Universidade de Lisboa
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AN INNOVATIVE STRATEGY FOR THERAPEUTIC ANGIOGENESIS: LOW DOSES OF IONIZING RADIATION

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ABBREVIATIONS

ALK - Activin receptor like kinase

ANG - Angiopoietin

BW – Body weight

CLI - Critical limb ischemia

CVD - Collateral vessel density

Dll - Delta like ligand

DM – Diabetes *mellitus*

EC - Endothelial cell

ECM – Extracellular matrix

ENGR - Endoglin receptor

EPC - Endothelial progenitor cell

EphB - Ephrin type B receptor

ESC - Embryonic stem cell

FGF - Fibroblast growth factor

FGFR - FGF receptor

FZD - Frizzled

HIF - Hypoxia inducible factor

HMGB – High mobility group box

iPC - Induced pluripotent stem cell

LDIR – Low doses of ionizing radiation

MMP - Metalloproteinase

NRP - Neuropilin

PD - Postnatal day

PAD - Peripheral arterial disease

PDGF - Platelet-derived growth factor

PlGF - Placental growth factor

PPAR – Peroxisome proliferator activated receptor

RAGE – Receptor advanced glycation end product

ROI - Region of interest

ROP - Retinopathy of prematurity

RT - Room temperature

SD – Standard deviation

SMC - Smooth muscle cell

STZ - Streptozotocin

TGF - Transforming growth factor

TIE - tyrosine kinase with immunoglobulin-like and EGF-like domains

VEGF - Vascular endothelial growth factor

VEGFR- VEGF receptor

VPF - Vascular permeability factor

vSMC - Vascular smooth muscle cell

INDEX

1. INTRODUCTION

1.1. ANGIOGENESIS

During embryogenesis and adulthood, vertebrate blood vessels have essential functions. The circulatory system (blood and lymphatic vessels) is responsible for the distribution of oxygen and nutrients through the tissues and for the filtration of metabolites and waste products like carbon dioxide [1]. This system permits the communication between distant tissues and plays a role in the regulation of body temperature and of pH [1,2]. The structure and different levels of permeability of vessels vary on their size because of their location and function. They can be formed and remodelled by three distinct mechanisms: vasculogenesis, angiogenesis and arteriogenesis [3].

Vasculogenesis consists in the *de novo* formation of blood vessels by recruitment and differentiation of multipotent mesodermal cells into the endothelial lineage and occurs especially during embryo development, but also in adulthood, in tumor vascularization [4]. This process is completed with formation of the primary vascular plexus (Figure 1) [3,5].

In angiogenesis, new vessels are formed from the existing vasculature in a complex and extremely coordinated process driven by endothelial cell (EC) proliferation (Figure 1) [3]. This is more relevant in adult life to maintain homeostasis and may occur by sprouting or non-sprouting mechanisms. During sprouting, new vessels are formed by branching of pre-existing ones. On the contrary, in non-sprouting angiogenesis, the vessels are enlarged by the proliferation of ECs within the wall of a pre-existing vessel, followed by its splitting and fusion. This process requires a precise spatial and temporal coordination of multiple steps by a series of “on” and “off” regulatory switches. Angiogenesis is regulated by different pro- and anti-angiogenic factors and the balance between them determines if the ongoing angiogenesis will be brief (physiological) or prolonged (pathological). In adult organisms, angiogenesis is necessary for wound healing, growth and action of female reproductive organs including ovulation, follicular development and formation of a fully vascularized tissue for implantation and placentation during pregnancy [6].

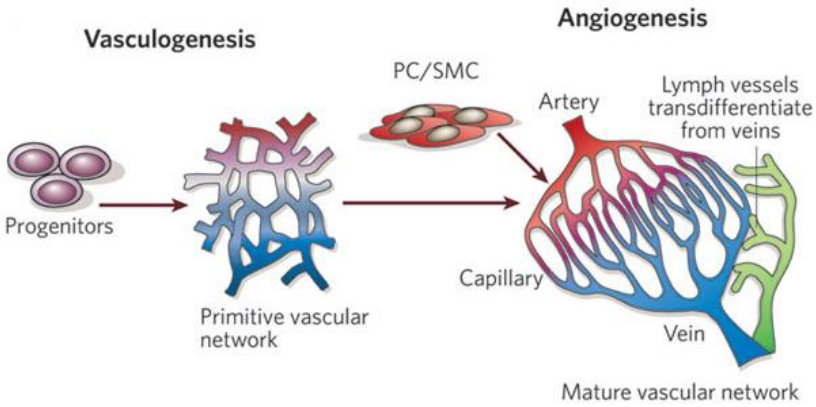

Figure 1- Development of the vascular system. During vasculogenesis, endothelial progenitors give rise to a primitive vascular of arteries and veins. During angiogenesis, the network expands, pericytes (PCs) and smooth muscle cells (SMCs) cover nascent endothelial channels, and a stereotypically organized vascular network emerges. Lymph vessels develop via transdifferentiation from veins. (adapted from: Carmeliet, 2005)

Arteriogenesis or collateral growth is the organization of mature arteries from pre-existing arterioles after arterial obstruction of a major artery. After this occlusion, the distal arterial blood pressure falls resulting in a pressure gradient along pre-existent collateral vessels. Consequently, a unidirectional flow in these vessels will be established. In the proximal arteries feeding the collateral vessels flow will increase. In the receiving artery, the direction of blood flow may reverse and the arterial blood in these vessels is fully saturated with oxygen. This process is able to fully compensate a blocked artery because vessels can grow and proliferate enough even to take over the role of this occluded artery, improving local blood flow. This growth process goes along with a diameter and wall thickness increase in the blood vessels. Frequently, these vessels assume a gradually tortuous course. Today little is known about the triggers and mediators of this mechanism in ischemic vascular diseases [3,7,8].

The formation and maintenance of a complex, highly organized and functionally competent vascular system is therefore a key component to homeostasis [6].

1.2. ANGIOGENIC MECHANISMS

During angiogenesis, new vessels form from pre-existing ones and invade avascular ischemic areas, where tissues experience hypoxia and nutrient deprivation [9,10]. This process is modulated by the secretion of various growth factors and the endpoint of this phase is the formation of a highly branched and poorly perfused network of capillary connections [10,11]. This process is responsible for the formation of

an efficient vascular tree, containing defined arteries and veins and an optimized vascular capillary network [9,10].

The angiogenic process is composed by the following steps: (1) A structural alteration of the basement membrane occurs in a dilated mother vessel, followed by a partial degradation of the basement membrane at places where EC processes are projecting into the connective tissue; (2) migration of ECs, which are arranged in parallel, maintaining their basal-luminal polarity and forming a slit-like lumen, takes place continuously with the lumen of the mother vessel and sealed by intact inter-endothelial; (3) Basement membrane is deposited continuously by the polarized EC; (4) Pericytes proliferate and migrate along the basement membrane, resulting in a complete coverage of the new vessel (Figure 2) [10,12,13].

Initiation of sprouting requires the specification of two different EC states: tip and stalk cells, which have distinct phenotypes based on their gene expression profiles and the functional specifications of ECs within a newly formed sprout [14]. Perturbations of any of these cell states compromise vessel development and function [15].

Tip cells occupy the leading position while new vessels grow regulated by a vascular endothelial growth factor – A (VEGF-A) gradient that specifies the direction of their migration, and move forward towards the capillary-free zone [14,16]. These cells have low Notch activity, high vascular endothelial grow factor receptor – 2 (VEGFR-2) and low VEGFR-1 expression, which results in higher levels of delta-like ligand-4 (Dll4) expression and, hence through a higher production of Dll4 than in neighbouring cells, an increased ability to suppress its neighbouring cells from becoming tip cells [12,14-17]. Tip cells are migratory and polarized and they have invasive properties and activate secreted or membrane-bound proteases for remodelling of adjacent basement membrane. Tip cells extend numerous filopodia that serve to guide the new blood vessel in a certain direction toward an angiogenic stimulus, proliferate minimally and adopt a highly branched shape while moving [15,18].

Stalk cells produce fewer filopodia, proliferate contributing to sprout elongation and form the nascent vascular lumen cells. However, this type of cell has high levels of Notch signalling activity and elevated expression of Jagged1. This antagonizes Dll4 activity, reducing the induction of Notch signalling in the adjacent tip cell, which therefore maintains its responsiveness to VEGF stimulation and migrates outward to establish a new branch [12,14-16,19].

During embryonic development, selection of tip and stalk cells is regulated by Notch signalling, through Notch family receptor (Notch1) and their transmembrane ligands (Jagged1, Jagged2, Dll1, Dll3, and Dll4) [20]. In vascular ECs, these proteins are expressed on the surface of cell membranes. The interaction ligand-receptor

Notch/Dll4 permits the activation of an intracellular signalling cascade that determines the behaviour of ECs [19,21]. Inhibition of the active state of ECs due to stimulation of Dll4/Notch-associated transduction of intracellular signal is evidently caused by lowering of their sensitivity to VEGF-A and thus restricts an excessive number of tip cells. On the other hand, lowering the levels of Dll4 expression or blocking Notch-dependent signalling cascade enhances tip cell formation, resulting in a significant enhancement in the activity of formation of the new vessels [22]. In response to the action of VEGF-A, tip cells sprout in filopodia and begin to move forward. The direction and migration of these cells are regulated by the spatial distribution of VEGF-A in the tissue [20].

Angiogenesis initializes when a vessel senses a stimuli by an angiogenic factor released by a hypoxic, inflammatory or tumour cell. Pericytes first detach from the vessel wall (in response to angiopoetin-2 (ANG-2)) and release themselves, by matrix metalloproteinases (MMPs), from the basement membrane. ECs lose their junctions and the nascent vessel dilates. Next, VEGF increases the permeability of EC layer, which promotes the extravasation of proteins from plasma and lays down a temporary extracellular matrix (ECM) support. During this process, integrin signalling promotes the EC migration onto this ECM surface. Then, proteases release angiogenic factors stored in the ECM (VEGF, Fibroblast growth factor (FGF)) and remodel the ECM to allow ECs to escape from the original vessel walls. At this moment, tip cell is selected to lead the angiogenic sprouting in presence of VEGF receptors, neuropilins (NRPs) and the Notch ligands. In the neighbourhood, the other cells assume the position as stalk cells, responsible for the elongation process in presence of Notch, Wnts, placental growth factor (PlGF) and FGFs. Stalk cells are also responsible for the lumen formation and elongation mediated by VE-cadherin, CD34 and VEGF. During this process, ECs proliferate and migrate to form solid sprouts which connect with neighbouring vessels [10,20,23,24].

At this time, tip cells stop moving, the vessel lumen is formed and the blood flow contributes to stabilize the newly formed blood vessel, while oxygen supply by the blood flow lowers the local expression level of VEGF-A and other angiogenic factors induced earlier. The stalk cells proliferate even when a lumen is already formed, and because of this it is important that proliferation is tightly coordinated with EC junction formation in order to maintain patent and sealed vessels. During interactions of tip-stalk cells these sprouts form loops to establish a perfused neovessel. For this vessel to become functional, it must become mature and stable [9,23,24].

Vessel maturation occurs with a transition from actively growing vessel bed to the quiescent, fully formed and functional network. Inhibition of endothelium

proliferation and emergence of new capillaries take place, in this case, along with stabilization of already existing newly formed vascular tubules and incorporation of mural cells [23]. Finally, in the vessel stabilization occurs the recruiting of mural cells (pericytes and vascular smooth muscle cells (vSMCs)) that have important functions because disturbance of the correct formation of the wall causes an increase in vessel wall permeability. Pericytes are in contact with ECs and form walls of capillaries and immature blood vessels. The factors involved in this recruitment include (ANG1)-TIE2, platelet-derived growth factor β (PDGF-β)-PDBFR-B, transforming growth factor-β1 (TGF-β1) - activin receptor-like kinase 5 (ALK5; TGFβ-R1) and Notch pathway components. They have a vessel stabilising effect on newly formed vessels and arrest their growth via inhibition of angiogenic sprouting in ECs. Recruitment of pericytes and accumulation of ECM proteins in the adjacent basement membrane contribute to vessel maturation and its transition to the quiescent state [25].

Some mature blood vessels with large diameter, such as arteries and veins, are formed by several layers of smooth muscle cells (SMCs) separated from endothelium by a layer of basement membrane. The stage of maturation, associated with formation of the newly formed vessel walls, is often distorted in various pathological situations [23].

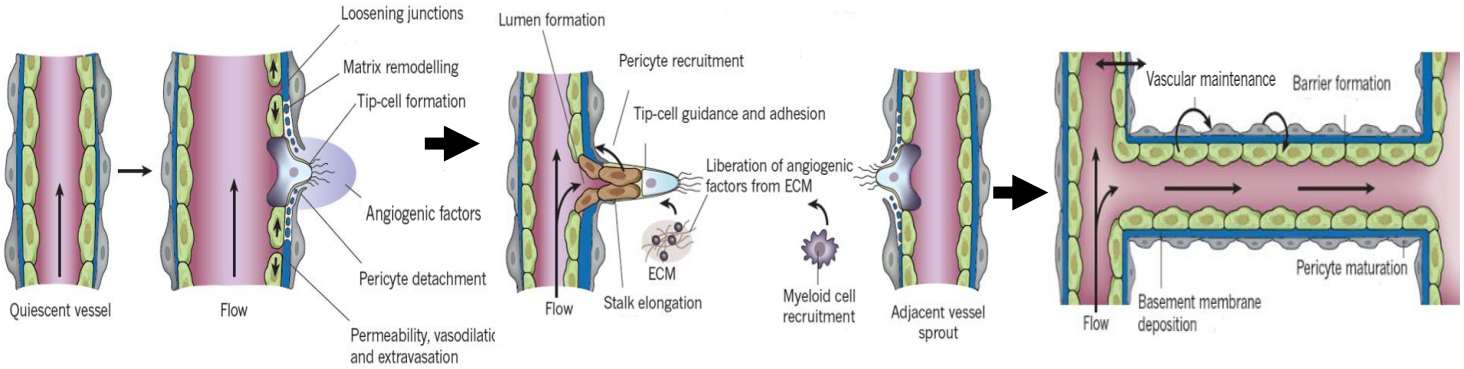

Figure 2 - Molecular mechanism of angiogenesis. After stimulation with angiogenic factors, the quiescent vessel dilates and an EC tip cell is selected to ensure branch formation. Tip cell formation requires degradation of the basement membrane, pericyte detachment and loosening of endothelial cell junctions. Increased permeability permits extravasation of plasma proteins to deposit a provisional matrix layer, and proteases remodel pre-existing interstitial matrix, all enabling cell migration. Then, tip cells navigate in response to guidance signals and adhere to the extracellular matrix to migrate. Stalk cells behind the tip cell proliferate, elongate and form a lumen, and sprout fuse to establish a perfused neovessel. Proliferating stalk cells attract pericytes and deposit basement membranes to become stabilized. Recruited myeloid cells such as tumour-associated macrophages and TIE-2-expressing monocytes can produce pro-angiogenic factors or proteolytically liberate angiogenic growth factors from the ECM. After fusion of neighbouring branches, lumen formation allows perfusion of the neovessel and the re-establishment of junctions, deposition of basement membrane, maturation of pericytes and production of vascular maintenance signals. (adapted from: Carmeliet and Jain, 2011a)

1.2.1 MOUSE RETINA MODEL OF ANGIOGENESIS

In humans, the retina begins to vascularize during embryogenesis. At week 40, the baby is born with fully developed retinal vessels and with regressed hyaloid vasculature [26].

In contrast to humans, the mouse retina is vascularized only after birth [27]. The fact that vascularisation occurs postnatally, makes this model very commonly used, because it permits imaging the planar vascular plexus with different antibodies and contrasts at high resolution microscopes [13], and observe the different stages of angiogenic network formation. Importantly, it allows to investigate developmental and pathologic vessel growth *in vivo*.

In mice, during embryonic periods, the retina has no proper vascular system [27]. Around embryonic day 17, astrocytes migrate into the retina from the optic nerve and form a fine meshwork extending towards the periphery [27,28]. Immediately after birth (Figure 3A), the retinal vascular system starts to develop as a sprout from the optic disc and initially forms a primitive vascular plexus, guided by the preformed astrocyte network [28,29]. The superficial vascular plexus is formed until postnatal day 8 (PD 8) (Figure 3E), and during this time retinal vessels continue to extend radially over the superficial layer from the optic nerve into the periphery of the retina, to form a two-dimensional vascular structure. The superficial layer is composed by distinct arteries, veins, and capillaries. Some of the capillaries are pruned whilst others are strengthened. Pruning can occur via migration and relocalization or via selective EC apoptosis and it is evident in the vicinity of arteries where capillary-free zones exist. At PD 7 (Figure 3D) the superficial capillaries start sprouting downwards into the retina to form additional plexuses: first the deep and then the intermediate vascular plexus. The deep plexus is formed at approximately PD 12 (Figure 3G), localized in the outer plexiform layer and it is formed faster and develops independently of retinal astrocytes. The intermediate plexus in the inner plexiform layer is formed between PD 12- PD 25. All three vascular layers are totally formed and matured by the end of PD 25 (Figure 3H) and at this stage vessels are found to be predominantly arteriolar in the superficial layer and predominantly venular in the deep capillary bed. The vascular layers are interconnected with perpendicular branches, but have no direct communication with the choroidal vascular system [27-30].

After the completion of angiogenesis, retinal vasculature enters in quiescent stages.

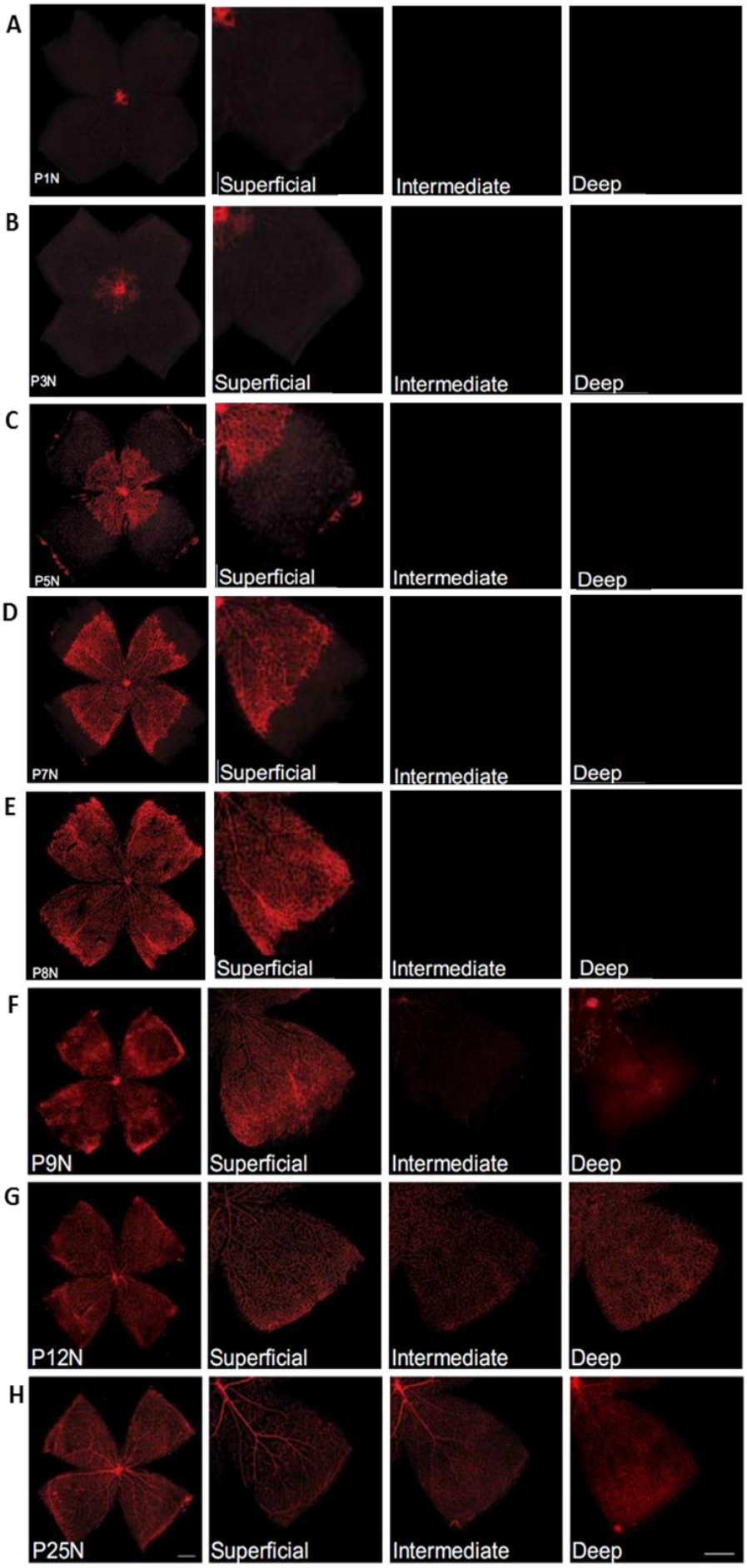

Figure 3- Development of the superficial, deep and intermediate vascular plexus in C57Bl/6 mouse retinas. Retinal whole mounts from P1 to P25 were stained for endothelial cells with isolectin B4-Alexa 594 (red). P signifies Postnatal day and N signifies normal development. **A, B, C, D, E)** At P1N, the mouse retina is almost completely devoid of blood vessels. The superficial vascular plexus can be seen originating from the optic nerve head. During the first week of postnatal development, the superficial plexus extends radially from the optic nerve head into the surrounding tissue, reaching the retinal periphery at P8N. **F)** At P9N, the superficial plexus has fully extended to the peripheral retina. The deep vascular plexus begins forming centrally from vertical vessels diving down from the superficial plexus. The intermediate vascular plexus has not yet begun to form. **G)** At P12N, the intermediate vascular plexus becomes visible on retinal whole mounts. The deep vascular plexus is nearly fully developed. **H)** Between P21N and P25N, further maturation of especially the intermediate plexus can be observed. (adapted from: Stahl et al., 2010)

Retina mouse model was used to study the VEGF [14,31] and its different isoforms expression [32], how this could influence this aspect of vascular network maturation [14] and how ECs respond to VEGF gradients by tip cell formation and guided migration of retinal vascular development. More recently, the important role of the Notch signalling pathway has also been extensively studied in this model. Also, it was seen that in retinal angiogenesis, most of the vascular sprouts are originated from venous vessels in the optic nerve and express ephrin type-B receptor 4 (EphB4). The expression of ephrinB2 is restricted to only a few vessels around the optic disc until PD 3 [19].

This model is frequently used to study ischemic diseases and to mimic the aspects of human retinopathy of prematurity (ROP) and diabetic retinopathy [13,20]. ROP is characterized by a temporal arrest in development of the retinal vasculature, followed by outgrowth of abnormal vessels in premature babies. On the other hand, in diabetic retinopathy, the retina's eye presents damage in the blood vessels caused by diabetes [20].

1.3. REGULATION OF ANGIOGENESIS

Angiogenesis requires a complex and precise coordination of multiple steps, which are regulated by a delicate balance between pro- and antiangiogenic factors.

1.3.1. ANGIOPOIETIN FAMILY

The ANG is a paracrine growth factor and consists of four members, ANG-1, ANG-2, ANG-3 and ANG-4, which bind to specific tyrosine kinase with immunoglobulin-like and EGF-like domains - 1 (TIE-1) and TIE-2 [33,34]. These factors maintain quiescence while still able to respond to angiogenic stimuli [23]. Knockout experiments in mice suggest a role for these receptors in blood vessel maturation. Both ANG-1 and ANG-2, expressed by a broad variety of cell types (ECs, SMCs, fibroblasts, pericytes, some tumor cell lines), play a role in angiogenesis by binding to TIE-2, which is mainly expressed in ECs. ANG-1 is expressed by mural and tumoral cells and stimulates mural coverage and basement membrane deposition, thereby promoting vessel tightness; ANG-2 is related to tip cells in the angiogenic process [35].

The ligands (ANG-1 and ANG-2) for TIE-2 were discovered recently, but only ANG-1 results in a signal transduction leading to stabilization and regulation of blood vessel maturation [33,34]. ANG-2 is an antagonist of ANG-1 that can block ANG-1 and induce autophosphorylation of TIE-2 in ECs. Overexpression of ANG-2 led to a fatal phenotype similar to ANG-1 and TIE-2 knockout mice. ANG-4 has not been as well studied but is thought to act like ANG1. In the presence of angiogenic stimulators,

sprouting ECs release ANG-2 which antagonizes ANG-1 and TIE-2 signalling to enhance mural cell detachment, vascular permeability and EC sprouting. Recently, it was proposed a complementary role between VEGF and ANG in vascular development and angiogenesis. During embryogenesis VEGF promotes differentiation and proliferation of ECs and the formation of immature vessels. ANG-1 bounds to TIE-2 receptor and induces the remodelling and stabilizing of the blood vessels in interaction with ECM. In an adult vessel, ANG-1 is associated with TIE-2 to keep the vessels in a stable state. In the presence of VEGF, ANG-2 is responsible for an increase in capillary diameter, migration and proliferation of ECs and sprouting of new blood vessels [33].

1.3.2. VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY

Initially VEGF was named Vascular Permeability Factor (VPF) because of its ability to induce vascular leakage and it is a heparin-binding homodimeric glycoprotein [36]. VEGF family in mammals includes VEGF -A, -B, -C, -D, -E (parapox virus VEGF) and PlGF [33,34]. VEGF-A (commonly named VEGF) is the best characterized member and the major mediator of normal and tumour angiogenesis during migration and mitosis of ECs and in the creation of blood vessel lumen. Vegf-a gene has an alternative splicing and produces 6 isoforms of VEGF-A [34]. VEGF-B is functional during embryonic angiogenesis in myocardial tissue and VEGF-C acts in lymphangiogenesis (the formation of new lymphatic vessels from pre-existing ones). VEGF-D is essentially for the development of lymphatic vasculature surrounding lung bronchioles and PlGF is dispensable for development but, in a disease situation of ischemia or cancer, it is important for vasculogenesis and angiogenesis [37].

VEGF is one of the most important angiogenic regulators for ECs *in vivo* and *in vitro.* In *in vitro* conditions, VEGF induces the expression of anti-apoptotic proteins and consequently prevents apoptosis of ECs [37].

This factor stimulates cellular responses by binding to transmembrane tyrosine kinase receptors (initial receptor names are given in parentheses): VERGFR-1 (FLT1), R-2 (Flk1/KDR) and -R3 (FLT4), expressed in ECs, and causing them to dimerize and become activated through transphosphorylation, although to different sites, times, and extents [34]. The affinity of biding in VEGF/VEGFR-1 is higher than in VEGF/VEGFR-2. Although VEGFR-1 was identified first, its functions are still not quite clear, but it is thought to act like a negative regulator during embryonic development. However, in adult VEGFR-1 is important in activation of VEGFR-2 and in angiogenesis binding of PlGF. VEGFR-2 binds to VEGF-A, -C and –D and it is important during proliferation and migration processes during vasculogenesis and angiogenesis [33]. Recent evidences suggest that the biological effect of this receptor signalling depends on its

subcellular localization. NRPs such as NRP1 and NRP2 are VEGF co-receptors and enhance the activity of VEGFR-2. VEGFR-1 and VEGFR-*2* are abundantly expressed on blood ECs. VEGFR-3 is necessary for the formation of vasculature during early embryogenesis, but later is found in lymphatic endothelium to regulate lymphangiogenesis [23,37].

VEGF is expressed in different tissues and is induced by several factors like hypoglycaemia, shear and cell stress, growth factors, oncogenes and hormones [23,33,37]. Importantly, VEGF levels are also regulated by tissue oxygen tension, since exposure to hypoxia induces VEGF expression (through hypoxia inducible factor 1, HIF1), while in normoxia a down-regulation of VEGF production is observed leading to regression of newly formed blood vessels. When overexpressed, this factor leads to diseases like cancer. Many of the human solid tumours express increased amounts of VEGF, stimulating the development of new vessels in the growing tumor tissue [33].

VEGF creates a gradient that is responsible for tip cells to upregulate Dll4 expression, which activates Notch pathway in stalk cells. Consequently this down-regulates VEGFR-2 expression, rendering stalk cells less responsive to VEGF and thereby ensuring that the tip cell takes the lead in the angiogenic sprouting process [37].

1.3.3. TRANSFORMING GROWTH FACTOR FAMILY

TGF family is composed by TGF-β and TGF-α and they are expressed in ECs, tumour cells and pericytes [23].

TGF is secreted in a biologically inactive form that can be activated *in vitro* by heat, acidification and proteases. *In vivo* the activation of this latent form by proteases could be a regulatory mechanism for mediating TGF-β activity [36].

TGF-β is a homodimeric polypeptide and include TGF-β1, -2, -3, -4 and -5 [34]. It binds to two types of serine-threonine kinase receptors: type 1 (TGF-βR1) and type 2 (TGF-βR2), interdependent. TGF-βR1 requires TGF-βR2 to bind to TGF-β and TGF-βR2 requires TGF-βR1 for signalling. ECs could also have another specific co-receptor (type 3 receptor), ENG, which is up-regulated during angiogenesis [38].

This factor is not an EC mitogen, so it is possible that it promotes angiogenesis by differentiating ECs after their proliferative phase has ended. This could happen by inducing the synthesis of the matrix or by stimulating angiogenesis in an indirect way, in which inflammatory cells release pro-angiogenic factors such as VEGF, FGF and PDGF [36]. It was found that its signalling regulates cell growth, differentiation, migration, adhesion, and apoptosis. Depending upon the dose and surrounding environmental conditions, TGF-β may promote pro- or anti-angiogenic functions [39].

TGF-β1 is the most well studied member of the TGFβ family. *In vitro* studies show that TGF-β1 activates upon making contact between ECs and pericytes progenitors. This activation results in inhibition of EC proliferation and migration and consequently inhibition of VEGFR-2 expression, inducing the differentiation of progenitor cells to pericytes to exert a stabilization effect on newly formed vessels and arrest their growth [23]. It also acts in the accumulation of ECM proteins in the adjacent basement membrane that contributes to vessel maturation [37]. Mouse studies have shown that the loss of the TGF-β receptors results in arteriovenous malformations, showing the importance of this factor to stimulate angiogenesis *in vivo* [36].

TGF-α is mitogenic for ECs and increases angiogenesis in an *in vivo* murine model. This binds to endoglin receptor (ENGR), decreasing apoptosis and inducing cell proliferation, angiogenesis and metastasis in cancer [34].

1.3.4. FIBROBLAST GROWTH FACTOR FAMILY

FGF family consists of at least 19 members of mitogenic polypeptides with high affinity for heparin [36]. Heparin induces oligomerization of FGF molecules promoting FGF receptor (FGFR) dimerization and activation. Receptor activation will then trigger an intracellular signal cascade leading to multiple biological responses like EC proliferation, migration and differentiation, protease production and angiogenesis [33]. One example of this process occurs in the heart where FGF stimulates angiogenesis indirectly by the release of hedgehog, ANG-2 and VEGF-B [23].

The most intensively studied are basic FGF (FGF-b or FGF-2) and acidic FGF (FGF-a or FGF-1). Both are potent angiogenic factors because they are chemotactic and mitogenic for ECs, acting directly in these cells [36]. These factors commonly bind to high affinity tyrosine kinase FGFRs, FGFR-1 and R-2, on the surface of target cells. This results in control of the angiogenesis and arteriogenesis processes. FGFR-1 is required for the development and maintenance of the vasculature in the embryo and FGFR-2 is important for vascular tone and maintenance of normal blood pressure and its absence could lead to embryonic death before gastrulation [23].

FGF-b was one of the first to be characterized and studied. It has multiple isoforms and *in vivo* induces cell proliferation and chemotaxis in cultured ECs, stimulating these cells to product proteases capable of degrading basement membrane. It also induces capillary ECs to migrate into three-dimensional collagen matrices to form capillary-like tubes [36]. Deficient mice develop normally without any evident phenotype, however, they present neuronal defects and delayed wound healing. This factor is produced by tumour cell lines *in vivo* and thought to play a role in the growth and revascularization of solid tumours [33].

FGF could be released during injury playing an important role in stimulating connective tissue growth and inducing angiogenesis. Low levels of this factor could maintain the vascular integrity, as inhibition of FGFR signalling in quiescent ECs causes vessel disintegration [23].

1.3.5. PLATELET-DERIVED GROWTH FACTOR FAMILY

The PDGF family has four members: PDGF -A, -B, -C and -D, binding with distinct selectivity to the receptor tyrosine kinases PDGFRα and PDGFRβ, expressed on ECs, fibroblasts and astrocytes. Initially, it was isolated from platelets and appears to be the major source of EC growth factor activity in those cells [37]. However, this factor is not secreted by these cells, but instead is sequestered intracellularly and, unlike FGF and VEGF, this factor does not bind to heparin to potentiate its activity [36].

It was described that through the expression and release of PDGF-B, endothelial tip cells recruit PDGF-Rβ-expressing pericytes to angiogenic vessels stimulating vSMC development and leading to vessel maturation [23,37].

In PDGF-B-deficient mice, several defects were found in the capillary wall formation due to insufficient content of pericytes like vessel leakage, tortuosity and bleeding. Knockout of the genes encoding the PDGF-B protein results in tumour vessel fragility and hyperdilation and PDGFR inhibition decreases tumour growth by causing pericyte detachment, leading to immature vessels that are prone to regression [23,37].

PDGF-C promotes vascular development in the embryo and in wound healing, as well as angiogenesis in avascular tissues. PDGF-D is involved in tumour revascularisation [23].

1.3.6. NOTCH AND WNT FAMILY

Notch signalling is a cell-cell communication pathway that controls multiple cell fate decisions, stem cell renewal and differentiation during embryonic and adult life.

Notch family is composed by receptors and their transmembrane ligands. In mammals, four NOTCH receptors (NOTCH-1, NOTCH-2, NOTCH-3 and NOTCH-4) and five ligands (Jagged-1, Jagged-2, Dll1, Dll3, and Dll4) are found, which are expressed on the surface of cell membranes [37,40,41].

Activation of the NOTCH receptor by delta- or jagged-type ligands on neighbouring cells results in proteolytic cleavage of the receptor and induces the release of the intracellular domain of the NOTCH receptor, which activates the transcription of NOTCH target genes [37,41].

Notch signalling is known for the “lateral inhibition” process. Initially, in this process, all progenitor cells are equivalent and express both NOTCH ligands and

receptors. Owing to intrinsic or extrinsic factors, one cell adopts a particular fate and inhibits its immediate neighbours from acquiring the same fate, because it starts expressing higher levels of ligand than its neighbours. Lateral inhibition utilises a feedback mechanism in which the activation of the NOTCH receptor reduces ligand expression, thereby amplifying the small differences in levels of ligand expression between the cells [20].

This pathway is essential for embryo polarity and somitogenesis; in central nervous system development and function; in cardiovascular and endocrine development and also during arterial/venous specification. In this last process during the angioblast phase occurs differentiation into ECs that acquire either an arterial or venous identity based on their expression of EphrinB2 or EphB4, respectively [41].

Recent evidence from zebrafish and mouse models indicates that VEGF and Notch signalling pathways play critical roles in the angiogenic process [12]. ECs use the Notch signalling pathway to coordinate cellular behaviours during blood vessel sprouting and these cells continuously compete for the tip-cell position by fine-tuning their expression of VEGFR-2 *versus* VEGFR-1, indicating that this signalling circuit is constantly re-evaluated as cells meet new neighbours [15].

During sprouting, VEGF up-regulates the expression of Dll4 in endothelial tip cells. Subsequently, Dll4/Notch signalling would induce the expression of VEGFR-1, including the soluble VEGFR-1 isoform that blocks VEGF signalling and thereby limit migration and/or proliferation. This provides an important mechanism to maintain the migratory activity of the leading tip cell in response to VEGF while reducing this response in the adjacent EC. In mural ECs, Dll4 is responsible for the stimulation of vessel maturation [12,23].

ECs also express various types of Wnt ligand and their frizzled (FZD) receptors, stimulating proliferation. Notch activates Wnt signalling in proliferating stalk cells during vessel branching and this process occurs in a reciprocal-feedback system [23].

Previous experiments show that Dll4 and its ligand of NOTCH-1 and NOTCH-4 receptors plays an important role in the regulation of angiogenesis [37]. Consequently, inhibition of Dll4 and Notch signalling induces the formation of more numerous but hypoperfused vessels, resulting in tumour hypoxia and growth inhibition [42].

1.4. PHYSIOLOGICAL *VERSUS* PATHOLOGICAL ANGIOGENESIS

After birth, angiogenesis still contributes to organ growth, although in the adult most ECs of the blood vessels have long-half lives and remain quiescent [43].

Physiological angiogenesis can occur only in specific situations in response to some stimuli, when ECs are activated and keep their capacity to invade tissues. These stimuli may be hypoxia, wound healing (blood coagulation and inflammation, new tissue formation and tissue remodelling), female reproductive organs that are undergoing physiological growth of injured tissue, regeneration of the endometrium during the menstrual cycle or in the placenta during pregnancy and skeletal growth [43,44].

Pathological angiogenesis happens when the dynamic equilibrium between pro- and antiangiogenic factors is disrupted.

Some examples of diseases characterized or caused by excessive angiogenesis are summarized in Table 1 [2].

Table 1- Diseases characterized or caused by excessive angiogenesis (adapted from: Carmeliet, 2005)

Organ	Disease in mice or humans
Numerous organs	Cancer and metastasis; infectious diseases
Blood and lymph vessels	Vascular malformations; cutaneous hemangioma; lymphatic malformations; atherosclerosis
Adipose tissue	Obesity
Skin	Psoriasis; allergic dermatitis; scar keloids; systemic sclerosis
Eye	diabetic retinopathy; retinopathy of prematurity; choroidal revascularization
Lung	asthma, nasal polyps; rhinitis; chronic airway inflammation, cystic fibrosis
Gastro-intestinal tract	periodontal disease; peritoneal adhesions; liver cirrhosis
Reproductive system	Endometriosis; uterine bleeding; ovarian cysts; ovarian hyperstimulation
Bone	Arthritis and synovitis; osteomyelitis; HIV-induced bone marrow angiogenesis
Kidney	Diabetic nephropathy

On the other hand, in other diseases such as ischemia heart disease hypertension, atherosclerosis, diabetes or preeclampsia, the angiogenic switch is insufficient causing EC dysfunction, vessel malformation or regression, or preventing revascularization, healing and regeneration (Table 2). In this situation there are more inhibitors than stimulating factors [2].

Table 2- Diseases characterized or caused by insufficient angiogenesis or vessel regression (adapted from: Carmeliet, 2005)

Organ	Disease in mice or humans
Nervous system	Alzheimer's disease; Amyotrophic lateral sclerosis; diabetic neuropathy; Stroke
Blood and lymph vessels	Diabetes; Hypertension; Atherosclerosis; Restenosis; Lymphedema
Skin	Hair loss; Systemic sclerosis; Lupus
Heart	Ischemic heart disease
Lung	Neonatal respiratory distress syndrome; Pulmonary fibrosis; emphysema
Gastro-intestinal tract	Gastric or oral ulcerations; Crohn's disease
Reproductive system	Preeclampsia; Menorrhagia
Bone	Osteoporosis
Kidney	Nephropathy; tubulointerstial fibrosis

1.4.1. PERIPHERAL ARTERIAL DISEASE

Peripheral arterial disease (PAD) is characterized by an obstruction of the arterial tree of the lower limbs resulting in diminished blood supply to tissues required during exercise or even at rest [45-47].

PAD is a manifestation of systemic atherosclerosis and it is an independent predictor of increased cardiovascular death and also an important cause of morbidity and mortality [48]. The prevalence for PAD is increasingly recognized as a health burden worldwide. The factors with the greatest impact for this disease are diabetes *mellitus* (DM), tobacco, age (>60/70 years), hyperlipidaemias and hypertension. Decreasing the level of any of these risk factors can improve the prognosis [45,48].

The severity of symptoms is dependent on the extent of the obstructive process and collateral circulation [48]. It is known that after the patient is diagnosed with PAD, 25% of patients will have a worsening ischemic condition with 5 to 10% progressing to critical limb ischemia (CLI). CLI patients have chronic ischemic rest pain, ulcers or gangrene attributable to objectively proven arterial occlusive disease. It is the end-stage of PAD and occurs when blood flow and distal perfusion pressure are insufficient to satisfy the rest nutritive needs of the limb. CLI is associated to endothelial dysfunction, white blood cell activation and inflammation (Table 3) [46-48].

Table 3- Pathophysiology of Critical limb ischemia (adapted from: Varu et al., 2010)

Macrovascular changes	Microvascular changes
Atherosclerosis	Decreased Nitric Oxide production
Arterial stenosis	Increased Reactive Oxygen Species
Angiogenesis	Increased platelet activation
Arteriogenesis	Microvascular thrombosis
Increased VEGF	Pre-capillary arteriole collapse
Arterial remodelling	Impaired oxygen exchange
Decreased wall thickness	
Decreased cross-sectional area	
Decreased wall-to-lumen area	

Treatment objectives in CLI include relieve of ischemic rest pain, limb salvage and improve patient function and quality of life, despite advances in surgery and interventional radiology. However, approximately 20%–30% of patients with CLI cannot be treated by conventional techniques and still require amputation [49]. Actually, amputation continues to be the recommended solution to the disabling symptoms, even if it is associated to morbidity and mortality [47,48].

Almost 50% of patients with limb ischemia have DM and this factor increases the risk of PAD approximately three- to five-fold [50]. PAD in patients with DM is more aggressive compared to non-diabetics, with early large vessel involvement coupled with distal symmetrical neuropathy. The need for a major amputation is five- to ten-times higher in diabetics than non-diabetics [48]. In patients with CLI, progression to gangrene occurs in 40% of diabetic patients compared with 9% of nondiabetic patients [51]. Further, limb salvage rates in diabetic patients with CLI have been reported to be lower than in nondiabetic patients, and DM is an independent risk factor for postoperative amputation and complications in CLI [48].

DM is a metabolic disorder of multifactorial etiology characterized by chronic hyperglycemia and changes in the metabolism of carbohydrates, lipids and proteins resulting from absolute or relative impairment in insulin secretion and/ or reduction in its biological activity [52]. This disease is also associated with a marked impairment in collateral formation and yet angiogenesis is markedly increased in several vascular beds in this disorder. The development of new vessels is significantly reduced in diabetic patients with coronary or PAD [53]. DM is also correlated with peripheral neuropathy and decreased resistance to infection, which leads to an increased risk of foot ulcers and foot infections [48,52].

1.5. THERAPEUTIC ANGIOGENESIS

The scientific advances achieved on the molecular mechanism that involves the angiogenic response, and develop disease, have triggered the path for the development of therapeutic strategies to promote or inhibit angiogenesis.

Actually, vasculogenesis and angiogenesis could be studied in numerous *in vitro* and *in vivo* research models. The *in vitro* models are best suited to examine specific aspects of particular processes involved in angiogenesis such as the biochemical interactions that regulate EC proliferation, motility, differentiation and apoptosis or lumen formation. However, these models are not able to recreate the microenvironment of an intact organism and the massive amount of influences on ECs *in vivo*. Thus, *in vivo* models have become crucial to understand the complex cellular interactions that enable the generation of functionally active blood vessel networks, capable of providing an appropriate blood supply and paracrine stimuli to organs. The full understanding of the angiogenic and vasculogenic processes would give us the ability to modulate blood vessels growth in a controlled manner [13].

Therapeutic angiogenesis includes pro- an anti-angiogenic therapies. An anti-angiogenic therapy aims at the inhibition of the new blood vessel growth in the treatment of many diseases, such as arthritis and cancer. In this therapy drugs act by: i) inactivating the agents responsible to activate and promote cell growth, migration and survival; ii) upregulating inhibitors or iii) directly blocking the receptors and/or their downstream signalling [54].

On the other hand, in a pro-angiogenic therapy, blood vessels are induced and improved to grow in a controlled manner. This therapy is clinically indicated for chronic wounds (diabetic lower extremity ulcers, venous leg ulcerations, and pressure ulcers), PAD and ischemic heart disease. There are several approaches to induce angiogenesis. The most used is the direct use of angiogenic growth factors and the inhibition of anti-angiogenic factors, but gene therapy and stem cells were also used to recover perfusion [55].

1.5.1. PRO-ANGIOGENIC THERAPY

The concept of "therapeutic angiogenesis" has become widely accepted during the past few years. Stimulation of vascular growth in a controlled manner can be achieved by exogenous administration of pro-angiogenic factors: pro-angiogenic therapy (Figure 4).

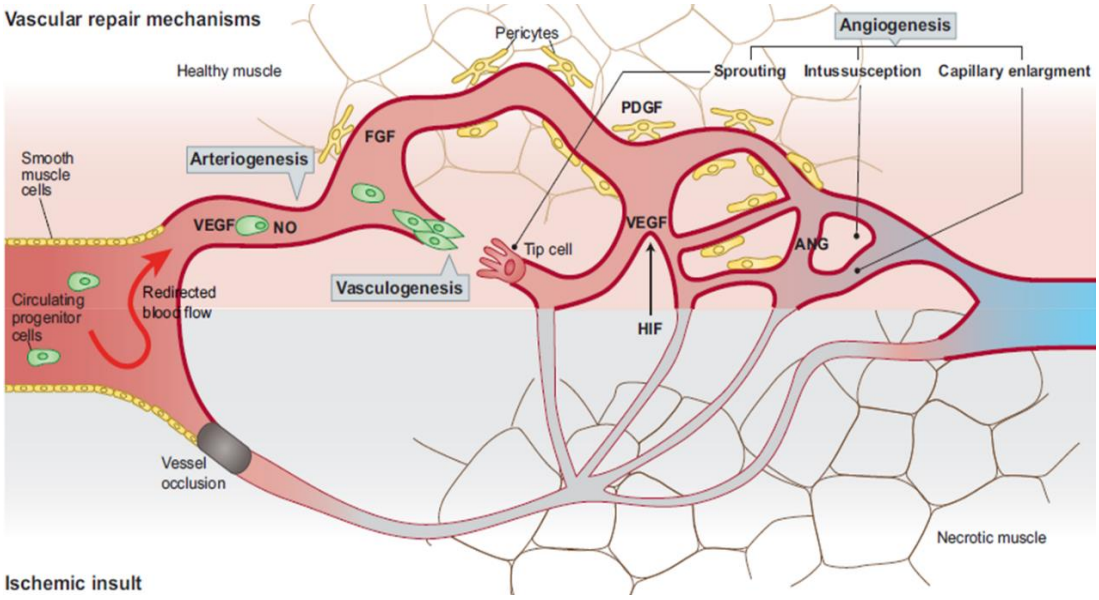

Figure 4 - Post-ischemic vascular repair mechanisms and the growth factors involved. The top half of the diagram shows angiogenic vascular repair processes that can take place after an ischemic insult (arterial occlusion), which is displayed in the bottom half of the diagram. Upon an arterial occlusion, arteriogenesis is induced by the redirection of blood flow, causing increased stress and subsequent cytokine production in the vascular endothelium. Factors such as VEGF and NO are responsible for the enlargement and growth of the collaterals, whereas factors such as PDGF and FGF mediate the stabilization of the vessels by recruiting pericytes. The hypoxic tissue distal to the occlusion (bottom half of the diagram) expresses transcription factors such as HIF, which enables the production of angiogenic proteins such as VEGF and ANG, which are involved in the modulation of the distal vasculature to make connections to the opening collaterals (angiogenesis). Angiogenesis can include sprouting, intussusception or capillary enlargement. Postnatal vasculogenesis might also contribute to post-ischemic vascular repair via the incorporation of circulating endothelial progenitor cells into the forming vascular structures. (adapted from: Dragneva et al., 2013)

The growth factors that have been more explored in this therapeutic option are members of the VEGF (VEGF-A, VEGF-B) [56,57] and FGF (FGF-2, FGF-1, FGF-4) [58] families. However many others have been studied, including PDGF (PDGFB) [59], HGF [60,61] and ANG (ANG-1) [62].

The potential of these therapies to revascularize tissues has been extensively studied in animal models of myocardial and peripheral ischemia.

Preclinical studies, using *in vivo* models and several individual angiogenic factors (VEGFs, FGFs, HIF-1α and HGF), showed significant improvements, with clinically relevant end points such as increased regional perfusion, improved exercise

tolerance and tissue energy metabolism, improved myocardial function and protection against ischemic damage [63].

Different animal models of peripheral ischemia were developed. For example, in ischemic rabbit hind limbs, administration of VEGF-a during neoangiogenesis, resulted in increased skeletal muscle perfusion [64,65]. Additionally, VEGF-A was shown to induce the growth of the vascular tree, including collateral arteries [66]. Furthermore, VEGF-A improved local perfusion, aerobic energy metabolism and exercise tolerance [65,67] and it was also reported that FGF-4 induces therapeutic angiogenesis and arteriogenesis in the local muscle perfusion [68].

Finally, proteins like HIF-1α and HGF that are responsible for recruiting an entire angiogenic response can simultaneously stimulate the expression of multiple growth factors involved in post-ischemic vascular response and tissue recovery [69,70]. A recent study demonstrated that the combined Vegf and Hgf gene therapy leads to a robust angiogenic effect in ischemic skeletal muscle [71]. Moreover, in diabetic mice it was demonstrated that pioglitazone, a peroxisome proliferator-activated receptor-γ (PPARγ) ligand, restores blood flow recovery and capillary density in ischemic muscle by using a hindlimb ischemia murine model. These data demonstrate that Akt-VEGF pathway is essential for the ischemia-induced angiogenic effect of pioglitazone and that pioglitazone exerts this effect in a PPARγ independent manner [72]. Other study shows that treatment with the receptor for advanced glycation end products (RAGE) significantly improved angiogenic response to ischemia in diabetic mice and was associated with increased high mobility group box-1 (HMGB-1) and VEGF levels in muscle tissues [73]. In both of these works, diabetes was induced with Streptozotocin (STZ). STZ is particularly toxic to the Islets of Langherhans, insulin-producing beta cells of the pancreas, in mammals. In medical research, a large dose of STZ is used to produce an animal model of type 1 diabetes [74].

Many experimental studies performed *in vitro* and *in vivo* are encouraging, but most of these factors have been tested in clinical trials and the promising preclinical potential has thus far not been translated into clinical success clinical trials [75-77]. Several factors could contribute to this failure: i) the maintenance of long lasting strong and functional vessels remains a challenge; ii) 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. Moreover, proangiogenic therapy still raises some questions regarding long term side effects and it is crucial to understand if these therapies can indirectly contribute to trigger dormant tumours and and/or accelerate atherosclerosis [75,78].

As an alternative to angiogenic factors administration, stem cell therapy is another promising therapeutic approach.

Of the available stem cell approaches, the induced pluripotent stem cell (iPSC) appears to have the greatest promise by reprogramming somatic cells. These cells can be easily accessible sources of tissue (donor's skin, fat or hair) and have high replicative capacity. It has been shown that these cells could differentiate into each of the major cardiovascular components, including SMCs, ECs, vSMCs, and cardiomyocytes, so they are patient-specific stem cells that do not face the immunologic barrier that confront cells derived from the pluripotent embryonic stem cells (ESCs) [79,80].

ESCs are appealing in the pro-angiogenic therapy because of their pluripotency and replicative capacity. These cells are derived from the inner cell mass of the blastocyst and, unlike adult stem cells, can differentiate into any cell type or any organ of endodermal, mesodermal, or ectodermal lineage [81]. However, this therapeutic option is not used due to ethical and immunologic concerns [82].

The use of "adult" stem cells, which include the endothelial progenitor cells (EPCs) is further along in clinical development than any of the other stem cell approaches. To date, therapies aimed at revascularization have included bone marrow– derived and circulating stem cells, since the mobilized EPCs enter the circulation and home to the site of ischemia [83,84]. Also, it has been shown that EPCs incorporate into the vasculature, differentiating into ECs, pericytes, or SMCs. This therapeutic option could potentiate local angiogenesis by a paracrine mechanism [85].

The different forms of therapeutic angiogenesis for patients with CLI still have to prove safety and efficacy before one can conclude on its role as an additional limb saving strategy. Despite a considerable number of ongoing clinical trials, it is still a long way to run in order to achieve the patient's benefit [46,86,87].

It is also relevant to note that ionizing irradiation has been shown to have angiogenic potential in multiple contexts, including the therapeutic one. Heissig B et al, show a novel mechanism of neovascularization and suggest that doses between 2 and 10 Gy of ionizing radiation may be used for therapeutic angiogenesis to augment vasculogenesis in ischemic tissues. However, long-term studies are needed to rule out negative side effects resulting from local ionizing irradiation administration, since potential adverse effects such as mast cell degranulation, increased melanization of the epidermis and allergic-like reactions might occur. To the best of our knowledge, to date the use of the high doses described has not been proposed for therapeutic angiogenesis [88].

Moreover, our research group found that low doses of ionizing radiation (LDIR), lower than 0.8 Gy, enhance EC migration without causing cycle arrest or apoptosis. These effects were shown in different *in vitro* and *in vivo* models: i) in human lung microvascular ECs, LDIR protect endothelium against cell death and promote EC migration inducing a rapid phosphorylation of VEGFR2 and VEGF production in hypoxia mimicking conditions; ii) in zebrafish, LDIR accelerate sprouting angiogenesis during development without causing excessive vessel formation and enhance the angiogenic response during caudal fin regeneration and iii) in different mice models, these LDIR promote angiogenesis and consequently accelerate tumour growth and metastasis [89].

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