no corredor e em noites de Tropical! Obrigada pelos bons momentos, apoio, opinião sincera, companheirismo, conversas e mimos.

Ao chefinho, co-orientador, professor. Por acreditar em mim e me ajudar, por me deixar crescer e criar o meu próprio caminho. Pela motivação, entusiasmo, orientação, discussão de ideias e ensinamentos. Pela tolerância e compreensão nos momentos de mau feitio, dúvidas e angústias, pelo apoio na hora de algumas lágrimas (temos tido muitos destes momentos, eu sei!)

Aos colegas de laboratório, Carla, Steve, Rui Baptista, Hugo e Inês Rodrigues pelos momentos e ajuda em tantas pequenas/grandes coisas.

Às amigas de sempre e para sempre, Sara e Micaela, porque me fazem sentir sempre próxima e estão sempre disponíveis para me ouvirem e partilharem comigo os momentos de dúvida e os momentos felizes.

Aos meus pais e irmãos, obrigada por tudo! Pela paciência e tolerância, pela palavra certa, confiança, incentivo e sensatez que transmitem. Espero que seja sempre motivo de orgulho.

Table of contents

Index of figures ix

Abbreviations xi

Resumo xiii

Abstract xv

Introduction 1

Molecular mechanisms of angiogenesis 3

Types of angiogenesis 7

Intussuspective angiogenesis 7

Sprouting angiogenesis 8

Material and methods 17

Cell culture 17

Cell counting assays 17

Determination of VEGF levels in supernatants 18

Endothelial sprouting assay 18

Matrigel plug assay 19

MTT cell viability assay 20

BrdU incorporation colorimetric assay 20

Results 23

Cell number as a function of time 23

Cell number as function of VEGF 26

Cell number as function of VEGF and serum concentration 28

Cell viability and proliferation 33

VEGF in the medium as function of cell number 35

VEGF in Solution 36

Sprouting assays 37

Matrigel plug assay 39

Discussion 41

References 49

Index of figures

Figure 1. Schematic representation of angiogenic factors and receptors. 6
Figure 2. Schematic illustration of intussusceptive angiogenesis. 8
Figure 3. Schematic illustration of sprouting angiogenesis. 9
Figure 4. Tip/stalk cell specification during sprouting angiogenesis.. 14
Figure 5. Cell number as function of time.. 24
Figure 6. VEGF in the medium as function of time and cell number. 25
Figure 7. Cell number as function of time and VEGF.. 27
Figure 8. VEGF in the medium as function of time... 27
Figure 9. Cell number as function of time and VEGF... 30
Figure 10. Cell number as function of time and VEGF. 31
Figure 11. Cell growth rate per hour in function of VEGF concentration.. 32
Figure 12. Cell viability assay.. 33
Figure 13. Cell proliferation assay.. 34
Figure 14. VEGF as a function of time.. 35
Figure 15. Concentration of VEGF in solution.. 36
Figure 16. Sprouting induction by VEGF. 38
Figure 17. Vessel formation *in vivo* induced by VEGF. 40

Abbreviations

Ang	Angiopoietin
BSA	Bovine Serum Albumin
Dll1	Delta-like ligand 1
Dll4	Delta-like ligand 4
EC	Endothelial Cell
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
HMEC	Human Microvascular Endothelial Cell
IA	Intussusceptive Angiogenesis
Jag-1	Jagged-1
PBS	Phosphate Buffered Saline
PC	Pericyte
PDGF	Platelet-Derived Growth Factor
RPMI	Roswell Park Memorial Institute 1640 medium
SA	Sprouting Angiogenesis
SMC	Smooth Muscle Cells
TGF-β	Transforming growth Factor-β
UNC5b	unc-5 homolog b
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
vWF	von Willebrand Factor

Resumo

A angiogénese, processo pelo qual se formam novos vasos sanguíneos a partir de outros já existentes, desempenha um papel crucial não apenas em condições fisiológicas, mas também na progressão de diversas patologias tais como cancro, artrite reumatóide e retinopatia diabética. Desta forma, uma melhor compreensão dos mecanismos celulares e moleculares envolvidos na angiogénese é de extrema relevância. Os modelos matemáticos da angiogénese constituem uma ferramenta valiosa na previsão do crescimento vascular, em órgãos e tecidos sujeitos a diversos estímulos ou insultos, podendo, desta forma contribuir para o desenho de novas abordagens terapêuticas, dirigidas especificamente a alvos ou factores potencialmente identificados e descritos no modelo. Apesar da potencial relevância destes modelos, os dados quantitativos validados, que podem ser usados directamente para os parametrizar são escassos.

São muitos os factores que estimulam e intervêm durante o processo de angiogénese, sendo o principal o Factor de Crescimento Endotelial Vascular (VEGF). Na verdade, este factor de crescimento tem sido amplamente descrito como responsável pela proliferação, migração e activação do fenótipo de *tip cell* nas células endoteliais (EC). Para além disso, o VEGF tem sido também associado a vasodilatação e aumento da permeabilidade de vasos já existentes, podendo desta forma contribuir para o complexo fenótipo de doença vascular.

Assim, o principal objectivo deste trabalho foi caracterizar e quantificar o papel do VEGF na angiogénese. Para este efeito, numa primeira fase, começou-se por determinar a influência da densidade celular inicial na taxa de proliferação das ECs. Os resultados obtidos mostram que, independentemente da densidade inicial de células, estas seguem um crescimento exponencial. Além disso, foi determinada a taxa basal de secreção e consumo de VEGF, pelas ECs, bem como a sua influência na taxa de proliferação celular. Ao medir a quantidade de VEGF ao longo do tempo, quer na presença ou ausência de VEGF externo, concluiu-se que, independentemente da quantidade de VEGF adicionada ao meio, após uma diminuição inicial, a concentração deste mantém-se aproximadamente constante ao longo do tempo. Os resultados mostram também que as

concentrações de VEGF necessárias para desencadear uma resposta proliferativa em ECs são superiores às suas taxas de produção. Por outro lado, a exclusão de outros estímulos externos, além do VEGF, alcançada pela depleção de soro do meio de cultura, mostra que a adição de VEGF provoca, numa fase inicial, um rápido crescimento celular, após o qual o crescimento celular diminui, sugerindo que o VEGF por si só não é suficiente para sustentar a proliferação das ECs. Para além da proliferação, alterações dos níveis de VEGF durante a angiogénese podem modelar o comportamento das ECs, incluindo *sprouting,* que pode, por sua vez, condicionar a estrutura final da rede vascular.

Os nossos ensaios de *sprouting* mostram que, dependendo da concentração em que está presente, o VEGF pode induzir *sprouting* ou proliferação das ECs. De facto os nossos dados demonstram que com baixas concentrações de VEGF, o *sprouting* é estimulado, no entanto quando presente em concentrações mais elevadas, a proliferação parece ser dominante sobre o processo de *sprouting*.

Para validar os dados obtidos *in vitro,* analisamos a formação de estruturas vasculares em ensaios *in vivo* em função da concentração de VEGF. Os resultados obtidos mostram que concentrações mais elevadas de VEGF resultam em mais vasos e de maior calibre, que, frequentemente levam, em condições patológicas, à formação de vasos anormais e de elevada permeabilidade. Contudo, os nossos resultados mostram estruturas vasculares bem formadas e ausência de extravasamento, levando-nos a concluir que as concentrações de VEGF usadas permitem a formação de uma vasculatura funcional e fiável.

Em resumo, os dados recolhidos neste trabalho vão permitir a parametrização da influência VEGF em processos essenciais para a angiogénese, incluindo a proliferação celular, migração, *sprouting* e formação de novos vasos sanguíneos. Além disso, estes resultados contribuem para melhorar os parâmetros matemáticos usados no modelo de angiogénese, permitidno, desta forma, aumentar a sua precisão e poder de previsibilidade. Assim, este estudo pode abrir novas perspectivas na avaliação da influência de factores que podem estar envolvidos na formação de vasos anormais geralmente associada a condições patológicas, fornecendo uma ferramenta poderosa para projectar novas estratégias terapêuticas na biologia vascular.

Abstract

Angiogenesis, the process by which new blood vessels grow from existing ones, plays a crucial role not only in normal physiological conditions but also on the progression of several diseases such as cancer, rheumatoid arthritis and diabetic retinopathy. Therefore, a better understanding of the cellular, molecular and mechanical events involved in angiogenesis is of utmost importance. Quantitative mathematical models for angiogenesis constitute a valuable tool in the prediction of vascular growth, and may provide a powerful instrument to design new therapeutic solutions. Despite its putative biological and pathophysiological relevance, the currently repository of validated quantitative data that can be directly used to parameterize predictive mathematical models of angiogenesis is scarce. The best characterized player in the stimulation of angiogenesis is Vascular Endothelial Growth Factor (VEGF). Indeed, this growth factor has been extensively described as a general activator of endothelial cells (ECs) proliferation, migration and activation of tip cell phenotype. Additionally, VEGF has been associated with vasodilation of the existing vessels an increased permeability of the vessel wall.

Therefore, the aim of this work was to characterize and quantify the influence of VEGF in angiogenesis.

For this purpose, we started by determining the influence of initial cell density in proliferation rates of HMEC. The results obtained show that regardless the initial cell density, cells have an exponential growth. Furthermore we established the basal rate of VEGF secretion and consumption, by ECs, as well as its influence in the cell proliferation rate. By measuring the amount of VEGF over time, either in the presence or absence of externally added VEGF, we concluded that regardless the amount of VEGF added to the medium, after an initial decrease in the concentration of VEGF remains approximately constant in time and also that VEGF concentrations necessary to trigger a proliferative response in the ECs are superior to the range of their own production. Furthermore the isolation of other external stimuli besides VEGF, by depletion of serum from the culture medium, shows that addition of VEGF triggers a rapid cell growth in an initial stage, after

which the cell growth decreases, suggesting that VEGF by itself is not enough to sustain ECs proliferation.

In addition to the initial trigger, modifications in the levels of VEGF during angiogenesis can model the behaviour of ECs, including sprouting and determine the final structure of the vascular network.

Indeed sprouting is strongly dependent on VEGF. Our sprouting assays show that, depending on its concentrations, VEGF can induce sprouting or proliferation. Indeed our data demonstrate that for low concentrations of VEGF, sprouting is stimulated, whereas for higher concentrations proliferation seems to be dominant over EC sprouting.

To validate the data obtain *in vitro* we also analysed vascular formation in *in vivo* matrigel assays as a function of the concentration VEGF. The results obtained show that higher concentrations of VEGF results into more and larger calibre vessels, that in pathological conditions often leads to the formation of abnormal and leaky vessels. However our results show well-formed vessels and absence of leakage, leading us to conclude that the VEGF concentrations used allow the formation of a reliable and functional vasculature.

In summary, the data gathered in this work allowed the parameterization of VEGF influence on processes essential for angiogenesis including cell proliferation, migration, sprouting and new blood vessel formation. Moreover, these results will allow the improvement of the mathematical model for angiogenesis, in terms of accuracy and predictability. Thus, this study may open new perspectives regarding the evaluation of the factors that might be involved in abnormal vessel formation usually associated with pathological conditions, providing a powerful tool to design new therapeutical strategies in vascular biology.

Introduction

In small and simple animal species such as the worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, oxygen is capable of diffusing throughout their small body to all cells. However in larger species, a diffusion mechanism is not sufficient, and is essential a vascular network capable of distributing oxygen and nutrients and collect the products resulting from the metabolic activity of all cells in the organism.

The importance of a well-organized and structured vascular network starts at early stages of embryo development where blood vessels provide the necessary oxygen and nutrients to support the proper development of the growing organs. Apart from their nutritive function, vessels also carry instructive trophic signals that regulate cell behaviour and promote organ morphogenesis.

The primitive vascular network arises from endothelial cells (ECs) precursors, which share an origin with hematopoietic progenitors. These progenitors ECs organize themselves in blood clusters which then assemble into a primary capillary plexus by autonomous migration, aggregation and alignment, through a process known as **vasculogenesis** (Carmeliet, 2005a). This primitive early vascular network is progressively expanded and remodelled by vessel sprouting, branching, splitting and differential growth of vessels through a process named **angiogenesis**, into a highly organized and hierarchical vascular network. Nascent ECs sprouts become covered by pericytes (PCs) and smooth muscle cells (SMCs), which provide strength and allow regulation of vessel perfusion.

The formation of new vessels during angiogenesis can occur by growing new capillaries from the existing vessels through sprouting angiogenesis (SA) or by dividing existing capillaries, in a process called intussusceptive angiogenesis (IA).

While angiogenesis intervenes at the embryonic stage by altering the initial capillary network into a mature and vascular structure, it also plays a pivotal role in specific moments of adult life, being essential in physiological processes like ovulation, pregnancy and wound healing. Nonetheless, angiogenic progression is also critical to the onset and progression of a large variety of different pathological conditions. For example, the aggressive outgrowth of blood vessels is involved in cancer, rheumatoid arthritis,

atherosclerosis and diabetic retinopathy, whereas insufficient blood vessel formation is associated with scaring following myocardial infarction and chronic wound healing failure (Carmeliet, 2005a; Carmeliet and Jain, 2000). The neo-vessels resultant from an aggressive pathological process such as the one occurring in solid tumor growth, grow in an environment where there is an imbalance in the relative quantities of pro- and anti-angiogenic factors that is not reversed. This imbalance leads to a highly disorganized vascular growth and to the failure of the vessel maturation process, resulting in the formation of fragile and leaky vessels, which are prone to rupture. Improving the knowledge about the cellular and molecular events in angiogenesis and the influence of the factors that stimulate or inhibit it may lead to better ways to control the nutrient delivery to tissues, and consequently the survival of the organism.

Molecular mechanisms of angiogenesis

Until the moment several endogenous biochemical factors capable of stimulating or inhibiting angiogenesis were identified. Among them are growth factors/cytokines and their receptors, extracellular matrix (ECM) constituents, matrix-degrading enzymes and cell-cell or cell-matrix molecules. These molecules have been show to participate and regulate various biological processes, such as gene transcription and signalling pathways controlling cellular movement, proliferation and phenotype alteration.

One of the most important growth factors in angiogenesis is Fibroblast Growth Factor (FGF), namely FGF-1 (acidic FGF) and FGF-2 (basic FGF). FGF is a pleiotropic mitogenic for growth and differentiation that acts on most cells derived from embryonic mesoderm and neuroectoderm, including fibroblasts, ECs and PCs. The cellular effects of FGFs, occur via specific binding to high-affinity receptors for FGF (FGFR), that triggers a signaling cascade that stimulates all major steps in the angiogenesis process. FGF is produced by various cells including macrophages and tumour cells and is further secreted into the ECM, where it is entrapped through the interaction with ECM proteins (Botta et al., 2000). At the early steps of angiogenesis, degradation of ECM permits the release of FGF that is further used by ECs to promote cell growth. There is an intricate interaction with other growth factors, such as Vascular Endothelial Growth Factor (VEGF), that results in a synergistic action in many EC functions (Zachary, 2005).

VEGF, the best characterized player in the stimulation of angiogenesis, is associated with vasodilation of the existing vessels and increase permeability of the vessel wall. It is also a general activator of EC proliferation and mobility. VEGF is a highly conserved homodimeric glycoprotein which is present in various forms, that exhibit different biological roles: VEGF-A, VEGF-B, VEGF-C and VEGF-D, being VEGF-A the main member of the VEGF family. The alternative splicing of VEGF-A gene results in three primary VEGF-A isoforms, namely VEGF121, VEGF165 and VEGF189, each presenting different properties, including unique ECM binding affinities based on the presence or absence of heparin-binding domains. This variable affinity for the ECM results in a proper spatial distribution of VEGF-A in the ECM. For example, VEGF189 the full-length protein, forms a homodimer, and has a limited biologic activity because of their membrane localization as a result of

heparin-binding sites, something that can be altered by proteolytic cleavage of a fragment of the protein. On the other hand, VEGF165, a splice variant rather than a proteolytic product of the full-length clone, although it maintains some heparin-binding capacity, this isoform of VEGF can also readily difuse, likely accounting for the majority of the angiogenic stimulatory properties of VEGF. Lastly, VEGF121 is a diffusible splice variant of VEGF that can no longer bind to the ECM (Ferrara et al., 2003). Although VEGF-A can bind both VEGF receptors 1 and 2 (VEGFR1 and VEGFR2), most of the data suggests that binding of VEGF-A to VEGFR2 accounts for the majority of the angiogenic stimulatory signal observed *in vivo*, being VEGFR1 a decoy receptor with limited signalling capacity (Ho and Kuo, 2007). VEGFR2, which is highly expressed in ECs, is a transmembrane protein, with kinase activity, that upon binding of VEGF undergoes dimerization and autophosphorylation of specific tyrosines in the cytoplasmic domain. Activated VEGFR2 sets off an intracellular cascade leading to the activation of multiple cellular responses namely EC migration, proliferation, differentiation, survival, as well as the translocation of endothelial progenitor cells from their place of origin, the bone marrow, to the blood circulation, in addition to an increase of vessel permeability and dilation (Figure 1) (Blanco and Gerhardt, 2013; Ferrara et al., 2003).

Another important player on angiogenesis is the Platelet-Derived Growth Factor (PDGF), which triggers a series of signalling pathways upon binding to PDGF receptors. This factor is mainly produced by platelets, fibroblasts, macrophages and ECs. In blood vessels, PDGF receptor can be found in PCs, SMCs and also ECs (Li et al., 2003). The signalling pathways triggered by PDGF, after the binding to its specific receptors promote proliferation and migration of PCs along angiogenic sprouts, which plays a central role in blood vessel maturation. Transforming growth factor-β (TGF-β) family comprises multifunctional cytokines whose effects are mediated by TGF-β receptors (TGFR) and includes cell proliferation, differentiation, migration, inflammation and survival of many cell types. However, the binding of TGF-β, to the TGFR, that constitutes a receptor-type serine/threonine kinases, can either promote or repress angiogenesis, depending on the cells it acts. Indeed when it recruits inflammatory cells that release VEGF, FGF and PDGF, TGF-β presents a pro-angiogenic effect. On the other hand, by regulating ECs proliferation

and the production of ECM proteases, and their inhibitors, by ECs, TGF-β may inhibit the initiation of angiogenesis, but is important in stabilizing immature blood vessels (Hillen et al., 2007).The angiopoietins (Ang) are ligands that mediate the communication of ECs with their surrounding mesenchyme by binding to an endothelial-specific receptor called Tie-2. Despite the receptor being always the same, different ligands can have opposite effects. Indeed, Ang-1 and Ang-4 activates the Tie-2 receptor while Ang-2 inhibits the Ang-1-induced Tie-2 phosphorylation, acting as an antagonist of Ang-1. The action of Ang-1, through the activation of the receptor, has a chemotactic effect in EC, also promoting the recruitment of PCs and smooth muscle cells therefore playing a role in stabilizing and maintaining vascular integrity. The action of this factor has also been described in embryonic vascular remodelling, promoting cell survival, sprouting and tube formation, and reducing inflammation. On the other hand Ang-2, that competes with Ang-1 for binding to Tie-2, is involved in postnatal angiogenic and vascular remodelling events, detaching SMCs, loosening the interactions of ECs with PCs and the ECM and therefore allowing ECs to migrate. Considering this data, Ang-2 may induce vessel regression, however in concert with VEGF contributes to the progression of angiogenesis (Jones et al., 2001). Angiogenesis is an highly regulated process that involves the coordinated and orchestrated action and participation of many factors that promote and/or control the process. The interaction and equilibrium of these factors define how the process proceeds, through one of two types: sprouting or intussusceptive angiogenesis.

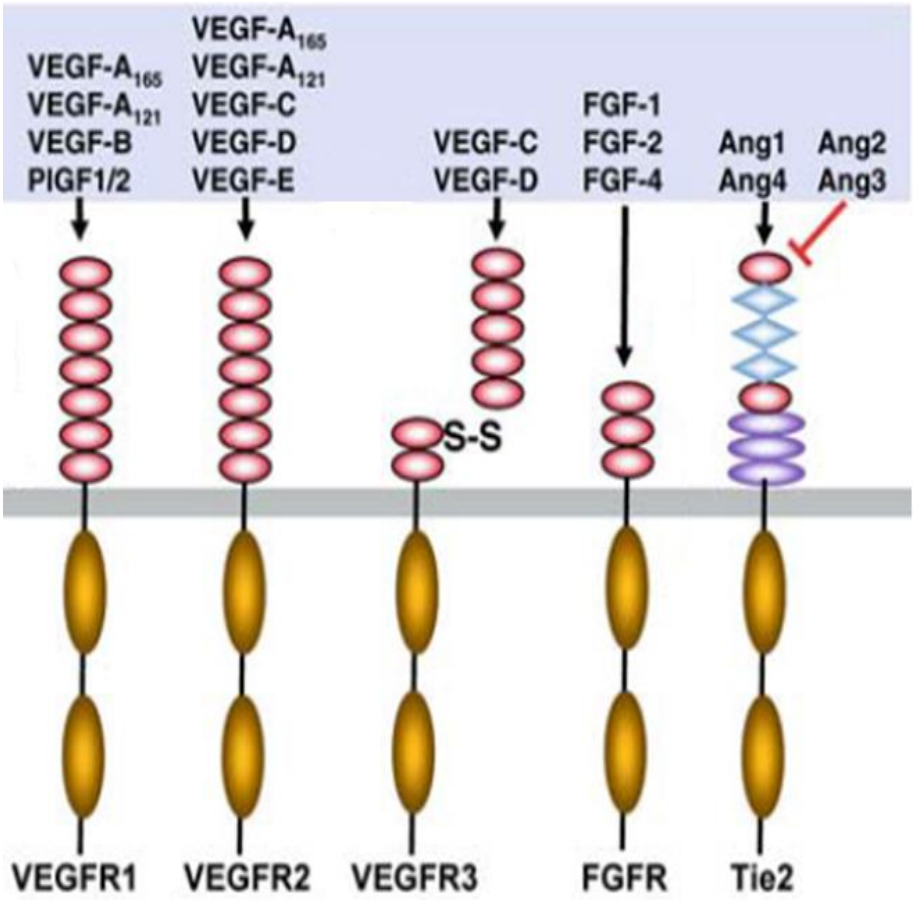

Figure 1. Schematic representation of angiogenic factors and receptors. Adapted from Zachary (2005).

Types of angiogenesis

Intussuspective angiogenesis

Intussusceptive angiogenesis (IA) (also named *splitting angiogenesis*) refers to the process characterized by the formation of so called intraluminal tissue pillars that arise from the invagination of the capillary walls into the vascular lumen, through a complex multistep process. It starts when the endothelial walls of the opposite sides of a vessel migrate towards each other forming an intraluminal pillar. The interendothelial junctions are reorganized and a central perforation is formed in the core of the pillar. In sequence, the pillar is invaded by PCs and myofibroblasts that deposit ECM. This pillar elongates in the direction of the vessel axis, leading to the generation of two parallel capillaries. Finally several pillars increase in size and fuse with each other, splitting up the initial capillary into new capillaries (Figure 2) (Burri and Djonov, 2002; Djonov et al., 2000).

The basement membrane remains intact during the course of this process, preventing the bloods vessels from becoming leaky. In addition, endothelial migration and proliferation are kept to a minimum as the endothelial cells do not necessarily proliferate but rather increase in size and change their shape(Styp-Rekowska et al., 2011).

This form of angiogenesis has been described in a wide range of developing tissues including in different developmental stages in the mammary gland, in developing glomeruli and in the ovary cycle (De Spiegelaere et al., 2012).

IA is regulated by a combination of hemodynamic forces and molecular actors. Studies using the chick chorioallantoic membrane show that blood flow establishes a hierarchical vasculature system, comprising venules and arterioles from a pre-existing capillary plexus. Furthermore the experiments revealed an increase in IA in blood vessels in which the blood flow is enhanced by clamping their side branches. However is still unclear whether the effect of hemodynamics on IA is mainly induced by changes in hydrostactic pressure, by cyclic stretch, by shear stress or by a combination of all factors (Styp-Rekowska et al., 2011).

Besides hemodynamic forces, IA can be also regulated by molecular mechanisms but due to lack of more accurate experimental assays, the molecular mechanisms and players involved in the control of IA remain largely unknown.

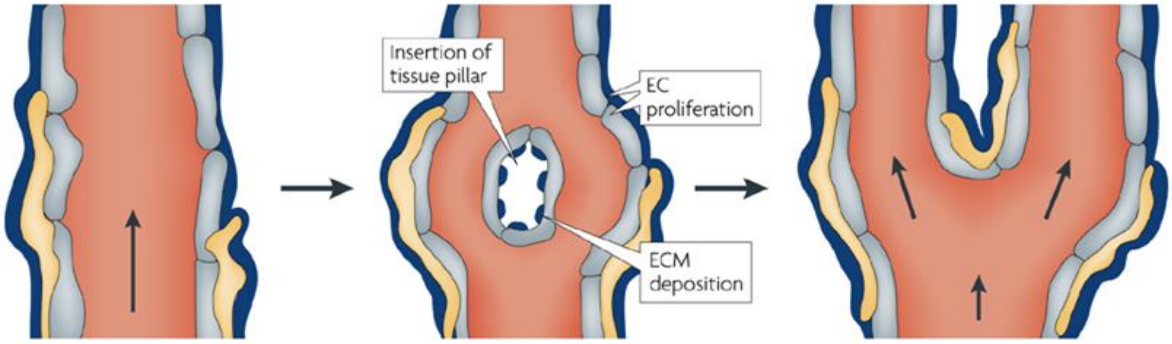

Figure 2. Schematic illustration of a small capillary undergoing intussusceptive angiogenesis. The opposite walls of this capillary start to migrate to each other, an intraluminal pillar is formed, and the cellular junctions of the opposing endothelial cells are rearranged. Subsequently, further growth of the pillar leads to spitting of the blood vessel into two new vessels. Adapted from Adams and Alitalo (2007).

Sprouting angiogenesis

Sprouting Angiogenesis (SA) (also called *tubular morphogenesis* or *sprouting angiogenesis*) refers to the process in which activated ECs branch out from an existing capillary (or venule), extending through the surrounding matrix to form a new vessel.

The process of sprouting occurs with a known sequence of events: first the basement membrane on the site of branching is degraded, the PCs detach and interendothelial contacts are weakened. Consequently ECs migrate into the connective tissue and form an endothelial sprout. Next, proximal to the migrating front, lumen starts to form and contiguous tubular sprouts anastomose originating functional capillary loops. In parallel occur the synthesis of the new basement membrane and the recruitment of PCs. Although initially the blood vessel sprouting progress without cells division, the sustainability of the growth requires cell proliferation (Figure 3) (Adams and Alitalo, 2007).

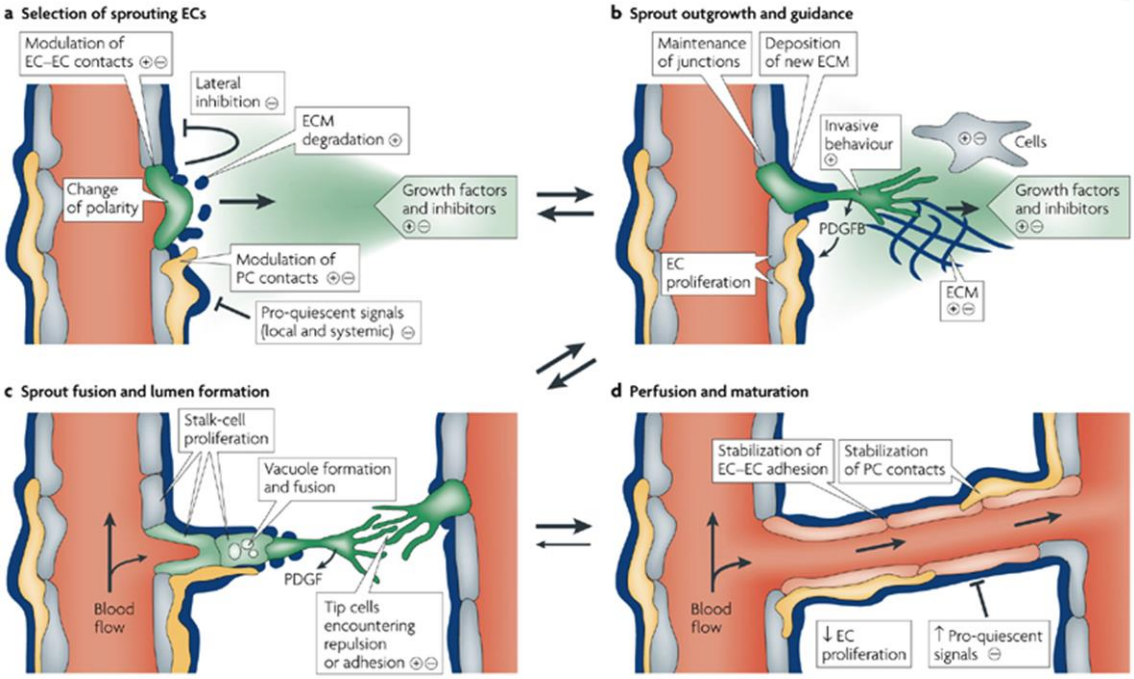

Figure 3. Schematic illustration of sprouting angiogenesis. (a) This process is controlled by the balance between pro-angiogenic signals (+), such as VEGF, and factors that promote quiescence (-), such as tight PC **(yellow)** contact, certain ECM molecules or VEGF inhibitors. In conditions that favor angiogenesis, some ECs can sprout **(green). (b)** Sprouting requires motile and invasive activity, the modulation of cell–cell contacts and local matrix degradation. The signals for sprouting include VEGF gradients, attractive (+) or repulsive (-) matrix cues and guidepost cells in the tissue environment. EC–EC junctions need to be maintained after lumen formation to prevent excessive leakage. **(c)** Adhesive or repulsive interactions that occur when tip cells encounter each other regulate the fusion of adjacent sprouts and vessels. **(d)** Fusion processes at the EC–EC interfaces establish a continuous lumen. Blood flow improves oxygen delivery and thereby reduces pro-angiogenic signals that are hypoxia-induced. Perfusion is also likely to promote maturation processes such as the stabilization of cell junctions, matrix deposition and tight PC attachment. Adapted from Adams and Alitalo (2007).

Blood vessel sprout

The initiation of sprouting requires the specialization of EC into tip and stalk cells with different morphologies and functional properties. Tip cell is migratory and polarized, while stalk cells proliferates during sprout extension and forms the nascent vascular lumen (Gerhardt et al., 2003).

Tip cells are specialized cells that respond to environmental stimuli directing the migration and consequently the direction of the sprouting. Although endothelial tip cells are characterized by their migration properties, with only a minimal proliferation activity, in contrast, endothelial stalk cells, present a high proliferation rate. Moreover, tip cells can be distinguishable from their neighbour stalk cells by the presence of extensive

filopodia and the highly branched shape while moving as well as the expression of specific markers such as delta-like ligand 4 (Dll4), platelet derived growth factor-b (PDGF-b), unc-5 homolog b(UNC5b), VEGFR2 and VEGFR-3/Flt-4, and low levels of Notch signalling activity (Claxton and Fruttiger, 2004; Gerhardt et al., 2003; Siekmann and Lawson, 2007). On the other hand, the stalk cells produce fewer filopodia, constitute tubes and branches and form a vascular lumen. They also form junctions with neighbour cells and produce the components of the basement membrane. The balance between migration of tip cells and the proliferation of stalk cells allows adequately shaped nascent sprouts (Geudens and Gerhardt, 2011).

This phenotypic specialization of endothelial cells in tip or stalk cells is transient, reversible and dependent on the balance between pro-angiogenic factors such as VEGF and Jagged-1 (Jag-1), and suppressors of EC activity such as delta-like ligand 4 (Dll4)-Notch activity.

As described above in some detail, the VEGF-A signalling pathway has been established as an essential regulator of angiogenesis. In ECs, this pathway is mainly mediated by the VEGF-A signalling receptor, VEGFR2, which regulates the mitogenic and chemotactic response of ECs to VEGF. Interestingly, the different cells that constitute the sprouting respond differently to the presence of VEGF. Tip cells are responsive to a gradient of VEGF, whereas stalk cells are regulated by VEGF concentration and respond increasing their proliferation (Gerhardt et al., 2003).

The leading tip cell responds to a VEGF gradient migrating outward from the main vessel up the gradient. This process involves the VEGF induced formation and extensions of filopodia, through the activation of the Rho GTPase Cdc42 , as well as the expression of DII4 protein in the tip cells. Tip cell filopodia surface presents, besides the high levels of VEGFR2 to detect the chemotactic VEGF-A, integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, and the α_v integrins to engage binding sites within ECM and facilitate migration along the scaffold (Hynes et al., 2002).

While in tip cells the effect of VEGF relies on the activation of the VEGFR-2, accumulated in filopodia, in stalk cells, the effect occurs predominantly through the activation VEGFR1, induced by Notch signalling. The presence of VEGFR1 reduces VEGF

ligand availability and is involved in guidance and limited tip cell formation as well as reducing tip cell outward migration. Consistently, the loss of VEGFR1 leads to an increase in sprouting and vascularization(Chappell et al., 2009; Chappell et al., 2011).

Overall, an emerging sprout integrates information from local guidance cues including soluble factors, ECM components and cell-cell contacts to initiate and maintain a proper trajectory away from the parent vessel.

The filopodia from tip cells extend and engage with those of a nearby tip cell to form a bridge and the formation of a new vessel. Sprout fusion may therefore result from filopodia interactions and adhesions, and this increased cell-cell contact potentially enhances Notch signalling to reduce tip cell motility and stabilize the connection for further maturation (Bentley et al., 2009). The stability of the sprouts is reinforced by the recruitment of PCs trough the release of PDGFβ by the tip cells (Adams and Alitalo, 2007).

During the process of vessel elongation and maturation, the stalk cells proliferate in response to VEGF, form a lumen, produce components of the basement membrane and associates with PCs, creating a more stable structure and increasing the mass and surface of the nascent vessel.

The establishment of blood flow requires the formation of a vascular lumen, which involves a complex molecular mechanism composed of endothelial cell repulsion at the cell-cell contacts within the endothelial cell cords, junctional rearrangement and endothelial cell shape change. Two different ways of lumen formation have been described: cell hollowing and cord hollowing. By cell hollowing ECs creates the lumen through the coalescence of vesicles/vacuoles intracellularly. These vesicles carry apical markers helping to distinguish the future luminal membrane from the basal membrane of ECs. Once the cell is “hollowed”, intracellular vacuoles in ECs fuse by exocytosis, opening the cell to the exterior at both ends to a membrane-bound lumen (Kamei et al., 2006; Tung et al., 2012). In the model of cord hollowing, lumen is generated extracellularly without cell loss when an external cue signals for polarization, and surfaces in contact with the ECM accumulate basal markers whereas apical markers move near to cell-cell junctions at the central axis via vesicles. This vesicles fuse at the central axis, separating

the cell surfaces leading to the formation of a lumen continuous within itself and the parent vessel (Lammert and Axnick, 2012; Tung et al., 2012).

The generation of a lumen is critical for stabilization of the newly formed vessel and the maintenance of the existing vasculature. Improved oxygen delivery lowers local VEGFA production, decreasing ECs migration and proliferation.

During differentiation, transition from active sprouting to quiescent ECs, the tip cell adopts a "phalanx" phenotype, characterized by lumenized, non-proliferative, and immobile state of the cells that promotes vessel integrity and stabilization of the vasculature through an increased cell adhesion and low response to VEGF.

Moreover the recruitment of mural cells (PCs and SMCs) and the deposition of ECM proteins into the subendothelial basement membrane also promote vessel maturation and quiescence. During this phase a major role is played by angiopoietins (Ang-1 and Ang-2), which results in the development of the simple endothelial tubes into a more elaborate vascular network composed of several cell types. The angiopoietins contribute to the maintenance of vessel integrity through the establishment of appropriate cell-cell and cell-matrix connections (Thurston, 2003).

After the formation of the vascular network the reorganization process starts, conducted by blood flow. The hemodynamic stimuli caused by shear stress determines if the vessel regress, in cases of low flow or the adaptation of the size of the lumen, and the remodeling of the ECM (Song and Munn, 2011).

Angiogenesis is a highly dynamic process and changes in the local balance between pro- and anti- angiogenic factors may lead to the elimination (pruning) of the new connections or the appearance of new ones.

Tip and stalk cell dynamics

This phenotypic specialization of endothelial cells in tip or stalk cells is transient, reversible and dependent on the balance between pro-angiogenic factors such as VEGF and Jagged-1 (Jag-1), and suppressors of EC activity such as delta-like ligand 4 (Dll4)-Notch activity. This dynamic of the molecular mechanisms that regulate tip-stalk cell selection is proven by the competition of ECs for the tip cell position (Jakobsson et al., 2010). The tip

cell has higher VEGF signalling than its neighbours, and Notch signalling controls the status of VEGF signalling among neighbouring cells.

The Notch signalling is a highly conserved pathway essential in a diversity of processes, such as cell fate, proliferation and differentiation, playing a crucial role in vascular development (Gridley, 2010; Holderfield and Hughes, 2008). Notch-1 and Notch-4 and three Notch ligands, Jag-1, Delta-like1 (Dll1), and Dll4 are expressed in ECs, where they participate in the induction of arterial cells fate as well as in the selection of endothelial tip and stalk cell phenotypes during SA (Blanco and Gerhardt, 2013; Gridley, 2010; Roca and Adams, 2007) . Since the Notch receptors and ligands are all trans-membrane proteins, Notch signal transduction pathway requires cell-cell contact. The Notch receptors, which are located at the cell surface, bind to ligands on adjacent cells, initiating the cleavage of the Notch intracellular domain and the activation of pathways regulated by Notch signalling. Evidence suggest that during angiogenesis the Notch-mediated lateral inhibition, achieved when a cell expressing the highest levels of ligand activates Notch in the surrounding cells, is important not in endothelial cell fate decisions, but in regulating tip and stalk cell phenotypes during angiogenesis (Blanco and Gerhardt, 2013).

While the activation of the Notch signalling inhibits sprouting, impairment in ECs of this pathway induces tip cell phenotype (Hellstrom et al., 2007). Cells with low Notch activity have high VEGFR2 and low VEGFR1 expression, which results in higher levels of Dll4 expression and, hence through a higher production of DII4 than in the neighbouring cells, an increased ability to suppress its neighbouring cells from becoming tip cells. ECs with higher levels of VEGF increase Dll4 expression which further increases the cell sensitivity to VEGF, and this cell becomes the tip cell selected for outward migration for the original vessel (Jakobsson et al., 2010; Ribatti and Crivellato, 2012). The stalk cells have high levels of Notch signalling activity and elevated expression of Jag-1. The ligand Jag-1 antagonizes Dll4 activity, reducing the induction of Notch signalling in the adjacent tip cell, which therefore maintains its response to VEGF stimulation and continues to migrate to establish a new vessel (Benedito et al., 2009). Cells dynamically compete for the tip position based on differential VEGFR levels, as cells with higher VEGFR signalling

express higher levels of DII4 and therefore inhibit their neighbour cells (Blanco and Gerhardt, 2013; Claxton and Fruttiger, 2004; Lobov et al., 2007). In this context, a refined feed-back mechanism between VEGF and Notch/Dll4 signalling pathways is established and a cross-talk between these pathways is essential for a proper vascular network formation (Figure 4).

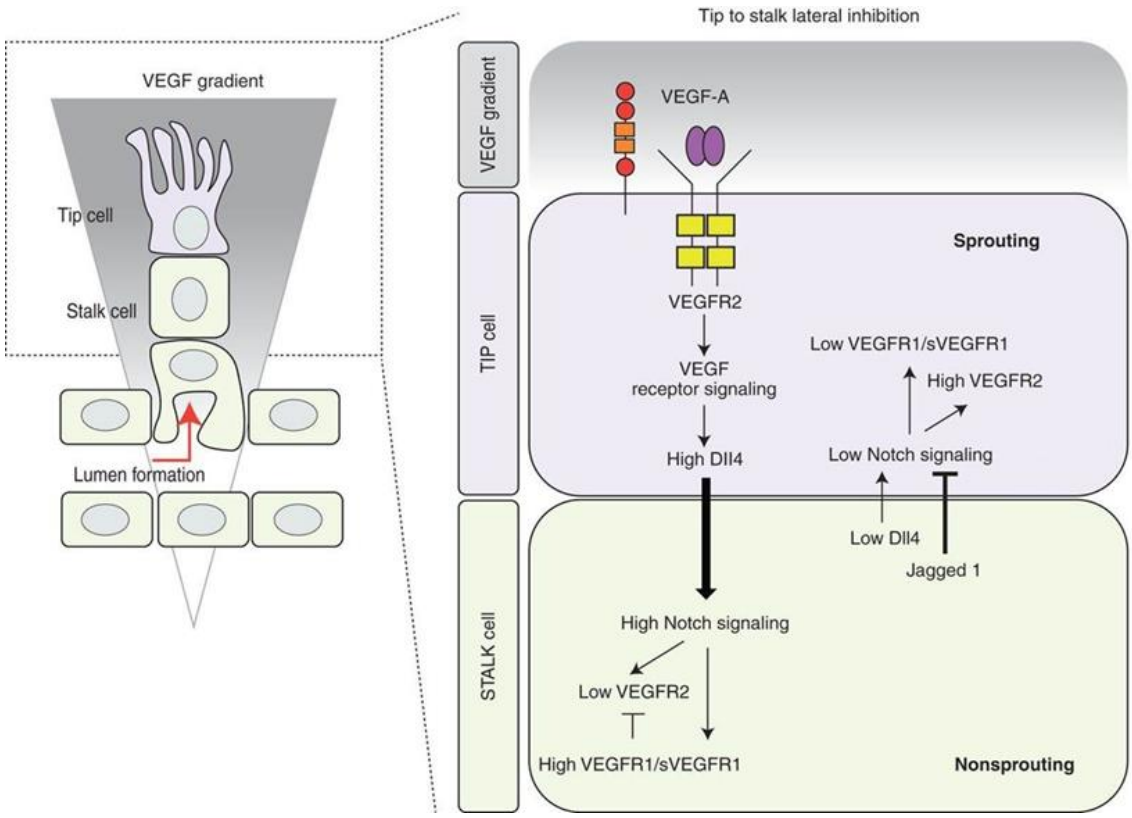

Figure 4. Tip/stalk cell specification during sprouting angiogenesis. During sprouting angiogenesis, VEGF and Notch signalling pathways are implicated in the specification of tip and stalk cells in the vascular endothelium. VEGF interacts with VEGFR2, expressed at the surface of the endothelial cells of the quiescent vessels. Under VEGF stimulation, Dll4 expression is up-regulated in the tip cells. In turn, Dll4 ligand activates Notch signalling in the stalk, consequently suppressing the tip cell phenotype. Notch signalling activation reduces VEGFR2 expression and increases VEGFR1 levels as well as the expression of different Notch target genes. In contrast, the tip cell receives low Notch signalling, allowing high expression of VEGFR2, but low VEGFR1. Contrary to Dll4, Jagged1 ligand is expressed by the stalk cells antagonizing Dll4–Notch signalling in the sprouting front. Adapted from Blanco and Gerhardt (2013).

The process of angiogenesis is extremely complex involving hundreds of proteins that regulate transcription and participate in signalling pathways controlling cellular movement, proliferation and phenotype alteration. Given its importance in a high variety of physiological and pathological processes angiogenesis has been a focus of research, including mathematical modelling. The ability to predict the formation and growth of a vascular bed provides and improves the current understanding of fundamental aspects of angiogenesis, constituting a valuable tool in the prediction of vascular growth, motivating the exploration of new therapeutic solutions. Combined experimental and theoretical approaches inevitably lead to a better understanding of how the mechanical cues, signalling pathways and factors interact to control angiogenesis in a pathological scenario. However there is a substantial lack of published data that can be used to parameterize quantitative models of angiogenesis.

Angiogenesis is a widely studied theme and experimental studies have revealed the role of many different factors driving the formation of vascular networks, in physiological or pathological situations, however innumerable other processes, remain to be explained. The lack of full understanding on the biological process means that is difficult to modulate this process in a biological accurate form. Considering that, we focus in sprouting angiogenesis since it is the most studied type of angiogenesis where the biological and mechanical processes are more clearly identifiable.

This work had the aim of quantifying vascular growth, and other processes associated with it, such as endothelial cell proliferation, VEGF consumption/production and formation of sprouting and vascular structures *in vitro* and *in vivo*. The data gathered by this project can be used by different groups in the task of constructing mathematical models of angiogenesis with predictive capabilities. We took advantage of existing protocols for qualitative and quantitative angiogenesis experiments to create this repository of quantitative data.

Material and methods

Cell culture

The human dermal microvascular endothelial cell line (HMEC-1, refered to hereafter as HMEC) (Ades et al., 1992) was developed by the Center for Disease Control and Prevention (Atlanta, GA, USA) and kindly provided by Dr. João Nuno Moreira. HMEC line was cultured in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), GlutaMax (1x), 10ng of epidermal growth factor/mL (Benton-Dickinson, Bedford, MA, USA) and 1µg hydrocortisone/mL (Sigma-Aldrich, St. Louis, MO, USA). All media, GlutaMax and antibiotics were purchased from Invitrogen (Carlsbad, CA, USA). The cells were maintained at 37^{o}C in a humidified chamber with 5% CO_2.

Cell counting assays

After cell confluence has been reached the culture medium was changed and cells were left overnight in medium without serum to induce G0 synchronization.

Cells were then seeded at the plating densities indicated inside 24 well culture dishes with 1mL medium containing no serum or either 2% or 10% of FBS. When indicated, cells were treated with recombinant VEGF165 (R&D Systems, Minneapolis, MN, USA). The evolution of cell density was followed by cell counting realized at successive intervals. Medium was removed and stored at -20^{o}C for posterior VEGF quantification, and the cells were gently rinsed with phosphate buffered saline (PBS) solution. Then, all adherent cells were incubated in trypsin/EDTA solution at 37^{o}C for 2 minutes. After suspension in fresh medium and mechanical dispersion adherent alive cells were counted using a TC10™ Automated Cell Counter (BioRad, Hercules, CA, USA). The automated cell counter was chosen after validation of the method through comparison with manual cell counting.

Counting assays have been conducted without substitution of the cell culture medium and were performed in duplicates.

Determination of VEGF levels in supernatants

The concentration of diffusible VEGF in the cell culture supernatants was measured by quantikine enzyme-linked immunosorbent (ELISA) assay kit using a monoclonal antibody direct against human VEGF, according the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Briefly, cell culture supernatant samples or standards were added to a 96-well plate, previously coated with a mouse monoclonal antibody anti-human VEGF and incubated for 2 hours at room temperature. Subsequently, samples were aspirated and wells washed three times. In the last wash step, plate was inverted and blotted against clean paper towel to efficiently dry the wells. Wells were then incubated with a biotinylated goat anti-human VEGF antibody for 2 hours at room temperature. After incubation, wells were aspirated and washed three times, as mentioned before. Subsequently, wells were incubated with Streptavidin horseradish-peroxidase for 20 minutes at room temperature. After incubation, wells were aspirated and washed three times, as mentioned before. Next, wells were incubated with the substrate solution, which is a 1:1 mixture of a hydrogen peroxide solution and a tetramethylbenzidine chromogenic solution, for 20 minutes and protected from light. The reaction was stopped by adding 2N (g/L) of H_2SO_4. Absorbance was measured at 450 nm, with wavelength correction at 570 nm, on a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA), using the Gen 5 software to monitor the results (Biotek, Winooski, VT, USA).

Endothelial sprouting assay

Endothelial sprouting was assessed by a modification of the procedure described in Nakatsu et al. (2007). Briefly, microcarrier beads coated with gelatin (Cytodex® 3; Sigma-Aldrich, St. Louis, MO, USA) were seeded with HMEC cells. When cells reached confluence on the beads, equal numbers of HMEC-coated beads were embedded in fibrin gel in 24-well plates. For preparation of fibrin gels, bovine fibrinogen type I (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS in a concentration of 2.0mg/mL. Aprotinin (Sigma-Aldrich, St. Louis, MO, USA) was added at a concentration of 0.15 Units/mL, and the

solution was then filtered through a 0.22 µm-pore-size filter. The fibrinogen solution was supplemented with VEGF (R&D Systems, Minneapolis, MN, USA) in the indicated concentrations. As a control, fibrinogen solution without angiogenic factor was used. Next, after the beads were ressuspended in the fibrinogen solution at a concentration of ~500 beads/ mL, the solution was transfered to 24-well plates and clotting was induced by the addition of 0.625 Units/mL of thrombin (Sigma-Aldrich, St. Louis, MO, USA). After clotting was complete, gels were equilibrated with RPMI-2%FBS at 37^{o}C. Following 60 minutes of incubation, the overlying medium was substituted by RPMI-2%FBS either alone or containing VEGF.

Individual beads were imaged after 24 hours by using phase contrast. For each condition reported, at least n=3 independent experiments were performed, and ~10 beads per device were imaged in each experiment.

Matrigel plug assay

Male Wistar rats (6 months old) were obtained from local breeding colonies maintained at the animal facility of the Faculty of Medicine – University of Coimbra by the group of Dr. Raquel M. Seiça (Physiology Group). The animals were subjected to a constant daily cycle of 12 hours of light and 12 hours dark with constant temperature (22 - 24°C) and humidity (40 – 70%). Rats were given free access to water and to standard commercial pellet chow diet (AO4; Panlab, Barcelona, Spain).

To model VEGF-driven *in vivo* angiogenesis, a subcutaneous matrigel plug assay was performed, as previously described {Malinda, 2009 #81}, with modifications. Briefly, growth factor reduced Matrigel (BD Biosciences, Bedford, MA, USA) was thawed at 4°C overnight to become liquid. After anesthetized, each rat was injected subcutaneously in the back, 1 cm off the midline, with 0,6 mL of liquid Matrigel supplemented with heparin (Sigma, St Louis, MO, USA), with or without VEGF (R&D Systems, Minneapolis, MN, USA) in the indicated concentrations.

Seven days after implantation animals were anesthetized and injected through the jugular vein with an Evans Blue (Sigma-Aldrich, St.Louis, MO, USA) – PBS solution (100mg/Kg of body weight). Evans blue is a dye that combines with plasma albumin,

allowing for detection of functional vessels. Thirty minutes later, the animals were killed by cervical dislocation and the matrigel plugs were enucleated.

Removed matrigel plugs were fixed in with 4% paraformaldehyde for 10 minutes, washed, embedded in OCT (Tissue-Tek, Histolab, Sweden) and stored frozen at -80°C until further analysis. Vascular density was determined by immunofluorescence on 5µm cryosections. The samples were permeabilized with 1% Triton X-100 (v/v) for 10 minutes and blocked with 5% Bovine Serum Albumin (BSA) for 1 hour prior the incubation with a marker for endothelial cells, primary antibody rabbit Anti- von Willebrand Factor (vWF) (Dako, Glostrup, Denmark) overnight at 4°C. The samples were then rinsed three times with PBS and incubated with 1:5,000 DAPI (Invitrogen, Carlsbad, CA, USA) and 1:100 Alexa Fluor® 488-conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. The samples were then washed with PBS and mounted with Mowiol® 4-88 Reagent (Calbiochem, San Diego, CA, USA). The images were collected by confocal microscopy using a Zeiss LSM 710 Confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

MTT cell viability assay

After the treatments, HMEC cells seeded onto 24-well plates were washed twice with PBS and incubated with 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, Carlsbad, CA, USA] in RPMI-1640 for 2 hours at 37°C in a cell culture incubator. Subsequently, supernatants were removed and the precipitated dye was dissolved in 300 µL 0.04 M HCl (in isopropanol) and quantified at a wavelength of 570 nm, with wavelength correction at 620 nm, using a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA).

BrdU incorporation colorimetric assay

Cells seeded onto a 96-well plate were incubated with 5-bromo-2'-deoxyuridine (BrdU; Roche Applied Science, Indianapolis, IN, USA) labelling solution to a final concentration of 10 µM for 24 hours at 37°C. Subsequently, the labelling solution was

removed by taping-off the plate and 200 µl of FixDenat was added to the cells for 30 minutes at room temperature. FixDenat induces cell fixation and DNA denaturation, making BrdU accessible for binding to an anti-BrdU antibody. Thirty minutes later, FixDenat was removed and 100 µl of peroxidase-conjugated anti-BrdU antibody (1:100 dilution) was added per well. Antibody was incubated for 90 minutes at room temperature. Subsequently, cells were washed three times and the substrate solution tetramethyl-benzidine was added. Cells were then incubated at room temperature until colour development was sufficient for photometric detection (30 minutes). The entire procedure was performed as described by the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA) and absorbance was measured at 370 nm (reference wavelength at 492 nm) on a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA).

Results

Cell number as a function of time

A main objective of this study is to establish how initial cell density, VEGF concentration and nutrient availability determine EC growth.

We start by investigate how cell proliferation varies in function of initial cell number.

To identify how plating cell number would affect cell proliferation estimation, plating experiments were performed with seeding of $1x10^4$, $3x10^4$ or $5.04x10^4$ cells and cell counting was performed at successive intervals of 12 hours. The mean of the triplicates for each condition was calculated and plotted in the following graphics (see Figure 5 A, B and C).

The data allowed us to determine if in these conditions the cells follow a simple exponential growth, how expressed in $\frac{dN(t)}{dt} = \alpha N(t)$, where α is the growth rate. If this is the case, then $N(t) = N_0 e^{\alpha t} \Rightarrow \ln(N(t)) = \ln(N_0) + \alpha t$, where N_0 is the initial cell number, and so, the growth rate will be the slope of the plot of ln(N(t)) as a function of time (see Figure 5).

Indeed we observe that the data falls in approximately straight lines.

Putting the three slopes together (Figure 5D) we can infer that the growth rate is $\alpha = 0.0159 \pm 0.0011$ /hour, being independent of the initial number of cells.

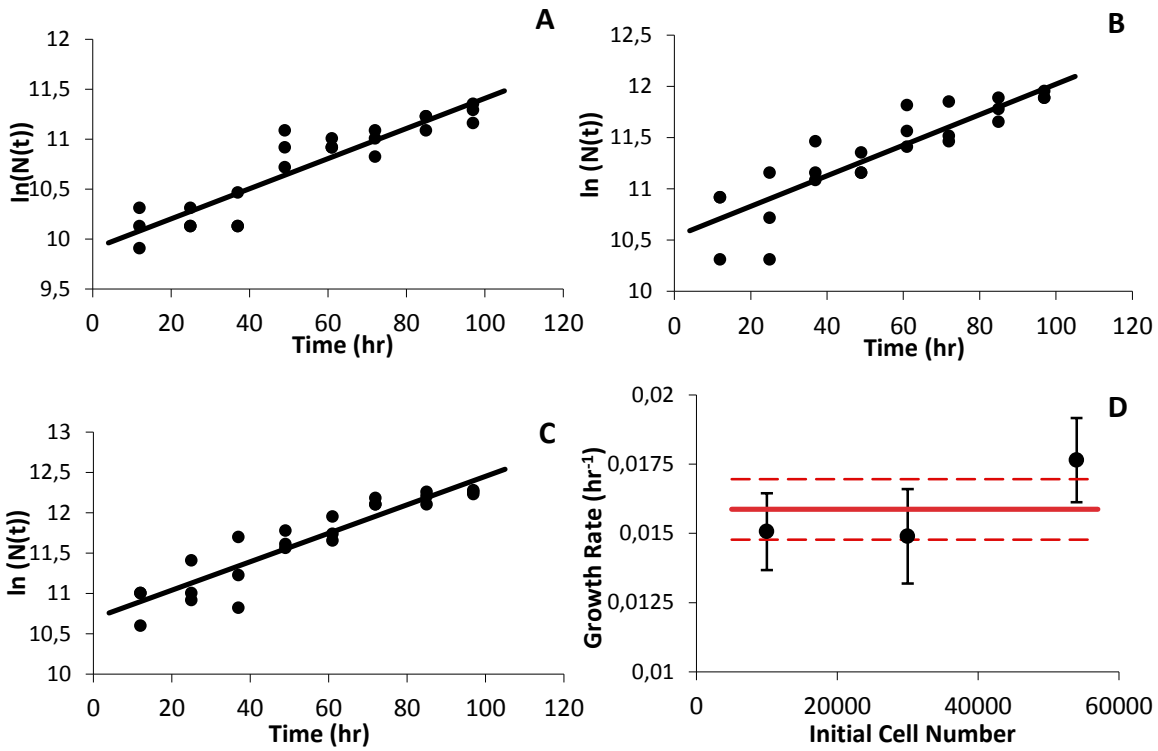

Figure 5. Cell number as function of time. 1x10^4**(A)**, 3x10^4 **(B)** or 5.04x10^4 **(C)** cells were platted and cell counting was performed at 12 hour interval, logarithm of cell number as function of time was plotted (dots). The cells followed an exponential growth and growth rate in function of initial cell number was calculated and is represent in **(D).**

The cell growth is determined, among other factors, by the concentration of VEGF in the medium. Therefore, in order to establish if there is an important regulation of cell proliferation by the VEGF produced by the ECs themselves, we quantified the amount of VEGF present in the culture medium at various time points (Figure 6 A, B and C).

If VEGF is produced independently, then $\frac{dV(t)}{dt} = \gamma N(t) = \gamma N_0 e^{\alpha t}$. The result of this equation $V(t) = V_0 + \frac{\gamma N_0}{\alpha}\left(e^{\alpha t} - 1\right) = V_0 - \frac{\gamma N_0}{\alpha} + \frac{\gamma}{\alpha} N(t)$ where γ is the VEGF production rate and V_0 is the initial concentration of VEGF. Therefore, the slope of the line of VEGF concentration *V(t)* as a function of *N(t)* should give the ratio between the VEGF production rate of a cell, γ, and the cell growth rate α.

Considering the data from these two experiments we can estimate the amount of VEGF produced per cell. As demonstrated in figure 6, the value for the slope

$(3.3 \pm 0.5) \times 10^{-5}$pg gives finally the production rate of $\gamma = (5.2 \pm 1.1) \times 10^{-6}$ pg/hour/cell, also independent of the initial cell number.

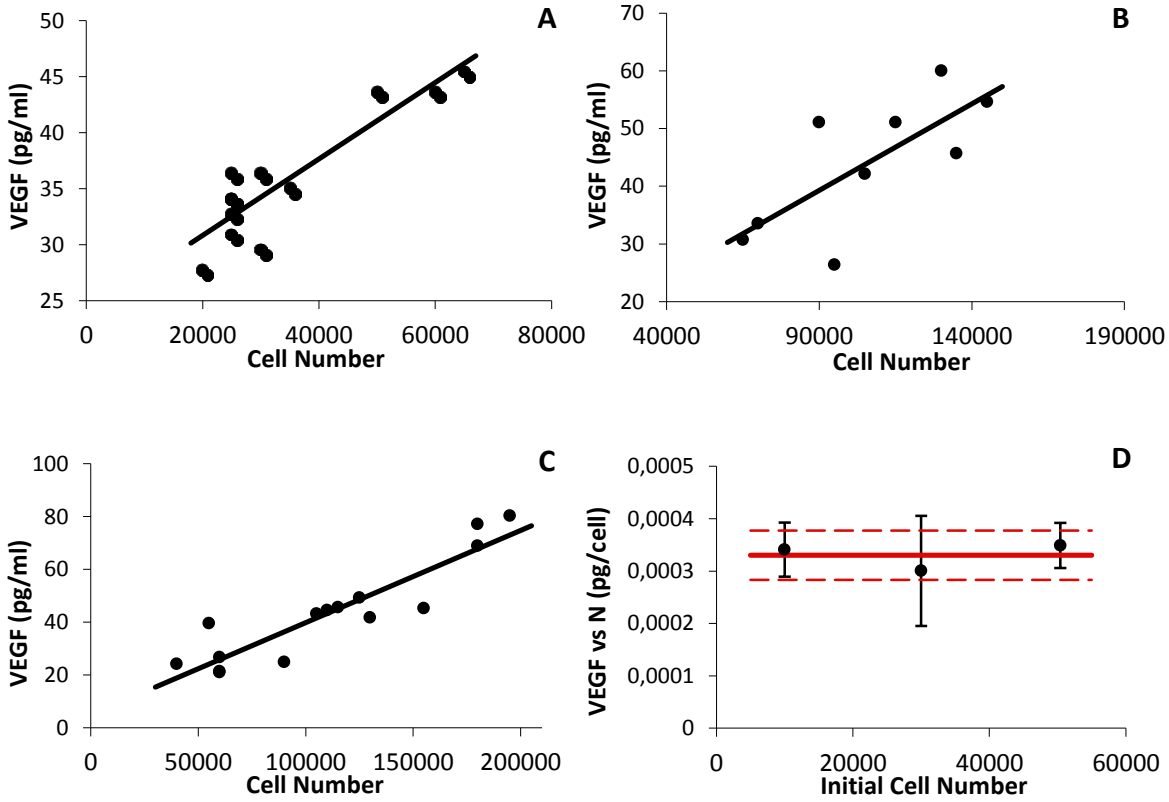

Figure 6. VEGF in the medium as function of time and cell number. 1x10^4**(A)**, 3x10^4 **(B)** or 5.04x10^4 **(C)** cells were platted and the medium was collected, at 12 hour interval, for VEGF quantification (dots). Slopes values of the three lines that allowed the calculation of VEGF production rate γ **(D).**

Cell number as function of VEGF

Once we have established the ECs VEGF production rate, we investigated how these levels of VEGF influence cell growth. For this purpose, we evaluated cell number for different amounts of VEGF added to the medium. Importantly, the concentrations of VEGF used in this experiment were in the range of those observed in the previous experiment. To access those effects 1.5x104 cells were plated and VEGF was added to medium in the beginning of the experiment in the concentrations of 50, 100 and 200 pg/mL. Medium without external VEGF was used as a control. The cell growth was followed by counting assay at successive intervals of 12 hours during 10 days.

The graph presented in figure 7 shows that, independently of the concentration of VEGF tested, it is possible to observe an increase in the cell number as a function of time, with a similar pattern of increase, decrease and stabilization in the number of cells. Indeed, the number of cells over time does not significantly vary with the amount of VEGF added to the medium, at the order of magnitude of the amounts of VEGF produced by ECs. Since, the amount of VEGF in the medium changes over time, that is dependent on the balance between its production and degradation/consumption, we next quantified the levels of VEGF in the conditions 100pg/mL and 200pg/mL. The results presented in figure 8, show that regardless the concentration of VEGF added to the medium, the concentration decreases in the first hours, after which stabilizes. We observe again this behaviour when we analyse systematically the levels of VEGF in solution for cells incubated with different concentrations of VEGF.

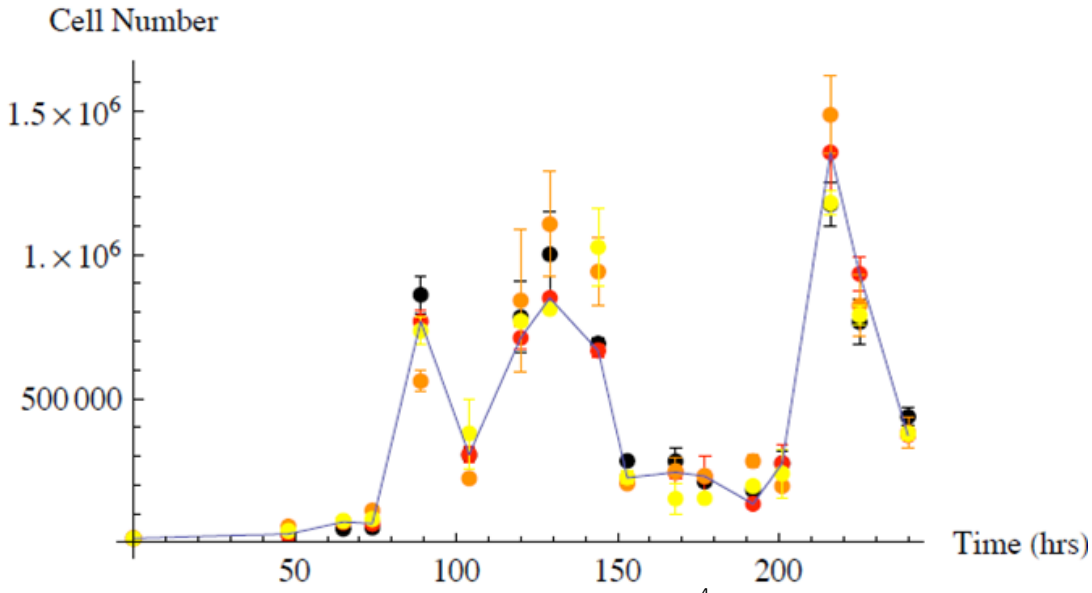

Figure 7. Cell number as function of time and VEGF. 1.5x10^4 cells were plated and VEGF was added to the medium in the concentrations of 50 **(red)**, 100 **(orange)** and 200 pg/mL **(yellow).** Medium without external VEGF was used as a control **(black).** Cell counting was performed at 12 hour interval. Each data point represents the mean of cell number ± S.D..

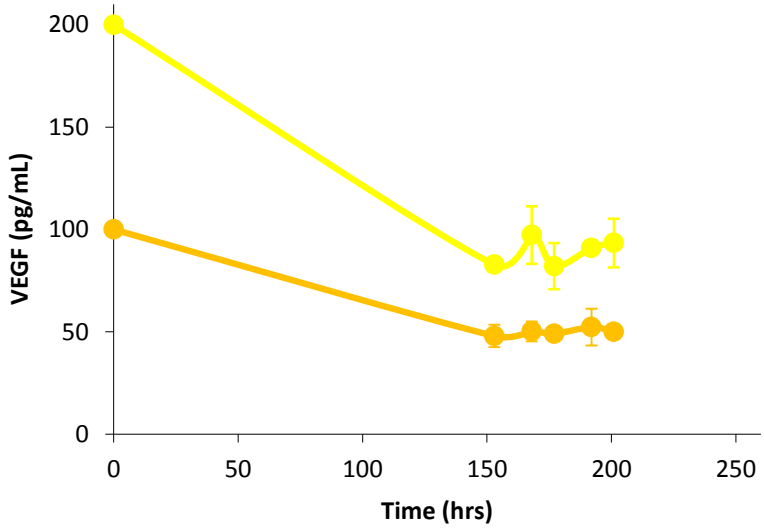

Figure 8. VEGF in the medium as function of time and initial concentration of VEGF. 1.5x10^4 cells were plated and VEGF was added to the medium in the concentrations of 100 **(orange)** and 200 pg/mL **(yellow**), the medium was collected, at 12 hour interval, for VEGF quantification. Quantification was performed in the samples from the last time-points of the assay and VEGF concentrations were plotted in a graph (dots) Each data point represents the mean ± S.D..

Cell number as function of VEGF and serum concentration

The experimental approach used in the assays described above used complete cell culture medium that included serum. Since serum is known to contain various growth factors, including VEGF, a more accurate study of the effect of external added VEGF to cell proliferation, should be performed in cells cultured in the absence of serum. Moreover, under these conditions, the cells are all synchronized at the same stage of the cell cycle that additionally allows a more rigorous evaluation of the effect of VEGF in cell proliferation.

The cell number chosen to perform this assays was the cell number that better translate for optimal cell density to maintenance of cell function evaluated through tubular formation assays (data not show).

To address this question, $1.2x10^5$ cells were plated in 24-wells plates, in complete medium (10% FBS) or medium without serum with different concentrations of VEGF: 0.5, 1, 2.5, 5, 7.5 and 10 ng/mL; in the control no VEGF was added in the medium. The cell number was determined by cell counting at successive intervals of 12hours, after which the results were plotted in a graph ln(N(t)) as a function of time for different values of VEGF concentrations. The slopes obtained in these graphs represent the growth rates.

The results obtained show that cells incubated in medium containing 10% of serum (complete medium), present a cell growth that follows a linear and exponential growth, Figure 9. This approach allows us to calculate the growth rate as function of VEGF concentration. The results presented in Figure 9H demonstrate that all VEGF concentrations investigated, the number of cells increases over time. However, cells incubated with 1ng/mL of initial VEGF present the highest growth rate, with an increase of 60%, with respect to the growth rate in the absence of VEGF.

To further determine the effect of serum, we used the same experimental conditions described above, with the exception of serum that is absent from the culture medium. In this case the amount of nutrients present in solution is can't to sustain a constant growth rate. Therefore, not surprisingly, during the time of the experiment the growth rate decreases.

Therefore the simpler hypothesis that can be made is that the HMEC growth rate varies linearly in time: $\frac{dN(t)}{dt} = (\alpha_1 t + \alpha_2) N(t)$, with α_1 negative. The solution for this equation is $N(t) = N_0 e^{\frac{\alpha_1}{2} t^2 + \alpha_2 t} \Rightarrow \ln(N(t)) = \ln(N_0) + \frac{\alpha_1}{2} t^2 + \alpha_2 t$. Hence the plot of ln(N(t)) vs time can be fitted by a quadratic curve has represented in Figure 10. In Figure 10H is plotted the growth rate at 8 hours.

In Figure 11is plotted in the same graph the growth rate either in the presence (blue markers) or absence of serum (red markers).

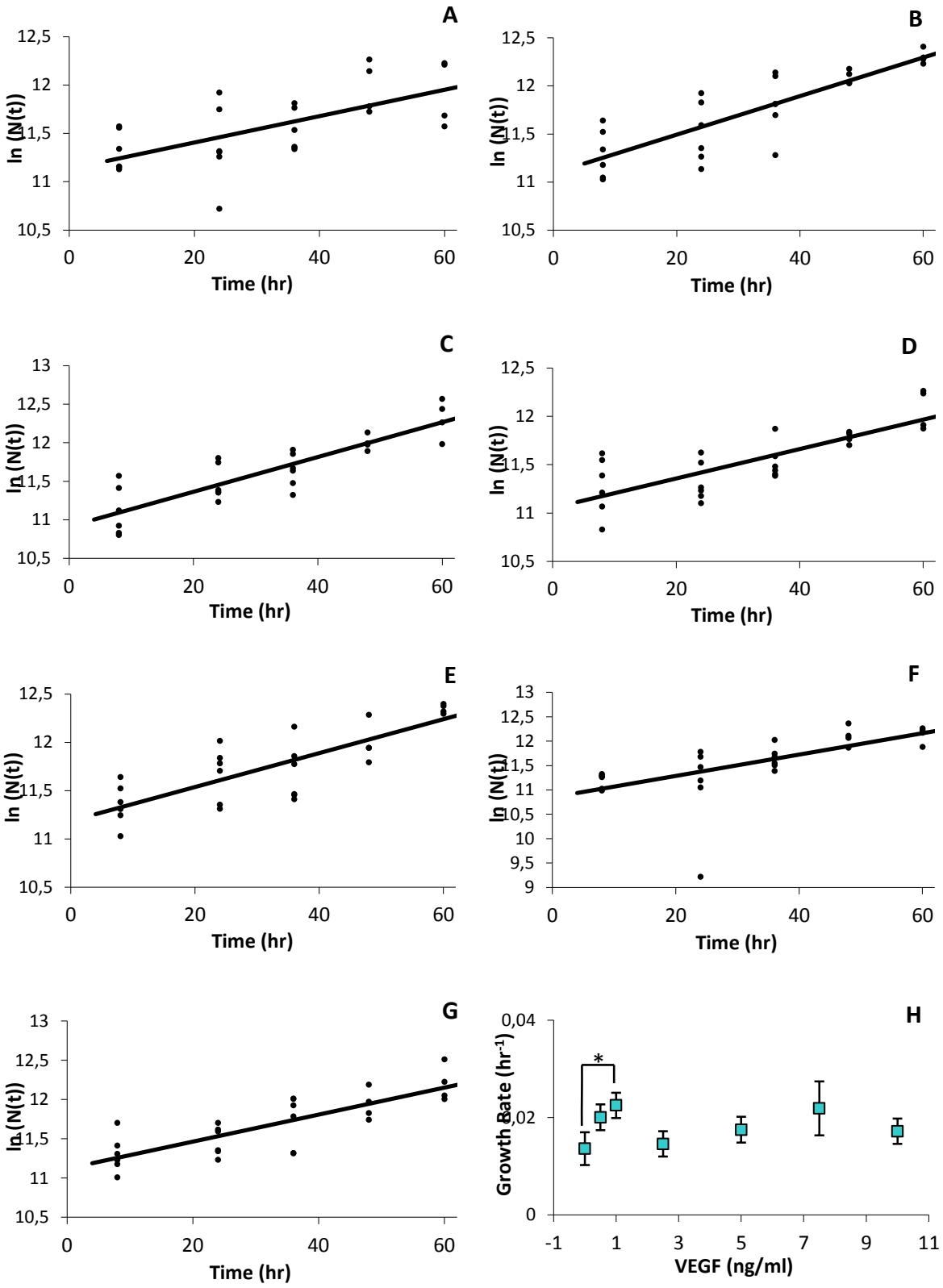

Figure 9. Cell number as function of time and VEGF. 1,2x10^5 cells were plated in complete medium with different concentrations of VEGF. Cell counting was performed at intervals of 12hours and results of ln(N(t)) were plotted. VEGF added to the medium (in ng/mL): 0 **(A)**, 0.5 **(B)**, 1 **(C)**, 2.5**(D)**, 5 **(E)**, 7,5 **(F)** and 10 **(G).** The increase in the growth rate per hour as function of initial VEGF concentration was significant with 1ng/mL **(H).** ***** p<0,05. Each data point represents the mean ± S.D..

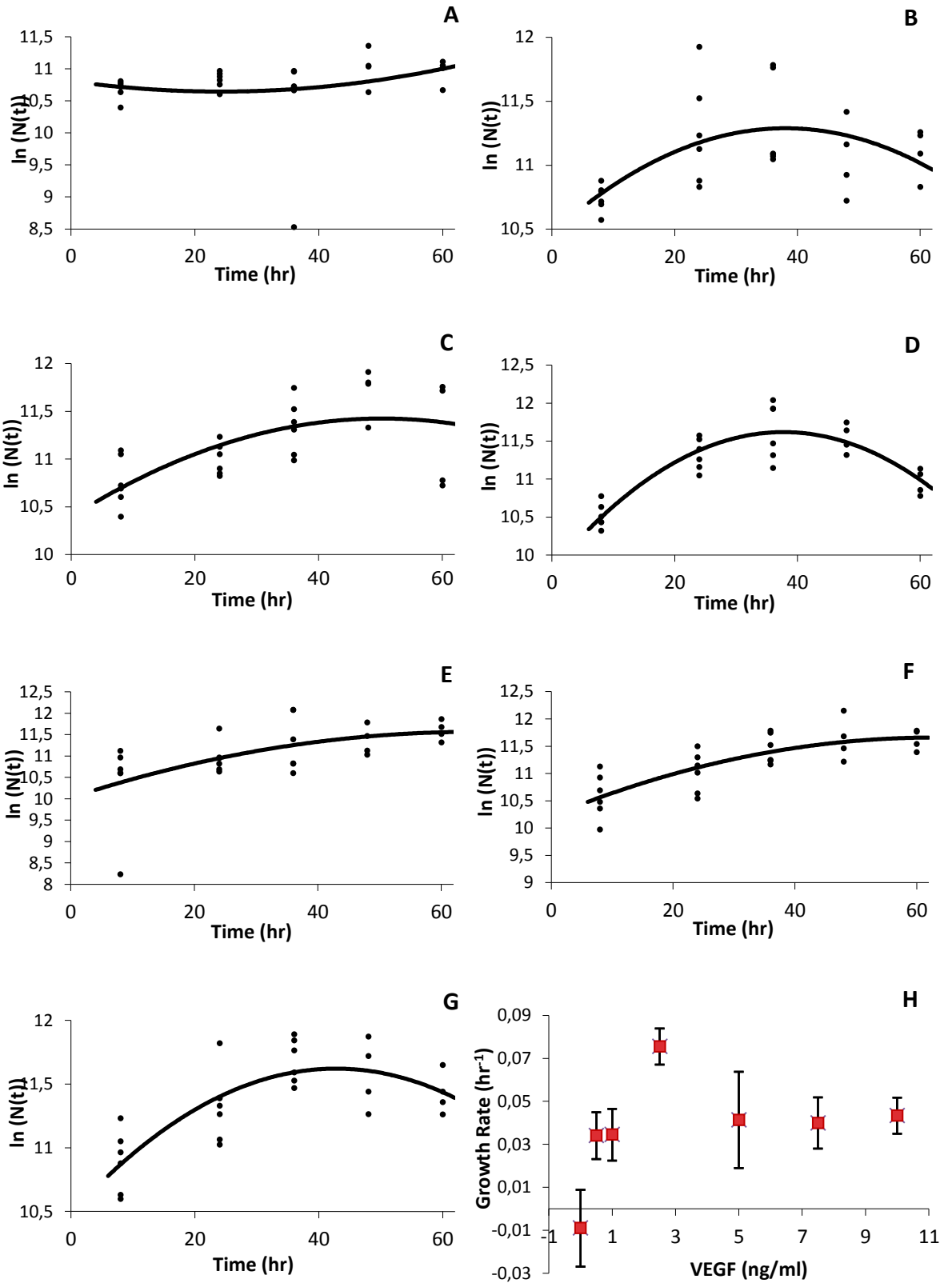

Figure 10. Cell number as function of time and VEGF. 1,2x10^5 cells were plated in medium without serum and different concentrations of VEGF were added to the medium. Cell counting was performed at intervals of 12hours and results of ln(N(t)) were plotted. VEGF added to the medium (in ng/mL): 0 **(A),** 0.5 **(B),** 1 **(C),** 2.5**(D)**, 5 **(E)**, 7,5 **(F)** and 10 **(G).** Cell growth rate per hour in function of VEGF concentration had no significant differences, each data point represents the mean ± S.D. **(H).**

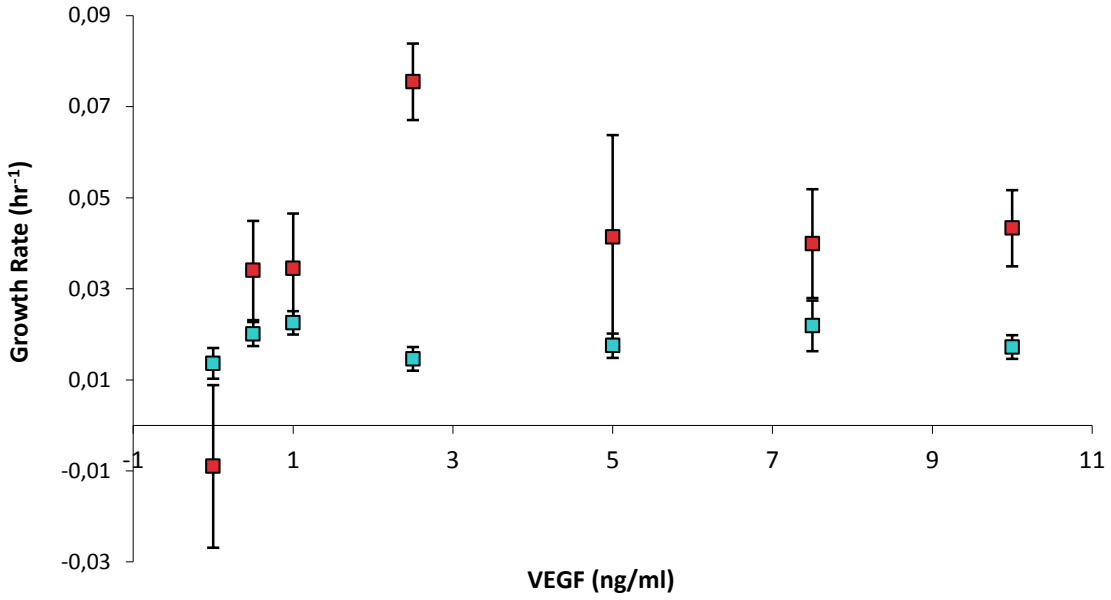

Figure 11. Cell growth rate per hour in function of VEGF concentration. Growth rate in the presence (blue markers) or absence of serum (red markers). Each data point represents the mean ± S.D..

Cell viability and proliferation

The data from the counting assays were supplemented with MTT and BrdU assays.

MTT assay is a colorimetric assay that is used to measure cellular metabolic activity and may, under defined conditions, reflect the number of viable cells present. MTT is added directly to the culture medium and is reduced by metabolically active cells to insoluble purple formazan dye crystals.

To perform this assay HMEC cells were platted in similar conditions to the counting assays. As described previously, $1.2x10^5$ cells were plated in 24-wells plates, in medium without serum or with 10% FBS, and supplemented with VEGF in the following concentrations: 0.5, 1, 2.5, 5, 7.5 and 10 ng/mL. In the control no VEGF was added in the medium. The assay was done 24 and 48 hours after platting, in duplicates.

After normalization of the absorvance values with cell number media at that time-point for each condition, results show that cell viability wasn't significantly altered in any of the experimental conditions. As visible in Figure 12, the values of MTT are in or near the range of the values of MTT in the normal conditions (medium 10% FBS and without addition of VEGF, Figure12H in panel A and B) as visible the red lines that define the range of values covered by the error in this conditions. Similar results are obtained at 24 and 48 hours.

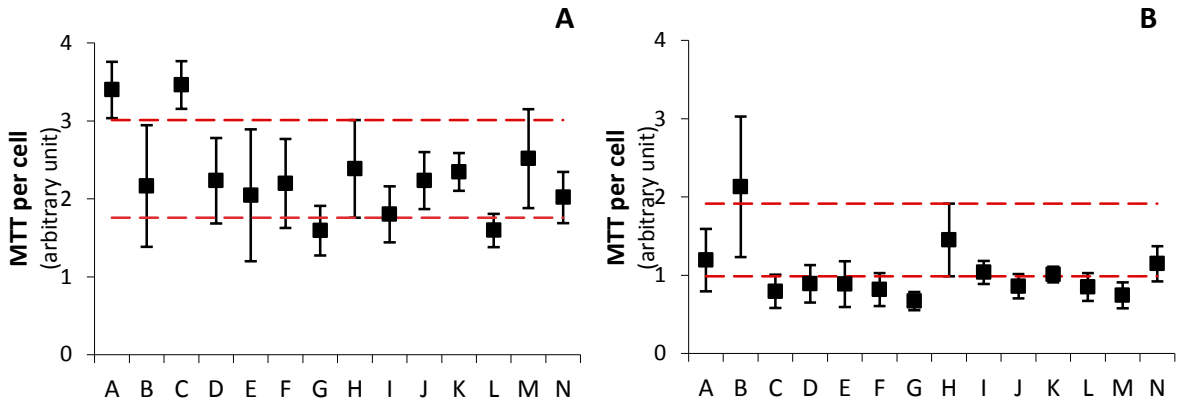

Figure 12. Cell viability assay. $1,2x10^5$ cells were plated in medium without serum **(A–G in both panels)** and with 10% FBS **(H-N in both panels).** Different concentrations of VEGF were added to the medium (in ng/mL): 0**(A and H)**, 0.5 **(B and I),** 1 **(C and J)**, 2.5**(D and K)**, 5 **(E and L), 7,5 (F and M)** and 10 **(G and N**). MTT assay was performed 24 **(Panel A)** and 48hours **(Panel B)** after plating. Results were normalized by cell number and each data point represents the mean ± S.D..

The BrdU is a common reagent used for cell proliferation assays and for the detection of apoptotic cells. BrdU is a uridine derivative and a structural analog of thymidine, that is incorporated into DNA during the synthesis-phase of the cell cycle, phase S, as a substitute for thymidine, thereby serving as a marker for proliferation. That marker was detected by anti-BrdU antibodies, after cell fixation.

For this assay the cells were platted in the same experimental conditions of serum and VEGF concentration described previously, for the counting and viability assays. However to the assay be performed in 96wells plates, the number of cells was adjusted to $2.0x10^4$ cells per well, to respect the cell density (number of cells per cm^2), used in the other assays, namely cell counting and MTT. The assay was done 24 and 48 hours after platting, in triplicates.

The results show that, after normalization of the absorvance values with cell number media at that time-point for each condition, results show that incorporation of BrdU was not significantly altered in any of the experimental conditions. As visible in Figure 13, the values of BrdU, similar to what happened in MTT assay, are in or near the range of the values of incorporation of BrdU in the normal conditions (medium 10% FBS and without addition of VEGF, Figure13H in panel A and B), as visible the red lines that define the range of values covered by the error in this conditions. Similar results are obtained at 24 and 48 hours. At 48 hours the incorporation of BrdU is higher in the conditions without serum, Figure 13B, in concordance with the results of growth rate in Figure 11, that showed that the growth rate in the conditions without serum were superior from the conditions with 10%FBS.

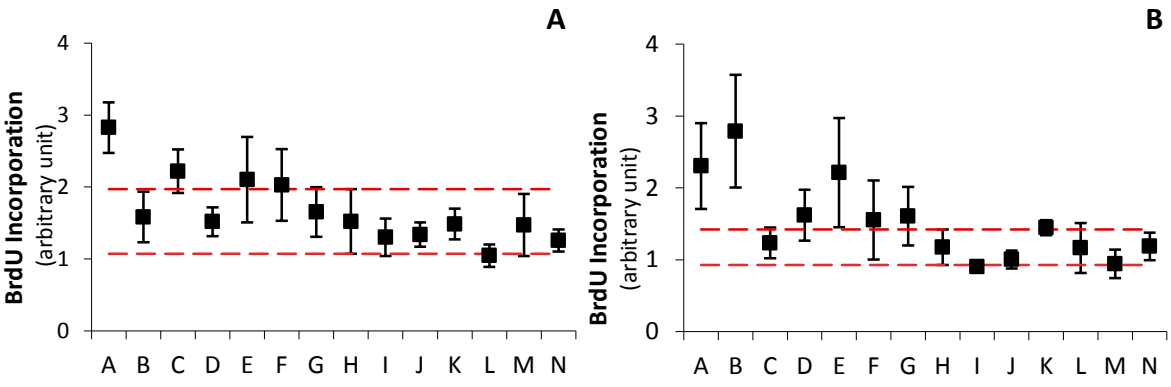

Figure 13. Cell proliferation assay. $2x10^4$ cells were plated in medium without serum **(A–G in both panels)** and with 10% FBS **(H-N in both panels).** Different concentrations of VEGF were added to the medium (in ng/mL): 0**(A and H)**, 0.5 **(B and I)**, 1 **(C and J)**, 2.5**(D and K)**, 5 **(E and L),** 7,5 **(F and M)** and 10 **(G and N).** BrdU incorporation assay was performed 24 **(Panel A)** and 48hours **(Panel B)** after plating. Results were normalized by cell number and plotted, each data point represents the mean ± S.D..

VEGF in the medium as function of cell number

In an attempt to clarify the amount of VEGF produced by the cells, VEGF in the medium was quantified through ELISA assay in the samples without addition of external VEGF, without serum.

In the condition of medium without serum and when no VEGF is added, the number of cells is approximately constant: $N = (5.0 \pm 0.3) \times 10^4$ cells. Therefore, $\frac{dV(t)}{dt} = \gamma N_0$, which implies that $V(t) = V_0 + \gamma N_0 t$. And so, the graphic of the concentration of VEGF in the medium as a function of the time should fall on a straight line (Figure 14).

From this slope we obtain a production of $\gamma = (2.0 \pm 0.9) \times 10^{-5}$ pg/hour, approximately 5 times larger than the production at 10%. However, the levels of VEGF observed in solution in this case are still very low to produce a significant alteration in the ECs growth rate.

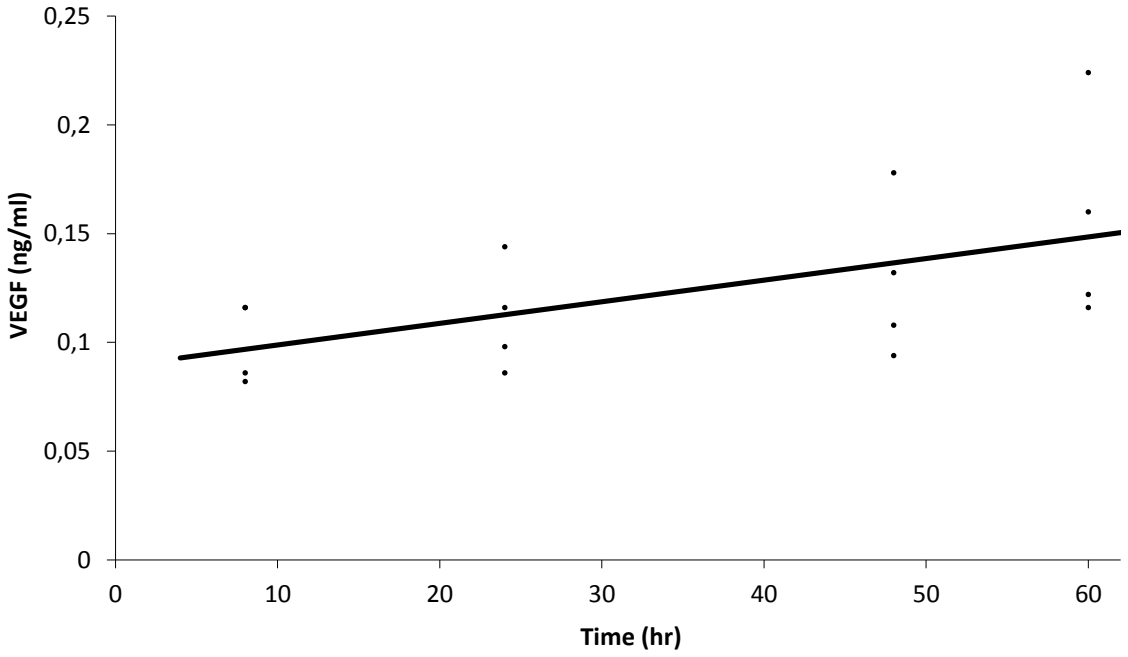

Figure 14. VEGF as a function of time. 1,2x10^5 cells were plated in medium without serum, the medium was collected and VEGF concentration was determined by ELISA assay.

VEGF in Solution

Putting the data of all assays together we conclude that when the initial VEGF concentration is higher than 500 pg/mL we do not have the precision in our measurements to observe alterations in the concentration of VEGF in solution, i.e. to observe the production of VEGF by the endothelial cells. Hence, we observe an approximately constant amount of VEGF in solution.

In the Figure 15 we plot the concentration of VEGF in solution as a function of the initial concentration of VEGF. For the larger concentrations we observe a very fast internalization of VEGF. No statistically differences were found in the internalization rate of VEGF when comparing medium with or without serum.

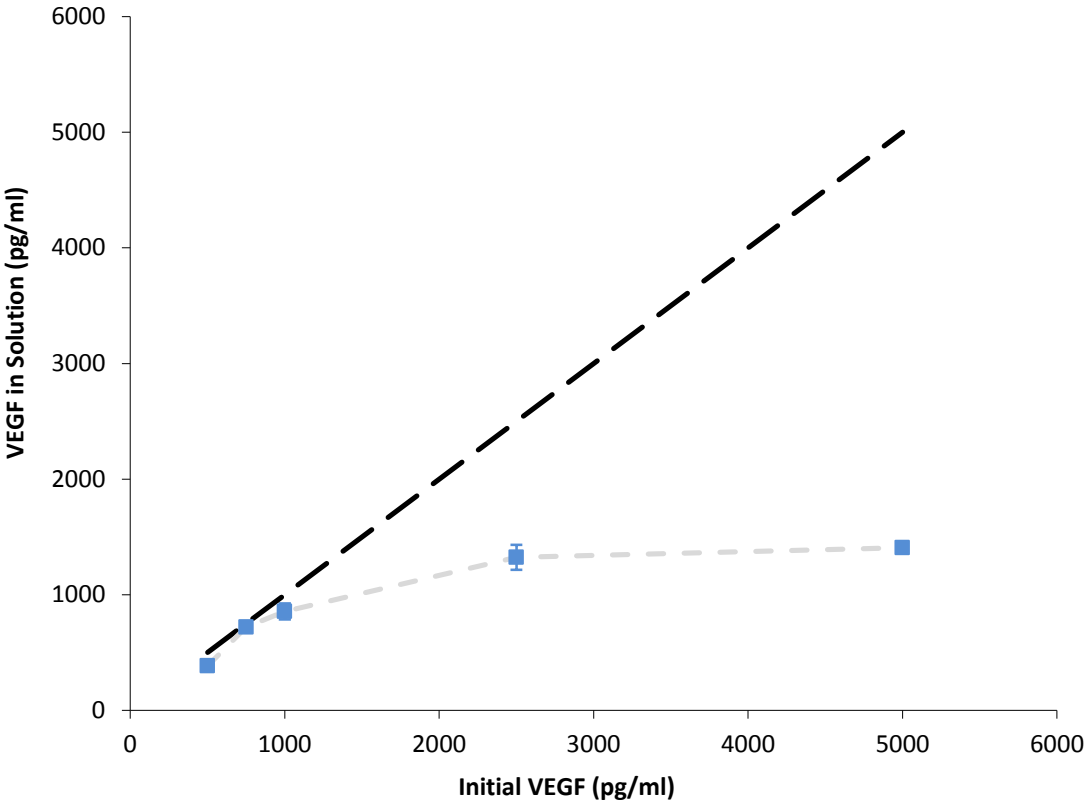

Figure 15. Concentration of VEGF in solution as function of initial concentration of VEGF. Representation of expected concentration if internalization rate were null (y=x) (black line). **Error bars represent the error in the average of the measure concentration of VEGF in solution.**

Sprouting assays

One of the functions of VEGF is to modulate ECs sprouting. Therefore, in a subsequent stage of this work we evaluated the effects of VEGF concentration on VEGF-induced endothelial sprouts. To address this question HMEC cells seeded on cytodex beads were cultured within 3D fibrin gel, containing different amounts of VEGF. All matrices were supplemented with aprotinin, a protease inhibitor typically added to slow fibrinolysis (Smith et al., 2007).

As described in the literature, VEGF induces sprouting angiogenesis. In an attempt to quantify the influence of VEGF in endothelial sprouting we performed sprouting assays in fibrin gels using different VEGF concentrations diffuse in the medium: 0.5, 1 and 5 ng/mL, medium without VEGF was used as a control.

As observed in Figure 16, VEGF induce higher number and longer sprouts. It is also observable that in the higher concentrations of VEGF (Figures 16C and 16D) the number of cells near the beads is higher, suggesting an increase in the ECs proliferation as a function of VEFG concentration.

However more assays are needed to perform a quantitative and standardized analysis.

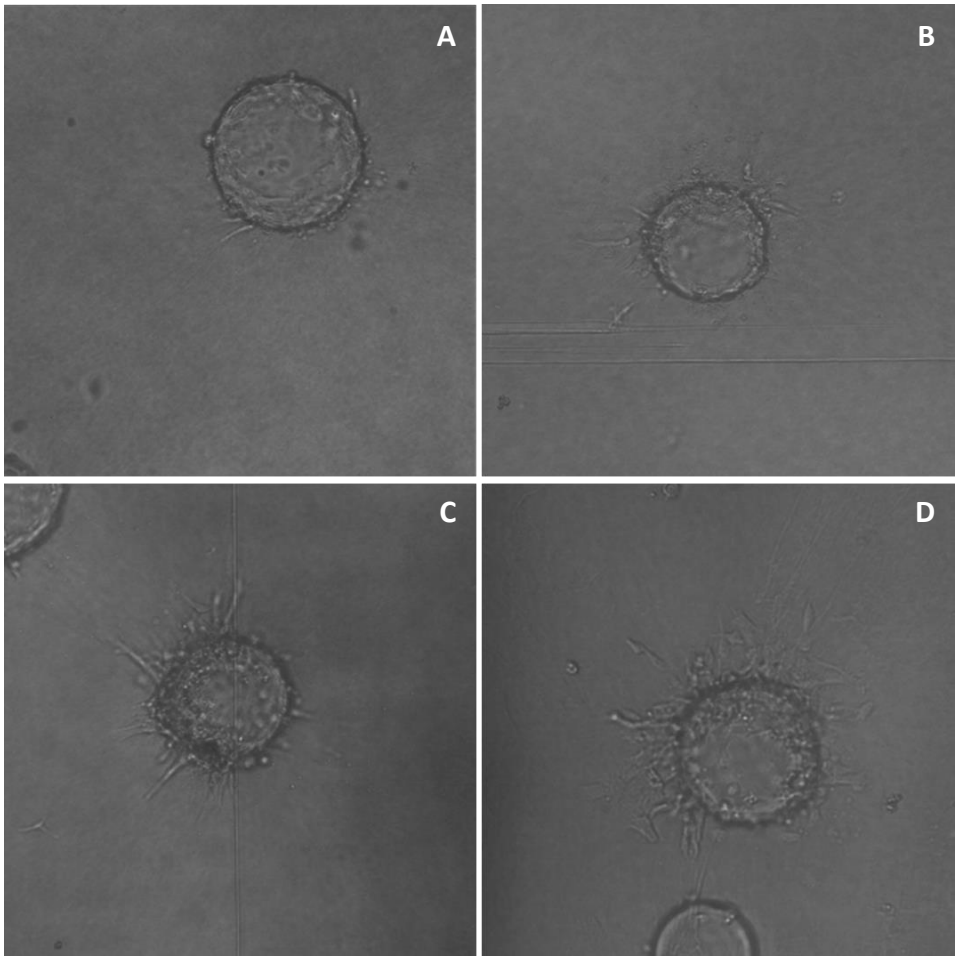

Figure 16. Sprouting induction by VEGF. Cytodex beads coated with HMEC cells were embedded within fibrinogen/thrombin polymerized gels and incubated without VEGF **(A)** or with different VEGF concentrations: 0.5ng/m**L (B)** 1ng/mL **(C)** 5ng/mL **(D).** Control Phase-contrast images were taken using an inverted light microscope 24 hours after embebbing (100x of magnification) and images are representative of each condition..

Matrigel plug assay

Once we have established the effect of VEGF concentration in ECs growth rate, in a second stage of this work we intended to decipher the effect of VEGF on endothelial cell function *in vivo*. For this purpose, we used a matrigel plug assay, in which matrigel with heparin was supplemented with VEGF in different concentrations. Then, the matrigel with various concentrations of VEGF was injected subcutaneously in rats. After seven days, the animals were sacrificed and the vascularization of the matrigel evaluated by the presence of Evans blue, a dye known to combine with plasma albumin, which was injected systemically into the rat before the sacrifice. Furthermore, the ECs were identified using immunofluorescence staining for vWF, a specific marker of ECs. Representative images of each condition are present in Figure17. The results obtained show a low signal for Evans blue and vWF in control conditions, in which we injected matrigel without VEGF. on the other hand plugs carrying any of the VEGF concentrations showed a strong signal, thus demonstrating angiogenesis *in vivo*. Moreover it was evident an increase in the vessel density when comparing intermediate concentrations of VEGF, 10 and 35 ng/mL, Figure 17B and 17C, respectively. Importantly higher caliber vessels are present predominantly in the highest concentration of VEGF, 60ng/mL (Figure 17D).

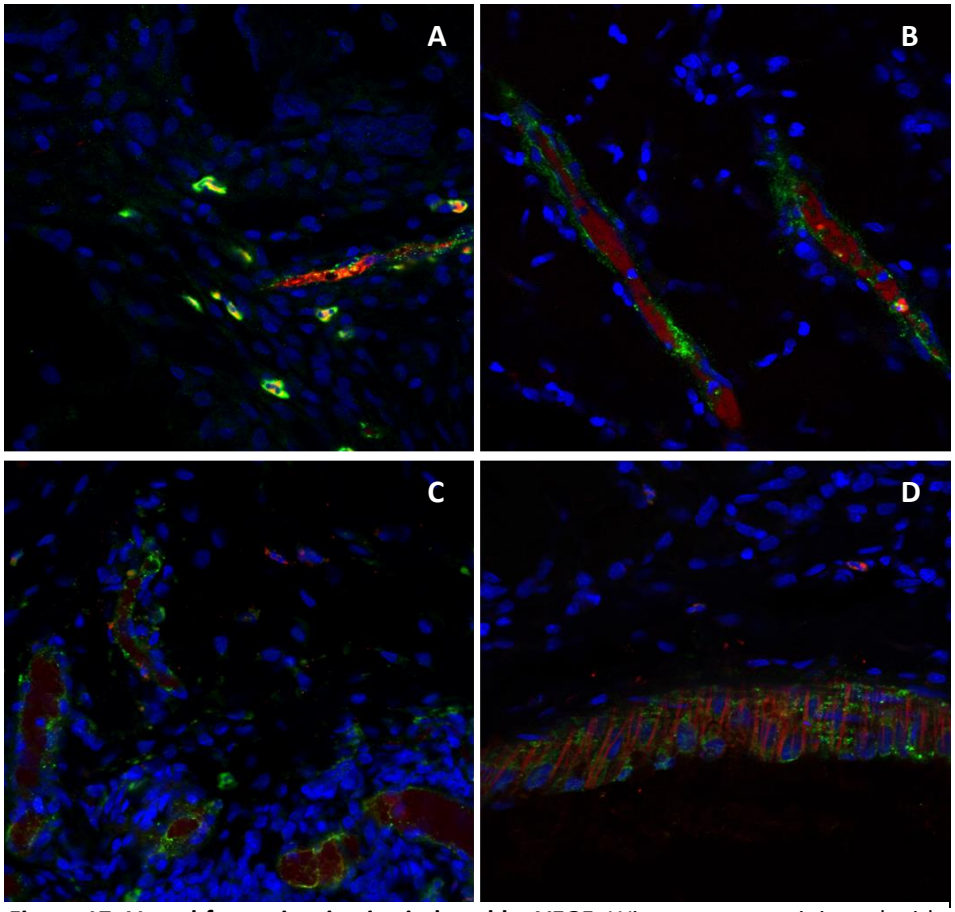

Figure 17. Vessel formation *in vivo* induced by VEGF. Wistar rats were injected with matrigel without VEGF **(A)** or containing different concentrations of VEGF: 10ng/mL **(B)** 35ng/mL **(C)** 60ng/mL **(D).** A week after matrigel implantation, rats were injected with Evans blue solution through the jugular vein and sacrificed. Matrigel slices 5µm thickness were used to perform immunofluorescence using specific antibody against vWF. Matrigel were imaged by confocal microscopy and images are representative of each condition (400x magnification); vWF – green, Plasma albumin (Evans blue) – red, Nuclei (DAPI) – blue.

Discussion

Understanding angiogenesis is essential to the potential control of the blood delivered to a tissue in order to prevent or promote its development, especially in a pathological condition. Considering the complexity of the angiogenesis process, the concurrence of sophisticated image-tracking systems and advanced mathematical models, would provide a relevant tool that integrates all the mechanical cues, signalling pathways and factors that interact and control angiogenesis. This would be particularly helpful to perform early prediction and diagnosis, and could help to define the proper therapies to be followed. However the construction of biological accurate mathematical model is dependent of well-defined biological parameters.

The final morphology of a vascular network depends on both phenomena which can occur at cell level, with activation and subsequent sprouting of new branches and on the large scale collective movements of the cells due to endothelial cell proliferation and the tissue properties. The sprouting angiogenesis constitutes a multifactorial and complex process, involving the orchestrated participation of hundreds of proteins that regulate transcription and participate in signal pathways involved in cell movement, proliferation and phenotype alteration. Among these factors is VEGF, a major regulator of angiogenesis that exerts its function upon binding to the major VEGF receptor, VEGFR2. VEGF-A is an important chemoattractant and mitogen factor, that a triple role in its vascular activity: 1) it is responsible for triggering vessel permeability, encouraging the deposition of proteins in the ECM and, consequently, activating the tip cell phenotype; 2) it also foments the migration of endothelial tip cells, which follow its gradient; and 3) fuels the proliferation of stalk cells and the survival of phalanx cells (Eilken and Adams, 2010; Gerhardt, 2008; Ho and Kuo, 2007).

Given the vital importance of VEGF in angiogenesis, it is of utmost relevance to better elucidate the conditions and factors that govern and determine the effect of VEGF on proliferation and behavior of ECs in the presence of VEGF *in vitro* and *in vivo*. This would allow us to improve the mathematical model, in order to make more accurate and predictable regarding the angiogenic process.

One of the objectives of this work was to quantify and parameterize cell proliferation according to the ECs microenvironment. Cell proliferation, one of the most important biological phenomena occurring during angiogenesis, is very mutable and dependent of slightly changes in the cells microenvironment. One of the factors that is known to modulate the cell proliferation rate is the initial cell density.

Therefore, in a first stage of this work we intended to determine the influence of initial cell density by evaluating cell proliferation rates using 3 different initial numbers of cells. Results obtained in these conditions demonstrate that cells have an exponential growth that is independent of the initial cell density. Moreover, this data enabled us to calculate a growth rate per cell is 0.0159±0.0011 per hour. Therefore the doubling time of a cell is 43.6±3.0 hours.

Another important parameter that determines the growth rate of ECs is the presence of VEGF and its concentration. As a starting-point we define the amount of this factor in the medium over time, in the absence of external VEGF, and therefore we monitor the amount of VEGF produced by ECs. Through these results we could estimate the production rate of ECs in basal conditions, i. e. without any external stimuli, that is approximately 5.2 ±1.1 $x10^{-6}$pg/hour/cell. Once we have established the basal rate of VEGF secretion, we further determine the influence of VEGF in the proliferation rate of ECs.

To address this question we started by incubating the ECs with various VEGF concentrations in the order of magnitude of the amounts produced by ECs and determined in the previous experiment. Although VEGF constitutes an important mitogenic in angiogenesis, the results obtained is this study demonstrate that incubation of cells with external VEGF concentrations between 50 and 200pg/mL did not induce significant changes in cell proliferation when compared to basal conditions (without addition of VEGF). This result can be explained by the fact that the concentrations used are probably too low to influence in a significant way the ECs proliferation and consequently angiogenesis. Moreover, the simplicity of our *in vitro* model, involving a single type of cells, does not reproduce the *in vivo* biological environment of ECs, where they are in contact with other types of cells and external stimuli. Among these *in vivo*

stimuli is VEGF that during angiogenesis is produced, not only be ECs, but also by other cell types such as fibroblasts, inflammatory cells, and others often in response to increasing hypoxia via the HIF-1α pathway (Carmeliet, 2005b). Additionally, it is likely that in vivo local high concentrations of VEGF, confined to specific and restricted cell niches, differentially affects cell proliferation. These experiments indicate that at the observed cell densities, ECs behave independently, not influencing one another's proliferation rate or VEGF production rate. Their growth is exponential and the growth rate depends on their medium, and not on cell density. This is certainly not the case for higher cell densities (see Figure 7), where we observe events of very high proliferation and cell death rates. This fact is not surprising considering that these cells form monolayers in culture, and that contacts between cells can regulate their number at high densities. The study of the proliferation rate at these high cell densities is out of the scope of this work.

Considering these facts and the results from the previous experiment we decided to increase the concentrations used to test the correlation between VEGF and ECs proliferation. For this experiment we used a range of VEGF concentrations between 0.5 and 10ng/mL. The results show that regardless the concentrations of VEGF, EC proliferation follows a similar exponential growth. Indeed we show that in this range of concentrations the mitogenic effect of VEGF in cell proliferation is not dose-dependent, that is in agreement with described for other cells types (Bernatchez et al., 1999; Rafiee et al., 2004), in others words, the increase in VEGF concentration is not always associated with an increase in growth rate. In fact with this experiment we demonstrate that VEGF elicits a biphasic proliferative response in cultured HMECs. Surprisingly, while the growth rate is higher in VEGF concentrations up to 1 ng/ml, and the response declined at 5-10 ng/ml. These observations suggest that there is a narrow window of desensitization to VEGF which may be important in vascular homeostasis. This is supported by the previous studies by Takagi et al., 1996, that showed a significant increase in VEGF expression in association with a transient decrease in VEGFR2 expression and VEGF binding. Based on these observations the authors proposed that when angiogenesis is not immediately needed, an increase from angiogenic stimulation of VEGF concentration may lead to downregulation of VEGFR2 thus protecting from angiogenic stimulation of VEGF.

Although our data consistently suggests that for the concentrations tested, the error associated with these experiments is high. Therefore more experiments are required not only to diminish the experimental error but also to test more concentrations of VEGF to perceive if the proliferation rate recovers or increases with higher concentrations (Cai et al., 2006).

Since serum present in the cell culture medium contains VEGF and other factors that can interfere on the analysis of the effect of external VEGF, we further evaluate the effect of VEGF in cells incubated in the absence of serum. In these conditions, the concentration of VEGF that leads to a higher growth rate is 2.5ng/ml. However, with higher concentrations of VEGF, the growth rate diminishes, but is always higher than the in the absence of external VEGF. However, when comparing growth rates of cells incubated either in the presence or absence of serum, for the same concentration of VEGF it is possible establish that, at 8 hours, the growth rate is superior in the absence of serum. Despite this fact the growth rates decreases after this time-point. In the absence of serum the external added VEGF constitutes the only source of growth factors present in the medium, and an abrupt increase in the levels of this factor prompt a rapid cell growth. After this initial period, VEGF is consumed, being the remaining VEGF together with the absence of nutrients from the serum not sufficient to sustain cell proliferation.

Changes in cell growth can be due to variations in the concentrations of VEGF in the culture medium. To test this hypothesis we evaluated the levels of VEGF in the medium over time. The results obtained show that, regardless the amount of VEGF added to the medium, after an initial decrease in the concentration of VEGF remains approximately constant in time. Based on this data, we suggest that the abrupt decrease in the amount of VEGF present in the medium, in the first 2 hours after platting, is due to the binding of VEGF to its receptor, VEGFR, followed by internalization of the ligand. Moreover, it is conceivable to speculate that the effect of VEGF in cell behavior and proliferation is mostly after its binding to the receptor.

Besides being an important mitogenic, VEGF also plays a role inducing and sustaining endothelial sprouting. For that reason we decided to perform sprouting assays to understand the influence of VEGF in the formation and extension of sprouting. In a first

approach we used ECs coated beads that were embedded in a fibrin gel, and incubated with different concentrations of soluble VEGF in the medium that diffuses equally in the fibrin matrix. In a qualitative analysis is possible to infer that VEGF induces more and longer sprouts, when compared with the control. However with the increase of VEGF concentration, the sprouts appear to loose linearity and direction, and the number of ECs around the carrier increases, likely due to the proliferation induced by VEGF. Taken together these results suggest that low concentrations of VEGF induces cell sprouting, however for higher concentrations of VEGF we observe a significant proliferation of ECs, with no effect on sprouting.

As described before the balance between migration of tip cells and the proliferation of stalk cells is essential for the formation of adequately shaped sprouts (Geudens and Gerhardt, 2011). However each of this population of cells responds differently to VEGF. In our experimental conditions, the VEGF is soluble and is equally diffuse in the matrix, which does not allow a VEGF gradient. That fact compromises the direction of sprouting considering that the tip cell responds to a gradient, migrating outward from the starting point. However, stalks cells response to VEGF is not dependent of a gradient, but yet to a concentration of VEGF that induces their proliferation (Gerhardt et al., 2003), this might explain our results in which we show an increase in cell sprouting with low concentrations of VEGF, when a gradient is likely produced by the consumption of VEGF by the ECs, while for higher concentrations this growth factor induces proliferation of ECs. Besides soluble growth factors, properties of the ECM, like protein composition and stiffness, also plays a role in organizing ECs during sprouting morphogenesis (Davis et al., 2011; Mason et al., 2013). Recent studies have also shown that the mechanism whereby VEGF is presented from the ECM to ECs may affect the architecture of sprouting and consequently of the blood vessels formed (Anderson et al., 2011). Taking this into account, we are now performing sprouting assays altering the presentation form of VEGF, using for that VEGF-coated beads or VEGF trapped in the matrix, that are distributed randomly in different densities. This will allow us to understand the positioning and directionality of sprouting under influence of different gradients created by the ECs trough the secretion of matrix metalloproteinases that degrade the matrix and release the VEGF. Strikingly due to the

magnetic properties of the VEGF-coated beads we will be able to manipulate their positioning.

In the most part of this study we used cell culture systems to investigate various biological aspects of VEGF modulated HMEC proliferation and growth. Therefore, on a subsequent stage of this work we intended to validate the results obtained *in vitro* assays, using for this purpose *in vivo* experiments with Growth Factor Reduced Matrigel, in which VEGF, among other angiogenic cytokines, have been significantly depleted. Addition of recombinant VEGF caused higher recruitment of blood vessels to the Matrigel compared to PBS-loaded controls, thus demonstrating an angiogenic effect. Moreover, despite the increase in the recruitment and formation of the new vessels the vascular integrity was maintained and is not visible vessel leakage, translated by the absence of evans blue in the matrix, in any of the experimental conditions. Previous studies show that process of formation of new vessels is VEGF dose-dependent, and the phenotype of the new blood vessels is governed by the local concentration of this factor (Blanco and Gerhardt, 2013; Carmeliet, 2005b; Ferrara et al., 2003). Greater concentrations of VEGF produce more abundant blood vessels and exaggerate vessel abnormalities, whereas lower concentrations of VEGF drive less angiogenesis and promote thinner and more stable vessels. Our results lead us to conclude that the concentrations used in this experiment are within the range of concentrations that allow the formation of normal and functional vessels.

The analysis performed in the sprouting and in matrigel assays was only qualitative, however in the future these assays will be complemented with a more methodic and accurate analysis that will allow the transposition of these results to quantitative data.

In summary this work allowed us to test and understand better some biological phenomena already known that occur during angiogenesis. However the quantification and the parameterization these biological processes increase our knowledge about angiogenesis and will allow the improvement of a mathematical model of angiogenesis.

The collaboration between biology and mathematical modeling can lead to a better understanding of the process of angiogenesis and properties of newly formed blood vessels will lead to even more informative assays and biomarkers. These in turn will help

in screening and evaluating of new, more efficacious drugs and other novel tools in vascular biology. Together these advances will further the exploitation of vascular abnormalities as targets for drug delivery and the control of blood vessel growth and regression in health and disease.

References

Adams, R. H., and Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol *8*, 464-478.

Ades, E. W., Candal, F. J., Swerlick, R. A., George, V. G., Summers, S., Bosse, D. C., and Lawley, T. J. (1992). HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol *99*, 683-690.

Anderson, S. M., Siegman, S. N., and Segura, T. (2011). The effect of vascular endothelial growth factor (VEGF) presentation within fibrin matrices on endothelial cell branching. Biomaterials *32*, 7432-7443.

Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M., and Adams, R. H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell *137*, 1124-1135.

Bentley, K., Mariggi, G., Gerhardt, H., and Bates, P. A. (2009). Tipping the balance: robustness of tip cell selection, migration and fusion in angiogenesis. PLoS Comput Biol *5*, e1000549.

Bernatchez, P. N., Soker, S., and Sirois, M. G. (1999). Vascular Endothelial Growth Factor Effect on Endothelial Cell Proliferation, Migration, and Platelet-activating Factor Synthesis Is Flk-1-dependent. Journal of Biological Chemistry *274*, 31047-31054.

Blanco, R., and Gerhardt, H. (2013). VEGF and Notch in tip and stalk cell selection. Cold Spring Harb Perspect Med *3*, a006569.

Botta, M., Manetti, F., and Corelli, F. (2000). Fibroblast growth factors and their inhibitors. Curr Pharm Des *6*, 1897-1924.

Burri, P. H., and Djonov, V. (2002). Intussusceptive angiogenesis--the alternative to capillary sprouting. Mol Aspects Med *23*, S1-27.

Cai, J., Jiang, W. G., Ahmed, A., and Boulton, M. (2006). Vascular endothelial growth factor-induced endothelial cell proliferation is regulated by interaction between VEGFR-2, SH-PTP1 and eNOS. Microvasc Res *71*, 20-31.

Carmeliet, P. (2005a). Angiogenesis in life, disease and medicine. Nature *438*, 932-936.

Carmeliet, P. (2005b). VEGF as a key mediator of angiogenesis in cancer. Oncology *69 Suppl 3*, 4-10.

Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. Nature *407*, 249-257.

Chappell, J. C., Taylor, S. M., Ferrara, N., and Bautch, V. L. (2009). Local guidance of emerging vessel sprouts requires soluble Flt-1. Dev Cell *17*, 377-386.

Chappell, J. C., Wiley, D. M., and Bautch, V. L. (2011). Regulation of blood vessel sprouting. Semin Cell Dev Biol *22*, 1005-1011.

Claxton, S., and Fruttiger, M. (2004). Periodic Delta-like 4 expression in developing retinal arteries. Gene Expr Patterns *5*, 123-127.

Davis, G. E., Stratman, A. N., Sacharidou, A., and Koh, W. (2011). Molecular basis for endothelial lumen formation and tubulogenesis during vasculogenesis and angiogenic sprouting. Int Rev Cell Mol Biol *288*, 101-165.

De Spiegelaere, W., Casteleyn, C., Van den Broeck, W., Plendl, J., Bahramsoltani, M., Simoens, P., Djonov, V., and Cornillie, P. (2012). Intussusceptive angiogenesis: a biologically relevant form of angiogenesis. J Vasc Res *49*, 390-404.

Dempke, W. C. M., and Zippel, R. (2010). Brivanib, A Novel Dual VEGF-R2/bFGF-R Inhibitor. Anticancer Research *30*, 4477-4483.

Djonov, V., Schmid, M., Tschanz, S. A., and Burri, P. H. (2000). Intussusceptive angiogenesis: its role in embryonic vascular network formation. Circ Res *86*, 286-292.

Eilken, H. M., and Adams, R. H. (2010). Dynamics of endothelial cell behavior in sprouting angiogenesis. Curr Opin Cell Biol *22*, 617-625.

Ferrara, N., Gerber, H. P., and LeCouter, J. (2003). The biology of VEGF and its receptors. Nat Med *9*, 669-676.

Gerhardt, H. (2008). VEGF and endothelial guidance in angiogenic sprouting. Organogenesis *4*, 241-246.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., and Betsholtz, C. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol *161*, 1163-1177.

Geudens, I., and Gerhardt, H. (2011). Coordinating cell behaviour during blood vessel formation. Development *138*, 4569-4583.

Gridley, T. (2010). Notch signaling in the vasculature. Curr Top Dev Biol *92*, 277-309.

Hellstrom, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A. K., Karlsson, L., Gaiano, N.*, et al.* (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature *445*, 776-780.

Hillen, F., Melotte, V., van Beijnum, J. R., and Griffioen, A. W. (2007). Endothelial Cell Biology. In Angiogenesis Assays, (John Wiley & Sons, Ltd), pp. 1-38.

Ho, Q. T., and Kuo, C. J. (2007). Vascular endothelial growth factor: biology and therapeutic applications. Int J Biochem Cell Biol *39*, 1349-1357.

Holderfield, M. T., and Hughes, C. C. (2008). Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis. Circ Res *102*, 637-652.

Hynes, R. O., Lively, J. C., McCarty, J. H., Taverna, D., Francis, S. E., Hodivala-Dilke, K., and Xiao, Q. (2002). The diverse roles of integrins and their ligands in angiogenesis. Cold Spring Harb Symp Quant Biol *67*, 143-153.

Jakobsson, L., Franco, C. A., Bentley, K., Collins, R. T., Ponsioen, B., Aspalter, I. M., Rosewell, I., Busse, M., Thurston, G., Medvinsky, A.*, et al.* (2010). Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat Cell Biol *12*, 943-953.

Jones, N., Iljin, K., Dumont, D. J., and Alitalo, K. (2001). Tie receptors: new modulators of angiogenic and lymphangiogenic responses. Nat Rev Mol Cell Biol *2*, 257-267.

Kamei, M., Saunders, W. B., Bayless, K. J., Dye, L., Davis, G. E., and Weinstein, B. M. (2006). Endothelial tubes assemble from intracellular vacuoles in vivo. Nature *442*, 453-456.

Lammert, E., and Axnick, J. (2012). Vascular Lumen Formation. Cold Spring Harbor Perspectives in Medicine *2*.

Li, H., Fredriksson, L., Li, X., and Eriksson, U. (2003). PDGF-D is a potent transforming and angiogenic growth factor. Oncogene *22*, 1501-1510.

Lobov, I. B., Renard, R. A., Papadopoulos, N., Gale, N. W., Thurston, G., Yancopoulos, G. D., and Wiegand, S. J. (2007). Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci U S A *104*, 3219-3224.

Mason, B. N., Starchenko, A., Williams, R. M., Bonassar, L. J., and Reinhart-King, C. A. (2013). Tuning three-dimensional collagen matrix stiffness independently of collagen concentration modulates endothelial cell behavior. Acta Biomater *9*, 4635-4644.

Nakatsu, M. N., Davis, J., and Hughes, C. C. (2007). Optimized fibrin gel bead assay for the study of angiogenesis. J Vis Exp, 186.

Passaniti, A., Taylor, R. M., Pili, R., Guo, Y., Long, P. V., Haney, J. A., Pauly, R. R., Grant, D. S., and Martin, G. R. (1992). A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. Lab Invest *67*, 519-528.

Rafiee, P., Heidemann, J., Ogawa, H., Johnson, N. A., Fisher, P. J., Li, M. S., Otterson, M. F., Johnson, C. P., and Binion, D. G. (2004). Cyclosporin A differentially inhibits multiple steps in VEGF induced angiogenesis in human microvascular endothelial cells through altered intracellular signaling. Cell Commun Signal *2*, 3.

Ribatti, D., and Crivellato, E. (2012). "Sprouting angiogenesis", a reappraisal. Dev Biol *372*, 157-165.

Roca, C., and Adams, R. H. (2007). Regulation of vascular morphogenesis by Notch signaling. Genes Dev *21*, 2511-2524.

Siekmann, A. F., and Lawson, N. D. (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. Nature *445*, 781-784.

Smith, J. D., Chen, A., Ernst, L. A., Waggoner, A. S., and Campbell, P. G. (2007). Immobilization of aprotinin to fibrinogen as a novel method for controlling degradation of fibrin gels. Bioconjug Chem *18*, 695-701.

Song, J. W., and Munn, L. L. (2011). Fluid forces control endothelial sprouting. Proc Natl Acad Sci U S A *108*, 15342-15347.

Styp-Rekowska, B., Hlushchuk, R., Pries, A. R., and Djonov, V. (2011). Intussusceptive angiogenesis: pillars against the blood flow. Acta Physiol (Oxf) *202*, 213-223.

Takagi, H., King, G. L., Ferrara, N., and Aiello, L. P. (1996). Hypoxia regulates vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A2 receptors in retinal capillary endothelial cells. Invest Ophthalmol Vis Sci *37*, 1311-1321.

Thurston, G. (2003). Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. Cell Tissue Res *314*, 61-68.

Tung, J. J., Tattersall, I. W., and Kitajewski, J. (2012). Tips, stalks, tubes: notch-mediated cell fate determination and mechanisms of tubulogenesis during angiogenesis. Cold Spring Harb Perspect Med *2*, a006601.

Zachary, I. (2005). Signal transduction in angiogenesis. In Mechanisms of Angiogenesis, M. Clauss, and G. Breier, eds. (Birkhäuser Basel), pp. 267-300.