MOLECULAR MECHANISMS AND RELEVANCE
OF ANGIOGENESIS INDUCED BY
LOW-DOSE IONIZING RADIATION

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MESTRADO EM BIOQUÍMICA

(Bioquímica Médica)

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2011
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# Table of Contents

## Chapter I: Introduction
- Radiotherapy ................................................................. 1
- Radiotherapy and Metastasis ............................................. 3
- Blood vessels and Angiogenesis ........................................... 5
- Tumor angiogenesis and Metastasis ..................................... 9
- Angiogenic factors .......................................................... 11
  - VEGF ........................................................................ 12
  - Angiopoietins .............................................................. 12
  - FGFs ........................................................................ 13
  - TGFβ ........................................................................ 13
  - CYR61 ....................................................................... 14
- Cell migration and Focal adhesions ...................................... 14

## Chapter II: Objectives .......................................................... 18

## Chapter III: Material and Methods ...................................... 19
- Irradiation ........................................................................ 19
- Cell culture ...................................................................... 19
- Western blot ..................................................................... 20
  - Cell lysate preparation .................................................. 20
  - Whole cell extracts ....................................................... 20
  - Cytoskeletal extracts ...................................................... 20
  - Protein quantification ..................................................... 20
  - Western blot running ...................................................... 21
  - Protein transfer to nitrocellulose ...................................... 22
  - Western blot analysis ..................................................... 22
- Quantitative real-time PCR ................................................. 23
  - RNA extraction ........................................................... 23
  - Cells ........................................................................... 23
  - Zebrafish larvae ........................................................... 24
  - DNase treatment ........................................................... 24
  - cDNA synthesis ........................................................... 25
  - Quantitative real-time PCR assay ..................................... 25
# Table of Contents

- Immunofluorescence staining ........................................................................................................ 27
- p53 mutated BRAF<sup>V600E</sup> zebrafish ...................................................................................... 28

## Chapter IV: Results
- Microarray validation .................................................................................................................... 29
- Low-dose IR and zebrafish development ......................................................................................... 33
- Low-dose IR and tumor development in a p53 mutated BRAF<sup>V600E</sup> zebrafish model ....... 35

## Chapter V: Discussion .................................................................................................................. 38

## Bibliography .................................................................................................................................. 41

## Appendix ....................................................................................................................................... 47
- Attach 1 ......................................................................................................................................... 47
- Attach 2 ......................................................................................................................................... 48
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>aFGF</td>
<td>Acidic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Angiopoietin 1</td>
</tr>
<tr>
<td>Ang-2</td>
<td>Angiopoietin 2</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related protein 2/3</td>
</tr>
<tr>
<td>BBE</td>
<td>Bovine Brain Extract</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTV</td>
<td>Clinical Target Volume</td>
</tr>
<tr>
<td>CYR61</td>
<td>Cysteine-rich protein 61 (also known as CNN1)</td>
</tr>
<tr>
<td>DLL4</td>
<td>Delta like ligand 4</td>
</tr>
<tr>
<td>dpf</td>
<td>days post-fertilization</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Breaks</td>
</tr>
<tr>
<td>EBM-2</td>
<td>Endothelial Basal Medium</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EPCs</td>
<td>Endothelial Progenitor Cells</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FAs</td>
<td>Focal adhesions</td>
</tr>
<tr>
<td>FBs</td>
<td>Fibrillar adhesions</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FGFRs</td>
<td>Tyrosine kinase FGF receptors</td>
</tr>
<tr>
<td>FLT1</td>
<td>fms-related tyrosine kinase 1 (VEGFR1)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor-associated macrophages</td>
</tr>
<tr>
<td>TβRI</td>
<td>Serine-threonine kinase receptors I</td>
</tr>
<tr>
<td>TβRII</td>
<td>Serine-threonine kinase receptors II</td>
</tr>
<tr>
<td>TEMs</td>
<td>TIE-2-expressing monocytes</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>Tie</td>
<td>Tyrosine kinase with immunoglobulin and EGF homology domains</td>
</tr>
<tr>
<td>TV</td>
<td>Treatment Volume</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated Phosphoprotein</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular Endothelial Growth Factor A</td>
</tr>
<tr>
<td>VEGFB</td>
<td>Vascular Endothelial Growth Factor B</td>
</tr>
<tr>
<td>VEGFC</td>
<td>Vascular Endothelial Growth Factor C</td>
</tr>
<tr>
<td>VEGFD</td>
<td>Vascular Endothelial Growth Factor D</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>Vascular Endothelial Growth Factor Receptor 1 (FLT1)</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular Endothelial Growth Factor Receptor 2 (KDR)</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
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Resumo

A radioterapia é usada no tratamento de tumores malignos caracterizados por um crescimento descontrolado e capacidade de invadir tecidos adjacentes e metastizar. Todavia, observações clínicas e experimentais sugerem que a radiação ionizante (RI) pode aumentar a agressividade do tumor e a metastização. Contrariamente ao que se pensou durante muito tempo, a progressão tumoral durante ou após a radioterapia não resulta necessariamente do desenvolvimento de células tumorais resistentes. Diferentes estudos identificam os mecanismos pelos quais a RI activa alvos celulares, contribuindo de forma significativa para a invasão e metastização. As doses de RI que causam estes efeitos 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 distribuição homogénea das curvas de isodose em radioterapia de feixe externo contribuem para que existam ainda menores doses de RI fora da área a tratar. Recentemente foi demonstrado que baixas doses de RI, (menor ou igual a 0.8 Gy), unicamente presentes nos tecidos que rodeiam a área tumoral, induzem angiogénese e, consequentemente, promovem o crescimento tumoral e o aparecimento de metástases.

Durante este estudo, procurámos identificar os mecanismos moleculares pelos quais baixas doses de RI induzem uma resposta pró-angiogénica e avaliar a sua contribuição na progressão tumoral. Os nossos resultados mostram que, quer in vitro, quer in vivo, baixas doses de RI induzem a expressão de factores pro-angiogénicos, como por exemplo o receptor 1 e 2 do factor de crescimento de endotélio vascular (VEGF), Angiopoietin-2, Cyr61, FGF2, TGFβ2, mas modulam também a expressão e localização subcelular de proteínas do citoesqueleto e envolvidas na migração celular, como a vinculina e a paxilina.

Usando peixes zebra, mostramos igualmente que baixas doses de RI induzem angiogénese e consequentemente aceleram o desenvolvimento de larvas. Finalmente, e utilizando como modelo peixes zebra com a mutação p53 BRAF^{V600E}, estudámos o efeito de baixas doses de RI no desenvolvimento do melanoma. Os resultados obtidos sugerem que as baixas doses de RI aceleram o desenvolvimento de melanoma e modulam a agressividade do tumor, sendo os melanomas em peixes zebra expostos a radiação mais invasivos do que os desenvolvidos em animais não irradiados.

Os resultados obtidos contribuem para uma melhor compreensão do potencial pró-metastático da RI, levando a progressos significativos no conhecimento desta área e sendo da maior importância para a optimização dos actuais protocolos de radioterapia.
Abstract

Radiotherapy is a protocol of choice for the treatment of malignant tumors. However, there is evidence suggesting that ionizing radiation (IR) may enhance tumor aggressiveness and promote metastasis. Contrary to what we thought before, tumor progression during or after radiotherapy is not necessarily due to the appearance of resistant tumor cells. Different studies show the mechanisms by which IR activates cellular targets potentially contributing to invasion and metastasis. Doses of IR causing such stimulating effects are classically delivered inside the tumor target volume in daily small fractions, in order to limit the damage to healthy tissues and until a potentially curative dose has accumulated inside the tumor volume. Furthermore, the delivery in small fractions and the isodose distribution of external beam radiotherapy result in even lower doses of IR outside the tumor target volume. It was demonstrated that low doses of IR (ranging from zero to 0.8 Gy), only present on the surrounding tumor target, can enhance angiogenesis and consequently promote tumor growth and metastasis.

We investigated the molecular mechanisms by which low-dose IR induces a pro-angiogenic response and its contribution to tumor progression. We show in vitro and in vivo that exposure to low doses of IR up-regulate the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) receptor 1 and 2, Angiopoietin-2, CYR61, FGF2, TGFβ2, as well as the expression and subcellular localization of proteins implicated in the cytoskeleton, like paxillin and vinculin.

We also demonstrate that low doses of IR accelerate angiogenesis and consequently zebrafish larvae development. Finally, we used a p53 mutated BRAFV600E zebrafish model to study low-dose IR effects on tumor development. Our results suggest that low doses of IR accelerate melanoma development, enhancing its invasive capacity.

Taken together, the results obtained provide new contributions to understand the potential pro-metastatic effect of IR, leading to significant breakthroughs, advancing the state of the art in the field and being of utmost importance to improve current radiotherapy protocols.
I Introduction

Radiotherapy

Radiation therapy has been used for the treatment of malignant tumors for more than 100 years, with its earliest roots traced from the discovery of x-rays in 1895. This can be used as palliative treatment, for local disease control and symptomatic relief when cure is not possible or as therapeutic treatment, to increase survival rate and with curative purposes. Additionally, radiotherapy can be used as a primary or adjuvant modality, with the possibility to combine it with surgery, chemotherapy, hormone therapy and immunotherapy.

Virtually, all kinds of tumors can be treated using radiotherapy, although they differ in radiosensitivity. Besides metastatic spread, this sort of treatment can fail due tumor localization, size, inadequate supply (hypoxia) or genetic factors (such as p53 mutations, that may result in radioresistant cellular phenotypes) (Pawlik and Keyomarsi, 2004).

Radiotherapy can be applied by different methods: internal radiation therapy (or brachytherapy), systemic radiation therapy and external beam radiation therapy. In the first, the radiation is delivered from radiation sources (radioactive materials) placed inside or on the body. The second method, systemic therapy, uses radioactive substances, such as radioactive iodine or a radioactive substance bound to a monoclonal antibody, which patient swallows or receives by injection. At last, in the external beam therapy, the most frequently applied, radiation is delivered by a machine outside the body according to a previous specified plan (Joiner and van der Kogel, 2009; Lawrence et al., 2008).

Ionizing radiation (IR) consisting of electromagnetic radiation (X-rays and γ-rays) is the type of radiation most commonly used in radiotherapy, being photon its basic unit (Joiner and van der Kogel, 2009; Lawrence et al., 2008). In general, in external beam therapy, mainly high energy photons are applied, generated by MV-linear accelerators (Joiner and van der Kogel, 2009). Usually, this treatment method is carried out with more than one shaped radiation beam in order to achieve a uniform dose distribution inside the target volume and minimize the dose in the normal surrounding tissue (Steel, 2002). However, the surrounding healthy tissues are also exposed to doses lower than the tumor area dose (Figure 1.1). The molecular and biological effects of these low doses of IR on the healthy tissues surrounding the tumor, and in particular on the vasculature, remain largely to be determined.
Figure 1.1 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 the 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 tissue that will receive 50% or 30% of the total daily dose (0.9-1.0 Gy or 0.5-0.6 Gy).

Radiation treatments of human cancer through external beam therapy are 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. Fractionation schedules allows normal cells to recover from the effects of radiation, protecting healthy tissues, since these cells are more proficient in radiation damage repair than tumor clonogens (Connell et al., 2004; Steel, 2002).

To understand better the benefits of fractionated radiation it is necessary to know the molecular mechanism of action of IR. IR effects include alterations on signaling transduction pathway, mitochondria, cell cycle and apoptosis, due to DNA damage, but also primarily effects (Lehnert, 2007; Pawlik and Keyomarsi, 2004). Nevertheless, DNA damage is the main
cause of observed macroscopic effects (Joiner and van der Kogel, 2009). IR damages are caused by ionization or excitation of atoms and could be either direct or indirect. In the first case, the ionized/excited atoms belong to key molecules of the cell, like DNA (Lehnert, 2007; Steel, 2002). In indirect action, damage occurs due the formation of free radicals, consequence of water ionization. Although H* and OH*, formed by water hydrolysis, have short lifetimes, in the presence of oxygen, free radicals with greater stability and longer lifetimes will be produced (O2• −, H2O2, •OH and •NO), enhancing the cell damage. For that reason, tumor hypoxia is associated with greater radioresistance (Connell et al., 2004; Lehnert, 2007).

DNA damage can occur in different extents: single strand breaks (SSB), double strand breaks (DSB) and altered bases (Joiner and van der Kogel, 2009). From these, DSB prove to be the most efficient to cause cell death (Connell et al., 2004). After DNA damage by IR, varied pathways can be activated, namely: programmed cell death, DNA repair or temporary (or permanent) cell cycle arrest (Joiner and van der Kogel, 2009). Cancer cells divide more often and have diminished ability to repair sub-lethal damages compared to normal cells. Therefore, single-strand DNA damage is passed on through cell division of tumor cells, accumulating damages that retard cell cycle or cause their death, while normal cells recover from IR-induced damages between fractions administration (Connell et al., 2004; Steel, 2002). Since one of the key factors that will determine the cell radiosensitivity is its capability of repair DNA damage, this will vary with the access of repair complexes and, consequently, DNA conformation. As a result, cells in the S phase are, in general, the most resistant and cells in late G2 and mitosis are the most sensitive to IR (Joiner and van der Kogel, 2009; Pawlik and Keyomarsi, 2004). Besides, IR can retard the rate of cell proliferation by activation of several checkpoints. This, in combination with fractionated schemes, leads to cell accumulation in the G2 phase and partial synchronization (Pawlik and Keyomarsi, 2004).

**Radiotherapy and Metastasis**

The therapeutic effects of IR are correlated to the death of tumor cells and are also consequence of the interactions between the tumor cells themselves and tumor-associated host cells (endothelial cells – ECs –, leukocytes, macrophages, fibroblasts, myofibroblasts and nerve cells). It is generally assumed that tumor progression towards metastasis, during or after
radiotherapy, is due the appearance of resistant tumor cells through a combination of therapy-induced genetic instability and mutations and subsequent clonal selection of the most fitted cells. However, there are clinical and experimental observations indicating that IR may promote a metastatic behavior of cancer cells (Barcellos-Hoff et al., 2005; Madani et al., 2008; von Essen, 1991). This data is not contradictory with the concept that the anti-proliferative and cytotoxic effects of radiation on ECs contribute in anti-tumoral treatment, as previous reported, but suggest that at certain doses and time frames, the IR enhances the build of new vessels, supporting invasion and metastasis. In fact, tumor recurrences within a pre-irradiated field are associated with higher risk of local invasion and metastasis (O’Brien et al., 1986; Suit, 1992; Vicini et al., 2003). In 1991, von Essen (von Essen, 1991), considered a number of mechanisms for the pro-metastatic activity of IR, 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.

Focusing in the vascular context, IR may exert its anti-angiogenic activity through its direct pro-apoptotic effect on ECs via ceramide pathways (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). Accordingly, it was demonstrated that IR itself induces the production of pro-angiogenic molecules by the tumors, such as TGFβ, FGF, IL-1Ra, IL-10, IL-3, IL-4 and IL-5, that may activate the microenvironment, including the vasculature, and that anti-angiogenic approaches can enhance IR-induced tumor growth inhibition (Gorski et al., 1999; Lee et al., 2000; Mauseri et al., 1998; Teicher et al., 1995).

Many works have been developed in order to investigate the effects of therapeutic doses of IR in the tumor area and to prevent the putative pro-metastatic effects of radiotherapy associated with angiogenesis. However, not much attention has been given to the tissues that surround the tumor area and that are exposed to doses lower than the tumor area dose (see Figure 1.1). Recently, Vala et al. (Vala et al., 2010) show that, in vitro, IR doses lower than 0.8 Gy do not cause cell cycle arrest or apoptosis. Instead, low-dose IR led to the phosphorylation of several cellular proteins, including VEGF receptor-2 (VEGFR2), enhancing endothelial cell migration and preventing endothelial cell death. Moreover, in zebrafish, low-dose IR accelerates sprouting angiogenesis during development and enhances the

---

1 Ability of radiotherapy to cause effects beyond the radiation field
angiogenic response during caudal fin regeneration (Vala et al., 2010). Using different mouse models, low-dose IR promotes angiogenesis resulting in accelerated tumor growth and metastasis formation in a VEGFR-dependent manner (Vala et al., 2010).

**Blood vessels and Angiogenesis**

Vertebrate blood vessels have essential functions, both during embryogenesis and adulthood. The circulatory system, which includes blood vessels and lymphatic vessels, is responsible for the distribution of oxygen and nutrients through the tissues, as also for the filtration of metabolites and waste products (Adams and Alitalo, 2007; Lawson and Weinstein, 2002; Nagy et al., 2008). Furthermore, it acts as a route of communication between distant tissues and plays a role in the regulation of body temperature and systemic pH (Carmeliet, 2005; Lawson and Weinstein, 2002).

The walls of larger vessels are composed of specialized layers that ensure the transport of blood to and from the heart (Eichmann et al., 2005). The inner layer of arteries and veins, tunica intima, is composed of ECs surrounded by a basement membrane. A middle layer named tunica media is composed of smooth muscle cells (SMCs) and elastic fibers that control the vessel diameter and the blood flow. The outer layer, tunica adventitia is composed of connective tissue, collagen fibers and nerves and has its own blood supply named vasa vasorum (Jain, 2003).

The capillaries are composed of ECs surrounded by a basement membrane and a sparse layer of pericytes (Jain, 2003). The capillary bed is the largest surface of the vascular system and due to its wall structure these vessels form the main site for exchange of gases and nutrients with the surrounding tissues (Eichmann et al., 2005; Jain, 2003). The capillary endothelial layer presents distinctive characteristics depending on the different organs or tissues, conferring different levels of permeability. The endothelial layer is continuous in the muscle tissue and discontinuous in the liver sinusoids, while in the kidney is fenestrated. The blood-brain barrier has further specialized ECs that are impermeable to various molecules (Jain, 2003). Arterioles and venules have an increased coverage of pericytes and SMCs compared to capillaries. The vascular SMCs from the precapillary arterioles are tightly packed with the endothelial layer and form their own basement membrane (Jain, 2003).
Blood vessels can be built by two different mechanisms: vasculogenesis and angiogenesis. Vasculogenesis consists on a de novo formation of blood vessels by endothelial differentiation of multipotent cells (Figure 1.2 b) and occur especially during embryo development, but also in adulthood (particularly during tumor vascularization) (Bussolati et al., 2011; Carmeliet and Jain, 2011). In the case of angiogenesis, new vessels are formed from the existing vasculature in a complex and extremely coordinated process (Owen et al., 2009). Angiogenesis can occur by sprouting or non-sprouting mechanisms. In the first, new vessels are formed by branching of preexisting ones (Shahi and Pineda, 2008; Figure 1.2 a). In non-sprouting angiogenesis, also called intussusception, the vessels are enlarged by the proliferation of endothelial cells within the wall of a preexisting vessel, followed by its splitting and fusion (Carmeliet and Jain, 2011; Shahi and Pineda, 2008; Figure 1.2 c). In pathologic conditions, namely in tumors, vessels can still be formed by co-option or vascular mimicry. In vessel co-option, tumor cells hijack the existing vasculature (Figure 1.2 d), whereas in the second these cells line vessels (Figure 1.2 e) (Carmeliet and Jain, 2011). Tumor cells can still redifferentiate in ECs and integrate the vessel wall (Figure 1.2 f).

![Figure 1.2 Mechanisms of vessel formation](image)

*Figure 1.2 Mechanisms of vessel formation, in normal tissues and tumors. Adapted (Carmeliet and Jain, 2011).*
In sprouting angiogenesis, when quiescent microvessels sense an angiogenic signal, the stable association between ECs and pericytes is disrupted. Instead, activated pericytes are loosely attached to vessels and can change its expression profile, becoming highly proliferative and capable of differentiate into other pericytes, matrix-forming cells, SMCs or adipocytes (Raza et al., 2010). Thereby, in response to an angiogenic signal, pericytes detach from the vessel wall and release themselves from the basement membrane by proteolytic degradation, mediated by matrix metalloproteinases (MMPs) (Carmeliet and Jain, 2011). Consequently, ECs loosen their junctions, vessel permeability increases and plasma proteins extravasate, which serve as a provisional extracellular matrix (ECM) for ECs and pericytes migration (Carmeliet and Jain, 2011; Raza et al., 2010). In addition, proteases liberate angiogenic molecules stored in the ECM, guiding ECs migration (Carmeliet and Jain, 2011). To prevent ECs from moving en masse towards the angiogenic signal and to build a perfused tube, at the front of the vessel sprout one EC is selected to lead the tip towards the angiogenic stimulus, the “tip cell” (Figure 1.3 a). Tip cells are highly polarized and motile, extend filopodia and proliferate minimally. Immediately behind the leading tip cell, cells assume subsidiary positions as “stalk cells”, which proliferate to elongate the vessel branch and establish the lumen (Figure 1.3 b) (De Bock et al., 2009; Carmeliet and Jain, 2011). Myeloid bridge cells assist fusion with another vessel branch, establishing blood flow, and ECs return into a dormant “quiescent”, non-proliferating, immobile state (“phanlax cells”) (De Bock et al., 2009; Carmeliet and Jain, 2011). For vessels stabilization, several signals cause the cells to become covered by pericytes, protease inhibitors permit the deposition of a basement membrane and junctions are re-established to ensure optimal flow distribution (Figure 1.3 c) (Carmeliet and Jain, 2011).
Figure 1.3 Molecular basis of vessel branching. The consecutive steps of blood vessel branching are shown, with the key molecular players involved denoted in parentheses. 

a) After stimulation with angiogenic factors, the quiescent vessel dilates and an endothelial cell tip cell is selected (DLL4 and JAGGED1) to ensure branch formation. Tip-cell formation requires degradation of the basement membrane, pericyte detachment and loosening of endothelial cell junctions. Increased permeability permits extravasation of plasma proteins (such as fibrinogen and fibronectin) to deposit a provisional matrix layer, and proteases remodel preexisting interstitial matrix, all enabling cell migration. For simplicity, only the basement membrane between endothelial cells and pericytes is depicted, but in reality, both pericytes and endothelial cells are embedded in this basement membrane. 

b) Tip cells navigate in response to guidance signals (such as semaphorins and ephrins) and adhere to the extracellular matrix (mediated by integrins) to migrate. Stalk cells behind the tip cell proliferate, elongate and form a lumen, and sprouts fuse to establish a perfused neovessel. Proliferating stalk cells attract pericytes and deposit basement membranes to become stabilized. Recruited myeloid cells such as tumor-associated macrophages (TAMs) and TIE-2-expressing monocytes (TEMs) can produce pro-angiogenic factors or proteolytically liberate angiogenic growth factors from the ECM. 

c) After fusion of neighbouring branches, lumen formation allows perfusion of the neovessel, which resumes quiescence by promoting a phalanx phenotype, re-establishment of junctions, deposition of basement membrane, maturation of pericytes and production of vascular maintenance signals. Other factors promote transendothelial lipid transport. Adapted (Carmeliet and Jain, 2011).
Tumor Angiogenesis and Metastasis

Expansion of tissues, both normal and neoplastic, requires the concomitant growth, infiltration and elaboration of a supporting vasculature network. In 1971, Folkman proposed that blocking angiogenesis would be an effective strategy to arrest tumor growth and treat human cancer (Folkman, 1971). However, only after 33 years, the first anti-angiogenic drug was approved by the Food and Drug Administration (FDA) (Kerbel, 2008).

Avascular tumors undergo diffusion-limited growth and, as a result, are typically at most several millimeters in radius (Owen et al., 2009); once exceeding a critical volume, tumor cell proliferation and metabolic demands outpace the supply of O₂, leading to hypoxia (De Bock et al., 2009).

During evolution, molecular O₂ sensing mechanisms were developed and, therefore, in hypoxia cells secrete angiogenic factors. Tumors try to overcome O₂ deficiency by upregulating further angiogenic factors, in addition to the upregulation achieved by oncogenes (De Bock et al., 2009). The tumor microenvironment also have a role in promoting tumor angiogenesis; many angiogenic factors and cytokines are secreted by stromal cells, such as infiltrated macrophages, inflammatory cells or even endothelial progenitor cells (EPCs) (Fong, 2008; Kopfstein and Christofori, 2006; Ribatti and Vacca, 2008; Ruegg, 2006). However, angiogenesis depends on a fine balance between angiogenic factors and inhibitors, leading the excessive production of angiogenic factors to an abnormalization effect (Otrock et al., 2007; De Bock et al., 2009).

Tumor vasculature typically lacks hierarchy, with tortuous and mal-shaped vessels presenting a highly dysfunctional, leaky EC layer. Tumor vessels have hypermotile and loosely connected ECs, with irregular shapes that form pseudostratified layers, leave gaps and obstruct the lumen by extending multiple protrusions (De Bock et al., 2009; Shchors and Evan, 2007). Abnormal tumor vessels also have a defective basement membrane and pericyte coverage, being immature. In normal conditions, feedback mechanisms ensure that inhibitors will eventually dominate, ceasing angiogenesis. However, in tumor environment, EC and vascular changes impair perfusion and the supply of oxygen, which aggravates hypoxia and upregulate more abnormalization factors (De Bock et al., 2009; Owen et al., 2009).

Metastasis are formed by a series of steps in which cancer cells leave the original tumor site and migrate to other parts of the body via the bloodstream or the lymphatic system (Figure 1.4)(Mierke, 2008). It has been postulated that the metastatic potential of solid tumors
can be related to tumor vasculature and angiogenesis (Raza et al., 2010; Zetter, 1998). As a matter of fact, tumor angiogenesis increase the density of immature, highly permeable blood vessels, which have little basement membrane and fewer intercellular junctional complexes than normal mature vessels. So, the most important way in which angiogenesis can facilitate tumor metastasis may be by providing an efficient route of exit for tumor cells to leave the primary site and enter the blood stream (Raza et al., 2010; Zetter, 1998). On the other hand, altered pericytes may assist circulating tumor cells in the process of extravasation (Raza et al., 2010). Equally, severe hypoxia may enhance metastasis by switching tumor cells to a more invasive and metastatic program, to escape the hostile hypoxic microenvironment, thereby accelerating tumor cell invasiveness and malignancy (De Bock et al., 2009). These findings raise questions in respect to the best approach for angiogenic therapies.

**Figure 1.4 Vascular components of tumor metastasis.** The steps of the metastatic pathway that involve interactions with blood vessels: (a) small primary tumors (< 2 mm) remain avascular until they (b) invade the local epithelial basement membrane. If the tumor cells produce angiogenic factors (c) angiogenesis will occur, allowing 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 endothelial cells 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) Micrometastases 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).
Classically, anti-angiogenic therapies were focused on inhibiting the growth of new blood vessels, by targeting angiogenic factors that promote cell growth, survival and/or migration, or by up-regulating or delivering endogenous inhibitors (Carmeliet and Jain, 2000). Anti-angiogenic agents should normalize vessels, by pruning the immature and inefficient ones and remodeling the remaining (Hillen and Griffioen, 2007; Jain, 2005; Siemann et al., 2005).

Vessel regression is a physiological mechanism that takes place when the nascent vasculature comprises too much vessels. This can be promoted not only by the presence of anti-angiogenic factors, but also by removal of angiogenic stimuli, insufficient perfusion of blood or absence of pericytes; that is to say, when vessels are immature and have an inadequate function (Carmeliet, 2003; Risau, 1997).

However, excessive vessel pruning and growth arrest by anti-angiogenic agents could aggravate tumor invasiveness and metastasis. By contrast, anti-tumor vessel abnormalization strategies that focus on vessel perfusion and not on their density could be an important approach in angiogenic therapies (Carmeliet and Jain, 2011; De Bock et al., 2009). When combined with radiotherapy and chemotherapy, anti-angiogenic agents enhance their anti-tumor effects (Owen et al., 2009). This synergistic or additive anti-tumor effect results from the improvement on the delivery of chemotherapeutic agents to the tumor mass (Shahi and Pineda, 2008). Moreover, anti-angiogenics, by inducing organizational and functional vessel normalization, will improve the delivery of oxygen, contributing to radiotherapy’s success (Carmeliet and Jain, 2011; De Bock et al., 2009).

Angiogenic factors

As referred previously, angiogenesis depends on a complex balance between pro- and anti-angiogenic factors which are tightly regulated both temporally and spatially. Angiogenesis inducers include the members of the vascular endothelial growth factor (VEGF) family, angiopoietins, transforming growth factors (TGF), platelet-derived growth factor, tumor necrosis factor-α, interleukins and members of the fibroblast growth factor (FGF) family. Additionally, many factors regulate angiogenesis, including soluble growth factors, membrane-bound proteins, cell-matrix and cell-cell interactions and many interacting systems (Otrock et al., 2007).
**VEGF**

VEGF family includes VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PIGF), which have largely non-redundant roles (Carmeliet and Jain, 2011; Kerbel, 2008). VEGF (VEGFA) is the best characterized member of the family and the major mediator of normal and tumor angiogenesis, acting especially on ECs from new blood vessels (Kerbel, 2008; Kuwano et al., 2001; Otrock et al., 2007). It is produced by the majority of cells in the body and is up-regulated by hypoxia. Hypoxia-inducible factor 1 (HIF1) is an oxygen-regulated transcriptional activator that functions as a master regulator of oxygen homeostasis (Semenza, 2001). HIF1 is a heterodimer, composed of HIF1α and HIF1β subunits. Whereas HIF1β is constitutively expressed, HIF1α expression is induced in hypoxic cells with an exponential increase in expression as cells are exposed to low O₂ concentrations (Semenza, 2001). In non-hypoxic conditions, HIF1α is ubiquitinated and subjected to proteasomal degradation (Semenza, 2001). Under hypoxic conditions, the fraction of HIF1α that is ubiquitinated decreases dramatically, resulting in an accumulation of the protein (Semenza, 2001). A functional hypoxia response element (HRE) in the 5’ region of the VEGFA human promoter was found to bind the heterodimer HIF1α/HIF1β (Forsythe et al., 1996). Moreover, the expression of VEGF is also modulated by inflammatory mediators, growth factors, oncogenes and mechanical forces of shear stress and cell stretch (Kuwano et al., 2001). VEGF family members exert their biologic effect through interaction with transmembrane tyrosine kinase receptors: VEGFR1 and VEGFR2, selectively expressed on vascular ECs; and neuropilin receptors (NP-1 and NP-2), expressed on vascular ECs and neurons. VEGFR2 (KDR) is the major responsible for VEGF signaling and its activity may be enhanced by neuropilins co-receptors, which also may signal independently. The precise role of VEGFR1 (FLT1) in angiogenesis is still unclear (Carmeliet and Jain, 2011; Kerbel, 2008; Kuwano et al., 2001; Otrock et al., 2007).

**Angiopoietins**

Another receptor tyrosine kinase signaling pathway is mediated by tie-2, which is expressed mainly on vascular endothelium. The two major ligands of tie-2 are angiopoietin-1 (ang-1) and angiopoietin-2 (ang-2) that act as an agonist and an antagonist, respectively. Ang-1 and ang-2 are expressed by several cell types, including ECs, SMCs, fibroblasts, pericytes and
some tumor cell lines (Carmeliet and Jain, 2011; Kerbel, 2008; Otrock et al., 2007). The fourth member of this family is the tie-1 receptor, with no known ligands (Otrock et al., 2007). Tie-1, tie-2, ang-1 and ang-2 are secreted proteins. These molecules operate as a binary system that allows vessels to maintain quiescence, while remaining able to respond to angiogenic stimuli (Carmeliet and Jain, 2011). In confluent endothelium, ang-1 induces tie-2 clustering in trans at cell-cell junctions to maintain ECs quiescence and stimulate mural coverage and basement membrane deposition, thereby promoting vessel tightness. In the presence of angiogenic stimulators, sprouting ECs release ang-2, which antagonizes ang-1 and tie-2 signaling and enhances pericytes detachment and vascular permeability. The destabilized vessels will undergo sprouting in the presence of angiogenic factors such as VEGF (Carmeliet and Jain, 2011). Ang-2 may also act to prevent excessive sprouting and branching of vessels by enhancing their regression in the absence of growth factors (Lobov et al., 2002; Otrock et al., 2007).

**FGFs**

Fibroblast growth factors, acidic (aFGF) and basic (bFGF), are heparin-binding protein mitogens that exert their activities mainly by binding to high affinity tyrosine kinase FGF receptors (FGFRs) (Otrock et al., 2007). They can also interact with other receptors non-tyrosine kinase, such as αvβ3 integrin and syndecan-4 (Murakami et al., 2008). FGFs stimulate ECs proliferation and migration, collagenase and plasminogen activator production through FGFR1, the main FGFR expressed in ECs (Javerzat et al., 2002; Milkiewicz et al., 2006; Otrock et al., 2007; Turner and Grose, 2010). Activation of FGFR2 increases only cell motility (Milkiewicz et al., 2006). FGFs also stimulate angiogenesis indirectly by inducing the release of angiogenic factors from other cell types (Carmeliet and Jain, 2011) and contribute to the proliferation of tumor cells either by autocrine or paracrine signaling (Beenken and Mohammadi, 2009; Javerzat et al., 2002).

**TGFβ**

TGFβ represents a family of highly conserved cytokines, being TGFβ1 the most well studied member of this family (Dickson et al., 1995; Otrock et al., 2007). TGFβ stimulates two types of interdependent serine-threonine kinase receptors (TβR-I and TβR-II), regulating cell
growth, differentiation, migration, adhesion and apoptosis of various cell types (Distler et al., 2003; Fonsatti et al., 2010; Otrock et al., 2007; Pepper, 1997). A third type of (co)receptor for TGFβ is present on ECs, endoglin, which is up-regulated during angiogenesis (Fonsatti et al., 2010). Both TGFβ and its receptors are expressed in several cell types, including tumor cells, pericytes and ECs (Otrock et al., 2007).

TGFβ may promote angiogenesis indirectly, inducing the release of pro-angiogenic factors by inflammatory cells (Distler et al., 2003; Kaminska et al., 2005), or directly, through the binding to two types of TβR-1 (activin receptor-like kinase-1 and -5) and consequent activation of the downstream signaling Smads (Bertonilo et al., 2005; Otrock et al., 2007). TGFβ may act also as an anti-angiogenic agent, according to dose and microenvironment conditions (Distler et al., 2003).

**CYR61**

CYR61 (CCN1) is a member of the CCN family, expressed and capable of acting in a wide range of cell types (e.g. fibroblasts, SMCs, tumor cells, EPCs and ECs) (Chen and Lau, 2009). It regulates proliferation, adhesion, migration, survival, chemotaxis and gene expression (Brigstock, 2002; Chen and Lau, 2009; Yu et al., 2008). CYR61 transcription is activated by several cytokines and growth factors (Chen and Du, 2007), then regulating angiogenesis by direct and indirect mechanisms (Leask and Abraham, 2006). In direct signaling, CYR61 regulates the expression of VEGF, MMPs and TIMs and the activation of targets involved in adhesion and survival (Brigstock, 2002; Leask and Abraham, 2006; Leu et al., 2002) through an integrin dependent pathway. In addition, CYR61 can increase growth factors activity by direct binding or by HSPG (heparin sulfate proteoglycan) binding with release of FGF2 from ECM (Brigstock, 2002) and may promote a higher level of cross-talk between integrin and growth factor receptors as VEGFR2 (Somanath et al., 2009).

**Cell migration and Focal adhesions**

Cell migration is a coordinated and complex process, where integrin-mediated adhesions provide traction points by linking cells to the ECM (Kaverina et al., 2002). Integrin adhesions also participate on environmental sensing (Geiger et al., 2001). These structures
contain more than 100 different types of scaffolding and signaling proteins that physically connect the integrin receptors to the actin cytoskeleton (Critchley, 2000; Geiger et al., 2001; Zamir and Geiger, 2001), including vinculin, paxillin, talin, α-actinin, zyxin, VASP, FAK and p130Cas (Fraley et al., 2010).

According to their molecular composition, dynamics and function, three types of integrin adhesions are defined (Figure 1.5) (Zamir and Geiger, 2001). Focal complexes (FXs) are dot-like adhesions that assemble under the lamellipodium in a hierarchical manner (Kaverina et al., 2002; Zaidel-Bar et al., 2003). Within less than a minute of their formations, FXs either turn over or undergo a force-dependent transformation into focal adhesions (FAs), which are considerably larger structures associated with actin- and myosin-containing stress fibers (Critchley, 2000; Geiger et al., 2001; Zaidel-Bar et al., 2003). FAs are maintained and develop into fibrillar adhesions (FBs) in response to actomyosin contractility (Bershadsky et al., 2003; Wierzbicka-Patynowski and Schwarzbauer, 2003). Formation of these adhesions is regulated by members of the Rho GTPase family: Rac induces FXs formation, whereas Rho leads to the induction and growth of FAs and enhances FBs formation (Wierzbicka-Patynowski and Schwarzbauer, 2003). FAs assembly and turnover are also regulated by tyrosine phosphorylation of some of their proteins, such as focal adhesion kinase (FAK), paxillin and p130Cas (Panetti, 2002). The main kinases that may be involved are pp60^{C-src} (Src) and FAK (Frame, 2004; Schlaepfer and Mitra, 2004). Integrin activation and growth factor stimulation lead to phosphorylation of tyrosine and serine residues of paxillin. Accordingly, vinculin activity regulates paxillin recruitment, which, depending on cosignals, may lead to additional modifications of FAs and cell migration (Turner, 2000; Zaidel-Bar et al., 2006).
The adaptor protein vinculin is a key regulator of FAs, having a profound role on cell adhesion and motility (Jockusch and Rudiger, 1996; Zamir and Geiger, 2001). Vinculin has a closed ‘inactive’ conformation and an open ‘active’ conformation, located within the cytoplasm and FAs, respectively. In the active integrin adhesion, vinculin interacts directly with talin, α-actinin, ponsin, vinexin, vasodilator-stimulated phosphoprotein, Arp2/3, actin, PIP2 and paxillin (Chen et al., 2005; Zamir and Geiger, 2001; Ziegler et al., 2006). Recently, a new model for FAs regulation by vinculin was proposed by Humphries et al. (Figure 1.6) (Humphries et al., 2007).
Figure 1.6 Model of vinculin action. Vinculin is recruited via low-affinity binding to talin or neck-binding proteins to focal complexes at the cell front. At this stage, if vinculin establishes associations with PIP2 or actin it becomes activated (open state); if not, adhesion complexes turn over rapidly. Activated vinculin stabilizes an active conformation of integrins in FAs, resulting in reduced FA turnover and growth. Subsequently, vinculin links to the contractile actomyosin machinery, which allows effective transmission of forces and information between the inside and outside of the cell. To complete the cycle, FAs may be destabilized via further retrograde flow, leading to the removal of vinculin from FAs, or, alternatively, if actomyosin-mediated forces play a role in maintaining vinculin activity, via the transition of FA to areas of low actomyosin activity, with vinculin adopting a low affinity for talin upon refolding to an inactive state. Adapted (Humphries et al.)

Another model, focused on paxillin’s role in integrin adhesion assembly, proposed that paxillin’s phosphorylation state is the key regulator (Zaidel-Bar et al., 2006). Zaidel-Bar et al. suggests that the assembly of FXs is driven by the recruitment of phosphopaxillin (probably phosphorylated by Src), which serves as a docking site for FAK. Then, FAK up-regulates the FXs turnover by an unknown mechanism. It is possible that FAK activates calpains, which cleave several FA proteins (e.g. paxillin, vinculin and talin), or regulates actomyosin contractility by inhibiting Rho. So, the assembly and turnover of FXs is maintained by a negative feedback between FAK and phosphopaxillin. Application of mechanical force induces paxillin dephosphorylation, leading to FAK dissociation and transformation of FXs into FAs. Finally, paxillin dephosphorylation enables FB formation.
II Objectives

It is generally assumed that tumor progression towards metastasis, during or after radiotherapy, is due to the appearance of resistant tumor cells. However, several studies indicate that IR creates a microenvironment that might exert tumor-promoting effects. Several studies have been developed to investigate the effects of therapeutic doses of IR in the tumoral area and to prevent the putative pro-metastatic effect of radiotherapy. However, during radiation treatment, surrounding healthy tissues are also exposed to an IR gradient, ranging from the daily dose to zero. The biological effects of these low doses of IR remain largely undetermined. Recently, Vala et al. (Vala et al., 2010) showed for the first time that this low-dose IR, present in the vicinity of the tumor volume target, enhances angiogenesis, supporting invasion and metastasis.

The primarily aim of this work was to discover the molecular targets and cellular mechanisms by which low doses of IR induce a pro-angiogenic response. The gene expression profile of irradiated and non-irradiated human microvascular ECs (Attach 1 and 2) was investigated in a microarray study, where several proteins required for angiogenesis show an increased fold change upon low-dose IR delivery. We chose the genes whose expression is significantly altered by low doses of IR and that represent the best candidates for a pro-angiogenic response and confirmed their expression by quantitative real-time PCR (qRT-PCR) and western blot, in Lung Human Microvascular Endothelial Cells (HMEC-L) and in Fli1:EGFP zebrafish larvae. In the case of proteins whose role is implicated in the cytoskeleton, we performed western blot analysis of soluble and insoluble cytoskeleton fractions and immunofluorescence and confocal studies.

We also investigated the effects of low-dose IR in zebrafish growth and the role of VEGFR2. We used the Fli1:EGFP zebrafish model and assessed to development stage over time by confocal imaging.

Finally, we studied the effects of low doses of IR in the spontaneous development of melanoma in a p53 mutated \textit{BRAF}\textsuperscript{V600E} zebrafish model.

Based on the results obtained during this project, we expect to provide a better understanding of the potential pro-metastatic effect of IR, leading to the improvement of current radiotherapy protocols.
III Material and Methods

IRRADIATION

HMVEC-L cultures or anesthetized 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 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 cm³ 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.

CELL CULTURE

Lung Human Microvascular Endothelial Cells (HMEC-L) were grown at 100 mm tissue culture plates treated with gelatin from porcine skin 0.2% (Sigma), containing EBM-2 supplemented with 5% FBS (fetal bovine serum), hydrocortisone, hFGF-B (human fibroblast growth factor B), VEGF (vascular endothelial growth factor), R3-IGF-1 (recombinant long R insulin-like growth factor 1), ascorbic acid, hEGF (epidermal growth factor human, recombinant in a buffered BSA saline solution), GA-1000 (gentamicin, amphotericin B), heparine and BBE (bovine brain extract) (Lonza), in a humidified incubator (HERAcell CO₂, Heraeus) at 37ºC with 5% CO₂, until reach 90-100% confluence. Cells were used up to passage 6.
WESTERN BLOT

Cell lysate preparation

7 x 10^5 cells were passed to a 40 mm culture plate the day before irradiation, to reach monolayer coverage. In the case of cytoskeletal extracts and just before irradiation, three parallel wounds had been done with a micropipette yellow tip.

Whole cell extracts

Whole cell extracts were prepared by resuspending the cell pellets in the following buffer: 1% NP40, 50 mM Tris pH 7.5, 10% (v/v) Glycerol, 150 mM NaCl, 1 mM EDTA supplemented with protease and phosphatase inhibitors. After incubation 30 min on ice, the cells were centrifuged, for 15 min, at 4°C and 13000 rpm and the supernatants were recovered.

Cytoskeletal extracts

Culture medium was removed and cells were washed two times with PBS 1x. Two hundred microliters of PHEM buffer (60mM PIPES, 2mM HEPES, 10 mM EGTA and 2mM MgCl₂) containing protease inhibitors (protease inhibitor cocktail tablets – Roche), phosphatases inhibitors (vanadate, Na₂VO₄ – Sigma) and 0.1% of Triton X-100 were added to each plate and incubated in ice for 2 min. Lysate was centrifuged for 30 min, at 13500 rpm and 4°C. The supernatant was collected as soluble fraction and 100 μL of SDS 3x buffer (3.5 mL Tris/SDS buffer1, 1.8 mL glycerol, 0.5 g SDS for 10 mL final volume) was added. Pellet was washed with PHEM buffer to eliminate traces of the soluble fraction, resuspended in 100 μL of PHEM buffer (without triton X-100) plus 50 μL of SDS 3x buffer and incubated for 5 to 10 min at 100°C. Finally, the sample was centrifuged for 10 min at 13500 rpm, 4°C and the supernatant was collected as the insoluble fraction. Samples were stored at -80°C.

Protein quantification

Protein quantification was performed with the Bio-Rad Dc Protein Assay. The method is based on the Lowry assay. The standard samples were prepared as a 1:10 dilution of 10 mg/mL stock solution of BSA. Two microliters of each sample were transferred for a 96 well plate. For each well was added 24.5 μL of reagent A, 0.5 μL of reagent S and 200 μL of reagent B. Samples were incubated at room temperature for 15 to 60 min and absorbance was measured

1 0.5 M Tris, pH 6.8 + 0.4% SDS
at 690 nm with Infinite M200. Protein concentration in unknown samples was extrapolated from the linear part of the standard curve.

<table>
<thead>
<tr>
<th>BSA (1 mg/mL)</th>
<th>0 μL</th>
<th>1 μL</th>
<th>2 μL</th>
<th>4 μL</th>
<th>6 μL</th>
<th>8 μL</th>
<th>10 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>24.5 μL</td>
<td>24.5 μL</td>
<td>24.5 μL</td>
<td>24.5 μL</td>
<td>24.5 μL</td>
<td>24.5 μL</td>
<td>24.5 μL</td>
</tr>
<tr>
<td>Reagent S</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>Reagent B</td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
</tr>
</tbody>
</table>

Western blot running

All gels were prepared freshly and for a western blot, resolving and stacking gel. Eight resolving gels were made first, as follows:

- 11.5 mL H₂O
- 6.7 mL Acrylamide 30%
- 6.3 mL Tris 1.5 M (pH 8.8)
- 250 μL SDS 10%
- 250 μL APS 10%
- 15 μL TEMED

Stacking gel was prepared as follows:

- 5.55 mL H₂O
- 1.7 mL Acrylamide 30%
- 2.5 mL Tris 0.5 M (pH 6.8)
- 100 μL SDS 10%
- 100 μL APS 10%
- 10 μL TEMED

The running buffer was prepared as a 1:10 dilution of 10x Tris/Glycine/SDS buffer pH 8.3 from Bio-Rad. Gels were placed in the running rig, samples and protein markers were added to the gel and ran at 30 mV.
Protein transfer to nitrocellulose

Nitrocellulose membrane (Protran® 0.45 μm pore), sponges and filter paper were soaked in 1x transfer buffer (500 mL of 10x Tris/Glycine buffer pH 8.3 – Bio-Rad – plus 1 L of absolute ethanol, made up to 5 L with sterile water). Once the gel had finished running, the transfer “sandwich” was assembled and slotted into the rig filled with transfer buffer. Proteins were transferred at 100 V for 1 h and 30 min. Protein transfer was checked with Ponceau S red stain (0.5% Ponceau S in 5% acetid acid – Merck) and then washed with PBS 1x/Tween-20 0.1% (Sigma) for 5 min.

Western blot analysis

Membranes were blocked for 1 h at room temperature with 5% milk powder in PBS 1x/Tween-20. Membranes were further incubated 1 h (or at 4ºC overnight) with antibodies against several targets (Table 3.2). After three washes of 10 min in PBS/Tween, the membranes were incubated for 1 h with anti-mouse, anti-rabbit or anti-goat antibody, which were horse-radish peroxidase-conjugated. Detection was made in a dark room using Pierce ECL Western Blotting Substrate or Super Signal® West Pico Chemiluminescent Substrate (Pierce) and Fuji Medical X-ray film (Fujifilm). The film was developed and fixed using Curix 60, AGFA. The images were scanned and the amount of protein in samples was estimated using image analysis (ImageJ).

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR2</td>
<td>Rabbit polyclonal – CS</td>
<td>1:1000</td>
</tr>
<tr>
<td>Ang2</td>
<td>Mouse monoclonal – SC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cyr61</td>
<td>Rabbit polyclonal – SC</td>
<td>1:1000</td>
</tr>
<tr>
<td>FGF2</td>
<td>Goat polyclonal – SC</td>
<td>1:200</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>Rabbit polyclonal – SC</td>
<td>1:1000</td>
</tr>
<tr>
<td>βTubulin</td>
<td>Mouse monoclonal – CS</td>
<td>1:500</td>
</tr>
</tbody>
</table>
Table 3.3 List of secondary antibodies used in western blot.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine anti-goat IgG-HRP SC</td>
<td>1:5000</td>
</tr>
<tr>
<td>Goat anti-mouse IgG-HRP SC</td>
<td>1:10000</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG-HRP SC</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

1 SC – Santa Cruz Biotechnology

**QUANTITATIVE REAL-TIME PCR**

**RNA extraction**

**Cells**

Total RNA was prepared using the RNeasy Mini kit (Qiagen) in accordance with the manufacture’s protocol. Briefly, cells were pelleted and resuspended in RLT buffer. The mixture was applied onto a QIAshredder spin column. The column was centrifuged at 13200 rpm for 2 min. The resulting eluent was applied to an RNeasy spin column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted using RNase-free water.
Zebrafish larvae

Heterozygous embryos were obtained by crossing wt zebrafish with Fli1:EGFP. Three days post-fertilization, larvae were irradiated or not for three consecutive days with 0.5 Gy. Larvae were collected at different time points post-irradiation (20 larvae for total RNA extraction, per experimental condition), with the minimum medium volume possible. Larvae were euthanized with tricaine 0.25 M (Sigma) and washed twice with PBS 1x. The dead larvae were transferred to Petri dishes and the larger amount of medium was removed. Trypsin 0.5% - EDTA.4Na 5.3 mM (Invitrogen) was added and samples were incubated at 37°C for around 45 min, resuspending occasionally with a P200 to allow better tissue disaggregation. When all the larvae tissue seemed to be disaggregate, cell suspension was passed through a 70 μm cell strainer (BD Falcon), pelleted and ressuspended in PBS 1x+5%FBS. Samples were stored at -80°C.

RNA isolation was made using the Total RNA isolation Trizol reagent protocol (Invitrogen). Samples with Trizol were thawed at room temperature or in ice. For 1 mL of Trizol, 200 μL of chloroform was added, followed by 15 sec of vigorous agitation and 5 min of incubation at room temperature. The mixture was centrifuged during 10 min, at 13000 rpm and 4°C. The aqueous phase was removed to a clean tube and RNA was precipitated with 0.5 mL of isopropanol (incubate for 10 min at room temperature for total RNA precipitation). A new centrifugation was performed for 10 min, at 13000 rpm and 4°C. Supernatant was removed and the RNA pellet washed with 1 mL of 75% ethanol and centrifuged for 10 min, at 13000 rpm and 4°C. The dried RNA pellet was resuspended in 20-30 μL of RNase free DEPC(diethylpyrocarbonate)-treated H2O and incubated at 65°C for 10 min.

Determination of RNA concentrations in the samples was done with NanoDrop ND-1000 spectrophotometer, with measurements at 260 nm. Samples purity was evaluated based on A260/A280 ratio. RNA samples were stored at -80°C.

DNase treatment

Ten micrograms of RNA from each sample was treated to make sure no genomic DNA was left in the samples. A 25 μL reaction contained the following components:

- 10 μg of RNA
- 1 μL of DNase (2 U/ μL) (Ambion)
- 2.5 μL of buffer (Ambion)
- Made up to final volume with RNase-free water (Ambion)
The mixture was incubated at 37ºC for 20 to 30 min. Five microliters of DNase inactivation reagent (Ambion) were added, followed by 2 min incubation at room temperature, with occasionally agitation. Samples were centrifuged for 1.5 min, at 13000 rpm and 4ºC. Supernatant was transferred to a clean tube, RNA was re-quantified with NanoDrop ND-1000 spectrophotometer and stored at -80ºC.

**cDNA synthesis**

Complementary DNA (cDNA) was synthesized using SuperScript™ II Rnase H-Reverse Transcriptase (Invitrogen) and Biometra thermocycler. In our studies, random hexamers, pdN(6) (Roche), were used. For each synthesis was used 1 µg of treated RNA and 1 µL of pdN(6) (3 µg/µL), for a final volume of 21 µL. The reactional mixture was incubated for 5 min at 65ºC. Afterward, the follow components were added to the sample:

- 6.75 µL RNase free water
- 9 µL Buffer 5x (Invitrogen)
- 2.25 µL DNTPs 10 mM (Fermentas)
- 4.5 µL DTT 0.1 M (Invitrogen)
- 0.5 µL RNase Out (40 U/µL) (Invitrogen)
- 1 µL SuperScript II RT (200 U/µL)

The mixture was incubated for 50 min at 42ºC and the reaction was stopped with 10 min incubation at 65ºC. Samples were stored at -20ºC.

**Quantitative real-time PCR assay**

The relative quantity of selected genes was measured by performing SYBR green-based (Applied Biosystems) quantitative real-time PCR using Applied Biosystems 7500 Fast Real-time PCR System. The housekeeping genes used to normalization were 18S in the case of HMEC-L and elongation factor 1 for zebrafish larvae, for an 1:100 cDNA dilution. All genes were analyzed in triplicate. Each plate included “no template control” reactions, where cDNA was not added to the reaction mixture. The running protocols used are presented below (Figure 3.1), as well as primer sequences for HMEC-L and zebrafish larvae (Tables 3.4 and 3.5).
Figure 3.1 qRT-PCR protocol used with HMEC-L (A) and zebrafish larvae mRNA (B).

Table 3.4 Primer used for qRT-PCR of HMEC-L.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR1</td>
<td>5'-CCCTCGCGGAAGTTGTAT-3'</td>
<td>5'-GTCAATAGCGAGCAGATTTCTCA-3'</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>5'-ATTCCTCCCCCGCATCA-3'</td>
<td>5'-GCTCGTGGGCAGCCTCTT-3'</td>
</tr>
<tr>
<td>CYR61</td>
<td>5'-AATGGAGGCTCGCATCTAT-3'</td>
<td>5'-CCCTTTTCAGGCTGCTGTA-3'</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>5'-AGGACACACCACAATGGCATCTA-3'</td>
<td>5'-TGAATATTGTCCACCCGCCTCCT-3'</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>FGF2</td>
<td>5'-AAGAGCGGACCTCACATCAAGCTA-3'</td>
<td>5'-TAGCCAGGTAACGGTTAGCACACA-3'</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>5'-GCTTTGGATGCGGCCTATTGCTTT-3'</td>
<td>5'-CTCCAGCAGAACAGTTGGCATTTG-3'</td>
</tr>
<tr>
<td>CLTC</td>
<td>5'-TTTGGCATCTACCTGGTTCACCT-3'</td>
<td>5'-TCCACACATCGAGCAAGACGGGA-3'</td>
</tr>
<tr>
<td>TUBB</td>
<td>5'-CATTGGCAATAGCAGCCATCCA-3'</td>
<td>5'-ACGAGGTCGTTCATTTGCTCTCA-3'</td>
</tr>
<tr>
<td>18S</td>
<td>5'-GCCCTATCACTCTCGATGTTAGT-3'</td>
<td>5'-CCGGAATCGAACCCTGATT-3'</td>
</tr>
</tbody>
</table>

Table 3.5 Primer used for qRT-PCR of zebrafish larvae.

**IMMUNOFLUORESCENCE STAINING**

The day before irradiation, 2 x 10⁵ cells were passed to 12-well plates, each one with 10 mm cover slips treated with gelatin, to achieve monolayer coverage. Immediately before irradiation, a wound was made through all the monolayer with a micropipette yellow tip. At different time points post-irradiation, medium was removed and cells were fixed with 4% paraphormaldehyde (PFA), for 10 min at room temperature. Cells were rinsed three times in PBS 1x and then blocked with 5% normal goat serum (DakoCytomation) in PBS 1x with 0.1% Triton X-100 and 0.1% BSA. Cells were incubated for 1h at room temperature with primary
antibody (Table 3.2) in PBS 1x/0.1% BSA and washed thrice for 10 min with PBS 1x/0.1% BSA. The process was repeated with the secondary antibody (Table 3.3). The last wash was made in PBS 1x. Microscope preparations were assembled using VectaShield® Mounting Medium for Fluorescence with DAPI H-1200 (Vector). Slides were imaged on Confocal Laser Point-Scanning Microscope Zeiss LSM 510 Meta.

**P53 MUTATED BRAF<sup>V600E</sup> ZEBRAFISH**

p53 mutated BRAF<sup>V600E</sup> zebrafish with approximately 5 months of life were irradiated for 18 consecutive days with 0.5 Gy, under anesthesia with tricaine 10 mM. These animals were followed over time, until a significant part of the population develop apparent melanoma. Zebrafish were euthanized with tricaine 0.25 M and fixed with PFA 4%. Samples were then cut with microtome (Leica RM2145) and stained with hematoxylin and eosin staining, to confirm tumor development and aggressiveness. Slides were imaged on Brightfield Microscope Leica DM2500.
Microarray validation

With the purpose of investigating the cellular mechanisms and molecular targets by which low doses of IR induce angiogenesis, we started by validating the results obtained in an in vitro microarray study. We showed that low-dose IR activates receptor-2 of VEGF, critical to the angiogenic process (Vala et al., 2010). It was also described that the activation of VEGFR-2 leads to rapid activation of different cellular proteins and consequently to de novo mRNA synthesis and protein expression of angiogenic mediators. This is supported by the data we obtained in an in vitro microarray study: from 28,869 genes represented on the array, 4,042 significantly changed with low dose IR (Attach 1 and 2). The genes significantly altered by low doses of IR, which represent the best candidates for a pro-angiogenic response, were selected and their expression validated by quantitative real-time PCR (qRT-PCR) and Western blot. HMEC-L were irradiated or not with 0.1 and 0.3 Gy using a linear accelerator and processed for analysis at different times post-stimulus (Figure 4.1).
Figure 4.1 Low doses of IR modulate expression of pro-angiogenic targets and cytoskeleton-related proteins in HMVEC-L cells. (A) HMVEC-L cells were exposed or not to 0.3 Gy and incubated for the time indicated. The mRNA expression was quantified by qRT-PCR and normalized to 18S expression. Data represent the fold change in mRNA expression in irradiated cells relative to non-irradiated ones in triplicate measurements. (B) HMVEC-L cells were irradiated or not with 0.1 or 0.3 Gy and 8h post-irradiation, protein extracts were collected every 2 h until 24h post-irradiation. Protein expression was assessed by western-blot analysis, using actin as endogenous control. Data represents the highest fold change in the expression of each protein in irradiated cells relative to non-irradiated ones, obtained by ImageJ.

We have also investigated the validity of these results in *Fli1:EGFP* zebrafish larvae. Heterozygous zebrafish larvae were irradiated or not with 0.5 Gy 3 days post-fertilization (dpf) until they reached 5 days of age. This time frame ensures that IR exposure does not interfere with vasculogenesis, but just with angiogenesis (Referencia). Larvae tissue dissociation and RNA isolation were performed 4 and 8 h post-irradiation. In accordance with the *in vitro* results, low-dose IR enhances the expression of angiogenic factors in zebrafish larvae (Figure 4.2). Presently, we are performing the same analysis in sorted ECs of *Fli:EGFP* zebrafish larvae.

![Graph](image)

Figure 4.2 Low doses of IR modulate expression of pro-angiogenic targets and cytoskeleton-related proteins in *Fli1:EGFP* zebrafish larvae. Live *Fli1:EGFP* zebrafish larvae were exposed or not to 0.5 Gy at 3 dpf, for 3 consecutive days. Total mRNA expression was quantified by qRT-PCR and normalized to elongation factor 1. Data represent the fold change in mRNA expression in irradiated larvae relative to non-irradiated ones, in triplicate measurements.
To find out the effect of low-dose IR not only in the expression, but also in the subcellular localization of cytoskeleton proteins, we performed immunofluorescence assays and western blot analysis of soluble and insoluble fractions of HMEC-L. We used an *in vitro* wound healing model, where confluent EC monolayers suffer mechanical wounding. Immediately after wound, cells were irradiated or not with 0.3 Gy. Cells were incubated at 37°C and 5% CO₂ for wound healing and samples were processed for analysis. Immunofluorescent staining of HMEC-L with anti-paxillin monoclonal antibody was used to assess focal adhesion density in pro-migratory cells at leading edge, which is significantly higher in irradiated ones, 1 h and 3.5 h post-irradiation when compared to non-irradiated (Figure 4.3). Levels of Rac1, vinculin and paxillin are also significantly increased in insoluble fractions when cells are exposed to low doses of IR (Figure 4.4).
**Figure 4.3 Low doses of IR enhance protein targeting to focal adhesions.** Paxillin targeting to focal adhesions was assessed by immunofluorescence staining. (A) Representative images of focal adhesions in irradiated cells when compared to non-irradiated ones, at the indicated time post-irradiation. (B) Five pro-migratory cells of each experimental condition were quantified for focal adhesion density using ImageJ.

**Figure 4.4 Low doses of IR enhance levels of Rac1, vinculin and paxillin on insoluble fractions.** Confluent monolayers of HMVEC-L were subjected to an *in vitro* wound healing assay and exposed or not to 0.3 Gy of IR. Soluble and insoluble extracts were prepared and protein expression was determined by western blot analysis, using actin as an endogenous control. Data represent the fold change in protein levels in soluble and insoluble fractions in irradiated cells relative to non-irradiated cells, determined with ImageJ.
Low-dose IR and zebrafish development

Previously, we showed that low-dose IR accelerates angiogenic sprouting during the development of the sub-intestinal vessels in Fli1:EGFP zebrafish (Vala et al., 2010). Moreover, our unpublished results show that low doses of IR accelerate vasculature formation and consequently the larval development without causing abnormal patterns (Figure 4.5).

![Figure 4.5](image_url)

**Figure 4.5 Low-dose IR accelerates zebrafish development.** Live Fli1:EGFP zebrafish larvae were exposed or not to 0.5 Gy at 3 dpf and photographed over-time. Representative images are presented. Scale bars, 1mm.

Since we have found that low doses of IR activate VEGFR-2, we decided to investigate whether VEGFR activation could be involved in the acceleration of larval development induced by low doses of IR. With this purpose, Fli1:EGFP zebrafish larvae were treated or not with the VEGF receptor tyrosine kinase inhibitor (PTK/ZK) and 30 min later exposed to 0.5 Gy, whole body IR. After irradiation, PTK/ZK was removed by washing and transferring the larvae to new medium. This process was repeated during three consecutive days. Larvae growth was assessed 33 dpf. Irradiated larvae treated with PTK/ZK showed earlier stages of vascular formation in comparison to larvae irradiated without PTK/ZK (Figure 4.6). According to this, we are now confirming these results by performing a similar experience with a larger population and four experimental groups: non-irradiated without PTK/ZK, non-irradiated with PTK/ZK, irradiated without PTK/ZK and irradiated with PTK/ZK.
Figure 4.6 Accelerated development of zebrafish larvae induced by low doses of IR is abolished by PTK. Live FlII:EGFP zebrafish larvae were exposed to 0.5 Gy at 3, 4 and 5 dpf, in presence or not of PTK/ZK, and photographed at 33 dpf. (A) Representative images of the vasculature at 33 dpf. Scale bars, 1 mm. (B) Development stage was established by quantification of caudal fin area, using ImageJ. Representative images of the most relevant phenotypes are showed next to the graph. Scale bars, 100 μm.
Low-dose IR and tumor development in a p53 mutated BRAF\textsuperscript{V600E} zebrafish model

We found that low-dose IR promotes acceleration of tumor growth in two different mouse models by enhancing angiogenesis. Here, we decided to study the effect of low doses of IR in melanoma development in a zebrafish model. According to the literature (Patton et al., 2005), p53 mutated BRAF\textsuperscript{V600E} zebrafish spontaneously develop melanoma after 5 months of life. Melanoma arises from the malignant transformation of melanocytes, the black pigmented cells carrying melanin. These transgenic zebrafish develop melanoma at the sites of melanocyte proliferation, namely as fish (f)-nevi. We irradiated or not the all body of these transgenic animals with 0.5 Gy, for 18 consecutive days, at approximately 5 months of age, before nevi appearance. Animals were followed over-time. Four months after the beginning of the experiment, 9 zebrafish with apparent melanoma and/or infection were euthanized for ethical reasons (4 non-irradiated and 5 irradiated). The experiment was ended 6 months post-irradiation, when a significant part of the population exhibited atypical nevi that resemble melanoma. Adult fish were sectioned at the f-nevi and sections were stained with hematoxylin and eosin. Our results suggest that some zebrafish did not present melanoma, but just a melanotic hyperpigmentation (Figure 4.7). Moreover, we found that low doses of IR accelerate the melanoma development (Figure 4.8).

![Figure 4.7](image-url) (A) Representative image of melanoma in p53 mutated BRAF\textsuperscript{V600E} adult zebrafish. (B) Representative image of a melanotic hyperpigmentation (100x) and (C) melanoma (40x) after hematoxylin and eosin stain.
Figure 4.8 Low-dose IR promotes the development of melanoma in p53 mutated \textit{BRAF}^{V600E} zebrafish. Five months-old zebrafish were irradiated (IR) or not (NIR) with 0.5 Gy for 18 days and followed over time. The percentage of animals that develop or not menaloma is presented. n=32 for non-irradiated animals and n=28 for irradiated ones.

It was reported that \textit{p53} mutated \textit{BRAF}^{V600E} zebrafish developed highly invasive melanomas (Patton \textit{et al}., 2005). According to our results we also observed that low doses of IR modulate tumor aggressiveness (Figure 4.9). By histopathology, we found that non-irradiated zebrafish exhibit smaller, melanocytic and/or exophytic\footnote{In oncology, proliferating on the exterior or surface epithelium of an organ or other structure in which the growth originated.} melanomas, when compared to irradiated animals, which present larger tumors with a highly invasive phenotype, infiltrating the deeper subcutaneous and muscular layers of the skin. In the future the mitotic index will be investigated.
Figure 4.9 Low-dose IR is associated with a more invasive melanoma. Non-irradiated zebrafish develop smaller, melanocytic melanomas (bottom) and/or exophytic (top). Irradiated animals show a highly aggressive phenotype, with larger, poorly demarcated and invasive tumors. Images were taken with an amplification of 40x.
V Discussion

Radiotherapy is a widely used local treatment for malignant tumors, characterized by uncontrolled growth and the ability to invade adjacent tissues and metastasize. However, clinical and experimental observations indicate that ionizing radiation (IR) might promote a metastatic behavior of cancer cells (O’Brien et al., 1986; Suit, 1992; Vicini et al., 2003). 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. It is generally assumed that tumor progression towards metastasis, during or after radiotherapy, is due to the appearance of resistant tumor cells. However, a growing number of evidence is now being found that support the role of microenvironment modification by IR (Barcellos-Hoff et al., 2005; Madani et al., 2008; Morgan, 2003; von Essen, 1991). Therefore it was demonstrated that IR exerts a pro-angiogenic function (Abdollahi et al., 2005; Garcia-Barros et al., 2003; Madani et al., 2008; McBride et al., 2004), which is supported by the benefits of anti-angiogenic approaches in tumor treatment (Gorski et al., 1999; Lee et al., 2000; Mauceri et al., 1998; Teicher et al., 1995). In the last years many studies have focused on the mechanisms by which IR activates the tumoral vasculature, stimulating angiogenesis, and in the contribution of the irradiated Endothelial Cells (ECs) in invasion and metastasis after radiotherapy. The doses of IR used in those studies correspond to the doses classically delivered inside the tumor target volume.

Recently, Vala et al. (Vala et al., 2010) studied for the first time the relevance of doses of IR, lower than those delivered inside the tumor mass, to the disease progression. That work focused, in an innovative way, in the vasculature that surrounds the tumor and receives relatively low doses of IR. Their results show that low-dose IR might disrupt the balance between survival and apoptosis through the activation of pro-survival signaling proteins and so induce angiogenesis. Given that tumor angiogenesis contributes not only to tumor growth, but also to metastasis by facilitating tumor cell entry into circulation (Raza et al., 2010; Zetter, 1998), it is of great importance take into account these new data.

In the present work, we have investigated the molecular targets that might be involved in the pro-angiogenic response induced by low-dose IR and its contribution to metastasis as well as physiological angiogenesis during development.

We began by validating our microarray data for several pro-angiogenic factors and found that, both in vitro and in vivo, low doses of IR up-regulate their mRNA expression. Moreover, we also demonstrated that their protein expression was increased in response to low-dose IR.
Our in vitro experimental evidence shows that low-dose IR activates VEGFR expressed in mature ECs inducing angiogenesis. However, we cannot exclude the possibility that in vivo low-dose IR might activate other cells. As we know, many angiogenic factors and cytokines are secreted by infiltrated macrophages and inflammatory cells (Fong, 2008; Kopfstein and Christofori, 2006; Ribatti and Vacca, 2008; Ruegg, 2006) and several angiogenic factors are up-regulated by inflammatory mediators, such as VEGF (Kuwano et al., 2001). Moreover, it is possible that IR might activate other cells that express VEGFR, such as bone marrow-derived hematopoietic progenitors (HPCs) that express VEGFR1 or endothelial progenitor cells (EPCs) that express VEGFR-2, contributing to neovascularization.

We also show that low doses of IR not only regulate angiogenic factors expression, but also protein subcellular localization. When irradiated with low doses, pro-migratory ECs present a higher density of focal adhesions at lamellipodium, which can explain why ECs exposed to low doses of IR have a faster wound healing (Vala et al., 2010). These data are in agreement with western blot analysis of insoluble extracts, where irradiated cells show increased levels of Rac1, paxillin and vinculin, in comparison with non-irradiated ECs.

A second point of interest in our study was the potential of low-dose IR in accelerating zebrafish growth. According to prior results, low doses of IR accelerate angiogenic sprouting during zebrafish development (Vala et al., 2010). Here we demonstrate that irradiated animals were in more advanced development stages relatively to non-irradiated ones, during the first weeks of life. Moreover, we show that this low-dose IR effect in accelerating larval development is abrogated by PTK/ZK. In the early stages of development, nutrients and oxygen supply is the most relevant limiting factor. Therefore, the capacity of low-dose IR to accelerate development is easily understandable taking into account its effect in the enhancement of angiogenesis.

Finally, we studied the effect of low doses of IR on melanoma development in a p53 mutated BRAF^{V600E} zebrafish model. Our data show that low-dose IR accelerates melanoma development, enhancing its invasive capacity. These results are according to Vala et al. data (Vala et al., 2010), where it was demonstrated that low doses of IR promote tumor growth and metastasis. Considering the pro-angiogenic effect of low-dose IR and the vasculature’s role in tumor progression, we may hypothesize that irradiation facilitates tumor invasion by establishing new routes for tumor cells escape.

Overall, our results show that low doses of IR, present in the vicinity of the tumor target volume, enhance angiogenesis by increasing the expression of several proteins required for
neovascularization. Moreover, our results suggest that low doses of IR promote tumor progression and invasion. We believe that the data generated in this work will reveal new mechanisms to understand the pro-metastatic effects of IR and will provide a new rationale basis to the improvement of current radiotherapy protocols. In the future, we aim to validate the obtained results using material from patients with rectal cancer that received preoperative radiotherapy. Tissue samples from the distal area (irradiated with low doses), tumor area (irradiated with the therapeutic dose) and the proximal area (not irradiated) will be collected and analyzed.

We also demonstrate that low-dose IR induces physiologic angiogenesis during zebrafish development. Therefore, the present study may have repercussions not only in medicine, but also in other industries such as, hypothetically, aquaculture.
Bibliography


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Appendix

Attach 1. Principal component analysis (PCA) of irradiated and non-irradiated 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 non-irradiated populations, while the second principal component shows the separation between the experimental replicates. The red (non-irradiated, 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.
Attach 2. 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.