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UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Low Doses of Ionizing Radiation: A New Approach For Therapeutic Angiogenesis.

Adriana de Fátima Dias Lisboa Correia

DISSERTAÇÃO

Mestrado em Biologia Molecular e Genética

2014

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Dissertação orientada pela Profª. Doutora Susana Constantino (Unidade de Angiogénese, Faculdade de Medicina da Universidade de Lisboa), e pela Profª. Doutora Maria Margarida Perestrello Ramos (Faculdade de Ciências da Universidade de Lisboa)

INTRODUCTION

1. The Circulatory System and Homeostasis

The survival of any metazoan specie is dependent upon continuous oxygen supply. In more primitive organisms, consisting of several hundred up to a few thousands of cells (e.g. *C.elegans*), oxygen is distributed by simple diffusion. In more complex species the increased biological complexity brought upon by selection and evolution, lead to the need for specialized systems of conduits capable of delivering oxygen and nutrients to distant organs, along with the removal of metabolic wastes[1]. In higher vertebrates, these vital functions are carried out by the circulatory system, which comprises the cardiovascular system, distributing blood, and the lymphatic system, circulating the lymph[2]. A network of blood vessels containing carrier molecules assemble a closed circuit where blood is pumped around the body through arteries and returned though veins, both of which ramify into arterioles and venules, respectively, and further into capillaries[2]. Capillaries form interweaving networks called capillary beds, that surround the tissues and establish a surface for the exchange of gases and nutrients, closing the circulatory loop between the arterial and the venous systems[2]. Due to the arterial pressure imposed by the system, blood plasma continuously leaks from the capillaries into the extracellular space[3]. In order to maintain the fluid balance in the tissue, the extravasated fluid (interstitial fluid) that cannot be osmotically re-absorbed at site, is drained by the lymphatic system (as lymph), via lymphatic vessels and capillaries, and ultimately returned back to the venous circulation[3]. This lymph, rich in proteins, lipids, immune cells, and any foreign material present in it, is filtered along the way through a chain of lymph nodes, granting the lymphatic system a crucial role in the immune defence[4].

The formation and further maintenance of a complex, highly organized and functionally competent vascular network is therefore a key component to homeostasis.

2. Early Blood Vascular Development and Maturation of Blood Vessels

The entire circulatory system is lined by interconnected, tube-forming, endothelial cells (ECs). In a gastrulating embryo, ECs differentiate from mesodermal-derived EC percursors, or angioblasts[5–7], to form a primitive network of rather uniformly sized EC channels in a process known as vasculogenesis[8–10]. This primitive vascular network then progressively expands through the formation of new vascular segments originating from existing vessels, remodelling into a highly organized network of larger vessels that ramify into smaller ones[9,11]. There are

two processes by which new vessels are formed from the pre-existing vasculature: i) by intussusception, the splitting of vessels through the insertion of tissue pillars and ii) by (sprouting) angiogenesis whereby new vessels sprout from the ends and sides of the pre-existing ones; either of which may then split and branch into lower calibre vessels[8,9,11–13]. After expansion of the nascent capillary bed, the EC channels mature into a system of stable vessels. Maturation of the neovessels involves recruitment of mural cells (pericytes and smooth muscle cells) to form the surrounding vessel wall, granting stability to the vessel, a process named arteriogenesis[8,14,15]. This process depends highly on the physiological function of the vessel, regarding the tissue it nurtures. Small blood vessels consist of only ECs, whereas larger vessels are surrounded by mural cells: pericytes in medium-sized and smooth muscle cells (SMCs) in large vessels[15]. Additionally, collateral growth denotes the expansive growth of pre-existing vessels, forming collateral bridges between arterial networks[14,16].

During the maturation process, ECs become phenotypically and functionally specialized in order to assure proper integrity and architecture of the circulatory system, as well as to perform their necessary functions[17,18]. It is a combination of hard-wired genetic programming and extrinsic signals that determines the specialization of the endothelium to arterial, venous, hemogenic and lymphatic subtypes[19]. For instance, arteries are supported by layers of vascular SMCs and a specialized matrix, whereas veins are thinner and surrounded by fewer SMCs[13,15]. Their structures reflect the differences in hemodynamic load to which they are subject. Arteries face high pressure gradients enabling transportation of blood to capillaries whereas veins face low pressure gradients[13]. Moreover, arterial and venous ECs possess specific molecular identities[17]. For instance, Notch pathway components are highly expressed in arteries but low in veins[20–22]. Disruption of Notch signalling causes loss of arterial markers and re-expression of venous signature genes, suggesting that Notch promotes arterial specification by repressing venous identity[23–28].

Disrupting the normal program of vascular development often results in disease phenotypes or even embryonic lethality.

3. Angiogenic sprouting

The first step in the angiogenic process requires the activation of ECs by angiogenic signals, such as vascular endothelial growth factors (e.g. VEGF, VEGFC), angiopoietins (e.g. ANGPT2), fibroblast growth factors (FGFs) and/or chemokines[29]. When a quiescent vessel

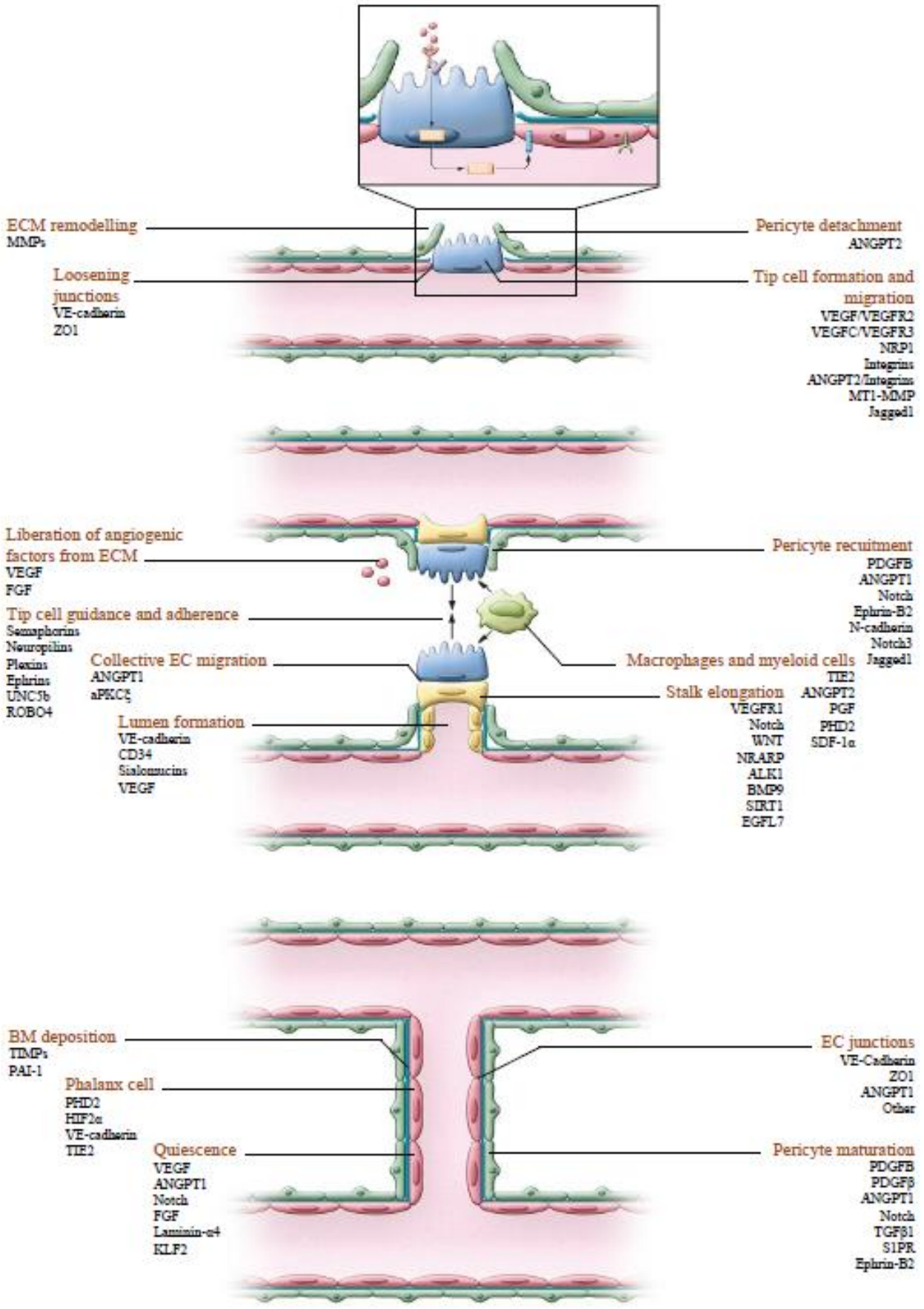

Figure [1] Molecular basis of vessel branching. *Adapted from* Welti *et al.* 2013 (*detailed in page 3*).

senses an angiogenic signal, cell-cell junctions and the basement membrane (BM) are remodelled[30] in part by matrix metalloproteinases (MMPs) and mural cells detach allowing an activated EC, tip cell, to migrate in response to guidance signals[31]. ECs become motile and invasive and protrude filopodia[32]. Trailing behind tip cells, stalk cells extend fewer filopodia but establish a lumen and proliferate to support sprout elongation[33–35]. Tip cells anastomose with cells from neighbouring sprouts to build vessel loops[36,37]. The initiation of blood flow, the establishment of a basement membrane, and the recruitment of mural cells stabilize new connections[13,38]. The sprouting process iterates until proangiogenic signals abate, and quiescence is re-established[13,14,39–41] (Figure 1).

Figure [1] Molecular basis of vessel branching. a. Initial steps of tip cell selection. Vascular sprouting is initiated by proangiogenic factors (e.g. VEGF). ECs at the leading edge of the vascular sprout extend filopodia and migrate toward angiogenic signals. VEGF activates VEGFR-2 to stimulate tip cell migration. The coreceptor NRP1 complexes with and enhances VEGFR-2 signalling. ECs become either the migratory vessel-leading tip cell or the proliferating stalk cell, but their phenotype is fluid; Notch regulates this specification. ECs with activated VEGFR-2 signalling compete for the tip cell position by increasing their expression of DLL4, which binds to Notch receptors on neighbouring ECs, releasing the transcription regulator NICD. NICD transcriptionally downregulates VEGFR-2 and NRP1 expression while increasing VEGFR-1, a VEGF trap, thus enhancing the stalk cells' unresponsiveness to VEGF. The tip cell is not a fixed position, and fluidity at the front occurs depending on the VEGFR-1/VEGFR-2 ratio. Tip cell migration requires BM degradation (in part due to MMP), EC junction loosening (caused by VE-cadherin, ZO1, and others), and pericyte detachment (regulated by ANGPT2). VEGF increases the permeability of the vessel, allowing the extravasation of plasma proteins (e.g., fibronectin and fibrinogen) that are deposited as a provisional matrix layer while the pre-existing interstitial matrix is remodelled by proteases; these events enable tip cell migration. **b.** Tip cell guidance and stalk elongation. The growing sprout moves along a VEGF gradient. Tip cells adhere to the ECM, mediated by integrins, and migrate toward guidance signal molecules (e.g., semaphorins and ephrins). Stalk cells trail behind the tip cell and proliferate to allow sprout elongation and lumen formation. While Notch signalling inhibits proliferation, expression of NRARP at branch points allows Wnt signalling to maintain stalk cell proliferation. This system allows vascular migration/directionality (by tip cells) and elongation of the shaft (by proliferating stalk cells). When two tip cells meet, they fuse (anastomose); this mechanism is assisted by macrophages, which accumulate at sites of vascular anastomosis to act as bridge cells by interacting with the neighbouring tip cells' filopodia. Once contact between the tip cells has been established, VE-cadherin–containing junctions further strengthen the connection. Perivascular macrophages further stimulate sprouting by producing angiogenic factors or proteolytically liberating them from the ECM. The stalk cells also deposit BM and recruit pericytes, thus stabilizing the forming vessel. Pericyte precursors are attracted to vessels by EC-expressed PDGF. Once at the vessel, these mesenchymal precursor cells differentiate to pericytes in response to TGFβ and decrease EC migration, proliferation, and vascular leakage, resulting in nascent vessel stabilization. **c.** Maturation through resolution of quiescent phalanx cells. Once fusion has occurred, a connected lumen is formed to allow blood flow through the new vessel. This perfuses the hypoxic tissue, and the resultant oxygen and nutrient delivery leads to decreased levels of angiogenic signals, inactivation of EC oxygen sensors, and increased proquiescent molecules that lead to EC quiescence. Establishment of the blood flow remodels vessel connections, which are regulated by the shear stress–responsive transcription factor KLF2. ECs resume a quiescent phalanx phenotype in a tightly apposed monolayer with a streamlined surface that conducts the blood flow and regulates tissue perfusion. Perfusion induces vascular maturation by reestablishment of cell-cell junctions, pericyte maturation, and BM deposition. Autocrine and paracrine signalling from ECs and surrounding support cells by VEGF, FGF, ANGPT1, and Notch, among others, maintain a quiescent EC phenotype and protect the vessel from environmental stresses. Reduced growth factor signalling can lead to vessel retraction and EC apoptosis. Once stabilized and matured, the vessel forms a barrier between the blood and surrounding tissue, controlling the exchange of fluids and solutes. *Adapted from* Welti *et al*. (2013).

4. Angiogenic Regulators

Functional angiogenesis requires a complex coordination of steps, which are regulated by a fine-tuned balance of angiogenic inducers and inhibitors[42–46]. Table [1] for a selective list of the main stimulators and inhibitors that take part in the angiogenic response.

Table [1] List of the main angiogenic stimulators and inhibitors.

Angiogenic Stimulators	Angiogenic Inhibitors
Angiogenin	Angiopoietin (ANGPT) -2
Angiopoietins (ANGPTs)	Angiostatin
Angiotropin	Arresten
Cysteine-rich protein 61 (CYR61)	Endostatin
Ephrin	Interferon (IFN) -α, -β, -γ
Epidermal growth factor (EGF)	Interleukin (IL) -12
Erythropoietin (EPO)	Platelet factor (PFA) -4
Fibroblast growth factors (FGFs)	Thrombospondin (TSP) -1, -2
Hepatocyte growth factor (HGF)	Tissue inhibitors of metalloproteinase (TIMP) -1, -2, -3
Insuline-like growth factor (IGF) -1	Transforming growth factor (TGF) -β *
Integrins	
Interleukin (IL) -8	
Matrix metalloproteinases (MMPs)	
Nitric Oxide (NO)	
Platelet endotelial cell adhesion molecule (PECAM) -1	
Platelet-derived endothelial growth factor (PDGF)	
Transforming growth factor (TGF) -β *	
Vascular endothelial (VE)–cadherin	
Vascular endothelial growth factor (VEGF)	

* Can show opposite effects depending upon doses and environmental conditions

4.1. The angiopoietin family

The human angiopoietin (ANGPT) family consists of four members, ANGPT1, ANGPT2, ANGPT3 and ANGPT4, which bind to specific tyrosine kinase receptors with immunoglobulin-like and EGF-like domains (TIE) -1 and -2 (TIE1 and TIE2)[44].

ANGPT1 induces EC survival, capillary sprouting and pericyte recruitment[47]. By increasing the interaction between ECs and pericytes, ANGPT1 is known to stabilize blood vessels[48]. *In vivo,* studies suggest that ANGPT1 is essential for maturation and stabilization of the developing vasculature and for normal remodelling, since mice lacking *Angpt1* start to develop a primary vasculature which fails to stabilize or remodel leading to embryonic lethality[49]. On the other hand, ANGPT2 can either promote or inhibit vessel growth, depending on the presence of other growth factors, such as VEGF[47]. ANGPT2 was first described to block ANGPT1-mediated TIE2 receptor activation, acting as an antiangiogenic factor capable of promoting EC

apoptosis and blood vessels regression *in vivo*[50]. Intriguingly, subsequent studies have shown that higher expression levels of ANGPT2 are associated to sites of vascular remodelling in adults, in particular in the female reproductive tract and in highly vascularised tumours[51,52]. In fact, it has been suggested that, by antagonizing the stabilizing influence of ANGPT1, ANGPT2 might provide a key destabilizing signal reverting vessels to a more plastic state[48]. Such destabilized vessels could then be prone to: regression in the absence of growth factors; or alternatively, angiogenic sprouting mediated by available angiogenic factors such as VEGF. Further investigations have demonstrated that, in the presence of VEGF, ANGPT2 is responsible for an increase in capillary diameter, migration and proliferation of ECs, and sprouting of new blood vessels[53]. Additionally, high levels of ANGPT2 can induce TIE2 phosphorylation in human umbilical vein endothelial cells (HUVEC), stimulating cell proliferation, cell differentiation and protection against induced cell death[54,55]. ANGPT2-induced TIE2 phosphorylation has also been demonstrated in murine brain capillary ECs, promoting migration and tube-like structure formation[56].

4.2. The fibroblast growth factor family

The fibroblast growth factor (FGF) family consists of at least 18 members, to which four high-affinity tyrosine-kinase receptors (FGFRs) have been described[57]. The most studied forms are FGF2 (or basic FGF, bFGF) and FGF1 (or acidic FGF, aFGF), which most commonly bind to FGFR1 and FGFR2, on ECs[57].

In vitro studies have shown that stimulation of FGF1 and FGF2 induces proliferation, migration, survival, and differentiation of ECs[58,59]. The angiogenic activity of recombinant FGF1 and FGF2 proteins has been demonstrated in various *in vivo* models, including the avascular rabbit or mouse cornea[60], mice subcutaneous matrigel injection[61], and the chicken chorioallantoic membrane (CAM) assay[62]. FGF2 has also been reported to up-regulate the expression of several proangiogenic molecules, such as MMPs, αvβ3 integrin, VEGF and HGF[44,58]. Studies using knockout mice have demonstrated essential functions for FGFR1 and FGFR2 in early development[63]. Moreover, *Fgf2* and *Fgf1/Fgf2* knockout mice exhibit delays in the remodelling of damaged blood vessels during wound healing and tumour angiogenesis[58]. Additionally, tube formation stimulated by VEGF is totally abolished when neutralizing antibodies to FGF2 are added to the system, showing that in this particular setting, VEGF requires the presence of FGF2 for promoting vessel assembly[64]. FGF signalling also contributes to the proliferation of tumour cells either by an autocrine or paracrine mechanism[57,64]

4.3. The hepatocyte growth factor family

The hepatocyte growth factor (HGF) is a mesenchyme-derived pleiotropic factor[65], which regulates cell growth[66], cell motility[66,67], and morphogenesis of various types of cells and is thus considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis[68,69]. Although HGF was originally identified as a potent mitogen for hepatocytes[70], it has also been identified as a member of the angiogenic factors.

HGF, together with its tyrosine kinase receptor, C-MET[71], have been associated with most types of human cancers. Their expression often correlates with poor prognosis and metastases[72,73]. Multiple biological outcomes of HGF–Met signalling account for its role in cancer, among which the most critical are cell proliferation, tumour cell invasion, and angiogenesis[74]. HGF is angiogenic: the ligand–receptor interaction stimulates ECs to proliferate and migrate *in vitro*[66], induces blood vessel formation *in vivo*[75], and induces the VEGF expression in human cancer cells. It was shown that HGF–Met signalling operates as a key switch, turning on VEGF and turning off TSP-1 expression and thus invoking angiogenesis[76]. Recent studies have demonstrated the potential application of HGF to treat cardiovascular diseases such as peripheral vascular disease, myocardial infarction and cerebrovascular disease[77].

4.4. The platelet-derived growth factor family

The platelet-derived growth factor (PDGF) family comprises four members: PDGF -A, -B, -C, -D, binding with distinct selectivity to the receptor tyrosine kinases PDGFRα and PDGFRβ, expressed on ECs and SMCs[78]. The PDGFB/ PDGFRβ signalling is an important regulator of the blood vessel maturation[38]. PDGFB secreted by ECs in response to VEGF recruits mural cells to the growing vessels by signalling through PDGFRβ[79,80]. In agreement, *Pdgfβ -/-* and *Pdgfrβ -/-* knockout mice are embryonic lethal presenting severe haemorrhage and oedema due to the failure of immature vessels to attract pericytes[80]. PDGFC promotes vascular development in the embryo and in wound healing, as well as angiogenesis in avascular tissues[81], whereas PDGFD is involved in tumour neovascularisation[82].

4.5. The transforming growth factor-β family

The transforming growth factor-β (TGFβ) and its receptors are expressed in a broad spectrum of cell types, including tumour cells, pericytes and ECs, acting both in a paracrine and autocrine fashion.

TGFβ binds to two different types of serine-threonine kinase receptors, known as type I (TGFβR1) and type II (TGβR2). TGFβR1 and TGFβR2 are interdependent, meaning that TGFβR1 requires TGFβR2 to bind TGFβ and TGFβR2 requires TGFβR1 for signaling[44,83].

The most well studied member of this family is the TGFβ1, being its biological function highly dose- and microenvironment-dependent[84]. At low doses it contributes to the angiogenic switch, either indirectly, by inducing the upregulation of angiogenic factors (e.g. VEGF, FGF and PDGF, and proteinases)[44,85]; or directly, through the binding to the two types of TGFβR1: ALK1 and ALK5 (activin receptor-like kinase -1 and -5, respectively) and consequent activation of proangiogenic or maturation-specific genes[84]. At high doses it inhibits EC proliferation and migration through down-regulation of angiogenic factors and up-regulation of TIMPs[44,83].

4.6. The vascular endothelial growth factor family

The vascular endothelial growth factor (VEGF) family includes VEGF(-A), -B, -C, -D, and the placenta growth factor (PGF)[86–88]. VEGF (VEGFA) is the best characterized member of the family and the major mediator of normal and tumour angiogenesis[45,89]. Genetic studies have further demonstrated the importance of VEGF for normal vascular development since the loss of a single VEGF allele results in mice embryonic lethality[90,91]. VEGF is expressed in different tissues and circumstances[88], with mRNA transcription reportedly induced by a number of factors including hypoglycaemia, mechanical forces of shear stress and cell stretch[45], other growth factors and cytokines (e.g. PDGF, EGF, TNFα, TGFβ and IL1β to name a few)[91,92], hormones (e.g. estrogen and progesterone), but mostly and above all by hypoxia[93].

Hypoxia inducible factor 1 (HIF1) is an oxygen-regulated transcriptional activator that functions as a master regulator of oxygen homeostasis[94]. HIF1 is a heterodimer, composed of HIF1α and HIF1β subunits[94]. Whilst HIF1β is constitutively expressed, HIF1α expression is highly induced in hypoxic cells upon low O_2 concentrations in the microenvironment [94].In non-hypoxic conditions, HIF1α is ubiquitinated and subjected to proteosomal degradation[94]. Under hypoxic conditions, the fraction of HIF1α that is ubiquitinated decreases dramatically, resulting in an accumulation of the protein[94]. A functional hypoxia response element (HRE) in the 5' region of the *VEGF* human promoter was found to bind the heterodimer HIF1α/HIF1β[95].

VEGF family members exert their biologic effect through binding to transmembrane tyrosine kinase receptors: VEGFR-1 and VEGFR-2, selectively expressed on vascular ECs[96]. Additionally, VEGF interaction with a class of co-receptors, neuropilin 1 (NRP1) and NRP2,

initially described as semaphorin receptors involved in axon guidance in the nervous system, has been shown to modulate the VEGF binding to the main receptors[97].

The binding affinity of VEGFR-1 for VEGFA is at least 10 fold higher than that of VEGFR-2[98]. However, despite binding VEGF with high affinity, VEGFR-1 presents weak tyrosine kinase phosphorylation activity following VEGF stimulation[98]. Experiments with mice lacking *Vegfr-1* show that this receptor acts as a negative regulator of angiogenesis during embryonic development, as animals exhibit uncontrolled EC proliferation which results in the obstruction of vessel lumen and early lethality[99]. However, in adult, VEGFR-1 plays a role in activating VEGFR-2 and thereby in angiogenesis, binding of PGF[100]. This mechanism gains importance in angiogenesis-associated pathologies, where PGF has often been described upregulated[101]. Furthermore, VEGFR-1 is involved in the preparation of the metastatic niche, since VEGFR-1-positive haematopoietic progenitor cells were shown to colonize tumour specific pre-metastatic sites prior to the arrival of tumour cells[102].

VEGFR-2 is considered the major mediator of the VEGF signalling during vasculogenesis and angiogenesis. Phosphorylated VEGFR-2-tyrosine residues serve as docking sites for molecules that initiate different signalling cascades leading to cellular responses such as proliferation, migration, survival and permeability. In pathologic conditions, VEGFR-2 promotes tumour angiogenesis, being highly expressed by several human cancer cells[103]. It has been shown that the blockage of VEGF activity leads to an inhibitory effect on the growth of many tumour cell lines in nude mice[104,105].

5. Physiological Angiogenesis

In the embryo, blood vessels provide the growing organs with the necessary oxygen and nutrients to develop. After birth, despite still contributing to organ growth, most blood vessels remain quiescent and angiogenesis occurs only in specific situations, such as wound healing, the regeneration of the endometrium during the menstrual cycle or in the placenta during pregnancy[1,106].

Healthy adult ECs have long-half lives and are rendered quiescent by the action of maintenance signals. However, when an insult arises and physiological stimuli are released by environmental cues, such as hypoxia for blood vessels or/and inflammation for lymph vessels, such phenotype is rapidly shifted towards a motile, proliferative and evasive cell population, sending out sprouts in a coordinated directional manner[14]. ECs must therefore be equipped with a set of molecules that allow them to communicate with other cells within the vessel wall, and with cells inside

and outside of the lumen of the vessel, allowing them to sense changes in the blood flow and dynamically interact with the internal cytoskeleton and surrounding extracellular matrix, all in an integrated manner.

6. Pathological Angiogenesis.

When the dynamic equilibrium between pro- and antiangiogenic factors is further disrupted, vessel growth is deregulated, which may in turn have a major impact in health contributing to the pathogenesis of many disorders[1,14,107]. Impairment of blood vessel supply characterizes diseases like heart ischemia, hypertension, atherosclerosis, and diabetes[1]. Excessive growth on the other hand, has been implicated in diseases like cancer (and metastasis), age-related macular degeneration and rheumatoid arthritis[1,107]. Several congenital or inherited diseases have also been described to be caused by abnormal vascular remodelling[14,46]. Table [2] for a list of diseases characterized by excessive and insufficient angiogenesis.

Table [2]. Diseases characterized by de-regulated Angiogenesis. *Adapted from* Carmeliet (2003) *and* Bhadada *et al.* (2010).

Affected organ	Diseases characterized by excessive angiogenesis	Diseases characterized by insufficient angiogenesis
Adipose tissue	Obesity.	
Bone	Synovitis; Osteomyelitis.	Osteoporosis; Impaired fracture healing.
Blood vessels	DiGeorge syndrome; Hereditary hemorrhagic teleangiectasia (HHT); Cancer Metastasis; Cavernous hemangioma.	Atherosclerosis; Hypertension; Diabetes; Peripheral arterial disease (PAD).
Eye	Persistent hyperplastic vitreous syndrome; Diabetic retinopathy; Retinopathy of prematurity; Aged-related macular degeneration; Choroidal neovascularisation.	
(Gastro)-intestinal	Inflammatory bowel and periodontal disease; Peritoneal adhesions; Ulcerative colitis.	Gastric or oral ulcerations; Crohn disease.
Heart		Myocardial Ischemia; Myocardial hypertrophy.
Lung	Primary pulmonary hypertension; Asthma; Nasal polyps.	Neonatal respiratory distress; Pulmonary fibrosis; Emphysema.
Nervous system	Von Hippel-Lindau disease (VHL).	Alzeihmer disease; Amyotrophic lateral sclerosis; Diabetic neuropathy; Cerebral Ischemic Stroke.
Reproductive system	Endometriosis; Ovarian cysts/hyperstimulation and cysts.	Preeclampsia.
Skin	Psoriasis; Scar keloids; Wound healing.	Hair loss; Lupus.

6.1. Peripheral Arterial Disease

Peripheral arterial disease (PAD) is characterized by an occlusion in arterial beds, exclusive of the coronary, aortic arch or brain, restricting blood flow[108,109]. As a consequence, the nutritive requirements of the tissues beyond the occlusion point can only be met to a certain extent. PAD is a typical manifestation of systemic atherosclerosis, greatly affecting the circulation of the lower limbs[108,109].

The risk factors for PAD include diabetes mellitus, cigarette smoking, advanced age (>60/70), hyperhomocysteinemia, hyperlipidemia, hypercholesterolemia and hypertension[110]. These same risk factors are described for cardiovascular disorders, explaining in part the higher risk of cardiovascular morbidity and mortality associated with PAD patients[111]. The prevalence for PAD is high[112–114] and is increasingly recognized as a health burden worldwide[115].

Critical limb ischemia (CLI) is the term used for patients with chronic ischemic rest pain, ulcers, or gangrene, attributed to inadequate blood flow or arterial occlusive disease[109]. It is the progressive evolution and clinical manifestation of peripheral arterial disease (PAD)[109].

CLI occurs when arterial lesions impair blood flow and distal perfusion pressure, to a level that is insufficient to satisfy the nutritive needs of the limb. This usually results from the presence of multilevel occlusive disease or occlusion of critical collaterals[110]. Moreover, in CLI, there is an inappropriate response of the microcirculatory flow regulatory mechanism due to microcirculatory defects that might include endothelial dysfunction, altered hemorheology and white blood cell activation and inflammation[110].

The primary goals of in the treatment of CLI are to relieve ischemic pain, heal ulcers, prevent limb loss, improve patient function and quality of life, and to prolong overall survival[110].

Any kind of surgical/endovascular revascularization, the therapy of choice in CLI patients, should be done whenever technically possible. Attempts to manipulate and normalize the microcirculatory flow pharmacologically may enhance the results of revascularization. In patients with CLI not eligible for arterial revascularization, prostanoids are vasoactive drugs with proven efficacy[116,117]. However, recent trials do not support the benefit of prostanoids in promoting amputation-free survival[118]. In a large proportion of CLI patients, the extent and anatomic distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization[110]. Amputation is often recommended as a solution to the disabling symptoms, even if it is associated to morbidity and mortality[110]. The need for alternative treatment strategies in CLI patients is therefore compelling, and therapeutic angiogenesis is a promising tool to treat these patients.

7. Angiogenesis as a therapeutic target

The scientific advances achieved on the molecular mechanisms that involve the angiogenic response, and/or develop disease, have triggered the path for the development of therapeutic strategies to promote angiogenesis[119].

The administration of angiogenic growth factors or inhibition of antiangiogenic factors, gene therapy to improve perfusion, and mobilization of stem cell populations, are some of the approached strategies to induce angiogenesis[120–122]. Therapeutic angiogenesis not only must ultimately lead to an increased neovascularisation, as the neovessels should be fully functional, stable and long-lasting.

VEGF and FGF2 have been the growth factors mainly explored for this purpose; however many others have been studied (e.g. FGF1, FGF4, HGF). While many experimental studies are encouraging, randomized controlled clinical trials have produced less consistent results[120]. Although part of this failure is attributed to delivery procedure problems, the maintenance of long-lasting strong and functional vessels remains a challenge. It is not clear if a single growth factor is sufficient to initiate the entire cascade of events leading to a mature, functional and stable vascular network *in vivo* (most growth factors secondarily induce other factors), or if a mix of growth factors should be used and precisely orchestrated over time[106,120]. Since HIFs initiate an entire angiogenic response, they have also been considered for angiogenic therapy[123].

Transplantation of endothelial progenitor cells (EPCs), derived from the bone marrow or peripheral blood, have demonstrated to be an alternative to angiogenic factors contributing to postnatal neovascularisation in ischemic limbs and myocardium[124–127].

Finally, therapeutic interventions that focus on the inhibition of natural antineovascularisation mechanisms should be considered (e.g. HGF inhibits endostatin and TSP-1)[106,125].

Nevertheless, successful proangiogenic therapy still raises some questions regarding long term side effects. If these therapies are able to significantly contribute to vessel integrity and repair, can they indirectly contribute to trigger dormant tumours and/or accelerate atherosclerosis? The different forms of therapeutic angiogenesis still have to prove safety and efficacy before one can conclude on its relevance as an additional limb saving strategy. It is still a long way from bench to bedside and patient benefit, despite a considerable number of ongoing clinical trials.

Although the ability of IR in promoting therapeutic angiogenesis has never been investigated, it is interesting to note that doses of IR between 2 and 8 Gy were showed to induce the production of proangiogenic molecules, such as VEGF, TGFβ, FGF, IL-1R-a, IL10, IL3, IL4 and IL5, by the tumoural cells, that may activate the microenvironment, including the

vasculature[128–132]. In addition, we found that the exposure of lung microvascular endothelial cells (HMVEC-L) to doses lower than 0.8 Gy enhances EC migration without impinging on cell proliferation or survival[105]. Moreover, it was found that low-dose IR activates the endothelium by phosphorylating the receptor-2 of the VEGF[105], a critical player on the angiogenic process. It is described that the activation of VEGFR-2 leads to a rapid activation of different cellular proteins and consequently to the *de novo* mRNA and protein expression of mediators involved in the angiogenic response[96,133]. This is strongly supported by the data obtained in our lab from an *in vitro* microarray study where several transcripts encoding for proteins required for angiogenesis are induced upon low-dose IR delivery (unpublished results). The genes whose expression is significantly altered by low doses of IR and that represent the best candidates for a proangiogenic response were selected. Their expression was validated by quantitative real-time PCR (qPCR) and Western blot using irradiated and non-irradiated HMVEC-L. Unpublished results suggest that VEGFR-1, VEGFR-2, ANGPT2, FGF2, TGFβ have their expression increased in response to doses corresponding to 5 or 30 % of the therapeutic dose (100%). To validate these *in vitro* data, Sofia et al has shown that low doses of IR accelerates vessel formation by inducing angiogenic sprouting in fli1:EGFP embryos and increases vessel density in adult fli1:EGFP zebrafish.