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# THE EFFECTS OF LOW-DOSE IONIZING RADIATION ON ANGIOGENESIS

**INÊS SOFIA BATISTA VALA SILVA DE OLIVEIRA**

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# THE EFFECTS OF LOW-DOSE IONIZING RADIATION ON ANGIOGENESIS

**INÊS SOFIA BATISTA VALA SILVA DE OLIVEIRA**

**Dissertation submitted to obtain a PhD Degree in**
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*Ao Cláudio e ao Artur*

**PREFACE**

The present thesis embraces the data obtained during my PhD research project.

The experimental work was developed under the supervision of Prof. Dr. Susana Constantino Rosa Santos at the Angiogenesis Unit, Instituto de Medicina Molecular, Lisboa, Portugal.

This PhD was also supervised by Prof. Dr. Rita Maria Pulido Garcia Zilhão from the Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Universidade de Lisboa, Lisboa, Portugal.

The financial support was provided by the Fundação para a Ciência e Tecnologia, through a PhD fellowship grant SFRH/BD/27541/2006.

This dissertation is organized in five chapters, which are preceded by a summary, both in Portuguese and in English.
Chapter I consist of a general introduction to blood vessels, with particular emphasis on the angiogenic process, approached from the early embryonic development to adulthood, in physiology and pathology. A brief overview on radiotherapy and some cellular and molecular effects of ionizing radiation is also presented.
Chapter II specifically indicates the main objectives of the research proposal that led to the work presented in this thesis.
Chapter III and IV include the experimental work developed through the research project. Chapter II, *low doses of ionizing radiation promote tumor angiogenesis and metastasis by enhancing angiogenesis*, includes some already published work (presented in the publication format) and some complementary data to the article. Chapter III, *combined*

*effect of vasoprost® and low-dose ionizing radiation on angiogenesis*, presents some results from ongoing work that is currently being developed in our lab and has not yet been published. Each one of these chapters includes a specific introduction, the results obtained in the work developed, and a focused discussion, as well as the methods, acknowledgements and references.

Chapter IV comprises the concluding remarks and future perspectives.

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Quase sinto o barulho do tempo a passar por mim...
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**RESUMO**

A angiogénese é o processo de formação de novos vasos sanguíneos a partir de vasos pré-existentes. Em situações fisiológicas a angiogénese ocorre durante o desenvolvimento embrionário, crescimento de órgãos e, no adulto, em processos de cicatrização de feridas e ciclo reprodutivo. Nestas condições, o processo angiogénico é fortemente controlado por um equilíbrio complexo entre factores estimuladores (pró-angiogénicos) e inibidores (anti-angiogénicos).

A angiogénese pode, no entanto, ocorrer em situações patológicas onde há uma perda do equilíbrio entre factores pró- e anti-angiogénicos, resultando numa vascularização excessiva ou deficiente. O cancro é uma das patologias que se caracterizam por um excesso de angiogénese.

A radioterapia é frequentemente aplicada ao tratamento do cancro. Porém, tem vindo a ser observado que doentes submetidos a esta terapia têm um risco aumentado de desenvolver metástases. Esta situação constitui um desafio para a clínica e os mecanismos celulares e moleculares que estão na origem deste problema têm vindo a ser investigados.

É geralmente assumido que a metastização e recidiva tumoral após a terapia se devem ao aparecimento de células tumorais resistentes à radiação ionizante. No entanto, há evidências de que doses terapêuticas de radiação ionizante promovem alterações ao nível do microambiente tumoral, podendo também contribuir para o processo de radioresistência.

A vasculatura providencia oxigénio e nutrientes ao tumor, sendo essencial para o seu desenvolvimento. Contudo, favorece também a metastização, na medida em que as células tumorais entram em circulação através de vasos sanguíneos. A contribuição da vasculatura irradiada na invasão e metastização após radioterapia é, portanto, de extrema importância. Por este motivo, ao longo dos últimos anos, têm surgido diversos estudos com o objectivo de perceber através de que mecanismos, doses de radiação

ionizante induzem a angiogénese na área tumoral e qual poderá ser a sua contribuição no processo de invasão e metastização.

Estes estudos têm-se focado em doses de radiação ionizante que são administradas diariamente, em pequenas fracções, até que a dose potencialmente curativa seja acumulada no interior da área a tratar, com o objectivo de minimizar o dano provocado nos tecidos saudáveis. Para além disso, a administração em baixas doses e a convergência de diversos feixes que garantem a distribuição homogénea das curvas de isodose em radioterapia externa, contribuem para a existência de uma menor dose de radiação ionizante fora da área a tratar. Os efeitos biológicos e moleculares destas baixas doses de radiação ionizante nos tecidos que rodeiam a área a tratar são ainda desconhecidos.

O nosso trabalho centrou-se, de forma inovadora, na vasculatura que rodeia o tumor e que recebe doses relativamente baixas de radiação ionizante. O principal objectivo, foi investigar o efeito destas baixas doses de radiação ionizante na angiogénese, e compreender a sua contribuição para a recidiva tumoral, invasão e metastização.

Investigámos assim o efeito das baixas doses de radiação ionizante *in vitro*, em células endoteliais humanas de microvasculatura de pulmão (HMVEC-L, *lung human microvascular endothelial cells*) e células endoteliais de veia umbilical (HUVEC, *human umbilical vein endothelial cells*). Constatámos que doses iguais ou inferiores a 0.8 Gy promovem a migração de células endoteliais, sem afectar a sobrevivência e o ciclo celular, activam o receptor-2 do factor de crescimento endotelial vascular (VEGF, *vascular endothelial growth factor*) e, em condições de hipóxia, promovem o aumento da expressão do próprio VEGF.

A utilização do peixe-zebra como modelo de estudo permitiu-nos confirmar *in vivo* a indução da angiogénese em resposta a baixas doses de radiação ionizante. Observámos que doses de 0.5 Gy aceleram o processo angiogénico durante o desenvolvimento embrionário e promovem um aumento do número de vasos durante a regeneração da barbatana caudal dos adultos.

Para estudarmos a contribuição das baixas doses de radiação ionizante no crescimento tumoral e metastização, utilizámos dois modelos experimentais de ratinho: um modelo de leucemia, e um modelo metastático de cancro de mama. Verificámos que baixas doses de radiação ionizante promovem o crescimento tumoral e metastização através de um mecanismo dependente do receptor do VEGF.

O efeito das baixas doses de radiação no desenvolvimento tumoral também foi estudado utilizando um modelo de melanoma em peixe-zebra. Neste modelo, os peixes-zebra mutantes em p53 (*protein 53*) e BRAF (*raf murine sarcoma viral oncogene homolog B1*) são expostos a baixas doses de radiação ionizante antes do melanoma ser detectado. De acordo com os nossos resultados, não publicados, é desenvolvido um maior número de melanomas em peixes zebra irradiados. Verificámos igualmente, que os melanomas nestes peixes irradiados apresentam um tamanho superior em relação aos melanomas desenvolvidos em peixes-zebra não irradiados. Estudos adicionais estão a ser efectuados com o objectivo de caracterizar os melanomas que surgem em ambos os grupos experimentais.

Finalmente, e com o objectivo de identificar os mecanismos através dos quais as baixas doses de radiação ionizante induzem uma resposta pró-angiogénica, investigámos o perfil de expressão génica de HMVEC-L irradiadas *versus* não irradiadas. Os nossos resultados indicam a modulação da expressão génica de mediadores moleculares envolvidos na resposta angiogénica.

No seu conjunto, o nosso trabalho permite compreender o efeito das doses de radiação ionizante que estão presentes nos tecidos que rodeiam a área tumoral e sua importância na angiogénese, e consequentemente na progressão tumoral e metastização, pelo que poderá ser um contributo importante na optimização dos actuais protocolos de radioterapia.

Assim, de acordo com os nossos resultados as baixas doses de radiação ionizante induzem angiogénese *in vivo*; não existe, contudo, prova de que induzam angiogénese terapêutica em doentes com doença isquémica, sendo este um dos objectivos de investigação do nosso laboratório.

A isquémia crítica dos membros inferiores é uma das manifestações clínicas da doença arterial periférica em que se descreve doentes com dor em repouso ou com lesões tróficas cutâneas, sejam elas úlceras ou gangrena. A Isquemia crítica dos membros inferiores envolve uma perturbação grave tanto ao nível da microcirculação como da macrocirculação.

O vasoprost® é frequentemente utilizado no tratamento da doença arterial periférica. O princípio activo do vasoprost® é a prostaglandina E1 (alprostadil), cujas propriedades hemodinâmicas e acção anti-agregante plaquetária justificam a sua indicação no tratamento da doença vascular periférica grave.

No entanto, a literatura não é unânime quanto à sua função como indutor angiogénico. Por este motivo, e através da realização de um conjunto de ensaios *in vitro*, em HUVEC, começámos por clarificar este assunto. Os nossos resultados sugerem que o vasoprost® funciona como um agente pró-angiogénico, induzindo a migração, proliferação e sobrevivência endotelial.

O uso de vasoprost® na clínica apresenta, contudo, limitações terapêuticas. Assim, a amputação surge como última alternativa terapêutica, apesar das taxas de morbilidade e mortalidade associadas. O objectivo de preservar o membro tem estimulado a investigação de tratamentos alternativos, incluindo a angiogénese terapêutica.

Propusemo-nos então a averiguar se baixas doses de radiação ionizante poderiam potenciar os resultados obtidos pelo tratamento com o vasoprost®. Avaliámos a acção combinada destes dois agentes e verificámos *in vitro* que doses de radiação ionizante inferiores a 0.8 Gy potenciam o efeito pró-angiogénico do vasoprost®.

Os nossos resultados sugerem deste modo, que a combinação da radiação ionizante e vasoprost® devem ser considerados em estudos futuros, de forma a avaliar o seu potencial terapêutico na doença arterial periférica.

**Palavras-chave:**

Angiogénese; Células endoteliais, Metástases, Radiação ionizante; Radioterapia; Vasoprost.

## ABSTRACT

Angiogenesis is the formation of new blood vessels from pre-existing ones. This process is regulated by a balance between pro- and anti-angiogenic molecules and is derailed in various diseases, such as cancer.

Radiotherapy is a commonly-used treatment for cancer. However, recent studies suggest that ionizing radiation (IR) doses delivered inside the tumor target volume during fractionated radiotherapy can stimulate invasion and metastasis through effects on cancer cells but also on other elements of the microenvironment. Furthermore, radiotherapy results also in the delivery of doses lower that the therapeutic ones to the tissues surrounding the tumor area, and the biological effects of these low IR doses remain largely undetermined.

Our overall goal was to investigate the effects of these low IR doses on angiogenesis, and consequently in tumor progression and metastasis.

We showed that low-dose IR induces an angiogenic response both *in vitro* and *in vivo*. Doses equal or lower than 0.8 Gy promote endothelial cell migration without causing cell cycle arrest or apoptosis, activate vascular growth factor (VEGF) receptor-2 and up-regulate the expression of VEGF. In zebrafish, low-dose IR accelerates sprouting angiogenesis during development and enhances angiogenesis during regeneration. In mice, we showed that low-dose IR promotes angiogenesis resulting in accelerated tumor growth and metastasis formation in a VEGFR-dependent manner. Additionally, we demonstrated that low-dose IR modulates the gene expression of molecular mediators involved in the angiogenic response.

Our observations provide novel insights into the biological effects of low-dose IR relevant to tumor biology, which may serve as basis for the prevention of possible tumor-promoting effects of current radiotherapy protocols.

Therefore, according to our findings low-dose IR induces angiogenesis *in vivo* but, there is no evidence that it produces therapeutic angiogenesis in ischemic disease patients. In the

second part of this work we showed that low-dose IR potentiates the pro-angiogenic effect of vasoprost®, commonly used in the treatment of peripheral arterial disease treatment (PAD).

Our results suggest that the combinatory use of both vasoprost® and low-dose IR should be considered for future studies concerning its clinical therapeutic potential in pathologies such as PAD.

**Keywords**

Angiogenesis; Endothelial cells; Ionizing radiation; Metastasis; Radiotherapy; Vasoprost.

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**ABBREVIATIONS**

| | |
|---|---|
| 5-FU | 5-fluorouracil |
| Akt | protein kinase B (also known as PKB) |
| ALK | activin receptor-like kinase |
| ANGPT | angiopoietin |
| ANOVA | analysis of variance |
| BM | basal membrane |
| BRAF | raf murine sarcoma viral oncogene homolog B1 |
| CAM | chicken chorioallantoic membrane |
| cDNA | complementary DNA |
| CHK | Csk-homologous kinase |
| CLI | critical limb ischemia |
| CLTC | clathrin |
| COUP-TFII | chicken ovalbumin upstream promoter transcription factor II |
| CSFs | colony-stimulating factors |
| CT | computed tomography |
| CTV | clinical target volume |
| CYR61 | cysteine-rich protein 61 (also known as CNN1) |
| DAPI | 4', 6-Diamidino-2-phenylindole |
| Dll4 | delta-like-4 |
| DNA | deoxyribonucleic acid |
| dpf | days post-fertilization |
| dpmd | days post-melanoma detection |
| DSB | double strand breaks |
| e.g. | *exempli gratia* (for example) |
| ECM | endothelial cell matrix |
| ECs | endothelial cells |
| EDG-1 | endothelial differentiation G-protein coupled receptor-1 |
| EGF | epidermal growth factor |
| EGFP | enhanced green fluorescent protein |

| | |
|---|---|
| ELISA | enzyme linked immunosorbent assay |
| eNOS | endothelial nitric oxide synthase |
| EPCs | endothelial precursor cells |
| EPO | Erythropoietin |
| ERK | extracellular signal-related kinase (also known as MAPK) |
| FACS | fluorescence activated cell sorting |
| FAK | focal adhesion kinases |
| FBS | fetal bovine serum |
| FCT | fundação para a ciência e tecnologia |
| FGF | fibroblast growth factor |
| FGFR | fibroblast growth factor receptor |
| FITC | fluorescein isothiocyanate |
| foxc | forkhead C |
| GFP | green fluorescent protein |
| GTV | gross tumour volume |
| HEY2 | hairy and enhancer of split-related protein 2 |
| HGF | hepatocyte growth factor |
| HIF | hypoxia inducible factor |
| HMVEC-L | microvascular endothelial cells |
| HR | homologous recombination |
| HSPG | heparin sulfate proteoglycan |
| HuR | hypoxic-induced stability factor |
| HUVEC | human umbilical vein endothelial cells |
| i.e. | *id est* (that is) |
| IGF | insulin-like growth factor |
| IL | interleukin |
| IMM | instituto de medicina molecular |
| IPA | ingenuity pathway analysis |
| IR | ionizing radiation |
| IVIS | *in vivo* imaging system |
| MAPK | mitogen-activated protein kinase (also known as ERK) |
| MEK | mitogen-activated protein kinase kinase (also referred to as MAPKK) |

| | |
|---|---|
| MMP | matrix metalloproteinase |
| mRNA | messenger RNA |
| NFkB | nuclear factor-kB |
| NHEJ | non-homologous end-joining |
| NO | nitric oxide |
| NP | neurpilin |
| PAD | peripheral arterial disease |
| PAK | p21-activated kinase |
| PCA | principal component analysis |
| PDGF | platelet-derived growth factor |
| PDGFR | platelet-derived growth factor receptor |
| PECAM | platelet endothelial cell adhesion molecule |
| PEDF | pigment epithelium-derived factor |
| PF | platelet factor |
| $PGE_1$ | Prostaglandin $E_1$ |
| PGs | prostaglandins |
| PI | propidium iodide |
| PI3K | phosphatidylinositol-3-kinase |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PlGF | placenta growth factor |
| PTEN | phosphatase and tensin homolog |
| PTK/ZK | PTK787/ZK222584 |
| PTV | planning target volume |
| qPCR | quantitative real time polymerase chain reaction |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| RT-PCR | real time polymerase chain reaction |
| S1P1 | Sphingosine-1-phosphate-1 |
| SIV | sub-intestinal vessels |
| SMCs | smooth muscle cells |
| SSB | single strand breaks |

| | |
|---|---|
| TGFβ | transforming growth factor-β |
| TIE | tyrosine kinase with immunoglobulin and EGF homology domains |
| TIMP | tissue inhibitor of metalloproteinase |
| TKI | tyrosine kinase inhibitor |
| TKR | tyrosine kinase receptors |
| TNFβ | tumor necrosis factor-α |
| TSP | thrombospondin |
| TUBB | β-tubullin |
| TV | treatment volume |
| TβR | transforming growth factor-β receptor |
| VCAM | vascular cell adhesion molecule |
| VE-Cadherin | vascular endothelial cadherin |
| VEGF | vascular endothelial growth factor |
| VEGFR1 | vascular endothelial growth factor receptor-1 (also known as Flt1) |
| VEGFR2 | vascular endothelial growth factor receptor-2 (also known as KDR or Flk1) |
| VHL | von Hippel Lindau |
| WB | western blot |

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**III. RESEARCH WORK:**

**LOW DOSES OF IR PROMOTE TUMOR GROWTH AND METASTASIS BY ENHANCING ANGIOGENESIS**

# I. GENERAL INTRODUCTION

This chapter contains a general introduction to the subjects approached during the experimental work presented in this thesis

## 1. CIRCULATORY SYSTEM AND STRUCTURAL PROPERTIES OF BLOOD VESSELS: AN OVERVIEW

All vertebrates require an efficient circulatory system, able to distribute oxygen and nutrients to tissues and, simultaneously remove carbon dioxide and other metabolic waste products. This task is carried out by two main networks: the blood vessels and the lymphatic vessels, both formed by endothelial cells (ECs) (Adams and Alitalo, 2007). Additionally to the gases, liquids and nutrients transport, the vascular system is also important in the regulation of body temperature and systemic pH (Carmeliet, 2005).

In the cardiovascular system, oxygenated blood is pumped from the heart through the arteries and capillaries to the tissues where exchanges occur. The blood is then returned to the heart *via* the venous system (Eichmann et al., 2005).

As a result of the high arterial pressure, blood plasma leaks from the capillaries into the extracellular space, becoming interstitial fluid. The majority of the extravasated fluid is reabsorbed by post-capillary venules driven by osmotic forces, but the remaining is drained by the lymphatic system, returning it into the venous circulation. Unlike the blood vascular system, the lymphatic system does not feature a central pump. Instead, interstitial fluid (lymph) is moved forward by skeletal muscle action and respiratory movement (Cueni and Detmar, 2008). The lymphatic system is also essential for the immune defense (Eichmann et al., 2005).

Both networks are essential for homeostasis of a healthy organism, and their malformation or dysfunction contributes to many diseases (Eichmann et al., 2005).

Blood vessels are divided into three main groups: arteries, veins, and capillaries. Arteries and veins are further divided, according to caliber, into large, medium, and small blood vessels. The vascular system is subjected to varying degrees of hydrostatic pressure, and the structure of vessels varies in an adaptive fashion. Blood vessels are thickest and their walls more complex in the immediate vicinity of the heart, where hydrostatic pressure is greatest (Nussenbaum and Herman, 2010). As blood vessels decrease in caliber, their wall becomes thinner and less complex.

Heterogeneity in vessel wall composition is evident between vessels of different sizes and between arterial and venous vessels (Figure 1). Large vessel vascular walls are composed of the *tunica intima*, which consists of the endothelium, basal membrane and an internal elastic layer; the *tunica media*, a thick layer of smooth muscle with reticular fibers, elastin and proteogycans; and the *tunica adventitia*, which consists of connective tissue with both elastic and collagenous fibers (Cleaver and Melton, 2003). Since veins conduct blood back to the heart, the pressure exerted by the heartbeat on them is much less than in the arteries. The middle muscular wall of a vein is therefore much thinner than that of an artery (Bergers and Song, 2005). Veins differ from arteries also in that they have semi- lunar valves, which prevent the blood from flowing backwards (Cleaver and Melton, 2003).

Small blood vessels are composed of ECs surrounded by a basal lamina covered by pericytes. Pericytes exhibit long cytoplasmic processes that not only can contact numerous endothelial cells and thus integrate signals along the length of the vessel, but can also extend to more than one capillary in the vasculature (Bergers and Song, 2005).

Pericytes are functionally significant; when vessels lose pericytes, they become hemorrhagic and hyperdilated, leading to conditions such as edema, diabetic retinopathy, and even embryonic lethality (Hellstrom et al., 2001). Recently, pericytes have gained new attention as functional and critical contributors to tumor angiogenesis and therefore as potential new targets for anti-angiogenic therapies.

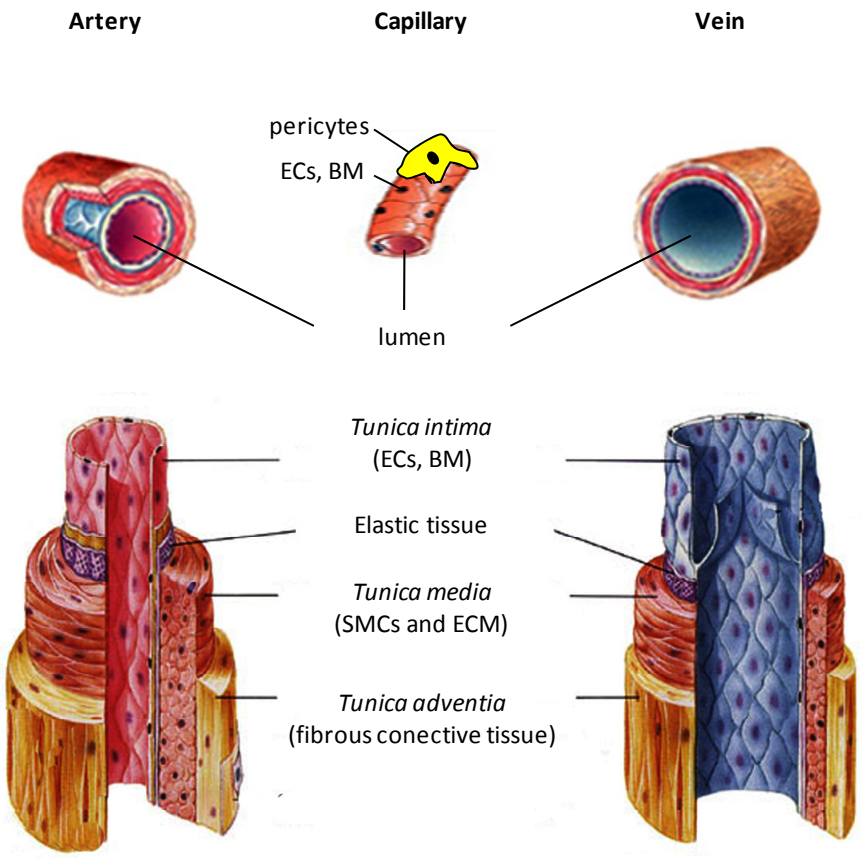


**Figure 1| Blood vessels.** Blood vessels are divided into three main groups: arteries, veins, and capillaries. They all present particular cellular differences, which are highlighted above.

## 2. VASCULOGENESIS AND ANGIOGENESIS DURING EMBRYONIC DEVELOPMENT

Two mechanisms account for the formation of blood vessels, vasculogenesis and angiogenesis. Unfortunately, the terms vasculogenesis and angiogenesis literally have the same general meaning, i.e., the genesis of blood vessels. Despite the nomenclature, the two processes are clearly distinct. Vasculogenesis is the process of *de novo* blood vessel formation driven by the recruitment and differentiated of mesodermal cells into the endothelial lineage and the *de novo* assembly of such cells into blood vessels. Angiogenesis is the generation of new blood vessels from pre-existing ones, a process driven by EC proliferation.

### 2.1. EARLY BLOOD VASCULAR DEVELOPMENT

Given the importance of the vascular system, in any organ and tissue, its establishment has to occur early in embryogenesis (Eichmann et al., 2005).

During development, the vasculature is established both by vasculogenesis and angiogenesis.

The first evidence of blood vessel development first appears on the extra-embryonic yolk sac, and intraembryonic tissue, where groups of splanchnic mesoderm cells, specified to become hemangioblasts, aggregate and condense, forming what is known as blood islands. Here, hemangioblasts, the precursors of blood cells and ECs, start to differentiate. Cells at the perimeter of the blood islands become angioblasts, the precursors of the ECs. Those at the center constitute the hematopoietic precursors of all the blood cells (Conway et al., 2001).

As the yolk sac begins to form, angioblasts migrate to distant sites, multiply and differentiate forming a primitive network of simple endothelial tubes, called primary vascular plexus. This process where blood vessels are created *de novo* from endothelial precursor cells (EPCs), in response to local signals such as growth factors, is known as vasculogenesis. During this process, ECs undergo specification, proliferation, migration,

differentiation and finally fuse to form the inside layer of nascent vessels (Conway et al., 2001; Jain, 2003; Risau, 1997).

The subsequence growth, expansion and remodeling of the primitive vessels into a mature vascular network, that develops into arteries, veins, and capillaries, is referred to as angiogenesis (Conway et al., 2001; Jain, 2003; Lin et al., 2007).

Vascular endothelial growth factor (VEGF), and its receptor-2 (VEGFR2) are the most critical drivers of embryonic vessel formation, since knock-out mice for either of these molecules result in embryonic lethality between 8.5 and 9.5 days post-coitum. Embryos deficient for the receptor tyrosine kinase VEGFR2 or VEGF fail to develop blood islands, and therefore ECs (Carmeliet et al., 1996; Shalaby et al., 1995). In contrast, in mice missing vascular endothelial growth factor receptor-1 (VEGFR1), vessels do form but exhibit excessive levels of ECs, which obstruct the lumen of abnormal vascular channels (Fong et al., 1995). Thus, although both VEGFR1 and VEGFR2 are expressed on hematopoietic stem cells and ECs, only VEGFR2 is absolutely critical for the earliest stages of vasculogenesis. VEGFR1 becomes involved later on, acting as a negative regulator of VEGF activity during early angiogenesis (Fong et al., 1995; Risau, 1997).

## 2.2. MATURATION OF BLOOD VESSELS

After the establishment of a primitive vascular plexus by vasculogenesis, sprouting angiogenesis starts, and new vessels form from the sides and ends of pre-existing ones. At this point, sprouting angiogenesis is facilitated by hypoxia, which up-regulates a number of genes involved in vessel formation, patterning and maturation, such as endothelial nitric oxide synthase (eNOS), VEGF and angiopoetin-2 (ANGPT2). Existing vessels dilate in response to nitric oxide (NO) (a product of eNOS), and become leaky in response to VEGF, which indirectly controls the redistribution of intracellular adhesion molecules (e.g. platelet endothelial cell adhesion molecule-1 (PECAM1) and vascular endothelial cadherin (VE-Cadherin). As the endothelial cell matrix (ECM) dissolves in response to activation of matrix metalloproteinases (MMPs) (e.g. MMP2, MMP3, MMP9)

and suppression of tissue inhibitor of metalloproteinases (TIMPs) (e.g. TIMP2), plasma proteins leaked from these nascent vessels serve as a provisional matrix. Since the physical barriers are dissolved, ECs are free to migrate establishing interactions between their integrins and matrix proteins, simultaneously proliferating in response to VEGF and other endothelial mitogens, as platelet-derived growth factors *(*PDGFs), fibroblast growth factors (FGFs), angiopoetin-1 (ANGPT1) and ANGPT2 (in the presence of VEGF) (Conway et al., 2001; Jain, 2003). The selection of ECs for sprouting is a highly regulated process, where Notch signaling also plays an important role (Adams and Alitalo, 2007).

The maturation of new vessels, involves the recruitment of mural cells and the expansion of the surrounding matrix. The regulation of this process involves 4 main molecular pathways: (1) PDGFB/PDGFRβ (PDGF receptor-β), (2) ANGPT1/TIE2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains), (3) S1P1 (sphingosine-1-phosphate-1)/EDG-1 (endothelial differentiation G-protein coupled receptor-1), and (4) TGFβ (transforming growth factor-β) signaling. PDGFB has an important role in the recruitment of mural cells. In response to VEGF, PDGFβ is secreted by ECs recruiting PDGFRβ positive mural cells around vessel sprouts, which will stabilize the new vessels by inhibiting EC proliferation and migration (Bergers and Song, 2005; Hellstrom et al., 2001). ANGPT1 is secreted by pericytes and interacts with TIE2, which is specifically expressed in ECs, mediating an appropriate interaction between ECs and pericytes (Bergers and Song, 2005; Loughna and Sato, 2001). S1P1/EDG1 and TGFβ have been shown to be involved in the recruitment and differentiation of pericytes (Jain, 2003; Pepper, 1997; Risau, 1997).

The last step in the maturation process is the organ-specific specialization, where ECs acquire highly specialized characteristics to provide the functional needs within specific tissues. This process includes arterial-venous determination, formation of homotypic and heterotypic junctions and EC differentiation to form organ-specific capillary structures (Conway et al., 2001).

Figure 2 represents a schematic summary of the major steps involved in the vasculogenesis and angiogenesis during embryonic development.

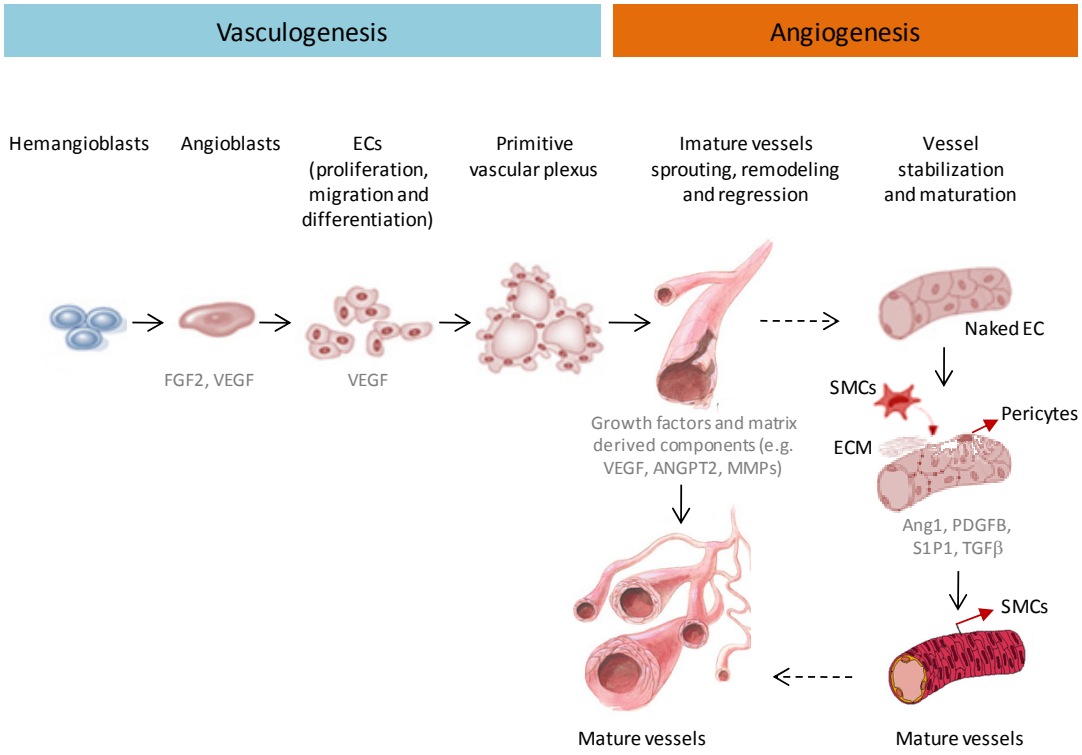


**Figure 2| Vasculogenesis and angiogenesis during embryonic development**. During vasculogenesis, hemangioblasts aggregate and condense, differentiating into angioblasts, which migrate, proliferate and differentiate into ECs, forming a primitive network of simple endothelium tubes (primary vascular plexus). Due to an orchestrated chain of growth factors and matrix remodeling molecules, ECs proliferate and migrate, giving rise to new sprouting vessels (angiogenesis). Maturation of blood vessels, involves the recruitment of mural cells (pericytes and SMCs), the development of the surrounding matrix, and specialization of ECs.

### 2.2.1. ARTERIAL AND VENOUS SYSTEMS

After endothelial progenitors differentiate into ECs to further form the primate vascular plexus, remodeling into a more complex network requires the discrimination of artery and vein boundaries.

Although the process of artery and vein specification is not well understood, it is known that most of the molecules that seem to be involved, are also expressed in the nervous

system, regulating cell fate decisions and axonal migration guidance (Eichmann et al., 2005).

It is known that the Notch pathway, with its ligands (Delta-like-4, Jagged-1, and Jagged-2) and receptors (Notch-1, Notch-3 and Notch-4), promotes arterial fate of ECs by repressing venous differentiation (Figure 3). Upstream of Notch, we find VEGF and Foxc1 and Foxc2 (forkhead C1 and C2), which activate Notch pathway, while HEY2 (hairy and enhancer of split-related protein 2) and EphrinB2 act downstream to determine arterial fate (Adams and Alitalo, 2007; Carmeliet, 2003; Lin et al., 2007).

Moreover, COUP-TFII (chicken ovalbumin upstream promoter transcription factor II), specifically expressed in venous endothelium, and phosphatidylinositol-3-kinase (PI3K) / protein kinase B (Akt) signaling have also been implicated in the specification of vein identity. The first by suppressing the Notch pathway, and the second, by blocking ERK (extracellular signal-related kinase) signaling, which is preferentially detected in angioblasts that are fated to become arteries (Lin et al., 2007).

EphrinB2 ligand and its receptor, EphB4, also contribute for the formation of arterial-venous anastomoses, since EphrinB2 marks only arteries and EphB4 is vein specific (Adams and Alitalo, 2007; Carmeliet, 2003).

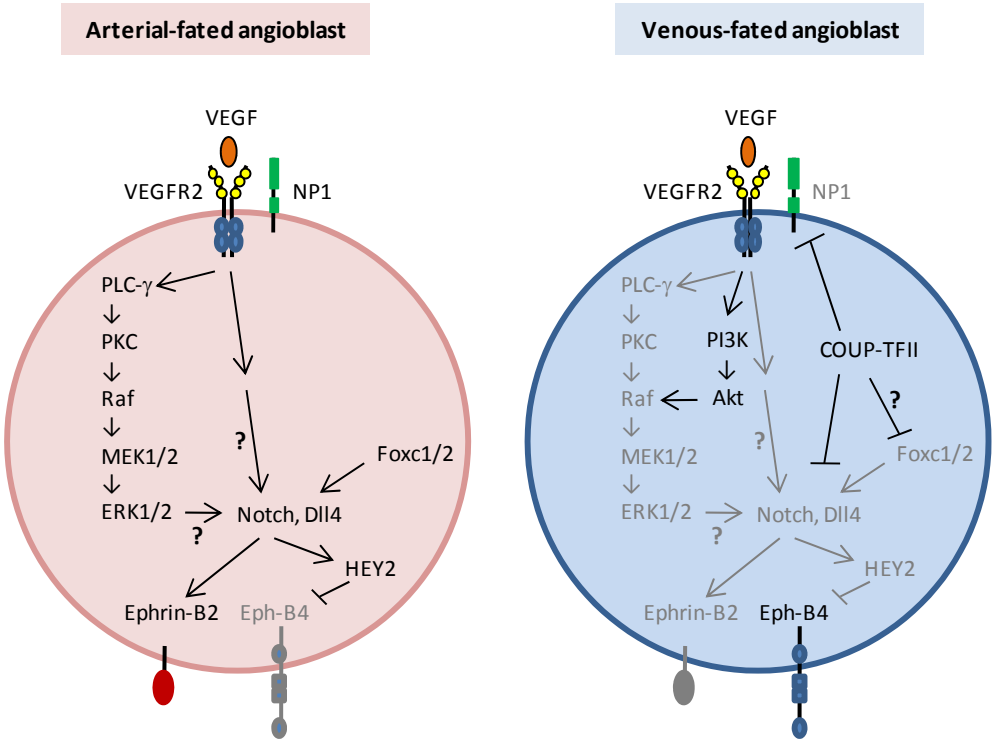


**Figure 3| Arterial-venous differentiation.** VEGF binds to VEGFR2/neurpilin-1 (NP1) complex, activating both ERK and Notch pathways, which results in the expression of Ephrin-B2 and thus, arterial fate. Foxc1 and Foxc2 also activate the Notch pathway through the expression of D*elta-like-4* (*Dll4*). However, COUP-TFII and PI3K/Akt signaling are able to inhibit arterial fate through the inhibition of both Notch and ERK signaling pathways, respectively, resulting in the expression of the venous marker EphB4. Question markers indicate unconfirmed interactions. Adapted (Lin et al., 2007).

### 2.2.2. HOMOTYPIC AND HETEROTYPIC JUNCTIONS

Tight-junctions (formed by occludins, claudins and zona occludens) and adherens junctions (formed by cadherins, with special attention to VE-cadherin), promote EC-EC communication (Dejana et al., 1995) and provide mechanical strength and tightness, thereby establishing a permeability barrier to plasma solutes and leukocytes (Carmeliet, 2003). In addition, gap junctions (clusters of connexins) provide both EC-EC and EC-perivascular cells communication, allowing the direct exchange of ions and small molecules between neighboring cells (Dejana et al., 1995; Jain, 2003). Other adhesion

molecules such as PECAM1 and integrins play also an important role by promoting both homotypic and heterotypic adhesion (Dejana, 2004). When ECs migrate during vessel sprouting, these contacts are transiently dissolved but later re-established.

### 2.2.3. LOCAL SPECIALIZATION OF ENDOTHELIAL CELLS

Capillaries in different tissues exhibit different cellular morphology (Figure 4), associated with distinct levels of permeability: (a) continuous capillaries that occur for instance in muscle and nervous tissue where ECs form a continuous internal lining without openings in their walls; (b) fenestrated capillaries that occur in endocrine glands and gastrointestinal mucosa where although the continuous BM, ECs are pierced by pores (fenestrations) allowing the rapid passage of macromolecules; (c) discontinuous capillaries found in spleen and liver with large openings and a discontinuous or absent BM (Cleaver and Melton, 2003).

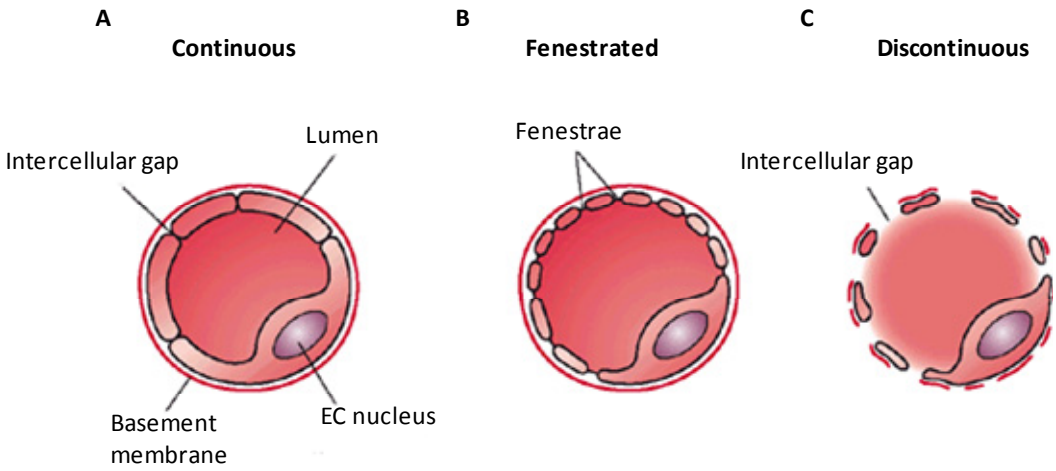


**Figure 4| Capillary wall morphology.** Capillaries in different tissues exhibit different cellular morphology. (A) Continuous capillaries have no openings in their walls and are lined continuously with the endothelial cell body. (B) Fenestrated capillaries have small openings, called fenestrae, of about 80−100 nm in diameter. Fenestrae are covered by a small, non-membranous, permeable diaphragm, and allow the rapid passage of macromolecules. The BM of ECs is continuous over the

fenestrae. (C) Discontinuous capillaries have a large lumen, many fenestrations with no diaphragm and a discontinuous or absent BM. Adapted (Cleaver and Melton, 2003).

### 2.2.4. VESSEL REGRESSION

Vessel regression is a physiological mechanism, and occurs when the nascent vasculature consists of too many vessels. Removal of angiogenic stimuli is, in many cases, enough to promote vessel regression, especially if they are still immature (Carmeliet, 2003). Insufficient perfusion of blood, absence of pericytes or presence of anti-angiogenic factors are other stimulus that can contribute to this process (Risau, 1997).

## 3. POSTNATAL NEOVASCULARIZATION

Until 1997, it was generally accepted that vasculogenesis could only occur during embryogenesis. More recently, the existence of a postnatal vasculogenesis has been supported by the evidence that EPCs circulate postnatally in the peripheral blood (Reed et al., 2007), and that may be recruited from the bone marrow and incorporated into sites of active neovascularization (Asahara et al., 1999; Shi et al., 1998). The recruitment and integration of EPCs involves chemoattraction, active arrest, migration to the interstitial space, incorporation into the vasculature, and differentiation into mature ECs (Hillen and Griffioen, 2007). Although this multistep process includes the active role of many molecules, once more, VEGF seems to be the most important factor in the control of this process in adulthood (Grunewald et al., 2006; Ribatti et al., 2001).

Although vasculogenesis may occur during adulthood, often associated with pathological conditions, new vessels in the adult arise mainly through angiogenesis (Conway et al., 2001; Jain, 2003; Lin et al., 2007).

### 3.1. ANGIOGENIC REGULATORS

As it was previously referred, angiogenesis requires a precise coordination of multiple steps (Figure 5), which are regulated by a delicate balance between pro- and anti-angiogenic factors. A selective list of some of the most important angiogenic stimulators and inhibitors is shown in Table 1 and Table 2, respectively.

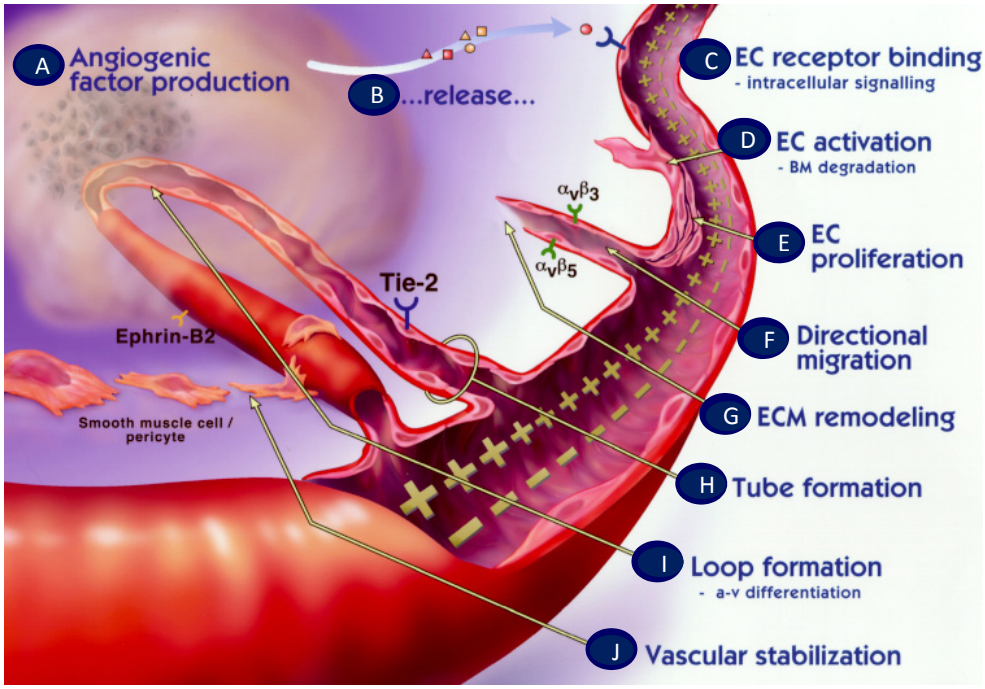


**Figure 5| The angiogenic sprouting occurs as a coordinated multistep process.** (A) diseased or injured tissues produce and release angiogenic growth factors (e.g. VEGF) that diffuse into the nearby tissues; (B) the angiogenic growth factors bind to specific receptors (e.g. VEGFR2) located on the ECs of nearby preexisting blood vessels; (C) once growth factors bind to their EC receptors, a cascade of intracellular signaling is activated; (D) pericytes detach and blood vessels dilate before the basement membrane and extracellular matrix is degraded by ECs produced enzymes (MMPs); (E) ECs proliferate and (F) migrate towards the growth factor producer tissue through the action of integrins (e.g. αvβ3, αvβ5) that serve as grappling hooks to help pull the sprouting new blood vessel sprout forward; (G) additional enzymes (MMPs) are produced to dissolve and remodel the ECM around the vessel; (H) sprouting ECs roll up to form tubes which (I) differentiate in arterial-venous systems and connect to form blood vessel loops that can circulate blood; (J) Newly formed blood vessel tubes are stabilized by SMCs and pericytes that provide structural support. Adapted (Angiogenesis Foundation website (http://www.angio.org/understanding/process.php).

**Table 1| Major stimulators of angiogenesis and their role in the formation of blood vessels**

| CLASS | FACTOR | BIOLOGICAL FUNCTIONS | REFERENCE |
|---|---|---|---|
| **Growth factors, Cytokines and Chemokines** | Angiotropin | ↑EC migration<br>Angiogenesis in vivo | (Hockel et al., 1988) |
| | Angiopoietin-1 (ANGPT1) | ↓EC apoptosis<br>EC sprouting<br>Vessel stabilization | (Loughna and Sato, 2001; Nussenbaum and Herman, 2010; Tait and Jones, 2004; Yancopoulos et al., 2000) |
| | Angiopoietin-2 (ANGPT2)* | ↑EC proliferation<br>↑EC migration<br>EC sprouting only in the presence of VEGF | (Loughna and Sato, 2001; Nussenbaum and Herman, 2010; Tait and Jones, 2004; Yancopoulos et al., 2000) |
| | Epidermal growth factor (EGF) | ↑EC proliferation<br>↑VEGF<br>Angiogenesis in vivo | (van Cruijsen et al., 2006) |
| | Erythropoietin (EPO) | ↑EC proliferation<br>Angiogenesis in vivo | (Yasuda et al., 2002) |
| | Fibroblast growth factors (FGFs) family | ↑Plasminogen activators<br>↑EC proliferation<br>↑EC migration<br>↑$\alpha v\beta 3$ integrin<br>↓EC apoptosis<br>Angiogenesis in vivo | (Beenken and Mohammadi, 2009; Cross and Claesson-Welsh, 2001; Distler et al., 2003; Presta et al., 2005; Turner and Grose, 2010) |
| | Hepatocyte growth factor (HGF) | ↑EC proliferation<br>↑EC migration<br>Angiogenesis in vivo | (Taniyama et al., 2001) |
| | Insulin-like growth factor-1 (IGF1) | ↑EC proliferation<br>↓EC apoptosis<br>↑VEGF<br>↑Plasminogen activators | (Delafontaine et al., 2004) |
| | Interleukin-8 (IL8) | ↑EC proliferation<br>↑EC migration<br>↓EC apoptosis | (Li et al., 2005) |
| | Platelet-derived growth factor (PDGF) | ↑SMCs and pericyte proliferation<br>↑VEGF<br>Vessel stabilization | (Distler et al., 2003; Hellberg et al., 2010; Nussenbaum and Herman, 2010) |
| | Transforming growth factor- | ↑EC proliferation<br>↑EC migration | (Bertolino et al., 2005; Distler et al., 2003; |

| | | | |
|---|---|---|---|
| | β (TGFβ)* | ↓EC apoptosis<br>↑PDGF and eNOS<br>Tube formation<br>Vessel stabilization<br>Angiogenesis in vivo | Kaminska et al., 2005; Laverty et al., 2009; Pepper, 1997) |
| | Vascular endothelial growth factor (VEGF) family | ↑Permeability;<br>↑Plasminogen activators<br>↑EC proliferation<br>↑EC migration<br>↓EC apoptosis<br>Angiogenesis in vivo | (Ferrara, 2001; Ferrara, 2002; Kuwano et al., 2001; Nussenbaum and Herman, 2010; Takahashi and Shibuya, 2005; Yancopoulos et al., 2000) |
| **Matrix proteins and adhesion molecules** | Cysteine-rich protein 61 (CYR61) | ↑EC proliferation<br>↑EC migration<br>↓EC apoptosis<br>Tube formation | (Brigstock, 2002; Chen and Lau, 2009; Chen and Du, 2007; Leask and Abraham, 2006) |
| | Integrins | EC attachment<br>↑EC migration<br>↓EC apoptosis<br>FGF induced angiogenesis | (Avraamides et al., 2008; Nussenbaum and Herman, 2010) |
| | Platelet endothelial cell adhesion molecule-1 (PECAM1) | EC aggregation<br>↑EC migration<br>Tube formation<br>Vessel stabilization<br>FGF induced angiogenesis | (Distler et al., 2003) |
| | Vascular endothelial-cadherin (VE-cadherin) | ↓EC apoptosis<br>Vessel stabilization<br>Angiogenesis in vivo | (Dejana et al., 1999) |
| **Proteases** | Matrix metalloproteinases (MMPs)* | ECM degradation | (van Hinsbergh and Koolwijk, 2008) |
| **Others** | Angiogenin | ↑EC proliferation | (Wiedlocha, 1999) |
| | Ephrin | ↑EC proliferation<br>↑EC migration<br>Vessel stabilization | (Mosch et al., 2010) |
| | *Nitric oxide* (NO) | ↑Permeability<br>↑EC proliferation<br>↑EC migration<br>↑FGF and VEGF | (Ziche and Morbidelli, 2000) |

* Can show opposite effects depending upon doses and environmental conditions

**Table 2 - Major endogenous inhibitors of angiogenesis and their role in the formation of blood vessels**

| CLASS | FACTOR | BIOLOGICAL FUNCTIONS | REF |
|---|---|---|---|
| **Growth factors, Cytokines and Chemokines** | Angiopoietin-2 (ANGPT2)* | ↑EC apoptosis<br>Vessel destabilization | (Loughna and Sato, 2001; Nussenbaum and Herman, 2010; Tait and Jones, 2004; Yancopoulos et al., 2000) |
| | Interleukin-12 (IL12) | ↓FGF mediated angiogenesis | (Kerbel and Hawley, 1995) |
| | Interferon–α, –β, –γ | ↓MMPs<br>↓FGF<br>↓IL8 mediated angiogenesis<br>↓Plasminogen activators | (Nussenbaum and Herman, 2010; Nyberg et al., 2005) |
| | Platelet factor-4 (PF4) | ↓FGF mediated angiogenesis | (Nussenbaum and Herman, 2010; Nyberg et al., 2005) |
| | *Transforming growth factor-β (*TGFβ)* | ↓EC proliferation<br>↓EC migration<br>↑EC apoptosis<br>↑TIMPs<br>↓Plasminogen activators | (Bertolino et al., 2005; Distler et al., 2003; Kaminska et al., 2005; Laverty et al., 2009; Pepper, 1997) |
| **Matrix proteins and adhesion molecules** | Arresten | ↓EC proliferation<br>↓EC migration<br>↓Tube formation | (Nyberg et al., 2005) |
| | Endostatin | ↓EC proliferation<br>↓MMPs | (Nussenbaum and Herman, 2010; Nyberg et al., 2005) |
| | Thrombospondin-1 and -2 (TSP1, TSP2) | ↓EC migration<br>↑EC apoptosis | ( Distler et al., 2003) |
| **Proteases** | Matrix metalloproteinases (MMPs)* | Generate angiostatin | (van Hinsbergh and Koolwijk, 2008) |
| | tissue inhibitors of metalloproteinases (TIMPs) | ↓MMPs | (Van Hinsbergh and Koolwijk, 2008; Nussenbaum and Herman, 2010) |
| **Others** | Angiostatin | ↓EC proliferation<br>↓EC migration<br>↑EC apoptosis<br>↓Tube formation | (Distler et al., 2003; Nussenbaum and Herman, 2010; Nyberg et al., 2005) |

* Can show opposite effects depending upon doses and environmental conditions

### 3.1.1. ANGIOPOIETINS

The human angiopoietin family consists in four members, ANGPT1, ANGPT2, Ang3 and Ang4, which bind to specific tyrosine kinase receptors (TKRs), TIE1 and TIE2 (Distler et al., 2003). Both ANGPT1 and ANGPT2, expressed by a broad variety of cell types (e.g. ECs, SMCs, fibroblasts, pericytes, some tumor cell lines), play a role in angiogenesis by binding to TIE2, which is manly expressed in ECs (Nussenbaum and Herman, 2010).

ANGPT1 is typically an angiogenic factor, inducing EC survival, capillary sprouting and pericytes recruitment (Loughna and Sato, 2001). By increasing the interaction between ECs and pericytes, ANGPT1 is known to stabilize blood vessels. Moreover, overexpression of ANGPT1 produces enlarged and leakage-resistant vessels in adult mice (Yancopoulos et al., 2000). *In vivo* studies also suggest that ANGPT1 is essential for maturation and stabilization of the developing vasculature and for normal remodeling, since mice lacking *ANGPT1* start to develop a primary vasculature which fails to stabilize or remodel leading to embryonic lethality (Suri et al., 1996). Furthermore, the importance of ANGPT1 on angiogenesis is also emphasized by the observation that its over-expression in transgenic mice promotes excessive hypervascularisation (Suri et al., 1998).

On the other hand, ANGPT2 can either promote or inhibit vessel growth, depending on the presence of other growth factors, such as VEGF (Loughna and Sato, 2001). ANGPT2 was first described to block ANGPT1-mediated TIE2 receptor activation, acting as an anti-angiogenic factor capable of promoting *in vivo* EC apoptosis and regression of blood vessels (Maisonpierre et al., 1997). Intriguingly, subsequent studies have shown that higher expression levels of ANGPT2 are associated to sites of vascular remodeling in adults, in particular in the female reproductive tract and in highly vascularized tumors (Tait and Jones, 2004; Thurston, 2003). In fact, it has been proposed that, by antagonizing the stabilizing influence of ANGPT1, ANGPT2 might provide a key destabilizing signal reverting vessels to a more plastic state (Yancopoulos et al., 2000). Such destabilized vessels could then be prone to two fates. On the one hand, these destabilized vessels would be prone to regression in the absence of growth factors. On the other hand, they would be prone to angiogenic sprouting induced by available angiogenic factors such as

VEGF. Further investigations demonstrated that, in the presence of VEGF, ANGPT2 is responsible for an increase in capillary diameter, migration and proliferation of ECs, and sprouting of new blood vessels (Lobov et al., 2002).

Additionally, high levels of ANGPT2 can induce TIE2 phosphorylation in human umbilical vein endothelial cells (HUVEC), stimulating cell proliferation, cell differentiation and protection to induced cell death (Kim et al., 2000; Teichert-Kuliszewska et al., 2001). ANGPT2-induced TIE2 phosphorylation has also been demonstrated in murine brain capillary ECs, promoting migration (Mochizuki et al., 2002).

Figure 6 highlights the major signaling transduction pathways involved in the TIE2-induced proliferation, migration and survival of ECs.

### 3.1.2. FGFs AND FGFRs

FGFs have a pleiotropic expression and stimulate proliferation in nearly all cells derived from embryonic mesoderm or neuroectoderm (Murakami et al., 2008a; Nussenbaum and Herman, 2010).

The angiogenic activity of recombinant FGF1 and FGF2 proteins has been demonstrated in various *in vivo* models, including the avascular rabbit or mouse cornea (Herbert et al., 1988), mice subcutaneous matrigel injection (Akhtar et al., 2002), and the chicken chorioallantoic membrane (CAM) assay (Ribatti et al., 2000). *In vitro* studies have also shown that FGF1 and FGF2 stimulation leads to an increased response in proliferation, migration, survival, and differentiation of ECs (Presta et al., 2005; Turner and Grose, 2010). Murakami et al. (Murakami et al., 2008b) have also shown that FGF signaling disruption in bovine aortic ECs led to a loss of function in the adherens and tight junctions, causing the loss of EC barrier function and disintegration of the vasculature. In addition, FGF2 has also been reported to up-regulate the expression of several pro-angiogenic molecules, such as MMPs, $\alpha v\beta 3$ integrin, VEGF, HGF (Distler et al., 2003; Presta et al., 2005).

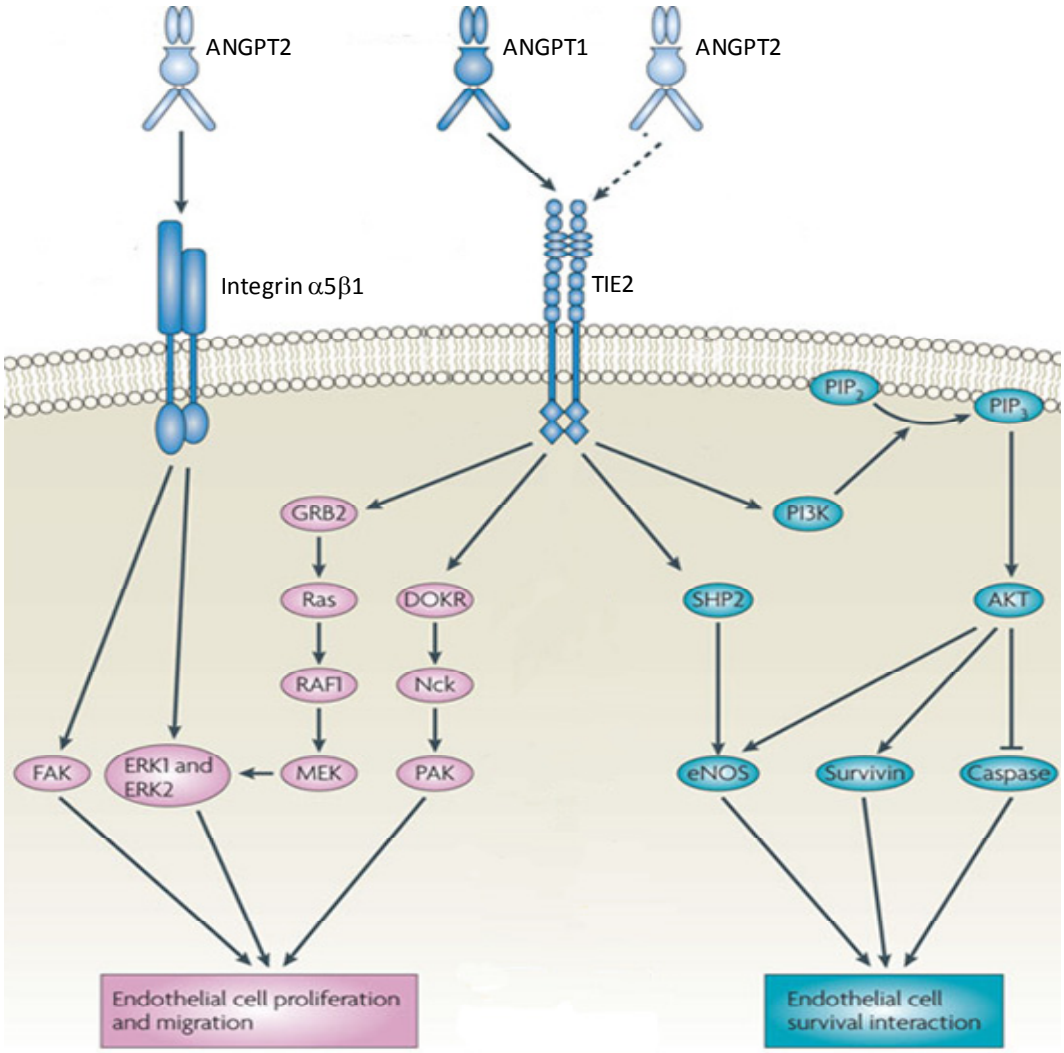


**Figure 6| Angiopoietin signaling in angiogenesis.** ANGPT1 and ANGPT2 both bind to TIE2 in ECs. Upon ligand binding, TIE2 dimerizes and is autophosphorylated, promoting the activation of multiple downstream signaling molecules. EC survival is stimulated through PI3K/Akt and eNOS pathway, as proliferation and migration depend upon the activation of PAK (p21-Activated Kinase) and MAPK (mitogen-activated protein kinase) /ERK signaling pathways. Moreover, ANGPT2 is also able to stimulate an integrin-mediated response, enhancing its canonical signaling pathway effects. Adapted (Huang et al., 2010).

FGFs mainly exert their biological activities by binding to specific TKRs (FGFRs) on the surface of target cells, however, recent evidences show that they can also interact with non-TKRs, such as αvβ3 integrin and syndecan-4 (Figure 7) (Murakami et al., 2008a).

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 (Javerzat et al., 2002; Turner and Grose, 2010).

Stimulation of FGFR1 in ECs leads to proliferation, migration, protease production and tubular morphogenesis, whereas activation of FGFR2 increases only cell motility (Milkiewicz et al., 2006). Although most of these effects are transduced through mitogen-activated protein kinase (MAPK) activation (Murakami et al., 2008a), protein kinase C (PKC) and PI3K activation are also required for FGF-induced EC proliferation and migration (Daviet et al., 1990; Presta et al., 1991). Studies using knockout mice have demonstrated essential functions for FGFR1 and FGFR2 in early development and roles for FGFR3 in skeletal morphogenesis.

Studies of 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 (Murakami et al., 2008b). 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 (Miller et al., 2000). Moreover, *FGF2* and *FGF1*/*FGF2* knockout mice exhibit delays in the remodeling of damaged blood vessels during wound healing and tumor angiogenesis (Presta et al., 2005).

Additionally, tube formation stimulated by VEGF is totally abolished when neutralizing antibodies to FGF2 are added to the system, showing that in this particular setting, VEGF requires the presence of FGF2 for promoting vessel assembly (Javerzat et al., 2002).

FGF signaling also contributes to the proliferation of tumor cells either by an autocrine or paracrine mechanism (Beenken and Mohammadi, 2009; Javerzat et al., 2002). Additionally, it has been reported that oral squamous carcinomas and gastric cancers

display increased FGFR1 (Freier et al., 2007) and FGFR2 (Kunii et al., 2008) molecules on tumor cells surface, which can either aberrantly respond to FGF ligands or establish a ligand-independent signaling (Turner and Grose, 2010).

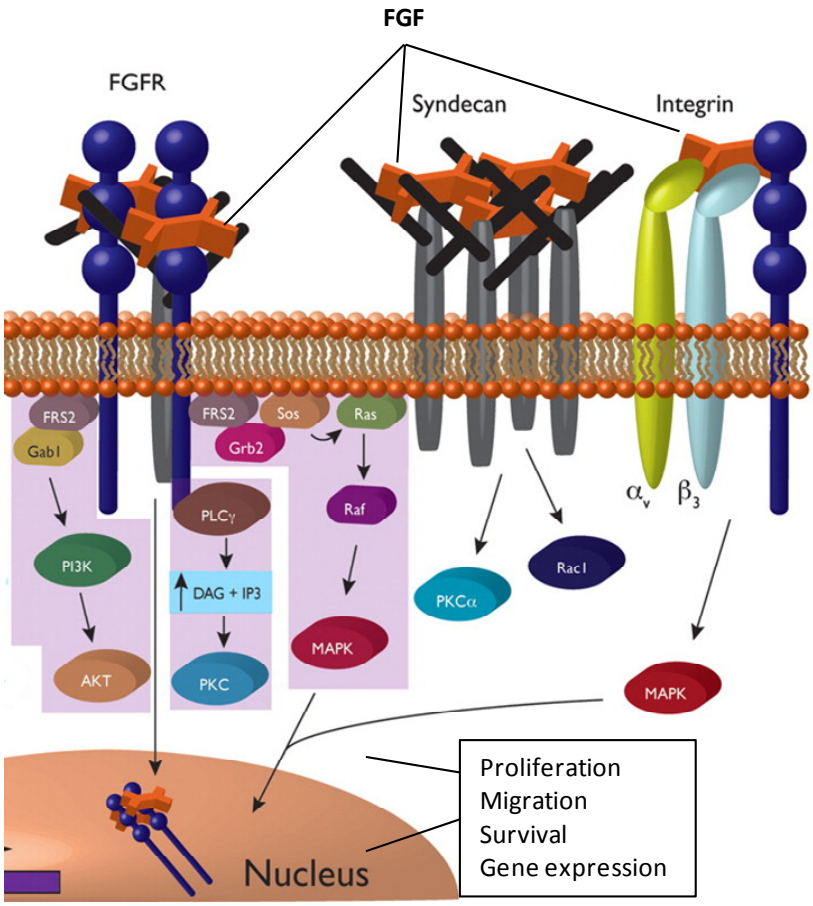


**Figure 7| FGF signaling pathway overview.** The canonical FGF signaling pathway (highlighted in purple) is elicited by FGF binding to heparin sulfate and FGFR. The canonical pathway includes the activation of PI3K/Akt, MAPK/ERK, and phospholipase-C-γ *(*PLCγ)/PKC signaling pathways, which lead to EC survival, proliferation, migration and up-regulation of growth factors (e.g. VEGF, HGF), adhesion molecules (e.g. integrins) and proteases (MMPs). Non-canonical pathways include FGF binding to syndecans and $\alpha_v\beta_3$ integrin. Adapted (Murakami et al., 2008a).

### 3.1.3. TGFβ

TGFβ and its receptors are expressed in a broad spectrum of cell types, including tumor cells, pericytes and ECs, acting both in a paracrine and autocrine fashion.

TGFβ binds to two different types of serine-threonine kinase receptors, known as type I (TβR-I) and type II (TβR-II). It was found that TGFβ signaling regulates cell growth, differentiation, migration, adhesion, and apoptosis of various types of cells (Distler et al., 2003; Pepper, 1997). TβR-I and TβR-II are interdependent, meaning that TβR-I requires TβR-II to bind TGFβ and TβR-II requires TβR-I for signaling. ECs also present another specific type III (co)receptor, called endoglin, which is up-regulated during angiogenesis (Fonsatti et al., 2010).

Angiogenesis stimulation by TGFβ is mostly due to indirect mechanisms, in which inflammatory cells release pro-angiogenic factors such as VEGF, FGF and PDGF (Distler et al., 2003; Kaminska et al., 2005). However, a direct action is also possible through the binding to two different TβR-I: ALK1 and ALK5 (activin receptor-like kinase-1 and-5, respectively). These kinases induce the expression of pro-angiogenic genes or maturation-specific genes, respectively (Figure 8) (Bertolino et al., 2005).

Although TGFβ1 and TGFβ2 have been demonstrated to be actively synthesized by cells located in places of active angiogenesis and vascular remodeling, such as wound healing (Frank et al., 1996) and tumor microenvironment (Kaminska et al., 2005), it seems that TGFβ may promote both pro- or anti-angiogenic functions, depending upon the dose and surrounding environmental conditions (Distler et al., 2003).

TGFβ1 is the most well studied member of the TGFβ family. *Tgfβ1*-null mice experiments have demonstrated that, during development, this cytokine is an important regulator of EC differentiation and cell adhesion. Transgenic animals presented defective capillary tube formation with increased wall fragility probably due to the lost of contact between ECs (Dickson et al., 1995). Different experiments showed that TGFβ1 actively induces angiogenesis when administered subcutaneously in mice (Frank et al., 1994), or applied to chick embryo CAM (Yang and Moses, 1990), or when tested in the rabbit cornea (Phillips et al., 1993). In contrast, the same cytokine seems to inhibit the growth of

vascular tumors in mice (Dong et al., 1996) and inhibits FGF-induced vessel formation in subcutaneous matrigel models (Passaniti et al., 1992).

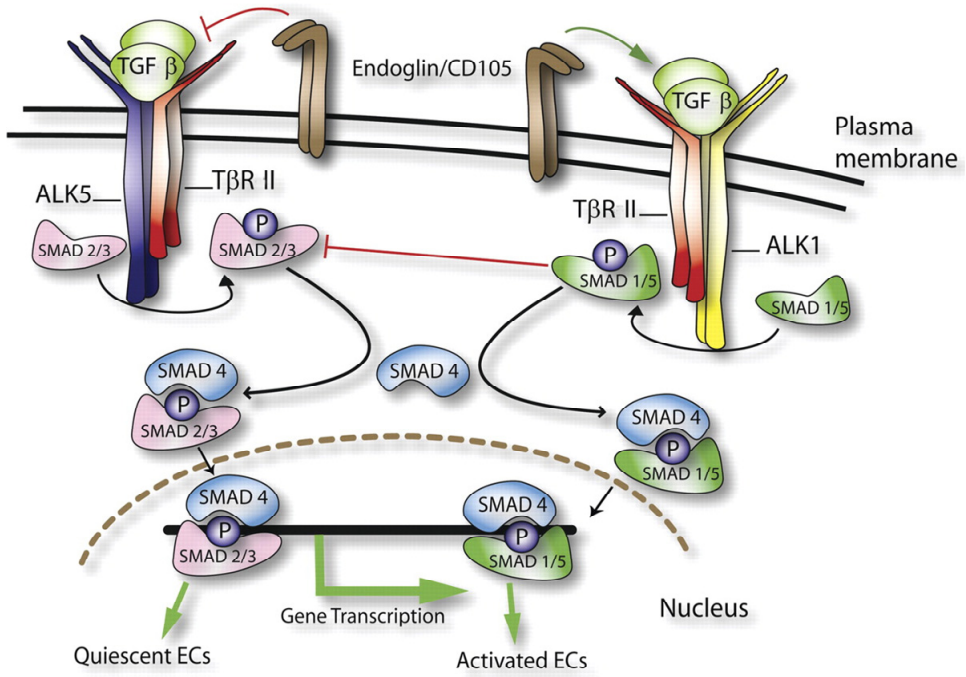


**Figure 8| TGFβ/ALK1 and TGFβ/ALK5 signaling pathways in EC**s. TGFβ dimmer binds first to TβR-II receptors which leads to association with TβR-I receptors. In ECs, TGF-β can activate two TβR-I pathways with opposite effects: ALK5 inducing Smad 2/3 phosphorylation and ALK1 promoting Smad 1/5 phosphorylation. Endoglin binds TGFβ by associating with TβR-II, and is needed for efficient TGFβ/ALK1 signaling. Upon activation, phosphorylated Smads form heteromeric complexes with the common mediator Smad 4, which in the nucleus act as transcription factor complexes regulating the transcriptional activity of target genes. ALK1 and ALK5 have opposite effects on EC migration and proliferation: ALK1 pathway induces the expression of pro-angiogenic genes, while the activation of ALK5 pathway results in the expression of maturation-specific genes. In addition, ALK1 can indirectly inhibit ALK5-induced Smad-dependent transcriptional responses. Adapted (Fonsatti et al., 2010). TGFβ also seems to be able to induce the activation of several other signaling targets in a Smad-independent way (e.g. MAPK/ERK and PI3K/Akt pathways) (Kaminska et al., 2005).

*In vitro*, low doses of TGFβ1 initiate the angiogenic switch by up-regulating angiogenic factors (e.g. VEGF) and proteases, while high doses inhibit EC growth and migration, through down-regulation of angiogenic factors and up-regulation of TIMPs (reviewed in Pepper, 1997). This anti-angiogenic effect might be overcome by the addition of other growth factors, such as FGF or HGF (Baird and Durkin, 1986; Taipale and Keski-Oja, 1996). TGFβ2 have also shown to activate ECs (Distler et al., 2003).

Besides its role in physiological angiogenesis, TGFβ is also associated to pathological conditions. In the later stages of tumor development, TGFβ1 and TGFβ2 are actively secreted by tumor cells or stromal cells and contribute to cell growth, invasion, metastasis, and to a decrease in host anti-tumor immune responses (Kaminska et al., 2005). Despite the role of TGFβ3 remains to be clarified, some studies indicate that this kinase may actually play a protective role against tumorigenesis in a wide range of tissues (Laverty et al., 2009).

### 3.1.4. VEGF AND VEGFRS

VEGF (VEGFA) is produced by the majority of cells in the body and is up-regulated by different mechanisms, such as hypoxia (through hypoxia inducible factor 1, HIF1), inflammatory mediators (IL1, IL6 and Prostaglandin-2), growth factors (EGF, IGF1, FGF, PDGF and TGFβ), oncogenes (e.g. *RAS*), and mechanical forces of shear stress and cell stretch (Kuwano et al., 2001). VEGF is the most important molecule that controls angiogenesis and acts mainly on ECs from newly formed blood vessels, promoting their survival, permeability, proliferation and migration (Kuwano et al., 2001). Although the endothelium of mature blood vessels in adults was believed not to require VEGF for maintenance, recent evidence indicates that at least the fenestrated capillaries of endocrine glands are dependent on continuous VEGF signaling (Kamba et al., 2006). Deletion of even a single V*egf* allele leads to embryonic lethality due to abnormal formation of blood islands and blood vessels, which are even more impaired in embryos lacking both V*egf* alleles, demonstrating a remarkably strict dose-dependence of early

blood vessel development for VEGF (Carmeliet et al., 1996; Ferrara et al., 1996). Furthermore, the essential role of regulated VEGF signaling during development is also demonstrated by the early embryonic lethality of mice with moderate over-expression of VEGF (Miquerol et al., 2000).

Besides its role in ECs, VEGF is also involved in the chemotaxis and differentiation of EPCs, chemotaxis of monocytes, growth of tumor cells, increased production of B cells and myeloid cells, among others (Kuwano et al., 2001). Therefore, VEGF has an important role not only in physiologic angiogenesis but also in several pathologies, such as in tumor angiogenesis (reviewed in Ferrara, 2002).

Tumor and tumor-associated stroma express VEGF (and/or VEGF-inducible molecules), which will interact with ECs (leading to the fast growth of new vessels), EPCs (contributing to their chemotaxis), or tumor cells itself (promoting cell growth) (Ferrara, 2002).

Other molecules of the VEGF family are: VEGFB, VEGFC, VEGFD, and placenta growth factor (PlGF) (Ferrara, 2001; Takahashi and Shibuya, 2005). The VEGF ligands mediate their angiogenic effects through different receptors: VEGFR1 (FLT1), VEGFR2 (KDR, Flk1) and VEGFR3 (primarily involved in lymphangiogenesis). In addition, VEGF also interacts with a family of co-receptors called neuropilins (NP), NP1 and NP2, which seem to enhance the effectiveness of VEGFR2 mediated signal transduction rather than induce intrinsic signaling pathways (Neufeld et al., 2002). All VEGFA isoforms (reviewed in Ferrara, 2001 and Kuwano et al., 2001) bind to VEGFR1 and VEGFR2, although the most angiogenic effects attributed to VEGF result from the activation of VEGFR2, which is autophosphorylated after binding, promoting a cascade of intracellular signaling pathways (Figure 9).

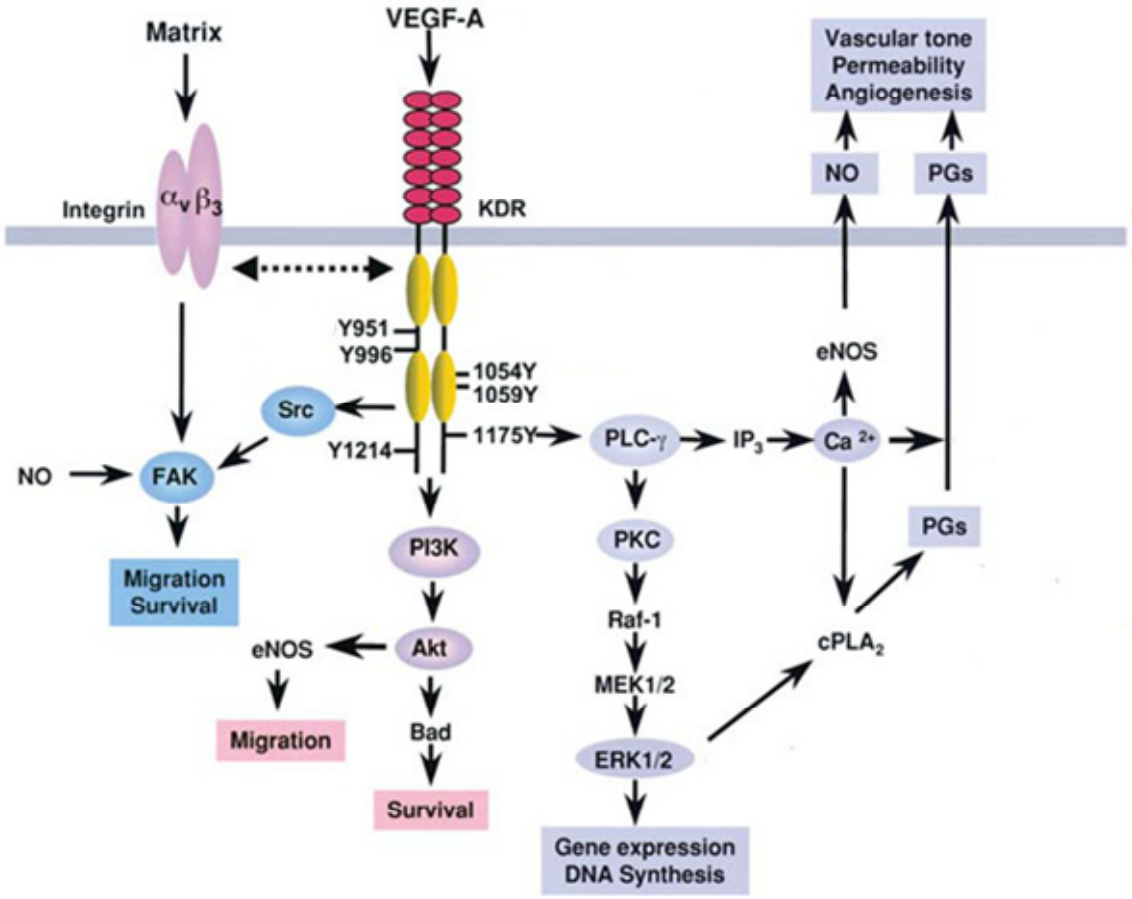


**Figure 9| VEGF pathway overview.** VEGF promotes the majority of it angiogenic effects through its binding to VEGFR2. Upon ligand binding, tyrosine residues of VEGFR2 are phosphorylated, resulting in the activation of a number of downstream signaling molecules. VEGF-dependent EC survival is mediated in part *via* PI3K/Akt pathways which results in the inhibition of the pro-apoptotic protein Bad; Akt also causes independent eNOS activation, playing also a role in EC migration. Increased tyrosine phosphorylation of focal adhesion kinases (FAK), mediates survival and migration signaling, which is enhanced by direct interactions between integrin αvβ3 and VEGFR2. VEGF signaling also induces the PLC-γ pathway resulting in PKC and, consequently, MAPK/ERK signaling activation, promoting EC proliferation and induced expression. $Ca^{2+}$ signaling is also important for eNOS activation and NO generation, that, in combination with prostaglandins, will regulate the vascular tone and permeability. Adapted (Zachary, 2003).

As mentioned above, embryos lacking *VEGFR2* fail to develop blood islands, ECs, and major vessel tubes (Carmeliet et al., 1996; Shalaby et al., 1995), which indicate its essential role in growth, survival, and differentiation of EPCs. Later, VEGFR2 becomes the major mediator of proliferation, migration and survival signals in ECs (Distler et al., 2003; Ferrara, 2001; Shibuya and Claesson-Welsh, 2006; Takahashi and Shibuya, 2005). Although its expression declines during later stages of vascular development, it becomes again up-regulated in physiological and pathological angiogenesis in adults (Ferrara, 2002).

The role of VEGFR1, which has lower kinase activity, in VEGF-mediated cellular responses remain to the clarified. Experiments with mice lacking *VEGFR1* show that this receptor acts as a negative regulator of angiogenesis during embryonic development, as animals exhibit uncontrolled EC proliferation which results in the obstruction of vessel lumen and early lethality (Fong et al., 1995). However, in adult, VEGFR1 plays a role in activating VEGFR2, and thereby in angiogenesis, by the binding of PlGF (Autiero et al., 2003). This mechanism gains importance in angiogenesis-associated pathologies, where PlGF is up-regulated (Carmeliet et al., 2001). Furthermore, VEGFR1 is involved in the preparation of the metastatic niche, since VEGFR1-positive haematopoietic progenitor cells were shown to colonize tumor specific pre-metastatic sites prior to the arrival of tumor cells (Kaplan et al., 2005).

### 3.1.4. CYR61

CYR61 (CCN1) belongs to the CCN family, and was first identified as a member of growth factor-inducible immediate-early genes (Chen and Du, 2007). CYR61 is expressed and capable of acting in a wide range of cell types, including fibroblasts, SMCs, some tumor cells, EPC, and ECs, where it plays a role in diverse cellular functions such as proliferation, adhesion and migration, gene regulation, survival and chemotaxis (Brigstock, 2002; Chen and Lau, 2009; Yu et al., 2008). Potent pro-angiogenic properties of CYR61 were demonstrated *in vivo* in a rat cornea model and in a rabbit ischemic hind limb model,

where the protein was found to induce neovascularization (Babic et al., 1998; Fataccioli et al., 2002). Also, *Cyr61*-null mice suffer embryonic death due to loss of vascular integrity in the embryo (Mo et al., 2002).

*In vitro*, CYR61 has been shown to actively stimulate migration, proliferation, adhesion, survival and tubule formation of HUVEC (Kireeva et al., 1996; Leu et al., 2002). Neutralizing antibody experiments have demonstrated the importance of different players in mediating CYR61 transduction signal, with special attention to the role of $\alpha v\beta 3$ and $\alpha 6\beta 1$ integrins (see below).

*CYR61* is transcriptionally activated in response to different cytokines and growth factors (e.g.TGF$\beta$1, FGF2, VEGF) (Chen and Du, 2007), regulating angiogenesis during development and adulthood both in physiological and pathological context. This regulation may happen either by direct or indirect mechanisms (Leask and Abraham, 2006). CYR61 initiates its own intracellular signaling cascade *via* an integrin dependent pathway. By this mechanism, CYR61 directly regulates the expression of *VEGF*, *MMPs* and *TIMPs*, as well as the activation of other intracellular targets involved in adhesion and survival (Figure 10), therefore leading to migration, survival and tube formation (Brigstock, 2002; Leask and Abraham, 2006; Leu et al., 2002). Moreover, CYR61 increases the activity of several growth factors, either by its direct binding (e.g. PDGF, VEGF, TGF$\beta$), as by HSPG (heparin sulfate proteoglycan) binding leading to FGF2 displacement from the ECM (Brigstock, 2002). In addition, CYR61 modulates the expression and function of $\alpha v\beta 3$ potentially through an alternative receptor (Leu et al., 2002; Monnier et al., 2008), possibly promoting a higher level of cross-talk between integrin and growth factor receptors as VEGFR2 (Somanath et al., 2009).

Although CYR61 can play different roles in different kinds of tumors, CYR61 over-expression is often associated with tumor development and growth (Leask and Abraham, 2006), higher invasion capacity and resistance to treatment due to its function in promoting cell survival (Menendez et al., 2003; Monnier et al., 2008).

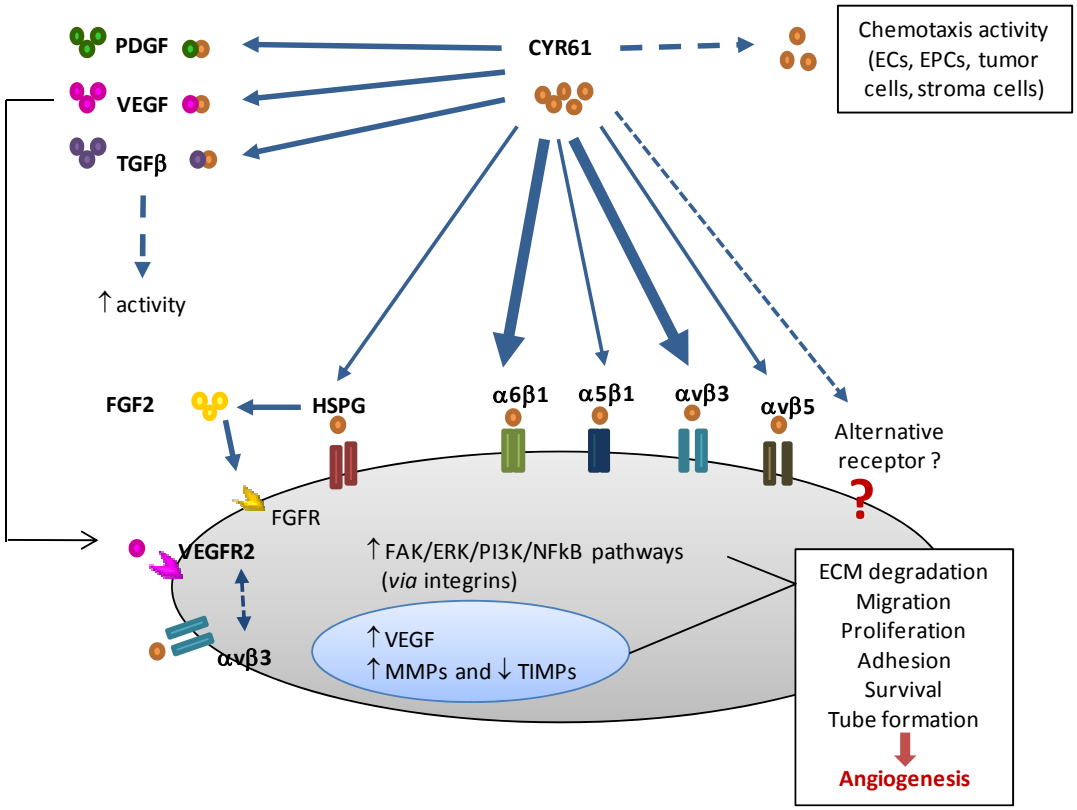


**Figure 10| CYR61 regulates angiogenesis both by direct and indirect mechanisms.** Besides its chemotaxis activity, CYR61 regulates the expression of VEGF, MMPs and TIMPs, as well as the activation of FAK, ERK, PI3K and nuclear factor-kB (NFkB) through an integrin-dependent pathway. CYR61 increases the activity of several growth factors, either by its direct binding (e.g. PDGF, VEGF, TGFβ), as by HSPG binding, leading to FGF2 displacement from the ECM. The integrin-VEGFR2 cross-talk induced response is also enhanced by CYR61 activity.

## 3.2. PHYSIOLOGICAL ANGIOGENESIS

Although after birth angiogenesis still contributes to organ growth, during adulthood most of the blood vessels remain quiescent. However, ECs keep their ability to divide in response to a physiological stimulus such as hypoxia. Consequently, in adulthood, angiogenesis is activated only in response to a small number of non-pathological

processes, such as wound healing and reproductive functions (ovulation, development of the *corpus luteum*, repair of the menstruating uterus, and development of the placenta) (Cao et al., 2005; Carmeliet, 2005).

Besides sprouting angiogenesis, there is another variant of angiogenesis where blood vessels are formed by the split of the pre-existing ones – intussusceptive angiogenesis – although little is known about its molecular regulation (Adams and Alitalo, 2007; Conway et al., 2001). It occurs during the first 2 years in humans during lung growth and it is an extremely quick process since, instead of proliferating, ECs are remodeled (Hillen and Griffioen, 2007). The process is initiated by the projection of opposing microvascular walls into the capillary lumen creating a contact zone between ECs. Next, the endothelial bilayer is perforated, intercellular contacts are reorganized, and a transluminal pillar with an interstitial core is formed, which is soon invaded by fibroblasts and pericytes leading to its rapid enlargement by the deposition of collagen fibers, and partition into two separated vessels (Figure 11) (Burri et al., 2004).

Molecules involved in vessel formation and maturation during embryonic development, seem to be also involved in the same process in the post-natal period. However, based on antibody blocking and gain of function studies, it appears that its spatial-temporal pattern of expression and concentration may differ slightly (Jain, 2003; Risau, 1997).

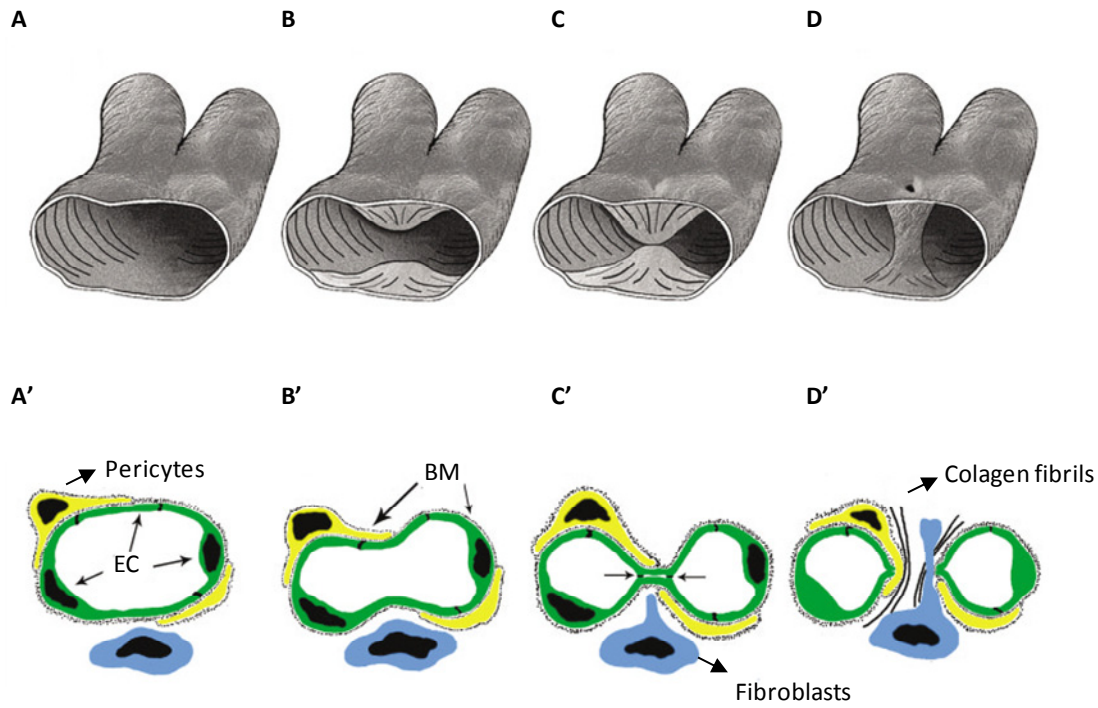


**Figure 11| Intussusceptive angiogenesis**. Three-dimensional (A–D) and two-dimensional (A'-D') representation of the process: projection of opposing capillary walls into the vessel lumen creating a contact zone between ECs where a transluminal pillar is formed and invaded by fibroblasts and pericytes, which lead to its rapid enlargement by the deposition of collagen fibers; the process finishes with the separation into two separated vessels. Adapted (Burri et al., 2004).

### 3.3. PATHOLOGICAL ANGIOGENESIS

Physiological angiogenesis depends on a complex balance between pro- and anti-angiogenic factors which are tightly regulated both temporally and spatially. In many disorders, however, this balance becomes unstable and the equilibrium between inducers and inhibitors is twisted, resulting in either deficient or excessive uncontrolled neovascularization (Carmeliet, 2003; Carmeliet, 2005; Carmeliet and Jain, 2000). In diseases such as ischemic heart disease, angiogenic inhibitors gain weight against stimulators, resulting in EC dysfunction, vessel malformation or regression (Carmeliet, 2005). On the other hand, cancer is a clear example of a pathology characterized by an

excessive angiogenesis, creating structurally and functionally abnormal vessels, highly disorganized, tortuous, dilated and excessively branched (Carmeliet and Jain, 2000).
Table 3 shows some examples of angiogenesis-dependent diseases (for a complete list of diseases see Carmeliet, 2005).

**Table 3 – Selected list of diseases characterized or caused by abnormal/excessive or insufficient angiogenesis.** Adapted (Carmeliet, 2005).

| ORGAN | DISEASES CHARACTERIZED OR CAUSED BY EXCESSIVE ANGIOGENESIS | DISEASES CHARACTERIZED OR CAUSED BY INSUFFICIENT ANGIOGENESIS |
|---|---|---|
| **Multiple Organs** | Cancer and metastasis<br>Infectious diseases<br>Auto-immune disorders | |
| **Blood vessels** | DiGeorge syndrome<br>Cavernous hemangioma | Peripheral arterial disease<br>Atherosclerosis<br>Diabetes<br>Hypertension |
| **Adipose tissue** | Obesity | |
| **Eye** | Diabetic retinopathy<br>Age-related macular degeneration | |
| **Nervous system** | | Alzheimer disease<br>Diabetic neuropathy<br>Amyotrophic lateral sclerosis |
| **Heart** | | Ischemic heart disease |
| **Reproductive system** | Endometriosis<br>Ovarian hyperstimulation Ovarian cysts | Preeclampsia |
| **Bone** | Synovitis<br>Osteomyelitis | Osteoporosis<br>Impaired bone fracture healing |
| **Skin** | Psoriasis<br>Scar keloids | Hair loss<br>Lupus |

### 3.3.1. TUMOR ANGIOGENESIS

Tumors, as normal tissues, require an adequate supply of oxygen and nutrients, and an effective way to remove waste products. As so, solid tumor progression beyond a volume of approximately 1-2 $mm^2$ requires angiogenesis (Nussenbaum and Herman, 2010).

Tumors are described as "wounds that never heal", contributing to the development of blood vessels that fail to become quiescent, and therefore the constant growth of new tumor blood vessels (Bergers and Benjamin, 2003). Tumor blood vessels are architecturally different from normal blood vessels, as they are irregularly shaped, dilated, tortuous, and can have dead ends (Figure 12) (Bergers and Benjamin, 2003).

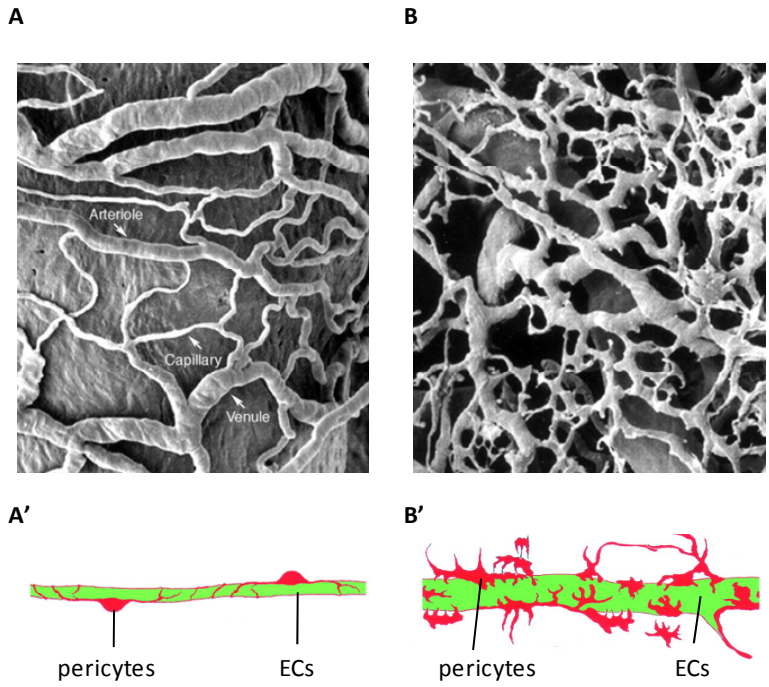


**Figure 12| Contrast between normal and tumor vasculature.** (A, B) Scanning electron microscopic imaging of rat vascular casts showing (A) a normal microvasculature with organized arrangement of arterioles, capillaries, and venules, versus (B) a tumor microvasculature, with disorganized and lack of conventional hierarchy of blood vessels where arterioles, capillaries, and venules are not identifiable as such. (A', B') Diagram comparing the structure and close EC association of pericytes in a normal capillary (A') versus a loosely association in tumor vasculature (B'). (A, B) Adapted (McDonald and Choyke, 2003). (A', B') Adapted and modified (Morikawa et al., 2002).

Moreover, overproduction of VEGF and the absent or abnormal association of pericytes, promotes the formation of leaky vessels (Bergers and Song, 2005; Morikawa et al., 2002). As a consequence, blood flows in a very irregular pattern, moving more slowly and in an oscillating way (Bergers and Benjamin, 2003; Jain, 2005).

The angiogenic switch seems to result mainly from the action of mutated oncogenes (e.g. c-myc, Ras family, HER2) and tumor suppressor genes (e.g. p53, BRACA1/2, APC) that deregulate the angiogenic balance by promoting the disproportionate expression of angiogenic factors (in favor of angiogenic stimulators) (Bergers and Benjamin, 2003). In this process, hypoxia plays also an important role. Tumor progression associated to a dysfunctional vasculature leads to an insufficient oxygen supply to the tumorigenic tissue, causing the induction of HIFs and, consequently, neovessels formation (Dewhirst et al., 2008) – see below.

Many angiogenic factors and cytokines are also secreted by stromal cells, such as infiltrated macrophages, inflammatory cells or even EPCs (Kopfstein and Christofori, 2006; Ribatti and Vacca, 2008; Ruegg, 2006). Thus, tumor microenvironment has also an essential role in promoting tumor angiogenesis through the release of molecules that directly bind to ECs promoting their activation or, indirectly, by stimulating the tumor cells to produce pro-angiogenic factors (Figure 13) (Jung et al., 2002; Lorusso and Ruegg, 2008). Like in the physiological angiogenic process, tumor angiogenesis depends not only of VEGF and its receptors, but from many other molecules, such as angiopoietins, FGF, PDGF, TGFβ, IL8, MMP2 and PlGF (which gains special weight) (Kuwano et al., 2001; Nussenbaum and Herman, 2010).

Although most of the studies regarding tumor angiogenesis are related to the progression of solid tumors, the angiogenic process has also been associated to a higher destructive potential and poor prognosis in a subset of hematological diseases, such as acute leukemia and multiple myeloma (Moehler et al., 2003).

There are, however, some tumors that do not required angiogenesis or, in parallel, use different mechanisms (reviewed in Auguste et al.,2005). Astrocytomas are an example of brain tumors that acquire their blood supply by co-option, meaning that they grow along

already formed blood vessels, without requiring neo-vascularization (Bergers and Benjamin, 2003; Hillen and Griffioen, 2007). Vasculogenic mimicry was also described in some aggressive melanomas, where tumor cells differentiate to an endothelial phenotype and make tube-like structures, providing a secondary circulation system (Hendrix et al., 2003).

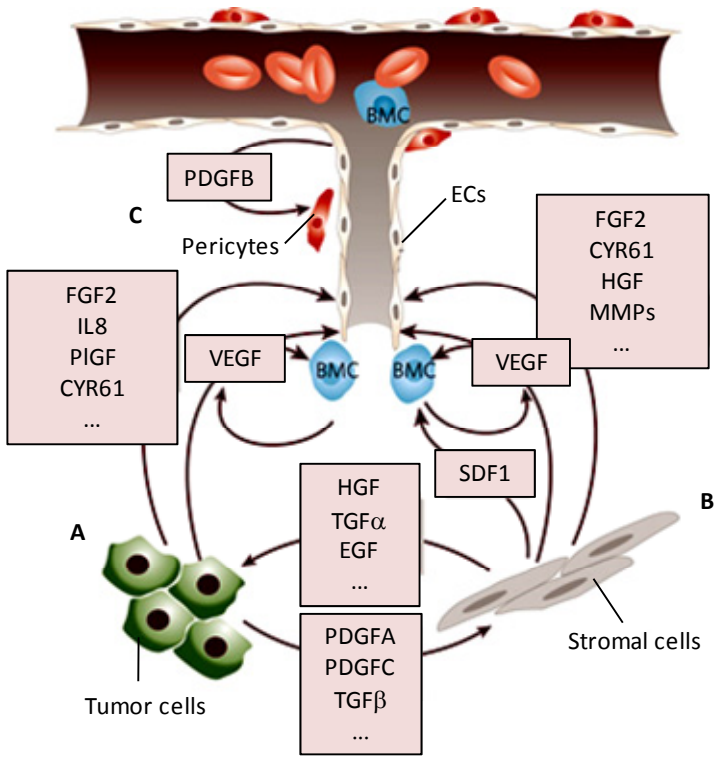


**Figure 13| A few of the molecular and cellular players in the tumor/microvascular microenvironment.** (A) Tumor cells produce VEGF and other angiogenic factors such as FGF2, angiopoietins, IL8, PlGF and CYR61, capable of stimulating resident ECs to proliferate and migrate; besides, tumor cells may also release factors capable of recruiting stromal cells (e.g. PDGFA, PDGFC, TGFβ) and bone-marrow-derived angiogenic cells (BMCs, EPCs) (e.g. PLGF, VEGF). (B) An additional source of angiogenic factors is the stroma, comprising fibroblastic, inflammatory and immune cells; tumor-associated fibroblasts also produce chemokines (e.g. SDF1) capable of recruiting EPCs and growth/survival factors for tumor cells (e.g. EGF, HGF, TGFα). (C) ECs produce PDGFB, which promotes recruitment of pericytes in the microvasculature, and a set of other pro-angiogenic factors able of promote its own growth and proliferation, by an autocrine way, or the

tumor cells progression through a paracrine fashion. Adapted and modified (Ferrara and Kerbel, 2005).

#### 3.3.1.1. Role of the endothelium in tumor cell metastasis

Many studies have postulated angiogenesis as an indicator of metastatic potential in human solid tumors (reviewed in Zetter, 1998). Besides contributing to tumor growth, tumor angiogenesis plays also an essential role in tumor metastasis due to the formation of additional highly permeable vessels that provide an efficient route of exit for tumor cells to leave the primary site and enter the blood stream (Zetter, 1998).

The metastasis formation process begins with the dissociation of single or clustered tumor cells from the primary tumor and is followed by extracellular matrix invasion, entrance into blood or lymph vessels (intravasation), and transport to other tissue sites of the body (Figure 14). Additionally, it is also possible for a tumor to grow inside the vessels on the EC layer and form the metastasis at these sites (intravascular metastasis), since tumor cells never extravasate (Mierke, 2008).

The intravasation and extravasation process involves adhesion of tumor cells to ECs where cell-matrix receptors (integrins) and cell-cell adhesion molecules (integrins, cadherins, immunoglobulins and selectins) play a central role (Mierke, 2008). Besides promoting tumor growth and angiogenesis, cytokines, chemokines and growth factors have also an important function in the formation of metastasis. They can regulate adhesion molecules and their receptors in tumor and ECs and play a role in the chemotaxis of tumor cells to other tissues (Kopfstein and Christofori, 2006; Opdenakker and Van Damme, 2004).

There is apparent site selectivity in the formation of secondary tumors: primary tumors in certain organs tend to metastasize to preferred site. For example, breast adenocarcinoma generally metastasizes to the regional lymph nodes and then to the liver, lungs and bone, while lung cancers frequently metastasize to the brain (Oppenheimer, 2006). The mechanism underlying this selection is almost unknown, but some possibilities have been advanced related to the geometry of the primary and

secondary sites, response to organ derived chemotactic factors, adhesion between tumor cells and the target organ components, and response to specific host tissue growth factor (Opdenakker and Van Damme, 2004; Ribatti and Vacca, 2008). Another possibility involves the interaction between metastatic cells and endothelial organ-specialized cells since, depending on the organ type, ECs might express specific surface receptors and growth factors that interact in a different way with diverse cancer cells types (Ribatti and Vacca, 2008).

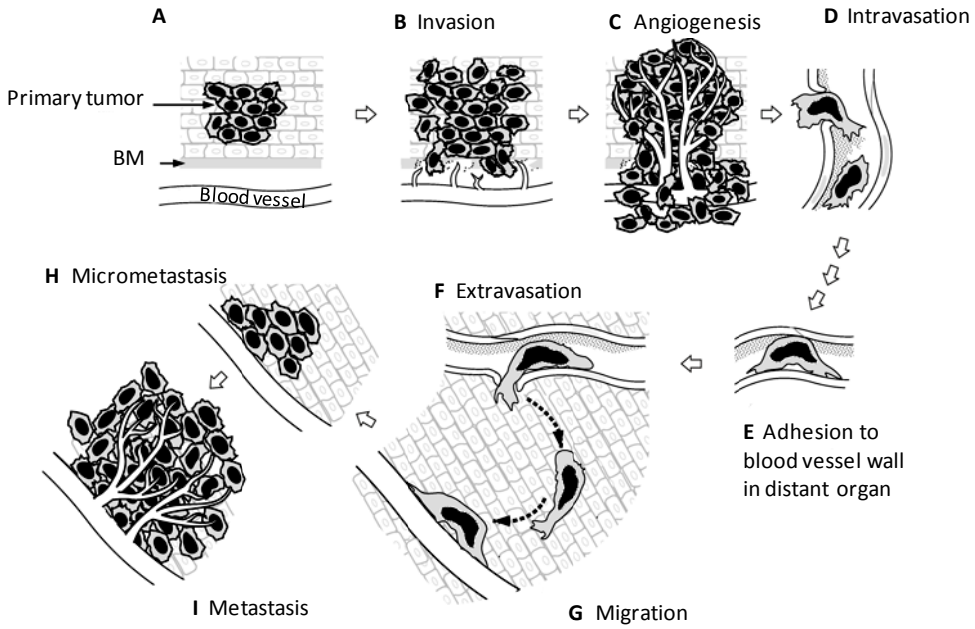


**Figure 14| Tumor metastasis formation: interactions with blood vessels.** (A) Small primary tumors (< 2 mm) remain avascular. After that, (B) tumor cells invade the basement membrane and produce angiogenic factors, promoting the (C) angiogenic switch, which allows the expansion of the primary tumor. (D) The new blood vessels provide a route of entry into the bloodstream and the tumor cells circulate until they die or (E) attach specifically to ECs in the vessels (usually venules) of downstream organs. (F) The tumor cells extravasate through the vessel wall and then (G) migrate to sites proximal to arterioles, where their growth is enhanced. (H) Micrometastasis can remain dormant for extended time periods, during which angiogenesis is suppressed. (I) Initiation of angiogenesis at the secondary site releases the metastatic colonies from dormancy and allows rapid growth. Adapted (Zetter, 1998).

The production and release of a variety of cytokines by the tumor, promotes microenvironmental changes even at distant sites, often stating the pattern of metastatic spread – "premetastatic niche" (Kaplan et al., 2006). These early changes, even before the arrival of tumor cells, include inflammatory response, matrix remodeling and increase in reactive oxygen species (ROS) (Kaplan et al., 2006). Some evidence to this process came from the observations of an up-regulation of fibronectin and clustering of VEGFR1-positive hematopoietic progenitor cells in common sites of metastasis formation prior to the detection of tumor cells (Kaplan et al., 2005). After the establishment of the metastatic cells, two paths are possible: (1) tumor growth and expansion at the second site; or (2) formation of dormant metastasis, that sometimes become activated by the removal of the first tumor (Bergers and Benjamin, 2003).

#### 3.3.1.2. Role of hypoxia in tumor angiogenesis and metastasis

As referred previously, continuous tumor progression associated to a dysfunctional vasculature generally leads to a hypoxic environment. Hypoxia affects cancer prognosis by 3 different biological processes: angiogenesis, metastasis and genetic instability, which finally leads to tumor aggressiveness.

Hypoxia affects cell behavior through HIF1 stabilization (reviewed in Pouyssegur et al., 2006). HIF1$\alpha$ is constitutively expressed in cells; however, it is degraded in the presence of oxygen *via* proteosome. In hypoxia, HIF1$\alpha$ join to its subunit β, becoming stable. The complex HIF1$\alpha$-HIF1β binds to DNA, and promotes the expression of different target genes actively involved in angiogenesis, cell survival/death, anaerobic metabolism, pH regulation, and invasiveness (Figure 15) (Brahimi-Horn et al., 2007; Dewhirst et al., 2008). *VEGF*, *ANGPT2*, *NOS* and *VEGFR1* are HIF1 direct transcriptional targets, contributing to the angiogenic switch on tumor vasculature (Pugh and Ratcliffe, 2003). However, the neo-vessels formed in response to hypoxia are also generally tortuous, functionally less efficient, and chaotic, facilitating tumor spread and poorly contributing to tumor normoxia (Brahimi-Horn et al., 2007; Pouyssegur et al., 2006). HIF1 activation is also

associated with loss of E-cadherin, and an up-regulation of chemokines (e.g. CXCR4) and MMPs (e.g. MMP2) involved in tumor invasion (Chan and Giaccia, 2007).

It is interesting to notice that HIF1 is overexpressed in many types of cancer (e.g. colon, lung, pancreatic cancer), and especially in aggressive metastasis (Zhong et al., 1999).

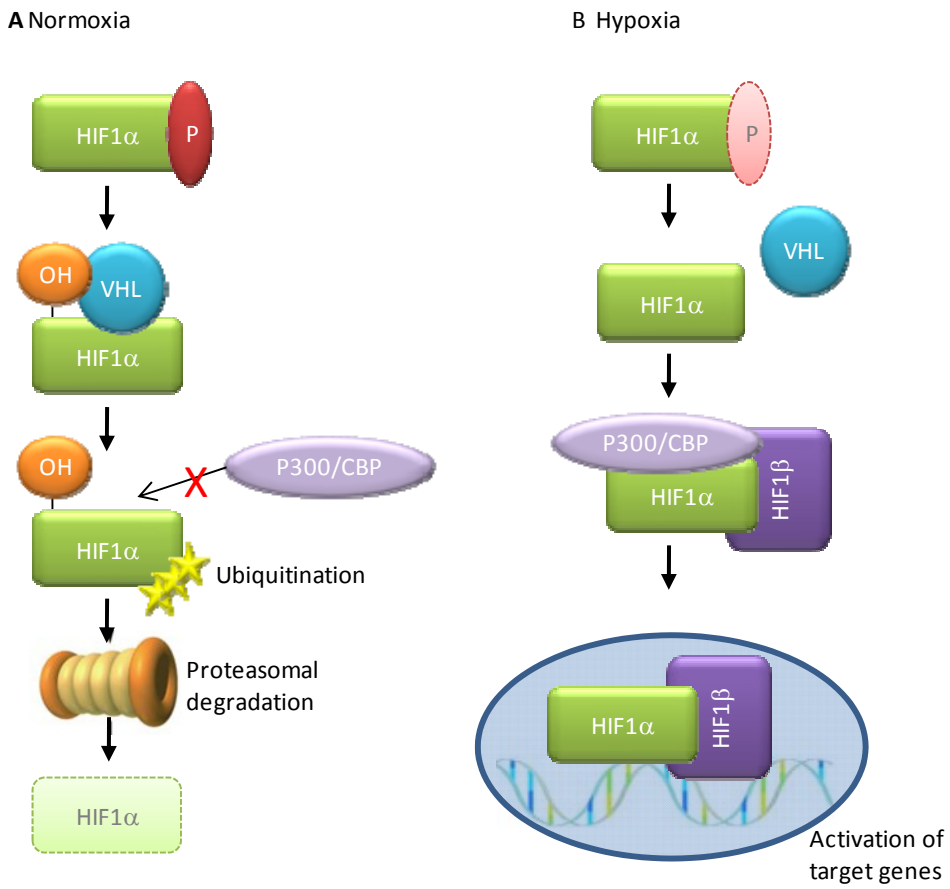


**Figure 15| HIF1α regulation in normoxia and hypoxia.** (A) During normoxia, $O_2$-dependent proline hydroxylases (P) modify HIF1α promoting hydroxylation, which decreases HIF1α interaction with the p300/CBP transcriptional co-activators and promotes VHL (von Hippel Lindau) binding; VHL ubiquitinates HIF1α and thereby targets it for proteasomal degradation. (B) During hypoxia, when proline hydroxylases are not active, HIF1α is stabilized through its binding to HIF1β; HIF1 complex binds also to p300/CBP, resulting in the transcription of hypoxia-inducible genes involved in angiogenesis (e.g. *VEGF*, *NOS*, *ANGPT2*), invasiveness (e.g. E-cadherin, *MMPs*), cell survival/death, anaerobic metabolism, pH regulation. Adapted and modified (Zarember and Malech, 2005).

### 3.3.2. PERIPHERAL ARTERIAL DISEASE

Peripheral arterial disease (PAD) involves obstruction of blood flow in arteries other than the coronary and intracranial vessels, most commonly affecting the circulation of the lower limbs due to atherosclerosis related problems (Baumgartner et al., 2005; Ouriel, 2001).

PAD is a progressive illness and the most severe manifestation is termed “critical limb ischemia” (CLI). CLI describes patients with chronic ischemic rest pain or patients with ischemic skin lesions either ulcers or gangrene (Ouriel, 2001). CLI occurs when arterial lesions impair blood flow and distal perfusion pressure to a level insufficient to satisfy the nutritive needs of the limb. This usually results from the presence of multilevel occlusive disease or occlusion of critical collaterals (Norgren et al., 2007). Moreover, in CLI, there is an inappropriate response of the microcirculatory flow regulatory mechanism due to microcirculatory defects including endothelial dysfunction, altered hemorheology and white blood cell activation and inflammation (Norgren et al., 2007).

The primary goals of the treatment of CLI are to relieve ischemic pain, heal ulcers, prevent limb loss, improve patient function and quality of life and prolong overall survival (Norgren et al., 2007).

Any kind of surgical/endovascular revascularization, the therapy of choice in CLI patients, should be done whenever technically possible. Attempts to manipulate and normalize the microcirculatory flow pharmacologically may enhance the results of revascularization or be one option in patients in whom revascularization is impossible or has failed. In patients with CLI not eligible for arterial revascularization, prostanoids are vasoactive drugs with proven efficacy (Brock et al., 1990; Diehm et al., 1989; Norgren et al., 1990; Severe Limb Ischaemia Study Group, 1991; The Ciprostene Study Group, 1991).

Prostaglandin $E_1$ ($PGE_1$, alprostadil, vasoprost®) is commonly used as a vasodilator for the treatment of PAD, especially in cases of critical limb ischemia, however, has to be repeatedly administrated due to its short half-life (Esaki et al., 2009). $PGE_1$ has been shown to induce vasodilatation and to inhibit platelet aggregation (Schror and Hohlfeld, 2004). In addition, some recent studies have also reported its pro-angiogenic effect (Diaz-

Flores et al., 1994), through the up-regulation of CYR61 (Schror and Hohlfeld, 2004), HGF (Makino et al., 2004), VEGF and eNOS (Haider et al., 2005) in ECs. However, this effect has been questioned by Cattaneo et al. (Cattaneo et al., 2003), whose work suggests that $PGE_1$ has a strong inhibitory effect on angiogenesis.

Moreover, recent trials do not support the benefit of prostanoids in promoting amputation-free survival (Brass et al., 2006). In a large proportion of CLI patients, the extent and anatomic distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization (Norgren et al., 2007). Amputation is often recommended as a solution to the disabling symptoms even if it is associated to morbidity and mortality (Norgren et al., 2007). Therefore, the need for alternative treatment strategies in CLI patients is compelling and therapeutic angiogenesis is a promising tool to treat these patients.

## 4. ANGIOGENESIS AS A THERAPEUTIC TARGET

The recognition that both vasculogenesis and angiogenesis are essential processes in both normal developmental, adulthood and in several pathologies, has lead to the establishment of numerous *in vitro* and *in vivo* research models. While many *in vitro* systems facilitate ascertaining specific information regarding EC biology, such as migration (e.g. wound healing assay), proliferation (e.g. cell count, cell cycle analysis) or differentiation (e.g. 3D gel), they are not able to recreate the microenvironment of an intact organism and the massive amount of influences on ECs *in vivo*. Thus, *in vivo* models have become crucial, although most of these systems do not permit the detailed visualization and functional dissection of the cellular processes under defined conditions that allow optimal reproducibility and interpretability of results (Staton et al., 2009; Staton et al., 2004). *In vivo* models include a wide variety of assays (reviewed in Staton et al., 2004), such as matrigel plug assay. In matrigel plug assays, angiogenesis-induced factors (e.g. FGF2) are introduced into cold liquid matrigel which, after subcutaneous injection, solidifies and permits penetration by host cells and the formation of new blood vessels, allowing to evaluate angiogenesis by scoring selected regions of histological sections for vascular density (Akhtar et al., 2002). The zebrafish model has also become a tool of choice to study vasculogenesis and angiogenesis, both in development and adult, due to its unique characteristics: small size, large offspring number, low cost, optical clarity, and external development of embryos and larvae (Kidd and Weinstein, 2003). Besides, the generation of transgenic zebrafish lines expressing green fluorescent protein (GFP) within vascular ECs (e.g. *Fli1:EGFP*) has open new perspectives in high optical resolution image, for studying the formation of the vasculature *in vivo (Lawson and Weinstein, 2002a; Lawson and Weinstein, 2002b).*

The full understanding of the angiogenic process, would give us the ability to induce or inhibit blood vessels growth in a controlled manner. Since angiogenesis is involved in several pathological conditions, this would provide an additional strategy for the

management and treatment of vascular dependent diseases. As so, many efforts have been done in order to develop angiogenic therapeutic strategies.

### 4.1. PRO-ANGIOGENIC THERAPY

Several approaches to induce angiogenesis are already in clinical trials, including (1) the direct use of angiogenic growth factors or (2) gene therapy to improve perfusion, (3) inhibition of anti-angiogenic factors, and (4) mobilization of regenerative stem cell populations. Functional and stable blood vessel formation, that will efficiently supply the ischemic region with oxygenated blood, is important for successful therapeutic neovascularization. There are three major conditions for which angiogenic therapies are clinically indicated: chronic wounds (e.g. diabetic lower extremity ulcers, venous leg ulcerations, pressure ulcers, arterial ulcers), peripheral arterial disease, and ischemic heart disease (Prabhu et al., 2011).

VEGF and FGF2 have been the growth factors majority explored for this purpose, however many others have been studied (e.g. FGF1, FGF4, HGF). While many experimental studies are encouraging, randomized controlled clinical trials have produced less consistent results (Simons, 2005). Although part of this failure is attributed to delivery procedure problems, the maintenance of long-lasting strong and functional vessels remains a challenge. It is not clear if a single growth factor is sufficient to initiate the entire cascade of events leading to a mature, functional and stable vascular network *in vivo* (most growth factors secondarily induce other factors), or if a mix of growth factors should be used and precisely orchestrated over time (Cao et al., 2005; Simons, 2005).

Because HIFs initiate an entire angiogenic response, they have also been considered for angiogenic therapy (Sarkar et al., 2009). Transplantation of EPCs, derived from the bone marrow or peripheral blood, have demonstrated to be an alternative to angiogenic factors contributing to postnatal neovascularization in ischemic limbs and myocardium (Asahara et al., 1999; Ferrara and Kerbel, 2005; Milkiewicz et al., 2006; Reed et al., 2007).

Finally, therapeutic interventions that focus on the inhibition of natural anti-neovascularization mechanisms should be considered (e.g. HGF inhibits endostatin and TSP1) (Cao et al., 2005; Milkiewicz et al., 2006).

However, successful pro-angiogenic therapy still raises some questions regarding long-term side effects. If these therapies are able to significantly contribute to vessel integrity and repair, can they indirectly contribute to trigger dormant tumors and, or accelerate atherosclerosis? The different forms of therapeutic angiogenesis still have to prove safety and efficacy before one can conclude on its role as an additional limb saving strategy.

It is still a long way from bench to bedside and patient benefit, despite a considerable number of ongoing clinical trials.

### 4.2. ANTI-ANGIOGENIC THERAPY

Anti-angiogenic therapy inhibits the growth of new blood vessels, being an important weapon in the treatment of many diseases, such as blinding disorders, arthritis, and cancer. Anti-angiogenic drugs exert their effects either by (1) disabling the agents that activate and promote cell growth, survival, and/or migration; (2) up-regulate or deliver endogenous inhibitors; or (3) directly blocking the growing ECs in its receptors or (4) downstream signaling (Carmeliet and Jain, 2000).

Most of the cancer therapies target tumor cells directly but, although their success, limitations are evident (Siemann et al., 2005). Since, the majority of tumors depend on a vascular supply in order to grow and metastasize, anti-angiogenic agents represent a new promising therapy for a number of reasons: (1) normal ECs are quiescent in physiological conditions, whereas tumor ECs and EPCs have a high rate of proliferation with an abnormal phenotype, lowering the possible toxicity of the angiogenic inhibitors; (2) in opposition to tumor cells, ECs are genetically stable; and (3) tumor endothelium expresses high levels of specific molecules that can be targeted by selective inhibitors (Bergers and Benjamin, 2003).

Anti-angiogenic therapy is frequently combined with conventional cytotoxic agents (chemotherapy) or radiation therapy (radiotherapy), commonly resulting in additive or synergistic anti-tumor effects (Gasparini et al., 2005). Note that tumor vasculature is structurally and functionally abnormal when compared to normal vasculature, causing interstitial hypertension and poor blood flow (Bergers and Benjamin, 2003; Jain, 2005). Thus, the aim of using anti-angiogenic agents in the treatment of cancer is to normalize vessels by pruning the immature and inefficient vessels and remodeling the remain, contributing to an effective delivery of chemotherapeutic agents to tumor cells and, by lowering hypoxia, enhance its cell death induced-radiation therapy (Figure 16) (Hillen and Griffioen, 2007; Jain, 2005; Siemann et al., 2005).

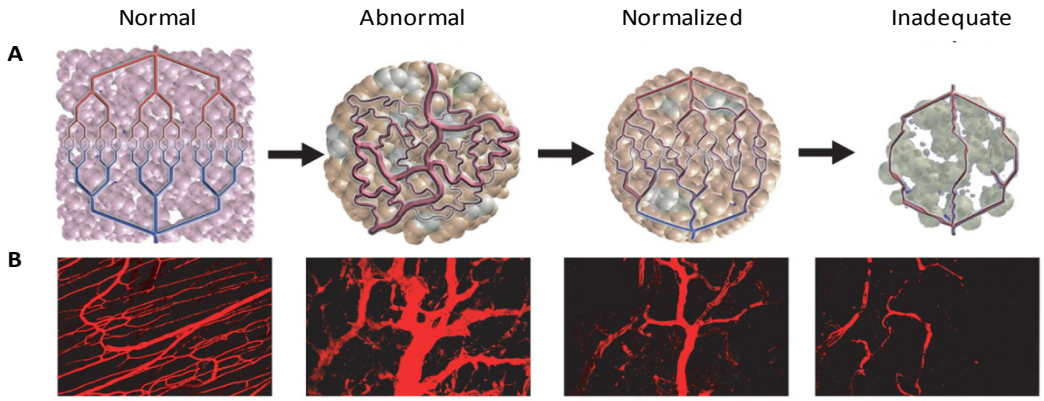


**Figure 16| Proposed role of vessel normalization in the response of tumors to anti-angiogenic therapy.** (A) Tumor vasculature is structurally and functionally abnormal. It is proposed that anti-angiogenic therapies initially improve both the structure and the function of tumor vessels; however, sustained or aggressive anti-angiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is inadequate for the delivery of drugs or oxygen. (B) Dynamics of vascular normalization induced by VEGFR2 blockade. On the left is a two-photon image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0, day 3, and day 5 after administration of VEGR2-specific antibody. Adapted (Jain, 2005).

Furthermore, since surgery, radiation therapy and chemotherapy may induce an up-regulation of VEGF expression by the tumor cell following its administration (Fan et al., 2008; Gorski et al., 1999; Gupta et al., 2002; Hormbrey et al., 2003; Riedel et al., 2004), VEGF-target therapy may also be beneficial by counteracting this response.

Due to the importance of VEGFA and its receptor VEGFR2 in angiogenesis (VEGFA alone is capable of initiating angiogenesis), several strategies to inhibit VEGF pathway have been developed. Bevacizumab (Avastin®), a monoclonal anti-VEGF antibody, and selective TKR inhibitors sunitinib (Sutent®) and sorafenib (Nexavar®), have conquered special attention in the treatment of metastatic colorectal cancer, advance renal cell carcinoma and hepatocellular carcinoma, respectively (Ellis and Hicklin, 2008; Ferrara and Kerbel, 2005; Siemann et al., 2005). Other VEGF receptor tyrosine kinase pathway inhibitors such as AG-013736 (Axitinib®) (Kelly and Rixe, 2010) and PTK787/ZK22584 (Vatalanib®) (Drevs et al., 2002), have also shown promising results in metastatic renal cell carcinoma and colorectal cancer patients, respectively (Ferrara and Kerbel, 2005).

VEGF-targeted therapy inhibits neovessel growth by blocking the activation of intracellular signaling pathways, and promotes EC apoptosis, since VEGF is also a mediator of different cell pro-survival pathways. Besides, VEGF also influences the chemotaxis of EPCs and the direct growth of tumor cells being, for that reason, expected a partial reduction in the incorporation of EPCs in the vasculature and a reduction of tumor growth (Ellis and Hicklin, 2008).

However, clinical experience has revealed that VEGF-targeted therapy often prolongs overall survival of cancer patients only by months, being metastasis the principal cause of death (Loges et al., 2009).

Consistent with the clinical observations (Grothey et al., 2008; Zhang et al., 2010), many studies have shown that VEGF-targeted therapies efficiently inhibited primary tumor growth in different biological models (reviewed in Ferrara and Kerbel, 2005). However, Ebos et al. (Ebos et al., 2009) shown that pre-treatment of healthy animals with VEGF inhibitors prior to the injection of tumor cells lead to the development of more aggressive metastasis, as well as in orthotopic tumor models lead to vaso-invasion

phenotype. These, apparently inconsistent, results might be explained by different hypothesis: (1) Replacement of VEGFA effects by other angiogenic pathways or other members of the VEGF family; (2) hypoxia, caused by exacerbated disruption of vessels, which can contribute to the selection of more resistant cancer cells; (3) Recruitment, by the tumors, of EPCs from the bone marrow; (4) co-option of existing vasculature; (5) production of pro-inflammatory (angiogenic) molecules due to the VEGF inhibitors-induction of a chronic "inflamed" state; and (6) facilitated intravasation of tumor cells in the destabilized vessels (Hillen and Griffioen, 2007; Loges et al., 2009).

Since VEGF-targeting therapy might not be enough to promote tumor inhibition, other strategies such as drugs blocking matrix break-down or the target of other angiogenic factors/receptors have also to be considered (e.g. cetuximab (Erbitux®), a monoclonal EGFR-antibody; interferon-$\alpha$ (Roferon®), a cytokine responsible for the inhibition of FGF2, IL8 and MMPs) (Carmeliet, 2005; Carmeliet and Jain, 2000; Siemann et al., 2000).

## 5. RADIOTHERAPY

Radiotherapy, either alone or combined with surgery and chemotherapy, is used as a treatment of choice for malignant tumors, characterized by uncontrolled growth and the ability of invading adjacent tissues and metastasize.

Ionizing radiation (IR) consisting of electromagnetic radiation (X-rays and $\gamma$-rays), is the type of radiation most commonly used in radiotherapy. Radiotherapy may be applied by two different methods: brachytherapy and external beam therapy. The first involves surgically implanting radioactive pellets into the tumor, and is often used to treat prostate cancer since the arrangement of critical structures in this region makes it difficult to use the second option without causing damage to these organs. The second method, external beam therapy, the most frequently applied, uses an external radiation source and forms a beam that is aimed at the patient according to a previous specified plan. Generally, for this standard treatment, mainly high energy photons are applied, generated by MV-linear accelerators (Joiner and van der Kogel, 2009).

Radiation treatment of human cancer, through external beam therapy, is classically delivered by fractionated schemes, consisting of a daily small dose (typically of 1.8 to 2.0 Gy in adults and 1.5 to 1.7 in children), repeated until a potentially curative tumor-specific dose has accumulated. Fractionated radiation has been favored because it affords protection of normal tissues relative to tumors. Normal cells appear to be more proficient in radiation damage repair during the inter-fractional intervals than tumor clonogens, which more likely accumulate IR-induced damages (Connell et al., 2004; Steel, 2002). Moreover, external photon beam radiotherapy is usually carried out with more than one shaped radiation beam in order to achieve a uniform dose distribution inside the target volume, minimizing the dose in the normal surrounding tissues (Steel, 2002). However, these surrounding healthy tissues will still be exposed to doses that increase gradually from 0 Gy to the maximal (Figure 17), and its biological effects must also be taken in consideration.

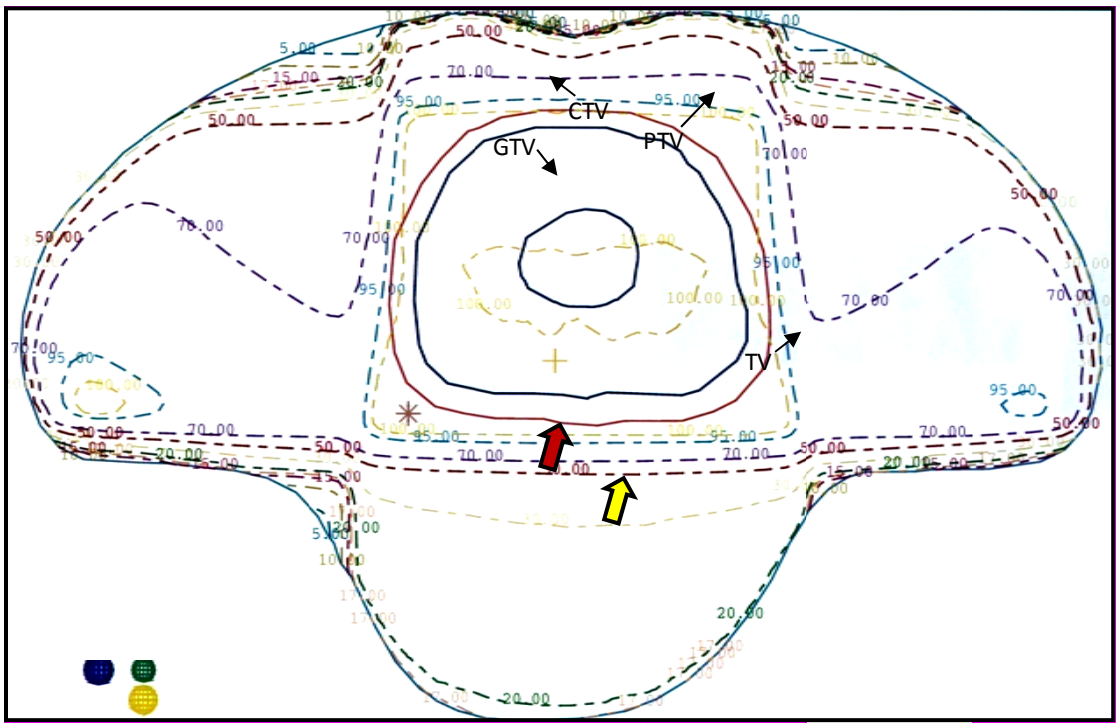


**Figure 17| Isodose curves on a pelvic axial slice.** Isodose (dashed) lines show the contour of the radiation levels in the anatomy and provide spatial information about the deposition of radiation. GTV (gross tumor volume) corresponds to the detectable tumor volume; CTV (clinical target volume) indicates the sum of GTV with volumes with expected subclinical spread; and PTV (planning target volume) is based in the CTV plus a safety margin for movements or deformations. TV (treatment volume) corresponds to the tissue that will receive the prescribed dose, thus the isodose curve of 100%. Red and yellow arrows indicate the isodose curves correspondent to the tissues that will receive 50% or 30% of the total daily dose (0.9-1.0 Gy or 0.5-0.6 Gy).

## 5.1. MOLECULAR BASIS OF IONIZING RADIATION RESPONSE

IR-induced transformations at the molecular level include signaling transduction pathway changes, mitochondrial changes, cell cycle, and apoptotic changes (Lehnert, 2007). Is the status of these signal transduction cascades that determines the cellular response to radiation. Although many of these can be mediated through DNA damage signals, other targets such as cell membrane and mitochondria can be primarily affected (Lehnert,

2007; Pawlik and Keyomarsi, 2004). However, DNA is the most radiation-sensitive biomolecule in the living tissue, and so, it is DNA damage what generally leads to the most observed macroscopic biological effects (Joiner and van der Kogel, 2009).

This damage might occur either directly or, in most cases, indirectly. Direct action in cell damage by IR occurs due to the ionization or excitation of atoms on key molecules of the biological system (e.g. DNA), leading to a chain of physical and chemical events that eventually produce a biological response by the inactivation or functional alteration of the molecule (Lehnert, 2007; Steel, 2002). Indirect action occurs mainly through the ionization of water (which represents 80% of the cell) and consequent formation of free radicals that can damage DNA (Lehnert, 2007; Steel, 2002). Although these free radicals created by the radiolysis of the water molecule have short lifetimes ($H^{\bullet}$ and $OH^{\bullet}$), oxygen can modify the reaction by enabling the creation of other free radicals with greater stability and longer lifetimes ($O_2^{\bullet -}$, H2O2, $^{\bullet}$OH, and $^{\bullet}$NO), enhancing cell damage by approximately 2.5 to 3.5 fold (Connell et al., 2004; Lehnert, 2007). On the other hand, tumor hypoxia leads to greater radioresistence, also by selecting more aggressive tumors (Connell et al., 2004).

The time scale involved between the breakage of chemical bounds and the biological effect may be from hours to years, depending on the type of damage (Figure 18) (Steel, 2002).

To give an idea to the scale of damage, 1 Gy of X-rays produces approximately 1000 single strand breaks (SSB), 40 double strand breaks (DSB) and 1000 altered bases (Joiner and van der Kogel, 2009). From these possible damages created by IR to DNA, is the number of DSB that better relates to cell killing (Connell et al., 2004). However, the number of induced lesions is far greater than those eventually leading to cell kill, which happens due to mechanisms of DNA repair: (1) Base-excision repair, for base damage and SSB; (2) homologous recombination (HR), for DSB only during the S phase; and (3) Non-homologous end-joining (NHEJ), for DSB in any phase of the cell cycle (Joiner and van der Kogel, 2009).

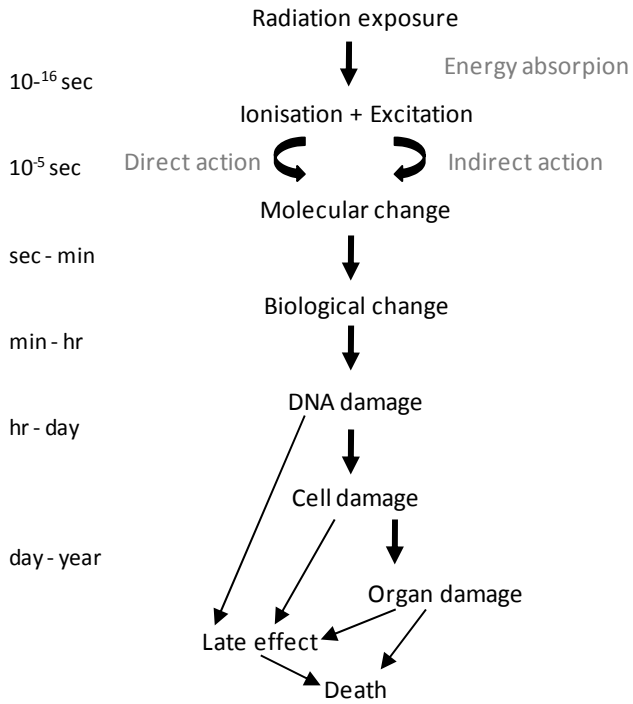


**Figure 18| Time-scale of the effects of radiation exposure.** Adapted (Steel, 2002).

After DNA damage by IR, a group of "sensor" proteins, that actively survey the genome for the presence of damage, signals these lesions to three main effectors pathways that together determine the outcome for the cell: (1) programmed cell death pathways that kill damaged cells, (2) DNA repair pathways that physically repair DNA breaks, and (3) pathways that temporary (or permanently) block the progress of cells through the cell cycle (Joiner and van der Kogel, 2009).

In summary, DSB detection in response to IR promotes the recruitment and activation of the MRN (Mre11/Rad50/NBS1) protein complex and ATM (ataxia telangiectasia mutated), which then phosphorylates H2AX (γH2AX) within only 5 to 30 minutes post-IR (Joiner and van der Kogel, 2009). This γH2AX sites then spread over relatively large chromatin regions causing the modification of the chromatin structure, allowing the access of other repair factors (H2AX can also be activated in response to DNA replication

defects in a mechanism independent of ATM and radiation-induced) (Joiner and van der Kogel, 2009). Besides H2AX, many other proteins act as substrates of ATM in response to DNA damage (e.g. p53, MDM2 and CHK2), acting as signals to trigger the various downstream effectors of DNA repair, namely by inducing the activation of cell checkpoints (Joiner and van der Kogel, 2009). Thus, DNA damage repair mechanisms, determine not only the sensitivity of cells to die following irradiation, but also the type of cell death that occurs, and the timing of cell death.

Variation of cellular radiosensitivity can be linked to the different cell cycle stages. There is a general tendency for cells in the S phase to be the most resistant and for cells in late G2 and mitosis to be the most sensitive. The reason for this variation seems to result from the DNA conformation, which facilitates the easier access of repair complexes in the S phase and the possible mechanisms of DNA repair: HR, which is error free (only in the S phase due to the availability of the undamaged sister template), or error prone NHEJ (Joiner and van der Kogel, 2009; Pawlik and Keyomarsi, 2004). This different radiosensitivity during the different stages of the cell cycle is also a cause for the use of fractionated schemes in radiotherapy since, by promoting the activation of several checkpoints, IR can retard the rate of progression of proliferating cell populations, causing cells to accumulate in the G2 phase and leading to a partial synchronization (Pawlik and Keyomarsi, 2004).

### 5.2. ROLE OF HYPOXIA IN RADIATION THERAPY

As previously mentioned, the presence of oxygen enhances ionizing radiation induced cell damage through the formation and stabilization of ROS with a longer life time (Connell et al., 2004; Lehnert, 2007). On the other hand, persistent severe hypoxia generally leads to the cellular activation of apoptotic pathways through the p53 pathway. However, about 60% of tumors are p53 mutated, resulting in a majority of cells resistant to hypoxic conditions, generally associated to a highly aggressive and metastatic potential (Chan and Giaccia, 2007; Lee and Bernstein, 1993).

Hypoxia inhibits the overall protein synthesis in an effort to preserve energy, selectively choosing which genes earn the priority to be made into proteins (e.g. VEGF). Thus, some HIF1 mediated transcripts are not immediately translated into protein, remaining in the cell in the form of stress granules. Upon radiation therapy, better oxygenated cells die and a temporary increasing tissue reoxygenation is achieved. At this point, the stress granules disaggregate, releasing HIF1-regulated mRNAs, which can then go on to be translated into proteins, promoting angiogenesis, tumor growth and upper invasion capacity (Dewhirst et al., 2008). Moreover, macrophages attracted to the dying tumor cells, become activated and release NO, which (in high levels) stabilizes HIF1, leading to the same outcome (Figure 19) (Dewhirst et al., 2008; Lee and Bernstein, 1993). Thus, it is important to consider that radiation-induced reoxygenation does not completely eliminate hypoxia and that there are oxygen gradients within the tumors and adjacent tissues.

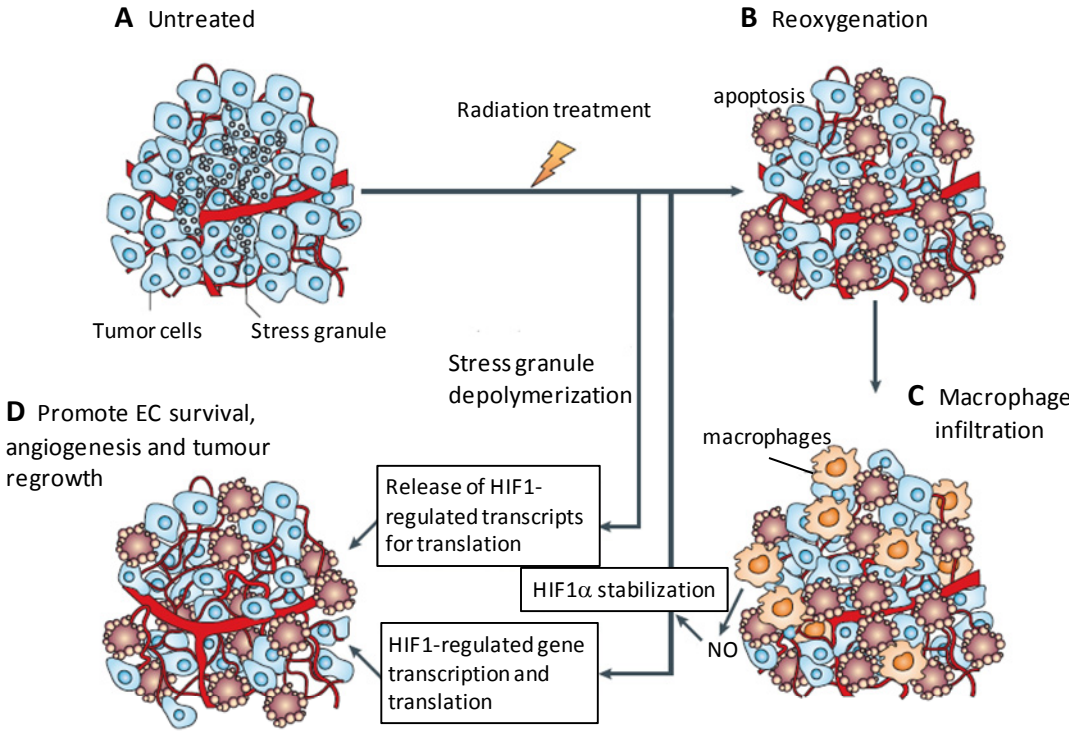

**Figure 19| Mechanisms for HIF1 up-regulation and consequences after radiation therapy.** A) Untreated tumors often have hypoxic sub-regions in which stress granules form containing HIF1-mediated transcripts. B) After radiation treatment, better oxygenated cells die and there is an increase in perfusion, leading to reoxygenation of the previously hypoxic cells; at this point stress granules disaggregate, releasing HIF1-regulated mRNAs that can then go on to be translated into protein. C) Macrophages are attracted to the dying tumor cells, become activated and release NO, which stabilizes HIF1$\alpha$. D) The increase in HIF1 activity increases the expression of pro-angiogenic factors, promoting EC survival, angiogenesis and tumor cell survival, proliferation and invasion. Adapted (Dewhirst et al., 2008).

## 5.3. UNEXPECTED EFFECTS OF RADIOTHERAPY IN BLOOD VESSELS AND METASTASIS

While radiotherapy has been classically viewed to exert its therapeutic effect by killing tumor cells, emerging evidence indicates that effect extends beyond cancer cell death.

The mechanisms behind IR response and the final outcome in tumor cells involve interactions between the tumor cells themselves and with their tumor-associated host cells (ECs, leukocytes, macrophages, fibroblasts, myofibroblasts, and nerve cells) (Barcellos-Hoff et al., 2005). These effects are not restricted to the irradiated cells present in the beam but also affect adjacent tissues, since they are able to communicate through cell junctions and soluble factors (Morgan, 2003).

IR changes the microenvironment, contributing to the anti-tumor effects of radiotherapy (Barcellos-Hoff et al., 2005). However, there are clinical and experimental observations indicating that IR might promote a metastatic behavior of cancer cells and that the irradiated host microenvironment might exert tumor-promoting effects (Barcellos-Hoff et al., 2005; Madani et al., 2008; von Essen, 1991). In 1991, von Essen (von Essen, 1991), considered a number of mechanisms for the pro-metastatic activity, including: (1) alteration of the cancer cells by direct action of IR, (2) indirect change of the metastatic site by abscopal effect, and (3) modulation of tissues harboring the primary tumor.

Recently, more studies have focused on the mechanisms by which IR activates cellular targets contributing to invasion and metastasis (Abdollahi et al., 2005; Garcia-Barros et al., 2003; McBride et al., 2004; Nozue et al., 2001). Exposure of cells to IR induces cascades of growth factors, cytokines and chemokines, activating multiple signal

transduction pathways by which signals move from the inside of the cell to the outside and reverse, therefore modulating several cellular functions in the tumor microenvironment and potential metastatic environments. By promoting the up-regulation of key molecules at certain doses within certain time frames, IR enhances the activities of tumor-associated host cells that support invasion and metastasis, including the ECs (inducing angiogenesis), leucocytes and macrophages (causing inflammation), myofibroblasts (initiating desmoplasia), osteoblasts and osteoclasts (establishing bone metastasis), and nerve cells (producing efferent growth and invasion-promoting molecules) (Madani et al., 2008).

Focusing in the vascular context, IR may exert its anti-angiogenic activity through its direct pro-apoptotic effect on ECs *via* ceramide pathway (Garcia-Barros et al., 2003), as well as a pro-angiogenic function through signals that are released by irradiated cancer cells (Abdollahi et al., 2005; Madani et al., 2008; McBride et al., 2004). Moreover, conditions able to induce EC apoptosis during the first hours after radiotherapy are frequently followed by stimulation, creating a biphasic response. In this context it is important to take into consideration that total dose and fraction can induce completely different effects (Madani et al., 2008). Thus, IR itself induces the production of pro-angiogenic molecules by the tumors, such as VEGF, MMP2, TGF$\beta$, FGF2, IL1R$\alpha$, IL10, IL13, IL4 and IL5 (Abdollahi et al., 2005; McBride et al., 2004), indirectly contributing to the vasculature protection (Figure 20). Furthermore, IR is also able to directly act on ECs promoting the same outcome, namely by the up-regulation of VRGFR2 (Abdollahi et al., 2003), NO pathway (Sonveaux et al., 2003), $\alpha v\beta 3$ integrin (Abdollahi et al., 2005), PECAM1 (Quarmby et al., 2000), IL8 (Meeren et al., 1997), pro-angiogenic CXC chemokines (Chang et al., 2009), FGF2 and PDGFB (Haimovitz-Friedman et al., 1995).

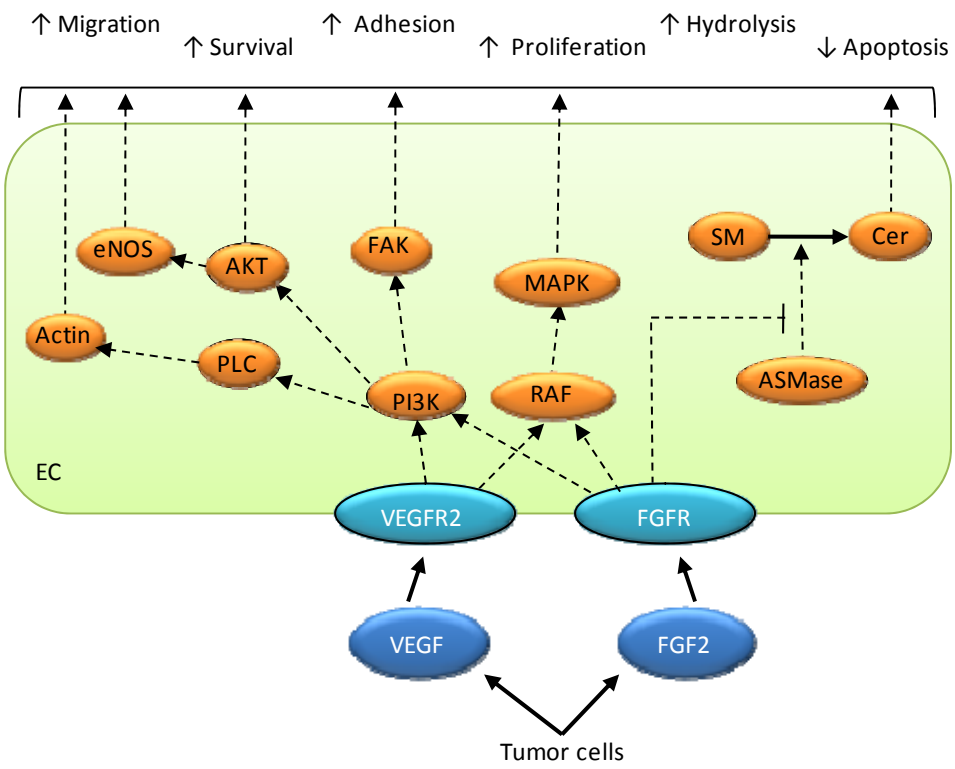


**Figure 20| Schematic representation of some major pro-angiogenic signaling from irradiated cancer cells to ECs.** At certain conditions, IR can induce the production of pro-angiogenic molecules by the tumor, which may activate ECs, promoting an angiogenic response. The diagram shows only some of the possibilities (for more detail see text). Adapted and modified (Madani et al., 2008).

## 5.4. IONIZING RADIATION COMBINED THERAPY

Although radiotherapy can be used to treat virtually all kinds of tumors, some are highly responsive to radiation (e.g. lymphomas) and others are typically very resistant (e.g. melanomas). Besides, treatment fails in a number of cases, not only due to metastatic spread but also because of local treatment site failure. This may happen due to tumor localization, size, inadequate supply (hypoxia) or genetic factors, such as p53 mutations, that may result in radioresistant cellular phenotypes (Pawlik and Keyomarsi, 2004). For

this reason, radiotherapy is often combined with other therapies, like surgery, chemotherapy and/or anti-angiogenic therapy, in an effort to improve cure rates.

Research during the last two decades has focused its attention on some classes of pharmacological agents for radiosensitization. Some of these agents, commonly used in a wide variety of tumor types are: paclitaxel (Taxol®), 5-fluorouracil (5-FU), gemcitabine (2',2'-difluorodeoxycytidine) and bevacizumab (Avastin®).

Paclitaxel is commonly used to treat breast, non-small cell lung, bladder, head and neck cancers (Wang et al., 2000). It binds and interferes with the normal function of microtubule growth by their hyper-stabilization, which results in the failure of chromosomes to segregate and thus, mitotic arrest (Blagosklonny and Fojo, 1999). Microtubules serve not only as the physical support for maintenance of the cell shape but also as an intracellular infrastructure for signal transduction pathways (Gundersen and Cook, 1999). So, the presence of paclitaxel will activate several signaling molecules that will induce, not only the cell cycle arrest, but also apoptosis pathways (reviewed in Wang et al., 2000). Thus, paclitaxel acts as a radiosensitizer by killing cancer cells through a p53-indeendent pathway (p53 mutated cells are resistant to radiotherapy), and by blocking cells at the radiosensitive G2/M phase of the cell cycle (Gupta et al., 1997; Wang et al., 2000). Moreover, the maximum radiosensitization by paclitaxel, is not necessarily at the time of highest mitotic arrest, but may occur 1 day after its administration (Milas et al., 1994), suggesting the involvement of other mechanisms, such as the reoxygenation of hypoxic tumor cell. In fact, some studies, have reported an anti-angiogenic activity of paclitaxel through the down-regulation of VEGF, which can promote normalization in tumor vasculature and thus, a higher oxygenation level (Grant et al., 2003; Lau et al., 2004; Lau et al., 1999; Naumova et al., 2006).

Gemcitabine is used in non-small cell lung, pancreas, bladder and breast cancers (Doyle et al., 2001; Pauwels et al., 2003) and 5-FU in the treatment of head and neck, rectal, breast, esophagus, and pancreatic tumors (Showalter et al., 2008). Gemcitabine and 5-FU are pyrimidine analogues (antimetabolites which mimic the structure of metabolic pyrimidines). In summary, they are transformed by phosphorylation inside the cell into

different cytotoxic metabolites which are then incorporated into the DNA or RNA, finally inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. In addition, gemcitabine also inhibits the ribonucleotide reductase activity, causing depletion of deoxynucleotide thriphosphates necessary to the DNA synthesis (Doyle et al., 2001). The main mechanism related to the induction of a more radiosensitive phenotype in tumor cells seems to be its ability to kill cells in the S phase, which generally show a higher resistance to radiation therapy (Lawrence et al., 2003; Pauwels et al., 2003). Moreover, additional mechanism have been described, such as the interference of gemcitabine with the HR mechanism of DNA repair after radiation-induced DSB (Wachters et al., 2003), or the capacity of both drugs to exert anti-angiogenic actions. Besides its cytotoxic effects in proliferating ECs (tumor vasculature), gemcitabine seems to be also involved in the down-regulation of VEGF (Kuwahara et al., 2004) and, as 5-FU, in the induction of TSB1 (Laquente et al., 2008; Ooyama et al., 2008).

Bevacizumab is a monoclonal antibody that binds to and inhibits the biologic activity of VEGF. In combination with intravenous 5-FU chemotherapy, it is indicated for the treatment of patients with metastatic carcinoma of the colon or rectum (Shannon and Williams, 2008). Although less commonly used in the clinical practice, there are also some studies indicating the use of bevacizumab in combination with paclitaxel in the treatment of breast cancer, in combination with paclitaxel and carboplatin for non-squamous non-small cell lung cancer, and in **c**ombination with interferon-$\alpha$ for metastatic renal cell carcinoma (information from industry in http://www.avastin.com/avastin/hcp/index.html). As discussed before, instead of obliterating tumor blood vessels, anti-angiogenics tend to destroy immature vessels, inducing an organizational and functional normalization and, consequently, an improvement in oxygen delivery, which will contribute to radiotherapy's success.

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# II. OBJECTIVES

This chapter specifies the main objectives of the research proposal that led to the work presented in this thesis.

## OBJECTIVES

Angiogenesis, the formation of new vessels from pre-existing ones, is a physiological process orchestrated by a variety of pro- and anti-angiogenic signals. However, their imbalance, either promoting excessive or insufficient angiogenesis, can lead to disease.

Therefore, the possibility of modulating blood vessel growth with anti-angiogenic therapy and therapeutic angiogenesis has become an area of vast interest and it has been proposed for the treatment of cancer and ischemic diseases, respectively.

Radiotherapy is one of the most important treatment options in cancer. However, recent studies suggest that doses of ionizing radiation (IR) delivered inside the tumor target volume during fractionated radiotherapy, can promote tumor invasion and metastasis. Importantly, in addition to the tumor target volume, the surrounding healthy tissues will also be exposed to doses lower than the therapeutic ones. The biological effects of these low doses of IR remain largely undetermined.

The overall goal of the work presented in this thesis was to investigate the effects of these low IR doses on angiogenesis. Therefore, instead of focusing on the endothelial cells located in the tumor area, which may survive the daily dose radiation and undergo the angiogenic phenotypic shift, we focused on the vasculature that surrounds the tumor and receives relatively low doses of IR.

Firstly, we aimed to evaluate if low IR doses were able to activate the vasculature inducing a pro-angiogenic response.

Secondly, considering the tremendous relevance of angiogenesis in cancer progression, we questioned whether low-dose IR could contribute to tumor re-growth and metastasis.

Thirdly, we aimed to identify the molecular targets and cellular mechanisms whereby low-doses of IR induce a pro-angiogenic response.

Altogether, we expected to provide a rationale basis to improve current radiotherapy protocols by better understanding the mechanisms that contribute to the potential pro-metastatic effect of IR.

Finally, the observation that low doses of IR induce angiogenesis *in vivo*, allows a new perspective: may low IR doses be used in the future as a new strategy for therapeutic angiogenesis? With this objective, we investigated if low-dose IR could be used synergistically to vasoprost®, commonly used in the treatment of peripheral arterial disease, in order to potentiate its pro-angiogenic effect.

# III. RESEARCH WORK:

## Low Doses of Ionizing Radiation Promote Tumor Growth and Metastasis by Enhancing Angiogenesis

Inês Sofia Vala, Leila R. Martins, Natsuko Imaizumi, Raquel J. Nunes, José Rino, François Kuonen, Lara M. Carvalho, Curzio Ruëgg, Isabel Monteiro Grillo, João Taborda Barata, Marc Mareel, Susana Constantino Rosa Santos

Part of the research work contained in this chapter was published in: Sofia Vala et al. (2010) Low Doses of Ionizing Radiation Promote Tumor Growth and Metastasis by Enhancing Angiogenesis. PLoS ONE 5(6): 11222. The remaining experiments represent complementary data to the already published article and ongoing work.

**INTRODUCTION OF THE CHAPTER**

Radiotherapy is widely used to treat human cancer. However, patients with locally recurring tumors after radiotherapy have an increased risk of metastatic progression and poor prognosis.

It is generally assumed that tumor progression toward metastasis, during or after therapy, is due to the appearance of resistant tumor cells. However, there are evidences showing that radiotherapy is also able to change the tumor microenvironment, interfering at this level. While some studies indicate that these changes might contribute to the anti-tumor effects of radiotherapy (Barcellos-Hoff et al., 2005), there are also clinical and experimental observations attesting that, under certain circumstances, the irradiated stroma might exert tumor-promoting effects (Barcellos-Hoff et al., 2005; Madani et al., 2008; von Essen, 1991).

Tumor vessels provide the primary tumor with oxygen and nutrients and are essential for its growth and survival, but also favor metastasis by facilitating tumor cell entry into circulation. Therefore, many studies have focused on the mechanisms by which ionizing radiation (IR) is able to activate the tumor vasculature stimulating angiogenesis, and on the contribution of the irradiated endothelial cells (ECs) to invasion and metastasis after radiotherapy.

While some authors have demonstrated that IR directly acts on ECs promoting the up-regulation of several pro-angiogenic targets, such as VEGFR2 (Abdollahi et al., 2003), NO pathway (Sonveaux et al., 2003), $\alpha v\beta 3$ integrin (Abdollahi et al., 2005), PECAM1 (Quarmby et al., 2000), IL8 (Meeren et al., 1997), pro-angiogenic CXC chemokines (Chang et al., 2009), FGF2 and PDGFB (Haimovitz-Friedman et al., 1995), others emphasized its indirect effects mediated by the action of IR-induced tumor release molecules (i.e. MMP2, VEGF, MMP2, TGFβ, FGF2, IL1R$\alpha$, IL10, IL13, IL4 and IL5), also leading to the vasculature's activation (Abdollahi et al., 2005; McBride et al., 2004). Accordingly, anti-angiogenic approaches enhance anti-tumor effects of IR (Gorski et al., 1999; Lee et al.,

2000; Mauceri et al., 1998). These finding are not in conflict with the concept that the cytotoxic effects of radiation on ECs contribute to anti-tumor treatment (Abdollahi et al., 2003; Garcia-Barros et al., 2003; Paris et al., 2001), but suggest that at certain doses and time frames, IR might enhance the formation of new blood vessels, thus favoring metastatic spreading.

External x-ray radiotherapy is usually carried out in daily fractionated schemes (typically of 1.8 to 2.0 Gy in adults), using more than one convergent beam to achieve a dose uniformly distributed throughout the target tissue, and minimizing the exposure of the normal surrounding tissues. Most of the studies on the effect of IR on target tissues have focus on the therapeutic doses that can be observed inside the tumor volume during fractionated radiotherapy. However, during each radiotherapy session, the healthy tissues surrounding the tumor area are also exposed to lower doses of IR, which range from 100% to 0% of the daily small dose delivered to the tumor mass. The molecular and cellular effects that these low IR doses have on the vasculature that surrounds the tumor remain to be determined.

Our goal was to investigate the effects of these low IR doses on angiogenesis and their contribution to tumor re-growth, invasion and metastasis after radiotherapy. Our work focused, in an innovative way, in the vasculature that surrounds the tumor and receives relatively low doses of IR.

To study the effects of low-dose IR, we used two EC types: lung human microvascular endothelial cells (HMVEC-L) and human umbilical vein endothelial cells (HUVEC). We showed that IR doses of 0.8 Gy or lower are able to promote a pro-migratory response *in vitro*, conferring a pro-angiogenic advantage.

In addition, we showed that this low-dose IR accelerates *in vivo* embryonic angiogenic sprouting during zebrafish development, and promotes adult angiogenesis during zebrafish fin regeneration and in the murine matrigel assay.

We also investigated the contribution of the low-dose IR to tumor re-growth and metastasis after radiotherapy, using two different mouse models: a leukemia and a

metastatic breast cancer model. With these experiments, we found that low doses of IR promote tumor growth and metastasis in a VEGFR-dependent manner.

Additionally, we used p53 mutated $BRAF^{V600E}$ zebrafish model to study the effect of low-dose IR in melanoma development. According to our preliminary data, irradiated animals seem to present a higher percentage of detectable melanomas as well as bigger tumors. However, further investigation is required in order to confirm our results.

Together, our *in vitro* and *in vivo* results indicate that these low IR doses promote angiogenesis and this effect may have a strong impact in tumor burden and metastasis. Therefore, our findings provide novel insights into the mechanisms involved in the pro-metastatic effects of IR, and add a clinically relevant element to the determination of dosimetry in radiotherapy.

In order to identify the mechanisms by which low doses of IR induce a pro-angiogenic response, we investigated the gene expression profile of irradiated and unirradiated HMVEC-L. Therefore, we performed the Affymetrix GeneChip® human gene 1.0 ST array. We have already selected some targets that are currently being analyzed and we expect to identify the genes and the mechanism involved in the angiogenic phenotypic shift after IR.

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# Low Doses of Ionizing Radiation Promote Tumor Growth and Metastasis by Enhancing Angiogenesis

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## Abstract

Radiotherapy is a widely used treatment option in cancer. However, recent evidence suggests that doses of ionizing radiation (IR) delivered inside the tumor target volume, during fractionated radiotherapy, can promote tumor invasion and metastasis. Furthermore, the tissues that surround the tumor area are also exposed to low doses of IR that are lower than those delivered inside the tumor mass, because external radiotherapy is delivered to the tumor through multiple radiation beams, in order to prevent damage of organs at risk. The biological effects of these low doses of IR on the healthy tissue surrounding the tumor area, and in particular on the vasculature remain largely to be determined. We found that doses of IR lower or equal to 0.8 Gy enhance endothelial cell migration without impinging on cell proliferation or survival. Moreover, we show that low-dose IR induces a rapid phosphorylation of several endothelial cell proteins, including the Vascular Endothelial Growth Factor (VEGF) Receptor-2 and induces VEGF production in hypoxia mimicking conditions. By activating the VEGF Receptor-2, low-dose IR enhances endothelial cell migration and prevents endothelial cell death promoted by an anti-angiogenic drug, bevacizumab. In addition, we observed that low-dose IR accelerates embryonic angiogenic sprouting during zebrafish development and promotes adult angiogenesis during zebrafish fin regeneration and in the murine Matrigel assay. Using murine experimental models of leukemia and orthotopic breast cancer, we show that low-dose IR promotes tumor growth and metastasis and that these effects were prevented by the administration of a VEGF receptor-tyrosine kinase inhibitor immediately before IR exposure. These findings demonstrate a new mechanism to the understanding of the potential pro-metastatic effect of IR and may provide a new rationale basis to the improvement of current radiotherapy protocols.

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## Introduction

Radiotherapy is a widely used local treatment for malignant tumors, characterized by uncontrolled growth and the ability of invading adjacent tissues and metastasize. While radiotherapy has been classically viewed to exert its therapeutic effect by killing tumor cells, emerging evidence indicates that effects extend beyond cancer cell death. Ionizing radiation (IR) changes the microenvironment, contributing to anti-tumor effects of radiotherapy [1]. However, there are clinical and experimental observations indicating that IR might promote a metastatic behavior of cancer cells and that the irradiated host microenvironment might exert tumor-promoting effects [1,2]. Therefore, a careful analysis of the putative tumor-promoting and pro-metastatic effect of IR is imperative, as radiotherapy is an essential part of cancer treatment. Several tumor-associated host cells including endothelial cells, leukocytes, macrophages, fibroblasts, myofibroblasts and nerve cells populate the tumor microenvironment. Recently, different studies have focused on the mechanisms by which IR activates cellular targets potentially contributing to invasion and metastasis [3,4,5,6]. Doses of IR causing such stimulating effects are classically delivered inside the tumor target volume in daily small fractions in order to limit damage of healthy tissues and until a potentially curative dose has accumulated inside the tumor volume. Furthermore, the delivery in small fractions and the isodose distributions of external beam radiotherapy result in lower doses of IR outside the tumor target volume. The biological effects of these low doses of IR on the healthy tissue

surrounding the tumor area, and in particular on the vasculature remain largely to be determined.

Here we report that, *in vitro*, IR doses lower than 0.8 Gy do not cause cell cycle arrest or apoptosis. We found that low-dose IR led to the phosphorylation of several cellular proteins including VEGF receptor-2 (VEGFR-2). By activating VEGFR-2, low-dose IR enhances endothelial cell migration and prevents endothelial cell death promoted by the VEGF-neutralizing antibody bevacizumab. Also, under hypoxic conditions low-dose IR upregulates VEGF. We further show that, in zebrafish, low-dose IR accelerates sprouting angiogenesis during development and enhances the angiogenic response during caudal fin regeneration. Using different mouse models, we show that low-dose IR promotes angiogenesis resulting in accelerated tumor growth and metastasis formation in a VEGFR-dependent manner.

These observations provide novel insights into the mechanisms involved in the pro-metastatic effect of IR and open novel therapeutic perspectives for the improvement of current radiotherapy protocols.

## Results

### Low-dose IR promotes endothelial cell migration without causing cell cycle arrest or apoptosis

To investigate the biological effects of low doses of IR on endothelial cells, we exposed lung human microvascular endothelial cells (HMVEC-L) to doses lower than 2.0 Gy and assess effects on cell proliferation, survival and migration. We found that 0.5 and 0.8 Gy did not modulate HMVEC-L proliferation, while doses higher than 1.0 Gy caused a significant decrease of their proliferation rate (Figure 1A). Accordingly, detailed analysis of cell cycle profile did not reveal any significant changes in phase distribution in cells irradiated with 0.5 or 0.8 Gy (Figure 1B). The impact of low-dose IR on HMVEC-L survival was also evaluated. No effect on cell viability was detected at doses up to 1.0 Gy, whereas higher doses (i.e. 10.0 and 20.0 Gy) caused increased cell death (Figure 1C). Moreover, we assessed the migration of HMVEC-L exposed to low-dose IR by the scratch wound healing assay. Doses of 0.5 and 0.8 Gy stimulated HMVEC-L migration and wound closure, whereas cells irradiated with 1.0 Gy presented a migration rate similar to non-irradiated controls (Figure 1D). To examine whether low-dose IR nevertheless induced DNA double-strand breaks we monitored H2AX phosphorylation [7]. As early as 30 min after irradiation the γ-H2AX foci were detected in a dose dependent manner in the nuclei of irradiated cells (Figure S1). At 12 h post-irradiation, γ-H2AX was no longer detectable in cells irradiated at low doses reflecting effective DNA double-strand break repair.

Taken together these results indicate that doses of 0.5 or 0.8 Gy promote endothelial cell migration and, in spite of induction of DNA double-strand breaks they do not impinge on endothelial cell proliferation or survival.

### Low-dose IR activates the PI3K/Akt and MEK/ERK signaling pathways and prevents apoptosis induced by PI3K or MEK inhibition

Next, we investigated whether low-dose IR could activate intracellular signaling pathways associated with cell survival and migration. To this end, HMVEC-L were exposed to 0.5 Gy and global protein phosphorylation was analysed. A transient increase in tyrosine phosphorylation of multiple proteins was evident 5 min post-irradiation, but no longer detected after 10 min (Figure 2A and 2B), suggesting the rapid triggering of tyrosine kinase activity upon irradiation. Next, we evaluated the activation of PI3K/Akt and MEK/ERK signaling pathways. We observed a slight but significant increase in phosphorylation of Akt and ERK upon exposure to 0.1, 0.3 or 0.5 Gy (Figure 2C). To assess the functional relevance of IR-induced activation of Akt and ERK, HMVEC-L cultured in the presence of specific inhibitors of PI3K (Ly294002) or MEK (U0126) were exposed or not to 0.1, 0.3 or 0.5 Gy. Treatment with either of these inhibitors decreased HMVEC-L viability, although to different extents. Remarkably, low-dose IR treatment significantly protected HMVEC-L from death induced by the signaling inhibitors (Figure 2 D).

### Low-dose IR promotes endothelial cell migration and protects microvasculature from bevacizumab-induced cell death by activating VEGFR-2

The previous results incited us to test the effect of low-dose IR on HMVEC-L death induced by the inhibition of VEGF, a main survival factor for endothelial cells and a target for anti-angiogenic therapies. HMVEC-L were incubated with bevacizumab (Avastin®), a monoclonal antibody that inhibits the biologic activity of VEGF, before exposure to low-dose IR. Bevacizumab treatment reduced HMVEC-L viability and low-dose IR significantly protected against bevacizumab-induced death (Figure 3A). The same low-dose IR did not protect against cell death induced by different chemotherapeutic agents (Figure S2). Based on its ability of low-dose IR to induce protein phosphorylation, we hypothesized that low-dose IR counteracted the pro-apoptotic effect of bevacizumab by activating VEGF receptor-2 (VEGFR-2). Consistent with this hypothesis, we found a significant increase in VEGFR-2 phosphorylation in response to 0.1 Gy (Figure 3B). To test the functional impact of VEGFR-2 activation, HMVEC-L were cultured in the presence of bevacizumab and treated or not with a VEGFR-2 tyrosine kinase inhibitor (TKI) prior to 0.1 Gy of IR. We found that the protective effect was completely abrogated by treatment with VEGFR-2 TKI (Figure 3C). Molecularly, we demonstrated that the VEGFR-2 TKI, in contrast to bevacizumab, inhibited VEGFR-2 phosphorylation induced by 0.1 Gy. (Figure 3D). Since VEGFR-2 activation has been reported to promote endothelial cell migration [8], we investigated whether low-dose IR promotes endothelial cell migration (Figure 1D) through the activation of VEGFR-2. HMVEC-L were treated or not with VEGFR-2 TKI prior to 0.5 Gy of IR and cell migration was assessed by the scratch wound healing assay. We found that the pro-migratory effect promoted by low dose IR was significantly abrogated by treatment with VEGFR-2 TKI (Figure S3).

Taken together these results demonstrate that low-dose IR enhances endothelial migration and prevents endothelial cell death promoted by the VEGF-neutralizing antibody bevacizumab, by inducing VEGFR-2 phosphorylation.

### Low-dose IR induces VEGF expression in endothelial cells under conditions mimicking hypoxia

We next asked the question whether low-dose IR modulates the expression of VEGF in endothelial cells. Under normoxic conditions, endothelial cells constitutively express very low levels of VEGF [9]. Low-dose IR did not induce VEGF expression under normoxic conditions (data not shown). However, in HMVEC-L cultures first exposed to cobalt chloride ($CoCl_2$), which mimics hypoxic conditions, and subsequently treated with 0.3 Gy of IR, we observed a greater increase of VEGF mRNA levels compared to cells treated with $CoCl_2$ alone (Figure 4A). Consistent with increase VEGF mRNA expression, we also observed increased levels of secreted VEGF protein (Figure 4B).

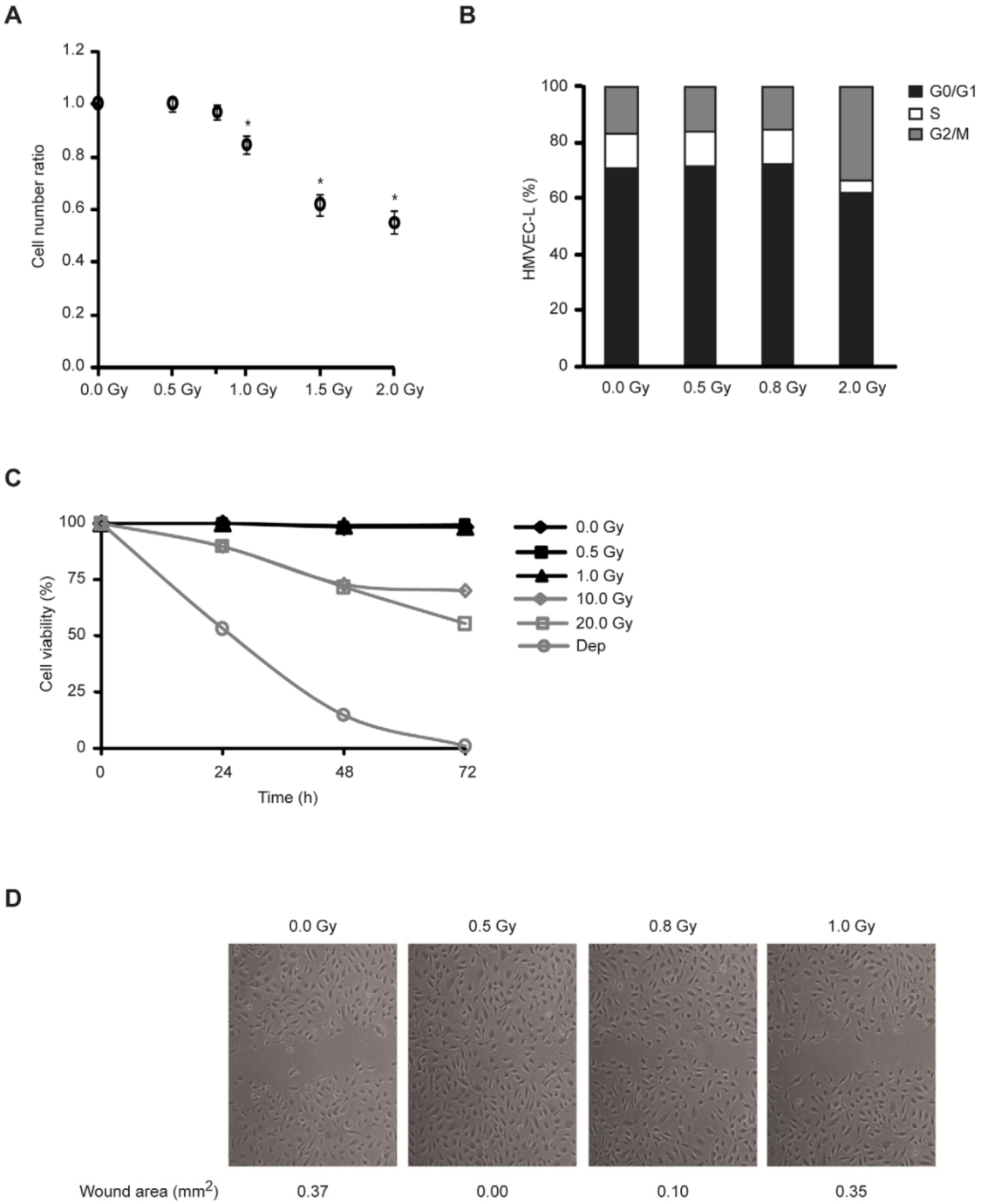


**Figure 1. Low-dose IR promotes endothelial cell migration without causing cell cycle arrest or apoptosis.** (A) HMVEC-L were plated at equal densities and, after 12 h, left untreated or exposed to 0.5, 0.8, 1.0, 1.5 and 2.0 Gy. After 72 h, the cells were counted using a Nucleocounter. The values (means ± s.d.) represent the ratio between cell number of irradiated and non-irradiated conditions and are derived from four independent experiments. * $P<0.02$. (B) HMVEC-L were exposed or not to 0.5, 0.8 and 2.0 Gy. Cell cycle profiles were assessed by flow cytometric analysis after 72 h of culture. Data are representative of three independent experiments. (C) The percentage of apoptotic cells was determined by flow cytometry at the indicated time. Cells cultured without serum (Dep) were used as cell death control. Values are given as the percentage of viable cells (Annexin V, PI negative) remaining in culture. Data are shown as mean in triplicate culture and are representative of three independent experiments. (D) Confluent monolayers of HMVEC-L

were subjected to *in vitro* wound healing and exposed or not to 0.5, 0.8 or 1.0 Gy. Photographs were taken immediately (not shown) and 9 h after wounding. Quantification of the wound area (in $mm^2$) is presented below the images. Data are representative of five independent experiments.
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These data indicate that low-dose IR enhances VEGF expression in endothelial cells under hypoxia-mimicking conditions.

## Low-dose IR accelerates angiogenic sprouting during zebrafish embryonic development and enhances angiogenesis during zebrafish fin regeneration

To ascertain whether low-dose IR was able to enhance angiogenesis *in vivo*, we used the transgenic zebrafish fli1:EGFP, which allow intra vital imaging of the vasculature through fli1 gene promoter-driven expression of GFP in endothelial cells. The first blood vessels formed in zebrafish embryo arise through vasculogenesis. The smaller vessels are then formed through angiogenic sprouting from these large vessels [10]. Fli1:EGFP zebrafish embryos were exposed to 0.5 Gy IR 3 days post-fertilization (dpf), a time of angiogenic sprouting, and the formed vasculature was examined 7 dpf. All non-irradiated embryos (n = 70) showed vertical, parallel-oriented and regularly spaced Sub-Intestinal Vessels (SIV), while 52 out of 70 irradiated embryos (74%), showed more and irregularly-shaped vessels between larger SIV (Figure 5A). We also found that these numerous vessels were formed by sprouting angiogenesis (Figure 5B). At a later time point (17 dpf), SIV vascular pattern was identical in non-irradiated embryos (Figure 5C), indicating that low-dose IR accelerated angiogenic sprouting without causing excessive vessel formation.

To test whether low-dose IR also stimulated angiogenesis in adult animals, adult fli1:EGFP zebrafish were subjected to caudal fin amputation at mid-fin level, exposed or not to 0.5 Gy of IR and then allowed to recover. Zebrafish caudal fin growth is accompanied by active angiogenesis [11]. Results showed a striking increase in the inter-ray vessel density in the irradiated regenerating fin (Figure 5D).

Taken together, these data demonstrate that low-dose IR accelerates sprouting angiogenesis from SIV during zebrafish embryonic development, and enhances the angiogenic response during fin regeneration.

## Low-dose IR promotes angiogenesis in the murine Matrigel plug assay

To test whether low-dose IR also stimulates angiogenesis in mammals, we used the murine Matrigel plug angiogenesis assay [12]. Athymic Swiss *nu/nu* mice were locally irradiated (lower-right back side) with 0.3 Gy, and growth-factor-depleted Matrigel plugs supplemented with fibroblast growth factor 2 (FGF2) were implanted 24 h later, within irradiated or non-irradiated tissue (the contralateral non-irradiated side was used as matched controls). Matrigel plugs were analyzed 5 days later, a time point where angiogenesis is naturally heterogeneous and not fully formed, thereby facilitating the detection of stimulatory effects, while at later times (7 to 10 d) angiogenesis is more homogenous and robust, which may mask stimulatory effects. Consistently, the degree of angiogenesis ranged from high to low across different plugs. Tissue pre-irradiation enhanced angiogenesis, albeit to different extents in the individual mice (Figure 6A and 6B).

These data demonstrate that low-dose IR significantly promotes angiogenesis in adult mice.

## Low-dose IR promotes acceleration of tumor growth and metastasis in a VEGF receptor-dependent manner

We asked whether low-dose IR had an impact in promoting tumor growth and dissemination. Six weeks-old NOD-SCID mice were irradiated or not with 0.3 Gy and subsequently injected intravenously with MOLT-4 cells. After 14 d, irradiated mice showed a significant increase in MOLT-4 tumor burden when compared to non-irradiated animals (Figure 7A).

Next, we investigated whether VEGFR activation could be involved in the acceleration of tumor growth promoted by low-dose IR. To this purpose, we administered the VEGF receptor tyrosine kinase inhibitor, PTK/ZK, 2h before irradiation with 0.3 Gy, and MOLT-4 cells were injected 24 h later. At this time point PTK/ZK is no longer active *in vivo*, thereby excluding possible direct effects on leukemia cells. After 14 d, leukemia burden in irradiated mice treated with PTK/ZK was significantly lower than in irradiated animals treated with vehicle only, and was similar to the tumor burden observed in non-irradiated mice (Figure 7B).

We also investigated whether low-dose IR could also promote tumor metastasis by using the 4T1 orthotopic implantation mouse model of spontaneous breast cancer metastasis to the lung [13]. NOD-SCID IL-2Rgamma$^{null}$ mice were treated or not with PTK/ZK and 2 h later exposed or not to 0.3 Gy, whole body IR. After 22 h, $5\times10^4$ 4T1 cells were injected into the 4$^{th}$ mammary fatpad of the mice in all four groups. Non-irradiated mice treated with PTK/ZK were also used as a control, to ensure that the single PTK/ZK treatment administered 24 h before 4T1 cell injection would not affect tumor growth. Twenty days post-transplantation, no difference in primary tumor growth between the groups of mice was found (data not shown). Primary tumors were surgically removed and a significantly higher bioluminescent signal was detected in the chest region of the irradiated group when compared to other groups, where the signal was similar (Figure 7C). By Computed Tomography (CT) scan, we found more nodules in both lobes of irradiated lungs, when compared to other groups (Figure 7D). To confirm the bioluminescent and CT results, lungs were analysed by histology at necropsy. Accordingly, number of metastasized 4T1 cells in the lungs of experimental mice groups was similar, except in the irradiated one, in which we observed an increased number and size of lung metastases (Figure 7E).

Taken together, these results indicate that low-dose IR promotes acceleration of tumor growth and enhances the metastatic spreading in a VEGFR-dependent manner.

# Discussion

There is growing evidence in the literature indicating that IR may have pro-metastatic effects [2]. It is generally assumed that tumor progression towards metastasis, during or after therapy, is due to the appearance of resistant tumor cells. However, several studies indicate that radiotherapy also rapidly and persistently alters the tumor microenvironment. IR induces the production of pro-angiogenic molecules [4], which may activate the microenvironment, including the vasculature. In accordance, anti-angiogenic approaches enhance anti-tumor effects of IR [14,15,16,17]. On the other hand, while adjuvant radiotherapy significantly improves local tumor control, recurrences within a preirradiated field are associated with higher risk of local invasion and metastasis and with poor prognosis [18,19,20].

Furthermore, the tissues that surround the tumor area are also exposed to low doses of IR that are lower than those delivered inside the tumor mass, because external radiotherapy is delivered

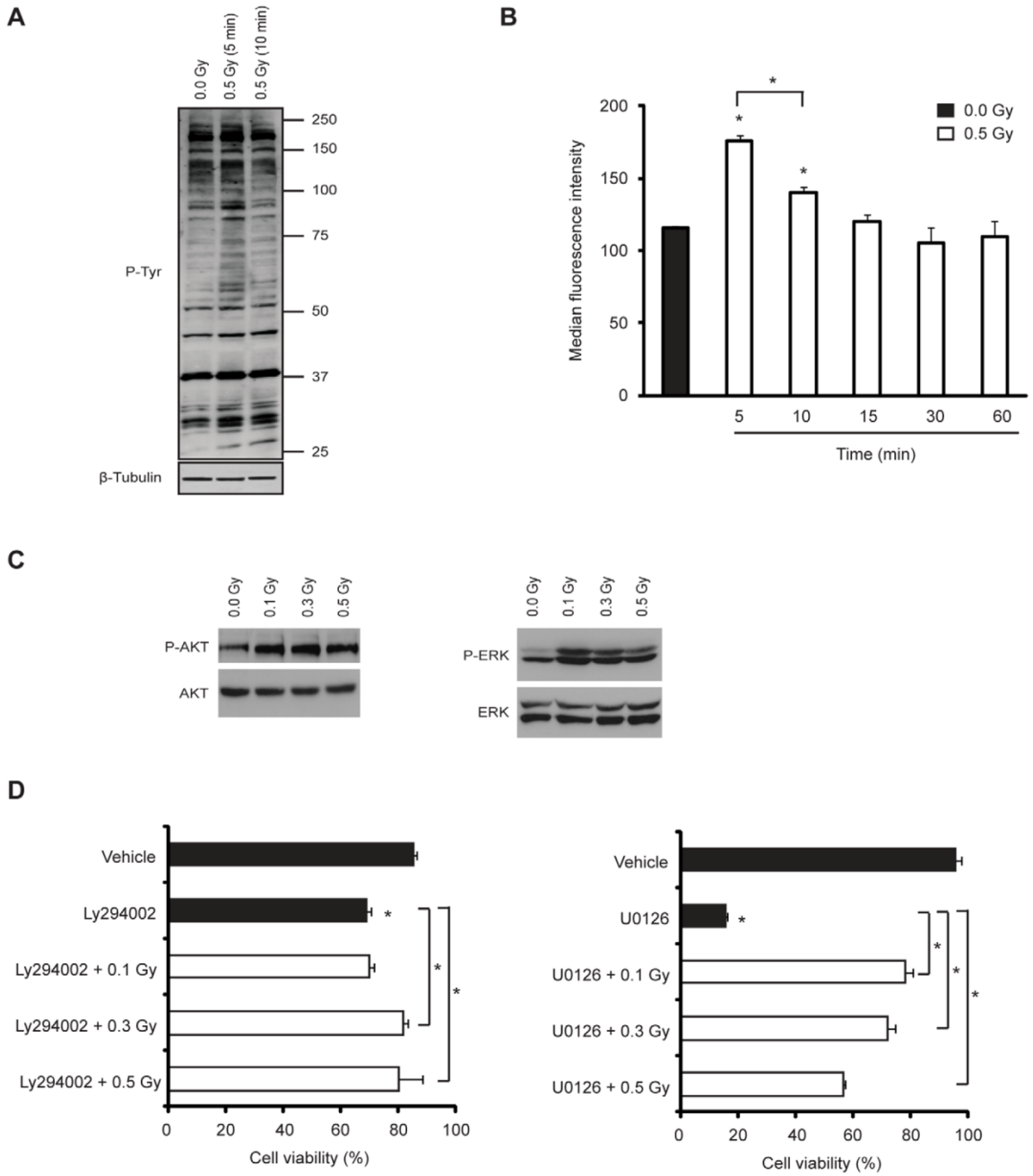


**Figure 2. Low-dose IR activates PI3K/Akt and MEK/ERK pathways and prevents apoptosis induced by their inhibition.** (A and B) HMVEC-L were exposed or not to 0.5 Gy and incubated for the time indicated. (A) Representative blots from three independent experiments. Top, Tyrosine phosphorylation Western blot; Bottom, β-tubulin Western blot to verify equal sample loading. (B) Tyrosine phosphorylation levels were assessed by flow cytometry. The values (means ± s.d.) represent the ratio between median fluorescence intensity of irradiated cells and non-irradiated cells and are derived from four independent experiments. (C) Representative blots from three independent experiments. Western blot analysis of total-, phospho-Akt (P-AKT) (left) and total-, phospho-ERK (P-ERK) (right) of HMVEC-L exposed or not to 0.1, 0.3 or 0.5 Gy. The levels of Akt and ERK phosphorylation were assessed after 5 or 60 min post-irradiation, respectively. (D) HMVEC-L were incubated in the presence or absence of a specific inhibitor of PI3K (Ly294002-50 μM) (left) or MAPK (U0126-10 μM) (right) and then exposed or not to 0.1, 0.3 or 0.5 Gy. Cells cultured with vehicle alone were used as a control. Cells were double stained with Annexin-V and propidium iodide at 36 h post-irradiation. The percentage of apoptotic cells was assessed by flow cytometry. The values (means ± s.d.) are given as the percentage of viable cells and are derived from four independent experiments. $^{*}P<0.03$.
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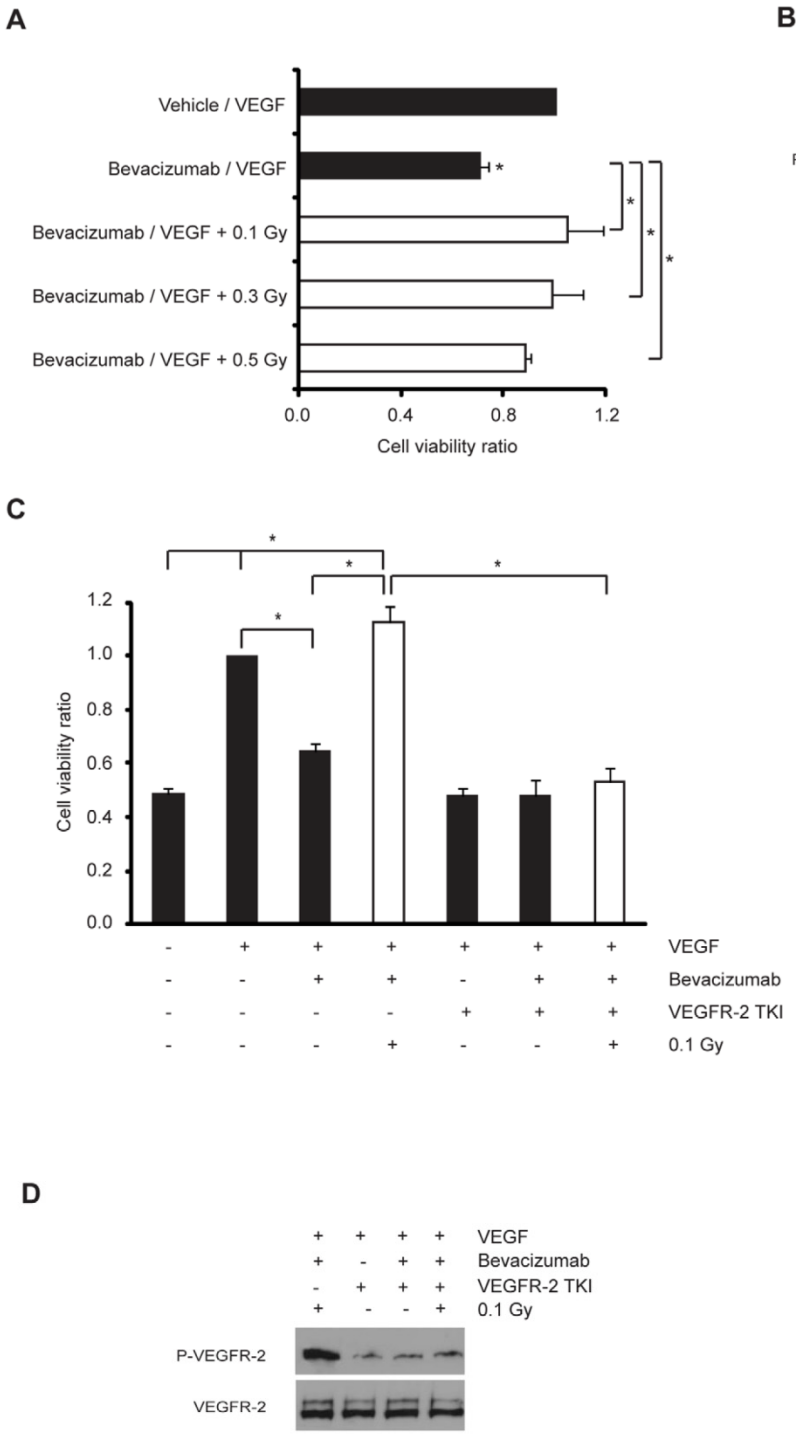


**Figure 3. Low-dose IR protects microvasculature from bevacizumab-induced cell death by inducing VEGFR-2 activation.** (A) Cells were cultured without serum for 12 h and incubated with vehicle/VEGF (20 ng/ml) or bevacizumab (0.25 mg/ml)/VEGF (20 ng/ml) mixtures. Then, cells were exposed or not to 0.1, 0.3 or 0.5 Gy and the percentage of apoptotic cells was assessed by flow cytometry at 48 h post-irradiation. Data (means ± s.d.) represent the ratio between cell viability percentage of each experimental condition and control condition and are derived from four independent experiments. (B) Representative blots from four independent experiments. Cells were exposed or not to 0.1 Gy. Western blot analysis of

total- and phospho-VEGFR-2. (C) Cells were cultured without serum for 12 h and treated or not with VEGFR-2 tyrosine kinase inhibitor (TKI at 300 nM) for 2 h and stimulated or not with VEGF (20 ng/ml) or bevacizumab (0.25 mg/ml)/VEGF (20 ng/ml) mixture. Then, cells were exposed or not to 0.1 Gy. The percentage of apoptotic cells was assessed by flow cytometry at 48 h post-irradiation. Data (means ± s.d.) represent the ratio between cell viability percentage of each experimental condition and control condition and are derived from four independent experiments. $^{*}P<0.03$. (D) Representative blots from four independent experiments. Western-blot analysis of total- and phospho-VEGFR-2 (P-VEGFR-2) of HMVEC-L cultured without serum for 12 h and treated or not with VEGFR-2 tyrosine kinase inhibitor (TKI at 300 nM) for 2 h and stimulated with VEGF (20 ng/ml) or bevacizumab (0.25 mg/ml)/VEGF (20 ng/ml) mixture. (B and D) The level of VEGFR-2 phosphorylation was assessed after 15 min post-irradiation.
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to the tumor through multiple radiation beams, in order to prevent damage of organs at risk. Our present data suggest that these low doses of IR promote angiogenesis and consequently tumor burden and metastasis. We found that doses of 0.8 Gy or lower do not cause cell cycle arrest or apoptosis. Molecularly, they led to rapid phosphorylation of several cellular proteins including Akt and ERK, which play relevant roles in microvascular endothelial cell function. Interestingly, our results suggest that there is not a specific low IR-dose that is most effective in activating specific cellular proteins and molecular response induced by low dose-IR, since effects are dependent not only on the IR dose but also on the molecular target. Doses of 0.1 and 0.3 Gy induce a similar phosphorylation of AKT that is greater than the phosphorylation induced by 0.5 Gy. On the other hand 0.1 Gy is more effective in inducing phosphorylation of ERK, compared to 0.3 and 0.5 Gy doses. Moreover, low-dose IR can protect endothelial cells against apoptosis induced by inhibiting these signaling pathways. Thus, low-dose IR might disrupt the balance between survival and apoptosis by activating pro-survival signaling proteins, thereby favoring angiogenesis. These findings do not contradict the

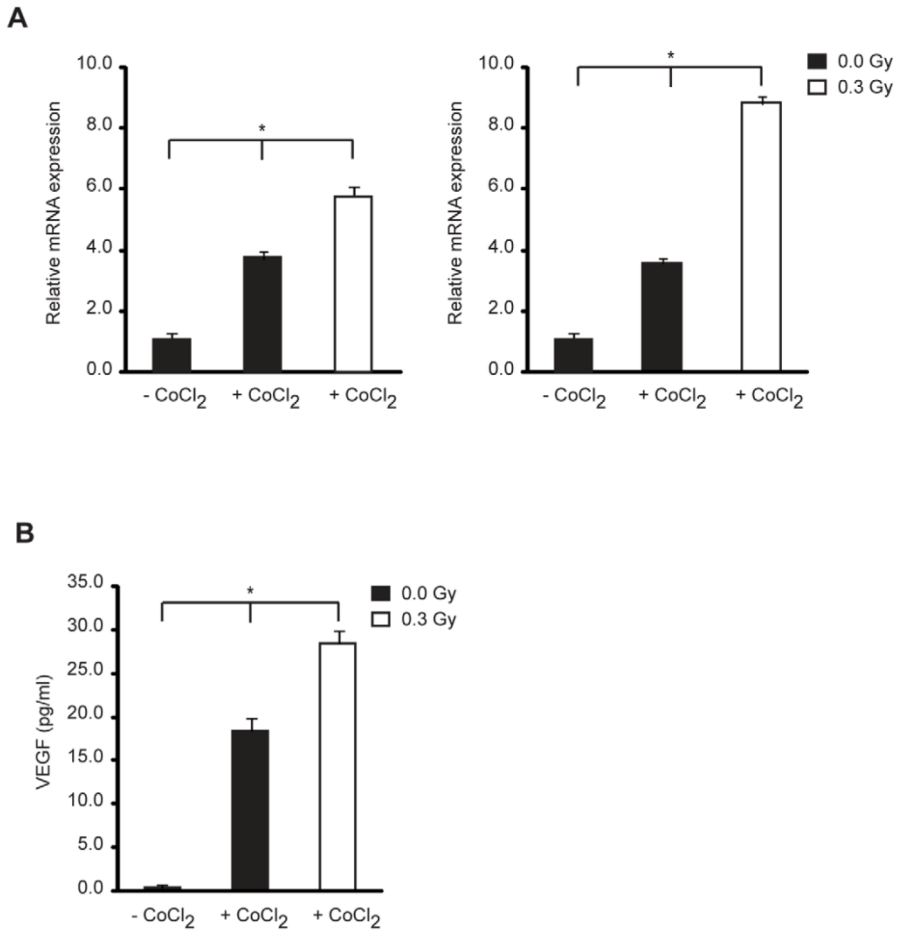


**Figure 4. Low dose IR enhances hypoxia-induced VEGF expression.** Cells were cultured with or without $CoCl_2$ (150 μM) in normoxia to mimic hypoxic conditions and immediately exposed or not to 0.3 Gy of IR. (A) 4 h (left graph) and 12 h (right graph) post-irradiation, *VEGF* mRNA was quantified by qRT-PCR. Data (means ± s.d.) represent the fold change in gene expression relative to the internal calibrator (−CoCl2) in triplicate measurements and are representative of three independent experiments. (B) 72 h post-irradiation, VEGF protein was assessed by VEGF ELISA Kit. Data (means ± s.d.) indicate the VEGF concentration in quadruplicate measurements and are representative of three independent experiments. $^{*}P<0.03$.
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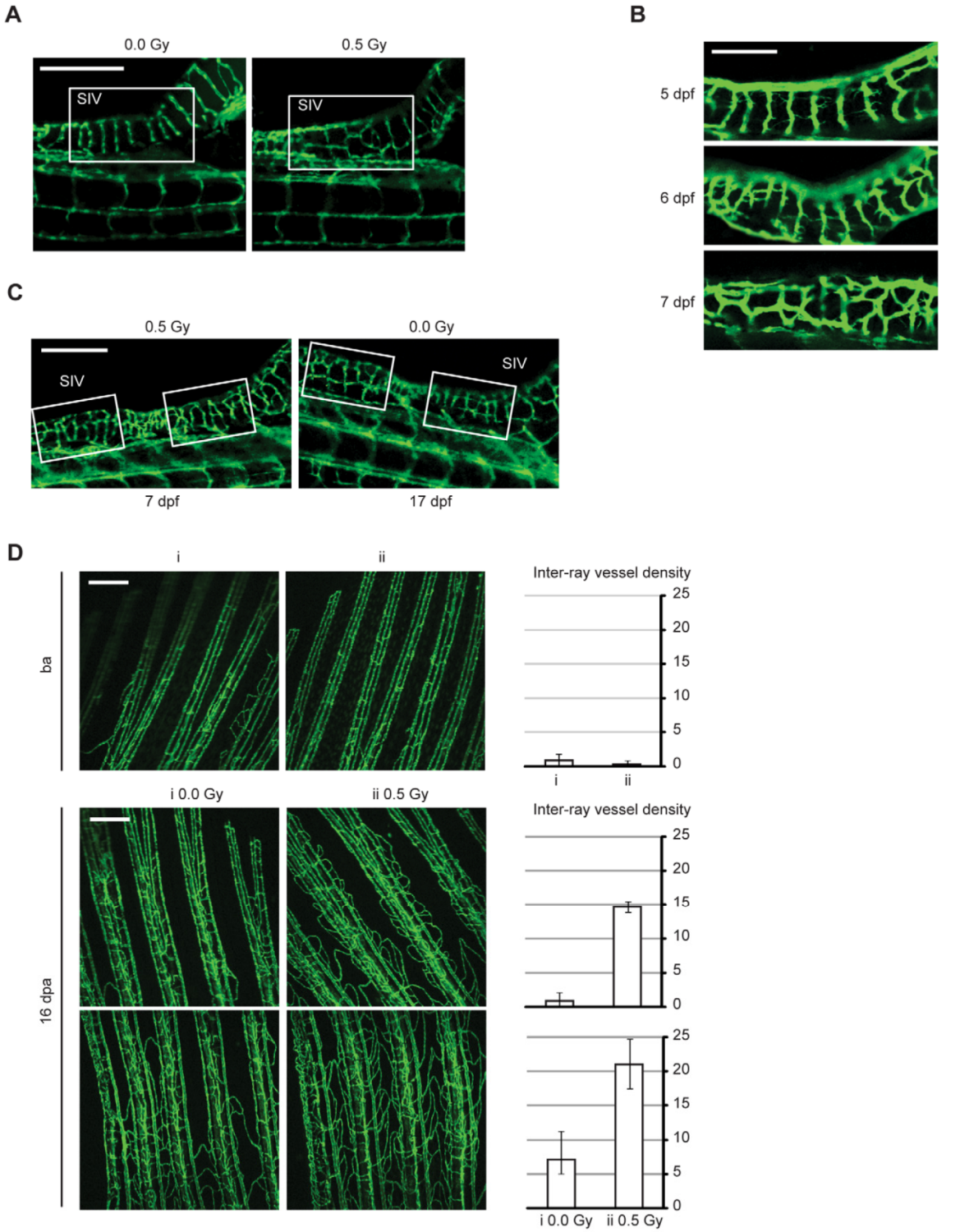


**Figure 5. Low-dose IR accelerates angiogenic sprouting during zebrafish embryonic development and enhances angiogenesis during fin regeneration.** (A–C) Live zebrafish embryos were exposed or not to 0.5 Gy IR 3 d post-fertilization (dpf). Representative images of Sub-Intestinal Vessels (SIV), from (A) a non-irradiated and irradiated zebrafish at 7 dpf; (B) an irradiated zebrafish at 5 (top), 6 (middle) and 7 dpf (bottom);

(C) an irradiated zebrafish at 7 dpf and a non-irradiated zebrafish at 17 dpf. Scale bars, 250 μm (A and C), 100 μm (B). (D) Fli1:EGFP adult zebrafish caudal fins were amputated at mid-fin level, exposed or not to 0.5 Gy of IR and then allowed to recover. Representative images from vasculature of two zebrafish fins (i and ii) before amputation ensure they had identical vasculature (top). Representative images from vasculature of two different fin areas of the same zebrafish, 16 days post-amputation (dpa), with or without low-dose IR treatment (middle and bottom). Each image was quantified for inter-ray vessel density. Data are shown as mean and error bars indicate maximum and minimum values. Images are representative of 10 zebrafish in five independent experiments. Scale bars, 250 μm.
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concept that the pro-apoptotic effects of radiation on endothelial cells contribute to antitumoral treatment, since these effects occur only at high IR doses [21]. Rather, they suggest that at given doses and at time points during radiotherapy, IR might enhance the formation of new vessels, thereby favoring metastatic spreading.

We also demonstrated a protective effect of low-dose IR against endothelial cell death induced by bevacizumab, an anti-VEGF monoclonal antibody, currently used in the clinic as anti-angiogenic drug in combination with chemotherapy [22]. VEGF inhibition by bevacizumab can prevent binding to its receptors, in particular VEGFR-2 which is the main pro-angiogenic VEGF receptor on endothelial cells [22,23]. Consistently, we found that low-dose IR activates VEGFR-2 and the protective effect of IR against bevacizumab-mediated VEGF inhibition was completely abrogated by treatment with a VEGFR-2 TKI. Therefore, we propose that low-dose IR might prevent endothelial cells death promoted by bevacizumab through a mechanism involving VEGFR-2 phosphorylation. We observed that low-dose IR enhanced endothelial cell migration by activating VEGFR-2. While this result is partially consistent with previous reports

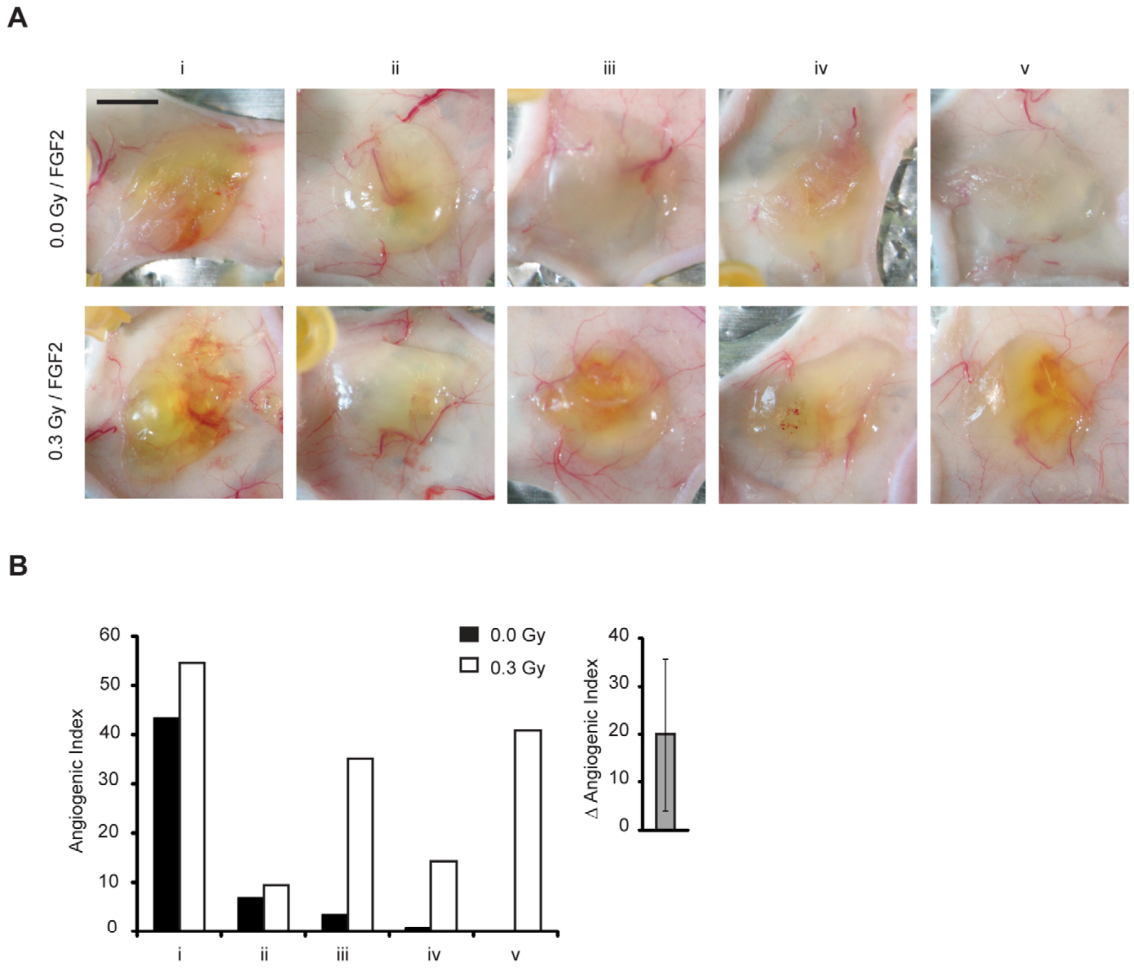


**Figure 6. Low-dose IR enhances angiogenesis in Matrigel plug assay.** Angiogenesis was induced by injection of growth factor-depleted Matrigel with FGF2. (A) Macroscopic evaluation of the Matrigel plugs explanted 5 d after injection in non-irradiated and 0.3 Gy preirradiated area. Scale bars, 500 μm. (B) Angiogenesis was quantified by determining the angiogenic index. Inset shows the mean difference of angiogenic index between paired mice. * $P<0.025$.
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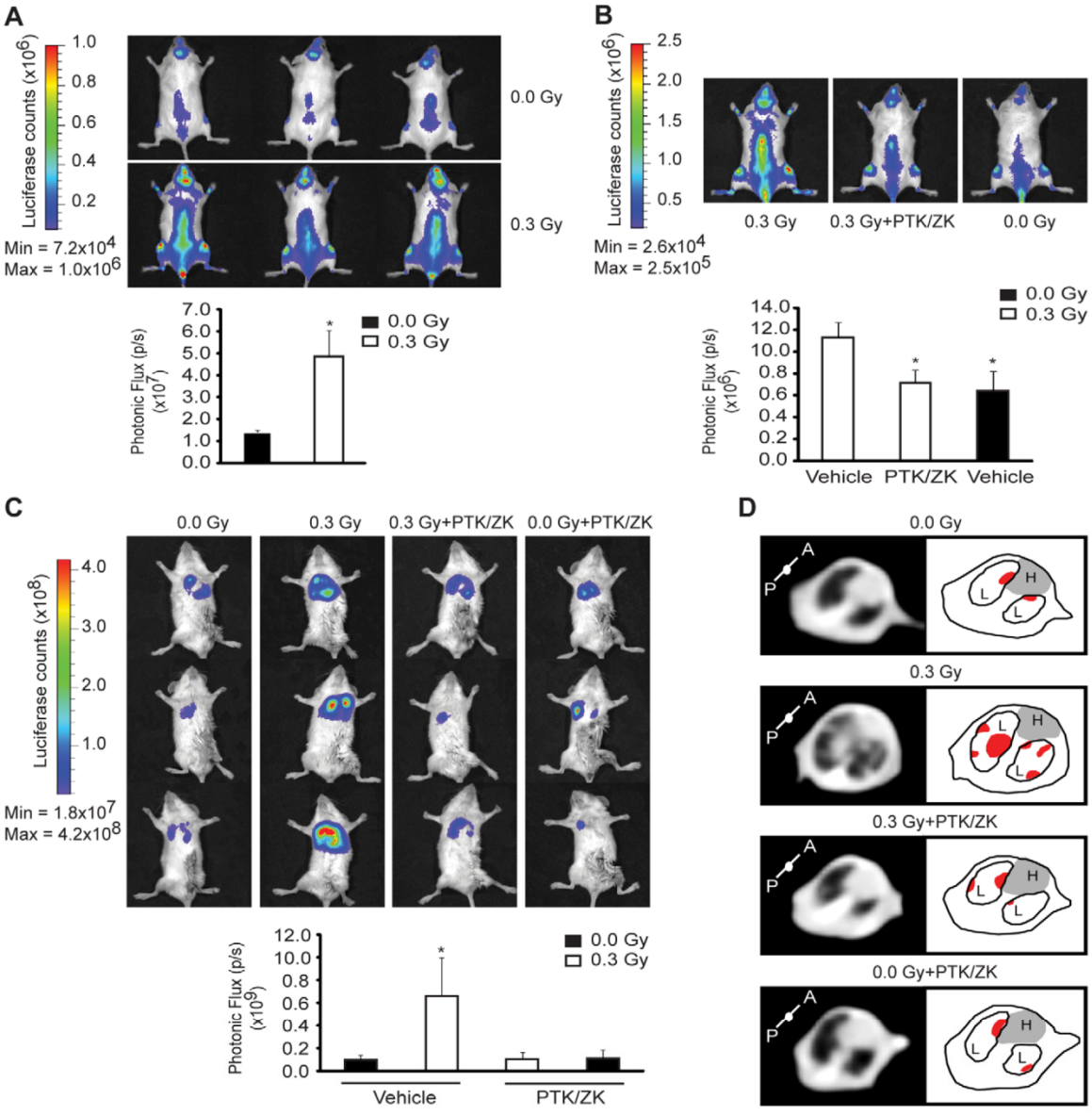
A
Luciferase counts (x10^6)
1.0
0.8
0.6
0.4
0.2
Min = 7.2x10^4
Max = 1.0x10^6
0.0 Gy
0.3 Gy
Photonic Flux (p/s) (x10^7)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
0.0 Gy
0.3 Gy
B
Luciferase counts (x10^6)
2.5
2.0
1.5
1.0
0.5
Min = 2.6x10^4
Max = 2.5x10^5
0.3 Gy
0.3 Gy+PTK/ZK
0.0 Gy
Photonic Flux (p/s) (x10^6)
14.0
12.0
10.0
0.8
0.6
0.4
0.2
0.0
*
*
0.0 Gy
0.3 Gy
Vehicle
PTK/ZK
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C
0.0 Gy
0.3 Gy
0.3 Gy+PTK/ZK
0.0 Gy+PTK/ZK
Luciferase counts (x10^8)
4.0
3.0
2.0
1.0
Min = 1.8x10^7
Max = 4.2x10^8
Photonic Flux (p/s) (x10^9)
12.0
10.0
0.8
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0.0 Gy
0.3 Gy
Vehicle
PTK/ZK
D
0.0 Gy
0.3 Gy
0.3 Gy+PTK/ZK
0.0 Gy+PTK/ZK
A
P
H
L
L
E
0.0 Gy
0.3 Gy
0.3 Gy+PTK/ZK
0.0 Gy+PTK/ZK

**Figure 7. Low-dose IR promotes acceleration of tumor growth and metastasis in a VEGF receptor-dependent manner.** (A) NOD-SCID mice were irradiated or not with 0.3 Gy and 22 h later injected with MOLT-4 cells (B) NOD-SCID mice were pre-treated with PTK/ZK (100 mg/Kg) or PTK/ZK vehicle, 2 h later irradiated or not with 0.3 Gy and after 22 h injected intravenously with MOLT-4 cells. (A and B) 14 d post-injection, the tumor burden was quantified by bioimaging. n = 3 mice per group. The values (means ± s.d.) are representative of three independent experiments. Dorsal images from representative mice are shown. $^{*}P<0.02$. (C) NOD-SCID IL-2R gamma$^{null}$ mice were treated with PTK/ZK (100 mg/Kg) or PTK/ZK vehicle, 2 h later irradiated or not with 0.3 Gy and after 22 h injected with 4T1 cells into the mammary fat pad. 20 d post-injection, the primary tumor was removed and lung metastases were quantified by bioimaging. n = 3 mice per group. The values (means ± s.d.) are representative of three independent experiments. $^{*}P<0.05$. Ventral images from representative mice were shown. (D) Representative images of CT cross section of lung area, from one mouse per experimental condition, showing pulmonary nodules in both lobes of lungs (left). Schematic illustration (right) of the pulmonary nodules (in red) located in both lobes (L) of the lungs. The gray area represents the heart (H). (E) Representative lung sections from one mouse per experimental condition stained with H&E. Scale bars, 1 mm (top), 0.5 mm (bottom).
doi:10.1371/journal.pone.0011222.g007

demonstrating that VEGFR-2 activation promotes endothelial cell migration [8], it also suggests that low-dose IR might modulate other molecules involved in endothelial cell migration since the treatment with a VEGFR-2 TKI does not completely suppress IR-induced migration.

Our data also showed that low-dose IR induces VEGF transcription and consequent protein expression in endothelial cells under hypoxia-mimicking conditions. $CoCl_2$ induces the expression of the transcription factor hypoxia-inducible factor 1 (HIF-1) $\alpha$ subunit, which leads to the increased transcription of genes involved in the initiation and progression of angiogenesis [9]. In normoxia, HIF1 activity is low and consequently the endothelial cells express basal levels of VEGF [9]. We found that these levels were not changed by low-dose IR. However, after $CoCl_2$ treatment and low-dose IR exposure, the expression of VEGF is higher than in non-irradiated cells treated with $CoCl_2$ alone. Although our data do not explain the mechanism by which low-dose IR upregulates VEGF under hypoxic conditions, this finding is of potential clinical relevance since hypoxic areas are naturally present both within tumor and in its periphery [24]. The exposure of hypoxic areas in the healthy tissues surrounding the tumor to low doses of IR may further enhance VEGF expression and favor tumor cell escape.

Taken together, our *in vitro* results suggest that low-dose IR promote endothelial cell events, including the activation of VEGFR, critical to the angiogenic process. Accordingly, we found that low-dose IR increases vessel density in adult fli1:EGFP zebrafish and mice and accelerates vessel formation by inducing angiogenic sprouting in fli1:EGFP embryos. This pro-angiogenic effect of low dose IR may be of clinical relevance, since low doses of IR promoted tumor spreading of leukaemic cells and lung metastasis formation of breast cancer in mice. According to our *in vitro* results where we demonstrated an activation of VEGFR-2 by low-dose IR, the VEGF receptor tyrosine kinase inhibitor PTK/ZK, was administered before irradiation in order to avoid the effect of low dose IR in inducing the phosphorylation of VEGFR in mice irradiated with 0.3 Gy. It is described that the activation of VEGFR leads to a rapid activation of different cellular proteins and consequently to the *de novo* mRNA and protein expression of mediators involved in the angiogenic response. This is strongly supported by the data we obtained in an *in vitro* microarray study, where several transcripts encoding for proteins required for angiogenesis are induced upon low-dose IR delivery (data not shown). However, since tumor cells also express VEGFR, PTK/ZK would directly interfere with their viability thereby confounding the results of the experiment. For that reason, the MOLT-4 or 4T1 cells were injected 24 h after PTK/ZK treatment, a time point this inhibitor is no longer active *in vivo*. We observed that in both mice models the experimental group treated with PTK/ZK presented tumor spreading and lung metastasis formation similar to the unirradiated experimental group, consistent with the hypothesis that IR promoted tumor spreading of leukaemic cells and lung metastasis through a mechanism involving the activation of VEGFR. Two putative vascular beds might be involved in this effect: the vascular beds that surrounds the primary tumor (which provides an efficient exit route for tumor cells); and the vasculature located in the metastatic organ (which will favor initial tumor cells homing and supply oxygen and nutrients necessary for the proliferation and survival of metastatic tumor cells).

In conclusion, our findings provide new insights into tumor radiobiology, identifying the pro-angiogenic effects that low-dose IR might have on the vasculature surrounding the tumoral mass, and thus adding a clinically relevant element to the interpretation of dosimetry in radiotherapy.

## Materials and Methods

### Ethics Statement

All animals experiments were carried out in accordance with protocols approved by the Animal Care Committee of the IMM (permit numbers AEC_004, AEC_005 and AEC_007).

### Cell culture and reagents

Lung Human Microvascular Endothelial Cells (HMEC-L) were purchased from Cambrex and cultured according to manufacturer's instructions. Cells were used up to passage 6. T-cell leukemia-derived MOLT-4 cells and 4T1 breast carcinoma cells (American Type Culture Collection), both stably expressing luciferase, were cultured in RPMI and 10% FBS. Ly294002 (50 μM), VEGF (20 ng/ml) and $CoCl_2$ (150 μM) were purchased from Sigma. U0126 (10 μM) was purchased from New England Biolabs. VEGF receptor-2 tyrosine kinase inhibitor (VEGFR-2 TKI) (70nM) was purchased from Calbiochem. PTK787/ZK222584 (PTK/ZK) (100 mg $kg^{-1}$) was provided by Novartis Pharma AG, Basel, Switzerland. Bevacizumab (0.25 mg/ml), 5-FU (5 μg/ml), gemcitabine (0.08 μM) and paclitaxel (3 nM) were provided by the Oncology Service of Santa Maria Hospital, Lisbon, Portugal.

### Irradiation

HMVEC-L cultures, anesthetized mice or zebrafish were transferred to an acrylic phantom box in order to achieve a certain thickness. A computed tomography (CT) scan (Somatom Sensation, Siemens) was performed and a volumetric acquisition was carried out; acquired images were reconstructed with axial slices 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 (0°, 180°) at 6 MV energy, normalized to a reference point. IR delivery was performed at room temperature using a linear accelerator x-rays photon beam (Varian Clinac 2100 CD) operating at a dose rate of 300 MU/min. A 0.6 $cm^3$ PTW farmer ionizing chamber, connected to UNIDOS electrometers, was used to validate the

IR doses calculated by PLATO, according to the IAEA TRS-398 protocol. We obtained, in average, differences lower than 2% between the experimental and the PLATO planning system dose values.

## Wound healing assay

HMVEC-L were plated to confluence and wounds created in the monolayer by scraping the plate with a pipette tip. Monolayers were irradiated or not and photographed immediately after wounding and 9 h later.

## Proliferation and cell cycle analysis

Cells were counted using a nucleocounter (ChemoMetec) according to manufacturer's instructions. Cell cycle assays were performed as described [25] and analyzed using a FACS Calibur (Beckton Dickinson) and ModFit LT 2.0 Software.

## Apoptosis analysis

HMEC-L were plated at equal densities, after 12 h incubated in the different experimental conditions and stained with Annexin V-FITC (Boehringer Manheim) and propidium iodide (PI) (Interchim). The percentage of apoptotic cells (Annexin V positive, PI negative and positive) was determined by flow cytometry (FACS Calibur, Beckton Dickinson) and Flowjo 6.4.7 Software. Results are shown as the percentage of viable cells (Annexin V, PI negative).

## Immunofluorescence analysis

HMEC-L were cultured on gelatin-coated glass coverslips. The cells were fixed, permeabilized, and incubated with antibody to γ-H2AX (from Upstate Biotechnology Inc.) followed by incubation with Alexa Fluor 488 (Molecular Probes). The samples were counterstained with DAPI and analyzed by fluorescence microscopy (Axioplan Microscope, Zeiss).

## Flow cytometry analysis

The cells were fixed, permeabilized and stained with the antibody to phospho-tyrosine (Santa Cruz Biotechnology) followed by incubation with Alexa Fluor 488 or 594 (Molecular Probes). We acquired cells using FACS Calibur (Beckton Dickinson) and analyzed data using Flowjo 6.4.7 Software.

## Western-blot analysis

Whole protein extracts were prepared as described [26]. Blots were incubated with antibodies to phospho-tyrosine, phospho-ERK (Tyr204), ERK, phospho Akt (Ser473), Akt (Santa Cruz Biotechnology), VEGFR-2 (Calbiochem) or β-tubulin (Sigma).

## Quantitative RT-PCR

For quantitative RT-PCR, total RNA was isolated using the QiaShredder and RNeasy (Qiagen) system. For each sample, 1 µg RNA was reverse transcribed into cDNA (Superscript II Kit, Invitrogen). *VEGF* mRNA levels were measured by RT-PCR (TAQMAN) on the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems) using specific primers and probes (forward primer: 5′-CCAGCACATAGGAGAGATGAGCTT-3′, reverse primer: 5′-CGCCTCGGCTTGTCACA-3′, probe: 6-FAM-5′-ACAGCACAACAAATGTGAATGCAGACCAAA-3′-TAMRA). The housekeeping gene used to normalize the samples in TAQMAN assay was the 18S (Human 18S rRNA-20×, Applied Biosystems). Real time PCR program consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The relative quantification of VEGF mRNA in HMEC-L was performed according to the comparative method (2 −DDCt; Applied Biosystems User Bulletin no. 2P/N 4303859), with −CoCl2 condition as internal calibrator. The formula used is 2 −DDCt = 2 −[DCt(sample)−DCt(calibrator)], where DCt(sample) = Ct(sample)−Ct(reference gene). For the internal calibrator, DDCt = 0 and 2 0 = 1. For the remaining samples, the value of 2 −DDCt indicates the fold change in gene expression relative to the calibrator. DCt value for each sample is the average of triplicates.

## ELISA assay

Conditioned medium from the different experimental conditions was stored at −80°C and VEGF concentrations were measured following manufacturer's instructions using the human VEGF-ELISA kit (Calbiochem).

## Zebrafish

The transgenic zebrafish fli1:EGFP were obtained from the Zebrafish International Resource Center and maintained under standard conditions [27] with institutional animal care. Before irradiation or imaging, adults and embryos zebrafish were anesthetized using 0.61 mM and 0.31 mM of tricaine, respectively. Caudal fins were acquired on a Zeiss LSM 5 Live microscope using a 10×/0.30 objective and a solid state 488 nm laser in conjunction with a LP 505 nm filter. Zebrafish embryos images were acquired on a Zeiss LSM 510 META using a 20×/0.80 objective and the 488 nm laser line of an Ar laser in conjunction with a LP 505 nm filter. The vessel density was calculated with Image J (NIH) using threshold segmentation and histogram analysis to measure blood vessel areas per total area of selected regions of interest (inter-ray regions).

## Mice and mouse procedures

Athymic Swiss *nu/nu*, NOD-SCID and NOD-SCID IL-2R gamma$^{null}$ mice were purchased from the Harlan Laboratories (Madison, WI, USA), the pathogen-free facility of the Instituto de Medicina Molecular (IMM) and the Jackson Laboratory (Bar Harbor, Maine, USA), respectively. Matrigel plug angiogenesis assay was adapted from the original method [12]. Briefly, 24 h after 0.3 Gy local radiation of 8 week-old Athymic Swiss *nu/nu* female mice (lower-right back side), two Matrigel plugs (400 µl/plug) supplemented with FGF-2 (500 ng/ml, PeproTech EC Ltd) and Heparin (3 U/ml, Sigma) were implanted in the irradiated dorsal region of the mice or in the contralateral non-irradiated side (control plug) of same mouse. Five days after Matrigel implantation, the plugs were removed and photographed using 0.56× magnification. Angiogenesis was reported as the angiogenic index (mean number of red pixels per the total number of pixels in the area of interest) using the Image J (NIH). 6-week-old NOD-SCID mice were injected intravenously (i.v.) with $20\times10^6$ MOLT-4 cells. 6 week-old NOD-SCID IL-2R gamma$^{null}$ female mice were injected with $5\times10^4$ 4T1 cells suspended in 50% PBS/50% Matrigel (from BD Biosciences) into the 4$^{th}$ mammary fat pad. PTK/ZK or its vehicle (polyethylene glycol-300 from Sigma) was administered by intragastric gavage. For imaging studies, mice were anesthetized and injected with D-luciferin at 150 mg kg$^{-1}$ (Xenogen) intraperitoneally (i.p). We imaged photonic emission with the *In Vivo* Imaging System (IVIS, Xenogen) with a collection time of 180 s for MOLT-4 experiments or 10 s for 4T1 experiment. We quantified tumor bioluminescence by integrating the photonic flux (photons per second) through a region encircling each tumor, as determined by the LIVING IMAGES Software package (Xenogen). We evaluated lung metastasis by Computed Tomography (CT) scan. Acquired images were reconstructed with

axial slices width of 1 mm. For histological analysis, the mice lungs were dissected, immersion-fixed overnight, at room temperature, in 4% paraformaldehyde and then embedded in paraffin. Paraffin-embedded sections with 3 μm thickness were counterstained with hematoxylin and eosin (H&E) using traditional methods.

### Statistical analysis

Numeric data were analyzed for statistical significance using Mann-Whitney test for comparison of means with GraphPad Prism 5 Software. Two-tailed unpaired and paired Student's *t*-test was also used for tumor and Matrigel plug assay experiments, respectively. $P<0.05$ was considered significant.

## Supporting Information

**Figure S1** Low doses of IR induce phosphorylation of H2AX. Cells were exposed or not to 0.3, 0.5, 1.0 and 2.0 Gy and *-H2AX foci, marking DNA damage, were visualized, by immunofluorescence microscopy, after 30 min and 12 h post-irradiation. *-H2AX foci are shown in green and nuclei are stained with DAPI (in blue). Magnification, 400×.
Found at: doi:10.1371/journal.pone.0011222.s001 (1.07 MB TIF)

**Figure S2** Low doses of IR do not protect the microvasculature from 5-FU-, gemcitabine- or paclitaxel-induced cell death. HMVEC-L were cultured for 12 h and treated or not with (A) 5-FU (5 * g/ml); (B) gemcitabine (0.08 * M); (C) paclitaxel (3 nM) and then exposed or not to 0.1, 0.3 or 0.5 Gy. Non-irradiated cells cultured with vehicle alone were used as a control. Cells were double stained with Annexin-V and propidium iodide at 48 h post-irradiation. The percentage of apoptotic cells was assessed by flow cytometry. Data (means ± s.d.) represent the ratio between the cell viability percentage of each experimental condition and the control condition and are derived from four independent experiments.
Found at: doi:10.1371/journal.pone.0011222.s002 (0.38 MB TIF)

**Figure S3** Low-dose IR promotes endothelial cell migration by activating VEGFR-2. Confluent monolayers of HMVEC-L were treated or not with VEGFR-2 tyrosine kinase inhibitor (TKI at 300 nM) for 2 h, subjected to in vitro wound healing and next exposed or not to 0.5 Gy. The quantification of the wound area (in mm2) was assessed 9h after wounding. Data (means ± s.d.) indicate the percentage of wound recovery in quadruplicate measurements and are representative of three independent experiments. *$P<0.05$.
Found at: doi:10.1371/journal.pone.0011222.s003 (0.13 MB TIF)

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## Author Contributions

Conceived and designed the experiments: CR IMG JTB MM SCRS. Performed the experiments: ISV LM NI RJN JR LC SCRS. Analyzed the data: ISV LM RJN JR IMG SCRS. Contributed reagents/materials/analysis tools: FK CR IMG JTB SCRS. Wrote the paper: SCRS.

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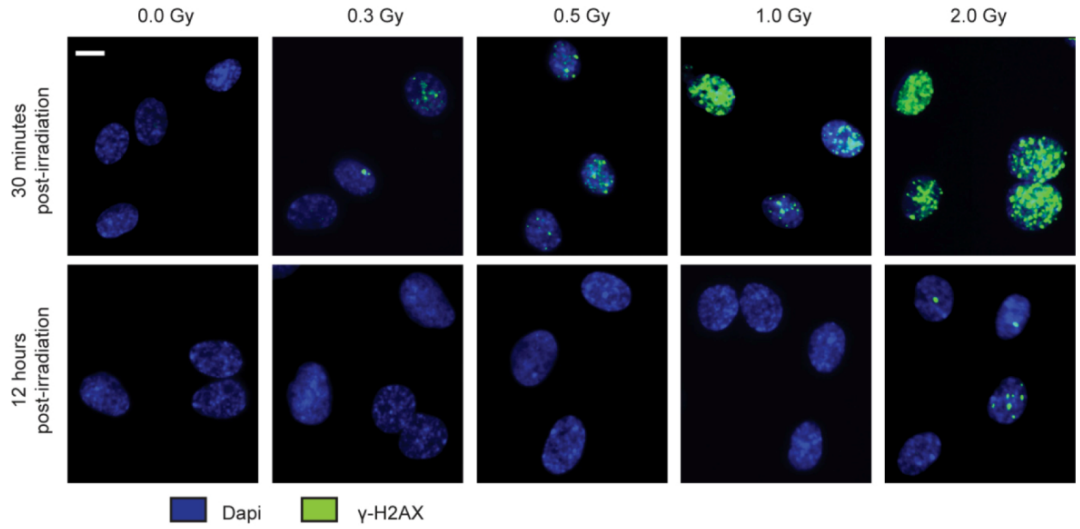


**Figure S1. Low doses of IR induce phosphorylation of H2AX.** Cells were exposed or not to 0.3, 0.5, 1.0 and 2.0 Gy and γ-H2AX foci, marking DNA damage, were visualized, by immunofluorescence microscopy, after 30 min and 12 h post-irradiation. γ-H2AX foci are shown in green and nuclei are stained with DAPI (in blue). Magnification, 400x

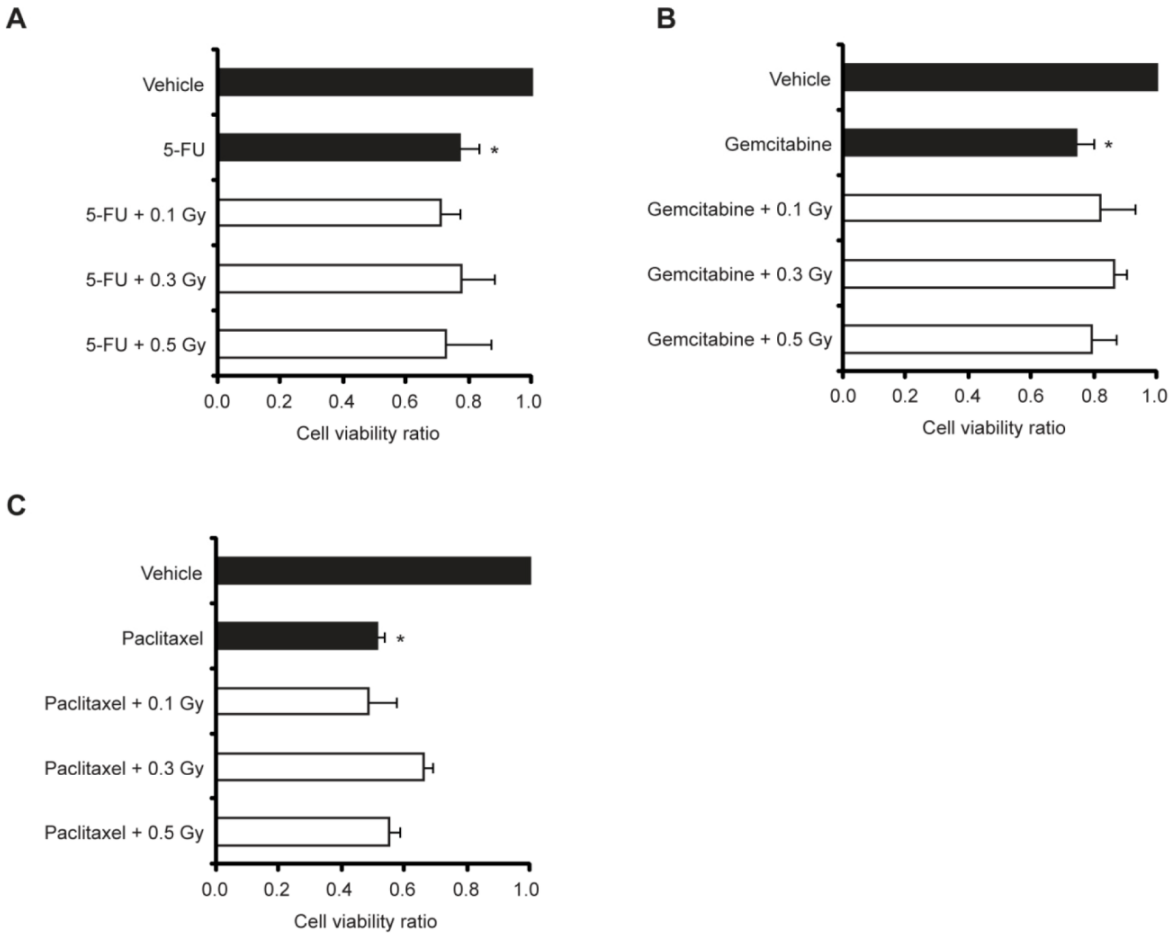


**Figure S2. Low doses of IR do not protect the microvasculature from 5-FU-, gemcitabine- or paclitaxel-induced cell death.** HMVEC-L were cultured for 12 h and treated or not with (A) 5-FU (5 μg/ml); (B) gemcitabine (0.08 μM); (C) paclitaxel (3 nM) and then exposed or not to 0.1, 0.3 or 0.5 Gy. Non-irradiated cells cultured with vehicle alone were used as a control. Cells were double stained with Annexin-V and propidium iodide at 48 h post-irradiation. The percentage of apoptotic cells was assessed by flow cytometry. Data (means ± s.d.) represent the ratio between the cell viability percentage of each experimental condition and the control condition and are derived from four independent experiments.

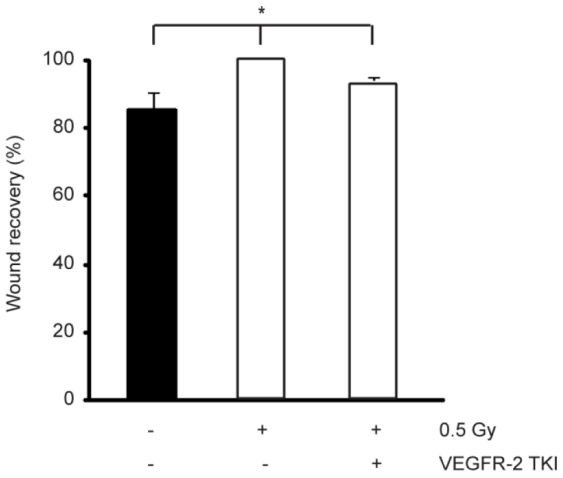


**Figure S3. Low-dose IR promotes endothelial cell migration by activating VEGFR-2.** Confluent monolayers of HMVEC-L were treated or not with VEGFR-2 tyrosine kinase inhibitor (TKI at 300 nM) for 2 h, subjected to in vitro wound healing and next exposed or not to 0.5 Gy. The quantification of the wound area (in mm2) was assessed 9 h after wounding. Data (means ± s.d.) indicate the percentage of wound recovery in quadruplicate measurements and are representative of three independent experiments. *P,0.05.

## COMPLEMENTARY RESULTS

### LOW-DOSE IR-INDUCED H2AX PHOSPHORYLATION IS TRANSITORY AND RAPIDLY RECOVERED WHEN COMPARED TO HIGHER DOSES

We have previously shown that low IR doses are responsible for the formation of γ-H2AX foci (Sofia Vala et al., 2010), indicating the presence of double-strand breaks (DSBs). We have also observed by immunofluorescence that, 12 hours (h) post-irradiation, γ-H2AX foci were no longer detected, reflecting effective DSBs repair. In order to validate these results and to obtain data quantitatively more comparable, we optimized a flow cytometry protocol. Cells were irradiated with doses equal or lower than 2 Gy and processed for analysis. As expected, the number of γ-H2AX foci increased in a dose dependent manner and, 12 h post-irradiation, γ-H2AX was no longer detected (Figure C1, panel A). We have also analyzed the effect of 10 Gy in H2AX phosphorylation, both by immunofluorescence and flow cytometry. At these conditions, the number of DSBs was extremely high and, 24 h after, some of them remained to be repaired (Figure C1, panel B).

Therefore, our results confirmed that low IR doses induce the formation of DSBs in a dose dependent manner, which are rapidly recovered when compared to higher doses.

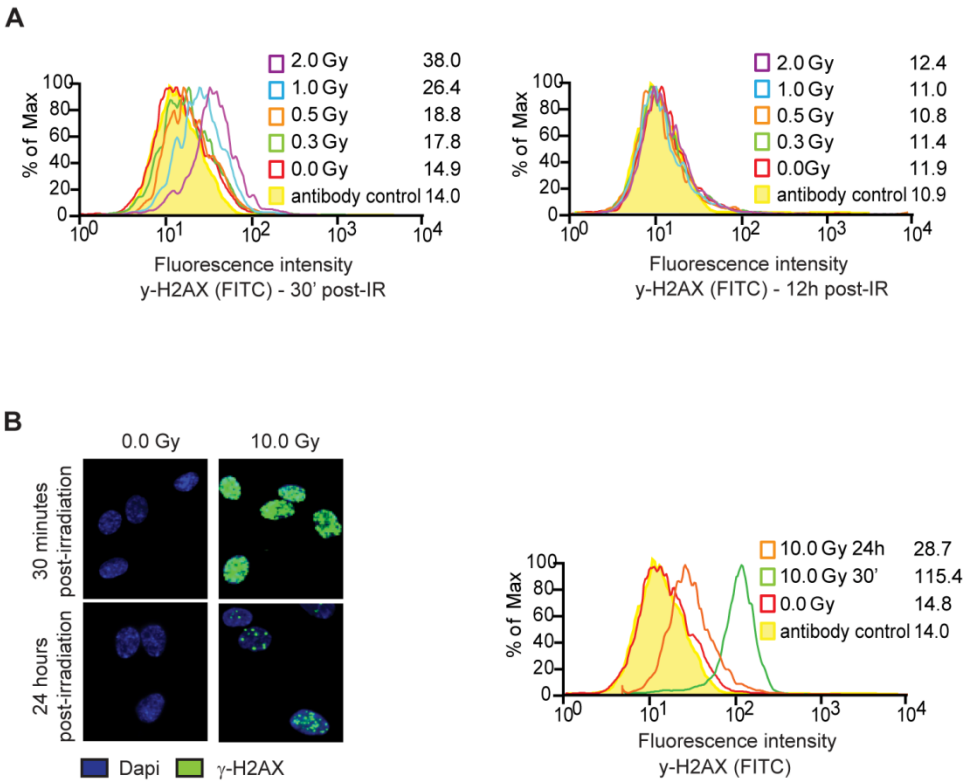


**Figure C1| Quantification of H2AX phosphorylation induced by IR in HMVEC-L.** (A) Cells were exposed or not to 0.3, 0.5, 1.0, 2.0 Gy and the level H2AX phosphorylation (γ-H2AX) marking DNA damage, was assessed by flow cytometry 30 min and 12 h post-irradiation. The values represent the median fluorescence intensity of irradiated cells and non-irradiated cells and are representative of three independent experiments. (B) Cells were exposed or not to 10.0 Gy and the level of γ-H2AX was quantified by flow cytometry (right) or visualized by immunofluorescence microscopy (left) 30 min and 24 h post-irradiation. γ-H2AX foci are shown in green and nuclei are stained with DAPI (in blue). Magnification, 400x.

### LOW-DOSE IR INDUCES THE MIGRATION OF ECS IN THE PRESENCE OF LY294002 AND U0126 BY PHOSPHORYLATING AKT AND ERK

We have previously described that low-dose IR activates the PI3K/Akt and MEK/ERK signaling pathways using a WB approach (Sofia Vala et al., 2010). We now confirmed these results by flow cytometry using cells exposed to 0.1, 0.3 and 0.5 Gy (Figure C2).

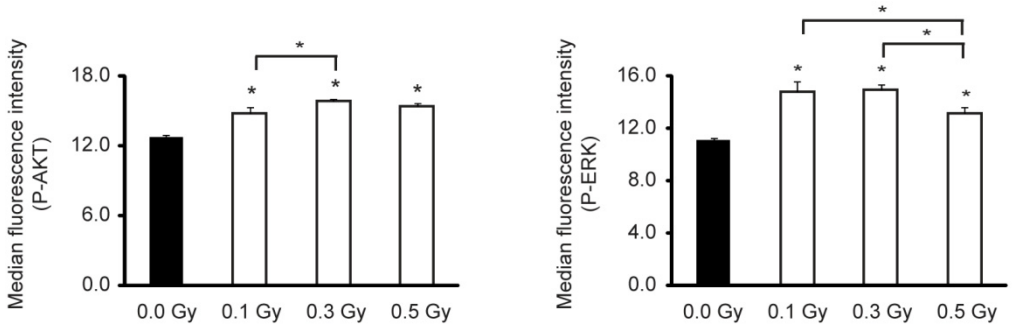


**Figure C2| Low-dose IR activates PI3K/Akt and MEK/ERK pathways in HMVEC-L.** Flow cytometry analysis of phospho-Akt (P-Akt) (left) and phospho-ERK (P-ERK) (right) of HMVEC-L exposed or not to 0.1, 0.3 or 0.5 Gy. The values (means ± s.d.) represent the median fluorescence intensity of irradiated cells and non-irradiated cells and are derived from four independent experiments. *P<0.03.

We have also assessed the functional relevance of IR-induced activation of PI3K/Akt and MEK/ERK signaling pathways by observing that low-dose IR treatment significantly protected cells from death induced by specific signaling inhibitors (Sofia Vala et al., 2010). However, in addition to their role in cell survival, PI3K/Akt and MEK/ERK pathways are also involved in EC migration (Dimmeler and Zeiher, 2000; Lawrence et al., 2003; Mavria et al., 2006). Consistently, treatment with Ly294002 and U0126 (inhibitors of Akt and ERK phosphorylation, respectively), decreased the migratory capacity of ECs (Figure C3).

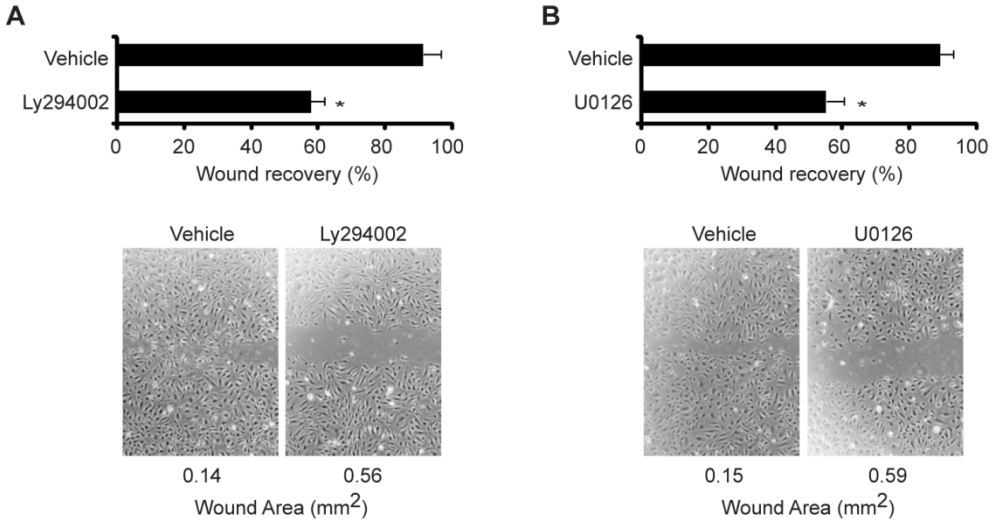


**Figure C3| Treatment with either Ly294002 or U0126 decreases the migration capacity of ECs.** Confluent monolayers of HMVEC-L were incubated in the presence or absence of a specific inhibitor of (A) PI3K (Ly294002 – 50 μM) or (B) MAPK (U0126 - 10 μM), and then subjected to an *in vitro* wound healing assay. Chart values (means ± s.d. of four fields of view) and photographs quantification (wound area in $mm^2$) represent the rate of wound recovery, 10 h after wounding; data are representative of two independent experiments. *P<0.01.

Since we have previously demostrated that low doses of IR were able to induce EC migration (Sofia Vala et al., 2010), we performed an *in vitro* wound healing assay, to investigate if IR was still able to induce the pro-migratory response of HMVEC-L in the presence of either Ly294002 or U0126. We observed that 0.3 Gy induced the migration of HMVEC-L in both conditions (Figure C4, panel A and B). Interestingly, we also observed that in the presence of Ly294002 or U0126, cells exposed to 0.3 Gy presented an increased phosphorylation level of Akt and ERK when compared to unirradiated cells (Figure C4, panel C).

Taken together, our results suggest that the IR-induced pro-survival and pro-migratory responses can be mediated through the phosphorylation of Akt and ERK.

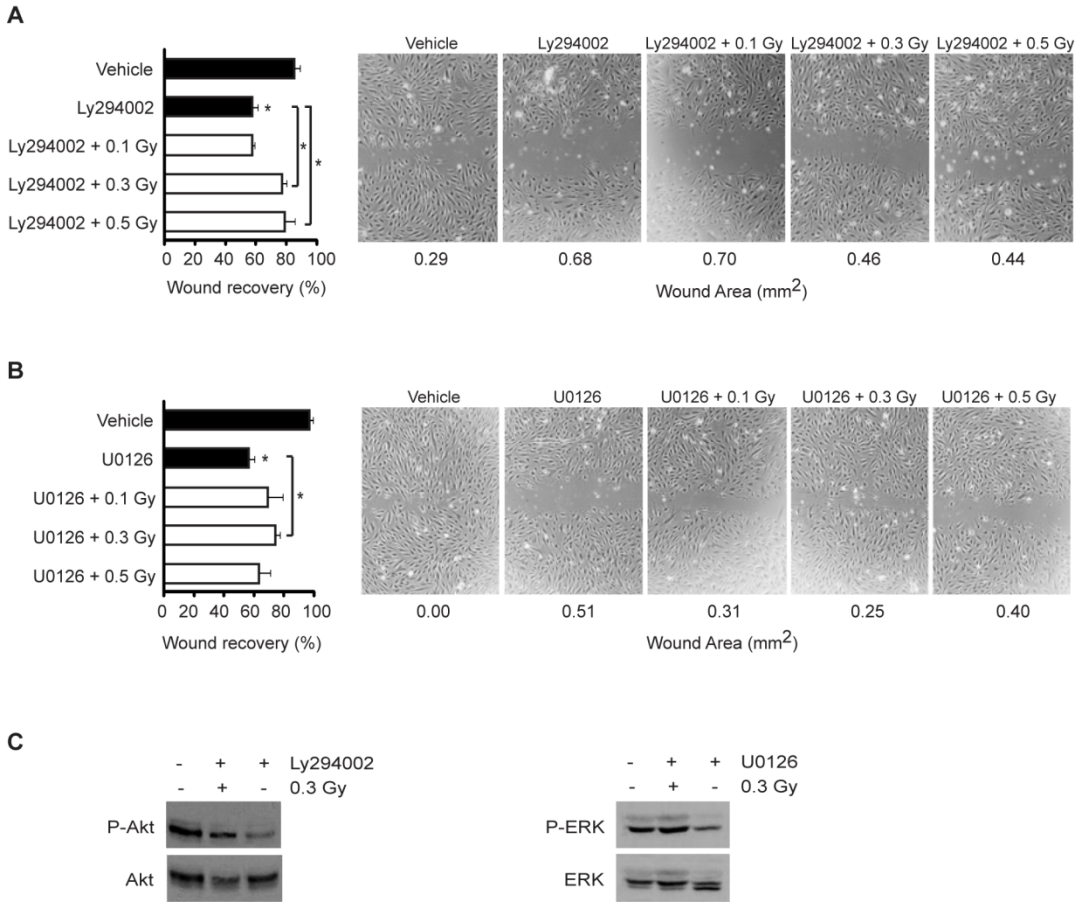


**Figure C4| Low-dose IR induce the migration of ECs in the presence of Ly294002 and U0126.** (A, B) Confluent monolayers of HMVEC-L were incubated in the presence or absence of a specific inhibitor of (A) PI3K (Ly294002 – 50 μM) or (B) MAPK (U0126 - 10 μM), exposed or not to 0.1, 0.3 or 0.5 Gy, and then subjected to an *in vitro* wound healing assay. Cells cultured with vehicle alone were used as a control. Chart values (means ± s.d. of four fields of view) and photographs quantification (wound area in $mm^2$) represent the rate of wound recovery, 10 h after wounding; data is representative of two independent experiments. *P<0.04. (C) Representative blots from two independent experiments. Western blot analysis of total-, phospho-Akt (P-AKT) (left) and total-, phospho-ERK (P-ERK) (right) of HMVEC-L exposed or not to 0.3 Gy, after treatment with a specific inhibitor of PI3K (Ly294002 – 50 μM) (left) or MAPK (U0126 - 10 μM) (right). The levels of Akt and ERK phosphorylation were assessed after 5 or 60 min post-irradiation, respectively.

**LOW DOSES OF IR PROTECT ECS FROM SERUM WITHDRAWAL-INDUCED CELL DEATH**

As previously referred, low doses of IR prevent EC death induced by specific inhibitors of diferent signaling pathways associated to cell survival. Thus, we decided to investigate the effect of low doses of IR in ECs cultured in serum-free medium. EC apoptosis is a natural consequence of the absence of serum, since cell maintenance and survival is dependent on essential cytokines and growth factors present in the culture medium. Interestingly, our results showed that low doses of IR (≤ 1.0 Gy) significantly protected ECs from serum withdrawal-induced cell death (Figure C5). As expected, the protective effect conferred by IR was no longer observed in ECs exposed to high IR doses (5.0 Gy).

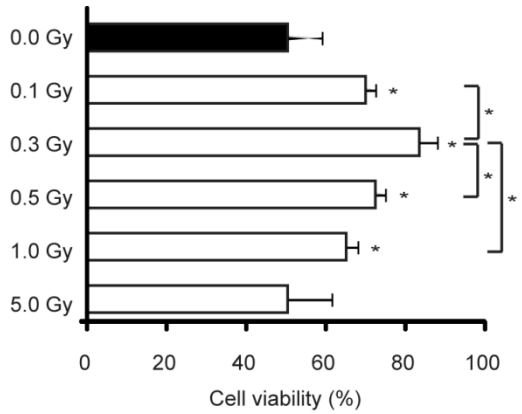


**Figure C5| Low doses of IR protect endothelial cells from serum withdraw-induced cell death.** HMVEC-L, in serum withdraw conditions, were immediately exposed to 0.1, 0.3, 0.5, 1.0 or 5.0 Gy. The percentage of apoptotic cells was determined by flow cytometry 36 h post-irradiation. Cells cultured in normal culture conditions were used as viability control (not shown). Values are given as the percentage of viable cells (Annexin-V, PI negative) remaining in culture. Data are shown as mean in triplicate culture and are representative of three independent experiments. *P<0.03.

### LOW-DOSE IR MODULATES THE ACTIVATION AND EXPRESSION OF ADHESION MOLECULES

Since cell-cell or cell-matrix interactions have an important role in angiogenesis and in tumor metastasis, we decided to investigate if low-dose IR was able to modulate the activation or expression of some adhesion molecules. We started by looking at PAK (p21 activated protein kinase) activation, which supports motility in ECs (Munoz-Chapuli et al., 2004), and VCAM1 (vascular cell adhesion molecule 1) expression, which can promote EC-tumor cell interaction, thus contributing to a higher metastatic potential (Ding et al., 2003; Slack-Davis et al., 2009). According to our results, 0.5 Gy increased the phosphorylation of PAK 30 min post-irradiation, and VCAM1 expression 12 h post-irradiation (Figure C6).

Our preliminary data indicates that low-dose IR modulates some adhesion molecules.

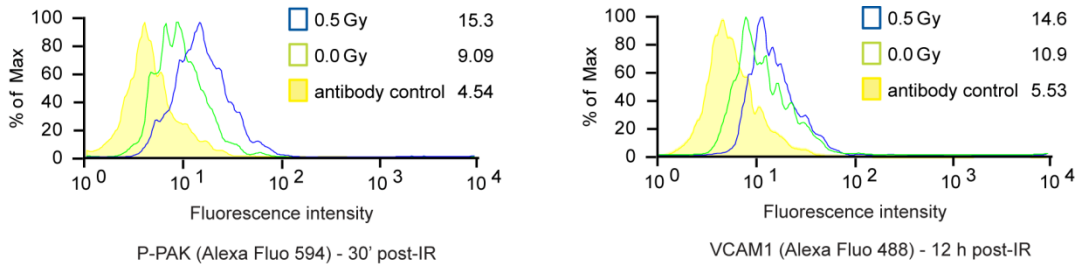


**Figure C6| Low-dose IR induces the activation or the expression of adhesion molecules in HMVEC-L.** The level of PAK phosphorylation (P-PAK) (left) and VCAM1 (right) expression was assessed, through flow cytometry analysis, in HMVEC-L previously exposed or not to 0.5 Gy. The levels of P-PAK and VCAM-1 were analyzed 30 min or 12 h post-irradiation, respectively. The values represent the median fluorescence intensity of irradiated cells and non-irradiated cells and are representative of two independent experiments.

### THE FUNCTIONAL AND MOLECULAR EFFECTS OF THE LOW DOSES OF IR ARE NOT HMVEC-L SPECIFIC

To test if the effects of low-dose IR observed in HMVEC-L were specific to these cells, we performed similar experiments using human umbilical vein endothelial cells (HUVEC).

In a first set of experiments, we confirmed that low doses of IR also promoted migration of HUVEC without causing cell cycle arrest or apoptosis (Figure C7). In these experiments, the best wound recovery was obtained at 0.8 Gy.

Moreover, by flow cytometry or WB analysis, we found that 0.8 Gy induces tyrosine phosphorylation of multiple proteins and specifically of Akt and ERK (Figure C8).

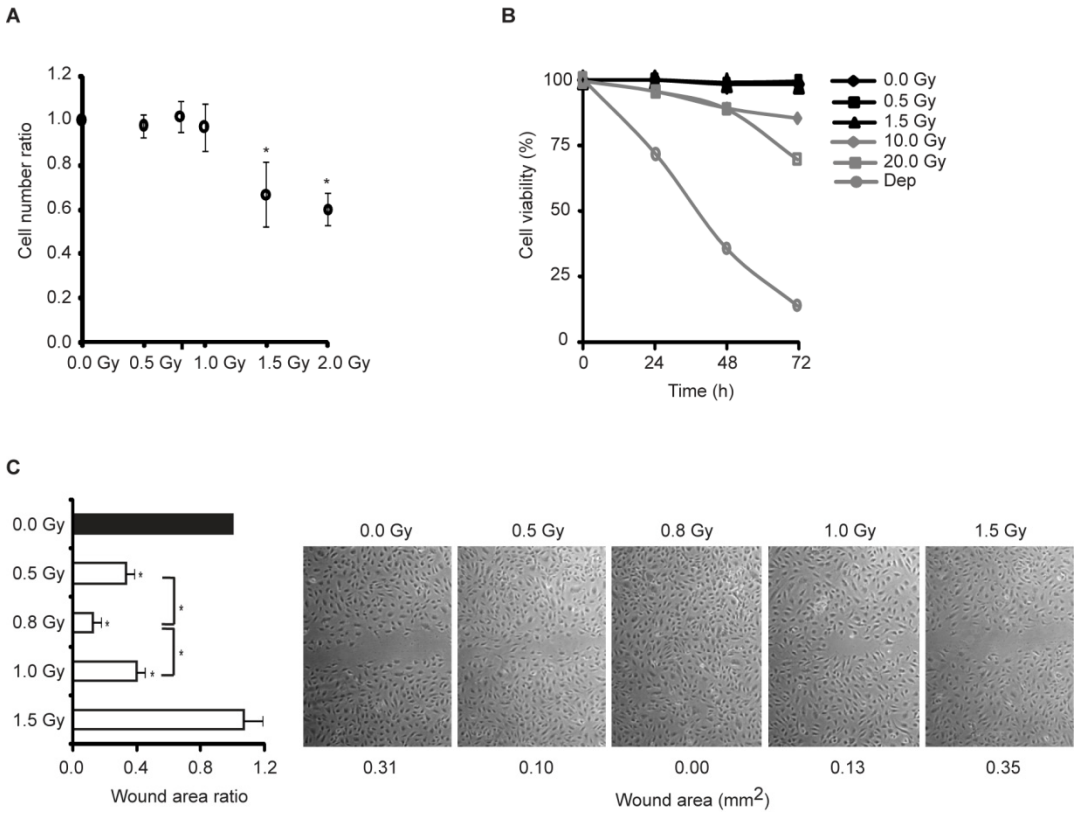


**Figure C7| Low-dose IR promotes HUVEC migration without causing cell cycle arrest or apoptosis.** (A) HUVEC were plated at equal densities and left untreated for 12 h, or exposed to 0.5, 0.8, 1.0, 1.5 and 2.0 Gy. After 72 h, the cells were counted using a Nucleocounter. The values (means ± s.d.) represent the ratio between cell number of irradiated and non-irradiated

conditions and are derived from four independent experiments. *P<0.05. (B) The percentage of apoptotic cells was determined by flow cytometry at the indicated time. Cells cultured without serum (Dep) were used as cell death control. Values are given as the percentage of viable cells (Annexin-V, PI negative) remaining in culture. Data are shown as mean in triplicate culture and are representative of three independent experiments. (C) Confluent monolayers of HUVEC were subjected to *in vitro* wound healing and exposed or not to 0.5, 0.8, 1.0 or 1.5 Gy. Photographs were taken immediately (not shown) and 7 h after wounding. Quantification of the wound area (in $mm^2$) is presented below the images. Data are representative of three independent experiments.

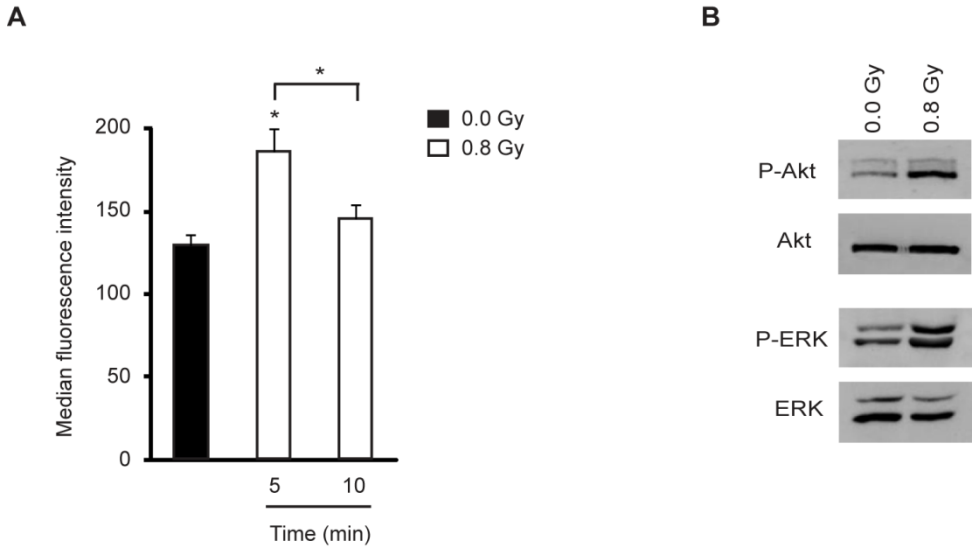


**Figure C8| Low-dose IR modulates tyrosine phosphorylation levels and activates PI3K/Akt and MEK/ERK pathways in HUVEC.** (A) HUVEC were exposed or not to 0.8 Gy and incubated for 5 or 10 min. Tyrosine phosphorylation levels were assessed by flow cytometry. The values (means ± s.d.) represent the median fluorescence intensity of irradiated cells and non-irradiated cells and are derived from four independent experiments. *P<0.05. (B) Representative blots from two independent experiments. Western blot analysis of total-, phospho-Akt (P-Akt) (up) and total-, phospho-ERK (P-ERK) (down) of HUVEC exposed or not to 0.1 or 0.8 Gy. The levels of Akt and ERK phosphorylation were assessed after 5 or 60 min post-irradiation, respectively**.**

We also tested the protective effects of low doses of IR against bevacizumab in HUVEC. Similar to what we observed in HMVEC-L, low-dose IR (0.5 and 0.8 Gy) significantly protected HUVEC against bevacizumab-induced cell death (Figure C9).

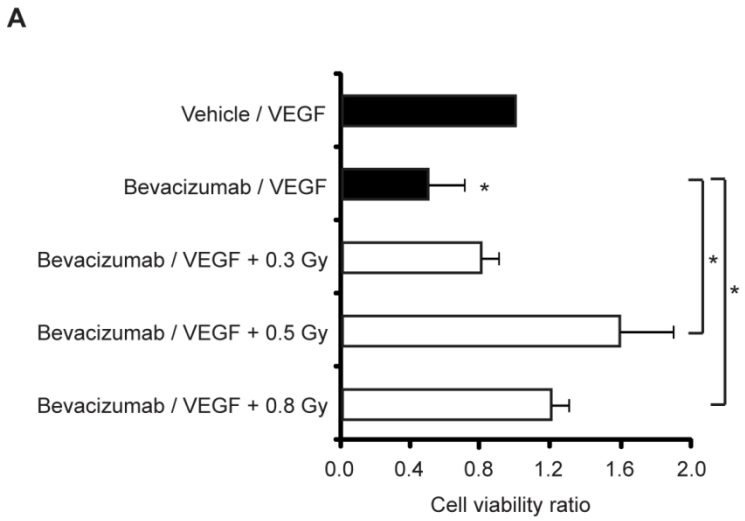


**Figure C9| Low-dose IR protects HUVEC from bevacizumab-induced cell death.** Cells were cultured without serum for 12 h and incubated with vehicle/VEGF (20 ng/ml) or bevacizumab (0.25 mg/ml)/VEGF (20 ng/ml) mixtures. Then, cells were exposed or not to 0.3, 0.5 or 0.8 Gy and the percentage of apoptotic cells was assessed by flow cytometry 40 h post-irradiation. Data (means ± s.d.) represent the ratio between cell viability percentage of each experimental condition and control condition and are derived from three independent experiments. *P<0.04.

5-FU, gemcitabine, and paclitaxel were also tested in HUVEC. Interestingly, and differently from what we observed in HMVEC-L (Sofia Vala et al., 2010), a statistically significant IR-induced protection was obtained for gemcitabine and paclitaxel-induced cell death (Figure C10). However, the protection level obtained was very low (approximately 10%). Considering that cell cycle arrest is the principal cause of cell death induced by both these drugs (Doyle et al., 2001; Lawrence et al., 2003), we performed a cell proliferation analysis to further understand the relevance of these results. We found that 5-FU, gemcitabine, and paclitaxel, induced cell cycle arrest, which was not changed by any of the IR doses tested (Figure C11). A similar result was obtained in HMVEC-L (Figure C12). Therefore, our results suggest that low doses of IR do not protect ECs from cell death induced by 5-FU, gemcitabine, or paclitaxel.

Taken together, we found that the functional and molecular effects of the low doses of IR, which may contribute to a pro-angiogenic response, are not specific to HMVEC-L and can also be observed in HUVEC.

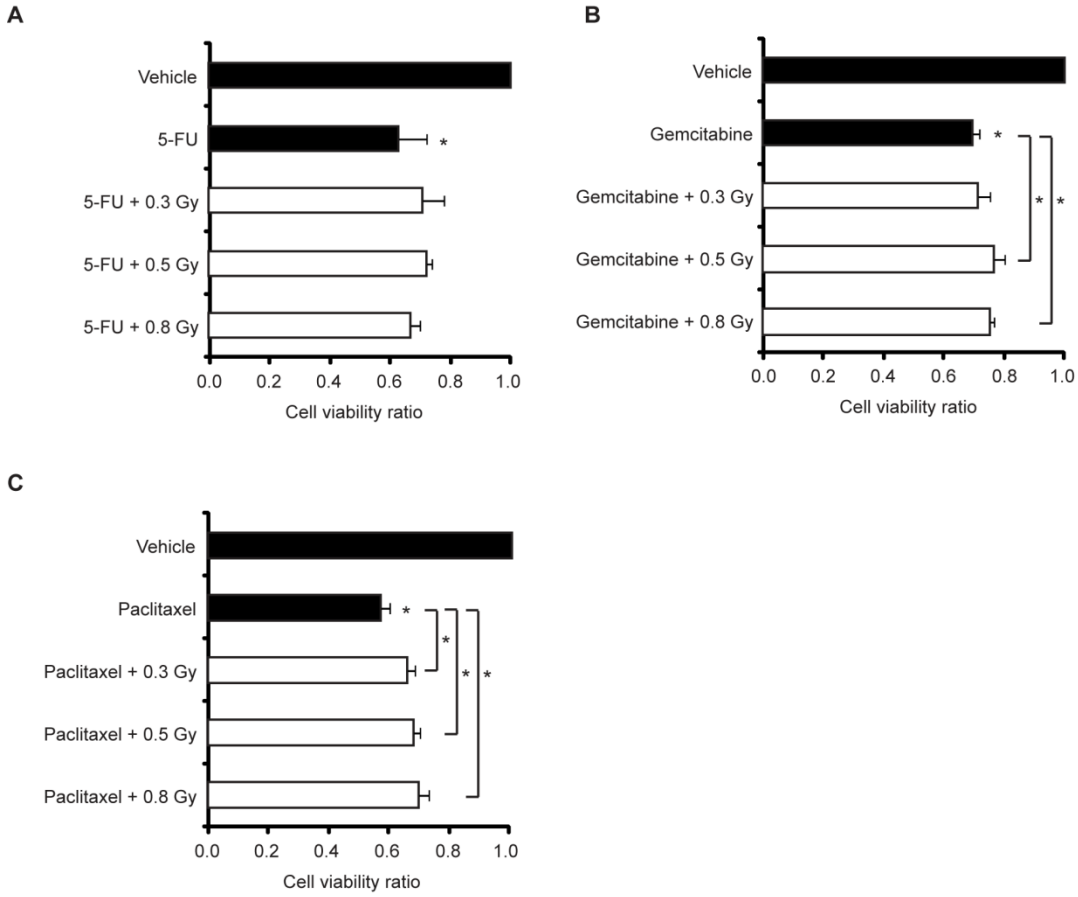


**Figure C10| Low doses of IR protect HUVEC from gemcitabine- and paclitaxel-induced cell death 60 h post-treatment.** HUVEC were plated at equal densities, cultured for 12 h, and treated or not with (A) 5-FU (5 µg/ml), (B) gemcitabine (0.08 µM) or (C) paclitaxel (3 nM); cells were then exposed or not to 0.3, 0.5 or 0.8 Gy. Non-irradiated cells cultured with vehicle alone were used as a control. Cells were double-stained with Annexin-V and PI 60 h post-irradiation, and the percentage of apoptotic cells was assessed by flow cytometry. The values (means ± s.d.) represent the ratio between the viability percentage of irradiated and non-irradiated conditions and are derived from three independent experiments. *P<0.04.

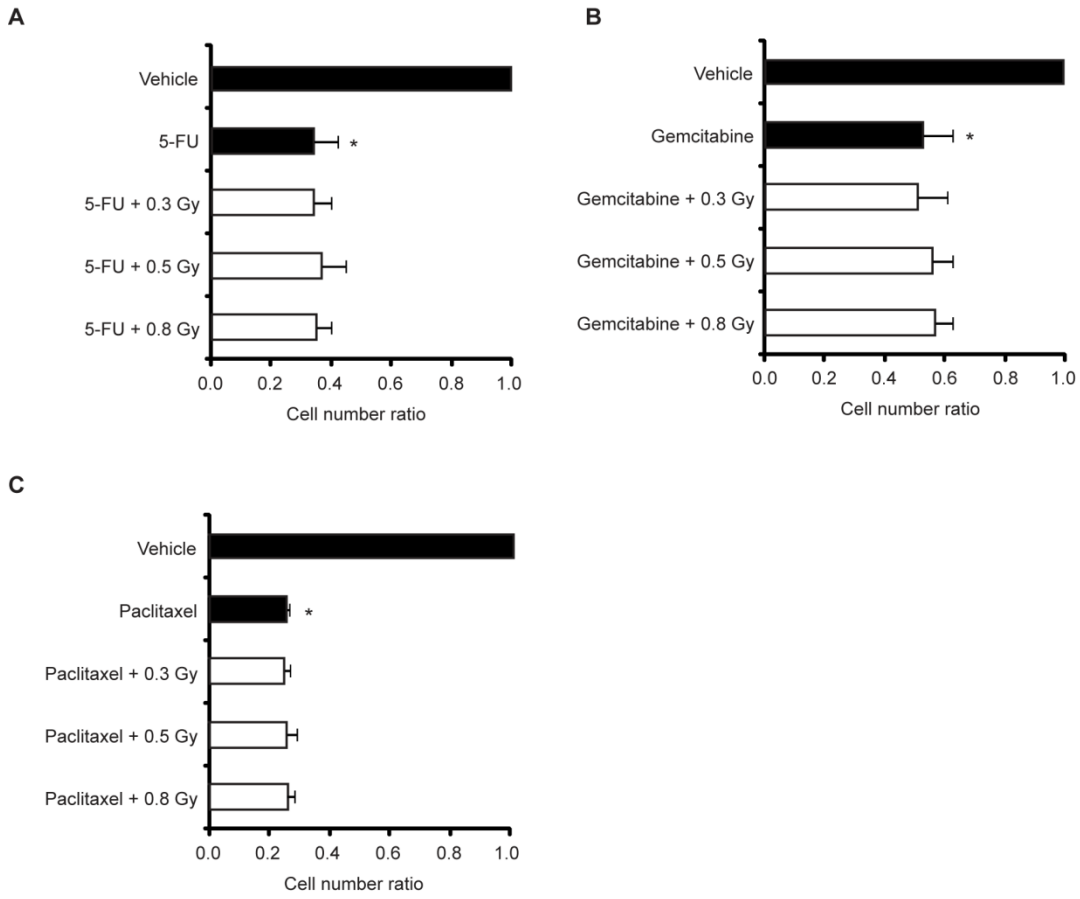


**Figure C11| Low doses of IR are not able to prevent the HUVEC arrest induced by 5-FU, gemcitabine or paclitaxel.** HUVEC were plated at equal densities, cultured for 12 h, and treated or not with (A) 5-FU (5 μg/ml), (B) gemcitabine (0.08 μM) or (C) paclitaxel (3 nM); cells were then exposed or not to 0.3, 0.5 or 0.8 Gy. Non-irradiated cells cultured with vehicle alone were used as a control. Cells were counted after 72 h of culture. The values (means ± s.d.) represent the ratio between the cell number of irradiated and non-irradiated conditions and are derived from three independent experiments. *P<0.01.

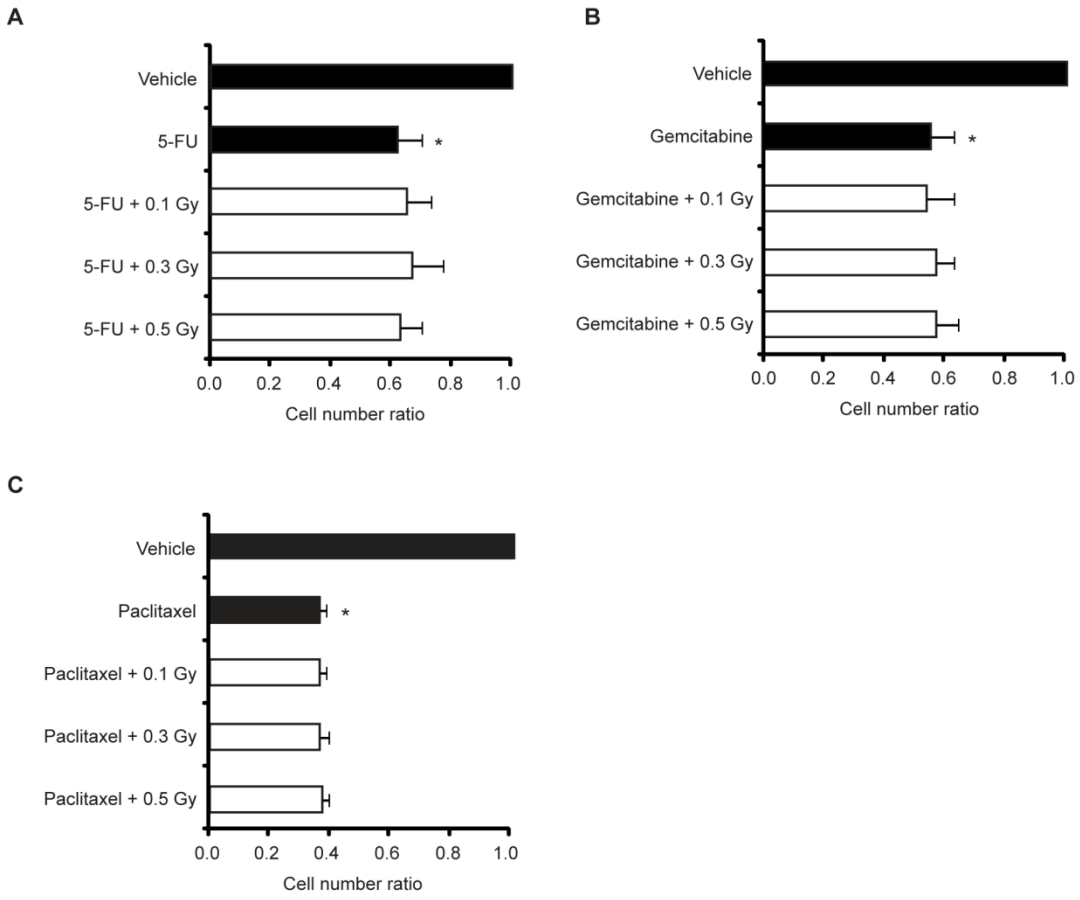


**Figure C12| Low doses of IR are not able to prevent the HMVEC-L arrest induced by 5-FU, gemcitabine or paclitaxel.** HMVEC-L were plated at equal densities, cultured for 12 h, and treated or not with (A) 5-FU (5 μg/ml), (B) gemcitabine (0.08 μM) or (C) paclitaxel (3 nM); cells were then exposed or not to 0.1, 0.3 or 0.5 Gy. Non-irradiated cells cultured with vehicle alone were used as a control. Cells were counted after 72 h of culture. The values (means ± s.d.) represent the ratio between the cell number of irradiated and non-irradiated conditions and are derived from three independent experiments. *P<0.01.

**LOW DOSES OF IR MAY BE INVOLVED IN A PRO-ANGIOGENIC RESPONSE BY MODULATING GENE EXPRESSION**

In order to identify the molecular targets involved in the mechanisms whereby low doses of IR induce a pro-angiogenic response, we investigated the gene expresion profile of irradiated and unirradiated HMVEC-L using the Affymetrix GeneChip human gene 1.0 ST array. Independent cultures of HMVEC-L were exposed or not to 0.3 Gy and RNA samples were prepared 4 h after treatment and sent to Affymetrix, who have processed them for hybridization. The 4 h time point was chosen in order to detect putative genes directly involved in the IR response, minimizing indirect late events.

According to one-way analysis of variance (ANOVA), with a cut-off value of $P<0.05$, from the 28 869 genes represented on the array, 3 459 genes were significantly changed in response to IR. As a quality control, we carried out principal component analysis (PCA) to estimate the sources of variability within the data. PCA relative to all the genes obtained by the array before ANOVA, is shown in Figure C13, panel A. The results show trends similar to those observed after ANOVA (Figure C13, panel B), where a statistically significant separation of samples is detected between the groups of unirradiated and irradiated cells.

To further investigate the biological relevance of the data obtained from the gene expression microarrays, we used the ingenuity pathway analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com). By this analysis, we explored the molecular interactions and biological processes in which the genes significantly modified by low doses of IR could be involved. The canonical pathway analysis allowed us to understand which well-characterized cell signaling pathways were being preferentially modified by low-dose IR. The functional network analysis indicated us which biological processes were mostly changed. The top twenty canonical signaling pathways that were most significantly associated with our dataset (list of genes that are significantly modified by low-dose IR, either by an up- or down-regulation) are illustrated in Figure C14.

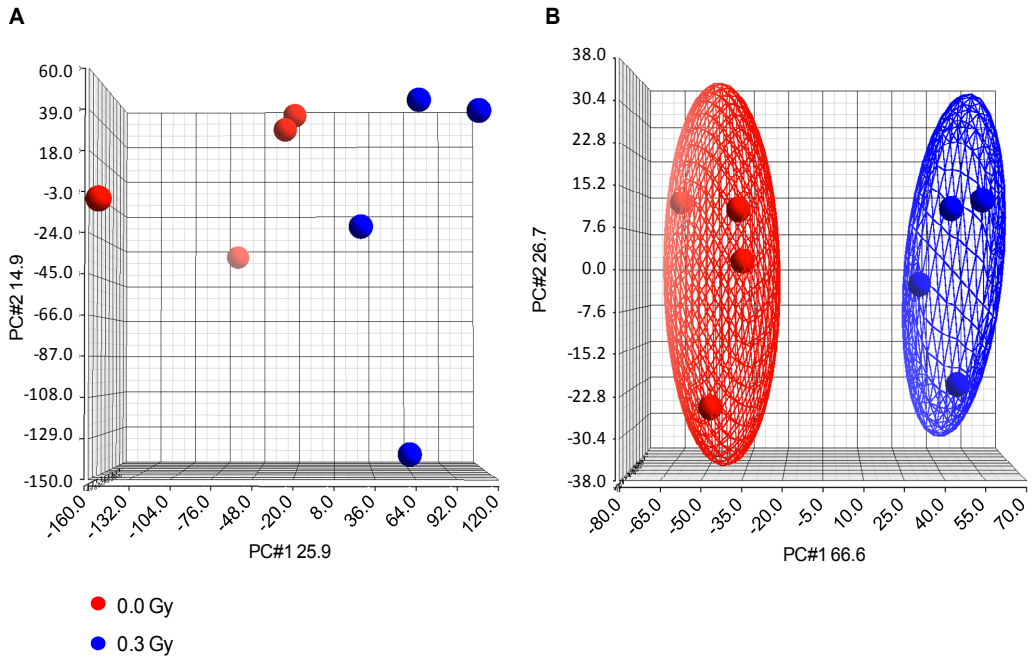


**Figure C13| Principal component analysis (PCA) of irradiated and unirradiated HMVEC-L.** Four RNA samples of each experimental group were obtained and processed for hybridization to Affymetrix Human Gene 1.0 ST arrays. PCA was performed (A) before and (B) after one-way ANOVA, using Partek Genomics Suite software. The percentage of variance attributed to principal components one (PC#1) and two (PC#2) is shown on the *x*- and *y*-axes, respectively. The closer two points are in the plot, more similar they are in terms of global gene expression profile. Thus, the first principal component separates irradiated from unirradiated populations, while the second principal component shows the separation between the experimental replicates. The red (unirradiated, 0.0 Gy) and blue ellipses (irradiated, 0.3 Gy) define the boundary of two standard deviations from the center of each cluster, indicating a statistically significant separation of samples based on the IR stimulus.

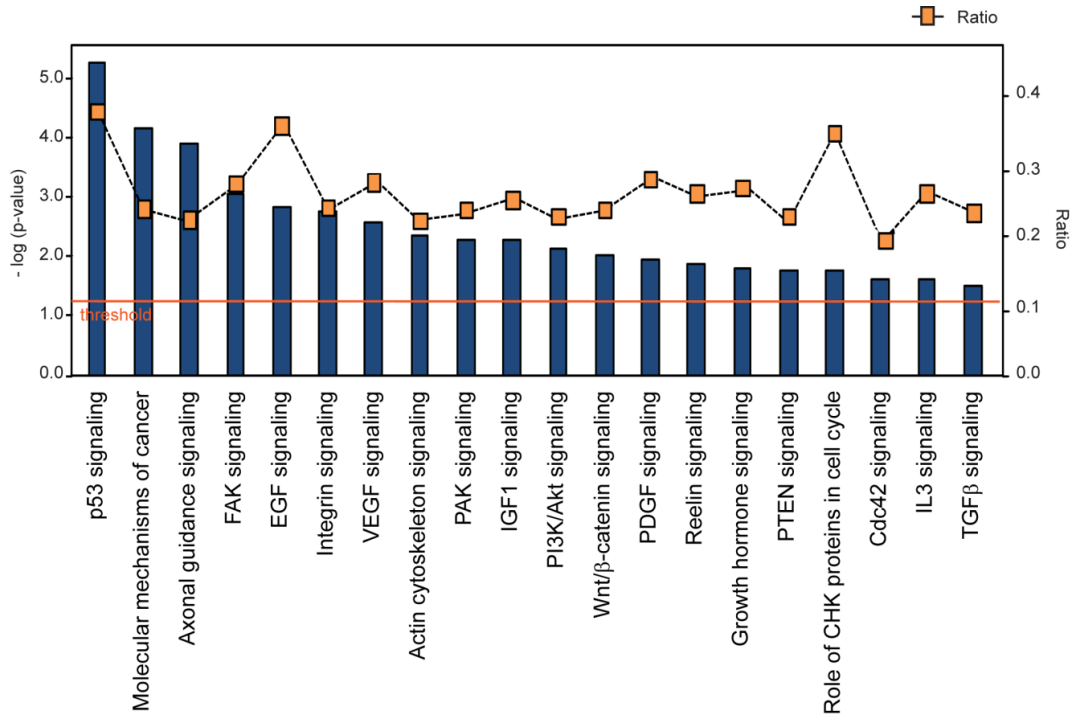


**Figure C14| Ingenuity pathway analysis showing canonical pathways significantly modulated by low-dose IR in HMVEC-L.** Only the 20 pathways with the most significant changes are shown. The P-value for each pathway is indicated by the bar and is expressed as negative log of P-value calculated using Fisher exact test; threshold is at 1.25 = −log (P = 0.05). The orange squares represent the ratio of the number of genes in a given pathway that meet the cutoff criteria divided by the total number of genes that make up that pathway.

Despite IPA also allowing us the direct visualization of the main biological processes linked to these target molecules (Figure C15), the most prominent signaling pathways associated to a molecular low-dose IR response were, in a general way, associated to three main groups: 1) growth factor and cytokine signaling which regulate cell proliferation, migration, survival and differentiation (EGF, VEGF, IGF1, Wnt, PDGF, growth hormone, IL3, and TGFβ2 signaling) (Distler et al., 2003; Kuwano et al., 2001); 2) cytoskeleton-related elements involved in cytoskeleton rearrangement, adhesion and migration (axonal guidance, FAK, integrin, actin, PAK, reelin, and cdc42 signaling (Adams and Eichmann, 2010; Chien et al., 2005; Hashimoto-Torii et al., 2008; Lamalice et al.,

2007; Munoz-Chapuli et al., 2004); and 3) stress-induced molecules, often associated to IR-induced damages, that play a central role in cell-cycle progression and DNA repair (CHK proteins, and p53 signaling) (Pawlik and Keyomarsi, 2004). PI3K/Akt signaling was also one of the canonical pathways with higher score, representing an intracellular signaling effector involved in signal transduction pathways of growth factors and cytokines (Munoz-Chapuli et al., 2004; Shiojima and Walsh, 2002).

Interestingly, many of the targets modulated by low-dose IR are also involved in the molecular mechanisms of cancer, promoting tumor growth as well as invasion.

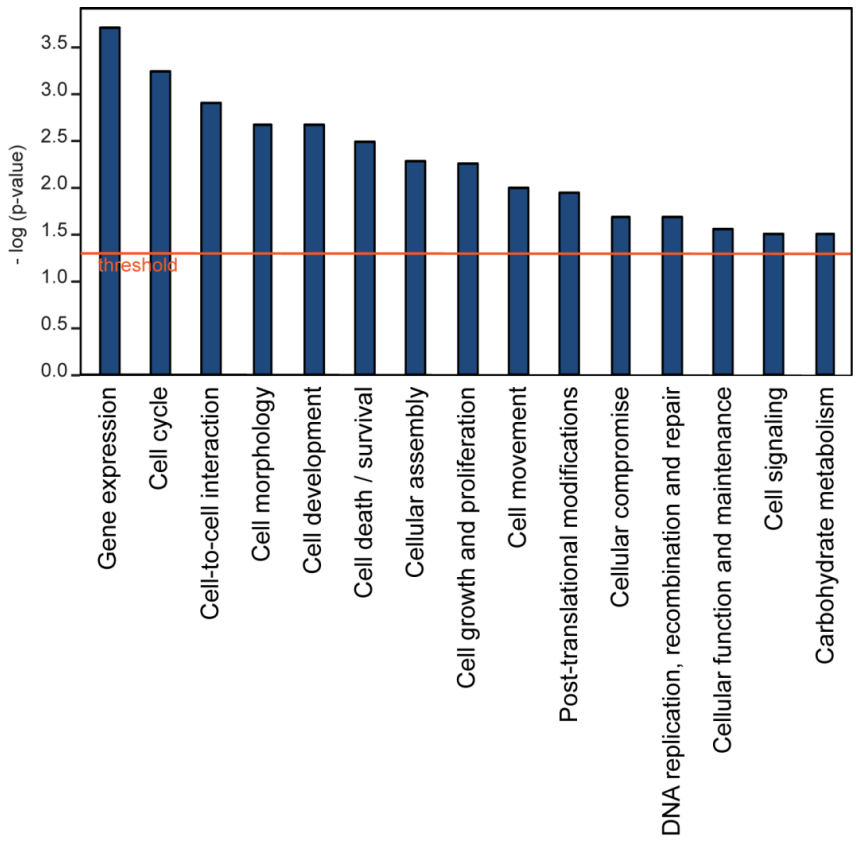


**Figure C15| Ingenuity pathway analysis showing the cellular biological functions significantly modulated by low-dose IR in HMVEC-L.** Only the 15 pathways with the most significant changes are shown. The p-value for each pathway is indicated by the bar and is expressed as negative log of P-value calculated using Fisher exact test; threshold is at 1.25 = −log (P = 0.05).

A careful analysis of the microarray data is now being done in our lab, in order to choose the genes whose expression is significantly changed by low-dose IR and that represent the best candidates for a pro-angiogenic response. However, some growth factors and endothelial receptors that are largely described as being associated to the EC activation and angiogenesis were already selected (*VEGFR1*, *VEGFR2*, *ANGPT2*, *FGF2*, *CYR61* and *TGFβ2*). As cytoskeleton-related elements were so well represented in our canonical signaling pathway analysis, and we have previously demonstrated that low-dose IR stimulates the pro-migratory activity of ECs, which involves cytoskeleton rearrangement (Lamalice et al., 2007), we decided to select also β-tubulin (*TUBB*) and clathrin (*CLTC*).

In a microarray analysis, some level of false positives is inevitable. For that reason, the expression of these selected genes was confirmed by quantitative RT-PCR (qRT-PCR). Analysis was performed at 4 h, 8 h and 12 h post HMVEC-L irradiation. According to our results, 0.3 Gy modulated the expression of all the selected pro-angiogenic targets and cytoskeleton-related proteins (Figure C16).

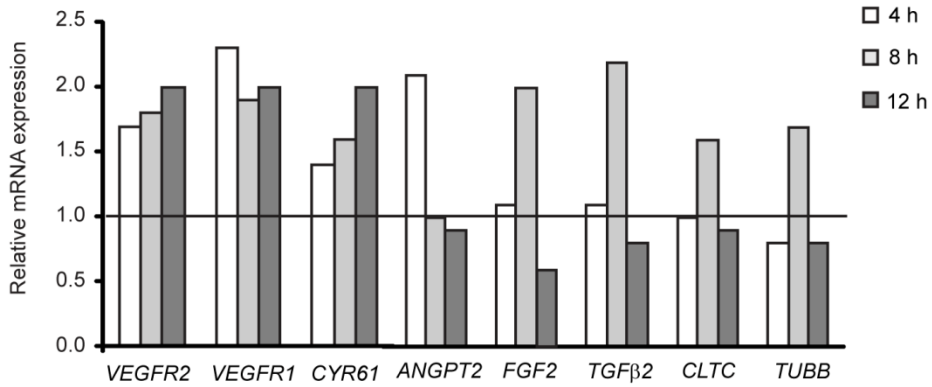


**Figure C16| Low doses of IR modulate the expression of several pro-angiogenic targets and cytoskeleton-related proteins in HMVEC-L.** HMVEC-L were exposed or not to 0.3 Gy and incubated for the time indicated. The mRNA expression of several pro-angiogenic targets (*VEGFR2*, *VEGFR1*, *CYR61*, *ANGPT2*, FGF2, *TGFβ2*) and cytoskeleton-related proteins (*CLTC*, *TUBB*) was quantified by qRT-PCR. Data (mean) represents the fold change in gene expression of each target in irradiated cells relative to the internal calibrator (non-irradiated) in triplicate

measurements; the error bars cannot be seen because the standard deviation (± s.d.) is negligible. Chart values are representative of three independent experiments.

In agreement to the microarray analysis, irradiated cells presented an up-regulation of *VEGFR2*, *VEGFR1*, *CYR61* and *ANGPT2* mRNA expression, 4 h post-irradiation. Additionally, an up-regulation of *VEGFR2*, *VEGFR1* and *CYR61* was also obtained at the remaining time points. Although our qRT-PCR analysis did not confirmed the microarray results concerning the up-regulation of *FGF2*, *TGFβ2*, *CLTC* and *TUBB* at 4 h post-irradiation, it is interesting to observe that this effectively occurs at 8 h post-irradiation.

There is a report from Lanza et al. (Lanza et al., 2005) that describes the transcriptional response of HUVEC to 0.2, 0.5, 1.0 and 2 Gy at 4 h post-irradiation (accessible by http://www.caspur.it/RadiationGenes, a recent web data base on IR response microarray screening). A significant change on the expression of *CYR61*, *VEGFR1* and *VEGFR2* was also reported by Lanza et al. (Lanza et al., 2005). However, they described the down-regulation of *VEGFR1* and *VEGFR2* in response to IR. Since their experiments were done in HUVEC and ours in HMVEC-L, we decided to test the expression of the 3 target genes (*CYR61*, *VEGFR1* and *VEGFR2*) also in HUVEC. HUVEC were exposed to 0.8 Gy and the mRNA levels of *CYR61*, *VEGFR1* and *VEGFR2* analyzed 4 h post-irradiation (Figure 17). According to our results, 0.8 Gy induced the expression of *CYR61*, *VEGFR1* and *VEGFR2* in HUVEC, which is consistent with our HMVEC-L data and the opposite of what was reported by Lanza et al. (Lanza et al., 2005).

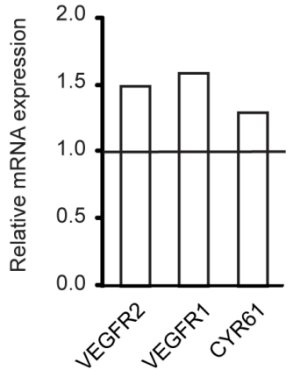


**Figure C17| Low doses of IR modulate the expression of *VEGFR2*, *VEGFR1* and *CYR61* in HUVEC.** HUVEC were exposed or not to 0.8 Gy and incubated for 4 h. The mRNA expression of *VEGFR2*, *VEGFR1* and *CYR61* was quantified by qRT-PCR. Data (mean) represents the fold change in gene expression of each target in irradiated cells relative to the internal calibrator (non-irradiated) in triplicate measurements; the error bars cannot be seen because the standard deviation (± s.d.) is negligible.

#### LOW DOSES OF IR ACCELERATE ZEBRAFISH GROWTH

We have previously shown that low-dose IR accelerates angiogenic sprouting during the development of the sub-intestinal vessels (SIV) in zebrafish (Sofia Vala et al., 2010). Additionally, we followed the effects of low-dose IR over time in these animals. Figure C18 illustrates live *Fli1:EGFP* zebrafish embryos that were exposed or not to 0.5 Gy at 3 days post-fertilization (dpf), and photographed over time. Our observations revealed that there was a clear advantage for growth in the irradiated animals at 27 dpf. Still, this difference was attenuated at 46 dpf.

Therefore, low-dose IR accelerates zebrafish growth speed without causing abnormal growth patterns.

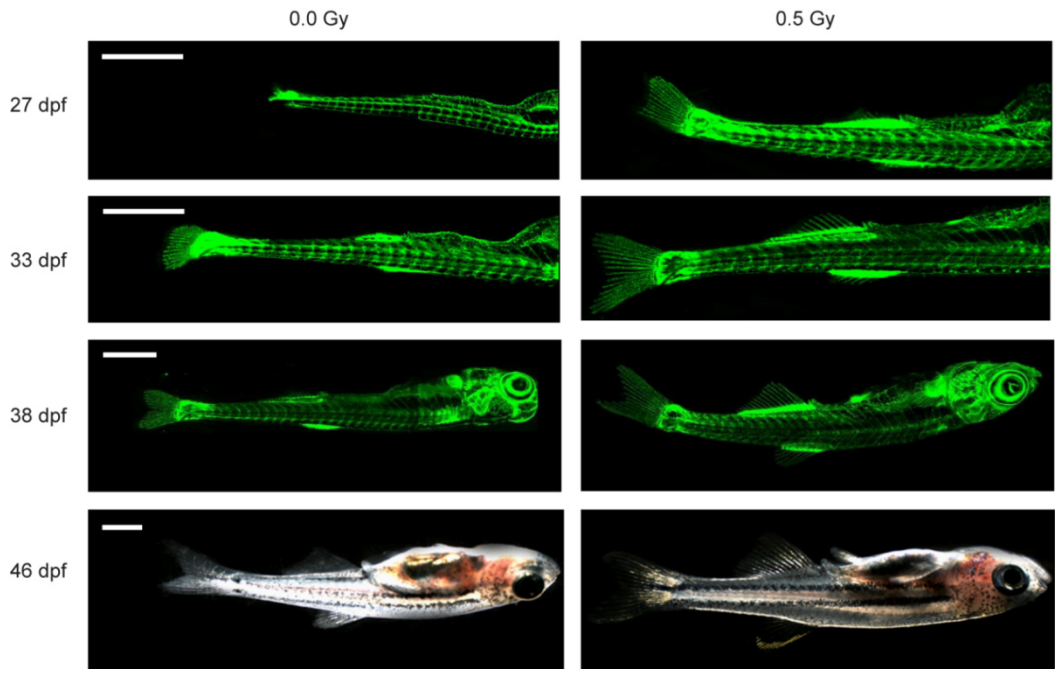


**Figure C18| Low-Dose IR accelerates zebrafish development.** Live *Fli1:EGFP* zebrafish embryos were exposed or not to 0.5 Gy at 3 dpf and photographed over-time. Scale bars, 1mm.

### *p53* MUTATED *BRAF*$^{V600E}$ ZEBRAFISH AS A MODEL TO STUDY TUMOR DEVELOPMENT

We have previously shown that low-dose IR promotes acceleration of tumor growth in two different mouse models by enhancing angiogenesis (Sofia Vala et al., 2010). Complementarily, a *p53* mutated *BRAF*$^{V600E}$ zebrafish model (Patton et al., 2005) was also used to study the effect of low-dose IR in melanoma development. According to the literature (Patton et al., 2005), these transgenic animals spontaneously develop melanoma within 4 months of life, which can be earlier detected as a constrained spot in the zebrafish body surface or head. To avoid irradiating the tumor cells, we performed all body low-dose irradiation, approximately at 3 months of age. Thus, *p53* mutated *BRAF*$^{V600E}$ zebrafish were exposed or not to 0.5 Gy during 4 consecutive days (4 x 0.5 Gy).

Animals were followed over-time. Four months later, 11 out of 37 (29.7 %) irradiated *p53* mutated *BRAF*$^{V600E}$ zebrafish *versus* 4 out of 29 (13.8 %) unirradiated *p53* mutated *BRAF*$^{V600E}$ zebrafish, presented naked-eye detectable melanoma. Melanomas were identified in head, body or zebrafish fins in both groups (Figure C19), which is consistent to what has been described previously (Patton et al., 2005). Moreover, our results showed that melanomas found in the irradiated group were bigger than those in the unirradiated group (Figure C20).


**Figure C19| *p53* -/- *BRAF*$^{V600E}$ zebrafish develop spontaneous melanomas.** Photographs are representative of advanced stage melanomas in (A) the head, (B) caudal fin, or (C) body surface of *p53* -/- *BRAF*$^{V600E}$ transgenic zebrafish. (*) indicates the place of the tumor.

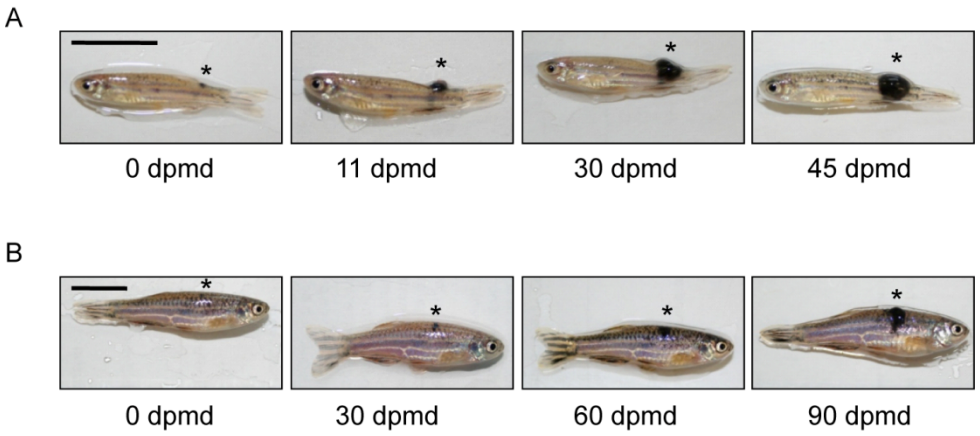


**Figure C20| *p53* -/- *BRAF*$^{V600E}$ zebrafish exposed to low-dose IR seem to present bigger tumors with an accelerated growth.** Animals were exposed or not to 4 x 0.5 Gy at 3.5 months old. First signs of tumor were detected 1 month after the beginning of the experiment. Some of the (A) irradiated zebrafish seem to develop bigger melanomas in a shorter time that (B) unirradiated

animals. Zebrafish were photographed at the day of melanoma detection (0 days post melanoma detection, dpmd), and followed over time. (*) indicates the place of the tumor.

A second similar experiment was performed. Here, we decided to maintain the daily dose and to increase the number of irradiation days. According to our preliminary results, adult *Fli1:EGFP* zebrafish exposed to 0.5 Gy during 15 days (15 x 0.5 Gy), present higher vascular density in their tail fin when compared to zebrafish exposed to 4 x 0.5 Gy or unirradiated zebrafish. Therefore, *p53* mutated $BRAF^{V600E}$ zebrafish were exposed to 15 x 0.5 Gy. After five months, 9 out of 22 (40.9 %) irradiated animals *versus* 3 out of 19 (15.8 %) unirradiated animals presented naked-eye detectable melanomas. These animals are now being histologically analyzed in order to compare the size and the invasion capacity of melanomas found in zebrafish of both experimental conditions (irradiated *versus* unirradiated).

Our results suggest that the development of melanoma may be potentiated in *p53* mutated $BRAF^{V600E}$ zebrafish previously exposed to low doses of IR.

## DISCUSSION OF THE CHAPTER

Radiotherapy is the treatment of choice for primary solid tumors, either alone or in combination with surgery and chemotherapy. However, patients with locally recurring tumors after radiotherapy have increased risk of metastatic progression and poor prognosis. The clinical management of this condition is a challenge, and the underlying cellular and molecular mechanisms are still a matter of investigation.

The goal of radiotherapy is to reduce or eliminate the primary tumor and its metastasis. Nevertheless, the mechanisms behind IR response and the final outcome in tumor cells involve interactions between the tumor and tumor-associated host cells (Barcellos-Hoff et al., 2005; Morgan, 2003). Therefore, it is important to consider not only the tumor cells' responsiveness, but also the relevance of IR-induced changes on the tumor microenvironment.

Considering the vascular context, IR may exert its anti-angiogenic activity through a direct pro-apoptotic effect on ECs *via* ceramide pathway (Garcia-Barros et al., 2003), as well as a pro-angiogenic function through signals that are released by irradiated cancer cells (Abdollahi et al., 2005; Madani et al., 2008; McBride et al., 2004). Since angiogenesis is crucial for tumor progression, the contribution of the irradiated vasculature in invasion and metastasis after radiotherapy is particularly relevant.

In addition, healthy surrounding tissues are also exposed to doses of IR lower than the therapeutic ones, whose effects have been mostly neglected so far.

Therefore, we aimed to investigate the effects of these low IR doses on angiogenesis, and their possible contribution to the pro-metastatic effect of IR treatment.

Since the IR-induced response can be considerably different depending on the irradiation conditions (e.g. quality and dose rate), often leading to inconsistent results (Chiani et al, 2009), we chose to perform our experiments using a linear accelerator x-ray photon beam operating at a dose rate of 300 MU/min, reproducing the most commonly used conditions in the clinical practice. The protocol was optimized in collaboration with the

Department of Radiotherapy from the Hospital de Santa Maria, assuring the accuracy of the radiation incidence.

### LOW DOSES OF IR ARE ABLE TO INDUCE AN ANGIOGENIC RESPONSE *IN VITRO* THROUGH THE ACTIVATION OF INTRACELLULAR SIGNALING PATHWAYS ASSOCIATED WITH CELL SURVIVAL AND MIGRATION.

To ascertain the functional and molecular effects of the low IR doses on ECs, we used two endothelial cell types, HMVEC-L and HUVEC. Although the optimal dose range may slightly fluctuate, our results showed that the IR-induced pro-angiogenic response is consistent in both cell types.

Exposure to IR is typically characterized by cellular DNA damage, and although the cell's fate is dependent on a complex set of signaling transduction pathways that regulate the cellular machinery, the number of DSBs is what better correlates with cell apoptosis (Connell et al., 2004). IR leads to the rapid phosphorylation of H2AX (γ-H2AX), that can be quantified as an intracellular marker directly associated to the number of DNA DSBs (Kuo and Yang, 2008).

As expected, we identified a high number of DSBs in HMVEC-L exposed to 10 Gy of IR, which were not completely repaired 24 h post-irradiation. Since it was described that γ-H2AX recruits repair enzymes to DNA damage sites (Joiner and van der Kogel, 2009), it is likely that these remaining nuclear foci represent non-repair DNA DSBs that may lead to subsequent cell lethality. On the other hand, we observed that even IR doses lower than the therapeutic ones (< 2 Gy) are able to induce DSBs in a dose dependent manner. However, these damages were completely repaired in less than 12 h post-irradiation. Interestingly, despite the complete repair of the IR-induced DSBs and the absence of changes in the survival rate of these cells, a careful analysis revealed a lower proliferation rate of cells exposed to 1.0 Gy. Furthermore, these cells seem to arrest at the G2/M phase, probably reflecting the activation of the cell cycle checkpoints.

Therefore, our results suggest that normal cells exposed to doses progressively lower than 2 Gy are able to activate their DNA repair mechanisms. Tumor cells, on the other hand, generally avoid some cell cycle checkpoints where the DNA repair mechanisms are activated, thereby accumulating several DNA damages over successive radiotherapy sessions, eventually leading to death.

Importantly, 0.8 Gy or lower doses, promote a pro-migratory response in ECs without causing cell cycle arrest or apoptosis, therefore conferring a pro-angiogenic advantage.

It is noteworthy that doses higher than 1.0 Gy do not induce EC migration and are responsible for a significant decrease in cell proliferation rate. These results are apparently contradictory to the data published by Sonveaux et al., showing a pro-migratory response of bovine aortic EC to 6.0 Gy of IR (Sonveaux et al., 2003). However, an orthovoltage equipment was used in this study and the different type of energy may explain the different molecular response found in both works.

At the molecular level, we have shown that low IR doses (≤ 0.8 Gy) may induce the phosphorylation of several cellular proteins including Akt, ERK and VEGFR2.

There are generally two proposed mechanisms that lead to the activation of membrane receptors and intracellular signaling pathways. The first involves a radiation-induced ATM-related mechanism that, besides activating cell cycle checkpoints and DNA repair, enhances the activity of growth factor receptors and signal-related molecules. The second is related to the generation of large amounts of ROS that inhibit protein tyrosine phosphatase activity, leading to increased tyrosine phosphorylation and activation of downstream signal transduction pathways (Valerie et al., 2007).

The activation of MAPK/ERK and PI3K/Akt pathways has already been cited as mediating different IR-induced effects. In tumor cells, radiation-induced ERK phosphorylation has been described to both enhance pro-survival and DNA repair mechanisms, or increase cell death (Golding et al., 2007; Grant et al., 2002; Park et al., 2001; Yacoub et al., 2001). The PI3K/Akt pathway has been widely associated to the radioresistance effect, playing a part in tumor cell survival, proliferation and mitogenic activity (Kargiotis et al., 2010; Schuurbiers et al., 2009). Similarly, in ECs, Akt and ERK both have a central role as

intracellular signaling players involved in the signal transduction pathways of several pro-angiogenic growth factors and cytokines (Munoz-Chapuli et al., 2004; Shiojima and Walsh, 2002), regulating functions of survival, proliferation and migration (Munoz-Chapuli et al., 2004; Shiojima and Walsh, 2002). Much fewer reports specifically associate the MAPK/ERK and PI3K/Akt pathways with IR-induced ECs response, and only globally discuss the role of these pathways in the signal transduction of VEGF and EGF signaling (Kargiotis et al., 2010).

Here, we demonstrate that low doses of IR induce the activation of Akt and ERK. Moreover, the functional relevance of this IR-induced activation was shown by the protective effect from cell death and impaired migration induced by pathway specific inhibitors.

Considering these results, we decided to focus in one of the most significant pathways in the angiogenic process (Kuwano et al., 2001), the VEGF/VEGFR signaling. Therefore, we evaluated if low-dose IR was also able to effectively protect ECs from the cell death induced by bevacizumab, a monoclonal anti-VEGF antibody which neutralizes VEGF, thus inhibiting its binding to specific receptors.

We demonstrated that low-dose IR protects ECs from bevacizumab-induced cell death through a VEGF-independent phosphorylation of VEGFR2, which is the key VEGF receptor involved in pro-angiogenic effects (Distler et al., 2003; Ferrara, 2001; Kuwano et al., 2001; Shibuya and Claesson-Welsh, 2006; Takahashi and Shibuya, 2005). Accordingly, the IR-induced protective effect was completely nullified by the specific inhibition of VEGFR2, which also plays a key role in the IR-induced pro-migratory effects.

Our observations do not go against the previously published studies that demonstrate the importance of anti-VEGF treatment in normalizing the vascular function, improving intratumoral oxygenation and consequently IR efficacy (Gorski et al., 1999; Grothey et al., 2008; Lee et al., 2000; McGee et al., 2010; Myers et al., 2010; Willett et al., 2010). Our data demonstrate that beyond the expected effects of anti-angiogenic therapy in the tumor target, low doses of IR may contribute to tumor progression during or after radiotherapy by activating VEGFR2 and, consequently, angiogenesis.

Additionally, we have also investigated whether low-dose IR was able to modulate the expression of VEGF in ECs. It is generally accepted that ECs do not express VEGF under normoxia conditions (Dewhirst et al., 2008; Lee et al., 2007). However, its expression has already been described under hypoxia (Liu et al., 1995), where mainly two different regulation mechanisms ensure increased VEGF expression. The first involves HIF1, which in hypoxia is stabilized and translocated into the nucleus where it binds to the *VEGF* promoter, mediating the increased transcription of *VEGF* mRNA (Levy et al., 1995; Pugh and Ratcliffe, 2003). The second mechanism involves the hypoxic-induced stability factor (HuR), which binds to a specific site at the *VEGF* mRNA and inhibits the enzymatic action of RNAses, resulting in a 3 to 8 fold increase of the half-life of this mRNA (Levy et al., 1996; Levy et al., 1998).

According to our results, low-dose IR is still not able to induce *VEGF* transcription under normoxia (data not shown). However, it is able to enhance the *VEGF* transcription and consequently protein expression in cells previously exposed to $CoCl_2$, which canonically induces hypoxia-mimicking conditions by promoting the expression and stabilization of HIF1 (Liu et al., 1999; Vengellur and LaPres, 2004; Vengellur et al., 2005).

Although our data do not explain the mechanism by which low-dose IR up-regulates VEGF under hypoxic conditions, it is interesting to consider that IR may play a role in the regulation of HuR, the main mechanism responsible for the stabilization of the *VEGF* mRNA under hypoxic conditions. HuR function is controlled in two ways: (1) by its mobilization from the nucleus to the cytoplasm, where it modulates the stability and translation of target mRNAs, and (2) by phosphorylation, which enhances its association with target mRNAs promoting their stabilization (Kim et al., 2010). It has recently been described that DNA damage regulates HuR function through an ATM-dependent mechanism, causing the accumulation of HuR in the cytoplasm that leads to an enhancement of its stabilization activity (Kim et al., 2010). Additionally, phosphorylated ERK and Akt seem also to influence HuR function, either by favoring its binding to the mRNA targets or by increasing its expression (Galban et al., 2008; Kang et al., 2008).

Therefore, since low IR doses induce DNA DSBs, and induce the phosphorylation of both ERK and Akt, it would be interesting to evaluate if the mechanism underling the observed enhanced VEGF expression may be explained through the stabilization of the mRNA whose expression was already been induced by HIF1.

Furthermore, this finding is clinically relevant since hypoxia occurs as a natural condition during cancer development both within the tumor and in its periphery (Zoula et al., 2003). Consequently, the exposure of these hypoxic areas surrounding the tumor area to low doses of IR may further enhance VEGF expression.

Altogether, our *in vitro* results, strongly suggest that low doses of IR induce the activation of several intracellular signaling pathways responsible for pro-angiogenic effects.

#### LOW DOSES OF IR ARE ABLE TO INDUCE AN ANGIOGENIC RESPONSE *IN VIVO*.

Since *in vitro* experiments are unable to recreate the microenvironment of an intact organism and the effects in ECs may be different from those found *in vivo*, we decided to use *Fli1:EGFP* zebrafish and a murine matrigel plug assay as models to ascertain the IR-induced effects on vasculature.

Because of its unique characteristics, and the recent availability of transgenic animals expressing GFP in ECs, zebrafish became a valuable model to study angiogenesis. During embryogenesis, the first vessels arise through vasculogenesis but, soon after that, it is possible to see some smaller vessels forming from sprouting angiogenesis. At day 3 post-fertilization, SIV start to take shape through angiogenesis (Isogai et al., 2001), representing an exceptional model to study sprouting. Here, we demonstrated that low-dose IR accelerates angiogenic sprouting of the SIV, without compromising the normal blood vessel pattern. Additionally, using adult *Fli1:EGFP* zebrafish subject to caudal fin amputation, we have also revealed the capacity of low-dose IR to enhance neo-vessel formation in adult animals during regeneration.

Furthermore, through the use of a murine matrigel plug assay, we have also confirmed the ability of low-dose IR to induce angiogenesis in mammals.

It is noteworthy that both development and regeneration zebrafish models attend an active angiogenic process, even before the exposure to radiation. This means that low-dose IR is able to increase the pro-angiogenic response in an already pre-activated system, similarly to what can be found in the vasculature that proximately surrounds the primary tumor and is continuously stimulated by the tumor microenvironment. On the other hand, the murine matrigel plug assay addressed in an adult stable vasculature, showed that exposure to a low IR dose changed the microenvironment enhancing a posterior pro-angiogenic stimulus, given by FGF2.

Therefore, considering the overall relevance of angiogenesis in tumor progression, we asked if these low IR doses could indeed affect tumor growth and metastasis *in vivo*.

### LOW-DOSE IR PROMOTES ACCELERATION OF TUMOR GROWTH AND METASTASIS IN A VEGFR-DEPENDENT MANNER.

In order to investigate the contribution of the low-dose IR in tumor re-growth and metastasis after radiotherapy, we used two different mouse models of cancer: a leukemia and a metastatic breast cancer model.

NOD/SCID mice injected with MOLT-4 cells is a recognized model for the study of acute lymphocytic leukemia (Siders, 2010). This model was used in order to investigate the effect of low IR doses in tumor growth and dissemination. Therefore, the whole body of 6 week-old NOD/SCID mice was exposed to 0.3 Gy, 22 hours before intravenous injection of MOLT-4 cells expressing luciferase, which allowed us to perform real-time bioluminescent analysis in live animals. By following this procedure, we guaranteed that IR was not acting directly on the tumor cells, and that only the microenvironment contribution was accountable for the final outcome. Moreover, the possibility of following tumor burden since the first moment after MOLT-4 injection allowed us to

identify any possible problems associated to the intravenous injection procedure early on, and to evaluate tumor progression over time. The results revealed a significant increase in MOLT-4 tumor burden in irradiated animals compared to the non-irradiated, confirming the role of low-dose IR in promoting tumor progression.

Considering our *in vitro* results showing that low-dose IR induces VEGFR2 activation, stimulating EC cell migration and protection from bevacizumab-induced cell death, we administrated a VEGFR tyrosine kinase inhibitor, PTK787/ZK222584 (PTK/ZK), 2 hours before irradiation. The inhibiting effect of PTK/ZK in neo-vessel formation had already been effectively shown in patients with advanced colorectal cancer and liver metastases, as well as in different mice tumor models (Drevs et al., 2002; Lee et al., 2006; Miyazawa et al., 2008; Wood et al., 2000). Since tumor cells also express VEGFRs, and are sensitive to treatment with PTK/ZK (Drevs et al., 2002; Lee et al., 2006; Wood et al., 2000), we assured that this was not a variable in our study by injecting the MOLT-4 24 h after the treatment. At this time, the inhibitor is no longer active *in vivo*.

Since the tumor burden of irradiated PTK/ZK-treated animals was significantly reduced when compared to the untreated irradiated group, we could successfully confirm the role of low-dose IR in promoting tumor spreading of leukemic cells through a mechanism involving the activation of the VEGFRs.

Cancer patients after radiotherapy have increased risk of metastatic progression. Therefore, by using the 4T1 orthotopic implantation mouse model of spontaneous breast cancer metastasis (Aslakson and Miller, 1992), we investigated the effect of low-dose IR in tumor metastasis' development.

After the direct injection of luciferase-expressing tumor cells into the mammary fat pad of NOD/SCID *IL2Rgamma*-null mice, the tumor grows rapidly at the primary site and is expected to form metastases in lungs, liver, bone and brain over a period of 3-6 weeks, being the lung the first site to detect nodules (Aslakson and Miller, 1992; Tao et al., 2008). The rapid and efficient establishment of metastases makes the 4T1 model an excellent choice to study the metastatic progression.

By using this model, we showed that low-dose IR enhances the metastatic spreading in a VEGFR-dependent manner, given that the treatment with PTK/ZK generates a significant reduction in the number and size of metastasis, enhanced by low-dose IR alone.

According to our data, it will be interesting to question if a VEGFR2 inhibitor should be given before the radiation treatment in order to prevent the angiogenic effects of low doses of IR in the surrounding tumor tissues. However, this is a complex issue since the VEGFR2 should be inhibited whenever a radiotherapy session occurs and this therapeutic will certainly be dependent of the costs and availability of the VEGFR2 inhibitors. Moreover, a VEGFR2 inhibitor should not be administered in all patients; diabetic cancer patients warrant special consideration and extra care in the design of anti-angiogenic treatments without adverse side effects.

Our findings are of utmost importance to improve radiotherapy protocols and the contribution of the angiogenic effects induced by low doses of IR in metastasis development after radiotherapy should be proven in patients.

In addition to these two cancer mouse models, we also used a *p53* mutated $BRAF^{V600E}$ zebrafish model (Patton et al., 2005) to approach the effects of low IR doses on melanoma development. This model presented some technical difficulties mostly related to the temporal unpredictability of the first signs of melanoma. Nevertheless, our preliminary results suggest that melanomas preferentially emerged in the group of irradiated animals, which also seemed to show bigger tumors. However, to confirm our data, it will be necessary to repeat this experiment with a higher number of animals. The histological analysis (currently being processed in our lab) will also help us to understand if only the number of animals with detectable melanoma was different between groups, or if the size and invasion capacity also changed.

**LOW DOSES OF IR MAY BE INVOLVED IN A PRO-ANGIOGENIC RESPONSE BY MODULATING GENE EXPRESSION.**

In order to identify molecular targets involved in the mechanisms whereby low doses of IR induce a pro-angiogenic response, we performed a transcriptomic analysis using the Affymetrix GeneChip® human gene 1.0 ST array.

DNA microarrays allow us to determine which genes are being expressed in a given cell type, at a particular time, and under particular conditions, with the great advantage that in a single experiment it is possible to address the expression level of thousands of genes within a cell. This is extremely important since it is the set of expressed genes and the interplay between the products encoded by them that determine the cell phenotype.

To study the gene expression pattern of ECs exposed or not to low-dose IR, we used HMVEC-L exposed or not to 0.3 Gy, from which RNA extraction was carried out 4 hours post-radiation. The 4 hours time point was chosen in order to obtain putative genes directly involved in the IR response, minimizing indirect late events.

In order to biologically interpret the data, we used the IPA software to explore the molecular interactions and biological processes in which our list of significant changed genes was involved.

IPA makes use of a non public bibliographic database, the Ingenuity® Knowledge Base, to integrate all the biological data, and it has been described as probably being the fastest and most powerful tool in the analysis of results of functional screens (Jimenez-Marin et al., 2009; Weeraratna and Taub, 2007). Still, we confirmed the results of IPA through the use of another software package, the Onto-Tolls (Intelligent Systems and Bioinformatics Laboratory, http://vortex.cs.wayne.edu/projects.htm), which relies on a large relational database that stores relevant sequence and annotation information from various public data sources including LocusLink and RefSeq, UniGene, dbEST, Gene Ontology, KEGG Genes, KEGG Ligand and KEGG Pathways. The results obtained were similar in both tools (data not shown); however, IPA provided a better presentation of the results and an

easier identification of the molecules implicated in each function within the interface of the software. For this reason, we proceeded using IPA.

We started by generating a canonical pathway and functional network analysis to investigate which well-characterized cell signaling pathways and biological processes are preferentially modified by low-dose IR. The most prominent signaling pathways associated to a molecular low-dose IR response are generally associated to three main groups: 1) growth factor and cytokine signaling which regulate cell proliferation, migration, survival and differentiation (Distler et al., 2003; Kuwano et al., 2001); 2) cytoskeleton-related elements involved in cytoskeleton rearrangement, adhesion and migration (Adams and Eichmann, 2010; Chien et al., 2005; Hashimoto-Torii et al., 2008; Lamalice et al., 2007; Munoz-Chapuli et al., 2004); and 3) stress-induced molecules, often associated with IR induce damages, that play a central role in cell-cycle progression and DNA repair (Pawlik and Keyomarsi, 2004). Therefore, it is possible to identify a unique gene pattern with function-related characteristics consistently associated to a pro-angiogenic response in ECs.

Interestingly, IPA also correlated many of the targets modulated by low-dose IR with molecular mechanisms of cancer, involved in tumor growth and invasion. Although, in the clinical context, cancer cells targeted for treatment do not receive doses lower than the therapeutic ones, it is important to have these results in consideration. On one side, we have the ECs activated by low-dose IR that will secrete growth factors that may contribute to tumor and endothelial cell precursors (EPCs) chemotaxis, dictating the pattern of metastatic spread. On the other hand, since it was described that dormant cancer cells can be located in the periphery of the planning target volume (PTV) (Bergers and Benjamin, 2003), we may hypothesize that, by being directly exposed to these low IR doses, dormant tumor cells will be activated.

Although a more careful analysis of the microarray data, now being done in our lab, is needed to choose the best candidates involved in the low-dose IR-induced pro-angiogenic response, some targets were already validated by qRT-PCR analysis. We thus confirmed the gene expression modulation of several important pro-angiogenic

targets and cytoskeleton-related proteins: *VEGFR1*, *VEGFR2*, *ANGPT2*, *FGF2*, *CYR61*, *TGFβ2*, *TUBB* and *CLTC*, which were found to be up-regulated in HMVEC-L exposed to 0.3 Gy. Additionally, we have also confirmed the up-regulation of *VEGFR1*, *VEGFR2* and *CYR61* in HUVEC exposed to 0.8 Gy.

Summarily, VEGFR1 and VEGFR2 are both VEGF receptors. VEGFR1 can also bind PlGF which, as ANGPT2, FGF2, CYR61 and TGFβ2, belong to a group of growth factors known to induce the *in vitro* proliferation, migration and survival of ECs, and to stimulate the angiogenic sprouting and/or vessel maturation *in vivo* (please see chapter I, section 3.1 for details). These molecules are generally associated to sites of active angiogenesis and vascular remodeling, such as wound healing, but also to the tumor microenvironment, activating both ECs and tumor cells. Furthermore, β-tubulin forms the microtubules involved in directional cell migration (Chien et al., 2005; Gordon and Buxar, 1997), and clathrin is involved in cell detachment and movement by promoting the direct endocytosis of integrins from focal adhesions (Ezratty et al., 2009), thus representing an important role in the angiogenic sprouting.

Although an up-regulation of *VEGFR2* (Abdollahi et al., 2003) and *FGF2* (Haimovitz-Friedman et al., 1995) have already been demonstrated in ECs exposed to high doses of IR, Criswell et al. (Criswell et al., 2003) highlighted the fact that studies involving extremely high doses of IR cannot be extrapolated for low-dose IR. There is however, to our knowledge, only one report describing the transcriptional response of ECs to low-doses of X-ray IR (Lanza et al., 2005). From all the eight target genes selected by us, only *CYR61*, *VEGFR1* and *VEGFR2* had already been reported by Lanza et al. to be significantly modulated by low IR doses. However, the variation they described for *VEGFR1* and *VEGFR2* was the opposite of what we observed, as they found these genes to be down-regulated in response to IR.

Although the reasons for this apparent contradiction are still unknown, by analyzing the methodology we found a difference between both works. In Lanza et al.'s work, an orthovoltage equipment working at 200 KV energy was used, which has only been clinically employed in superficial tumors (Tuddenhama and Flinton, 2006) and it is

practically never applied nowadays in cancer therapy. In contrast, we used a linear accelerator X-ray photon been operating at 6 MV energy, commonly used in the clinical practice for external beam radiation treatments of cancer patients. According to Chiani et al. (Chiani et al., 2009), the transcriptional response to radiation can be considerably different depending not only on the dose range and time window chosen for the analysis, but also on the quality and dose rate of the radiation. Therefore, the use of these different equipments, operating at different energy and dose rates, may be enough to explain the different molecular responses.

The IR-induced up-regulation response of these well known pro-angiogenic targets, corroborates our hypothesis that low-dose IR enhances angiogenesis.

In the future, it would also be interesting to focus our attention in some other cytoskeleton-related elements involved in adhesion and migration, since they represent one of the major functional groups changed by low-dose IR.

Therapeutic doses of radiation, have already been shown to modulate the expression and functional activities of several adhesion molecules, both in tumor cells and ECs (Baluna et al., 2006; Hallahan et al., 1995; Heckmann et al., 1998; Prabhakarpandian et al., 2001), possibly contributing to enhance the invasive and metastatic potential. Furthermore, our preliminary results have already confirmed that low IR doses modulate some adhesion molecules involved in EC migration and EC-tumor cell interaction (i.e. PAK and VCAM1 respectively), representing important players in the metastatic spreading (Ding et al., 2003; Munoz-Chapuli et al., 2004; Slack-Davis et al., 2009).

According to our data derived from the transcriptomic analysis, the integrins represent one of the families most up-regulated by low-dose IR (i.e. $\alpha$2, $\alpha$3, $\alpha$6, $\alpha$v, β1, β3, β8). Integrins consist of associated $\alpha$ and β subunits that form different integrin heterodimers to accomplish unique ligand-binding profiles (e.g. α1β1, α2β1, α4β1, α5β1, α6β1, α6β4, α9β1, αvβ3 and αvβ5) (Avraamides et al., 2008; Ruegg and Mariotti, 2003). Therefore, even though they are the main receptors for extracellular matrix (ECM) proteins (e.g. fibronectin, laminin, and collagen), integrins can additionally bind some matrix-related proteins (e.g. CYR61), cell surface molecules (e.g. VACM1) and several vascular growth

factors (e.g. VEGF, FGF2, TGFβ) (Alghisi and Ruegg, 2006). A cross-talk between integrin αvβ3 and VEGFR2 has also been reported, combining the individual potency of both receptors (Somanath et al., 2009). As a consequence, integrins have been largely described as key regulators of EC migration, proliferation, survival and differentiation, generally being up-regulated in ECs at active angiogenic sites (Avraamides et al., 2008; Hehlgans et al., 2007). αvβ3 and αvβ5 are the main integrins associated to tumor angiogenesis and, therefore, the main targets of clinical trials (Hehlgans et al., 2007). Moreover, recent studies demonstrated that other heterodimers may also play a part in angiogenesis and, consequently, in tumor progression (e.g. α1β1, α2β1, α5β1, α6β4) (reviewed in Alghisi and Ruegg, 2006, table 1). Interestingly, according to our microarray results, not only several integrin subunits seem to be up-regulated in response to low-dose IR, as many of their ligands (e.g. FGF2, TGFβ, CYR61, VCAM1, fibronectin, laminin) and downstream signaling pathways (e.g. FAK, PAK, PI3K/Akt and Cdc42 signaling) (for integrin signaling details see Alghisi and Ruegg, 2006 and Hehlgans et al., 2007, and Ruegg and Mariotti, 2003). Therefore, integrins may represent good candidates to the pro-angiogenic effects induced by low-dose IR, being worth to explore.

Another possible way to select the potential targets responsible for the low-dose IR-induced effects might be by looking at the genes with higher fold change. Interestingly, there are two specific gene families being highly up-regulated by low-dose IR, the Y RNAs and the small nucleolar RNAs (snoRNAs). The Y RNAs, as the snoRNAs, are small non-coding RNAs components. The literature about Y RNAs is very restricted; however they have been found to be functionally required for DNA replication (Christov et al., 2006). It has also been shown that Y RNAs are overexpressed in human tumors and essential for cell proliferation (Christov et al., 2008). snoRNAs are associated to specific proteins, snoRNPs (small nucleolar ribonucleoproteins), primarily guiding chemical modifications of ribosomal RNAs modulating both the biogenesis and the activity of ribosomes (Bachellerie et al., 2002; Puerta, 2008). In addition, they also target transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and messenger RNAs (mRNAs), possibly acting as modulators of gene expression by promoting alternative spicing (Bachellerie et al., 2002;

Puerta, 2008). Moreover, snoRNAs have been associated to the telomere's maintenance, through the stabilization of the telomerase RNA (Meier, 2005). The precise phenotypic effect of the nucleotide modifications directed by snoRNA is not clear. Although in most cases they appear to be dispensable for cell viability or growth (Bachellerie et al., 2002), recent *in vivo* studies reported an abnormal expression of snoRNAs in some diseases like cancer (Chang et al., 2002; Dong et al., 2009; Dong et al., 2008; Liao et al., 2010). Other studies have also revealed that some snoRNAs (e.g. snord116) may be essential for mice growth (Ding et al., 2008).

The identification of the mechanisms whereby low-doses of IR induce a pro-angiogenic response, not only provides potential new targets to combine with radiotherapy in order to abolish the pro-angiogenic effects of low IR doses, as reveals undiscovered pro-angiogenic mechanisms.

## COMPLEMENTARY MATERIAL AND METHODS

### FLOW CYTOMETRY ANALYSIS

The cells were fixed, permeabilized and stained with antibodies to γ-H2AX (Upstate Biotechnology Inc.), phospho-ERK (Tyr204), phosphor-Akt (Ser473), (Tyr204), phosphor-PAK (Ser), or VCAM1 (Santa Cruz Biotechnology), followed by incubation with Alexa Fluor 488 or 594 (Molecular Probes). We acquired cells using FACS Calibur (Beckton Dickinson) and analyzed data using Flowjo 6.4.7 Software.

### MICROARRAY AND DATA ANALYSIS

HMVEC-L were exposed or not to 0.3 Gy and incubated for 4 h before RNA extraction. Four biological replicas were used in each condition. Total RNA was isolated using the QiaShredder and RNeasy (Qiagen) system following the manufacturer's instructions. Total RNA was kept at -80°C and sent to the Affymetrix core facility (Instituto Gulbenkian de Ciência, Oeiras, Portugal), where quality-control analysis was carried out before cDNA synthesis; cDNA labeling and hybridization to the GeneChip human gene 1.0 ST array was then prformed. This array contains probes to query 28869 different genes.

Partek Genomics Suite Software (Partek Inc., www.patek.com) was used to normalize the array data and determine expression levels following correction for non-biological factors. Samples were represented three-dimensionally, according to the expression levels of all probe sets, by principal components analysis (PCA). PCA is a method that reduces the dimensionality of a high-dimensional data set (i.e. microarrays with thousands of genes) while retaining as much variability as possible, allowing us to visualize different patterns of expression profile. For the identification of differentially expressed genes we used ANOVA and a false discovery rate with a 0.05 threshold, which

originated our list of genes that are significantly modulated by low-dose IR (significant gene list).

To further investigate the biological relevance of the data derived from the gene expression microarrays, we used the ingenuity pathway analysis software (Ingenuity® Systems, www.ingenuity.com), a web-based application that enables the visualization, discovery and analysis of molecular interaction networks within gene expression profiles. Our list of significant changed genes, with respective fold-change and P-values was uploaded within the IPA database and a core analysis was carried out. Canonical pathways analysis identified the pathways from the IPA library that were most significantly associated with the data set. Genes from the data set that met the P-value cut-off of 5% and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that make that canonical pathway; 2) Fisher's exact test used to calculate a P-value, determining the probability that the association between the significant genes in the data set and the canonical pathway could be explained by chance alone. The pathways that are better candidates will always be the ones with very low P-value and high ratio. A functional analysis was also carried out in order to identify the biological functions that were most significant to the data set. Right-tailed Fisher's exact test was used to calculate a P-value determining the probability that each biological function assigned to that data set is due to chance alone.

**QUANTITATIVE RT-PCR**

For qRT-PCR, total RNA was isolated using the QiaShredder and RNeasy (Qiagen) system. For each sample, 1 mg RNA was reverse transcribed into cDNA (Superscript II Kit, Invitrogen). *VEGFR1*, *VEGFR2*, *CYR61*, *ANGPT2*, *FGF2*, *TGFβ*, CLTC, and *TUBB* mRNA levels

were measured by qRT-PCR, using Applied Biosystems' SYBR green PCR Master Mix, on a ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems), according to the manufacture' instructions. The housekeeping gene used to normalize the samples was the 18S (Human 18S rRNA). Specific primers were used (see Table 1). Quantification of VEGF mRNA in HMEC-L was performed according to the comparative method (2-ΔΔCt; Applied Biosystems User Bulletin no. 2P/N 4303859), with 0.0 Gy condition as internal calibrator. Measurements were done in triplicate.

**Table C1| Primers used for quantitative RT-PCR**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| ***VEGFR1*** | 5'-CCCTCGCCGGAAGTTGTAT-3' | 5'-GTCAAATAGCGAGCAGATTTCTCA-3' |
| ***VEGFR2*** | 5'-ATTCCTCCCCCGCATCA-3' | 5'-GCTCGTTGGCGCACTCTT-3' |
| ***CYR61*** | 5'-AATGGAGCCTCGCATCCTAT-3' | 5'-CCCTTTTTCAGGCTGCTGTA-3' |
| ***ANGPT2*** | 5'-AGGACACACCACGAATGGCATCTA-3' | 5'- TGAATAATTGTCCACCCGCCTCCT-3' |
| ***FGF2*** | 5'-AAGAGCGACCCTCACATCAAGCTA-3' | 5'- TAGCCAGGTAACGGTTAGCACACA-3' |
| ***TGFβ2*** | 5'-GCTTTGGATGCGGCCTATTGCTTT-3' | 5'-CTCCAGCACAGAAGTTGGCATTGT-3' |
| ***CLTC*** | 5'-TTTGGCATCTACCCTGGTTCACCT-3' | 5'-TCCACACATCTGAGCAAGACGGAA-3' |
| ***TUBB*** | 5'-CATTGGCAATAGCACAGCCATCCA-3' | 5'-ACGAGGTCGTTCATGTTGCTCTCA-3' |
| ***18S*** | 5'-GCCCTATCAACTTTCGATGGTAGT-3' | 5'-CCGGAATCGAACCCTGATT-3' |

### *P53* MUTATED *BRAF*$^{V600E}$ ZEBRAFISH

*p53* mutated *BRAF*$^{V600E}$ zebrafish were kindly provided by Dr. Leonard Zon (Children's Hospital, Boston, USA) and maintained under standard conditions. Animals were anesthetized before irradiation or photographs using 0.61 mM of tricaine.

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# IV. RESEARCH WORK

## COMBINED EFFECT OF VASOPROST® AND LOW-DOSE IONIZING RADIATION ON ANGIOGENESIS

The research work contained in this chapter has not yet been published and represents some preliminary results from ongoing work that is currently being developed in our lab

## INTRODUCTION OF THE CHAPTER

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a physiological event involved in embryonic development, organ growth and, during adulthood, in wound healing and reproductive functions (Cao et al., 2005; Carmeliet, 2005).

Excessive or deficient angiogenesis, can also be the cause or the consequence of several pathological conditions (Carmeliet and Jain, 2000). For this reason, the study of angiogenesis as a potential therapeutic target has captured tremendous interest in recent years. Much of these studies have focused on using anti-angiogenic therapeutics to treat diseases caused or characterized by an excessive angiogenesis, such as cancer. Furthermore, pro-angiogenic therapy (also referred to as therapeutic angiogenesis) has been introduced as a novel method for the treatment of ischemic diseases, in which there is deficient vascularization or insufficient angiogenesis. Functional and stable blood vessel formation, that will efficiently supply the ischemic region with oxygenated blood, is important for successful therapeutic neovascularization.

Peripheral arterial disease (PAD) is a progressive illness that involves obstruction of blood flow in arteries (Baumgartner et al., 2005; Ouriel, 2001). Advance disease is characterized by rest pain, ulceration, or gangrene of ischemic tissues in the lower limbs – critical limb ischemia (CLI) (Baumgartner et al., 2005; Ouriel, 2001). CLI involves a severe disturbance of both macrocirculation and microcirculation (Norgren et al., 2007). The limitations of surgical/endovascular revascularization (due to the extent and anatomic distribution of the arterial occlusive disease), and of pharmacological treatment, are well recognized.

Prostaglandin $E_1$ ($PGE_1$, alprostadil, vasoprost®) is commonly used as a vasodilator for the treatment of PAD, especially in cases of CLI (Esaki et al., 2009). $PGE_1$ has been shown to induce vasodilatation (Marchesi et al., 2003) and to inhibit platelet aggregation (Schror and Hohlfeld, 2004). Some recent studies have also reported its pro-angiogenic effect in rabbit cornea (Ziche et al., 1995), rat and mice hind limb ischemia models (Diaz-Flores et

al., 1994; Esaki et al., 2009; Moreschi et al., 2007), patients with ischemic cardiomyopathy (Mehrabi et al., 2002), and patients with PAD (Makino et al., 2004). In addition, studies with *in vitro* endothelial cell (EC) cultures have shown that PGE1 up-regulates the expression of CYR61 (Schror and Hohlfeld, 2004), HGF (Makino et al., 2004), VEGF (Haider et al., 2005; Mehrabi et al., 2002) and eNOS (Fang et al., 2010; Haider et al., 2005), all known powerful pro-angiogenic factors. However, this effect has been questioned by Cattaneo et al. (Cattaneo et al., 2003), whose work suggests that $PGE_1$ has a strong inhibitory effect on angiogenesis, reducing vessel formation in mice plug assays and *in vitro* EC migration, proliferation and tube formation.

Given that previously published reports described contradictory effects of vasoprost® on angiogenesis, our first goal was to clarify this issue through a set of functional assays in an EC population treated or not with vasoprost®.

Although our results suggest that vasoprost® induces a pro-angiogenic response in ECs, recent trials do not support the benefit of prostanoids in promoting amputation-free survival (Brass et al., 2006). Therefore, patients may require amputation despite its associated morbidity and mortality rates (Norgren et al., 2007). The goal of limb salvage has stimulated research into alternative methods, including therapeutic angiogenesis. Angiogenesis can be achieved either by local administration of pro-angiogenic growth factors in the form of recombinant protein, by gene therapy or by implantation of endothelial progenitor cells (EPCs) (Banai et al., 1994; Lopez et al., 1998; Luttun et al., 2002; Morishita et al., 1999; Takeshita et al., 1994). While the experimental studies are encouraging, the randomized controlled clinical trials have produced less consistent results (Collinson and Donnelly, 2004).

Very recently, we found that low doses of ionizing radiation (IR) promote tumor growth and metastasis by enhancing angiogenesis (Sofia Vala et al., 2010). Our work has focused, in an innovative way, in the vasculature that surrounds the tumor and receives relatively low doses of IR during treatment. We demonstrated that, in zebrafish, low-dose IR accelerates embryonic angiogenic sprouting during development and promotes adult angiogenesis during fin regeneration (Sofia Vala et al., 2010). Therefore, according to our

findings low-dose IR induces angiogenesis *in vivo;* however, there is no evidence that it produces therapeutic angiogenesis in ischemic disease patients.

Here we report that, *in vitro*, IR doses lower than 0.8 Gy could be used synergistically to vasoprost® in order to potentiate its effect as an inducer of endothelial migration, proliferation and survival. These observations open novel therapeutic perspectives for the improvement of vasoprost®, commonly used in the treatment of PAD.

## RESULTS

### LOW DOSES OF IR POTENTIATE THE MIGRATORY EFFECT OF VASOPROST® IN HUVEC

We have previously shown, through an *in vitro* wound healing assay, that low IR doses (≤ 1.0 Gy) were able to stimulate the migration of HUVEC (Chapter III, Figure C7, panel C). From the doses tested, 0.8 Gy induced the best recovery rate.

To examine if vasoprost® was also able to induce HUVEC migration, we preformed a similar wound healing assay, in which confluent monolayers were incubated with different doses of vasoprost® (0.5, 5.0 and 10 µM) immediately after injury. Our results showed that every vasoprost® dose tested was able to significantly induce an accelerated wound closure, comparing to untreated wounded monolayers (Figure 1). The best recovery rate after wounding was obtained using 10 µM of vasoprost®.

Next, we decided to investigate the migratory effects of vasoprost® when cells were simultaneously exposed to 0.8 Gy. Our results suggest that this dose potentiates the pro-migratory effect of vasoprost® (Figure 2).

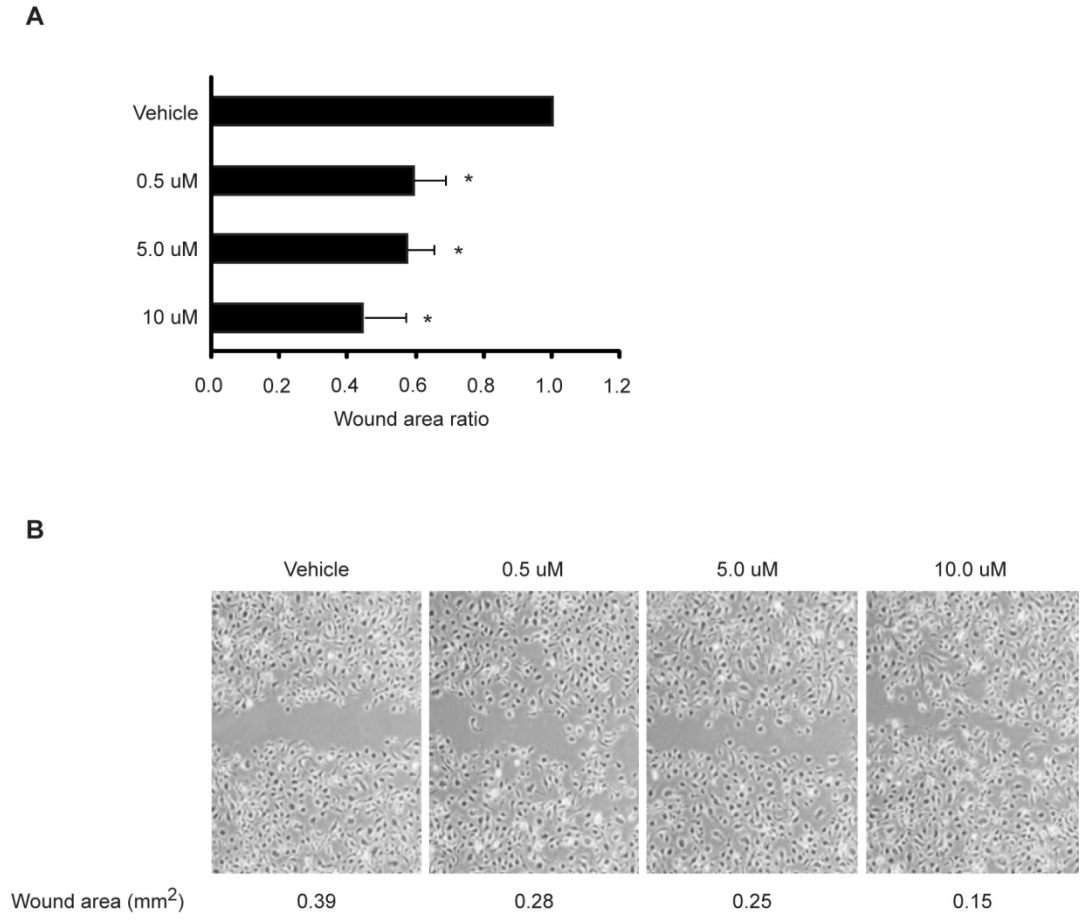


**Figure 1| Vasoprost® promotes HUVEC migration.** Confluent monolayers of HUVEC were subjected to *in vitro* wound healing and incubated with or without 0.5 µM, 5.0 µM or 10.0 µM of vasoprost®. (A) Values (means ± s.d. of four fields of view) represent the ratio between the wounded area of treated and untreated cells (vehicle), 7 hours (h) after wounding. *P<0.01. (B) Photographs, representative of four photographic fields, were taken immediately (not shown) and 7 h after wounding. Quantification of the wound area (in $mm^2$) is presented below the images. Data are representative of two independent experiments.

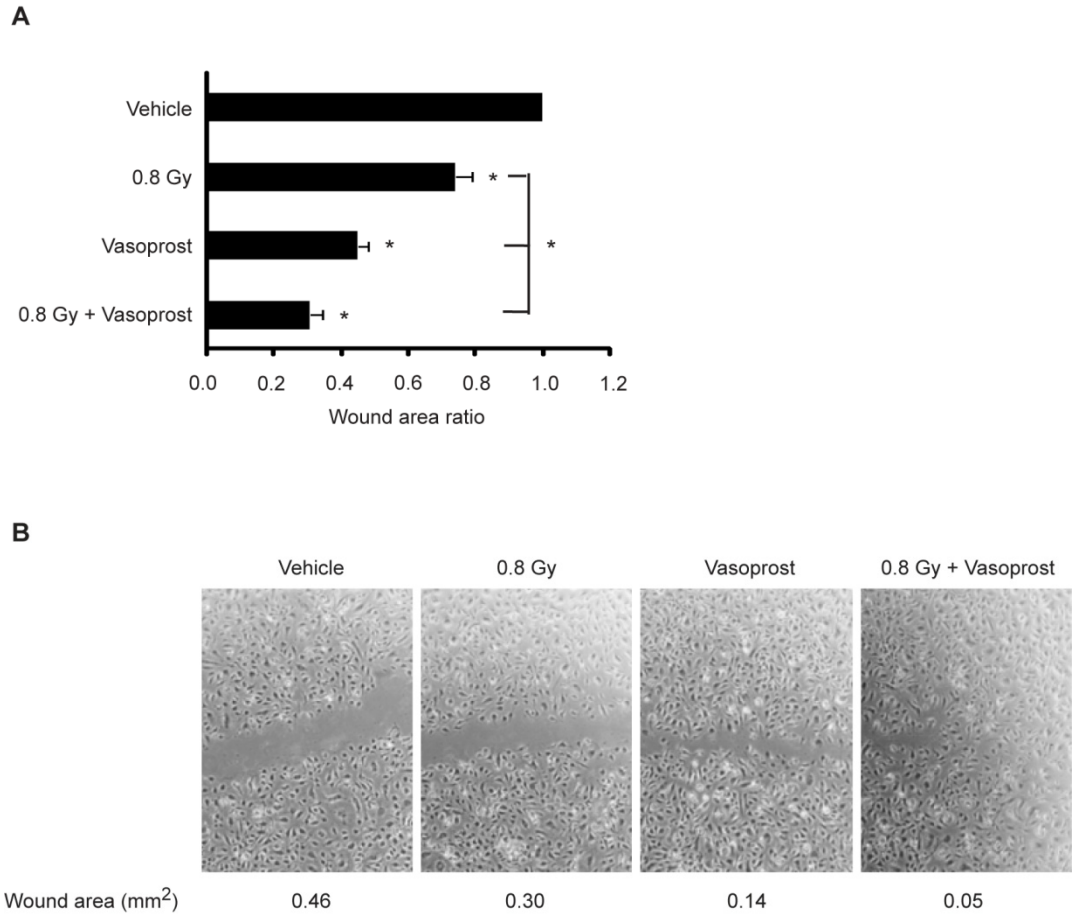


**Figure 2| Low doses of IR potentiate the migratory effect of vasoprost® in HUVEC**. Confluent monolayers of HUVEC were subjected to *in vitro* wound healing, exposed or not to 0.8 Gy, and incubated in the presence or absence of 10.0 µM vasoprost®. (A) Values (means ± s.d. of four fields of view) represent the ratio between the wounded area of treated and untreated cells (vehicle), 7 h after wounding, and are representative of two independent experiments. *P<0.01. (B) Photographs, representative of three independent experiments, were taken immediately (not shown) and 7 h after wounding. Quantification of the wound area (in $mm^2$) is presented below the images. Data are representative of three independent experiments.

### LOW DOSES OF IR POTENTIATE THE PROLIFERATIVE EFFECT OF VASOPROST® IN HUVEC

To study the effect of both vasoprost® and low doses of IR on EC proliferation, either separately or in combination, HUVEC were cultured in 0.5 % serum medium. Cells were exposed or not to 0.8 Gy and then incubated in the presence or absence of 10 µM vasoprost®. After 42 h, cell count revealed that exposure to either vasoprost® or low-dose IR resulted in increased EC proliferation. Nevertheless, we observed a significantly higher cell proliferation rate when both treatments were used in combination (Figure 3).

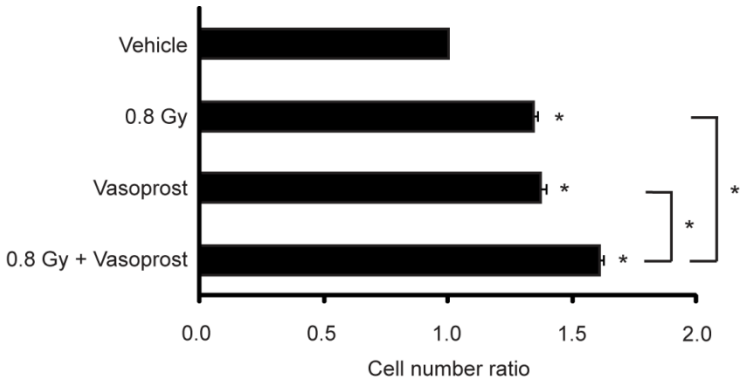


**Figure 3| Low doses of IR potentiate the proliferative effect of vasoprost® in HUVEC.** HUVEC were plated at equal densities and, after 12 h, incubated in 0.5 % serum medium. Cells were then exposed or not to 0.8 Gy and incubated in the presence or absence of 10.0 µM vasoprost®. After 42 h, cells were counted using a hemacytometer. Data (mean ± s.d.) represents the ratio between cell number of treated and untreated (vehicle) cells in quadruplicate culture, and are representative of three independent experiments. *P<0.01.

### THE COMBINATION OF LOW-DOSE IR AND VASOPROST® MAXIMIZES HUVEC PROTECTION FROM SERUM WITHDRAWAL-INDUCED CELL DEATH

Next, we investigated the ability of vasoprost® to promote endothelial survival, and the effect of low dose IR in its response. With this objective, HUVEC cultured in serum-free medium were exposed or not to 0.8 Gy and then incubated in the presence or absence of 10 µM vasoprost®. The percentage of apoptotic cells was assessed by flow cytometry. As expected, serum withdraw reduced HUVEC viability, and we verified that treatment with either of these agents (IR or vasoprost®) protected cells against cell death induced by serum withdraw. Interestingly, we observed a higher cell protection when both treatments were used in combination.

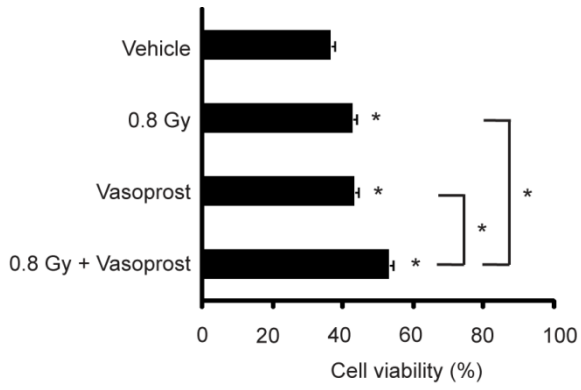


**Figure 4| The combination of low-dose IR and vasoprost® maximizes HUVEC protection from serum withdrawal-induced cell death.** HUVEC, in serum-free medium, were exposed or not to 0.8 Gy and then incubated in the presence or absence of 10.0 µM vasoprost®. Cells were double stained with Annexin-V and propidium iodide (PI) at 42 h post-irradiation. The percentage of apoptotic cells was assessed by flow cytometry. Data (mean ± s.d.) are given as the percentage of viable cells and are derived from three independent experiments. *P<0.03.

**DISCUSSION OF THE CHAPTER**

Angiogenesis, which is defined as the formation of new blood vessels from preexisting ones, occurs *via* activation of ECs, which proliferate and migrate to construct new capillary sprouts (Conway et al., 2001; Jain, 2003; Lin et al., 2007). The recognition that this process is involved in several diseases has lead to the study of angiogenesis as a source of potential therapeutic targets.

CLI is the term used for patients with chronic ischemic rest pain, ulcers, or gangrene attributed to inadequate blood flow or arterial occlusive disease (Norgren et al., 2007). It is the progressive evolution and clinical manifestation of PAD.

CLI occurs when arterial lesions impair blood flow to such an extent that the nutritive requirements of the tissues cannot be met. This is usually caused by multilevel arterial occlusive disease. In a large proportion of these patients the anatomic extent and distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization (Norgren et al., 2007). Amputation, despite its associated morbidity, mortality rates and functional implications, is often imposed as a last solution to the disabling symptoms (Norgren et al., 2007). Therefore, novel therapies are required to treat these patients. Preclinical studies, using lower limb ischemia animal models, have demonstrated that angiogenic growth factors augment collateral vessel formation, capillary density and blood flow (Asahara et al., 1995; Banai et al., 1994; Chae et al., 2000; Lopez et al., 1998; Luttun et al., 2002; Mack et al., 1998; Morishita et al., 1999; Ohara et al., 2001; Takeshita et al., 1994; Tsurumi et al., 1996). These studies administered pro-angiogenic factors in the form of recombinant proteins or gene therapy, a concept called therapeutic angiogenesis. While these experimental studies are encouraging, the randomized controlled clinical trials in PAD have produced less consistent results (Collinson and Donnelly, 2004).

More recently it was found that, in different animal models including ischemic hind limb model, circulating EPCs participate in new vessel growth (Asahara et al., 1999; Yamashita

et al., 2000). The possibility of using EPCs, both alone and in combination with different angiogenic growth factors, represents promising means of obtaining stable vessels. However, the different forms of therapeutic angiogenesis still have to prove safety and efficacy before one can conclude on its role as an additional limb saving strategy.

Vasoprost® is commonly used as a vasodilator for the treatment of PAD. It may enhance the results of revascularization or be an option in patients in whom revascularization is impossible or has failed (Esaki et al., 2009). Its role as a pro-angiogenic agent has however been controversial. While some studies report the *in vivo* and *in vitro* pro-angiogenic effects of alprostadil (Diaz-Flores et al., 1994; Esaki et al., 2009; Fang et al., 2010; Haider et al., 2005; Makino et al., 2004; Mehrabi et al., 2002; Moreschi et al., 2007; Schror and Hohlfeld, 2004), the prime component of vasoprost®, another report contest it, announcing alprostadil as a potent inhibitor of angiogenesis (Cattaneo et al., 2003).

In order to clarify this debate, we preformed a series of functional assays on *in vitro* cultures of ECs. Our results showed that vasoprost® stimulates EC migration and proliferation, which are important steps involved in the establishment of the angiogenic process. Additionally, vasoprost® protects ECs from cell-death induced by serum withdrawal conditions. Therefore, in agreement with the majority of the published studies, our results strongly suggest a pro-angiogenic function for vasoprost®.

According to our findings, low-dose IR induces angiogenesis *in vivo* (Sofia Vala et al., 2010), but there is no evidence that it produces therapeutic angiogenesis in ischemic disease.

The treatment with IR involves the risks of late skin damage, genetic damage or radiation-induced carcinogenesis. However, after weighing the pros and cons, radiation therapy is also accepted for treatment of certain benign or nonmalignant conditions either primarily, following surgery or when other modalities fail (Jha et al., 2008).

In this work, we proposed to investigate if low doses of IR, by inducing angiogenesis, would potentiate the pro-angiogenic effect of vasoprost®. Therefore, the migratory capacity, proliferation and survival were assessed in ECs treated with vasoprost® and simultaneously exposed or not to low dose-IR.

Taken together, our data suggest that the combinatory use of both agents should be considered in future studies, since the low-dose IR potentiates the effect of vasoprost® as an inducer of endothelial migration, proliferation and survival.

Although further studies are necessary to understand the molecular mechanisms involved in these biological processes, our research opens a new perspective and a significant contribution to CLI disease: the possible use of low doses of IR as a new method to stimulate the creation of blood vessels in ischemic organs.

## MATERIAL AND METHODS

### CELL CULTURE AND REAGENTS

HUVEC were kindly provided by Dr Shahin Rafii (Cornell University Medical College, NY, USA) and cultured according to manufacturer's instructions. Cells were used up to passage 7.

Vasoprost® (alprostadil) was kindly provided by Schwarz Pharma.

### IRRADIATION

HUVEC cultures were transferred to an acrylic phantom box in order to achieve a certain thickness. A computed tomography (CT) scan (Somatom Sensation, Siemens) was performed and a volumetric acquisition was carried out; acquired images were reconstructed with axial slices width of 1 mm, and cross sectional data were 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 (0º, 180º) at 6 MV energy, normalized to a reference point. IR delivery was performed at room temperature using a linear accelerator x-rays photon beam (Varian Clinac 2100 CD) operating at a dose rate of 300 MU/min. A 0.6 $cm^3$ PTW farmer ionizing chamber, connected to UNIDOS electrometers, was used to validate the IR doses calculated by PLATO, according to the IAEA TRS-398 protocol. We obtained, in average, differences lower than 2% between the experimental and the PLATO planning system dose values.

### WOUND HEALING ASSAY

HUVEC were plated to confluence and wounds created in the monolayer by scraping the plate with a pipette tip. Monolayers were irradiated or not, and incubated in the presence or absence of vasoprost®. Photographs were taken immediately after wounding and 7 h later.

### PROLIFERATION ANALYSIS

HUVEC were plated at equal densities and incubated in 0.5 % serum medium after 12 h. Cells were irradiated or not, and incubated in the presence or absence of vasoprost®. Cells were counted 42 h later using a hemacytometer.

### APOPTOSIS ANALYSIS

HUVEC were plated at equal densities, irradiated or not after 12 h, and incubated in the presence or absence of vasoprost®. 42 h later, cells were stained with Annexin-V-FITC (Boehringer Manheim) and propidium iodide (PI) (Interchim). The percentage of apoptotic cells (Annexin-V positive, PI negative and positive) was determined by flow cytometry (FACS Calibur, Beckton Dickinson) and Flowjo 6.4.7 Software. Results are shown as the percentage of viable cells (Annexin-V, PI negative).

### STATISTICAL ANALYSIS

Numeric data were analyzed, through GraphPad Prism 5 Software, for statistical significance using two-tailed unpaired or paired student's *t*-test for comparison of means with. $P<0.05$ was considered significant.

## ACKNOWLEDGMENTS

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# V. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This chapter encloses an overview of the key findings and future perspectives of the work developed.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Angiogenesis, the growth of new blood vessels from pre-existing ones, is a physiological event involved in embryonic development, organ growth and, during adulthood, in wound healing and reproductive functions (Cao et al., 2005; Carmeliet, 2005). This process is tightly regulated by a complex balance between pro- and anti-angiogenic factors. However, in many disorders this balance becomes unstable and the equilibrium between inducers and inhibitors is twisted, resulting in either deficient or excessive neovascularization (Carmeliet, 2003; Carmeliet, 2005; Carmeliet and Jain, 2000). The recognition that angiogenesis is also involved in several pathological diseases, has led to the emergence of a new and promising field of research: angiogenesis as a therapeutic target. Over the past 30 years, a number of cytokine growth factors and the corresponding receptors that appear to play a role in angiogenesis have been identified. Consequently, the search for methods and factors that modulate angiogenesis has become an area of great interest and excitement.

Anti-angiogenic therapy and therapeutic angiogenesis have been proposed as strategies for the treatment of cancer and ischemic diseases, respectively.

In the present work, we have explored the effects of low ionizing radiation (IR) doses on angiogenesis, and found evidence of important implications at the level of angiogenic therapy.

**During the first part of the research work presented on this thesis (chapter III), we focused on the tumor therapy context. We showed that low IR doses promote angiogenesis and that this effect may have a strong impact in tumor burden and metastasis.**

Cancer is clearly a pathologic condition characterized by excessive angiogenesis. Besides contributing to tumor growth, tumor angiogenesis also plays an essential role in metastasis development by facilitating tumor cell entry into circulation (Zetter, 1998).

Radiotherapy is frequently used in the treatment of malignant solid tumors, either alone or in combination with surgery and chemotherapy. Classically, it is administered as fractionated therapy, whereby small doses of IR are delivered to the tumor target volume until the potentially curative effect has been obtained. However, the delivery in small fractions and the isodose distribution of external beam radiotherapy results in the delivery of lower doses of radiation outside the volume targeted for therapy, whose biological effects remain unclear.

**The first key finding of this study** was the demonstration that low doses of IR are able to induce an angiogenic response in both *in vitro* and *in vivo* models. Doses equal or lower than 0.8 Gy promote endothelial cell (EC) migration without causing cell cycle arrest or apoptosis, activate vascular growth factor (VEGF) receptor-2 (VEGFR2) and up-regulate the expression of VEGF. We further show that, in zebrafish, low-dose IR accelerates sprouting angiogenesis during development and enhances the angiogenic response during caudal fin regeneration.

Consistent with these results, and by using different mouse models, we showed that low-dose IR promotes accelerated tumor growth and metastasis formation in a VEGFR-dependent manner, **the second key finding of this study**.

The **third key finding of this study** was the demonstration that low doses of IR modulate the gene expression of molecular mediators involved in the angiogenic response. It is described that the activation of VEGFR leads to a rapid activation of different cellular proteins and consequently to *de novo* mRNA and protein expression of mediators involved in the angiogenic response. This is strongly supported by the data we obtained in an *in vitro* microarray study where several transcripts encoding for proteins required for angiogenesis are induced upon low-dose IR delivery. According to our preliminary results, *VEGFR1*, *VEGFR2*, *ANGPT2*, *CYR61*, *FGF2*, *TGFβ2*, *CLT* and *TUBB* are some of the selected targets up-regulated by low doses of IR whose expression was already validated in our lab.

A careful analysis of the microarray data is now being carried out in our lab in order to choose the genes significantly modulated by low-dose IR, that represent the best candidates to a pro-angiogenic response.

In the future, it would be interesting to *in vivo* validate the pro-angiogenic potential of the molecular targets and cell pathways identified *in vitro*, by taking advantage of zebrafish caudal fin regeneration as a model. Using the transgenic *Fli1:EGFP* zebrafish, which allows intra vital imaging of the vasculature through *Fli1* gene promoter-driven expression of GFP in ECs, we found that low-dose IR enhances angiogenesis during zebrafish fin regeneration**.** Therefore, the expression of the several candidate molecules may be assessed by performing cell sorter, qRT-PCR and western-blot analysis in irradiated and unirradiated regenerated fin. While morpholino technology allows investigating the impact that the knock-down of several selected proteins has in the low-dose IR-induced angiogenesis.

Moreover, it is also possible to evaluate if the molecular targets, involved in the angiogenic phenotype promoted by low-dose IR, have their expression modulated in material exposed to low IR doses from patients with rectal cancer that received preoperative radiotherapy. The choice of this working model allows analyzing three different specimens of interest directly comparing our results in the same patient: (1) a tumor specimen after neo-adjuvant radiotherapy (irradiated with therapeutic doses); (2) a specimen from the proximal area (no tumor and unirradiated); and (3) a specimen from the distal margin (no tumor and irradiated with a range of IR doses lower than the therapeutic one). All tissue sections may be then analyzed using a laser microdissection microscopy (Emmert-Buck et al., 1996). Using this equipment, ECs from tissue sections can then be isolated followed by RNA preparation and qRT-PCR analysis.

Although our data has been obtained using ECs, we cannot exclude the possibility that *in vivo* low-dose IR might also activate other cells, such as fibroblasts, inflammatory cells, EPCs, or dormant cancer cells contributing to tumor progression.

Taken together, our observations provide novel insights into the biological effects of low doses of IR relevant to tumor biology, which may serve as basis for the prevention of possible tumor-promoting effects of current radiotherapy protocols.

**In the second part of the research work presented on this thesis (chapter IV), we focused on the pro-angiogenic therapy context. We showed that low IR doses potentiate the effect of vasoprost® as an inducer of endothelial migration, proliferation and survival.**

Considering our previous data, where we showed that low dose radiation stimulates angiogenesis, we asked whether these same low doses could be used to improve the results obtained by the already established therapeutic treatment of PAD. Therefore, we combined the action of low-dose IR with vasoprost®, a vasodilator commonly used in the treatment of PAD.

Here, **our first key finding** was the demonstration that vasoprost® is significantly able to stimulate HUVEC migration, proliferation and cell survival, suggesting a pro-angiogenic effect in ECs.

Importantly, the **second key finding** was the demonstration that low doses of IR could be used synergistically with vasoprost® in order to potentiate its effect as an inducer of endothelial migration, proliferation and survival.

Taken together, our results suggest that the combinatory use of both vasoprost® and low-dose IR should be considered for future studies concerning its clinical therapeutic potential in pathologies such as PAD.

Our lab is now interested in evaluating whether it is possible to promote therapeutic angiogenesis by means of low doses of IR in a mouse ischemia model, to examine the feasibility of therapy for critical limb ischemia (CLI). The preliminary results are extremely promising, since a significant increase in capillary density in ischemic irradiated muscles is observed when compared to ischemic unirradiated ones. Our lab aims to evaluate if the molecular targets identified as being involved in the angiogenic phenotype promoted by

low doses of IR have their expression modulated in the mouse ischemia model. With this objective, ECs from irradiated and unirradiated muscle sections will be isolated by a laser microdissection microscopy followed by RNA isolation and qRT-PCR analysis.

Since the treatment with IR involves the risks of genetic damage and radiation-induced carcinogenesis, the potential toxicological effects of low doses of IR are being assessed in collaboration with Vivotecnia (Spain), a Contract Research Organization with expertise in *in vivo* toxicological studies.

The results obtained have the potential to propose a new strategy for angiogenic therapy using low doses of IR, providing a significant contribution to CLI disease treatment.

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