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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Susana Constantino (Universidade de Lisboa) e da Professora Doutora Carmen Alpoim (Universidade de Coimbra)

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ABSTRACT

Radiotherapy is one of the most used approaches to treat cancer. It is known that ionizing radiation (IR) has the potential to induce DNA damage in cells and lead to cell cycle arrest and apoptosis. Therapeutic doses of IR delivered inside the tumor volume during radiotherapy block cancer cell proliferation and induce cancer cell death. However, during radiotherapy, not only the tumor area is exposed to IR, but also the tissues surrounding the tumor are exposed to doses of IR lower than the therapeutic dose. These low doses of IR may affect several types of stromal cells, including endothelial cells (ECs), and they have the potential to modulate the microenvironment that surrounds the tumor. *In vitro*, it was shown that low doses of IR do not decrease survival or proliferation of ECs and increase the migration of these cells by activating VEGFR2. Therefore, these low doses of IR activate VEGFR2 in a VEGF independent manner by promoting the auto-phosphorylation of this receptor tyrosine kinase. In both Zebrafish and mice models, it was demonstrated that low doses of IR enhance embryonic and adult angiogenesis. In a tumor context, using two different mice tumor models, these low doses of IR promote tumor growth and metastasis in a VEGFR dependent manner. In addition, by an *in vitro* microarray study, S. Constantino's unpublished results show that low doses of IR modulate the expression of several genes. From 28,869 genes represented on the array, 4,042 significantly changed with low dose IR, including several genes encoding angiogenic factors. These results were confirmed *in vitro* using microvascular lung EC targets. Our data suggest that the expression of several proangiogenic targets is upregulated 4 hours after IR exposure, followed by a decrease to levels similar to those found in non-irradiated cells. Our aim is to now validate these data in humans and evaluate if angiogenesis is promoted in tissues exposed to low doses of IR from patients with rectal cancer that received neoadjuvant radiotherapy. Our results show that ECs removed from specimens irradiated with low doses of IR present an overexpression of several pro-angiogenic factors 8 weeks after the end of the radiotherapy, when compared to the levels found in ECs removed from non-irradiated tissues. According to the clinical guidelines, the surgery should be done at the 8^{th} week post-radiotherapy, preventing us from obtaining biopsies at earlier or later time-points. Taken together our results suggest that ECs exposed to low doses of IR have their angiogenic balance skewed toward a proangiogenic phenotype. This shift represents a mark that those ECs were submitted to a stimulus that led to an angiogenic response.

These results have the potential to provide new contributions to understand the pro-metastatic effect of IR, leading to significant breakthroughs and advancing the state of the art in the field. Our findings will be of utmost importance to improve current oncology protocols.

Keywords: Radiotherapy, ionizing radiation, angiogenesis

RESUMO

A radioterapia é usada no tratamento de tumores malignos caracterizados por um crescimento descontrolado, capacidade de invadir tecidos adjacentes e metastizar. Sabemos que a radiação ionizante (RI) provoca danos no ADN, bloqueia o ciclo celular e induz morte celular. As doses terapêuticas de RI que são administradas no volume tumoral, durante a radioterapia, pretendem bloquear a proliferação das células tumorais e induzir a sua morte. Contudo, durante a radioterapia, não só a área tumoral é exposta a RI, mas também os tecidos que rodeiam o tumor são expostos a doses sub-terapêuticas de RI. Estas baixas doses podem afetar vários tipos de células do estroma, como as células endoteliais e poderão modular o microambiente que rodeia a área tumoral. Foi demonstrado *in vitro* que baixas doses de RI (menor que 0.8 Gy) não diminuem a sobrevivência ou proliferação de células endoteliais e que aumentam a migração destas células ativando o recetor 2 do VEGF (VEGFR2). Assim, estas baixas doses de RI ativam VEGFR2 independentemente da presença de VEGF, promovendo a auto-fosforilação deste recetor tirosina cinase. Em modelos de peixe-zebra e ratinho, foi demonstrado que baixas doses de RI induzem angiogénese em embriões e animais adultos. Num contexto tumoral, usando dois modelos de ratinho, as baixas doses de RI promovem o crescimento tumoral e a metastização através de um mecanismo dependente de VEGFR.

Para além disso, resultados de uma análise de microarrays do nosso laboratório mostram que baixas doses de RI modulam a expressão de vários genes. Dos 28,869 genes representados no array, a expressão de 4,042 encontra-se significativamente alterada pelas baixas doses de RI, incluindo vários genes que codificam para fatores angiogénicos. Estes resultados foram confirmados usando células endoteliais microvasculares do pulmão. Os nossos resultados sugerem que a expressão de vários alvos pró-angiogénicos é aumentada 4 horas depois da irradiação, seguida de uma diminuição para níveis semelhantes aos encontrados em células não irradiadas.

O nosso objetivo é agora validar estes resultados em humanos utilizando tecidos expostos, ou não, a baixas doses de RI de doentes com cancro do recto submetidos a radioterapia neoadjuvante. Os nossos resultados mostram que as células endoteliais removidas de amostras expostas a baixas doses de RI apresentam uma sobre-expressão de vários fatores pró-angiogénicos, 8 semanas depois do término da radioterapia, quando comparados com os níveis encontrados em células endoteliais de tecidos não irradiados. De acordo com aquilo que é definido na prática clínica, a cirurgia deve realizar-se na oitava semana pós-radioterapia, o que nos impede de obter biópsias em momentos mais precoces ou tardios.

Os nossos resultados sugerem que a balança angiogénica está em desequilíbrio havendo uma maior expressão de fatores pró-angiogénicos em células endoteliais expostas a baixas doses de RI. Este mesmo desequilíbrio poderá representar uma marca que essas células endoteliais foram submetidas a um estímulo que conduziu a uma resposta pró-angiogénica.

Estes resultados têm o potencial para contribuir para a compreensão do efeito pró-metastático da radiação ionizante, levando a avanços no estado da arte nesta área.

As nossas descobertas serão da maior importância para melhorar os actuais protocolos de oncologia.

Palavras-chave: Radioterapia, radiação ionizante, angiogénese

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LIST OF ABREVIATIONS

5-FU – 5-Fluorouracil

A-1 – Bcl-2 Associated Protein A1

ANG – Angiopoietin

BBE – Bovine Brain Extract

Bcl-2 – B-Cell lymphoma 2 protein

bFGF – basic Fibroblast Growth Factor

BMDCs – Bone Marrow Derived Cells

c-Myc – v-myc Avian Myelocytomatosis Viral Oncogene Homolog

CUB – Complement C1r/C1s, Uegf, Bmp1 domain

ECs – Endothelial Cells

ECM – Extracellular Matrix

EMT – Epithelial-Mesenchymal Transition

ERK – Extracellular-Signal-Regulated Kinase

FGF – Fibroblast Growth Factor

FGFR – Fibroblast Growth Factor Receptor

HGF – Hepatocyte Growth Factor

HIF – Hipoxya Inducible Factor

HLGAGs – Heparan-Like Glicosaminoglicans

HMVEC-L – Human Lung Microvascular Endothelial Cells

HPCs – Hematopoietic Progenitor Cells

HUVECs – Human Umbilical Vessel Endothelial Cells

IGF – Insulin-Like Growth Factor

IL – Interleukin

IR – Ionizing Radiation

LCM – Laser Capture Microdissection

MAPK – Mitogen-Activated Protein Kinase

MEK – Mitogen-Activated Protein Kinase Kinase

NRP – Neuropilin

OCT – Optimal Cutting Temperature compound

PAI – Plasminogen Activator Inhibitor

PCR – Polymerase Chain Reaction

PDGF – Platelet Derived Growth Factor

PDGFR – Platelet Derived Growth Factor Receptor

PI3K – Phosphoinositide 3-Kinase

PLC-γ – Phospholipase C- γ

PlGF – Placental Growth Factor

PTV – Planning Target Volume

qRT-PCR – quantitative Real Time-PCR

ROS – Reactive Oxygen Species

Shp-2 – Protein Tyrosine Phosphatase Non-Receptor 11

Src – Proto-Oncogene Tyrosine-Protein Kinase Src

TAFs – Tumor Associated Fibroblasts

TGF – Transforming Growth Factor

tPA – tissue Plasminogen Activator

uPA – urokinase Plasminogen Activator

VEGF – Vascular Endothelial Growth Factor

VEGFR – Vascular Endothelial Growth Factor Receptor

VHL – Von Hippel-Lindau tumor supressor

vWF – von-Willebrand Factor

Chapter I - Introduction

I.1 Blood vessels

The delivery of nutrients and oxygen to the whole body and the transport of carbon dioxide and waste products to be removed in excretory organs are critical mechanisms to maintain homeostasis. These processes are carried out by the vascular system, which is composed of blood and lymphatic vessels.

There are two major classes of blood vessels: arteries and veins. Arteries transport blood from the heart to the whole body providing oxygen and nutrients to the tissues and veins transport blood from the tissues back to the heart, allowing the removal of metabolites.

The structure of vessels varies a lot depending on their size, location and function. The larger vessels are composed of three layers: *tunica intima* (inner layer) composed of endothelial cells (ECs) surrounded by a basement membrane; *tunica media* (middle layer) composed of smooth muscle cells and elastic fibers and *tunica adventitia* (outer layer) composed of connective tissue, collagen fibers and nerves. Sometimes tunica adventitia has its own blood supply carried out by very small vessels named vasa vasorum [1].

In the tissues, arteries and veins branch into arterioles and venules, respectively, which in turn branch into smaller vessels, named capillaries. In contrast to larger vessels, capillaries are only composed of an EC layer surrounded by a basement membrane and a layer of sparse pericytes [1] and so they are very permeable to small molecules. When associated with each other, capillaries form the capillary bed, which represents the largest surface of the vascular system [1]. In addition, due to the special structural characteristics of capillaries, the capillary bed also represents the main site for exchange of gases and nutrients with surrounding tissues.

Depending on the tissue/organ, the endothelial layer of capillaries has specific characteristics conferring different levels of permeability. The level of permeability of vessels is very important to proper function of the organs. For example, in the brain, the endothelial layer is continuous and highly impermeable to several molecules, because the brain has a central role in regulating body homeostasis. On the other hand, liver sinusoids and capillaries of kidneys are very permeable, being discontinuous and fenestrated, respectively, because these organs are involved in processes of filtration and excretion of waste products.

Blood vessels are formed by two different mechanisms: vasculogenesis and angiogenesis. Vasculogenesis occurs especially during the embryonic development and consists in the *de novo* formation of blood vessels by ECs derived from progenitor cells in the blood. Angiogenesis is more relevant in adult life to maintain homeostasis and consists in the formation of new vessels from pre-existing ones.

I.2 Angiogenesis

Since the beginning of the twentieth century, the vascular system and especially the formation of new vessels have been related to tumor growth and metastasis [2], but it was only in 1971 that Judah Folkman proved for the first time that in fact tumor development is angiogenesis- dependent [3].

Angiogenesis can occur both in physiological and pathological conditions. In physiological processes such as the female reproductive cycle or wound healing, the quiescent ECs that line the adult blood vessels are induced to proliferate briefly and then they return to the quiescent state. However, in pathological conditions such as cancer, the "angiogenic switch" is permanently turned on, allowing the tumor to develop from a microscopic cluster with very low malignant potential to a rapidly growing mass favoring malignancy [4] and it is also involved in metastasis formation and further outgrowth of metastases [5].

Angiogenesis is a complex process involving several sequential steps. In tumors, angiogenesis starts with the activation of ECs by specific growth factors (pro-angiogenic) released from cancer cells or cancer associated stromal cells. These factors bind to their receptors in the surface of ECs and induce the activation of several signaling pathways [6], which lead to an increase in the expression of some proteolytic enzymes [7] that locally degrade the endothelium extracellular matrix (ECM) and basement membrane. This allows the ECs to invade the surrounding tissue and subsequently to proliferate and migrate through that matrix. Then migrating ECs polarize and create a lumen, leading to the formation of a new blood vessel [8]. Finally, immature vessels are stabilized by recruited mural cells, such as pericytes and by the formation of the ECM [8].

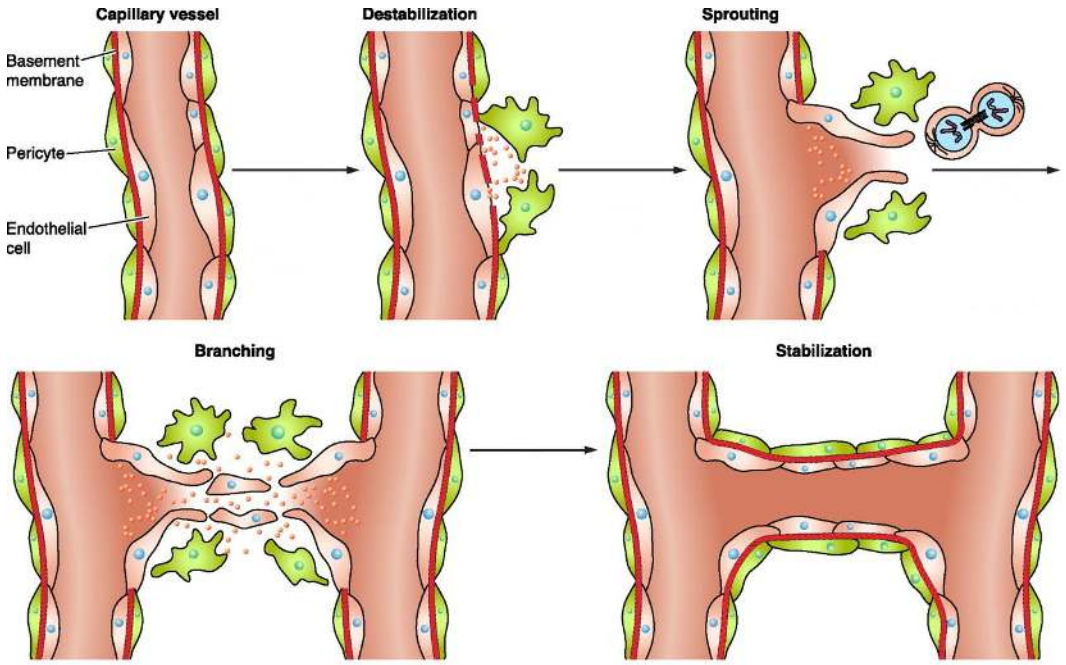

Figure 1 – **Angiogenesis**. The different steps in angiogenic process. Adapted from Clapp, C.; et al. *Physiological Reviews* (2009).

This process of angiogenesis is tightly 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).

The balance between pro- and anti-angiogenic factors can be disrupted by several signals, including metabolic stress, mechanical stress, inflammatory responses, genetic mutations and IR [9]. These signals act either by activating pro-angiogenic factors or blocking anti-angiogenic factors and all of these lead to the induction of angiogenesis [9].

I.2.1 Pro-angiogenic factors

Since the first experiments to isolate molecules capable of inducing angiogenesis in the 1970's [3], several angiogenic factors that directly or indirectly induce proliferation and differentiation of ECs have been found. These include VEGF, TGF-β, ANG2, FGF2, HGF, vWF, and PDGF-C.

I.2.1.1 Vascular Endothelial Growth Factor

Human vascular endothelial growth factor (VEGF) gene is composed by eight exons and seven introns, which by a process of alternative splicing can give rise to four different isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, having 121, 165, 189 and 206 aminoacids, respectively [6]. The most common isoform, $VEGF_{165}$, which lacks the residues encoded by exon 6, is a homodimeric glycoprotein of 45 kDa [6]. Normally, $VEGF_{165}$ is secreted but a significant fraction is sequestered in the ECM. To release and activate this fraction of VEGF, it is necessary the action of certain proteases.

The expression of VEGF can be regulated by different factors, such as oxygen tension, growth factors and oncogenes. Low oxygen tension induces the expression of VEGF under a variety of physiological and pathological conditions in a hypoxia inducible factor-1 (HIF-1) dependent manner. HIF-1 is an oxygen sensor: when oxygen tension is high, HIF-1 is hydroxylated at a specific proline residue recognized by Von Hippel-Lindau tumor suppressor (VHL), an ubiquitin ligase [10]. In this condition, VHL target HIF-1 to degradation in the proteasome. However, under low oxygen tension, HIF-1 is not hydroxylated, does not bind to VHL and is free to activate VEGF expression [10]. Several growth factors, such as transforming growth factor-α (TGF-α), TGF-β, keratinocyte growth factor, insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) also induce VEGF expression cooperating with local hypoxia to increase the release of this pro-angiogenic factor into the microenvironment [11]. In addition, some studies also suggest that certain oncogenes like Ras can induce VEGF expression [12][13].

The most studied and accepted functions of VEGF are the ability to promote growth and survival of ECs and the ability to increase vascular permeability [14]. *In vitro* VEGF prevents apoptosis of ECs by two different mechanisms: it promotes the activation of the PI3K/Akt signaling pathway that leads to survival signals and it increases the expression of some anti-apoptotic proteins, such as Bcl-2 and A-1. *In vivo*, VEGF effects depend on the developmental stage and maturation level of the vessels. In neonatal mice, vessels are very dependent on VEGF and the inhibition of this factor can lead to EC death and destruction of some vessels. However, in adult mice the inhibition of VEGF appears to have no significant effects in EC survival and vessels maintenance. In addition, newly formed tumor vessels are much more VEGF dependent than already established vessels. One explanation to this is the coverage of mature vessels by pericytes, which can somehow replace the role of VEGF and decrease the dependence of this factor. Although the mechanism by which pericytes modulate ECs is still unclear, there are several evidences that pericytes are involved in vessel maturation. Thus, the development of

drugs that inhibit the binding of pericytes to tumor vessels could be an interesting approach to cancer treatment.

The role of VEGF in protecting ECs is only possible by the activation of its receptors, VEGFR1, VEGFR2 and VEGFR3.

VEGFR3 has a central role in the lymphatic vasculature development and lymphangiogenesis. In the adult VEGFR3 expression is almost restricted to lymphatic vessels.

Gene-targeting studies demonstrated the essential role of VEGFR1 in vascular development. *Vegfr1-/-* mice die *in utero* between stages E8.5 and E9.0 exhibiting a severe disorganization of the vasculature and an increased number of ECs [15]. The phenotype observed was due to an increased mesenchymal to hemangioblast commitment resulting in an excess of the EC population that leads to the development of a disorganized vascular plexus [16]. Based on the biochemical and genetic data it was proposed that VEGFR1 could be a negative regulator of the VEGF activity, acting as a "decoy" receptor to sequester VEGF, thus rendering it less available for interacting with VEGFR2 [17].

VEGFR2 binds to VEGF with a lower affinity than VEGFR1, but in contrast to VEGFR1, it has a higher tyrosine kinase activity and it is responsible for the major biological effects of VEGF. As other tyrosine kinase receptors, upon binding to its ligand (VEGF) VEGFR2 dimerizes and auto-phosphorylates in specific tyrosine residues promoting its activation. Upon activation, this receptor has the ability to phosphorylate specific tyrosine residues in proteins involved in intracellular signaling that leads to survival and anti-apoptotic signals, such as PLC-γ, PI3k, Ras and Src. Knockout *Vegfr2-/-* embryos die *in utero* between stages E8.5 and E9.0 as a result of profound defects in vasculogenesis and angiogenesis. *Vegfr2-/-* embryos fail to develop yolk-sac blood islands and organized blood vessels and show a reduced number of hematopoietic and EC precursors [18]. These results suggested a pivotal role for VEGFR2 in vascular development and therefore it is considered the major mediator of the VEGF signaling during vasculogenesis and angiogenesis [19].

In addition to VEGFR1 and VEGFR2, VEGF can also bind to NRP1 and NRP2, initially described as mediators of neuronal guidance, which are also involved in angiogenesis [20]. Neuropilins are co-receptors for both the semaphorin family of axonal guidance molecules and the VEGF family [20]. The co-expression of NRP1 and VEGFR2 in porcine aortic ECs enhanced the binding and bioactivity of $VEGF_{165}$, suggesting that NRP1 acts as a co-receptor for VEGFR2 [21]. Genetic studies have shown that *Nrp1-/-* knockout mice die at stage E13 from cardiovascular defects and deficient neural vascularization [22]. Also, mice overexpressing NRP1 die *in utero* at stage E17.5 due to cardiac defects and excessive and hemorrhagic blood vessels [23]. *Nrp2-/-* knockout mice are viable and show a normal vascular phenotype [24]. However, double knockout mice (*Nrp1-/-*

Nrp2-/-) die *in utero* at stage E8.5 and show a severe vascular phenotype with greatly diminished yolk sac vasculature and disorganized blood vessels, resembling the *Vegfa-/-* and *Vegfr2-/-* knockout mice [25]. Thus, these genetic studies demonstrated a partial genetic redundancy between NRP1 and NRP2, and support an essential role for neuropilins in VEGF signaling [20].

I.2.1.2 Angiopoietin-2

Angiopoietins are a family of secreted oligomeric glycoproteins (ANG1, ANG2 and ANG4) sharing a conserved structure with 3 distinct domains: an N-terminal superclustering domain, a coiled-coil domain and a C-terminal fibrinogen homology domain [26][27].

All the angiopoietin proteins act by binding to a tyrosine kinase receptor, Tie-2, selectively expressed by ECs, some early hematopoietic cells and certain subsets of monocytes.

The most exhaustively studied angiopoietins are ANG1 and ANG2.

ANG1 is secreted mostly by pericytes and act in a paracrine manner to activate Tie-2 receptor in EC surface. ANG1 was shown to support EC survival and promote vascular stabilization, maintaining the vasculature in a quiescent state. Mice lacking ANG1 start do develop a primary vasculature which fails to stabilize and remodel leading to embryonic lethality. Therefore, ANG1 is essential for maturation and stabilization of the developing vasculature. Moreover overexpression of ANG1 produces enlarged and leakage-resistant vessels in adult mice. It was also found that ANG1 act synergistically with VEGF-A to promote angiogenesis [28].

On the other hand, ANG2 is specifically produced by ECs and stored in Weibel-Palade bodies, from which it can be rapidly released upon stimulation, acting in an autocrine manner (reviewed in [29]). ANG2 has been considered to have the opposite effect of ANG1 since it disrupts the connections between the endothelium and perivascular cells and promotes cell death and vascular regression by blocking ANG1-mediated Tie2 receptor activation. However, a number of studies of ANG2 function have suggested a more complex situation. Corneal pocket assays have shown that both ANG1 and ANG2 had similar effects acting synergistically with VEGF-A to promote the growth of new blood vessels, suggesting a pro-angiogenic role for ANG2 [30]. Moreover, it was found that *in vitro* and at high concentrations ANG2 can also be pro-angiogenic, suggesting the possibility that there was a dose-dependent endothelial response [31]. Furthermore, it was observed that the action of ANG2 could depend on EC differentiation state since the activation of Tie-2 by ANG2 was observed when ECs were cultivated on fibrin gel (a substrate that stimulates EC differentiation) [32]. In microvascular EC cultured in a three-dimensional collagen gel, ANG2 can also induce Tie-2 activation and promote formation of capillary-like structures [33]. Importantly, it was found that *in vivo*, ANG2 is expressed during

development at sites where blood vessel remodeling is occurring [34], as well as in highly vascularized tumors [35][36]. Finally, it was also demonstrated that in vivo, ANG2 can stimulate angiogenesis or capillary regression depending on the presence of VEGF [37]. In the presence of endogenous VEGF, ANG2 had a complex effect and efficiently induced an increased blood vessel diameter, remodeling of the basal lamina, EC proliferation, migration, and sprouting. If endogenous VEGF activity was inhibited, ANG2 effectively promoted capillary regression.

Its central role in the regulation of physiological and pathological angiogenesis makes the angiopoietin/Tie signaling pathway a therapeutically attractive target for the treatment of vascular disease and cancer.

I.2.1.3 Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is a 55kDa cytokine produced and secreted by most cell types in a latent form, which needs to be cleaved to become active and exert its proper function. TGF-β has 3 isoforms expressed in mammals: TGF-β1, TGF-β2 and TGF-β3, all of them binding to TGF-β receptors expressed by different cell types (reviewed in [38]). TGF-β receptors have a serine/threonine kinase activity and upon binding to TGF-β ligands, they become activated and phosphorylate some molecules involved in intracellular signaling pathways. TGF-β functions mostly by activating the intracellular Smad signaling pathway (canonical), but it also can activate other signaling pathways, such as PI3K/Akt, MEK/ERK and p38 MAPK pathways (non-canonical) (reviewed in [39]).

TGF- β has many distinct functions depending on its targets and cellular contexts. It regulates cell growth and differentiation, adhesion, migration, ECM production, bone remodeling, immune responses, apoptosis and angiogenesis [40][41].

The anti-inflammatory actions of TGF-β1 on ECs are well described. Moreover it was found that low doses of IR (0.3 Gy) induce a significant increase in TGF-β1 circulating levels and this effect may contribute to the anti-inflammatory effect mediated by low-dose IR [42].

In physiological conditions, TGF-β can influence the angiogenic process in different ways, depending on its concentration and the presence of other cytokines in the microenvironment. Therefore, it was shown *in vitro* that at low concentrations TGF-β potentiated the effect of VEGF and FGF2 in enhancing EC invasion, but at high concentrations it has the opposite role [43][44]. Moreover, it was found that at low doses, TGF-β1 up-regulates expression of angiogenic factors and ECM degrading proteases and consequently contributes to the angiogenic switch, whereas at high doses TGF-β1 inhibits EC growth, promotes the reorganization of the basement

membrane and stimulates smooth muscle cells differentiation and recruitment [45]. Gene studies in mice have shown that the loss of TGF-β signaling components results in frail vessels with decreased vessel wall integrity. Inactivation of the TGF-β1 caused lethality due to defects in the hematopoietic system and yolk sac vasculature [46].

In a tumor context, TGF-β signaling has been shown to act as a strong activator of tumor growth and metastasis by acting directly in tumor cells and local environment. TGF-β contributes for immunosuppression, modification of the ECM and induction of angiogenesis. TGF-β1 and β2 induce cancer cells to produce VEGF and PAI-1, promoting EC proliferation and vascular remodeling [47][48]. Some studies demonstrated that hypoxia and TGF-β signaling pathways can synergize in the regulation of VEGF gene expression at the transcriptional level and cooperate in the induction of the promoter activity of VEGF [49]. Blocking of TGF-β action inhibits tumor viability, migration, metastasis in mammary cancer, melanoma and prostate cancer. Reduction of TGF-β production and activity may be a promising target of therapeutic strategies to control tumor growth [39].

I.2.1.4 Fibroblast Growth Factor 2

The fibroblast growth factor (FGF) family is composed of over 20 members, sharing between them a much conserved central core of 140 amino acids and a strong affinity for heparin and heparan-like glicosaminoglicans (HLGAGs) (reviewed in [50]).

These growth factors induce proliferation and migration in several cell types, including ECs.

In this work we are especially interested in FGF2 (also known as bFGF), a well-known pro-angiogenic factor involved in the maintenance and activation of vascular endothelium.

In normal conditions, FGF2 is expressed in low levels and most of it, when released, is trapped in the ECM. However, during wound healing or some pathological conditions like cancer, FGF2 levels and its activity increase: its expression is up-regulated and the levels of some proteases that release FGF2 from ECM proteins also increase, allowing it to activate its receptors and exert its functions [50]. FGF2 binds to 4 membrane tyrosine kinase receptors (fibroblast growth factor receptor (FGFR)1, 2, 3 and 4) expressed by several cell types. FGFR1 is the main FGFR expressed in ECs, but small amounts of FGFR2 have also been found. FGFR3 and FGFR4 have never been reported in the endothelium [51][52].

When FGF2 binds to FGFR1, it dimerizes, auto-phosphorylates in specific tyrosine residues and phosphorylates other molecules, activating them. Stimulation of FGFR1 in ECs leads to proliferation, migration, protease production and tubular morphogenesis, whereas activation of

FGFR2 increases only cell motility [53]. Although most of these effects are transduced through MAPK activation [54], protein kinase C and PI3K activation are also required for FGF-induced EC proliferation and migration [55][56]. Studies using knockout mice have demonstrated essential functions for FGFR1 and FGFR2 in early development. Mice lacking individual FGFs revealed a variety of phenotypes which range from early embryonic lethality to very mild defects, most likely reflecting the redundancy of the FGF family of ligands or their uniqueness of expression in specific tissues [57]. Nevertheless, FGFs have been postulated to play a major role in wound healing, with particular focus on potential roles for FGF1, FGF2 and FGF7. Accordingly, topical application of FGF1 and FGF2 accelerates wound healing in a number of animal models [58]. Moreover, *FGF2* and *FGF1*/*FGF2* knockout mice exhibit delay in the remodeling of damaged blood vessels during wound healing and tumor angiogenesis [59].

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 to promote vessel assembly [51].

I.2.1.5 Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is a cytokine composed by two subunits: α-subunit with 55-65 kDa and β-subunit with 32-36 kDa. HGF is secreted by different cell types in an inactive form, which after being cleaved, originates 2 independent and active subunits (α and β), which in turn can activate target cells.

HGF acts in many different cell types (hepatocytes, ECs, melanocytes, etc) and consequently is involved in several physiological and pathological processes such as embryogenesis, wound healing, organ regeneration, inflammation, and tumor invasion. Since angiogenesis is a component of each of these processes, it was demonstrated that the *in vivo* biological action of HGF may be due to its effect on both epithelial and vascular ECs.

HGF activates a transmembrane tyrosine kinase receptor called c-MET. After binding to HGF, c-MET is activated and phosphorylates intracellular signaling proteins, such as Ras, PI3K, PLC-γ, Shp-2 and Crk-2 (reviewed in [60]).

HGF is an angiogenic factor because it is able to promote EC growth, survival, and migration both *in vitro* and *in vivo*. It was found that HGF induces the repair of wounds in EC monolayers. Moreover, HGF stimulates the scatter of ECs grown on three-dimensional collagen gels, inducing an elongated phenotype [61]. In the rabbit cornea, highly purified HGF promotes neovascularization at sub-nanomolar concentrations [61]. It was also found that HGF stimulates

EC expression of urokinase. Urokinase bound to its specific cell surface receptor mediates focal, directed, extracellular proteolysis, which is required for EC invasion and migration during the early stages of angiogenesis [62]. Moreover, it was found that combining HGF and VEGF results in a much more robust endothelial proliferative and chemotactic response than either growth factor alone [63].

Early studies of the proangiogenic actions of HGF attributed the effects of HGF to the induction of VEGF. It was also observed that HGF increased the expression of keratinocyte-derived VEGF and suggested that HGF might induce angiogenesis by a paracrine mechanism [64][65]. However, other studies suggested that the proangiogenic effects of HGF were independent of VEGF [66]. A gene expression profiling study clearly demonstrated that HGF and VEGF signal through discrete pathways in vascular ECs, and moreover the combination of the two growth factors synergistically induces a number of genes involved in cell cycle regulation [67].

Oncogenic activities of HGF have been proposed through its role in promoting angiogenesis in tumors. Data supporting this hypothesis came from examining the correlation of vessel density and HGF production in tumor samples and xenograft models [68-71].

I.2.1.6 Platelet Derived Growth Factor

Platelet derived growth factors (PDGFs) are a group of pleiotropic growth factors involved in many developmental processes (organogenesis, cell differentiation, axis formation, etc) and in some pathological conditions in adult life. However, in physiological conditions throughout the adult life, PDGFs functions remain unclear.

PDGF family is composed of 4 genes (PDGF-A, PDGF-B, PDGF-C and PDGF-D) which encode 4 polypeptide chains that homo- or hetero-dimerize to produce 5 different biologically active proteins (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD) (reviewed in [72]). One interesting aspect about these growth factors is the fact that they are structurally very similar to VEGF family. Accordingly, it was found that VEGF-A regulates adult mesenchymal stem cells migration and proliferation, by stimulating platelet derived growth factor receptors (PDGFRs). [73].

Within PDGF family, there are some differences between its members. The two newest members of PDGF family, PDGF-C and PDGF-D, differ from the others in that they are secreted in a latent form [74][75]. PDGF-C and PDGF-D possess a novel N-terminal domain among the PDGF/VEGFs, referred to as a CUB domain. In contrast to the N-terminal pro-peptides of PDGF-A and PDGF-B, the CUB domains of PDGF-C and PDGF-D appear not to be obligatorily removed by

intracellular proteolytic processing prior to secretion, but remain on the secreted PDGF-CC and PDGF-DD molecules. Proteolytic removal of the CUB domain is a prerequisite for binding of PDGF-CC and PDGF-DD to PDGF receptors. tPA and uPA were identified as the proteases capable of processing PDGF-C and PDGF-D, respectively [76][77]. Subsequent studies show that other proteases are able to process PDGF-C. The CUB domain exerts another level of regulation in these two growth factors, since, when cleaved, it can bind to active forms of PDGF-C and –D to inhibit them [72].

The PDGFs bind to 3 different tyrosine kinase receptors: PDGFR-αα, PDGFR-ββ and PDGFR-αβ. When PDGFs bind to their receptors, each half of the dimeric PDGF ligand recruits one receptor subunit to assemble the PDGFR dimer. Once dimerized, receptor subunits then cross-phosphorylate each other in specific tyrosine residues, activating the receptor, which in turn phosphorylates several target proteins in order to activate different signaling pathways (reviewed in [72]).

PDGFs play an important role in wound healing, stimulating cell proliferation, migration and angiogenesis.

In *in vitro* experiments, it was shown that heparin improves the binding of PDGFs to collagen, and the PDGF-heparin-collagen complex promotes proliferation of fibroblasts, cell migration and vascularization [78].

PDGF-B and PDGFR-β knockout mice die perinatally from vascular defects found in many organs [79][80]. Deletion of both receptors, PDGFR-α and PDGFR-β, led to a disruption in yolk sac blood vessels development in the transgenic mouse [81]. It was found that PDGFR expression in the yolk sac mesothelium is essential to promote vascular remodeling during blood vessel development through ECM deposition. These findings support the crucial contribution of PDGF signaling in vessel growth.

In vitro, it was shown that PDGF-B can directly induce ECs proliferation, migration and tube formation, whereas PDGF-A lacks such effects. Moreover, PDGFs stimulate not only ECs proliferation, but also VEGF secretion. Pro-angiogenic effects of different PDGF isoforms have been demonstrated *in vivo* in the chick embryo chorioallantoic membrane and in the mouse cornea pocket assay [82][83]. PDGF-B is produced by developing and quiescent ECs and PDGFR-β is expressed by perivascular cells and ECs [84]. When this paracrine signaling is disrupted, perivascular cells are not recruited and ECs proliferate irregularly, leading to improper vessel formation and hemorrhage [84]. During vasculogenesis and angiogenesis, PDGFs act in concert with other pro-angiogenic factors to induce formation and stabilization of new vessels by recruitment of perivascular cells.

PDGF-C is expressed in actively angiogenic tissues, like placenta, ovary, some embryonic tissues, and tumors [82]. In the developing chick embryo, PDGF-C induced sprouting of preexisting vessels and the angiogenic response is transduced by PDGFR-αα and -αβ. In tumor angiogenesis there is a complex interplay between cancer cells, ECs and other stromal cells. PDGF/PDGFR axis seems to be crucial in this interaction and thus, became an important target of novel anti-angiogenic therapies. It was shown that tumor associated fibroblasts (TAFs) increase the expression of PDGF-C, promoting angiogenesis by recruiting ECs to the surrounding tumor area and activating them [85]. In addition, PDGF-C produced by TAFs can act directly in tumor cells to promote cell proliferation [43].

I.2.1.7 von-Willebrand Factor

von-Willebrand Factor (vWF) is a large multimeric glycoprotein produced essentially by ECs, which are the main contributors to the plasma levels of this protein; however, megakaryocytes can also produce small amounts of it [86].

In ECs, vWF can be constitutively secreted or stored in Weibel-Palade bodies from where it can be released and secreted upon proper stimulation (reviewed in [87]). Interestingly, vWF is not simply stored in Weibel-Palade bodies, but it has an important role in their biogenesis [88]. Physiologically, vWF is an important regulator of blood homeostasis since it mediates the adhesion of platelets to the endothelium and sub-endothelial matrix, serves as a carrier for coagulation factor VIII in the plasma and is involved in EC adhesion to the vessel basal lamina.

However, vWF has also been linked to pathophysiological processes including angiodysplasia, smooth muscle cell proliferation and tumor metastasis.

Angiodysplasia is characterized by vascular malformations resulting from an impaired angiogenic process, and is often clinically manifested via gastro-intestinal bleedings. The manifestation of angiodysplasia is more observed in patients that lack high vWF multimers. In fact, vWF interacts in a multimer size-dependent manner with so far unidentified cellular receptors (expressed on ECs or other cells in the vascular wall) that are involved in maintaining the vascular integrity.

Regarding angiogenesis, it was found that the absence of vWF increases EC proliferation *in vitro*. Accordingly, vWF-deficient mice display an increased vessel density of the vasculature in the ears when compared to VWF-expressing mice.

According to the local cellular microenvironment, it was described that vWF may exert a proliferative effect. Upon damage of the vascular endothelial layer, vWF is able to penetrate

into the intima of large peripheral vessels and the deposition of vWF in the intima coincides with intimal thickening. This suggests that vWF plays a role in the pathogenesis of intimal hyperplasia by promoting smooth muscle cell proliferation. This is supported by the results obtained in *in vitro* experiments where it was found that vWF directly stimulates smooth muscle cell proliferation.

Concerning the influence of vWF in metastasis formation, opposite results seem to arise from studies where vWF was inhibited by pharmacological agents compared to genetically-altered mice. By using antibodies against vWF it was found that vWF promotes adhesion of tumor cells to platelets contributing to metastasis [89]. However, contradictory results were observed when vWF deficient mice were used [90]. In that work, it was found that the initial establishment of metastatic foci is increased in the knockout mice. *In vitro* studies showing that vWF induced tumor cell death corroborate the protective role of vWF in tumor metastasis [90]. Moreover, it was observed that ADAM28 cleaves and inactivates pro-apoptotic vWF in carcinoma cells and enhances lung metastasis, probably by promoting carcinoma cell survival within the blood vessels [91].

An association of vWF levels in blood and tissues and ADAM28 levels in patient tumor tissue with indolent vs aggressive cancers would be revealing to understand the role of vWF in metastasis. It is important to consider the fact that mouse lung metastasis occurs within weeks, whereas human metastases develop over years. Furthermore, the extensive proliferation of metastatic cancer cells intravascularly in mouse lungs [92] appears different from pathological examination of human metastases.

High plasma vWF concentrations are correlated with advanced tumor stage, the presence of multiple metastases and significantly poor prognosis of patients with metastatic colorectal carcinoma. High vWF plasma concentrations have also been reported in patients with various types of cancer, such as squamous cell carcinoma of the larynx and the cervix. This effect is associated with tumor-related angiogenesis and the metastatic process.

Importantly, it was found that FGF2 and VEGF, alone or in combination, up-regulate vWF mRNA and protein in human ECs. Therefore, the expression of this EC marker is controlled by angiogenic factors and this aspect makes vWF mRNA particularly useful to detect activation of the endothelium, an early sign of angiogenesis, in tumors [93].

I.2.2 Anti-angiogenic factors

All the pro-angiogenic pathways can be counter-acted by several endogenous inhibitors of angiogenesis.

In the last years, several anti-angiogenic factors were described, including thrombospondin-1, endostatin and angiostatin, the most studied ones. Most of anti-angiogenic factors are fragments of proteins: for example, endostatin and angiostatin are fragments of collagen XVIII and plasminogen, respectively. A great number of anti-angiogenic factors, including endostatin and thrombospondin-1, belong to the group of matrix derived angiogenic inhibitors [94]. In addition, some anti-angiogenic factors are growth factors or cytokines released from cells, such as interferons or interleukins, and others, like angiostatin, derive from the fragmentation of blood coagulation factors [95].

These anti-angiogenic factors act primarily in ECs blocking migration, reducing growth and proliferation and also inducing cell cycle arrest and apoptosis. However, Benelli, et al have shown that some anti-angiogenic factors, such as angiostatin, directly inhibit neutrophils and monocytes migration and inhibit angiogenesis by blocking chemokine- induced vascular recruitment [95].

The molecular mechanisms by which these factors inhibit angiogenesis are not well understood, but some studies have been elucidating how endostatin and angiostatin work. It was shown that endostatin down-regulates genes involved in the inhibition of EC migration by partly suppressing c-Myc expression, and also that it modulates intracellular calcium signaling in ECs (reviewed in [4]). Angiostatin is able to bind alpha and beta subunits of ATP synthase in ECs, probably making them more sensitive to hypoxic stress and it also inhibits ERK kinases activated by pro-angiogenic factors, such as FGF2 and VEGF (reviewed in [4]).

A better understanding of molecular mechanisms behind the effects of anti-angiogenic factors is needed, because they could represent a very useful tool to treat cancer and other pathologies related with unregulated angiogenesis. In fact, clinical trials using synthetic forms of angiostatin combined with radiotherapy to treat some types of cancer were already performed.

I.2.3 Angiogenesis and metastasis

As referred to before, the formation of new vessels by angiogenesis is an important factor in the progression of cancer since it facilitates metastasis formation by providing a way for cancer cells to spread from the region of the primary tumor to other organs in the body.

Metastasis is a very complex process comprising a sequence of several steps: invasion, intravasation, transport, extravasation and colonization (Figure 2). The metastatic process starts when cancer cells lose their epithelial morphology and change to a mesenchymal-like morphology, a process known as epithelial-mesenchymal transition (EMT). This transition occurs because some cancer cells suffer mutations or receive signals from stromal cells that block the expression of some adhesion molecules, such as E-cadherin, and induce alterations in cytoskeleton conformation. In addition, during this transition cancer cells increase their motility, improve their resistance to apoptosis and start expressing proteolytic enzymes that degrade ECM components [96]. This, results in the detachment of cancer cells from the primary tumor and the invasion of surrounding tissues. After invasion, cancer cells get closer to blood and lymphatic vessels and enter in the circulation by a process of intravasation. Once in circulation, cancer cells travel along blood and lymphatic vessels and rapidly spread to other regions of the body. Eventually, these cancer cells adhere to the endothelium and escape from the lumen of the vessels to the parenchyma of the tissues by a process of extravasation. In the new tissues, metastases can suffer a mesenchymal- epithelial transition, in which they lose their mesenchymal characteristics and return to their original epithelial phenotype. In this phase, cancer cells express again adhesion molecules, attach to the new tissue and start proliferating to form a secondary tumor.

Molecules such as VEGF or placental growth factor (PlGF) are also thought to mobilize into blood circulation bone marrow derived cells (BMDCs), which may subsequently be recruited to tumors and facilitate tumor growth and metastasis. A study suggested that the activation of VEGFR1 by PlGF is involved in the recruitment of VEGFR1-positive hematopoietic progenitor cells (HPCs) from the bone marrow [97]. These $VEGFR1^{+}$HPCs cells and other accessory cells released from the bone marrow can create a favorable microenvironment for cancer cell spreading. The migration and influx of these activated $VEGFR1^{+}$HPCs to distant tissues induces early changes in the local microenvironment, termed the “pre-metastatic niche”, priming the tissues for tumor cell implantation and proliferation. These $VEGFR1^{+}$HPCs clusters preserve the expression of primitive cell surface markers, rather than undergoing lineage committed maturation. The recruitment of $VEGFR1^{+}$HPCs might establish the metastatic signature, determining the tumor-pattern of metastatic spread and contributing for cancer cell

proliferation [98][99]. However, another study shows that blockade of VEGFR1 activity does not affect the rate of spontaneous metastasis formation in a clinically relevant and widely used preclinical model [100]. Therefore, alternative pathways probably mediate the priming of tissues for metastasis.

Furthermore, certain preclinical studies show enhanced metastasis in tumor-bearing mice treated with VEGF-blocking drugs, such as sunitinib [101-104]. However, these findings remain debated because other preclinical studies did not detect increased metastasis [105][106] and large meta-analysis have not shown more metastatic dissemination in patients [107].

The challenge is to develop agents that cause permanent tumor vessel normalization. Strategies combining i) anti-angiogenic agents with inhibition of metastasis or ii) VEGF-independent anti-angiogenic drugs with existing anti-angiogenic agents, might be useful to increase therapeutic efficacy.

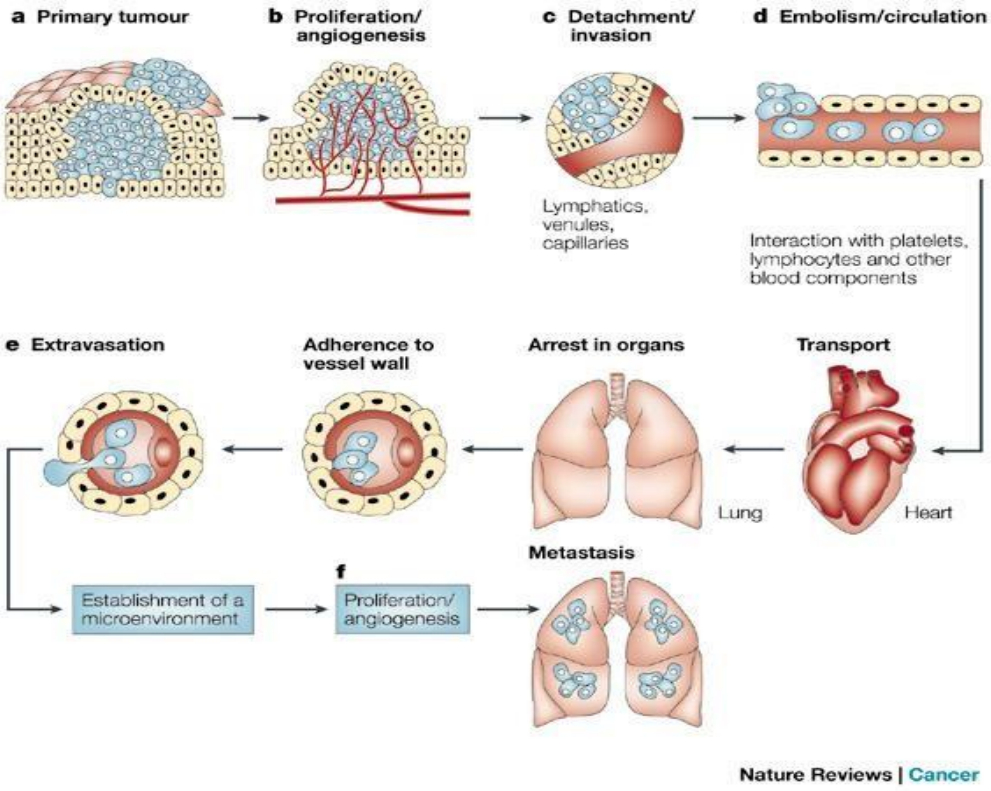

Figure 2 – **Metastatic process**. Sequential stages from the primary tumor to the formation of metastases in distant organs. Adapted from Fidler, I.J. *Nature Reviews Cancer* (2003).

I.3 Radiotherapy

Radiotherapy is one of the most used treatments for primary tumors and approximately 50% of all cancer patients will receive it, either alone or combined with chemotherapy and surgery [108]. Radiotherapy can be applied in two forms, external beam of IR or internal irradiation, depending on the type of cancer.

In the last decades, techniques to deliver IR improved a lot allowing a more precise deposition of the dose in the tumor and at the same time reducing doses impinging in surrounding healthy tissues. Despite these improvements that make radiotherapy one of the most effective forms to treat cancer, many patients still suffer from locally recurrent disease after radiotherapy. Moreover, clinically, while adjuvant radiotherapy significantly improves local tumor control, recurrences within a pre-irradiated field are associated with higher risk of local invasion and metastasis and poor prognosis when compared to recurrences outside the irradiated area [109-111].

It is known that the efficacy of radiotherapy is very dependent on some intrinsic biological factors whose understanding is critical to improve radiotherapy protocols [108]. The three main biological factors that affect the outcome after radiotherapy are: 1- the extent of hypoxia; 2- the ability of surviving cells to repopulate new regions during treatment; 3- the intrinsic radioresistance of tumor cells [108]. The understanding of these mechanisms is the key to achieve the ultimate goal of radiotherapy: to increase tumor cell kill at maximum and to decrease as much as possible the undesired side effects, leading to an improvement in cancer patients' quality of life.

IR induces some DNA damages, such as DNA double strand breaks, leading to cell cycle arrest, activation of repair mechanisms and eventually apoptosis [112]. Thus, radiotherapy is based in the fact that cancer cells have impaired repair mechanisms and so the damages caused by IR will be very efficient in killing these cells. However, IR can also affect normal cells near the tumor, but normal cells have functional repair mechanisms and are much more efficient in repairing damages than cancer cells. Thus, in the radiotherapy protocols, IR is always applied in fractionated doses to allow normal cells to recover from the damage while killing cancer cells. In addition to stress induced at cell level, IR also induces multicellular programs as a response to damage at tissue level [112]. These programs are executed by cytokines, chemokines and growth factors that modulate cell behavior both in the stroma (support cells) and the parenchyma (cancer cells) of the tumor. In the end, all of these processes lead to the remodeling of the ECM [112].

IR affects molecules by two major mechanisms: direct effects leading to alterations in molecule structure due to the accumulation of energy on it and indirect effects that lead to reactive oxygen species (ROS) production by the interaction of energy with water [112]. The most pronounced effect of IR is the production of ROS, because whereas DNA and damaged proteins can be quickly repaired or tagged for removal in proteasome, ROS can be itself a signal, amplified and persistent [112].

I.3.1 Ionizing radiation and angiogenesis

In the microenvironment many types of cells are affected by IR during radiotherapy, including fibroblasts, immune cells, nerve cells and ECs [113].

The effect of IR in the microenvironment is very complex and can be a little paradoxal, because on one hand it promotes a set of conditions non-permissible to tumor re-growth, but on the other hand it can also induce some alterations in stromal cells that contribute to tumorigenesis.

It was described that IR induces the production of VEGF by the tumor, which in turn may promote tumor re-growth [114][115]. It is generally assumed that tumor progression towards metastasis during or after radiotherapy is due to the appearance of resistant tumor cells through a combination of therapy-induced genetic instability, mutations and subsequent clonal selection of the most fitted cell. However, it was shown that radiotherapy also rapidly alters the tumor microenvironment and that anti-angiogenic approaches can enhance IR-induced tumor growth inhibition [114][116-118]. This data is not contradictory with the concept that the anti-proliferative and cytotoxic effects of radiation on ECs contribute in anti-tumor treatment, as previous reported [119], however at certain doses and time frames, IR enhances the build of new vessels, supporting invasion and metastasis.

It was found that high doses of IR induce HIF-1 expression in cancer cells, which in turn leads to the activation of VEGF and FGF-2 [120][114]. Once released, these two cytokines bind to its receptors in the surface of ECs and promote angiogenesis [112]. Moreover, the concept that IR itself induces the production of pro-angiogenic molecules by the tumors, such as TGF-β, FGF, IL-1Ra, IL-10, IL-3, L-4 and IL-5 [121] that may activate the microenvironment, including the vasculature, brings the need to new approaches in order to avoid tumor re-growth and metastasis enhancement after radiotherapy.

Since angiogenesis is crucial for tumor re-growth and metastasis and since IR may stimulate as well as inhibit angiogenesis, many works have been developed to investigate the effects of the therapeutic doses of IR in the tumor area and to prevent the putative pro-metastatic effect of

radiotherapy. However, we must take into consideration that, during the radiation treatment, not only the tumor area is exposed to a fractionated IR dose, but also the tissues surrounding the tumor area are exposed to doses, lower than the tumor area dose. The molecular and biological effects of these low doses of IR on the healthy tissue surrounding the tumor area, and in particular on the vasculature, remain to be determined.

A previous study in our lab has shown that low doses of IR, present in the vicinity of the tumor target, enhance angiogenesis [122]. Moreover, doses lower than 0.8 Gy induce DNA double strand breaks in ECs but they are rapidly repaired, not reducing the rate of survival or proliferation of these cells. Importantly, *in vitro* these doses of IR increase the migration of ECs [122].

In addition, it was shown that doses of 0.1, 0.3 and 0.5 Gy protect ECs from death induced by inhibitors of PI3k and MEK, two signaling proteins involved in survival signals. Low doses of IR also protected ECs from death induced by VEGF antibodies. Both EC migration and protection against cell death can be explained since IR activates VEGFR2 by promoting its auto-phosphorylation. Moreover, under hypoxic conditions low doses of IR also induce VEGF expression [122].

In both Zebrafish and mice models, it was demonstrated that low-dose IR accelerates embryonic and adult angiogenesis [122]. In a tumor context**,** these low doses of IR promote tumor growth and metastasis in a VEGFR dependent manner [122].

Having this in mind, the main goal of this work is to validate the data obtained in the animal models, in humans. This will be crucial to improve radiotherapy protocols and consequently cancer patients' quality of life.

Chapter II – Material and Methods

II.1 Patients

The samples used in this study were collected from rectal cancer patients that received pre-operative radiotherapy. Patients under 65 years old with a locally advanced rectal cancer in stage T2N0 or T3N1/2 without metastases that received a cumulative dose of 50.4 Gy participated in the present study with written informed consent. This study was performed in accordance with the Ethical Committee regulations of Centro Hospitalar Lisboa Norte

II.2 Radiotherapy

Radiotherapy was performed using an accelerator to produce an x-ray photon beam, operating at a dose rate of 300 MU/min. The treatment plan involved neoadjuvant radiotherapy in 28 fractions of 1.8 Gy, the cumulative dose being 50.4 Gy, in combination with chemotherapy (capecitabine – 5-FU). A dosimetric plan (Figure 3) was set for each patient.

Three measures were determined by evaluating the dosimetric plan and using the patient's iliac crest as reference. These measures were crucial to achieve a precise resection of the different specimens at the moment of surgery. Surgery was performed 8 weeks after radiotherapy ending.

This methodology was performed in a close collaboration with the Department of Radiotherapy and Surgery of the Hospital de Santa Maria.

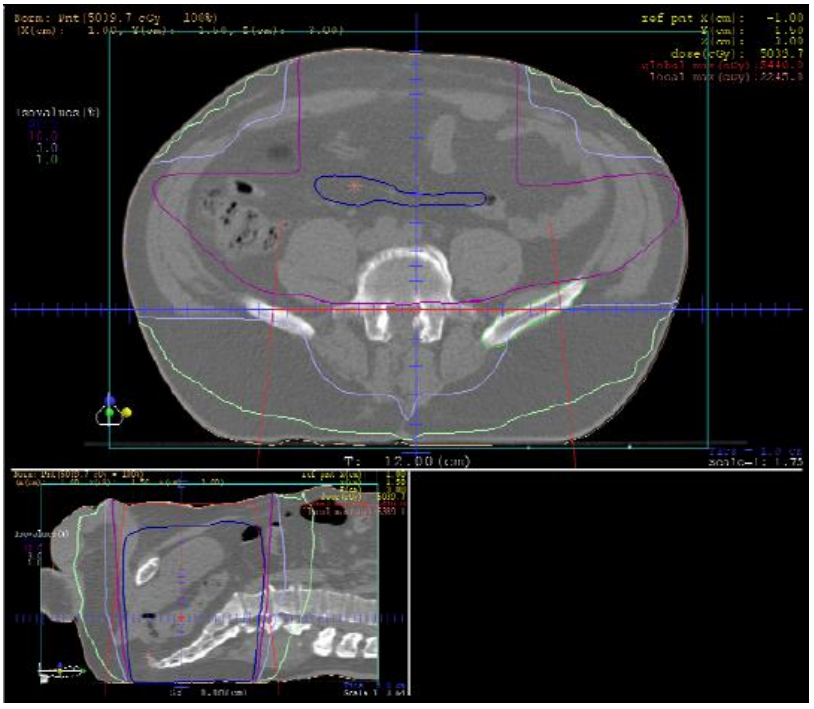

Figure 3 – Dosimetric plan/Isodose curves on a pelvic axial slice. The isodose curves represent the level of radiation to which different regions will be exposed during radiotherapy. The blue line delimits the region that will receive therapeutic doses of radiation (including tumor). The purple line delimits the region that will be irradiated with 5-30% of the therapeutic dose (low dose). The non-irradiated tissue will be removed outside of the green line.

II.3 Clinical Samples

According to the dosimetric plan, two samples were collected from the peritoneum of each patient: one non-irradiated sample (NIR) and one sample irradiated with 5-30% of the therapeutic dose (IRLD).

The samples were placed into cryomolds, embedded in optimal cutting temperature compound (OCT), rapidly frozen in cold isopentane and stored at -80ºC.

II.4 Immunohistochemistry

Using a cryostat, frozen samples were sliced in serial sections of 12µm, mounted in pre-cooled RNAse-free glass microscope slides (Carl Zeiss Microimaging) and stored at -80ºC until usage.

After thawed, samples were washed in ice cold RNAse-free water for 5 minutes, fixed in RNAse-free 70% ethanol for 5 minutes and washed again in ice cold RNAse-free water for another 5 minutes. Then samples were incubated with a primary antibody against CD31 (mouse anti-human; BD BioSciences), diluted 1:1500 in 2M NaCl phosphate buffered saline solution, for 45 minutes at 4ºC. Next samples were washed twice with an ice cold 2M NaCl phosphate buffered saline solution and incubated with a secondary antibody (biotinylated anti-mouse; Vector Laboratories), diluted 1:400 in a 2M NaCl phosphate buffered saline solution, for 30 minutes at 4ºC. After this, samples were washed twice again with an ice cold 2M NaCl phosphate buffered saline solution and incubated with an Avidin-Biotin complex (Vectastain® Elite ABC; Vector Laboratories) for 20 minutes at room temperature. Finally, the color development was performed by using diaminobenzidine (DAB+ - DAKO). After washing with an ice cold 2M NaCl phosphate buffered saline solution, the sections were dehydrated by their immersion in increasing concentrations of ethanol (90-100%).

II.5 ECs Isolation

The PALM Microbeam 4.2 microscope (Carl Zeiss MicroImaging) was used to collect the ECs previously labeled with the CD31 antibody 1. After EC's selection, the microscope laser cuts and catapults the cells to a tube with an adhesive cap (Figure 4). For each sample, an area of 1 500 000 μm^2 corresponding to the endothelium was collected.

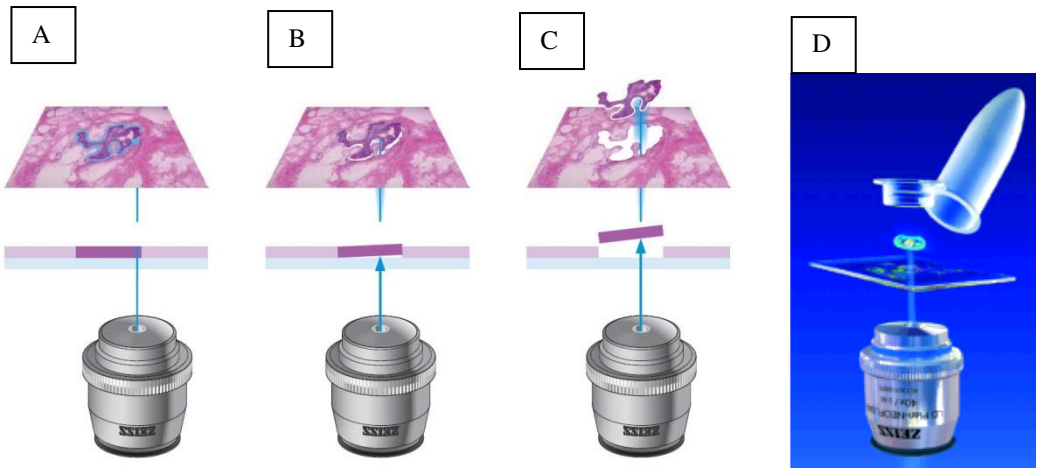

Figure 4 – Isolation of ECs. The stained tissue is selected using a software tool associated with PALM Microbeam 4.2 microscope (A). The selected area is cut (B) and catapulted (C) to a special tube with an adhesive cap (D).

II.6 Cell Culture and Irradiation

Human umbilical vein endothelial cells (HUVECs) were cultured in 0.02% gelatin-coated dishes in growing endothelial medium (basal EBM-2 medium supplemented with EGM-2 singlequots, BBE and 5% of Fetal Bovine Serum), as provided by the manufacturers (Lonza, USA). These cells were maintained in a humidified incubator at 37ºC, 5% CO_2.

Cells were irradiated at room temperature using a linear accelerator x-rays photon beam (Varian Clinac 2100 CD) operating at a dose rate of 300 MU/min. Previously, a computed tomography (CT) scan (Somatom Sensation, Siemens) was performed and a volumetric acquisition was carried out; acquired images were reconstructed with axial slices with a width of 1 mm, and cross sectional data was transferred to the image processing system work station for contouring the planning target volume (PTV). The radiotherapy plan was devised on a dedicated 3D planning system (PLATO, Nucletron) using an isocentric dose distribution of two opposite fields (0u, 180u) at 6 MV energy, normalized to a reference point.

II.7 RNA Extraction

Total RNA was extracted using the RNeasy® Micro (for ECs removed from patient samples) or Mini (for HUVECs) Kit (QIAGEN), following the protocol provided by the manufacturer.

II.8 cDNA Synthesis and Pre-amplifications

Using the RT^2 Nano PreAmp^{TM} cDNA synthesis Kit (SABiosciences, QIAGEN), RNA was reverse transcribed into complementary DNA (cDNA) with the First Strand cDNA synthesis, followed by three rounds of pre-amplifications for the targets VEGFR1, VEGFR2, ANG2, TGF-β2, vWF, FGF2, , PDGF-C, HGF and 18S. The sequences of primers used are shown in table 1.

VEGFR1	**Fw**: 5'-CCCTCGCCGGAAGTTGTAT-3' **Rev**: 5'-GTCAAATAGCGAGCAGATTTCTCA-3'
VEGFR2	**Fw:** 5'-ATTCCTCCCCCGCATCA-3' **Rev:** 5'-GCTCGTTGGCGCACTCTT-3'
ANG2	**Fw**: 5'-AGGACACACCACGAATGGCATCTA-3' **Rev:** 5'-TGAATAATTGTCCACCCGCCTCCT-3'
TGF-β2	**Fw**: 5'-GCTTTGGATGCGGCCTATTGCTTT-3' Rev: 5'-CTCCAGCACAGAAGTTGGCATTGT-3'
vWF	**Fw**: 5'- GTACAGCTTTGCGGGATACT-3' **Rev**: 5'- GCTCACTCTCTTGCCATTCT-3'
FGF2	**Fw:** 5'- GCAGTGGCTCATGCCTATATT-3' **Rev:** 5'- GGTTTCACCAGGTTGGTCTT-3'
PDGF-C	**Fw**: 5'- AGGTCTTCAATCGTGGAAAGAA-3' **Rev:** 5'-CAGAACCCAGCTAGTGGAATAC-3'
HGF	**Fw:** 5'- GGTAAAGGACGCAGCTACAA-3' **Rev:** 5'- AGCTGTGTTCGTGTGGTATC-3'
18S	**Fw**: 5' GCCCTATCAACTTTCGATGGTAGT-3' **Rev**: 5'-CCGGAATCGAACCCTGATT-3'

Table 1 - List of Primers. The table shows the primers sequences used to amplify each gene.

II.9 Quantitative Real Time PCR

The mRNA expression of the targets referred above was analyzed by qRT-PCR, which was performed using the Power SYBR® Green system (Invitrogen) following the manufacturer's protocol and an Applied Biosystems® RT-PCR 7500 Fast. The sequences of primers used were the same referred to in table 1. The Real Time PCR run method consisted of one holding stage of 2 minutes at 50ºC and 10 minutes at 95ºC, followed by 50 cycles of 15 seconds at 95ºC and 1

minute at 60ºC. 18S was used as a housekeeping gene to normalize the quantification. The relative quantification was performed according to the comparative method ($2^{-\Delta\Delta C}_T$; Applied Biosystems User Bulletin no. 2P/N4303859), with the non-irradiated sample as internal calibrator. The formula used is $2^{-\Delta\Delta C}_T = 2^{-[\Delta C}_T\text{(sample)}-\Delta C_T\text{(calibrator)}]$, where ΔC_T(sample)=C_T(sample) - C_T(reference gene). For the internal calibrator, $\Delta\Delta C_T$=0 and $2^{-\Delta\Delta C}_T$ =1. For the remaining samples, the value of $2^{-\Delta\Delta C}_T$ indicates the fold change in gene expression relatively to the calibrator. ΔC_T value for each sample is the average of triplicates.

Chapter III - Results

III.1 HGF, PDGF-C, FGF2 and vWF are overexpressed in HUVECs irradiated with 0.3 Gy,

According to previous results from a microarray analysis that were validated *in vitro*, several molecular targets involved in a pro-angiogenic response could be modulated by low doses of IR such as VEGFR1, VEGFR2, ANG2 and TGF-β (unpublished S. Constantino's data). Our objective was to analyze the effect of low doses of IR on other targets that according to the literature are described as being involved in an angiogenic response.

Therefore, HUVECs were irradiated or not with 0.3 Gy and the expression of HGF, PDGF-C, FGF2 and vWF was analyzed 4, 8 and 12 hours after irradiation by qRT-PCR. Our results suggest the levels of expression of HGF, PDGF-C, FGF2 and vWF were increased at 4 hours after irradiation with 0.3 Gy (Figure 5). At 8 or 12 hours the levels are similar to those found in non-irradiated HUVECs (Figure 5).

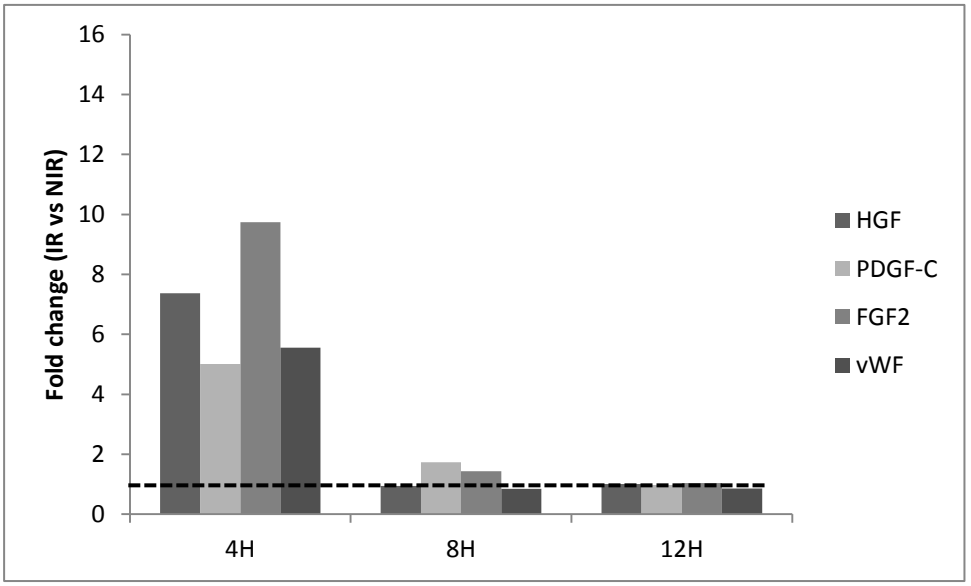

Figure 5 – **The expression of HGF, PDGF-C, FGF2 and vWF is induced by low doses of IR** The mRNA expression of HGF, PDGF-C and FGF2 was quantified by qRT-PCR in HUVECs non-irradiated or irradiated with 0.3 Gy at 4, 8 and 12 hours after irradiation. Values were normalized to 18S to obtain relative expression levels. The data represents the fold change in gene expression of each target in irradiated (IR) relatively to non-irradiated (NIR) cells (represented with a dashed black line).

III.2 Low doses of IR up-regulate the expression of pro-angiogenic factors in ECs removed from human samples

According to our results, low doses of IR induce the expression of several targets involved in a pro-angiogenic response *in vitro*. However, it is crucial to validate these data *in vivo*. With this objective, the levels of expression of several pro-angiogenic targets were evaluated in ECs removed from human tissues exposed to low doses of IR and compared to those obtained from ECs removed from non-irradiated tissues. Following this objective, samples from patients with rectal cancer that received preoperative radiotherapy were used. Therefore, human tissue sections exposed to doses from 5 to 30 % of the therapeutic dose (located in the vicinity of the tumor), were collected at the moment of the surgery, 8 weeks after the end of radiotherapy. Then, the ECs were stained with CD31 and removed from the tissues by using a laser capture microdissection (LCM) microscope. Then, RNA was extracted from these ECs in order to analyze the levels of expression of several pro-angiogenic targets by quantitative RT-PCR analysis. These levels of expression were compared to those obtained from ECs removed from the respective patient but from non-irradiated tissues by using a similar methodology. This process of calibration is fundamental since we are comparing patients with different aging, secondary diseases and genetic and environmental backgrounds.

According to the previous results obtained in S. Constantino's lab with 5 patients between 41 and 65 of age, ECs removed from specimens irradiated with low doses of IR present a consistent increase of VEGFR1, VEGFR2, TGF-β2 and ANG2 when compared to the levels found in non-irradiated ECs (Figure 6A).

Here our objective was to analyze the expression of other pro-angiogenic factors such as HGF, PDGF-C, FGF2 and vWF in the same 5 patients already evaluated for VEGFR1, VEGFR2, TGF-β2 and ANG2 (Figure 6B). Our results suggest that the levels of expression are higher in ECs removed from tissues irradiated with low doses of IR when compared to non-irradiated ECs (Fig 6B) for two patients (41 and 59 years old). These patients also presented increased levels for VEGFR1, VEGFR2, TGF-β2 and ANG2. For two patients 47 and 65 years old the levels of expression are similar to those obtained for non-irradiated ECs with the exception of FGF2, which presents a significant increase in ECs exposed to low doses of IR (Figure 6B). It is also important to refer that for one patient (44 years old), HGF, PDGF-C, FGF2 and vWF expression levels were lower relatively to those found in non-irradiated ECs (Figure 6B). Although, it is important to note that this patient presented high fold changes for VEGFR1, VEGFR2, TGF-β2 and ANG2.

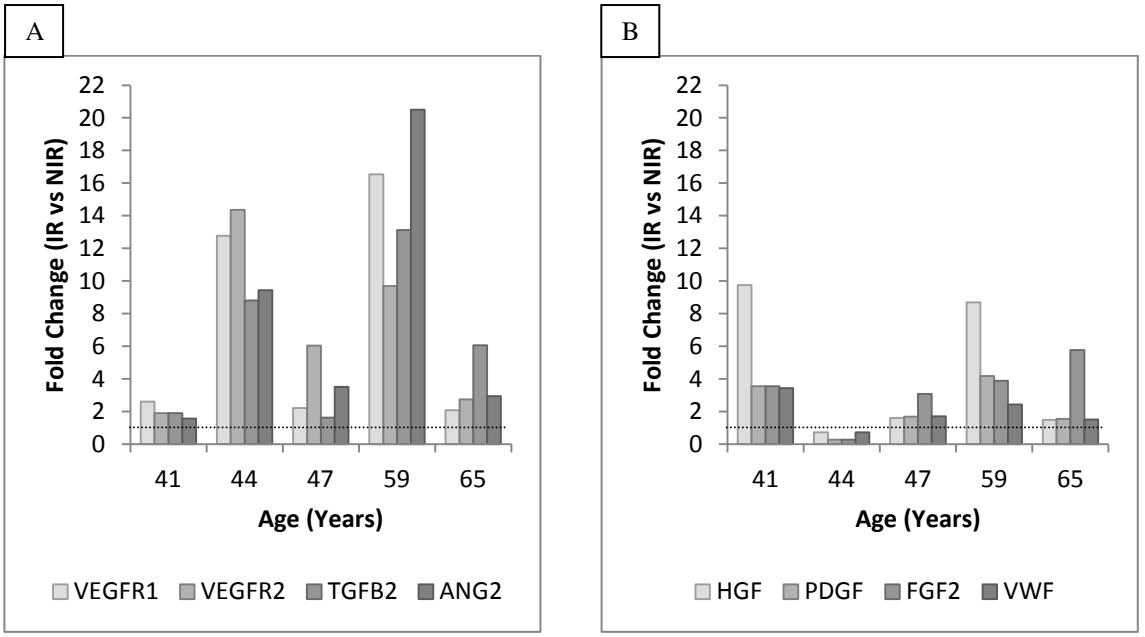

Figure 6 - **Low doses of ionizing radiation up-regulate the expression of several pro-angiogenic factors.** The mRNA expression of A) VEGFR1, VEGFR2, TGF-β2 and ANG2 and B) HGF, PDGF-C, FGF2 and vWF was quantified by qRT-PCR in ECs isolated from a specimen exposed to low doses of IR and a non-irradiated specimen. Expression values were normalized to 18S to obtain relative expression levels. The data represents the fold change in gene expression of each target in irradiated (IR) relatively to non-irradiated (NIR) cells (represented with a dashed black line). Each bar represents one different patient (A, B, C, D, E with 38, 46, 60 and 61 years old)

In order to corroborate our findings we evaluated the expression of these different molecular targets in 6 more patients. According to our results, all targets present an up-regulation of their relative gene expression levels in ECs exposed to low doses of IR for the two youngest patients (Figure 7). However, there are two patients 46 and 50 years old where the levels are not changed or present a very modest increase (Figure 7). The 60 years old patient present a significant increase in the levels of VEGFR2 relative expression, i.e., fold change levels of 5 while the remaining targets are slightly increased (Figure 7). In the 61 years old patient the levels of expression for VEGFR1, VEGFR2 and TGF-β2 are 2.6 fold change increased whereas levels of 1.6 are found for HGF (Figure 7). The remaining levels do not change or are lower (ex: ANG2) compared to those found in non-irradiated ECs (Figure 7).

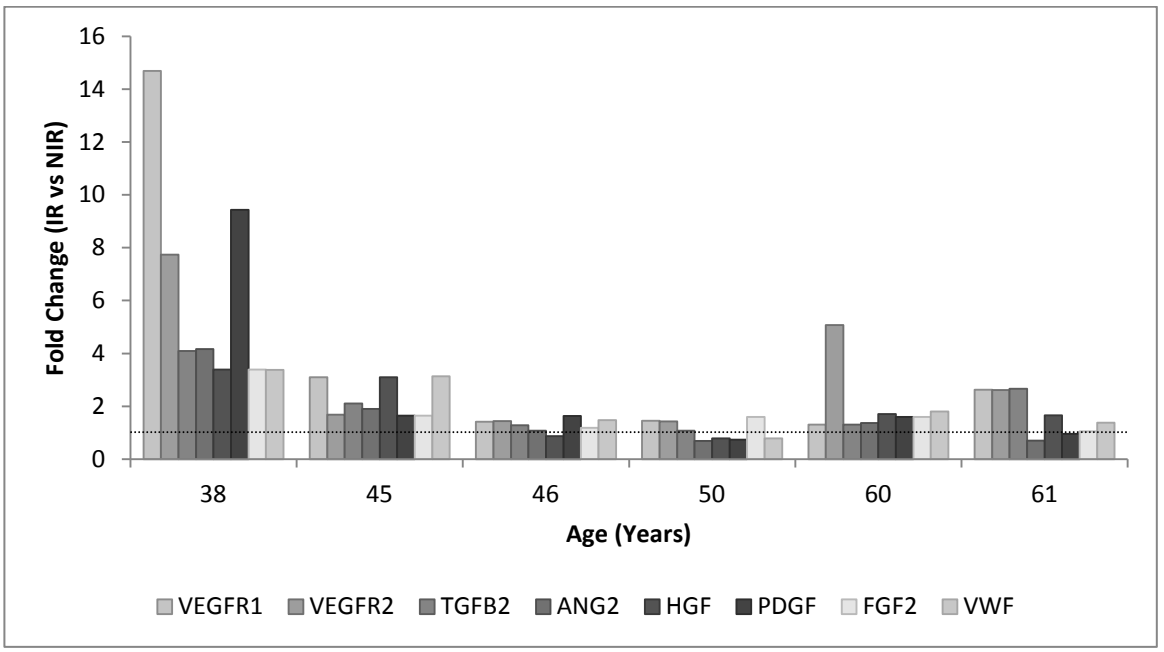

Figure 7 - **Low doses of ionizing radiation up-regulate the expression of several pro-angiogenic factors.** The mRNA expression of VEGFR1, VEGFR2, TGF-β2, ANG2, HGF, PDGF-C, FGF2 and vWF was quantified by qRT-PCR in ECs isolated from a specimen exposed to low doses of IR and a non-irradiated specimen. Expression values were normalized to 18S to obtain relative expression levels. The data represents the fold change in gene expression of each target in irradiated (IR) relatively to non-irradiated (NIR) cells (represented with a dashed black line). Patients 38, 45, 46, 50, 60 and 61 years old were analyzed.

A final figure (Figure 8) where all patients were represented was performed in order to better visualize the modulation of these pro-angiogenic targets in the different patients.

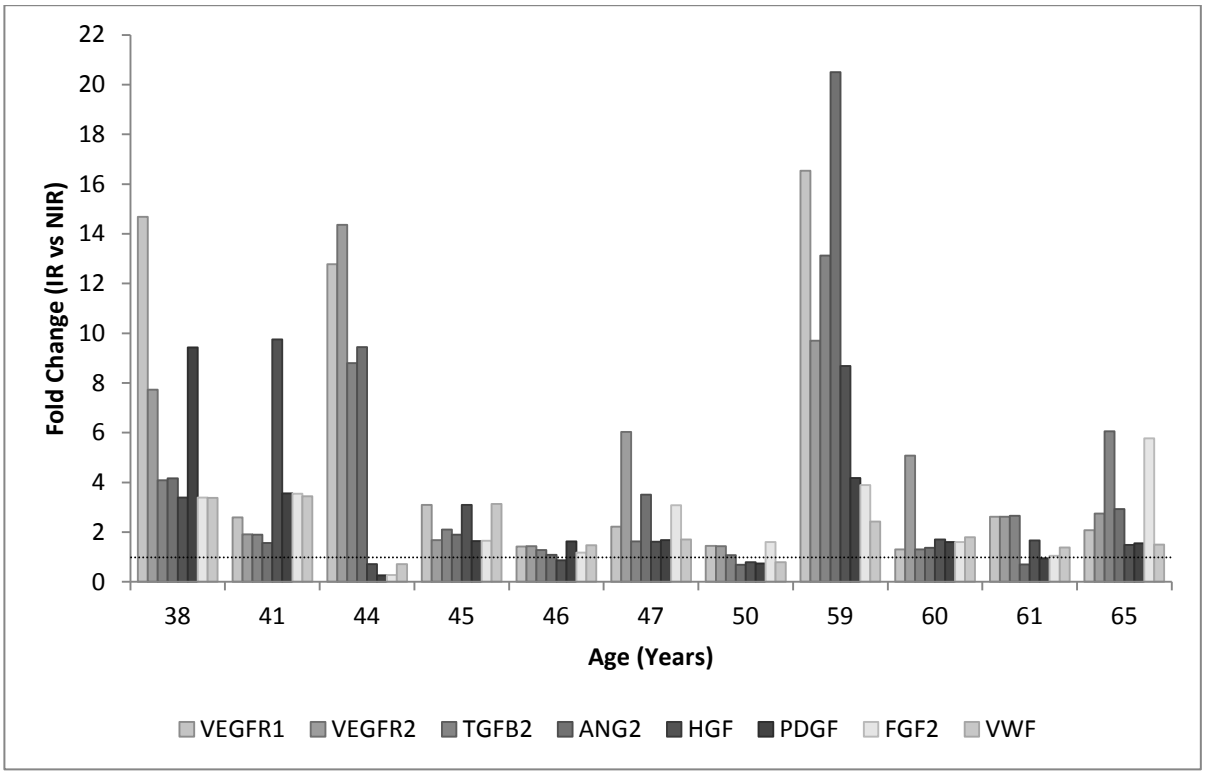

Figure 8 - **Low doses of ionizing radiation up-regulate the expression of several pro-angiogenic factors.** The mRNA expression of VEGFR1, VEGFR2, TGF-β2, ANG2, HGF, PDGF-C, FGF2 and VWF was quantified by qRT-PCR in ECs isolated from a specimen exposed to low doses of IR and a non-irradiated specimen. Expression values were normalized to 18S to obtain relative expression levels. The data represents the fold change in gene expression of each target in irradiated (IR) relatively to non-irradiated (NIR) cells (represented with a dashed black line). Patients 38, 41, 44, 45, 46, 47, 50, 59, 60, 61 and 65 years old were analyzed.

Chapter IV - Discussion

It has been previously shown that low doses of IR induce angiogenesis in different *in vitro* and *in vivo* models: i) in human lung microvascular endothelial cells (HMVEC-L), low doses of IR activate several signaling pathways involved in cell survival, protecting endothelium against cell death, and induce cell migration; in addition, by a microarray study performed in our lab, low-dose IR up-regulates the expression of several pro-angiogenic factors, including VEGFR1, VEGFR2, ANG2, TGF-β2 and FGF2; ii) in zebrafish, low doses of IR accelerate angiogenic sprouting during embryonic development and enhance angiogenesis during regeneration; iii) in mice, low-dose IR promotes angiogenesis in the murine Matrigel plug assay and iv) in mice tumor models, low doses of IR increase tumor growth and induce metastasis.

The data obtained from the microarray were previously validated *in vitro* by using HMVEC-L exposed or not to IR, with the exception of FGF2. Here, by using a similar approach, we assessed if the levels of FGF2 and other important pro-angiogenic factors, described in the literature, were modulated by low doses of IR. Therefore, by irradiating HUVECs with 0.3 Gy and analyzing the expression of HGF, PDGF-C, FGF2 and vWF at 3 different time points (4, 8 and 12 hours), we show that the expression of these targets is increased 4 hours after irradiation. However, 8 or 12 hours after irradiation the expression of these factors decreased to basal levels similar to those found in non-irradiated cells. This suggests that low doses of IR up-regulate the expression of HGF, PDGF-C, FGF2 and vWF in ECs and the levels are then restored back to basal ones.

Taken together, these data strongly suggest that low doses of IR activate ECs enhancing angiogenesis, although it is crucial to validate these findings in a human model. With this objective, we used peritoneal biopsies removed from rectal cancer patients that received pre-operative radiotherapy. We analyzed the expression of pro-angiogenic targets in ECs isolated from tissues exposed to 5-30% of the therapeutic dose (corresponding to low doses of IR) and in ECs isolated from non-irradiated tissues. The biopsy removed from the non-irradiated area served as an internal calibrator for each patient (paired control sample). This process of calibration is fundamental to compare patients with different age, metabolism, co-morbidities, and distinct genetic and environmental background.

According to the results obtained in our lab with 5 patients, low doses of IR up-regulate the expression of VEGFR1, VEGFR2, ANG2 and TGF-β2. Therefore, here we analyzed the effect of low doses of IR in the expression of HGF, PDGF-C, FGF2 and vWF for the same patients and we observed that some targets could be up-regulated in certain patients or not modulated in others. Moreover, it is important to note that one of the five patients that presented high levels of relative gene expression for VEGFR1, VEGFR2, ANG2 and TGF-β2, shows a down-regulation for all the new targets analyzed. It is also important to refer that FGF2 is the one that is up-regulated in

the other 4 patients. Taken together, our results suggest that the effect of low doses of IR seems to be more variable for the targets that were not selected from the microarray.

In order to corroborate our results, the same procedure was performed in 6 patients more. Our results suggest that 8 weeks after radiotherapy the modulation of pro-angiogenic factors by low-dose IR is no longer observed at least for 2 of the 6 patients. Moreover, we found that only one or three targets are up-regulated in other 2 patients. In addition, only 2 patients, from the 6, present an up-regulation of all the pro-angiogenic targets. These findings obligate to the analysis of a more representative number of patients in the future.

So far and taken together, our results suggest that at the 8th week after the end of radiotherapy, the relative levels of expression of pro-angiogenic targets in ECs removed from 3 (from a total of 11) patients were not changed by low doses of IR. The levels were similar to those obtained in non-irradiated ECs and in one of these 3 patients only one pro-angiogenic target, VEGFR2, was up-regulated. However, we must take into account that this analysis was done 8 weeks after the end of radiotherapy. According to the clinical guidelines, the surgery should be done at the 8th week, preventing us from obtaining biopsies sooner. We consider this a limitative aspect since it is important to investigate if the expression of molecular targets could be modulated by the time interval between the end of the radiotherapy treatment and surgery.

Importantly, our results also show that 8 (from a total of 11) patients present an up-regulation for all the pro-angiogenic targets (5 patients) or at least for all or for the majority (4 from 5) of those that were selected from our *in vitro* array. According to these findings we may hypothesize that 8 weeks after the end of radiotherapy, ECs that were exposed to low doses of IR have their angiogenic balance skewed toward a pro-angiogenic phenotype. This shift could represent a mark that those ECs were submitted to a stimulus that led to an angiogenic response. Also, this shift could be modulated overtime and previous results from our lab suggest that the number of weeks between the end of radiotherapy and the surgery is an extrinsic parameter that should be considered since it was observed a down-regulation of all pro-angiogenic factors by low doses of IR in ECs from two patients operated at the 11th week. Exceptionally, in these two patients it was not possible to perform the surgery at the 8th week.

As we cannot exclude the hypothesis that low dose of IR could be modulated overtime, we shouldn't exclude that they might not influence the activation of ECs in some patients, and a parameter that should also be taken into account considering that hypothesis is the influence of the microenvironment.

There are still open questions such as i) can the microenvironment influence the effect of low doses of IR in ECs? ii) If so, how? iii) Can low doses of IR modulate the microenvironment beyond ECs? The answer to these questions will be important to better understand the effect of low

doses in EC's activation and consequently in the putative modulation of angiogenesis in the peri-tumor area by low doses of IR and their relevance in the formation of metastasis after or during radiotherapy.

Chapter V – Conclusions and Future Perspectives

With the objective to validate in human our *in vitro* and *in vivo* data, demonstrating that low doses of IR enhance angiogenesis, we analyzed the expression of several pro-angiogenic factors in ECs removed from tissues that were exposed or not to low doses of ionizing radiation. Biopsies of rectal cancer patients that performed neo-adjuvant radiotherapy were used. Our findings suggest that in 8 patients (from a total of 11) and 8 weeks after the end of radiotherapy, the levels of relative gene expression of VEGFR1, VEGFR2, TGFB2, ANG2, FGF2 are consistently up-regulated in ECs exposed to low doses of IR, when compared to those found in non-irradiated ECs. In addition, some patients also present an up-regulation of HGF and vWF. The simultaneous activation of these pro-angiogenic factors in ECs suggests that low doses of IR may activate ECs, since the angiogenic balance is skewed toward a pro-angiogenic phenotype. According to the clinical guidelines, the surgery should be done at the 8^{th} week, preventing us from obtaining biopsies sooner. We consider this a limitative aspect since it is important to investigate if the expression of molecular targets could be modulated by the time interval between the end of the radiotherapy treatment and surgery. To address this question, a previously published syngeneic orthotopic mouse model of rectal cancer [123] will be used and mice will be sacrificed at different time points: immediately after ending radiotherapy and 4, 8 and 12 weeks later. Comprehensive necropsies will be performed and tumor margins (exposed to doses from 5 to 30 % of the therapeutic dose) will be collected and snap frozen. CD31 immunohistochemistry will be performed using a high salt buffer to stabilize RNA during prolonged antibody incubations and then ECs will be isolated by using a laser capture microdissection microscope. RNA will be extracted followed by cDNA synthesis and qRT-PCR analysis. The levels of expression of VEGR1, VEGR2, ANGPT2, TGFβ, FGF2, HGF, PDGF-C and vWF will be analyzed. These levels of expression will be calibrated with those obtained from ECs removed from non-irradiated tissues by using a similar methodology. Mice will be sacrificed immediately after and 4, 8 and 12 weeks post-radiotherapy and the ECs' profile expression for the target genes will be compared overtime. The use of a mouse model during this work will allow us to understand how the expression of these targets is modulated overtime after exposure to low doses of IR. The answer to this question will certainly be important for the interpretation of the results obtained in patients whose levels of pro-angiogenic gene expression are not influence by low doses of IR. Furthermore, it could be relevant to corroborate the gene expression data with protein levels. However, protein expression analysis by immunohistochemistry will be difficult to be performed since the majority of these pro-angiogenic targets are soluble factors, with the exception of the receptors for VEGF and vWF. By vWF immunohistochemistry it will be interesting to measure the microvascular density in tissues exposed to low doses of IR and compare those values with those obtained in non-irradiated areas. It is also possible to measure the levels of some of these soluble proteins in

patient's circulation by collecting blood samples before and after radiotherapy and ELISA. Besides these perspectives, we consider that it is crucial to increase the number of patients in our study and continue to analyze the data at an individual level. Moreover, one of the most important aspects of our work is the fact that the analysis is performed exclusively in ECs, since we consider it crucial to correlate the data with ECs' activation.

Chapter VI - References

[1] Eichmann, A. Vascular development: from precursor cells to branched arterial and venous networks. *Int J Dev Biol*. 2005; 49(2-3):259-67.

[2] Goldmann, E. The growth of malignant disease in man and the lower animals, with special reference to the vascular system. *Proc R Soc Med*. 1908; 1:1-13.

[3] Folkman, J. Tumor angiogenesis: therapeutic implications. *New England J Med*. 1971; 285:1182–6.

[4] Bisacchi, D.; et al. Anti-angiogenesis and angioprevention: mechanisms, problems and perspectives. *Cancer Detect Prev*. 2003; 27(3):229-38.

[5] Hanahan, D.; Weinberg, R. A. The hallmarks of cancer. *Cell*. 2000; 100:57–70.

[6] Ferrara, N.; et al. The biology of VEGF and its receptors. *Nature Medicine*. 2003; 9, 669-676.

[7] Chakraborti, S.; et al. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem*. 2003; 253(1-2):269-85.

[8] Hillen, F.; et al. Tumour vascularization: sprouting angiogenesis and beyond. Cancer Metastasis Rev. 2007; 26:489-502.

[9] Carmeliet, P.; Jain, R. K. Angiogenesis in cancer and other diseases. Nature. 2000; 407(6801):249-57.

[10] Maxwell, P.H.; Ratcliffe PJ. Oxygen sensors and angiogenesis. *Semin Cell Dev Biol*. 2002; 13(1):29-37.

[11] Ferrara, N. Davis-Smyth, T. The biology of vascular endotelial growth factor. *Endocr Rev*. 1997; 18(1):4-25.

[12] Grugel, S., et al. Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. *J Bio Chem*. 1995; 270(43):25915-9.

[13] Okada, F.; et al. Impact of oncogenes in tumor angiogenesis: mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. *Proc Natl Acad Sci U S A*. 1998; 95(7):3609-14.

[14] Dvorak, H. F.; et al. Vascular permeability factor/vascular endotelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Curr Top Microbiol Immunol*. 1999; 237:97-132.

[15] Fong, G.H.; Rossant, J.; Gertsenstein, M.; Breitman, M.L. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995; 376: 66-70.

[16] Fong, G.H.; Zhang, L.; Bryce, D.M.; Peng, J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knockout mice. *Development*. 1999; 126: 3015-3025.

[17] Takahashi, H.; Shibuya, M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci (Lond)*. 2005; 109: 227-241.

[18] Shalaby, F.; Rossant, J.; Yamaguchi, T.P.; Gertsenstein, M.; Wu, X.F.; et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995; 376: 62-66.

[19] Ferrara, N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*. 2004; 25: 581-611.

[20] Geretti, E.; Klagsbrun, M. Neuropilins: novel targets for anti-angiogenesis therapies. *Cell Adh Migr*. 2007; 1: 56-61

[21] Soker, S.; Takashima, S.; Miao, H.Q.; Neufeld, G.; Klagsbrun, M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell*. 1998; 92: 735-745.

[22] Kawasaki, T.; Kitsukawa, T.; Bekku, Y.; Matsuda, Y.; Sanbo, M.; et al. A requirement for neuropilin-1 in embryonic vessel formation. *Development.* 1999; 126: 4895-4902.

[23] Kitsukawa, T.; Shimono, A.; Kawakami, A.; Kondoh, H.; Fujisawa, H. Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development.* 1995; 121: 4309-4318.

[24] Chen, H.; Bagri, A.; Zupicich, J.A.; Zou, Y.; Stoeckli, E.; et al. Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. *Neuron*. 2000; 25: 43-56.

[25] l. Takashima, S.; Kitakaze, M.; Asakura, M.; Asanuma, H.; Sanada, S.; et al. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc Natl Acad Sci U S A*. 2002; 99: 3657-3662.

[26] Davis, S.; Papadopoulos, N.; Aldrich, T.H.; Maisonpierre, P.C.; Huang, T.; Kovac, L.; Xu, A.; Leidich, R.; Radziejewska, E.; Rafique, A.; et al. Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. *Nature Structural & Molecular Biology*. 2003; 10:38-44.

[27] Barton, W.A.; Tzvetkova-Robev, D.; Miranda, E.P.; Kolev, M.V.; Rajashankar, K.R.; Himanen, J.P.; et al. Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2-Tie2 complex. *Nature Structural and Molecular Biology*. 2006; 13:524–32.

[28] Yancopoulos, G. D.; Davis, S.; Gale, N. W.; Rudge, J. S.; Wiegand, S. J. & Holash, J. Vascular-specific growth factors and blood vessel formation. *Nature*. 2000; 407, 242–248.

[29] Gerald, D.; Chintharlapalli, S.; Augustin, H. G.; Benjamin, L. E. Angiopoietin-2: an attractive target for improved antiangiogenic tumor therapy. *Cancer Research*. 2013; 73(6):1649-57.

[30] Asahara, T.; Chen, D.; Takahashi, T.; Fujikawa, K.; Kearney, M.; Magner, M.; Yancopoulos, G. D. & Isner, J. M. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ. Res*. 1998; 83, 233–240.

[31] Kim, I.; Kim, J. H.; Moon, S. O.; Kwak, H. J.; Kim, N. G. & Koh, G. Y. Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3' –kinase/Akt signal transduction pathway. *Oncogene*. 2000; 19, 4549–4552.

[32] Teichert-Kuliszewska, K.; Maisonpierre, P. C.; Jones, N.; Campbell, A. I.; Master, Z.; Bendeck, M. P.; Alitalo, K.; Dumont, D. J.; Yancopoulos, G. D. & Stewart, D. J. Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. *Cardiovasc. Res*. 2001; 49, 659–670.

[33] Mochizuki, Y.; Nakamura, T.; Kanetake, H. & Kanda, S. Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. *J. Cell Sci*. 2002; 115, 175–183.

[34] Maisonpierre, P. C.; Suri, C.; Jones, P. F.; Bartunkova, S.; Wiegand, S. J.; Radziejewski, C.; Compton, D.; McClain, J.; Aldrich, T. H.; Papadopoulos, N.; et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science*. 1997; 277, 55–60.

[35] Eggert, A.; Ikegaki, N.; Kwiatkowski, J.; Zhao, H.; Brodeur, G. M. & Himelstein, B. P. High-level expression of angiogenic factors is associated with advances tumor stage in human neuroblastomas. *Clin Cancer Res*. 2000; 6, 1900–1908.

[36] Etoh, T.; Inoue, H.; Tanaka, S.; Barnard, G. F.; Kitano, S. & Mori, M. *Cancer Res*. 2001; 61, 2145–2153.

[37] Lobov, I.B.; Brooks, P.C.; Lang, R.A. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival *in vivo. Proc Natl Acad Sci USA*. 2002; 99(17):11205-10.

[38] Govinden, R.; Bhoola, K.D. Genealogy, expression, and cellular function of transforming growth factor-β. *Pharmacology & Therapeutics*. 2003; 98(2): 257– 265.

[39] Kaminska, B.; Wesolowska, A.; Danilkiewicz, M. TGF beta signalling and its role in tumour pathogenesis. *Acta Biochimica Polonica*. 2005; 52(2):329-37.

[40] Roberts, A.B.; Sporn, M.B. The transforming growth factor b's. Peptide Growth Factors and Their Receptors. *Springer-Verlag*, Berlin. 1990; 419–472.

[41] Massague, J. The transforming growth factor-b family. *Annu. Rev. Cell Biol.* 1990; 6:597–641.

[42] Arenas, M.; Gil, F.; Gironella, M.; Hernández, V.; Biete, A.; Piqué, J.M.; Panés, J. Time course of anti-inflammatory of low dose radiotherapy: correlation with tgfb1. *Radiother Oncol*. 2008; 86(3):399-406.

[43] Gajdusek, C.M.; Luo, Z.; Mayberg, M.R. Basic fibroblast growth factor and transforming growth factor beta-1: synergistic mediators of angiogenesis in vitro. *J Cell Physio*. 1993; 57(1):133-44.

[44] Pepper, M.S.; Vassalli, J.D.; Orci, L.; Montesano, R. Biphasic effect of transforming growth factor-beta 1 on in vitro angiogenesis. *Experimental Cell Research*. 1993; 204(2):356-63.

[45] Carmeliet, P. Angiogenesis in health and disease, Nat. Med. 2003; 9:653–660.

[46] Goumans, M.J.; Lebrin, F.; Valdimarsdottir, G. Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. *Trends Cardiovasc*. Med. 2003; 13:301–307.

[47] Sugano, Y.; Matsuzaki, K.; Tahashi, Y.; Furukawa, F.; Mori, S.; Yamagata, H.; Yoshida, K.; Matsushita, M.; Nishizawa, M.; Fujisawa, J.; Inoue, K. Distortion of autocrine transforming growth factor b signal accelerates malignant potential by enhancing cell growth as well as PAI-1 and VEGF production in human hepatocellular carcinoma cells. *Oncogene.* 2003; 22(15):2309-21.

[48] Sankar, S.; Mahooti-Brooks, N.; Bensen, L.; McCarthy, T.L.; Centrella, M.; Madri, J.A. Modulation of Transforming Growth Factor β Receptor Levels on Microvascular Endothelial Cells during In Vitro Angiogenesis. *The Journal of Clinical Investigation*. 1996; 97(6):1436-46.

[49] Sánchez-Elsner, T.; Botella, L.M.; Velasco, B.; Corbí, A.; Attisano, L.; Bernabéu, C. Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. *J Bio Chem*. 2001; 276(42):38527-35.

[50] Powers, C.J.; McLeskey, S.W.; Wellstein, A. Fibroblast growth factors, their receptors and signaling. *Endocrine-Related Cancer*. 2000; 7:165–197.

[51] Javerzat, S.; Auguste, P.; Bikfalvi, A. The role of fibroblast growth factors in vascular development. Trends Mol Med. 2002; 8(10):483-9.

[52] Turner, N.; Grose, R. Fibroblast growth factor signalling: from development to cancer. *Nat Rev Cancer*. 2010; 10(2):116-29.

[53] Milkiewicz, M.; Ipsanovic, E.; Doyle, J.L.; Haas, T.L. Regulators of angiogenesis and strategies for their therapeutic manipulation. *Int J Biochem Cell Biol.* 2005; 38(3):333-57.

[54] Murakami, M.; Simons, M. Fibroblast growth factor regulation of neovascularization. *Curr Opin Hematol.* 2008; 15(3):215-20.

[55] Daviet, I.; Herbert, J.M.; Maffrand, J.P. Involvement of protein kinase C in the mitogenic and chemotaxis effects of basic fibroblast growth factor on bovine cerebral cortex capillary endothelial cells. *FEBS Lett*. 1990; 259(2):315-7.

[56] Presta, M.; Rusnati, M.; Urbinati, C.; Sommer, A.; Ragnotti, G. Biologically active synthetic fragments of human basic fibroblast growth factor (bFGF): identification of two Asp-Gly-Arg-containing domains involved in the mitogenic activity of bFGF in endothelial cells. *J Cell Physiol*. 1991; 149(3):512-24

[57] Murakami, M.; Nguyen, L.T.; Zhuang, Z.W.; Moodie, K.L.; Carmeliet, P.; Stan, R.V.; Simons, M. The FGF system has a key role in regulating vascular integrity. *The Journal of Clinical Investigations.* 2008; 118(10):3355-66.

[58] Miller, D.L.; Ortega, S.; Bashayan, O.; Basch, R.; Basilico C. Compensation by Fibroblast Growth Factor1 (FGF1) Does Not Account for the Mild Phenotypic Defects Observed in FGF2 Null Mice. *Mol Cell Biol.* 2000; 20(6):2260-2268.

[59] Presta, M.; Dell'Era, P.; Mitola, S.; Moroni, E.; Ronca, R.; Rusnati, M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*. 2005; 16(2):159-78.

[60] Mizuno, S.; Nakamura, T. HGF–MET Cascade, a Key Target for Inhibiting Cancer Metastasis: The Impact of NK4 Discovery on Cancer Biology and Therapeutics. *International Journal of Molecular Sciences.* 2013; 14(1):888-919.

[61] Bussolino, F.; Di Renzo, M.F.; Ziche, M.; Bocchietto, E.; Olivero, M.; Naldini, L.; Gaudino G.; Tamagnone, L.; Coffer, A.; Comoglio, P.M. Hepatocyte Growth Factor Is a Potent Angiogenic Factor Which Stimulates Endothelial Cell Motility and Growth. *J Cell Bio*. 1992; 119(3):629-41.

[62] Grant, D.S.; Kleinman H.K.; Goldberg, I.D.; Bharqava, M.M.; Nickoloff, B.J.; Kinsella, J.L.; Polverini, P.; Rosen, E.M. Scatter factor induces blood vessel formation in vivo. *Proc Natl Acad Sci USA*. 1993; 90(5):1937-41.

[63] Gerritsen, M.E. HGF and VEGF: A Dynamic Duo. *Circ Res*. 2005; 96(3):272-3.

[64] Wojta, J.; Kaun, C.; Breuss, J.M.; Koshelnick, Y.; Beckmann, R.; Hattey, E.; Mildner, M.; Weninger, W.; Nakamura, T.; Tschachler, E.; Binder, B.R. Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells. *Lab Invest*. 1999*;* 79*:*427–438.

[65] Gille, J.; Khalik, M.; Konig, V.; Kaufmann, R.Hepatocyte growth factor/scatter factor (HGF/SF) induces vascular permeability factor (VPF/VEGF) expression by cultured keratinocytes. *J Invest Dermatol*. 1998*;* 111*:* 1160–1165.

[66] Sengupta, S.; Gherardi, E.; Sellers, L.A.; Wood, J.M.; Sasisekharan, R.; Fan, T.P. Hepatocyte growth factor/scatter factor can induce angiogenesis independently of vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol.* 2003; 23:69–75.

[67] Gerritsen, M.E.; Tomlinson, J.E.; Zlot, C.; Ziman, M.; Hwang, S. Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells. *Br J Pharmacol.* 2003; 140:595–610.

[68] Rosen, E. M.; Lamszus, K.; Laterra, J.; Polverini, P. J.; Rubin, J. S.; Goldberg, I. D. HGF/SF in angiogenesis. *Ciba Found. Symp.* 1997; 212*:* 215-226.

[69] Rosen, E. M.; Goldberg, I. D. Regulation of angiogenesis by scatter factor. *EXS*. 1997; 79*:*193-208.

[70] Laterra, J.; Nam, M.; Rosen, E.; Rao, J. S.; Lamszus, K.; Goldberg, I. D.; Johnston, P. Scatter factor/hepatocyte growth factor gene transfer enhances glioma growth and angiogenesis *in vivo*. *Lab Invest.* 1997; 76*:* 565-577.

[71] Lamszus, K.; Jin L.; Fuchs, A.; Shi, E.; Chowdhury, S.; Yao, Y.; Polverini, P. J.; Laterra, J.; Goldberg, I. D.; Rosen, E. M. Scatter factor stimulates tumor growth and tumor angiogenesis in human breast cancers in the mammary fat pads of nude mice. *Lab Invest*. 1997*;* 76*:* 339-*353.*

[72] Figg, W.; Folkman, J. Angiogenesis – An integrative approach from science to medicine. *Springer.* 2008; 9: 99-111.

[73] Ball, S.G.; Shuttleworth, C.A.; Kielty, C.M. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J Cell Bio*. 2007; 177(3):489-500.

[74] Fredriksson, L.; Li, H.; Eriksson, U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine & Growth Factor Reviews*. 2004; 15(4):197-204.

[75] Reigstad, L.J.; Varhaug, J.E.; Lillehaug, J.R. Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. *The FEBS Journal*. 2005; 272(22):5723-41.

[76] Fredriksson, L.; Li, H.; Fieber, C.; Li, X.; Eriksson, U. Tissue plasminogen activator is a potent activator of PDGF-CC. *The EMBO Journal*. 2004; 23(19):3793-802.

[77] Ustach, C.V.; Kim, H.R. Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells. *Mol Cell Biol*. 2005; 25(14):6279-88.

[78] Sun, B.; Chen, B.; Zhao, Y.; Sun, W.; Chen, K.; Zhang, J.; Wei, Z.; Xiao, Z.; Dai, J. Crosslinking heparin to collagen scaffolds for the delivery of human platelet-derived growth factor. *J Biomed Mater Res B Appl Biomater.* 2009; 91(1):366-72.

[79] Levéen, P.; Pekny, M.; Gebre-Medhin, S.; Swolin, B.; Larsson, E.; Betsholtz, C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* 1994; 8:1875–1887.

[80] Soriano, P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev.* 1994; 8:1888–1896.

[81] French, W.J.; Creemers, E.E.; Tallquist, M.D. Platelet-derived growth factor receptors direct vascular development independent of vascular smooth muscle cell function. *Mol Cell Biol*. 2008; 28:5646–5657.

[82] Cao, R.; Brakenhielm, E.; Li, X.; Pietras, K.; Widenfalk, J.; Ostman, A.; Eriksson, U.; Cao, Y. Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-alphaalpha and -alphabeta receptors. *FASEB J*. 2002; 16:1575–1583.

[83] Uutela, M.; Laurén, J.; Bergsten, E.; Li, X.; Horelli-Kuitunen, N.; Eriksson, U.; Alitalo, K. Chromosomal location, exon structure, and vascular expression patterns of the human PDGFC and PDGFD genes. *Circulation*. 2001; 103:2242–2247.

[84] Shih, A.H.; Holland, E.C. Platelet-derived growth factor (PDGF) and glial tumorigenesis. *Cancer Lett.* 2006; 232(2):139-47.

[85] Crawford, Y.; Kasman, I.; Yu, L.; Zhong, C.; Wu, X.; Modrusan, Z.; Kaminker, J.; Ferrara, N. PDGF-C Mediates the Angiogenic and Tumorigenic Properties of Fibroblasts Associated with Tumors Refractory to Anti-VEGF Treatment. *Cancer Cell.* 2009; 15(1):21-34.

[86] Kanaji, S.; Fahs, S.A.; Shi, Q.; Haberichter, S.L.; Montgomery, R.R. Contribution of platelet vs. endothelial VWF to platelet adhesion and hemostasis. *Journal of Thrombosis and Haemostasis*. 2012; 10(8):1646-52

[87] Rondaij, M.G.; Bierings, R.; Kragt, A.; van Mourik, J.A.; Voorberg, J. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. *Artherioscler, Thrombo and Vasc Biol*. 2006; 26(5):1002-7.

[88] Wagner, D.D.; Saffaripour, S.; Bonfanti, R., et al. Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell.* 1991; 64(2):403-13.

[89] Karpatkin, S.; Pearlstein, E.; Ambrogio, C.; Coller, B.S. Role of Adhesive Proteins in Platelet Tumor Interaction In Vitro and Metastasis Formation In Vivo. *J Clin Invest.* 1998; 81(4):1012-9.

[90] Terraube, V.; Marx, I.; denis, C.V. Role of von Willebrand factor in tumor metastasis. *Thromb Res.* 2007; 120 Suppl 2:S64-70.

[91] Mochizuki, S.; Soejima, K.; Shimoda, M.; Abe, H.; Sasaki, A.; Okano, H.J.; Okano, H.; Okada, Y. Effect of ADAM28 on Carcinoma Cell Metastasis by Cleavage of von Willebrand Factor. *J Natl Cancer Inst*. 2012; 104(12):906-22.

[92] Wong, C.W.; Song, C.; Grimes, M.M.; Fu, W.; Dewhirst, M.W.; Muschel, R.J.; Al-Mehdi, A.B. Intravascular location of breast cancer cells after spontaneous metastasis to the lung. *Am J Pathol*. 2002; 161(3):749-53.

[93] Zanetta, L.; Marcus, S.G.; Vasile, J.; Dobryansky, M.; Cohen, H.; Eng, K.; Shamamian, P.; Mignatti, P. Expression of von willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: a potential method for objective assessment of tumor angiogenesis. *Int J Cancer*. 2000; 85(2):281-8.

[94] Ribatti, D. Endogenous inhibitors of angiogenesis: a historical review. *Leuk Res.* 2009; 33(5):638-44.

[95] Benelli, R; et al. Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. *FASEB J.* 2002; 16(2):267-9.

[96] Hanahan, D; Weinberg, RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144(5):646-74.

[97] Hattori, K.; Heissig, B.; Wu, Y.; Dias, S.; Tejada, R.; Ferris, B.; Hicklin, D.J.; Zhu, Z.; Bohlen, P.; Witte, L.; Hendrikx, J.; Hackett, N.R.; Crystal, R.J.; Moore, M.A.; Werb, Z.; Lyden, D.; Rafii, S. Placental growth

factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med.* 2002; 8(8):841-9.

[98] Kaplan, R.N.; Psaila, B.; Lyden, D. Bone marrow cells in the "pre-metastatic niche": within bone and beyond. *Cancer Metastasis Rev*. 2006; 25(4):521-9.

[99] Kaplan, R.N.; Rafii, S.; Lyden, D. Preparing the "soil": the premetastatic niche. *Cancer Res.* 2006; 66(23):11089-93.

[100] Dawson, M.R; Duda, D.G.; Fukumura, D.; Jain, R.K. VEGFR1-activity-independent metastasis formation. *Nature.* 2009; 461(7262):E4

[101] Ebos, J. M. L. & Kerbel, R. S. Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nat Rev Clin Oncol.* 2011; 8**:**210–21.

[102] Loges, S.; Mazzone, M.; Hohensinner, P. & Carmeliet, P. Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. *Cancer Cell.* 2009*;* 15**:**167–70.

[103] Ebos, J. M. L.; *et al.* Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell.* 2009; 15: 232–9.

[104] Pàez-Ribes, M.; *et al.* Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell.* 2009*;* 15:220–31.

[105] Padera, T. P.; *et al.* Differential response of primary tumor versus lymphatic metastasis to VEGFR-2 and VEGFR-3 kinase inhibitors cediranib and vandetanib. *Mol Cancer Ther.* 2008*;* 7:2272–9.

[106] Singh, M. *et al.* Anti-VEGF antibody therapy does not promote metastasis in genetically engineered mouse tumour models. *J. Pathol.* 2012; 227:417–30.

[107] Miles, D.; *et al.* Disease course patterns after discontinuation of bevacizumab: pooled analysis of randomized phase III trials. *J. Clin. Oncol.* 2011*;* 29:83–8.

[108] Begg, A. C.; et al. Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer*. 2011; 11(4):239-53.

[109] O'Brien, C.J.; Smith, J.W.; Soong, S.J.; Urist, M.M.; Maddox, W.A. Neck Dissection With and Without Radiotherapy: Prognostic Factors, Patterns of Recurrence, and Survival. *Am J Surg*. 1986; 152(4):456-63.

[110] Suit, H.D. Local control and patient survival. *Int J Radiation Oncology Biol Phys*. 1992; 23:653-660.

[111] Vicini, F.A.; Kestin, L.; Huang, R.; Martinez, A. Does Local Recurrence Affect the Rate of Distant Metastases and Survival in Patients with Early-Stage Breast Carcinoma Treated with Breast-Conserving therapy?. *Cancer.* 2003; 97(4):910-9.

[112] Barcellos-Hoff, M. H.; et al. Radiation and the microenvironment – tumorigenesis and therapy. *Nat Rev Cancer*. 2005; 5(11):867-75.

[113] Madani, I.; et al. Does ionizing radiation stimulate cancer invasion and metastasis?. *Bull Cancer*. 2008; 95(3):292-300.

[114] Gorski, D.H.; Beckett, M.A.; Jaskowiak, N.T.; Calvin, D.P.; Mauceri, H.J.; Salloum, R.M.; Seetharam, S.; Koons, A.; Hari, D.M.; Kufe, D.W.; Weichselbaum, R.R. Blockade of the Vascular Endothelial

Growth Factor Stress Response Increases the Antitumor Effects of Ionizing Radiation. *Cancer Res.* 1999; 59(14):3374-8.

[115] Gupta, V.K.; Jaskowiak, N.T.; Beckett, M.A.; Mauceri, H.J.; Grunstein, J.; Johnson, R.S.; Calvin, D.A.; Nodzenski, E.; Pejovic, M.; Kufe, D.W.; Posner, M.C.; Weichselbaum, R.R. VEGF enhances endothelial cell survival and tumor radioresistance. *Cancer J.* 2002; 8(1):47-54

[116] Lee, C.; Heijn, M.; di Tomaso, E.; Griffon-Etienne, G.; Ancukiewicz, M.; Koike, C.; Park, K.R.; Ferrara, N.; Jain, R.K.; Suit, H.D.; Boucher, Y. Anti-Vascular Endothelial Growth Factor Treatment Augments Tumor Radiation Response under Normoxic or Hypoxic conditions. *Cancer Res*. 2000; 60:5565-5570.

[117] Mauceri, H.J.; Hanna, N.N.; Beckett, M.A.; Gorski, D.H.; Staba, M.J.; Stellato, K.A.; Bigelow, K.; Heimann, R.; Gately, S.; Dhanabal, M.; Soff, G.A.; Sukhatme, V.P.; Kufe, D.W.; Weichselbaum, R.R. Combined effects of angiostatin and ionizing radiation in antitumour therapy. *Nature*. 1998; 394(6690):287-91.

[118] Teicher, B.A.; Dupuis, N.; Kusomoto, T.; Robinson, M.F.; Liu, F.; Menon, K.; Coleman, C.N. Antiangiogenic Agents Can Increase Tumor Oxygenation and Response to Radiation Therapy. *Radiation Oncology Investigations*. 1995; 2:269-276.

[119] Paris, F.; Fuks, Z.; Kang, A.; Capodieci, P.; Juan, G.; Ehleiter, D.; Haimovitz-Friedman, A.; Cordon-Cardo, C.; Kolesnick, R. Endothelial Apoptosis as the Primary Lesion Initiating Intestinal Radiation Damage in Mice. *Science*. 2001; 293(5528):293-7.

[120] Moeller, B. J. Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell*. 2004; 5(5):429-41.

[121] McBride, W.H.; Chiang, C.S.; Olson, J.L.; Wang, C.C.; Hong, J.H.; Pajonk, F.; Dougherty, G.J.; Iwamoto, K.S.; Pervan, M.; Liao, Y.P. A Sense of Danger from Radiation. *Radiat Res*. 2004; 162(1):1-19.

[122] Sofia Vala, I. Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis. *PLoS One*. 2010; 5(6):e11222.

[123] Kishimoto, H.; Momiyama, M.; Aki, R.; Kimura, H.; Suetsugu, A.; Bouvet, M.; Fujiwara, T.; Hoffman, R.M. Development of a clinically-precise mouse model of rectal cancer. *Plos One*. 2013. 8(11):e79453.