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**The effect of irradiation on the Eph family of receptor tyrosine  
kinases in human lung adenocarcinoma**



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*To my family*  
*For all their kindness, support and*  
*sacrifices*

## Table of Contents

<b>Figure Index</b> .....	7
<b>Table Index</b> .....	9
<b>Abbreviations</b> .....	10
<b>PART I: REVIEW OF THE LITERATURE</b>	
<b>1. INTRODUCTION</b> .....	12
<b>2. ANGIOGENESIS</b> .....	14
2.1.1 Normal and Pathological Angiogenesis .....	14
2.1.2 Receptor Tyrosine Kinases and Growth factors in Angiogenesis.....	19
2.1.3 Angiogenesis and Lung Cancer.....	22
<b>3. THE EPH FAMILY OF RECEPTOR TYROSINE KINASES</b> .....	25
3.1.1. Structure of Eph receptors and their Ligands ephrins.....	25
3.1.2 The role of Eph and ephrins in Angiogenesis.....	28
<b>PART II: EXPERIMENTAL</b>	
<b>4.1 MATERIALS</b> .....	33
4.1.1 A549 and HUVEC cell culture Reagents.....	33
4.1.2 A549 and HUVEC cell culture Instruments and Equipment.....	33
4.1.3 Experimental Mice.....	34
4.1.4 In-vivo Laboratory Instruments and Software.....	34
4.1.5 Immunofluorescence and Immunohistochemistry Reagents.....	34
4.1.6 Immunofluorescence Processing and Image Acquisition.....	35
4.1.7 Metabolic Activity Assay Materials.....	35
4.1.8 RNA Isolation, cDNA Synthesis and Real-Time RT-PCR Materials.....	35
4.1.9 Electronic Equipment, Instruments and Software.....	35
4.1.10 Endothelial Cell Migration Assay Equipment.....	36
4.1.11 Buffers, Kits and Solutions.....	36
<b>4.2 METHODS</b> .....	37
4.2.1 A549 and HUVEC Cell Culture and Irradiation.....	37

4.2.2 RNA-extraction and cDNA synthesis.....	38
4.2.3 Real-time reverse transcriptase polymerase chain reaction (RT-PCR).....	38
4.2.4 Animals and Tumor treatment.....	38
4.2.5 Proliferation Assay.....	40
4.2.6 Immunofluorescence .....	40
4.2.7 Immunohistochemistry.....	41
4.2.8 Evaluation of EphA2 and ephrinA1 Immunohistochemical Expression.....	41
4.2.9 Matrigel Endothelial Cell Migration Assay.....	41
4.2.10 Statistical Analysis.....	42
<b>5. RESULTS.....</b>	<b>43</b>
5.1 Radiation induces activation of EphA2-ephrin A1 but not EphB4-ephrinB2.....	43
5.2 Irradiation of ECs <i>in vitro</i> does not affect transcriptional status of EphA2/ephrinA1 or EphB4/ephrinB2.....	45
5.3 Increased Immunofluorescent Expression of EphA2 in irradiated A549 cells <i>in vitro</i> .....	45
5.4 Increased Expression of EphA2 in irradiated A549 xenografts.....	47
5.5 Immunohistochemical Expression of EphA2 and ephrinA1 in irradiated A549 xenografts.....	49
5.6 EphA2 blockade does not alter cell viability of Irradiated A549 cells.....	51
5.7 EphA2-blockade significantly reduces migration of ECs induced by Irradiation of A549 cells.....	53
5.8 Unirradiated A549 cells can also induce EphA2-mediated Migration of ECs but in a less potent manner.....	53
5.9. Model of EphA2-mediated Interaction between Tumor and Vascular Compartment in response to IR.....	55
<b>6. DISCUSSION.....</b>	<b>56</b>
<b>7. SUMMARY.....</b>	<b>65</b>
<b>8. ZUSAMMENFASSUNG.....</b>	<b>67</b>
<b>9. REFERENCES.....</b>	<b>69</b>
<b>10. APPENDIX.....</b>	<b>78</b>
<b>10.1 ACADEMIC TEACHERS.....</b>	<b>78</b>

<b>10.2 DECLARATION .....</b>	<b>79</b>
<b>10.3 ACKNOWLEDGEMENT.....</b>	<b>80</b>

## Figure Index

Figure 1. Angiogenesis depends on the balance between pro-angiogenic and antiangiogenic factors.

Figure 2. Angiogenic and vasculogenic process are regulated by different mediators and growth factors playing an important role in blood vessel formation and maturation.

Figure 3. In contrast to normal blood vessels (left photo), tumor vasculature is characterised by abnormal, irregularly shaped, tortuous, leaky blood vessels with dead ends, sharing chaotic features (right photo).

Figure 4. A) model of avascular tumor initiation, B) model of tumor initiation involving host co-option.

Figure 5. The multistep process of angiogenesis in lung cancer.

Figure 6. Mechanism of action of Erlotinib and Bevacizumab on tumor and related endothelial cells.

Figure 7. The structure of Eph receptor and its ligand ephrin.

Figure 8. Steps in cell contact-dependent bidirectional signalling and mechanisms of Eph/ephrin-mediated cell adhesion and repulsion. The net effect of interaction between Eph receptors and their ligands Ephrins depends on the degree of clustering.

Figure 9. Model of ephrinB2-EphB4 signals in vasculoangiogenesis.

Figure 10. A 25 MV Elekta SL-25 linear accelerator (Elekta Oncology Systems, Norcross, GA).

Figure 11. Pathogen-free female Balb/c mice (6-8 weeks old).

Figure 12. Induction of EphA2 receptor and its ligand EphrinA1 in irradiated lung adenocarcinoma cells.

Figure 13. Effect of irradiation on EphB4/ephrinB2 mRNA expression in ECs.

Figure 14. The immunofluorescent expression of EphA2 receptor tyrosine kinase in A549 in vitro.

Figure 15. Immunofluorescence detection of EphA2 upon A549 irradiation in vivo.

Figure 16. Immunohistochemical expression of EphA2 and ephrinA1 in irradiated A549

xenografts.

Figure 17. Effect of EphA2 blockade on irradiated lung adenocarcinoma cell viability.

Figure 18. EphA2-blockade significantly reduces migration of ECs induced by IR

Figure 19. A suggested model for the function of EphA2 involved in the IR-induced interaction between tumor und microenvironment.

## **Table Index**

Table 1. Pathogenic angiogenesis represents a key process for several different diseases.

Table 2. Pro-angiogenic oncogenes and their effect on tumor angiogenesis.

Table 3. Summary of RTKs and their functional role in angiogenetic process.

Table 4. Molecules regulating interaction/communication between EC-EC and EC-ECM.

Table 5. Primers used for Real-Time RT-PCR.

.

## Abbreviations

AFB	Autofluorescence bronchoscopy
Ang	Angiopoietin
bFGF	basic Fibroblast growth factor
CEA	Carcinoembryonic Antigen
CT	Computer Tomography
DAPI	4', 6'-Diamidino-2-phenylindole
DLL4	Delta-like 4 receptor
DNA	Deoxyribonucleotide
ECs	Endothelial Cells
ECM	Extracellular matrix
EGFR	Epidermal Growth Factor Receptor
Eph	Erythropoietin producing hepatoblastoma
FCS	Fetal Calf Serum
FDG	Fluorodeoxyglucose
Flt-1	FMS-related tyrosine kinase 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEFs	Guanine Nucleotide Exchange Factors
GPI	Glycosylphosphatidylinositol
Gy	Gray
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HRP	Horseradish-conjugated
HSPG	Heparin Sulphate Proteoglycans
HUVECs	Human Umbilical Venous Endothelial Cells
IR	Ionizing Radiation
KDR	Kinase insert Domain-containing Receptor
MMP	Matrix Metalloproteinases
mRNA	Ribonucleic Acid
MVD	Microvascular density

NO	Nitric oxide
NSCLC	Non Small Cell Lung Cancer
PBS	Phosphate-Buffered Saline
PC	Pericytes
PDGF	Platelet-Derived Growth Factor
PDZ	Postsynaptic Density Zonula occludens domain
PI3K	Phosphatidyl Inositol 3 Kinase
PLGF	Placental Growth Factor
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse Transcriptase Polymerase Chain
RTKs	Receptor Tyrosine Kinases
SMC	Smooth muscle cells
TGF-alpha	Transforming Growth Factor-alpha
TSP-1	Thrombospondin-1
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

## **1. INTRODUCTION**

The most common cause of cancer-related mortality is lung cancer, causing more deaths than any other malignancy. It is estimated that around 75% of lung cancer cases are non-small cell lung cancer (NSCLC) and as many as 40% of all NSCLC patients present with locally advanced and/or unresectable disease (Jemal et al., 2006). Over the last two decades, several approaches to multimodality therapy have been investigated in patients with NSCLC (Le Chevalier et al., 2004). Postoperative chemotherapy, alone or in combination with radiation therapy, is considered the treatment of choice (Wagnern et al., 2005, Gandara et al., 2000). Adjuvant radiotherapy has been shown to improve local control of resected advanced NSCLC. Unfortunately, the latter has failed to translate in an improvement in patient survival due to the frequent recurrence and metastases appearing even after aggressive treatment schedules (Bogart et al., 2005, Taylor et al., 2003, Erman et al., 2004).

Tumor cells are greatly dependent on a proper, sufficient blood supply in order to survive and grow further (Carmeliet et al., 2000). In recent years an intense effort has been made in identifying new molecular markers with a role in tumor angiogenetic process. Eph proteins are the largest family of receptor tyrosine kinases (RTKs) consisting of 16 Eph receptors and 9 Ephrin ligands. Eph receptors are divided into class A, which bind to their corresponding GPI-linked class A ephrin ligands, and class B which bind to their transmembrane domain-anchored class B ephrin ligands (Poliakov et al., 2003). In contrast to other RTKs where the ligand functions as soluble molecule, Eph and ephrins are unique in that their ligands are tethered to the cell membrane and therefore interaction necessitates direct cell to cell contact (Pasquale et al., 2005). Moreover, these RTKs have the ability to induce both forward and reverse (bi-directional) signalling between adjacent interacting cells and Eph receptor signals of different intensities can produce opposite effects (Holland et al., 1996). Although initially recognised as modulators of embryonic development and neural axon guidance (Wilkinson et al., 2001, Davis et al., 1994), members of Eph family of RTKs are involved in tumor angiogenesis and vascular remodelling (Heroult et al., 2006, Erber et al., 2006, Hainaud et al., 2006, Ogawa et al., 2000) as well as invasion (Nakada et al., 2006) and metastatic potential of tumor cells (Brantely-Sieders et al., 2005). Additionally, increased expression of Eph family has been already described in several different tumor types,

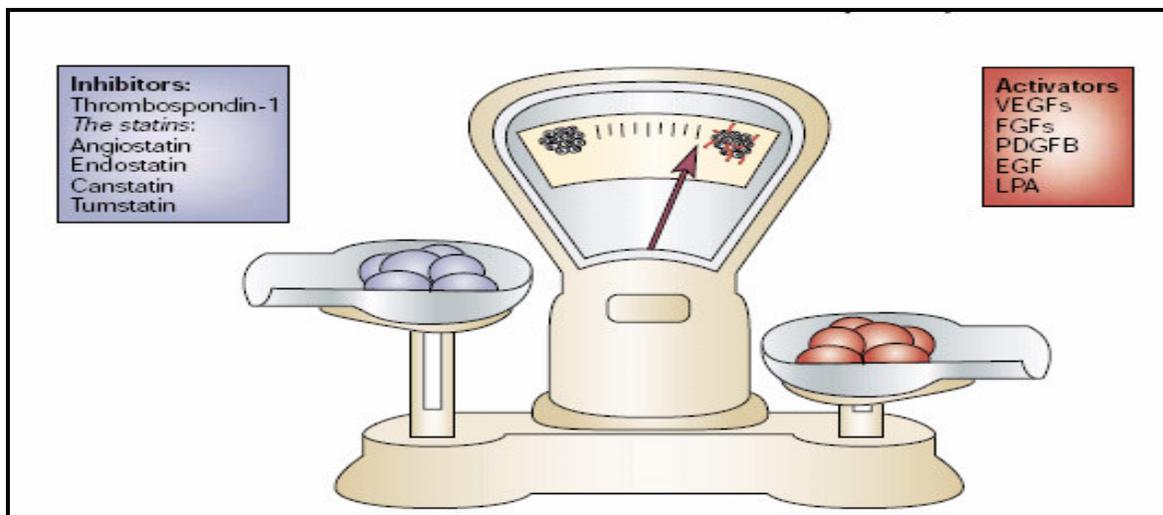
including lung cancer, both in vitro and in vivo (Abraham S et al., 2006, Kinch et al., 2003, Wykosky et al., 2005, Duxbury et al., 2004, Lugli et al., 2005).

The response of tumor to ionizing radiation (IR) is highly dependent on its blood supply and involves a series of complex biological interactions between tumor cells and vasculature as well as tumor stroma itself (Wachsberger et al., 2003). However, the effect of IR on the biggest family of RTKs, the Eph receptors and their ligands, remains still unknown. In the present study, the possible role of different members of the Eph family of RTKs in IR-induced tumor angiogenesis was investigated.

## 2. ANGIOGENESIS

### 2.1.1 Normal and pathological angiogenesis

The angiogenic process is a complex phenomenon characterised by the formation of new blood vessels that begins with an angiogenic stimulus followed by local degradation of the basement membrane surrounding the capillaries. The proliferation of cells at the edge of the migrating column occurs where endothelial cells migrate with formation of new capillary tubules occurs involving organization of endothelial cells three-dimensionally (Carmeliet et al., 2000).

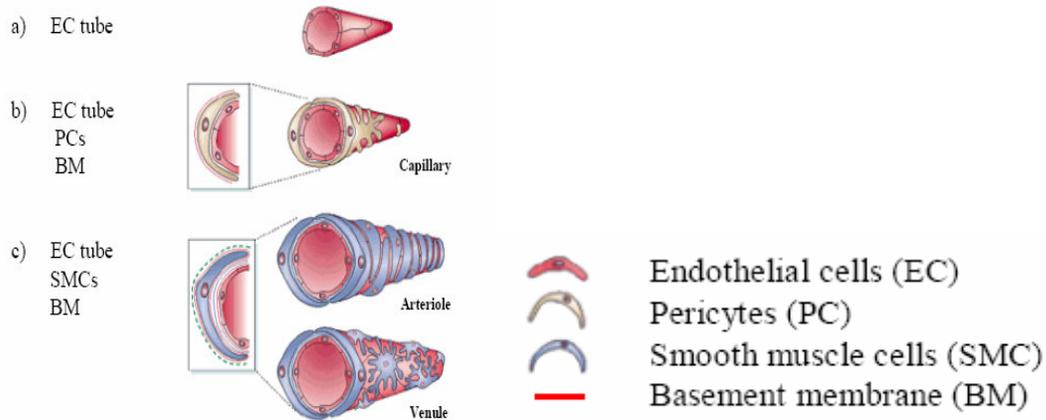
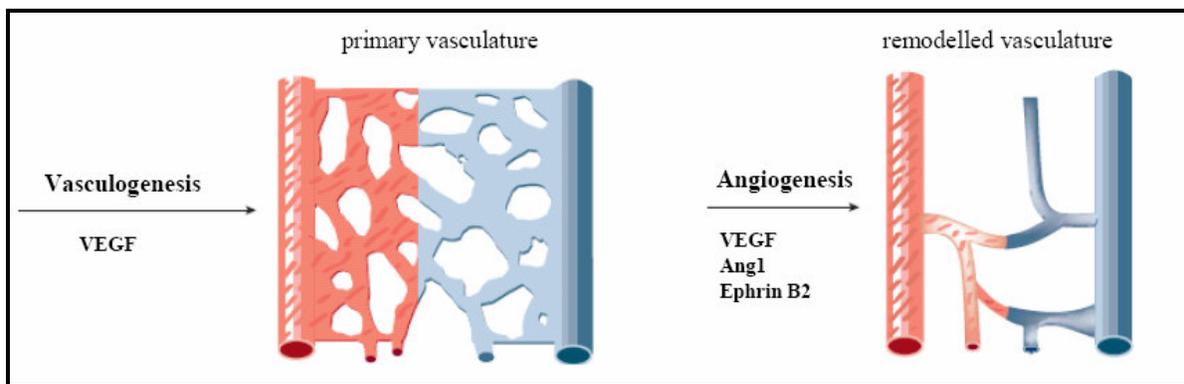


**Figure 1. Angiogenesis depends on the balance between pro-angiogenic and antiangiogenic factors (Berger et al., 2003).**

The interplay between pro-angiogenic and anti-angiogenic factors determines here the survival of endothelial cells (Bergers et al., 2003). The organ growth and repair is highly dependent on the angiogenic process. The metabolic processes and survival of normal as well as tumor cells need essential nutrients and oxygen with the latter found within the diffusion distance of blood vessels (100-180mm). Organ formation and embryonic development need oxygen as well as various growth factors secreted and released abundantly from blood vessels (shown in Figure 2). In the very first steps of embryonic angiogenesis,

blood vessels are formed by vasculogenesis as well as angiogenesis. While the first describes the process of de novo vessel formation from angioblasts or stem cells, the later collectively refers to the remodelling of the initial vessel network (Carmeliet, 2000).

The progressive expansion of vascular network begins with sprouting and remodelling of blood vessels into a highly organised architectural plexus of larger vessels constructed with ramification of smaller microvessel that ramify into smaller ones. This is followed by covering with pericytes (PC) and smooth muscle cells (SMC) of endothelial cells (EC) (Bergers et al., 2003, Carmeliet, 2000) and ensures proper perfusion and strengthening of vessels, a phenomenon known as arteriogenesis (Figure 2).



**Figure 2. Angiogenic and vasculogenic process-regulated by different mediators and growth factor, it mediates blood vessel formation and maturation (Bergers et al., 2003, Carmeliet et al., 2000).**

Cell-matrix receptors such as the  $\alpha$ v $\beta$ 3 and  $\alpha$ 5 integrins mediate cell spreading and migration. Maturation of nascent vessels involves formation of a new basement membrane and investment of new vessels with pericytes and smooth muscle cells. PDGF-BB recruits smooth muscle cells, whereas signalling by TGF- $\beta$ 1 and Ang1/Tie2 stabilizes the interaction between endothelial and smooth muscle cells. Proteinase inhibitors (for example, PAI-1) prevent degradation of the provisional extracellular matrix around nascent vessels. Maintenance of new vessels depends on the survival of endothelial cells. In a normal adult, quiescent endothelial cells can survive for several years. VEGF (through an interaction with VE-cadherin70) and Ang1 are vital survival factors (Bergers et al., 2003, Carmeliet, 2003). In contrast, most angiogenesis inhibitors cause endothelial apoptosis. By binding VEGF, soluble VEGF receptors (for example, VEGFR-1, neuropilin-1) reduce the angiogenic activity of VEGF. Molecules that initially induce angiogenesis are subsequently (proteolytically) processed to angiogenesis inhibitors, thereby providing a negative feedback. Most angiogenesis inhibitors suppress tumour angiogenesis; their role in normal vascular growth remains largely unknown (Yancopoulos et al., 2000).

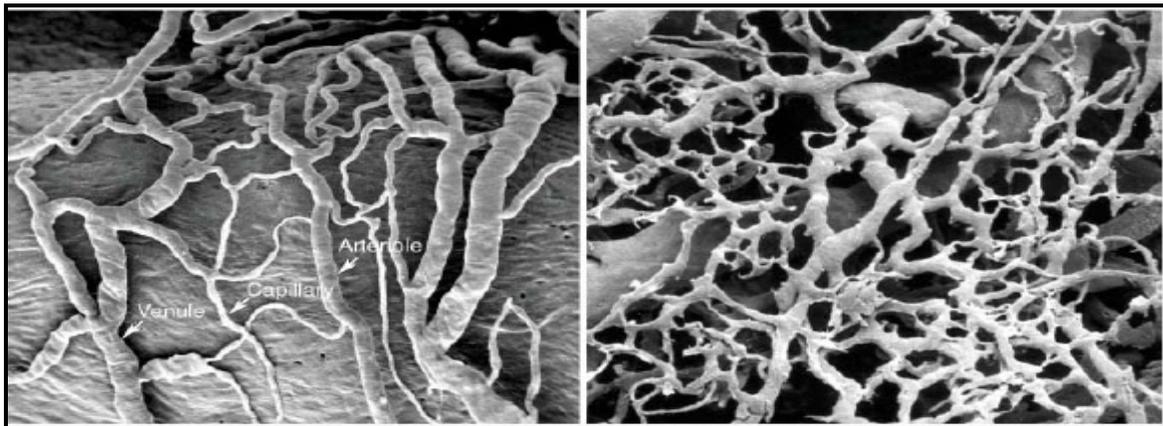
The phenomenon of hypoxia greatly affects the vascular compartment. The reduced oxygen tension stimulates various homeostatic mechanisms that aim to restore oxygen status. The progressive enlargement of tumors results in progressively increasing distances of cells from their vasculature, worsening the already existing deprivation of tumor cells from oxygen and nutrients. The HIF-1 transitionally controls various genes that participate in the formation of new blood vessels such as vascular endothelial growth factor (VEGF), angiopoietin 1 (ANGPT1) and ANGPT2, placental growth factor (PLGF), and platelet-derived growth factor B (PDGFB) and their various receptors, and genes involved in matrix metabolism, including matrix metalloproteinases, plasminogen activator receptors and inhibitors, and procollagen prolyl hydroxylase (Pouyssegur et al., 2006).

<p><b>Blood vessels:</b> atherosclerosis, haemangioma, haemangioendothelioma</p>	<p><b>Bone, joints:</b> rheumatoid arthritis, synovitis, bone and cartilage destruction, vascular malformations osteomyelitis,</p>
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	<p>pannus growth, osteophyte formation, cancer</p> <p>aseptic necrosis, impaired healing of fractures</p>
<p><b>Skin:</b> warts, pyogenic granulomas, hair growth, Kaposi's sarcoma, psoriasis (skin vessels enlarge and become tortuous) epithelia liver regeneration, cancer decubitus or stasis ulcers, gastrointestinal ulcers</p>	<p><b>Liver, kidney, lung:</b> inflammatory and infectious processes (hepatitis, pneumonia, scar keloids, allergic oedema, neoplasms ear and other glomerulonephritis), pulmonary hypertension, diabetes, systemic hypertension (vascular pruning) asthma, nasal polyps, transplantation,</p>
<p><b>Uterus, ovary:</b> dysfunctional uterine bleeding (contraception), follicular cysts, retinopathy of prematurity, diabetic retinopathy, choroidal and</p> <p><b>Placenta:</b> ovarian hyperstimulation, endometriosis, pre-eclampsia, placental insufficiency</p>	<p><b>Brain, nerves, eye:</b> stroke, vascular dementia, Alzheimer's disease, CADASIL Neoplasms, other intraocular disorders, leukomalacia, cancer</p>
<p><b>Peritoneum, pleura:</b> respiratory distress, ascites, peritoneal sclerosis (dialysis, pancreas transplantation patients), adhesion formation (abdominal surgery), metastatic organ spreading</p>	<p><b>Thyroid:</b> pseudocyst, thyroiditis, thyroid enlargement</p>
<p><b>Heart, skeletal:</b> work overload muscle, ischaemic heart and limb disease</p>	<p><b>Lymph vessels:</b> tumor metastasis, lymphoproliferative disorders, lymphoedema</p>
<p><b>Adipose tissue:</b> obesity</p>	<p><b>Haematopoiesis:</b> AIDS (Kaposi), haematologic malignancies</p>

**Table 1. Pathogenic angiogenesis represents a key process for several different diseases** (Bergers et al., 2003, Yancopoulos et al., 2000).

The architecture of tumor blood vessels is totally different from those of normal tissues. Tumors consist of irregularly shaped, dilated, tortuous blood vessels that can have dead ends. While normal vasculature is organised into arterioles, venules and capillaries, malignant ones do not and they are often chaotic. Greatly due to the overproduction of various growth factors (e.g. vascular-permeability factor), tumor blood vessels are leaky and haemorrhagic, with their walls having high permeability (Jain et al., 2002). Under normal conditions, the maturation and stabilization of new vessels occurs rapidly. In contrast, tumors have being characterised as “wounds that never heal” having irregular, slow and sometimes oscillating blood flow. This leads to dysfunctional capillaries (Figure 3). Tumours can be quite heterogeneous in their vascular patterns, and are able to overproduce their capillary networks (Rak et al., 2004).



**Figure 3. Tumor blood vessels:** In contrast to normal blood vessels [left], tumor vasculature is characterised by abnormal, irregularly shaped, tortuous, leaky blood vessels with dead ends, sharing chaotic features [right] (Carmeliet et al, 2001).

Oncogenes	Pro-Angiogenic Activity
kRAs, hRAS	VEGF↑ , TSP1↓
Src	VEGF↑ , TSP1↓
Bcl-2	VEGF↑
EGFR	VEGF↑, bFGF↑, IL-8↑
FOS	VEGF↑

*Table 2. Pro-angiogenic oncogenes and their effect on tumor angiogenesis (Rak et al., 2004).*

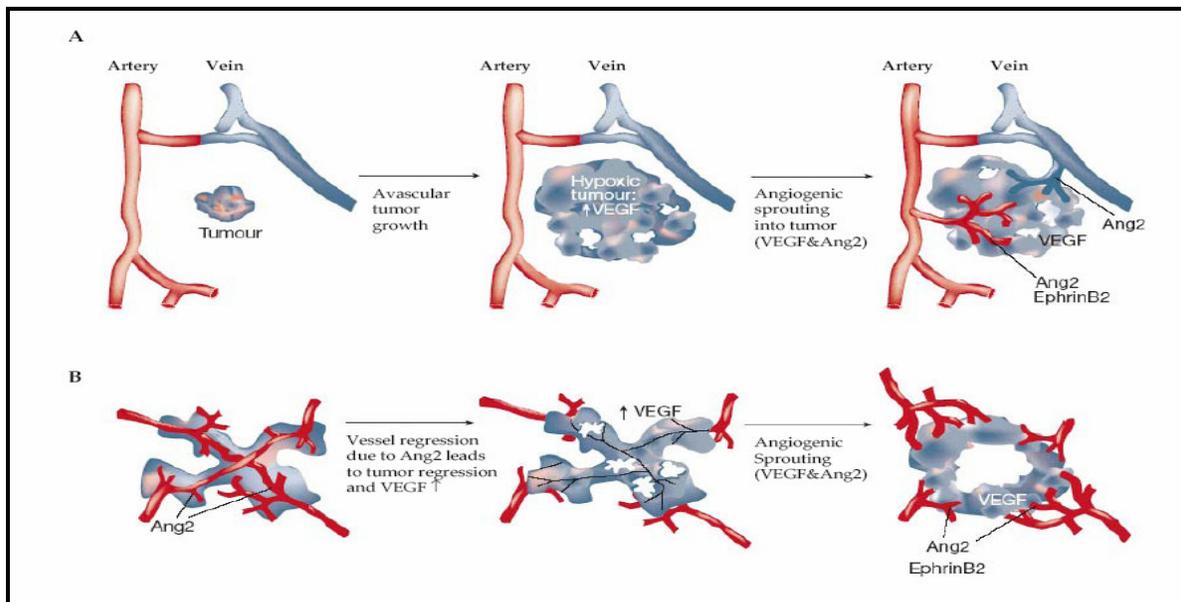
### 2.1.2 Receptor Tyrosine Kinases and Growth factors in Angiogenesis

Receptor tyrosine kinases (RTKs) are involved in signal transduction, and process a variety of environmental and intercellular cues. As central components of cell signalling networks, RTKs play crucial roles in physiological processes, such as embryogenesis, differentiation, neurite outgrowth, cell proliferation, anti-apoptotic and angiogenesis. RTKs are anchored in the plasma membrane at the transmembrane domain, while the extracellular domains bind growth factors. Activation of the kinase is effected by binding of a ligand to the extracellular domain, which induces dimerization of the receptors. Activated receptors autophosphorylate tyrosine residues outside the catalytic domain via cross-phosphorylation. The latter stabilizes the active receptor conformation and creates phosphotyrosine docking sites for proteins that transduce signals within the cell (Schlessinger et al., 2000).

The VEGF/VPF (vascular endothelial growth factor/vascular permeability factor) ligands and receptors are crucial regulators of vasculogenesis, angiogenesis, lymphangiogenesis and vascular permeability in vertebrates (Olsson et al., 2006). VEGF-A, the prototype VEGF ligand, binds and activates two tyrosine kinase receptors: VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1). VEGFR1, which occurs in transmembrane and soluble forms, negatively regulates vasculogenesis and angiogenesis during early embryogenesis, but it also acts as a

positive regulator of angiogenesis and inflammatory responses, playing a role in several human diseases such as rheumatoid arthritis and cancer (Table 1). VEGFR2 has critical functions in physiological and pathological angiogenesis through distinct signal transduction pathways regulating proliferation and migration of endothelial cells (Ferrara et al., 2003). VEGFR3, a receptor for the lymphatic growth factors VEGF-C and VEGF-D, but not for VEGF-A, regulates vascular and lymphatic endothelial cell function during embryogenesis. Loss-of-function variants of VEGFR3 have been identified in lymphedema. Formation of tumor lymphatics may be stimulated by tumor-produced VEGF-C, allowing increased spread of tumor metastases through the lymphatics. Mapping the signalling system of these important receptors may provide the knowledge necessary to suppress specific signaling pathways in major human diseases (Alitalo et al., 2005).

The second important family of RTKs in angiogenesis are the angiopoietins (Ang), the ligands for Tie receptors. To date, there are four known angiopoietin ligands that primarily bind to Tie2 receptor. The two most known ligands, Ang1 and Ang2, are regulated via Tie2 and end up in vessel stabilization or degradation. Pericytes and vascular SMC produce Ang-1 which is responsible for vessel maturation and wall stabilization. In contrast, only EC express Ang-2 and has exactly the opposite effects from Ang-1, inducing destabilization of blood vessels during angiogenetic onset. (Thurston et al., 2003).



**Figure 4. A) model of avascular tumor initiation, B) model of tumor initiation involving host co-option (Pouyssegur et al., 2006).**

FGFs are a family of heparin-binding growth factors. Through their interaction with different RTKs, integrins and heparin sulphate proteoglycans (HSPG) promote angiogenesis. FGFs are regulated by various proteins, either free or bound to extracellular matrix (ECM). Such a mechanism includes the cross-talk between VEGF/VEGFR (Presta et al., 2005).

The recruitment of PCs to newly formed vessels is regulated by PDGF-B and PDGFR- $\beta$  RTKs. Endothelial cells secrete PDGF-B during vessel sprouting of angiogenic sprouts. Binding of PDGF-B to HSPG is crucial for the proper localization of the ligand in the vicinity of developing vessels. PCs which express PDGFR- $\beta$  are stimulated by PDGF-B and proliferate and migrate towards the tip.

RTKs	Effect on Angiogenetic Process
VEGF/VEGF-R1 u. 2/	Blood wall permeability $\uparrow$ , Building of a provisional Matrix Release of proteolytic enzymes such as MMPs Endothelial cell proliferation $\uparrow$ and migration $\uparrow$ PDGF- $\beta$ $\uparrow$ for strengthening of blood vessel structure, Preventing EC's apoptosis
Ang-1/Tie-2/	Regulation of interactions between EC-Pericytes and ECs and ECM for stabilization of blood vessel wall Prevention of EC apoptosis Stimulation of blood vessel branching & ramification

Ang-2/Tie-2/ (EC)	Intercellular contacts↓ Enable migration of ECs in the absence of angiogenic factors, blood vessel regression↑
PDGF-β/PDGF-Rβ/ ↑	EC Proliferation↑, Migration↑, Recruitment of pericytes
TGF-β1/ TGF-βRII/	Production of ECM and Proteases↑ Differentiation of fibroblasts to pericytes

**Table 3. Summary of RTKs and their functional role in angiogenic process**

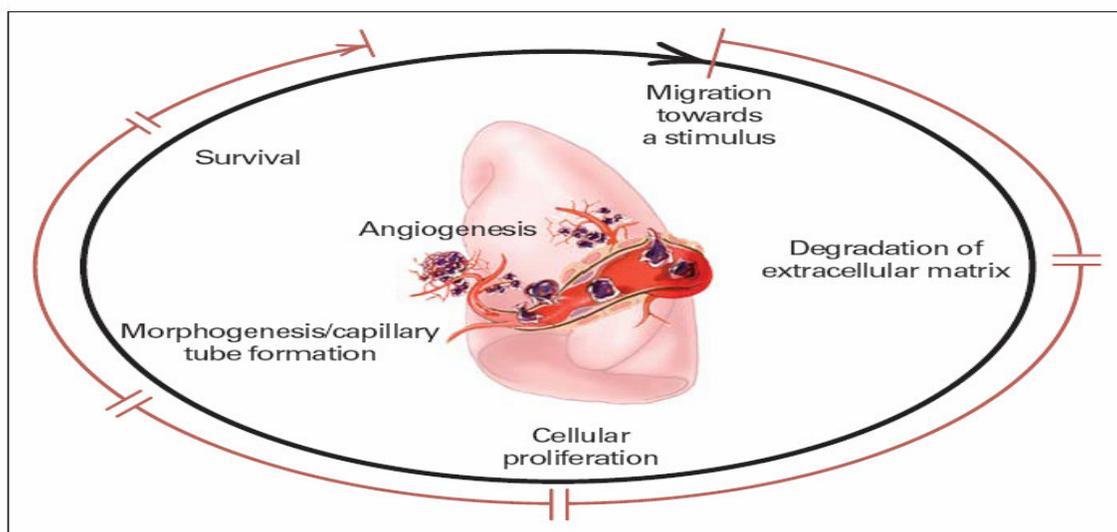
<b>Molecules</b>	<b>Effect on Angiogenetic Process</b>
VE-Cadherin	Mediates EC-EC interaction and binding
N-Cadherin	Mediates communication between EC and Pericytes
Integrins	Mediates EC-ECM binding Regulates apoptosis of ECs Role in EC migration and invasion
MMPs	Promotes EC migration and invasion through degradation of ECM
Protease Inhibitors (TIMP, PAI)	Stabilization of new blood vessel wall

**Table 4. Molecules regulating interaction/communication between EC-EC and EC-ECM** (Armulik et al., 2005, Cavallaro et al., 2003, Jeffrey et al., 2002).

### **2.1.3 Angiogenesis and Lung Cancer**

The word angiogenesis has dominated the scientific interest during the last decade in an intense effort to discover novel therapeutic targets and increase patient survival. The role of new blood vessel formation has also been studied in lung cancer. It has been found that microvessel density (MVD) in tumor samples from patients with lung cancer is closely

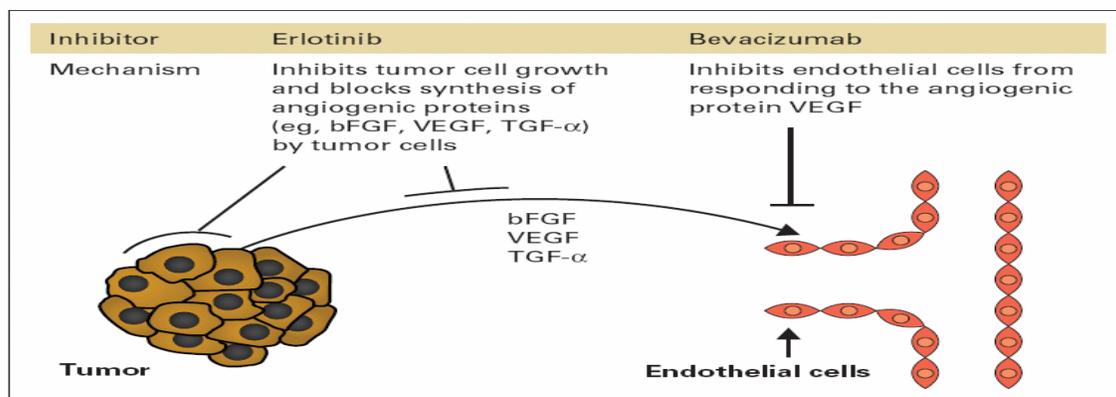
associated to disease progression and can be used as prognostic factor for metastasis and short survival (Meert et al., 2002). Overexpression of VEGF has been demonstrated in lung cancer and correlated as an independent prognostic factor with a poor patient prognosis (independent prognostic factor) (Fontanini et al., 1997). An interesting study has shown that in some cases NSCLC can present with a non-angiogenic phenotype. This study detected cases where tumors were actually filling pulmonary alveoli and utilized the vasculature of the trapped alveolar septa in order to ensure its blood supply (O'Byrne et al., 2000). Many other factors with an already proven role in the angiogenic process have been identified in lung cancer and can have be of predictive value. Some of these include bFGF, PDGF, HIF-1 and HIF-2. Herbst et al. have elegantly described the complex nature of angiogenesis in lung cancer. (Figure 5).



**Figure 5. The multistep process of angiogenesis in lung cancer (Herbst et al., 2005).**

VEGF and its receptor VEGFR play key role in promoting lung cancer growth and spread, not only by mediating tumor angiogenesis but also by promoting development of resistance to standard therapies. Recently, anti-angiogenic agents such as the monoclonal antibody Bevacizumab, have been studied and tried in clinical trials (O'Byrne et al., 2000). The addition of bevacizumab to paclitaxel-carboplatin regimen in selected patients with NSCLC had a

significant survival benefit with the risk of increased treatment-related deaths (Jain et al., 2006). In combination with paclitaxel-carboplatin regimen, treatment with bevacizumab provides significant survival benefit in selected NSCLC patients with risk of increased treatment-related deaths (Figure 6).



**Figure 6. Mechanism of action of Erlotinib and Bevacizumab on tumor and related endothelial cells.** bFGF, basic Fibroblast Growth Factor; VEGF, Vacular Endothelial Growth Factor; TGF- $\alpha$ , Transforming Growth Factor- $\alpha$  (Herbst et al., 2005).

The application of anti-angiogenic and vascular disrupting agents in combination with other medications has definitely scientific base. Tumor cells may produce and secrete/activate dozens of proangiogenic factors, suggesting that the combined action of more than one inhibitors can have more killing effects on tumor cells. Additionally, commonly used chemotherapeutic agents have been shown to possess anti-angiogenic effect when administered under the appropriate regimen (metronomic chemotherapy) (Kerbel et al., 2004). A matter of intense debate was the hypothesis that anti-angiogenic agents may induce regression of blood vessels, impeding in this way the proper delivery of chemotherapy to provide a reasonable explanation regarding the beneficial effect of antiangiogenic drug used together with chemotherapy (Jain et al., 2005). This “normalization” of blood vessel wall is caused by the ability of anti-angiogenic agents act to prune and normalize the tumor vascular supply, which is typically aberrant in tumors.

### **3. THE EPH FAMILY OF RECEPTOR TYROSINE KINASES**

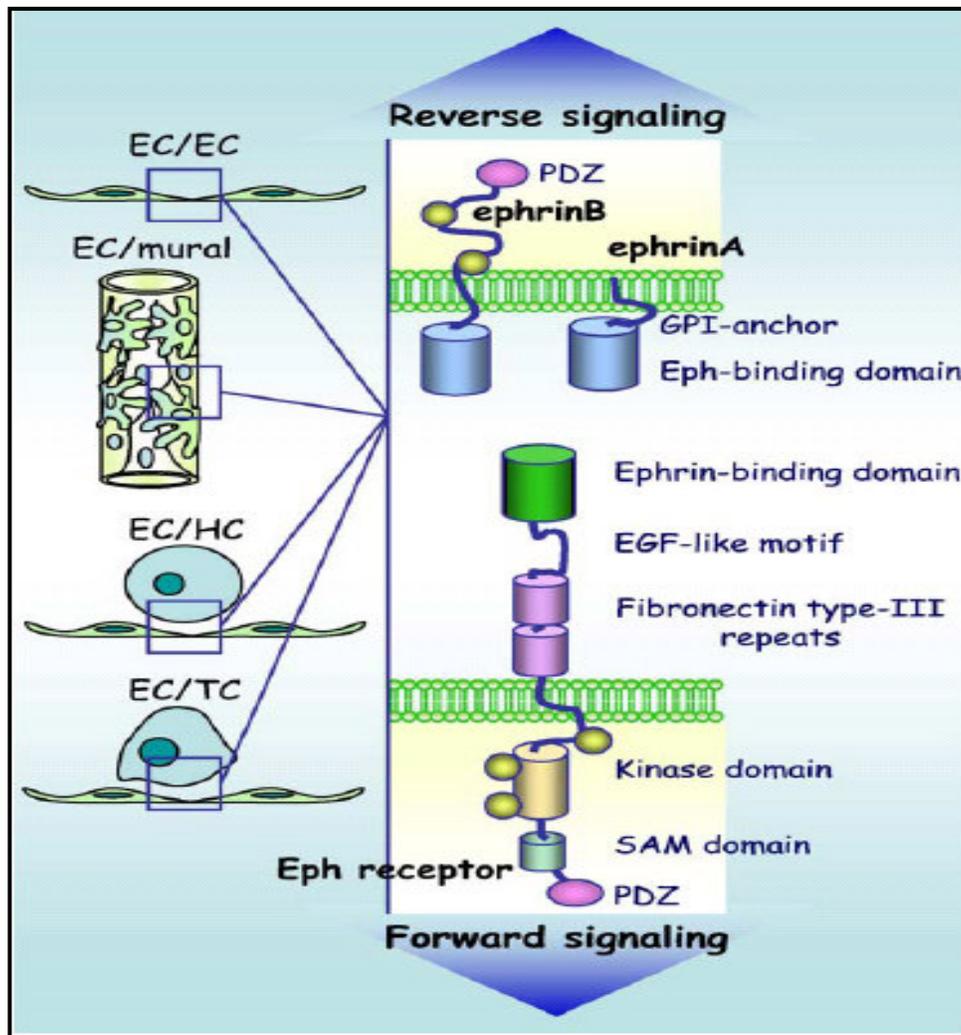
#### **3.1.1. Structure of Eph receptors and their ligands ephrins**

The Eph receptors consist the largest family of RTKs and were identified in the late 1980s in a human cDNA library screen for homologous sequences to the tyrosine kinase domain of the viral oncogene v-fps. It shares 65-90% sequence homology in the kinase domain and 30-70% in the extracellular domain. The Eph receptors mainly affect the dynamics of cellular protrusions and cell migration by modifying cytoskeletal organization and cell adhesion. Recent data indicate that receptors also influence cell proliferation and cell-fate determination (Wilkinson et al., 2001).

To date, totally ten EphA and six EphB receptors have been found: EphA1-EphA10 and EphB1-EphB6. The structure of Eph receptors is almost similar to other RTKS. They are composed of an extracellular globular domain responsible for ligand binding, a cysteine-rich region, two fibronectin type III repeats, a region spanning the cell membrane, and a tyrosine kinase domain (Davis et al., 1994). A sterile alpha motif and PDZ compose the carboxyl terminal. The PDZ domain is also known as the Post-synaptic Density-95/discs large/Zonula occludens-1 domain, It is involved in receptor clustering and organization of the receptors and acts through binding of selected proteins (C-terminal) at their carboxyl end. This protein binding is able to induce changes in PDZ structure inducing clustering of receptors upon ligand binding (Poliakov et al., 2004).

Similarly to the Eph receptors, there are 2 classes of ephrin ligands, class A and class B. While ephrinA ligands are glycosylphosphatidylinositol (GPI) anchored and bind to EphA receptors, ephrinB ligands contain a transmembranous domain and a short cytoplasmic region and bind to ephrinB ligands as it is shown in Figure 7 (Davis et al., 1994, Poliakov et al., 2004, Heroult et al., 2006).

The interaction between Eph receptors and an ephrin ligand is the first step in the formation of receptor-ligand signalling cluster and this interaction occurs on juxtaposed cell surfaces. Apart from this high affinity interface, there is also a low affinity interface on the opposite side of ephrin binding domain. Additionally, there are two binding interfaces in the ephrin ligand itself. The transphosphorylation of cytoplasmic domains depends on the proximity of the kinase domains (Davy et al., 2005).

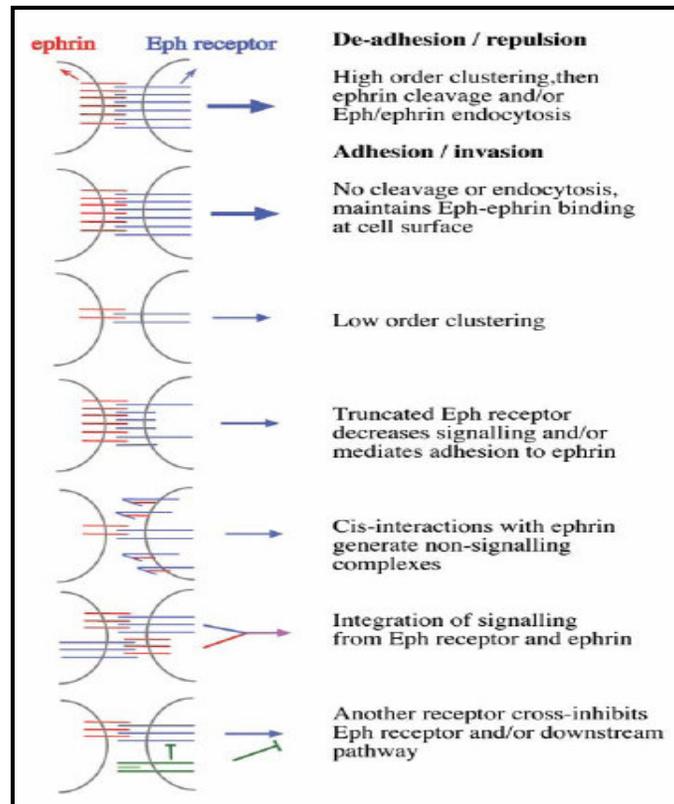


*Figure 7. The structure of Eph receptor and its ligand ephrin is shown here (Heroult et al., 2006).*

Upon ligand binding, the tyrosine domains of Eph receptors undergo transphosphorylation. Tyrosine residues can also become phosphorylated through Src kinases that are often associated with the receptors. Subsequently, disruption of intramolecular inhibitory interaction between the juxtamembrane segment and the kinase domain occurs (kinase) resulting in modification of the activation segment of the kinase domain that favours substrate binding. Similarly, phosphorylation induced by the Src kinases has been found to favour signalling (Kalo et al., 1999).

PDZ-domain-containing proteins and the Rho-family guanine nucleotide exchange factors (GEFs) can also interact with members of the Eph family of RTKs in manner independent of tyrosine phosphorylation of these receptors. Signal termination can occur through interaction with phosphotyrosine phosphatases and the ubiquitin ligase Cbl (Walker-Daniels et al., 2002). These interactions result in dephosphorylation of Eph receptor, internalization of the Eph-ephrin complex and protein degradation, respectively. An interesting phenomenon is the bidirectional signalling that occurs between different cells that reciprocally express ephrin ligands and Eph receptors. This function makes the Eph family of RTKs unique as the first time shown that ligands can also transfer a cell signal (Davy et al., 2005, Palmer et al., 2003). It is consisted of forward signalling (downstream of Eph receptors) and the reverse one (downstream of membrane-anchored ephrin ligands).

Repulsion of two cells bound to each other with a Eph-ephrin complex formation can occur via the process of membrane transendocytosis where both the ligand and receptor are internalised in a vesicular formation including their surrounding plasma membranes. The proteolytic degradation of ephrin ligands is another way to detach two adherent cells. The adhesion of two cells depends on the level of signalling that occurs after ligand binds to the receptor (Holmberg et al., 2000). Low levels of Eph signalling can end up in cell adhesion while high levels of Eph signalling results in repulsive movement. These suggested that clustering has direct effect on cytoskeletal organization and cell-to-cell contacts. In summary, the interaction of Eph receptors and ephrins can mediate contacts between cells to stabilize the cell adhesion, or to mediate repulsion, depending on the molecular density and degree of clustering of the complexes and the intensity of the signals generated (Davy et al., 2005, Holmberg et al., 2000, Kullander et al., 2002).



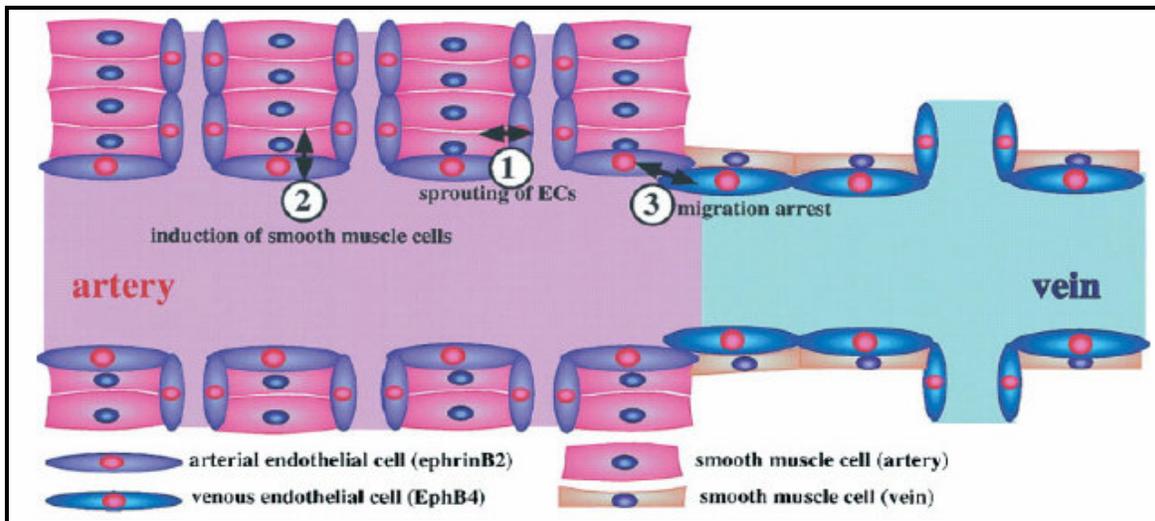
**Figure 8. Steps in cell contact-dependent bidirectional signalling and mechanisms of Eph-/ephrin- mediated cell adhesion and repulsion.** The net effect of interaction between Eph receptors and their ligands ephrins depends on the degree of clustering (Poliakov et al., 2004).

### 3.1.2 The role of Eph and ephrins in Angiogenesis

During embryonic development, vascular morphogenesis involves a continuous remodelling of primitive, relatively homogenous networks of embryonic and extraembryonic vasculature or primitive capillary plexus, into a complex, branching network of large and small mature, interconnected vessels. Different capillary plexuses are constructed by the de novo differentiation and coalescence of endothelial progenitors, or hemangioblasts, through vasculogenesis. Angiogenic remodeling occurs through sprouting of new branches, retraction of existing branches, joining of some capillaries and splitting of others. This complex of events includes endothelial cell proliferation, apoptosis, and migration.

Furthermore, vessel maturation and harmonised function are ensured by recruitment of mural supporting cells, pericytes (micro) and SMC (macrovesels) from surrounding mesenchyme (Carmeliet, 2000).

During embryonic development and angiogenesis, an impressive interplay between Eph receptors and ephrin ligands expressed in EC and associated tissues has great effect on the structure of new blood and lymphatic vessels. These include conformational changes of endothelial cell architecture such as lengthening, flattening and formation of microvascular tubules. Interaction between several EphB receptors and their ligands ephrins mediate capillary sprouting of ECs cultured in vitro (Gale et al., 1999). Analysis of mutant mice has indicated that the interplay between Eph receptors and ephrins expressed in endothelial cells and the surrounding tissues is physiologically important for the correct remodelling of the embryonic blood and lymphatic vascular systems (Kullander et al., 2002, Gale et al., 1999). Of relevance to the cardiovascular system is the involvement of ephrin ligands and Eph receptors in angiogenesis and myocardial trabeculation. These findings have significantly contributed to further define the hierarchical role of different regulators of arteriovenous specification and differentiation in which bi-directional EphB-ephrinB interactions play important roles as downstream effector molecules. The EphB4/ephrinB2 axis is the primary EphB/ephrinB interaction controlling vascular morphogenesis (Wang et al., 1998). The expression of ephrinB2 and EphB4 during development and specification of arterial and venous vessels appears to be influenced by external signals. Transplantation of chick embryonic arterial (ephrinB2-positive) or venous (ephrinB2-negative) endothelial cells into recipient embryos was performed and has shown that arterial or venous-derived cells maintained or induced expression of ephrin-B2 after into arteries. In contrast, cells incorporated into non-arterial vessels or other embryonic regions lost expression of ephrinB2 (Brantley-Sieders et al., 2004). It has been shown that in the human umbilical cord, EphB2 acts as a specific marker for arterial endothelial cells, whereas ephrinB2 can be also expressed by venous endothelial cells. The role of EphB4 and its ligand ephrinB2 in endothelial cells are not limited only to the topographical orientation of the latter towards each other (Fig. 9).



**Figure 9. Model of ephrinB2-EphB4 signals in vasculoangiogenesis.** (1) ephrinB2 on SMCs supports proliferation and sprouting of arterial ECs. (2) ephrinB2 ECs promote the recruitment of SMCs near ECs. Molecular cues for such induction are unknown; however, PDGF-BB and TGF- $\beta$  may be involved in this process. (3) When the ephrinB2 (arterial ECs) and EphB4 (venous ECs) face each other at the boundary of a capillary, cell proliferation of ECs may be suppressed, and the migratory ability may be arrested there (Zhang et al., 2006).

In contrast, they also participate in the regulation of the various interactions of endothelial cells with neighbouring stromal cells such as mural cells that result in reconstruction of vascular morphogenesis (Brantley-Sieders et al., 2004, Zhang et al., 2006). The expression of EphrinB2 in arteries as well as angiogenic endothelial cells can be regulated through shear stress (biomechanical forces). This major observation has raised several questions. The most important is whether the widely held view of angiogenic sprouting originating in postcapillary venules is correct indeed or whether angiogenesis is actually of arterial origin. The first protein from the Eph family of RTKS found to be of vascular origin was ephrinA1, a TNF $\alpha$ -regulated gene induced in endothelial cells. Subsequently, it was found that ephrinB2 is expressed in macrophage-like cells while EphB4 participates in recruitment of inflammatory cells to inflamed tissues in newly budding capillaries and postcapillary venules. The finding that EphB4 and ephrinB2 act antagonistically prompted investigators to

hypothesise an artery to vein pushes and pull model of invasive angiogenesis (Heroult et al., 2006). Given the angiogenic and arteriogenic expression of ephrinB2, activation of EphA4 or ephrinB1 synergizes with thrombin receptor activation to promote  $\alpha$ IIb $\beta$ 3 integrin-mediated adhesion of platelets to fibrinogen (Huynh-Do et al., 2002).

It has been previously reported that EphB4-deficient embryonic bodies display delayed expression of the hemangioblast marker VEGFR-2/Flk-1, as well as defective vascular morphogenesis in response to VEGF and bFGF *in vitro*. EphrinB2 expression has been detected in mesenchyme surrounding some blood vessels, and becomes increasingly extended to smooth muscle cells and pericytes surrounding vessels as development proceeds (Adams et al., 1999). Experiments in conventional knockout mice showed that depletion of ephrinB2 both in endothelium and endocardium was sufficient to recapitulate angiogenic remodeling defects. However, the full complement of vascular defects is produced by deletion of ephrinB2 in endothelium, mesenchymal expression of ephrinB2 remained intact suggesting that mesenchymal ephrin-B2 is not sufficient for vessel remodelling (Adams et al., 1999). The mesenchymal expression of EphB4 is known to inhibit vasculogenesis and it was assumed that this can be the net effect of the repulsive potential of this RTK. Inhibition of ephrinB2 ligand using a soluble ephrin-B2-Fc resulted in suppression of EphB4 (+) endothelial cells attachment, simultaneously detachment of three-dimensional spheroids and delamination of ECs from umbilical vein explants (Fuller et al., 2003). EphrinA1 is expressed in the developing vasculature, and promotes angiogenesis *in vitro* and *in vivo*. EphrinB1 is also expressed in embryonic vasculature, in both arteries and veins, as EphB3 RTK. In addition, EphB2 RTK is expressed in vascular-associated mesenchyme. ephrinB2 and ephrinA1 can also induce an angiogenic response from subcutaneous vessels *in vivo*. Surgical sponges impregnated with soluble ephrinA1 and implanted in the subcutaneous dorsal flank of wild-type mice induced sprouting of adjacent subcutaneous vessels and infiltration of new vessel sprouts into the sponges (Maekawa et al., 2003). Lung microvascular endothelial cells isolated from adult mice can also respond to ephrinA1, which induces assembly and migration *in vitro*. Soluble ephrinA1, ephrinB2, and the ectodomain of EphB1 induce corneal angiogenesis in adult mice, demonstrating that these mature endothelial cells have the capacity to respond to ephrin and Eph RTK signals (Heroult et al.,

2006, Brantley-Sieders et al., 2004). B-class Eph RTKs and ephrins may play a more direct role in endometrial disease, as EphB4/ephrin-B2 overexpression was recently reported in human endometrial hyperplasias and carcinomas (Berclaz et al., 2003). Ephrin-B2 expression has been observed in tumor arterioles infiltrating transplanted Lewis lung carcinomas and B16 melanomas in mice, suggesting that this ligand may regulate tumor neovascularisation (Shin et al., 2001). EphrinB1 overexpression has been reported in hepatocellular carcinoma, and overexpression of ephrinB1 enhances tumor neovascularization in vivo. Soluble ephrinB1 enhanced endothelial cell proliferation and migration in vitro, suggesting that at least one function of ephrinB1 in tumor progression involves recruitment of blood vessels through angiogenesis. While ephrinA1 stimulates chemotaxis in endothelial cells through Rac1 activation, treatment of vascular smooth muscle cells with ephrin-A1 produces the opposite effect, with inhibition of Rac1 and cell spreading (Deroanne et al., 2003). Additional studies demonstrated that ephrin-A1 treatment activates RhoA through EphA4 RTK-mediated activation of a novel guanine nucleotide exchange factor in vascular smooth muscle cells, Vsm-RhoGEF, which could also contribute to smooth muscle contractility (Ogita et al., 2003). EphrinA1 may therefore promote angiogenic remodeling not only through modulating endothelial cell migration and morphogenesis, but also perhaps by causing vascular smooth muscle cell retraction that might facilitate exposure of endothelium to angiogenic stimuli and assist movement of endothelial cells by disrupting the mural cell barrier. EphA2-deficient female mice displayed decreased tumor volume, tumor cell survival, microvascular density, and lung metastasis relative to tumor-bearing littermate controls (Brantley-Sieders et al., 2005). EphA2-deficient endothelial cells displayed impaired survival and failed to incorporate into tumor microvessels in vivo, and displayed impaired tumor-mediated migration in vitro relative to controls. These data suggest that host Eph receptor tyrosine kinase function is required in the tumor microenvironment for angiogenesis and metastatic progression.

## 4.1 MATERIALS

### 4.1.1 A549 and HUVEC cell culture Reagents

Endothelial Cell Growth Medium	Promocell, Germany
Endothelial Supplement Mix	Promocell, Germany
RPMI 1640	BA, Austria
L-Glutamine	PAA, Austria
Gelatin 1,5%	Bio-Rad Laboratories, USA
Penicillin/Streptomycin	Biochrom-Seromed, Germany
Fetal Calf Serum (FCS)	Sigma, Germany
Trypsin/EDTA	Invitrogen, Germany
PBS Buffer	PBS Dulbeccos, Gibco Karlsruhe

### 4.1.2 A549 and HUVEC cell culture Instruments and Equipment

Cell Number Plate Analyser System	BioRad, Germany
Microscope Olympus Optical Co.	Olympus, Germany
Autoflow CO <sub>2</sub> Incubator	Heraeus, Germany
IR Variolab W90 Safety Culture Chamber	Heraeus, Germany
Cell culture flasks 25cm <sup>2</sup>	Eppendorf, Germany
-20°C Refrigerator	BOSCH, Germany
-80°C Refrigerator	BOSCH, Germany
Cell culture flasks 75cm <sup>2</sup> -sterile with biofilter	Germany
Serological Pipettes	Beston-Dickinson Labware, USA
Water bath	Lauda, Germany
Table centrifuger	Heraeus, Germany

Primer	Orientation	Sequence
GAPDH	for	tggtcaccagggtgctt
	rev	agcttcccgttctcagcctt
EphA2	for	tcctgctgtgccatgct

	rev	ccctcagcggaagttgca
<b>ephrinA1</b>	for	cattcctgcctttaagccaaa
	rev	ctcagctcccactgtgccta
<b>EphB4</b>	for	tgtgttgagggaacctgttc
	rev	gggccctgttcaactg
<b>ephrinB2</b>	for	gatgtgaaattcattgtggcaat
	rev	cagaagtagctgtcaattgttt

*Table 5. Primers used for Real-Time RT-PCR (Invitrogen, Germany).*

#### 4.1.3 Experimental Mice

Balb/c Mice

Charles River, Germany

#### 4.1.4 In-vivo Laboratory Instruments and Software

Surgical instruments

Klingenfuss, Germany

Digital camera HP Photosmart M627

Germany

Dissection microscop Leica M690

Leica Instruments, Germany

Microfine Insulin syringes 0.3ml U-100

Beston-Dickinson, USA

Cell culture flasks 25 cm<sup>2</sup>

NUNG, Germany

Cell culture flasks 75 cm<sup>2</sup>- sterile with biofilter

NUNG, Germany

Serological Pipettes

Beston-Dickinson Labware, USA

#### 4.1.5 Immunofluorescence and Immunohistochemistry Reagents

Triton X100

Serva, Germany

Methanol-Ethanol 50%-50%

Roth, Germany

Roti-histol

Carl Roth, Germany

Jung Tissue Freezing Medium

Jung, Germany

Bovine Serum Albumin

Sigma, Germany

PBS

Invitrogen, Germany

Rabbit anti-EphA2	Santa Cruz, Germany
Donkey Anti-Rabbit Alexa 488	Invitrogen, Germany
4', 6'-Diamidino-2-phenylindole (DAPI)	Sigma, Germany
Fluorescent mounting medium	Dako, Germany
Distilled water	Millipore, Germany
Rabbit blocking serum	Sigma, Germany
Rabbit anti-ephrin A1	Santa Cruz Biotechnology, Germany
Streptavidin-HRP	Dako Cytomation, Germany
3,3-diaminobenzidine (DAB) solution	Sigma, Germany

#### **4.1.6 Immunofluorescence Processing and Image Acquisition**

DakoCytomation Pen (thick writing)	DakoCytomation, Germany
Staining slides and 4-well chamber slides	Dako, Germany
Mikroscope Zeiss Axiophot	Carl Zeiss, Germany
Prism 3.03 GraphPad Software	USA
AnalySIS® 3.1 Soft Imaging System	Germany

#### **4.1.7 Metabolic Activity Assay Materials**

96-well plates	Beston Dickinson, Germany
Soluble EphA2-receptor chimera	R&D Biosystems, Germany
WST-1 reagent	Roche, Germany
ELISA Reader	Biorad, USA

#### **4.1.8 RNA Isolation, cDNA Synthesis and Real-Time RT-PCR Materials**

RNEasy Mini Kit	Quiagen, Germany
cDNA synthesis kit	Fermentas, Germany
SYBRGreen Master Mix	Abgene, UK

#### **4.1.9 Electronic Equipment, Instruments and Software**

Taqman ABI 7700 real time PCR machine	Applied Biosystems, USA
Serological Pipettes	Beston Dickinson Labware, USA

Eppendorf tubes Germany  
96-well plates Beston Dickinson, Germany

#### **4.1.10 Endothelial Cell Migration Assay Equipment**

Matrigel Endothelial Cell Migration Chamber BD, Germany  
RPMI 1640 medium BA, Austria  
Endothelial Cell Growth Medium Promocell, Germany  
Endothelial Supplement Mix Promocell, Germany  
Soluble EphA2-receptor chimera RD, Germany  
Ethanol-Methanol 50%-50% Germany  
Haematoxyllin Dako, Germany

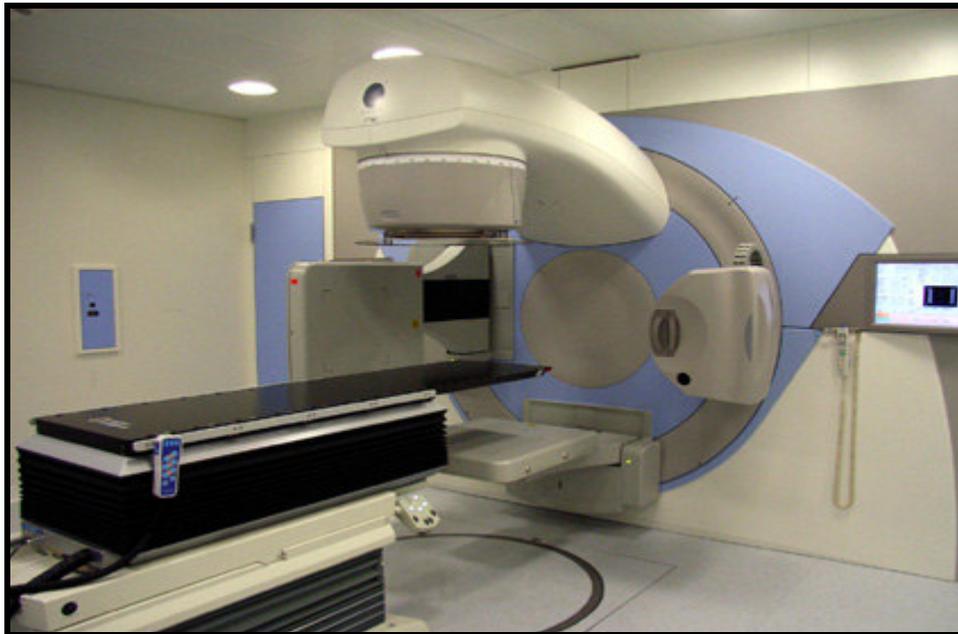
#### **4.1.11 Buffers, Kits and Solutions**

RPMI 1640 Invitrogen, Germany  
RNEasy Kit Quiagen, Germany  
cDNA Synthesis Kit Fermentas, Germany  
SYBRGreen Master Mix BioRad, Germany  
WST-1 reagent Roche, Germany

## 4.2 Methods

### 4.2.1 A549 and HUVEC cell culture and irradiation

Human lung adenocarcinoma cell line A549 was obtained from American Type Culture Collection, (Manassas, VA) and human umbilical vein endothelial cells (HUVEC) was purchased from BD Biosciences, (Bedford). A549 cells ( $5 \times 10^5/\text{ml}$ ) were maintained in 25  $\text{cm}^2$  culture flasks filled with 5 ml RPMI medium supplemented with penicillin, streptomycin (Sigma, Germany) and 10% fetal calf serum (FCS). HUVEC cells were maintained in EC medium with EC supplement (PromoCell, Germany) at 37°C and 5%  $\text{CO}_2$ . No growth factors were added in the medium. After incubation for 24 hours, cells were irradiated at room temperature. Single doses of 0.5, 2, 4 and 8 Gy with a rate of 450 cGy/min were given using an Elekta Synergy 18 MV linear accelerator at a room temperature of 20 °C (Fig. 10).



*Figure 10. The Elekta Synergy linear accelerator (Elekta Oncology Systems, Norcross, GA) was used for irradiation of tumor cells and xenografts.*

#### **4.2.2 RNA-extraction and cDNA synthesis**

RNAs were extracted from cultured cells collected at 1, 4, 8 and 24 hours after IR using RNEasy protect mini kit (Qiagen, Germany) according to manufacturer's instructions. First strand cDNA synthesis was performed using cDNA synthesis kit (Fermentas, USA). Briefly, RNA (0.5 µg) was added to 2.5 µl of random hexamer primer and sterile water to final volume of 11µl and incubated at 70°C for 5 min. Subsequently, 4µl of 5 x reaction buffer were added together with 1µl of ribonuclease inhibitor (Invitrogen Germany) and 2µl of 10mM deoxyribonucleotide triphosphate. After incubation at 37°C for 5 min, 2µl of moloney murine leukemia virus (MMLV) reverse transcriptase were added to a final volume of 20µl. The mixture was finally incubated at 37°C for 1h followed by 10 min in 70°C for inactivation of reverse transcriptase.

#### **4.2.3 Real-time reverse transcriptase polymerase chain reaction (RT-PCR)**

mRNA expression level of EphA2, ephrinA1, EphB4 and ephrinB2 genes was quantified by quantitative real-time RT-PCR. For the reaction, 5µl cDNA sample was mixed with 12.5µl SYBRGreen Master Mix (Abgene, UK), 1µl forward primer, 1µl reverse primer and 5.5µl sterile water to a final volume of 25µl. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences are shown on table 8. The reaction mixture was preheated at 95°C for 10 min and amplified for 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s followed 72°C for 2 min using the MyiQ real-time PCR detection system (Bio-Rad Laboratories, Munich). Experiments were performed three times and the results are the average of those three experiments. A change of more than 2.5-fold of relative expression was considered significant ( $p < 0.05$ ). A Ct value (cycle threshold) higher than 30 was considered non-significant.

#### **4.2.4 Animals and tumor treatment**

Animals were kindly provided from the Department of Internal Medicine, University Hospital Giessen and Marburg, Giessen and had the approval of the local ethical committee (Project number GI20/1049/2004). Pathogen-free female Balb/c mice (6-8 weeks old) were maintained in a pathogen-free animal facility for at least 1 week before each experiment.

Approximately,  $5 \times 10^6$  tumor cells were diluted in 200 $\mu$ l NaCl 0.9 % and injected subcutaneously into the right hind of the anesthetized mice using a 26G  $\frac{1}{2}$  syringe. Tumor dimensions were measured using a calliper (CD-15 CP, Mitutoyo, U. K. Ltd., England). Tumor volume was calculated using the following formula:

$$V (\text{mm}^3) = (w^2 \times l) / 2$$

where V: volume, w: width, l: length. Experiments were started when the tumors reached a mean diameter of approximately 0.8 cm. Approximately 10 min before irradiation, mice were intraperitoneal anesthetized with ketamine/ xylazine (60/6 mg/kg) combination. A single irradiation using a dose of 20 Gy at a dose rate of 2.5 Gy/min was given on tumor field of mice. Mice were sacrificed using cervical dislocation at 6, 10 and 30 hours after radiation, respectively. As control, unirradiated mouse was used for each time point. 4 mice were used for each group. Mice were also irradiated with a single dose of 2 Gy to analyse the effect of a clinically-relevant dose.



***Figure 11. Pathogen-free female Balb/c mice (6-8 weeks old) with A549 xenograft (frame).***

#### **4.2.5 Proliferation Assay**

Suspension of 5000 A549 cells in RPMI medium with or without EphA2-Fc soluble receptor (5 µg/ml) was plated in 96-well plate. Cells were irradiated with 4 Gy. An unirradiated (control) group with or without EphA2-Fc was used as well. The proliferation rate of A549 cells was assessed for a time interval of 5 days using proliferation reagent WST-1 according to manufacturer's instructions (Roche, UK). The viability was expressed as the percentage in relation to the viability in Fc-treated group on the first day of the experiments, for both irradiated and unirradiated cells. Values are shown as means ( $\pm$  SE). Statistical analysis was performed using the Students *t*-test

#### **4.2.6 Immunofluorescence**

Frozen sections of tumor tissue from A549 xenografts were cut (5 µm) for immunofluorescent staining. Fixation was performed in acetone-methanol mixture (1:1) at -20°C for 5 min. Slides were permeabilized with 1% Triton/PBS, rinsed thrice in PBS, and pre-incubated with blocking buffer (0.3% Triton X-100, 3% bovine serum albumin in PBS) for 1 hour. Following this, slides were incubated for 2 hours with rabbit anti-EphA2 (C-20, 1:200), purchased from Santa Cruz Biotechnology, Germany. After washing thrice with 0.1% BSA in PBS, the slides were incubated for 1 hour with donkey anti-rabbit Alexa 488 (1:800), purchased from Invitrogen, Germany. Slides were rinsed thrice again and counterstained for 5 min with 4', 6-Diamidino-2-phenylindole (DAPI) purchased from Sigma, Germany. Slides were washed thrice and mounted with fluorescent mounting medium (Dako, Germany). EphA2 expression in tumor sections as well as chamber slides was measured with a semi-automatic image recording system (Olympus Microscope, Germany). Magnification (200x and 640x) was used for quantification of EphA2 signal. The fraction of EphA2-positive cells to the total number of DAPI stained cells was calculated in 6 random tumor areas. The fraction of EphA2-positive cells (membranous staining) was calculated as well.

Immunofluorescent expression of EphA2 in irradiated A549 cells (6 Gy) in vitro was analysed similarly as described above for the in vivo investigation.

#### **4.2.7 Immunohistochemistry**

A549 xenografts were irradiated using different doses (0, 2 and 20 Gy) as mentioned above. Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue. Sample slides were passed through a sequence of Roti-histol (Carl Roth, Karlsruhe, Germany) and graded alcohol and then rinsed in phosphate-buffered saline (PBS). After rinsing with PBS, the slides were treated with 3% hydrogen peroxide in PBS for 15 min at room temperature in order to eliminate endogenous peroxidase activity. Subsequently, the slides were treated with 5% blocking serum for 1 h. Following this, the slides were incubated overnight at 4°C with a rabbit antihuman EphA2 or ephrin A1 polyclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Heidelberg, Germany). In the negative controls, the primary antibody was replaced with 1x PBS. The signal was enhanced by using biotinylated polyclonal goat anti-rabbit IgG with streptavidin-HRP (Dako Cytomation, Germany) for 30 min. The colour was developed after a 5 min incubation with 3,3-diaminobenzidine (DAB) solution (Sigma, Germany) and the sections were weakly counterstained with hematoxylin for 10 sec.

#### **4.2.8 Evaluation of EphA2 and ephrinA1 immunohistochemical expression**

The membranous and cytoplasmic expression of EphA2 on tumor cells was assessed at a x100 and x200 magnification. The assigned score first reflected the staining intensity A (0, negative; 1, weak; 2, moderate; 3, high) and second the percentage of positive cells B (0, no positive cells; 1, <25% positive cells; 2, 25 to 50% positive cells; 3, >50% positive cells). An overall score of 3 was defined as positive staining. The score of the EphA2 or ephrinA1 expression was scored using a scale between 0 and 3 (negative=0, low=1, moderate=2 and high=3, respectively). The scoring was performed separately by two independent observers. Any discrepancies in the scores were resolved on the conference microscope.

#### **4.2.9 Matrigel endothelial cell migration assay**

A Matrigel endothelial cell migration chamber (BD Bioscience, Germany) was used to examine the migration ability of ECs stimulated through irradiated tumor cells. A549 cells ( $6 \times 10^4$ ) were seeded on each of the 24-well plates in RPMI medium and irradiated with a

single dose of 4 Gy. RPMI medium was removed and fresh endothelial cell medium (PromoCell, Germany) was added. No growth factors such as VEGF, PDGF, bFGF were added in the medium. Unirradiated A549 cells were used as control. The Matrigel-coated transwell inserts (8- $\mu$ m pore size) that were added on the upper compartment had a reduced-growth factor membrane. ECs were seeded on the upper surface of the membrane and allowed to migrate towards the A549 cell compartment. Matrigel-coated transwell inserts contained  $2.5 \times 10^4$  ECs in endothelial cell medium and EphA2-Fc soluble receptor chimera at various concentrations (0.5, 1 and 10  $\mu$ g/ml) was added in endothelial cell medium to block EphA2 signalling. After incubation for 36 hours, ECs that had invaded and passed to the other site of the membrane were fixed with methanol-ethanol (1:1), stained with haematoxylin solution and sealed on slides. The number of migrated endothelial cells per field (magnification  $\times 50$ ) was counted by microscopy. The results represent the mean number of migrated endothelial cells as calculated from 4 fields.

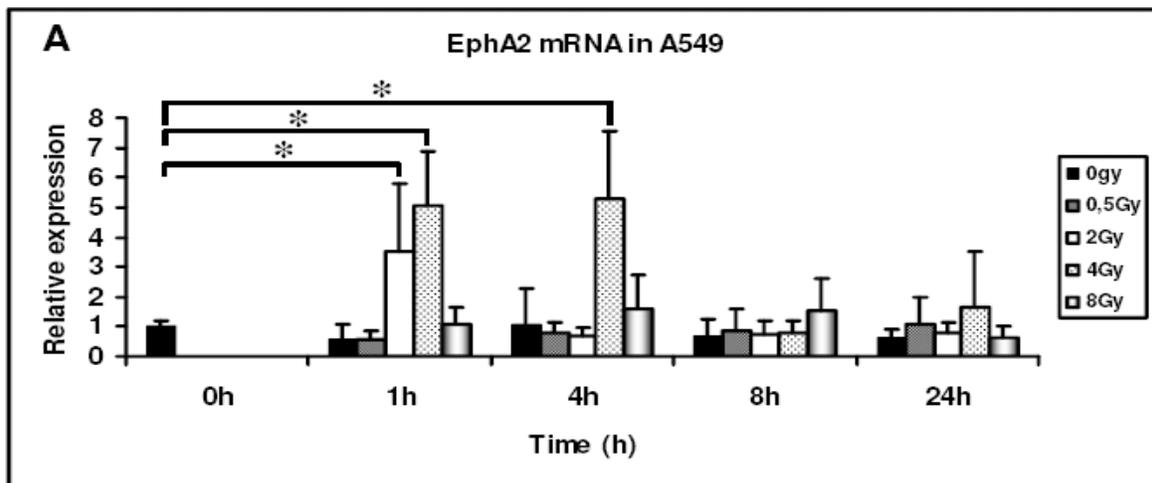
#### **4.2.10 Statistical analysis**

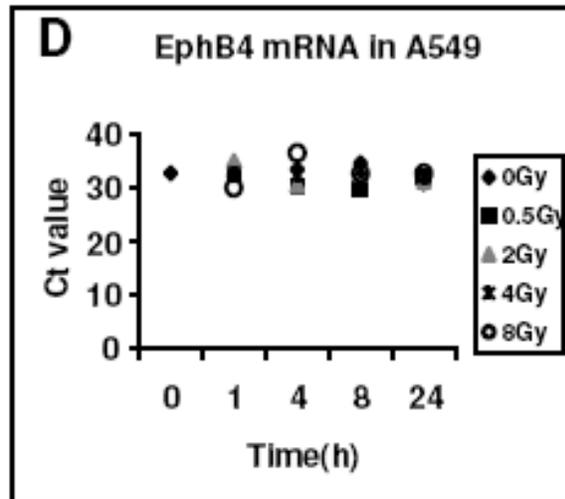
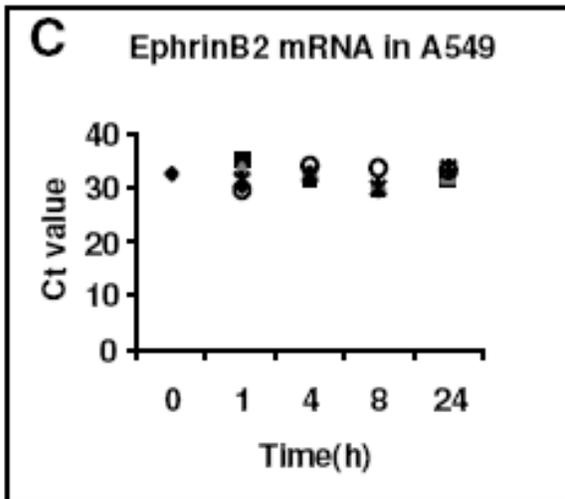
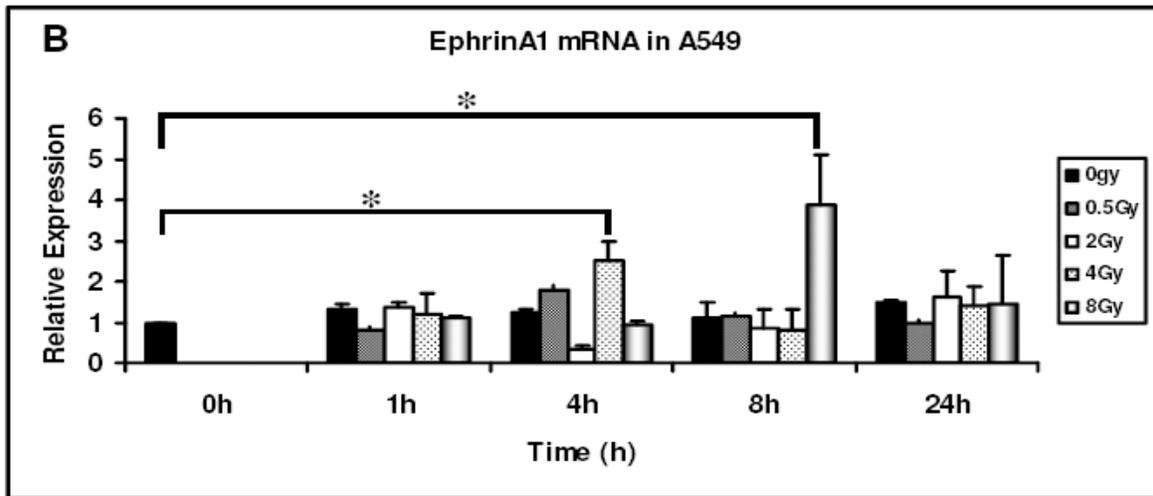
The significance of differences among the means of the irradiated and control group for all experiments was examined using the Student's *t*-test. Data are reported as means  $\pm$  SE. A value of  $p < 0.05$  was considered statistically significant.

## 5. RESULTS

### 5.1 Radiation induces transcriptional activation of EphA2-ephrin A1 but not EphB4-ephrinB2

Because the correlation between IR and the Eph family of RTKs has not been previously investigated, the first series of experiments were designed to study the effect of IR on key members of Eph genes. The high number of Eph receptors and ephrin ligands as well as the great diversity of functions among them prompt the author to study members, the importance of which, has already been confirmed in a number studies. Therefore, EphA2/ephrinA1 as well as EphB4/ephrinB2 were chosen to be analysed. The transcriptional expression of these members responding to irradiation was analysed by using Real time RT-PCR. As shown in Fig. 12A, EphA2 mRNA expression was induced at 1h upon irradiation with 2 Gy ( $3.5 \pm 2.29$  fold up-regulation as compared with control gene; mean  $\pm$  SE;  $p < 0.05$ ) while soon after returned back to baseline level. The most significant response was found at a dose of 4 Gy where EphA2 presented an induction after a period of 1h ( $5.02 \pm 1.85$ ; mean  $\pm$  SE;  $p < 0.05$ ) that persisted up to 4h ( $5.3 \pm 2.25$ ; mean  $\pm$  SE;  $p < 0.05$ ) but showed no effect at later time points. Fig. 12B shows upregulation of ephrinA1 mRNA expression at 4h after irradiation with 4 Gy ( $2.46 \pm 0.53$  fold; mean  $\pm$  SE;  $p < 0.05$ ). Similar induction was found with the dose of 8 Gy at 8h ( $4.76 \pm 1.22$ ; mean  $\pm$  SE;  $p < 0.05$ ). Analysis of mRNA expression of EphB4/ephrinB2 in A549 cells did not reveal any significant change ( $p > 0.05$ ) in their expression pattern, (Fig. 12C and 12D).

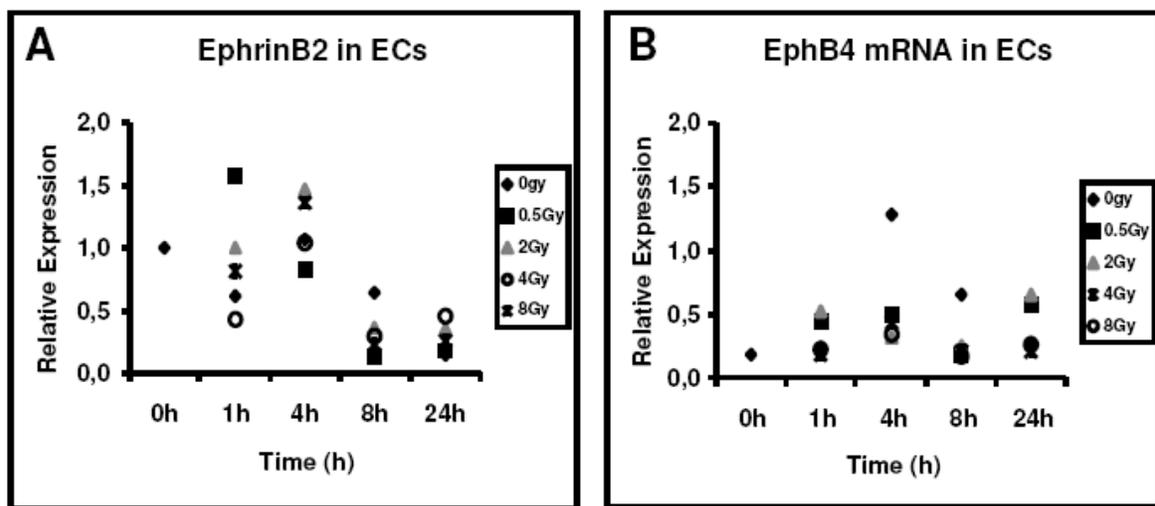




**Figure 12. Induction of EphA2 receptor and its ligand EphrinA1 in irradiated lung adenocarcinoma cells.** A and B, quantitative Real time-PCR analysis of EphA2 and ephrinA1 in irradiated A549 tumor cells. Bar graphs show relative mRNA expression (fold upregulation) in A549 cells in response to radiation (mean  $\pm$  SE). A549 cells were irradiated using single doses of 0.5, 2, 4 and 8 Gy. Unirradiated cells were used as control. The mRNA level of EphA/ephrin genes were compared to the housekeeping gene GAPDH (fold induction). Values represent averages and standard deviations of three different experiments (mean  $\pm$  SE; \*,  $p < 0.05$ ). C and D, EphB4 and EphrinB2 mRNAs were not significantly induced after irradiation of A549 cells.

## 5.2 Irradiation of ECs *in vitro* does not affect transcriptional status of EphA2/ephrinA1 or EphB4/ephrinB2

Endothelial cells were irradiated with different doses and gene response was monitored at 1, 4, 8 and 24h post-irradiation using Real-time PCR. No significant change of mRNA expression level was found in any of the four genes analysed at the level of mRNA expression ( $p>0.05$ ). Representative graphics from ephrinB2 and EphB4 are shown in Fig. 13A and B.

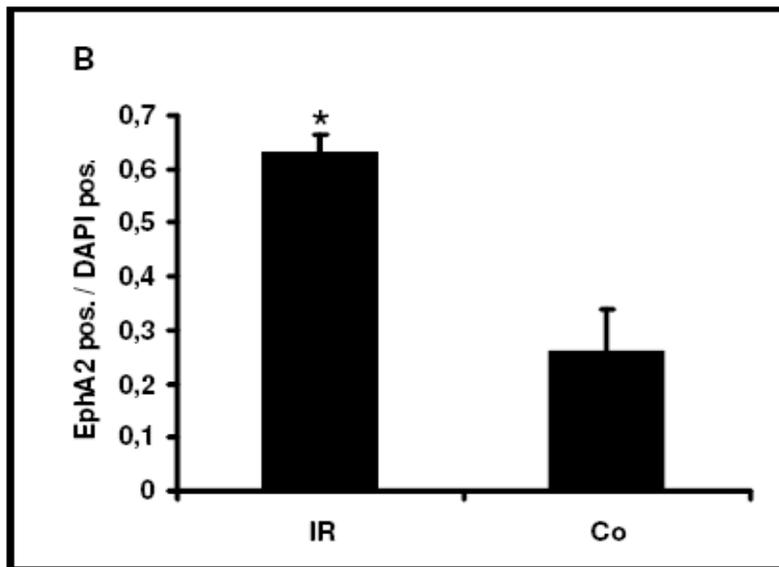
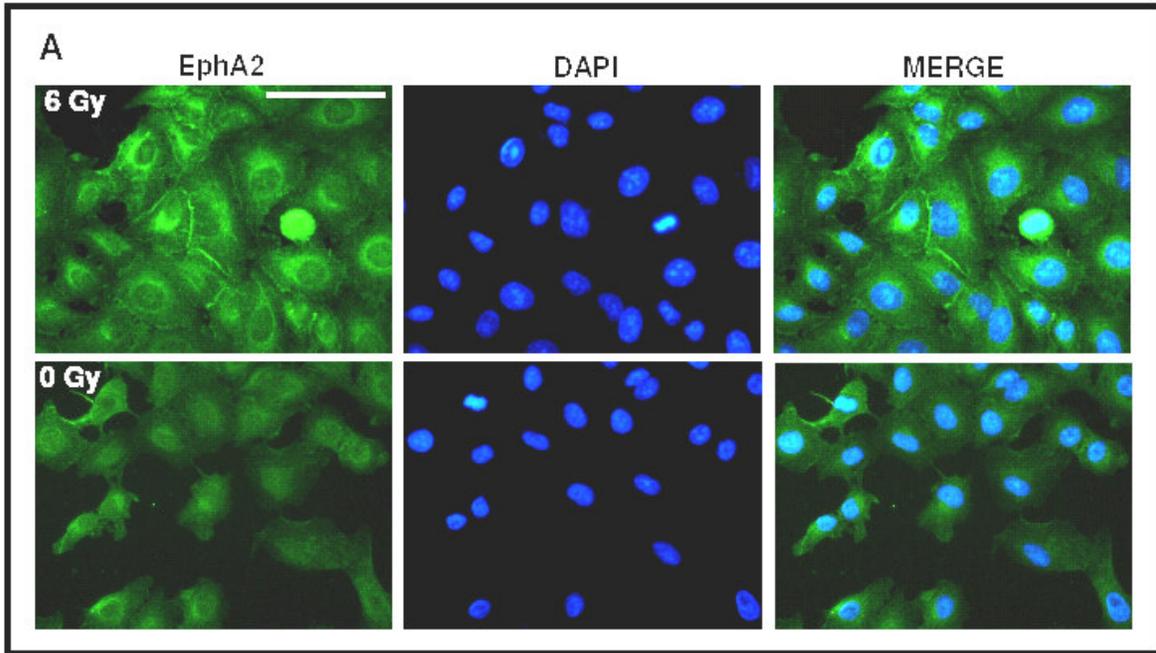


**Figure 13.** Effect of irradiation on EphB4/ephrinB2 mRNA expression in ECs. Quantitative Real time-PCR expression analysis of EphB4/ephrinB2 in irradiated HUVEC. Graph shows relative mRNA expression in HUVEC in response to radiation. ECs were irradiated, similarly to A549 cells, with 0, 0.5, 2, 4 and 8 Gy and gene response was monitored at 1, 4, 8 and 24h post-irradiation ( $p>0.05$ ).

## 5.3 Increased Immunofluorescent Expression of EphA2 in irradiated A549 cells *in vitro*

Due to the transcriptional activation of EphA2 and its well-known significance in tumor pathogenesis and angiogenesis, A549 cells were irradiated (6 Gy) and the immunofluorescent imaging of EphA2 protein was analysed. The investigation revealed an enhance expression of EphA2 in irradiated A549 cells (Fig.14B), which was approximately

2.5-fold higher than the control (unirradiated) A549 cells ( $p < 0.05$ ).

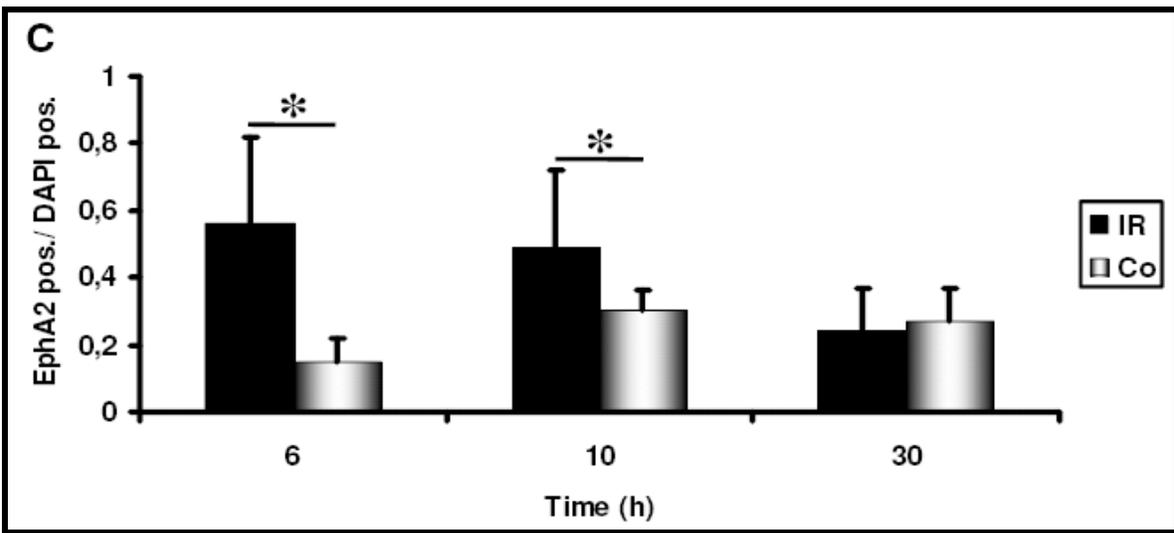
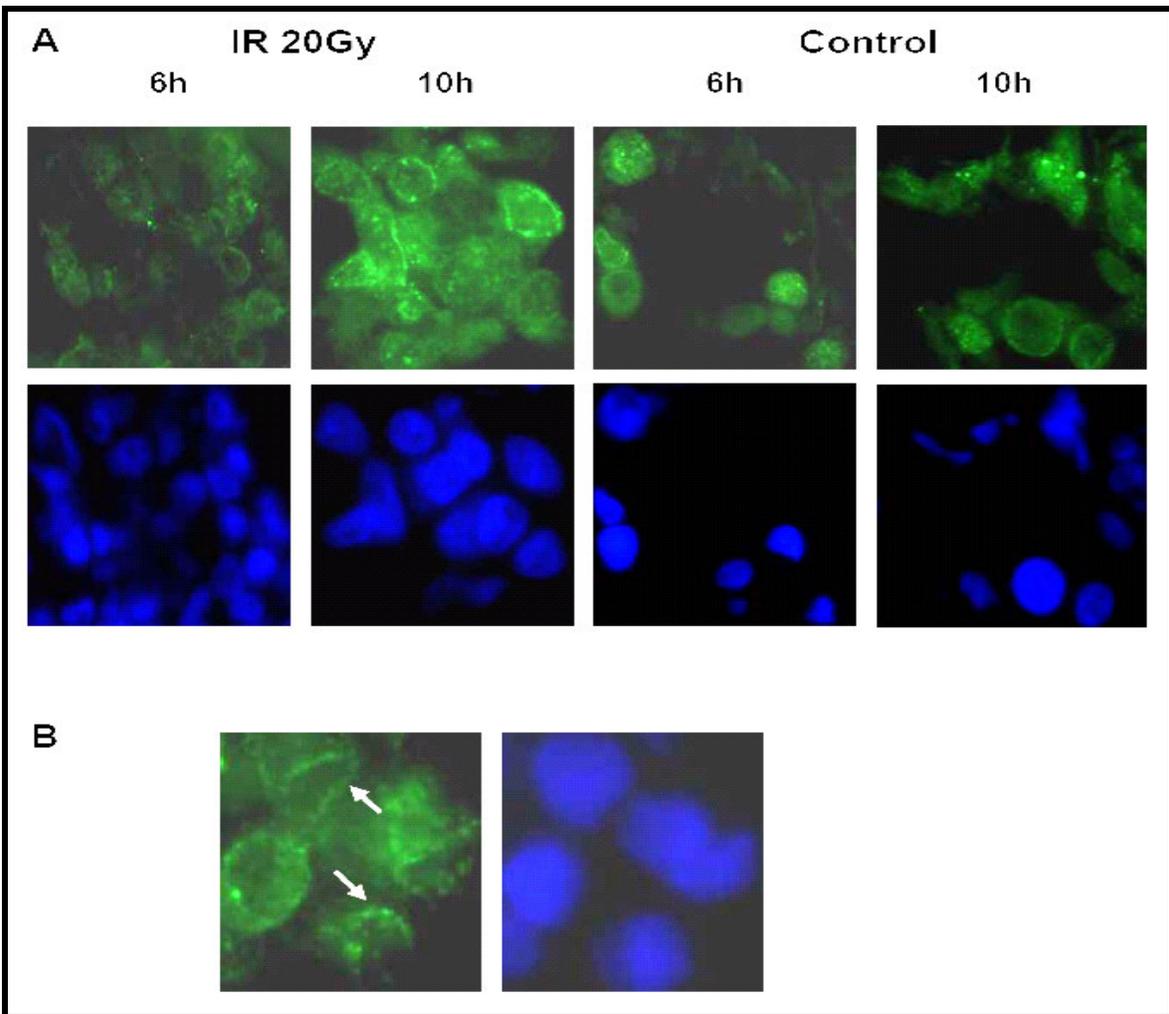


**Fig 14.** The immunofluorescent detection of EphA2 expression in A549 *in vitro*. A, representative pictures of EphA2 staining in both irradiated (IR) and control (Co) A549 cells *in vitro*. Cells were irradiated using a single dose of 6 Gy. Of note, EphA2-positive cells demonstrated the characteristic ring-line fluorescent staining, typical of the positive

*expression of a transmembrane protein. Bar=200  $\mu$ m. (Fig. 14A). Interestingly, unirradiated A549 cells also presented EphA2-positive cells but to a less extent. B, Increased expression of EphA2 in IR as compared to control A549 cells (Co). The fraction of EphA2-positive signal to DAPI signal (mean  $\pm$  SE; \*,  $p < 0.05$ ) is shown.*

#### **5.4 Increased Immunofluorescent Expression of EphA2 in irradiated A549 xenografts**

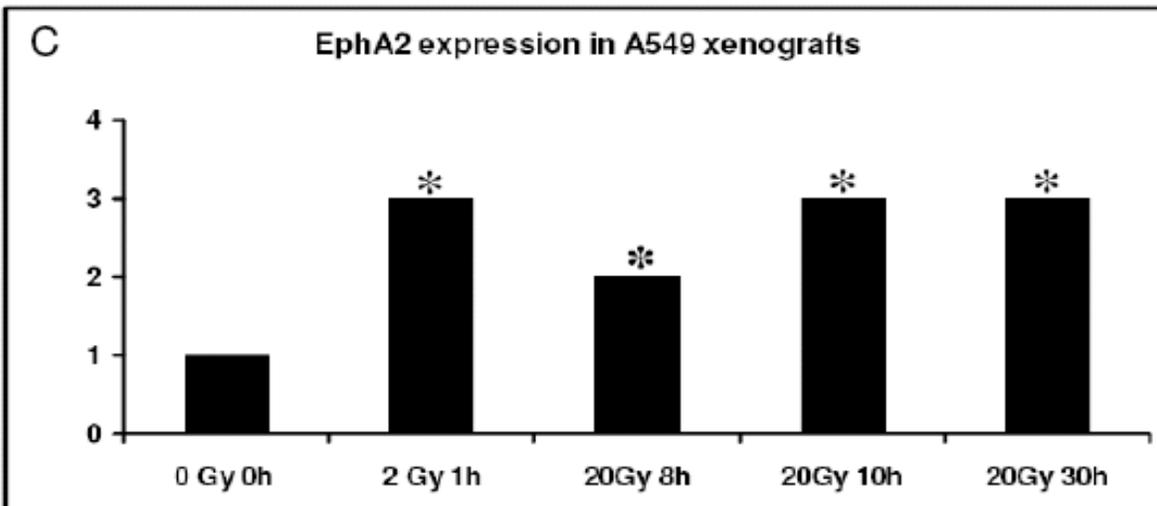
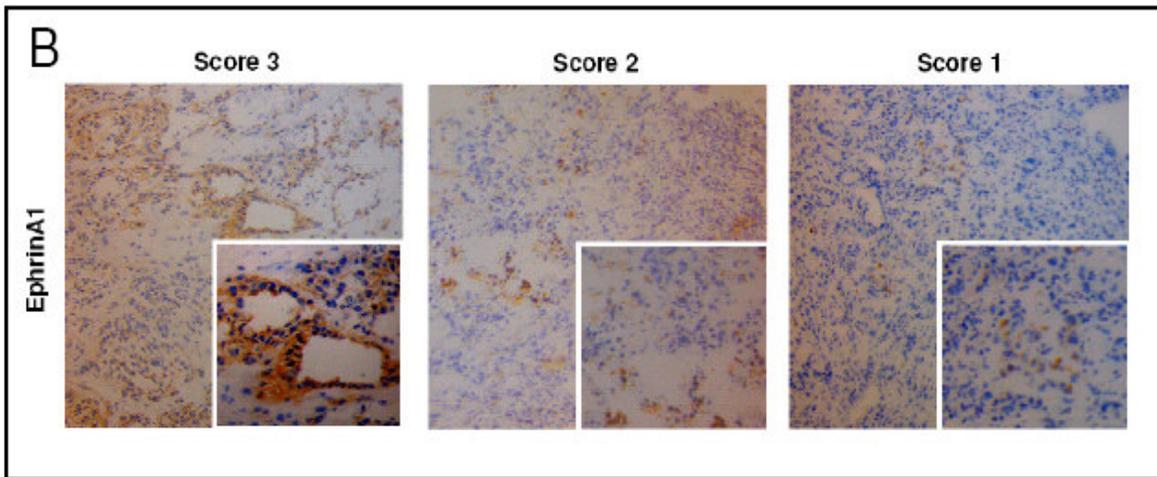
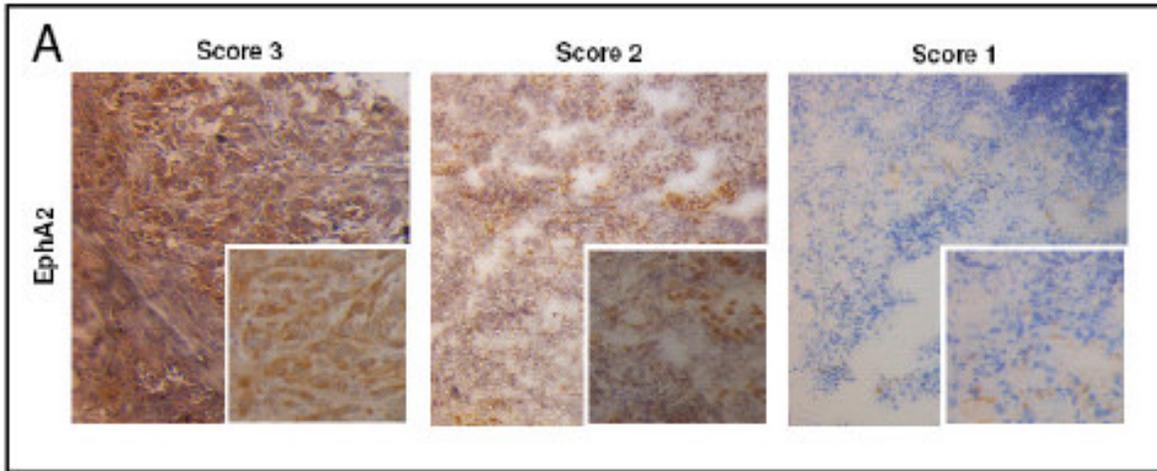
To further assess the effect of radiotherapy on EphA2 status in vivo, the expression of this receptor in A549 xenografts was analysed using immunofluorescent staining. In order to monitor the change in EphA2 profile at different time points post-irradiation, mice were irradiated with 20 Gy and sacrificed at 6, 10 and 30 hours, respectively. Unirradiated mice were used as control for each group (Fig 15 A and B). As it is shown in figure 15C, maximal specific staining of EphA2 was observed at 6 hours in the irradiated group indicating strong induction of EphA2 protein expression ( $0.56 \pm 0.31$ ; mean  $\pm$  SE;  $p < 0.05$ ) while abundant signal was still present at 10 hours ( $0.49 \pm 0.23$ ; mean  $\pm$  SE;  $p < 0.05$ ), showing a subsequent decrease 30 hours after irradiation ( $0.24 \pm 0.13$ , mean  $\pm$  SE;  $p > 0.05$ ). No major difference of EphA2 expression was noted at different time points in control group ( $0.15 \pm 0.07$ ,  $0.29 \pm 0.06$ ,  $0.27 \pm 0.1$ ; mean  $\pm$  SE;  $p < 0.05$ ) at 6, 10 and 30 hours sacrificed, respectively (Fig. 15C).

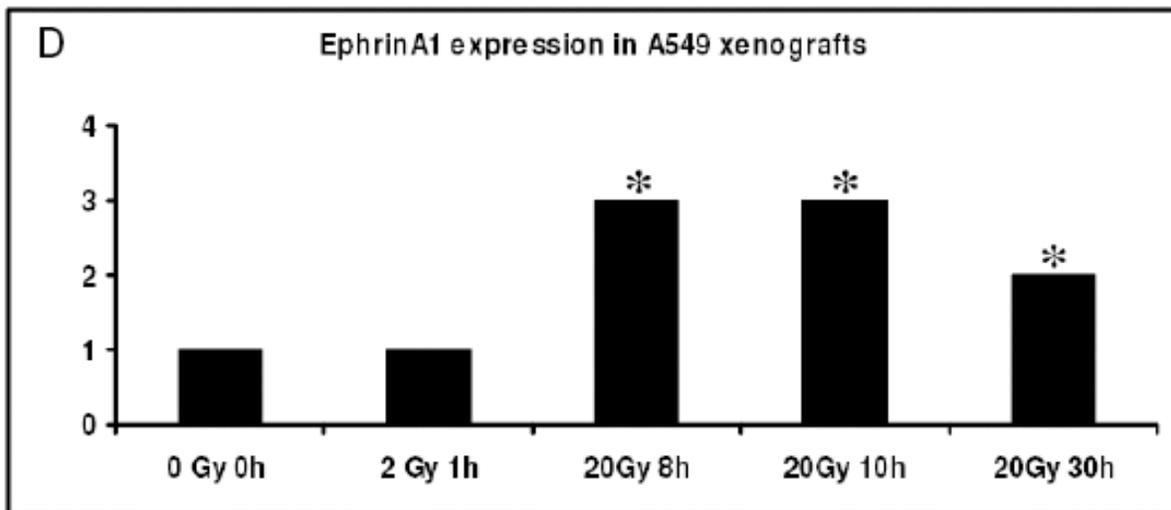


**Figure 15. Immunofluorescence detection of EphA2 upon A549 irradiation in vivo.** The expression of EphA2 receptor tyrosine kinase in A549 xenografts in vivo is shown using immunofluorescent staining. A549 cells were injected subcutaneously into pathogen-free female Balb/c mice and left to grow to a diameter of approximately 0.8 cm. Mice were irradiated using a single dose of 20 Gy. A, in order to monitor the change in EphA2 profile at different time points post-irradiation, 3 mice were treated and sacrificed at 6, 10 and 30 hours, respectively. For each time point, a control mouse was used as well (original magnification, x200). B, stronger membranous staining (white arrow) was observed in irradiated A549 xenografts as compared to the control group at 6h and 10h after irradiation. Green, monoclonal EphA2 antibody; Blue, DAPI, (original magnification, x640). C, the fraction of EphA2-positive signal to DAPI signal (mean  $\pm$  SE; \*,  $p < 0.05$ ) was calculated in 6 random tumor areas.

### **5.5 Immunohistochemical Analysis of EphA2 and ephrinA1 Expression in irradiated A549 xenografts**

In unirradiated A549 human lung adenocarcinoma xenografts (control), a weakly positive EphA2 expression (score 1) was observed (Fig. 16A). In contrast, in irradiated samples, a moderate to high expression was found (Fig. 16A). Specifically, EphA2 showed a moderate expression in tumors irradiated with 2 Gy (score 2). EphA2 was strongly induced in tumors irradiated with 20 Gy, confirming the results detected by using immunofluorescent staining (score 2-3). The EphA2 immunoreactivity was observed in both tumor cell membrane and cytoplasm as well as in endothelial cells. Notably, EphA2 immunoreactivity revealed no staining of adjacent connective tissues. EphrinA1 was also immunohistochemically studied. Similarly to EphA2, a weak expression for ephrinA1 (score 1) was detected in unirradiated tumor xenografts (Figure 16). The immunoreactivity of ephrinA1 was also low for tumors irradiated with 2 Gy. A strong expression was observed in tumors treated with 20 Gy in the early times points, which was reduced to moderate at late time points (Figure 16). A minimum staining of the connective tissues was found during the study (significant:  $p < 0.05$ ).

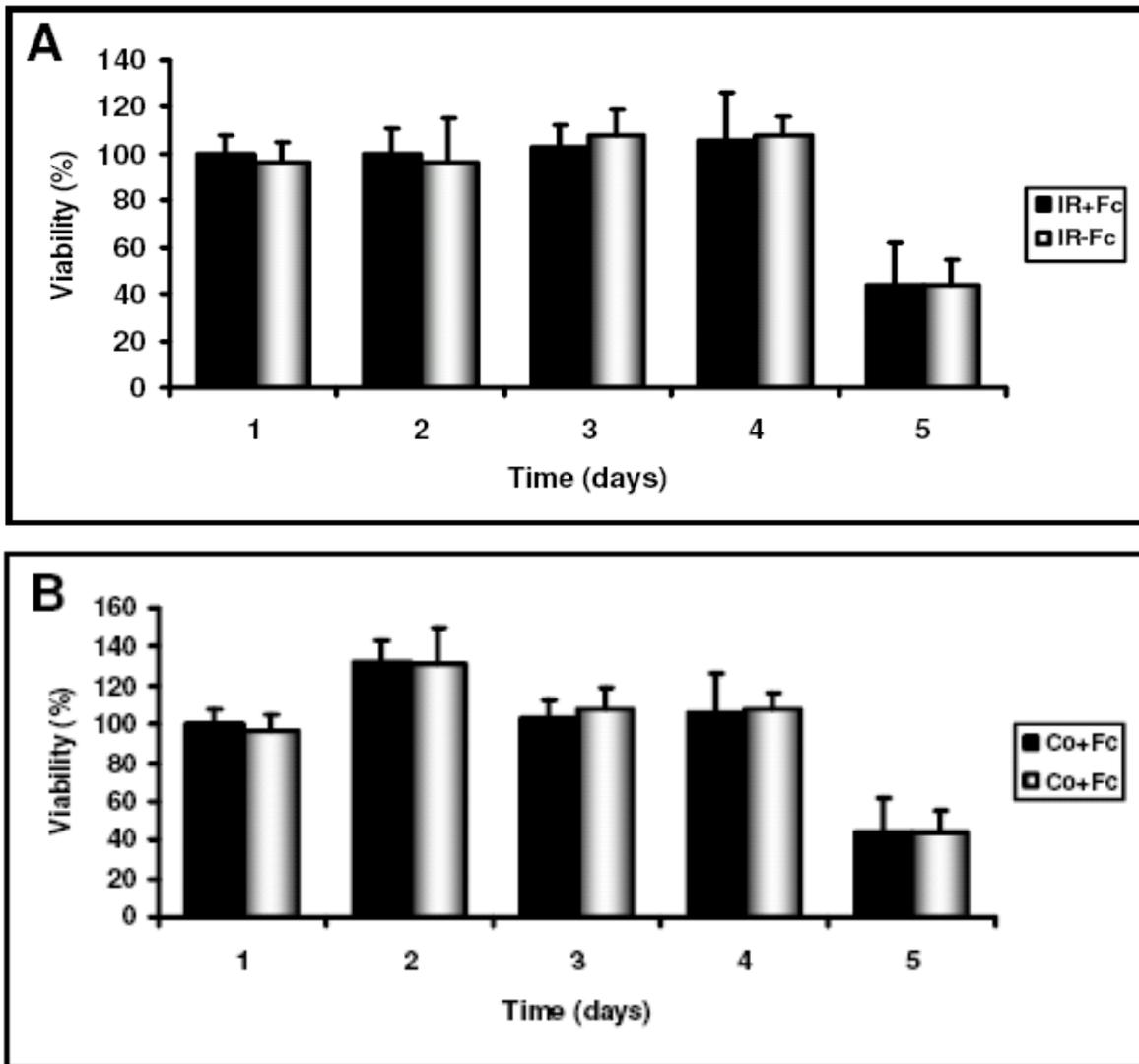




**Fig. 16. EphA2 and ephrinA1 expression in irradiated A549 human lung adenocarcinoma xenografts.** A, representative images of EphA2 staining with weak (score 1), moderate (score 2) and strong (score 3) expression. B, similar representative images for ephrinA1 are shown as well. Magnification, x10 and x 20 (inner photo). C and D, the average EphA2 and ephrinA1 expression scored on a scale of 1 to 3, respectively. Slides were analysed from n=4 mice per group (\*,  $p < 0.05$ ).

### 5.6 EphA2 blockade does not alter cell viability of irradiated A549 cells

To explore the role of EphA2 on tumor cell radiosensitivity, A549 cells were irradiated with a single dose of 4 Gy and RPMI medium was supplemented with or without the soluble EphA2-Fc receptor chimera (R&D Biosystems, Germany) at a concentration of 5  $\mu\text{g/ml}$  to block EphA2 signalling. Unirradiated cells with both inhibited and normal EphA2 status were used as a control. As it is shown in figure 17A, no statistically significant difference was observed in metabolic activity of A549 cells during the first 5 days post-irradiation upon EphA2 blockade in comparison to the unblocked cells analysed using proliferation assay ( $p > 0.05$ ). Similarly, in the unirradiated group, the viability of A549 cells remained unchanged after inhibition of EphA2 as compared to the unblocked cells (Fig. 17B).



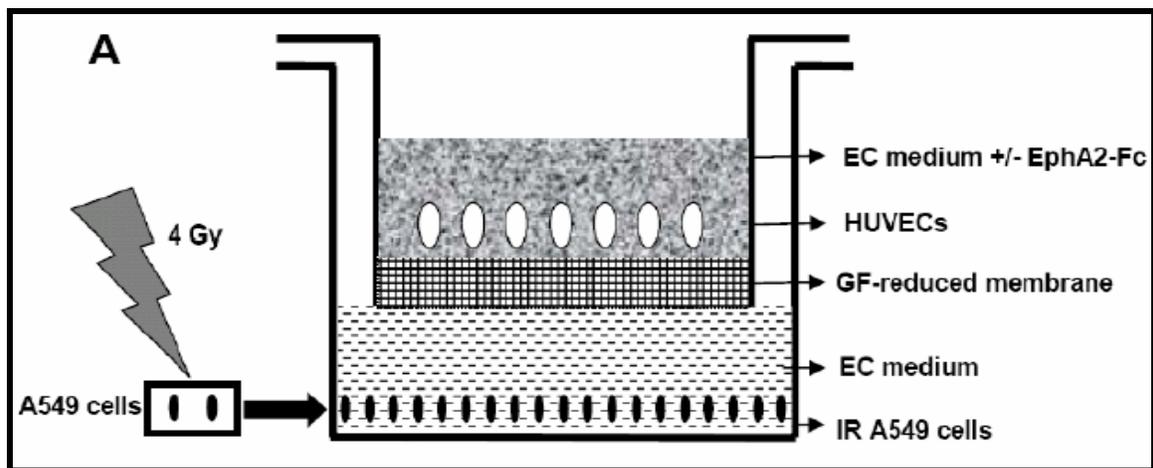
**Figure 17. Effect of EphA2 blockade on the viability of irradiated lung adenocarcinoma cell.** The effect of EphA2 on A549 cell radiosensitivity was assessed using WST proliferation assay. A549 cells were irradiated with 4 Gy in RPMI medium supplemented with or without the soluble EphA2-Fc receptor at a concentration of 5µg/ml to block EphA2 signalling. A control (unirradiated group) was used as well. Upon blocking of EphA2 RTK signalling, no major difference was observed in the viability of irradiated (4 Gy) and control (0 Gy) A549 cells. Points represent mean from one experiment done in quadruplicate (bars, mean ± SE; p>0.05)

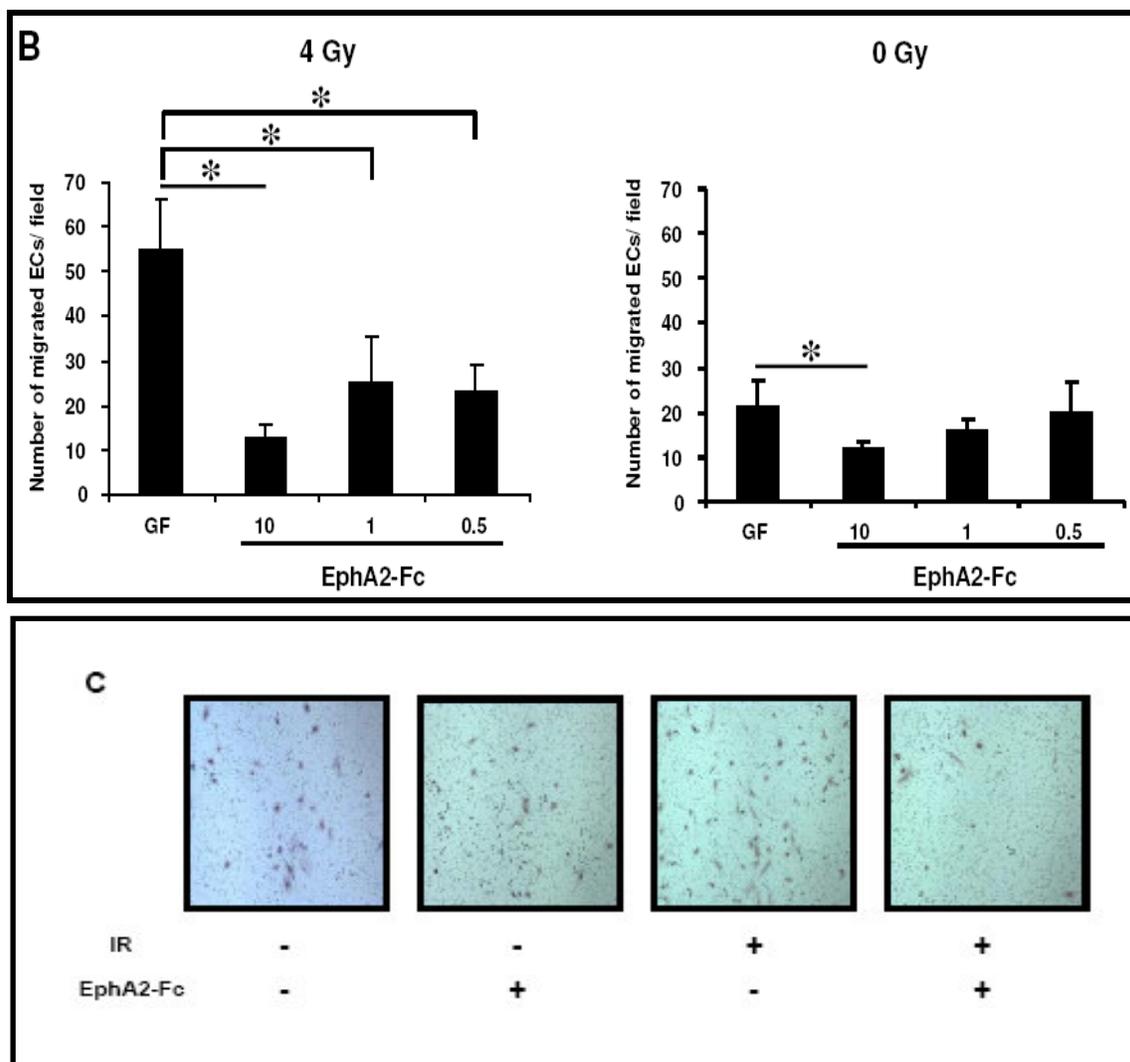
### 5.7 EphA2-blockade significantly reduces migration of ECs induced by irradiated A549 cells

One of the major characteristics of new blood vessel formation is migration of ECs. We performed a Matrigel migration assay after coculturing irradiated A549 cells together with ECs. The Matrigel membrane separating tumor from endothelial cells contained reduced growth factor in order to minimise external artificial chemotactic stimulation as shown in figure 18A. Irradiation of A549 cells was able to stimulate migration of ECs towards the tumor cell compartment. Blockade of EphA2 using the soluble EphA2-Fc receptor chimera strongly suppressed migration of ECs induced by irradiated A549 cells ( $p < 0.05$ , Fig. 18B and C). No growth factors such as VEGF, PDGF or bFGF were contained in the serum.

### 5.8 Unirradiated A549 cells can also induce EphA2-mediated migration of ECs but in a less potent manner

To investigate whether unirradiated tumor cells are also able to confer EphA2-mediated chemotactic stimulus to ECs, we cocultured the unirradiated A549 cells with ECs. The Matrigel migration assay of ECs cocultured with unirradiated A549 cells was performed similarly to the irradiated one. Matrigel migration assay revealed that unirradiated A549 cells stimulated migration of ECs but in a less potent manner. Blockade of EphA2 decreased migration of ECs induced by unirradiated A549 cells when  $10\mu\text{g/ml}$  soluble EphA2-Fc chimera antibody was used [ $p < 0.05$ ] (Fig. 18 B and C).



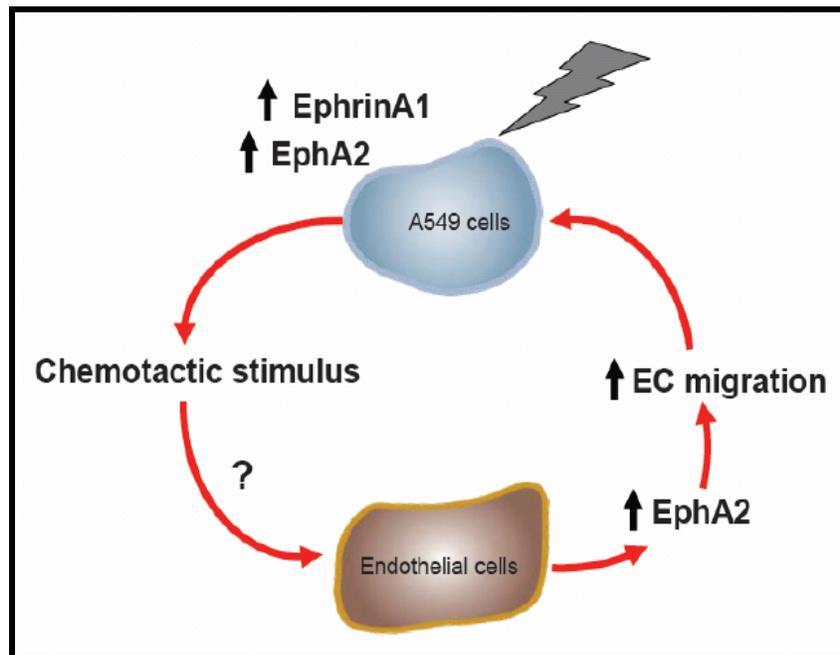


**Figure 18. EphA2-blockade significantly reduces migration of ECs induced by IR of A549 cells.** A, diagram of the co-culture model used for the migration assay. The Matrigel membrane separating tumor from endothelial cells contained reduced GF in order to minimise chemotactic stimulation. Migrated ECs are shown on the undersurface of the Matrigel membrane. Irradiated A549 cells were seeded in the lower compartment of the invasion chamber while ECs were added into the inserts of the transwells and allowed to migrate towards tumor cells. B, data are presented as the average number of ECs that migrated through Matrigel membrane in 4 different fields at 36 hours post-irradiation. Three different concentrations (0.5, 1 and 10μg/ml) of soluble EphA2-Fc receptor were used in order to suppress EphA2-signalling in ECs. Unirradiated A549 cells can also induce

*EphA2-mediated migration of ECs but in a less potent manner (mean  $\pm$  SE; \*,  $p < 0.05$ ), C, representative photos of ECs migrated to the underside of the Matrigel-coated insert were fixed and stained with Haematoxylin (magnification power x50). EphA2-Fc, soluble EphA2-Fc receptor chimera;*

### **5.9 Model of EphA2-mediated interaction between tumor and vascular compartment in response to IR.**

Irradiation of A549 cells activates EphA2 and its ligand ephrinA1. Additionally, it transmits a chemotactic stimulus to its associated vasculature leading to subsequent activation of EphA2 receptor. The latter results in recruitment of endothelial cells towards the tumor compartment, indicating EphA2-mediated endothelial cell migration as a new mechanism that tumor cells probably use to protect their vascular compartment from the cytotoxic effect of IR (Fig. 19).



**Figure 19.** A suggested model for the function of EphA2 involved in the IR-induced interaction between tumor and microenvironment.

## 6. DISCUSSION

Unequivocal evidence suggests that the Eph family of RTKs play an important role in tumor angiogenesis and pathogenesis. However, to the author's best knowledge, the effect of IR on these molecules in relation to tumor angiogenic response has not been previously investigated. This study demonstrates a novel relationship between EphA2 and IR in human lung adenocarcinoma and suggests the combined use of EphA2-blocking agents with radiotherapy in NSCLC.

Lung cancer is the most lethal form of malignancy and remains difficult to cure despite advances in surgery and adjuvant therapy (Jemal et al., 2006, Le Chevalier et al., 2004). The phenomenon of new blood vessel formation is one of the key events in lung cancer pathogenesis, associated with an adverse outcome (Herbst et al., 2005). Previous studies have shown high vascularity at the tumor periphery to be correlated with its progression while VEGF and PDGF were proven to have prognostic significance in NSCLC (O'Byrne et al., 2000). EphA2 receptor tyrosine kinase possesses a potent tumorigenic role (Zhang et al., 2006). Additionally, EphA2 can act as an oncoprotein and possess a powerful pro-angiogenic potential, directly involved in tumor cell growth and metastasis (Brantely-Sieders et al., 2005). Interestingly, high expression of EphA2 receptor in lung cancer closely correlated with brain metastases, predicting for an adverse patient outcome (Kinch et al., 2003).

Cheng *et al.* demonstrated a close relationship between EphA2 and VEGF in orchestrating tumor neovascularization (Cheng et al., 2002). In their study, a soluble EphA2-Fc receptor inhibited VEGF- but not basic FGF-induced endothelial cell survival, migration, sprouting, and corneal angiogenesis. Furthermore, EphA2 antisense oligonucleotides inhibited endothelial expression of EphA2 receptor and suppressed ephrinA1- and VEGF-induced cell migration (Cheng et al., 2002). VEGF-induced EphrinA1 expression in endothelial cells activated EphA2 receptor, while blockade of the latter inhibited VEGF-mediated endothelial cell survival, migration and invasion as well as VEGF-induced carcinogenesis, suggesting that EphA2 is a major downstream target of VEGF, mediating many of its effects (Cheng et al., 2003).

Surprisingly, the effect of IR on the Eph and ephrins has not been previously demonstrated, even though the latter consists the biggest family of RTKs. Intense research interest has

stemmed from the discovery that radiation prompts tumors to promote radioresistance in their vessels, and there is interest in blocking their response to improve treatment efficacy (Moeller et al., 2004, Jain et al. 2005). Radiotherapy is commonly used as an adjuvant tool for the treatment of NSCLC cancer (Le Chevalier et al., 2004), but has failed to display a survival benefit; despite the improvement in local control rate, patients often present with local recurrence and distant metastases (Wagner et al., 2005, Jeffrey et al., 2005). The importance of tumor microenvironment and vasculature in determining radiotherapy response and clinical outcome is increasingly highlighted. Still many factors remain to be elucidated regarding the interaction between cancer cells with tumor microenvironment upon IR. IR-induced tumor reoxygenation leads, through generation of reactive oxygen species (ROS), to activation of HIF-1- regulated cytokines (VEGF, bFGF) that stimulate tumor angiogenesis and survival, which way enhances radiation resistance of tumor cells (Moeller et al., 2004). Park et al. demonstrated an increase matrix metalloproteinase 2 (MMP-2) secretion and invasion of glioma cells post-irradiation through Src/EGFR-mediated p38/Akt and PI3K/Akt signalling pathways (Park et al., 2006). IR can mediate angiogenic mechanisms through up-regulation of VEGF, promoting in this way hepatic tumor cell growth (Chung et al., 2006). Sonveaux et al. provided evidence that IR alters the endothelial compartment and strongly activates tumor neovascularization through stimulation of nitric oxide (NO) pathway. In their study, the inhibition of NO production suppressed these provascular effects of irradiation, highlighting new potentials for the coordinated use of antiangiogenic strategies and radiotherapy in clinical practice. (Sonveaux et al., 2003).

The accumulating evidence supporting the pro-angiogenic role of IR formed the basis of the present study. The primary purpose was to investigate the impact of radiation therapy upon different members of Eph family of RTKs. The hypothesis that regulation of Eph/ephrin expression could be mediated by radiation therapy of tumor itself was investigated. In this present work, EphA2 receptor and its ligand ephrinA1 were up-regulated in A549 cells after irradiation. The most significant induction of EphA2/ephrinA1 in irradiated lung adenocarcinoma cells was noted for doses of 4 Gy and 8 Gy. In contrast, in HUVEC cells no prominent transcriptional activation of Eph family members was recognised.

The overexpression of EphA2 has been demonstrated in several malignancies (Kinch et al.,

2003, Wykosky et al., 2005, Duxbury et al., 2004, Lugli et al., 2005) and has been attributed in part to a decrease in c-CBL-mediated EphA2 degradation after binding to its ligand ephrinA1 (Walker-Daniels et al., 2002). In the present study, ephrinA1 revealed a strong induction in tumor cells upon IR. However, the fact that ephrinA1 ligand was upregulated in this study does not necessarily mean it is capable of binding to EphA2 receptor and lead to its degradation (Davis et al., 1994). Indeed, tumor cells can still contain ephrinA1 but they are probably present in a soluble, monomeric form, unable to bind EphA2 (Davis et al., 1994). Moreover, the net effect of EphA2-ephrinA1 interaction when two cells come in close contact to each other is strongly dependent on the degree of clustering (Pasquale et al., 2005). Indeed, low clustering degree has as a net effect an adhesion between adjacent cells while high clustering degree results in cell repulsion (Pasquale et al., 2005).

The second receptor-ligand pair was investigated EphB4/ephrinB2 in the present study. Numerous reports indicate EphB4/ephrinB2 as important Eph family members involved in tumor growth and angiogenesis. It has been shown that in the human umbilical cord, EphB2 acts as a specific marker for arterial endothelial cells, whereas ephrinB2 can be also expressed by venous endothelial cells. The role of EphB4 and its ligand ephrinB2 in endothelial cells are not limited only to the topographical orientation of the latter towards each other. EphB4 acts as a negative regulator of blood vessel branching and vascular network formation, switching the vascularisation program from sprouting angiogenesis to circumferential vessel growth. In parallel, EphB4 reduces the permeability of the tumor vascular system via activation of the angiopoietin-1/Tie2 system at the endothelium/ pericyte interface. Furthermore, overexpression of EphB4 variants in blood vessels during (i) vascularization of non-neoplastic cell grafts and (ii) retinal vascularisation revealed that these functions of EphB4 apply to postnatal, non-neoplastic angiogenesis in general. This implies that both neoplastic and non-neoplastic vascularization is driven not only by a vascular initiation program but also by a vascular patterning program mediated by guidance molecules (Erber et al., 2006). In venous endothelial cells, VEGF up-regulates DLL4 and presenilin, and increases the activation of Notch4, leading to an up-regulation of ephrinB2 with a downregulation of EphB4. The activation of Notch4 is required for VEGF-induced up-regulation of ephrinB2 and the differentiation of human venous endothelial cells in vitro.

Accordingly, the disruption of Notch4 signaling by pharmacologic inhibition of presenilin or addition of soluble DLL4 inhibited the effect of VEGF on human venous endothelial cell migration and differentiation. A coordinated activation of DLL4/Notch4 and ephrinB2 pathways downstream of VEGF plays a key role in the abnormal remodeling of tumor vessels (Hainaud et al., 2006). Previous studies did not prove any significant expression or role of EphB4/ephrinB2 in lung cancer, although there are numerous reports indicating them as important regulators of tumor growth and angiogenesis (Erber et al., 2006, Noren et al., 2004, Kertesz et al., 2006). Indeed, no significant response of EphB4/ephrinB2 expression was found in A549 cells upon irradiation. These findings are in line with previous reports confirming the absence of EphB4/ephrinB2 expression in lung adenocarcinoma.

IR of tumor vasculature leads to the release of various growth factors such as VEGF and bFGF from tumor cells, which can regulate important processes such as endothelial cell survival and determine in this way treatment response (Moeller et al., 2004). For this reason, the expression of EphA2/ephrinA1 and EphB4/ephrinB2 after irradiation of ECs was analysed. Importantly, no major expression of these genes was detected upon irradiation of ECs. Previous studies have demonstrated that all 4 members of the Eph family of RTKs might contribute to a certain extent to normal and tumor new blood vessel formation and can be expressed in endothelial cells (Heroult et al., 2006, Erber et al., 2006, Hainaud et al., 2006, 109, Cheng et al., 2002, Noren et al., 2004, Kertesz et al., 2006, Landen et al., 2006). IR alone, without additional growth factors, results in decreased proliferation, capillary tube formation, migration and survival of cultured endothelial cells (Bergers et al., 2003, Geng et al., 2001). On the other side, the process of angiogenesis is directly dependent on the interplay between pro-angiogenetic and anti-angiogenetic factors that finally determine the survival of endothelial cells (Bergers et al., 2003). Therefore, it is tempting to speculate that upon irradiation of ECs, the anti-angiogenic signals might overcome pro-angiogenic ones. The direct adverse effect of IR on endothelial cells reported in previous studies (Folkman et al., 2001, Bergers et al., 2003) is in line with this theory. At the same time, they are supported by the data in the present study, since no Eph RTK family member was activated in endothelial cells, at least in transcriptional level.

Furhtermore, the expression of EphA2 receptor in irradiated A549 tumor xenotransplants in

vivo was analysed in this study. Mice were treated with single dose of 20 Gy. The use of such single, high radiation dose is equivalent to a full curative dose applied in clinical radiotherapy. Several groups have previously emphasized the importance of using a high irradiation dose for studying parameters such as gene expression, hypoxia and angiogenic response in tumor xenografts (Schuuring et al., 2006, Znati et al., 1995, Schuuring et al., 2005). Additionally, A549 is a prominently radioresistant tumor line (Cocco-Martin et al., 1994) which was the rationale for the large radiation dose, suggesting that a lower IR dose would affect only the extent of the observed changes in the tumor microenvironment, with no effecting the kinetics of the cellular responses to irradiation (Schuuring et al., 2006). Interestingly, a more intense signal of EphA2 expression was detected in the irradiated group, in comparison to the unirradiated group. Previous study which has revealed the potential of IR to induce various growth factors such as VEGF in vivo conditions supported the present results. (Heissig et al., 2005). It should be emphasized here that the use for the in vivo experiments of a dose similar to the one that revealed induction of EphA2 in vitro (e.g. 4 Gy or 8 Gy) would probably yield different results. Indeed, irradiation of xenografts encapsulates a multi-orchestrated response involving not only tumor cells, as it is in monolayer cell culture in vitro, but also vascular and connective tissue and probably components of the immune system. Therefore, distinct differences in the molecular response of various genes to IR should be anticipated between in tumor cells and xenografts (Moeller et al., 2004, Tsai et al., 2007). Besides, changes in gene expression have been noted between single and fractionated irradiation between in vitro and in vivo in several cancer cells lines (Tsai et al., 2007). Of note, the increased EphA2 expression was also confirmed in xenografts irradiated with single fraction of 2 Gy, which is a clinically relevant dose.

Furthermore, the involvement of EphA2 in the viability of tumor cells response to IR was examined. However, no change was found in the proliferation of tumor cells after inhibiting EphA2 receptor with a soluble EphA2-Fc receptor, both in the irradiated and the untreated control group. These results are in agreement with previous studies which have shown EphA2 to play no major role in the proliferation of tumor cells in a monolayer cell culture (Wykosky et al., 2005, Landen et al., 2006). Moreover, they are in line with the common concept that potent antiangiogenic agents do not affect tumor cell viability, but instead,

endothelial cell viability, at least in vitro conditions (Wykowsky et al., 2005). These data indicate that proliferation of both irradiated and unirradiated A549 cells may be independent of the EphA2 status. The activation of EphA2 signaling pathway can probably affect tumor radiosensitivity in an indirect manner, through modulation of tumor vessel formation.

Tumor cells possess different mechanisms to escape cell death and protect the tumor from the cytotoxic effect of IR. Such a potential mechanism is the secretion and induction, upon irradiation, of several cytokines and growth factors from irradiated tumor cells, which may involved as proangiogenic factors in the formation of new blood vessels (Abdollahi et al., 2003, Geng et al., 2001). The neoangiogenetic process, in turn, enhances survival of tumor itself (Geng et al., 2001) and can render tumor cells more radioresistant (Geng et al., 2001, Lee et al., 2000). This impressive mechanism consisted a major rationale for using anti-angiogenic agents in combination with radiation therapy for the treatment of many types of tumors, including lung cancer, as performed in daily clinical practice (Jain et al., 2006). In this study, IR could induce EphA2 and EphrinA1 in tumor cells. Previous studies have shown that both of these members are powerful players in the angiogenetic field (Heroult et al., 2006, Erber et al., 2006, Hainaud et al., 2006, 109, Cheng et al., 2002, Noren et al., 2004, Kertesz et al., 2006, Landen et al., 2006, Brantley-Sieders et al., 2006). It was therefore asked whether irradiation-induced EphA2/EphrinA1 could consist another indirect mechanism to increase radiation resistance of tumor cells, through stimulation of angiogenetic process. The angiogenetic assay in this study revealed increased migration of ECs after co-culture with irradiated tumor cells. Moreover, the increased migration of ECs could be significantly reduced after blockade of EphA2 with a soluble EphA2-Fc receptor. This model of elevated activation of EphA2 may account for the observations that sublethal doses of tumor IR promote migration and invasiveness of glioblastoma in rats in vivo (Wild-Bode et al., 2001). These findings provide for the first time important information for the biological importance of EphA2 in radiation-induced angiogenetic mechanisms.

What makes EphA2/ephrinA1 RTKs an attractive source of study in tumor biology? Could the soluble EphA2-Fc receptor be a promising therapeutic strategy for cancer therapy? Co-expression of EphA2 RTK and its principle ligand, ephrinA1, in both tumor cells and tumor endothelium suggested that this receptor-ligand pair might contribute to tumor

angiogenesis (Sawai et al., 2003). Similar expression patterns were also observed in two independent mouse models of angiogenesis-dependent cancer, the RIP-Tag transgenic model of islet cell adenocarcinoma and the 4T1 transplantable model of mammary epithelial adenocarcinoma. Expression of ephrinA1 was predominantly detected in tumor cells, while the majority of EphA2 RTK protein localized to tumor associated endothelium, suggesting that ephrin-A1 might serve as a pro-angiogenic signal to attract EphA2-positive endothelial cells (Brantley et al., 2002). Soluble receptor (EphA2- or EphA3-Fc) treatment of 4T1 tumors transplanted into syngeneic Balb/c mice resulted in decreased tumor volume, proliferation, and survival accompanied by a decrease in microvascular density within the tumor. EphA-Fc proteins also impaired endogenous tumor progression in RIP-Tag mice. Soluble EphA-Fc proteins did not alter growth or survival of tumor cells in culture, suggesting that the effects observed in vivo were secondary, likely due to impaired recruitment of blood vessels supplying nutrients and oxygen necessary for growth and survival of the tumor RIP-Tag in cutaneous window assays (Brantley et al., 2002). Though soluble receptors do not affect proliferation or apoptosis in cultured endothelial cells, EphA-Fc proteins do impair ephrin-A1 and VEGF-induced cellular migration and sprouting, suggesting that these reagents might interfere with tumor angiogenesis at the level of endothelial migration (Cheng et al., 2002). Indeed, EphA2-Fc inhibited 4T1 and RIP-Tag tumor cell-induced migration of endothelial cells in co-culture assays. A recent study further confirmed the anti-angiogenic activity of soluble EphA2-Fc. In this report, soluble EphA2-Fc inhibited outgrowth of new vessel sprouts from explanted aortic rings, while EphB1-Fc and EphB3-Fc induced microvessel sprouting, suggesting that EphA and EphB RTKs might have different functions in angiogenesis (Dobrzanski et al., 2004). Soluble EphA2-Fc also inhibited VEGF/FGF induced neovascularization, progression, and metastasis of orthotopic human pancreatic ductal adenocarcinoma (Dobrzanski et al., 2004). EphA2-deficient mice display defective angiogenic remodeling of endogenous subcutaneous vessels in response to ephrinA1, suggesting that EphA2 RTK is necessary for post-natal angiogenesis and possibly for tumor angiogenesis (Brantley-Sieders et al., 2004). EphA2-Fc treatment not only impairs ephrinA1-induced corneal angiogenesis, and also significantly inhibits corneal neovascularization in response to VEGF Soluble EphA receptors also inhibit

VEGF mediated endothelial cell survival, sprouting, migration, and assembly in vitro Since VEGF, unlike ephrin ligands, is a soluble signal, VEGF signaling may initiate angiogenesis by activating host endothelial cells and inducing proliferation, a process not affected by EphA2-Fc (Cheng et al., 2003). Once host vessels infiltrate the tumor, membrane-bound ephrinA ligands may be able to bind to EphA2 RTK on adjacent endothelial cells to facilitate migration and assembly into functional tumor vessels. Alternatively or in addition to this mechanism of activation, VEGF may also modulate ephrin-A1 expression and subsequent function within endothelial cells (Zhang et al., 2006).

Treatment of cultured endothelial cells with VEGF enhances expression of ephrin-A1 and subsequent phosphorylation of EphA2. It is therefore possible that juxtacrine ephrin-EphA2 signalling initiated by VEGF could contribute to vascular remodeling. Studies performed in cultured cells suggest that ephrin-A1 may also mediate retraction of vascular smooth muscle cells in vascular remodelling (Cheng et al., 2002). The antivascular effects of EphA2 reduction were confirmed in an orthotopic ovarian cancer mouse model. Tumors were treated with an anti-EphA2 Ab (EA5) that resulted in reduced microvascular density, proliferation, and VEGF protein and mRNA levels and phosphorylated Src, paralleled by increased endothelial cell apoptosis. (Landen et al., 2006).

In contrast to the typical model of receptor tyrosine kinase activation where the ligand is a soluble factor secreted and subsequently binding to a transmembrane receptor, both Eph receptors and ephrin ligands are membrane-tethered and therefore activation of EphA2 by EphrinA1 prompts the close attachment of two cells to each other (Poliakov et al., 2005). In the migration experiment performed in this study, the membrane separating A549 cells from ECs has 8 $\mu$ m width and probably prevents this close attachment. A careful work recently published by Sieders et al. showed that ephrinA1-mediated modulation of VEGF pathway in tumor cell-conditioned medium can regulate angiogenic responses from initially distant host endothelium (Brantley-Sieders et al., 2006). Hence, irradiation-induced EphrinA1 found in this experiment could consist the first step to tumor angiogenesis induced by IR. Furthermore, our results on the tumor-endothelium communication suggest that the tumor compartment can produce survival factors for ECs, probably by paracrine signaling from radiation damage. It might be a chemotactic stimulus elicited by tumor cells that activates

EphA2 in ECs, with the most probable candidate being VEGF (Brantely-Sieders et al., 2006). The implication is that clinically radioresistant and radiosensitive tumors may differ, at least in part, because of differences in their ability to protect their vasculature and EphA2 can be a previously unrecognized contributing factor. With respect to the consequences of clinical radiotherapy, the co-culture data may also suggest that radiation effects may not necessarily be restricted to the site of physical radiation dose distribution, but can enhance tumor angiogenesis and tumor promotion outside the directly irradiated fields. The effects of radiotherapy can extend beyond the death of the target tumor cells, in that factor production/activation by such cells has been observed to influence the local environment for some time thereafter (Hlatky et al., 1996).

At present, there is great interest in combining antiangiogenic/vascular targeting strategies with conventional cytotoxic therapies such as radiotherapy to improve therapeutic gain. The mechanisms of tumor response to radiation, however, are not completely understood. Current approaches in clinical cancer therapy favor multimodal strategies. One rationale is the concept that the side effects of different therapies do not overlap. The combination of irradiation and chemotherapy has become a standard treatment and is associated with improved survival rates in many tumors. The simultaneous combination of radiotherapy and antiangiogenesis drugs has been used effectively in the clinic. These findings suggest a radiation-inducible protective role for tumor cells in the support of their associated vasculature that may be down-regulated by coadministration of angiogenesis inhibitors. These data suggest that, given the disparate modes of action, the proposed combination of irradiation and antiangiogenesis using an EphA2 receptor antagonist might have clinical potential as an anticancer strategy.

In conclusion, the first evidence of potential role of EphA2/ephrinA1 signaling pathway in IR-induced tumor angiogenesis is provided in the present study. These data propose a new mechanism that tumor cells might use to protect their vascular compartment from the cytotoxic effect of IR and suggest the use of EphA2 inhibitors in combination with radiation therapy for the treatment of NSCLC.

## **8. SUMMARY**

Lung cancer is the most lethal form of malignancy and prognosis remains poor despite recent advances in diagnostic and therapeutic modalities. The phenomenon of new blood vessel formation in tumors is one of the key events in lung cancer pathogenesis and metastasis. During embryonic development and angiogenesis, there is an impressive interplay between Eph receptors and Ephrin ligands expressed in EC and associated tissues that greatly affect the structure of new blood and lymphatic vessels. The Eph family of receptor tyrosine kinases (RTKs) and their ligands, ephrins, are dysregulated in different malignancies and play an important role in tumor blood vessel formation, remodelling and metastasis. The response of tumor to ionizing radiation (IR) is highly-dependent on tumor microenvironment and involves a series of complex biological interactions between tumor and vascular compartment. However, the influence of IR on the Eph family of RTKs remains unknown. During the last decades, radiobiological research has focused primarily on the cancer cell compartment. Indeed, much less is known about the effect of ionizing radiation on the endothelial cell (EC) compartment and the complex interaction between tumor cells and their microenvironment, consisting of extracellular matrix and ECs. In the present study, the effect of IR on the key members of the Eph family of RTKs in both A549 and Ecs was analysed both in vitro and in vivo. Moreover, the way the two-compartment system, consisting of tumor and ECs, intercommunicates in response to IR and the impact of Eph on this interaction was analysed.

The primary aim of this study was to elucidate the mechanisms of the combination of novel antiangiogenic agents, such as Eph-blocking agents, and IR for effective treatment of lung cancer. In this thesis, a critical review of angiogenesis and the biology of Eph and Ephrins was performed.

Furthermore, the interrelation between IR and Eph family of RTKs was investigated. The experimental results suggested that IR can transmit a proangiogenic stimulus to tumor associated vascular compartment through inducing different members of Eph/ephrins in tumor cells. IR promoted transcriptional activation of EphA2 and its ligand ephrinA1 but not EphB4/ephrinB2 in lung adenocarcinoma cells in vitro, while none of these members analysed was induced in irradiated ECs. Immunofluorescence detection of EphA2 revealed a

stronger membranous staining in irradiated lung adenocarcinoma xenografts. Immunohistochemical studies revealed in irradiated A549 xenografts revealed stronger expression for EphA2 and ephrinA1, as compared to unirradiated tissues. Furthermore, no difference was observed in the viability of lung adenocarcinoma cells *in vitro* after IR and EphA2 blockade using a soluble EphA2-Fc receptor chimera as analysed by an WST assay. IR of lung adenocarcinoma cells and immediate co-culture with ECs increased endothelial cell migration *in vitro*. EphA2 blockade significantly suppressed irradiated tumor-induced endothelial cell migration *in vitro*. This is the first demonstration to show the involvement of different members of the Eph/Ephrins in IR induced tumor angiogenesis both *in vitro* and *in vivo*. These data propose a new mechanism that tumor cells probably use to protect their vascular compartment from the cytotoxic effect of IR and rationalise the use of EphA2 inhibitors in combination with radiation therapy for the treatment of lung adenocarcinoma.

## **8. ZUSAMMENFASSUNG**

Das Lungenkarzinom ist weltweit die am häufigsten zum Tode führende Krebserkrankung in der westlichen Welt und hat nach wie vor eine sehr schlechte Prognose trotz moderner Fortschritte in Diagnostik und Therapie. Die angiogenetischen Prozesse sind für das lokale Wachstum und die systemische Metastasierung von Tumoren von essentieller Bedeutung. Angiogenetische Endothelzellen unterscheiden sich von ruhenden Endothelzellen durch die Expression einiger Rezeptoren auf ihrer Zelloberfläche wie die Eph Familie. Die Interaktion zwischen Eph-Rezeptoren und ephrin-Liganden, die in den Endothelzellen und zugehörigen Geweben exprimiert sind, hat großen Einfluss auf die Neubildung der Blut- und Lymphgefäße in embryonaler Entwicklung. Sowohl die Eph-Rezeptoren als auch die ephrin-Liganden sind häufig in verschiedenen bösartigen Tumoren dysreguliert und spielen eine wichtige Rolle in der Angiogenese und Metastasierung der Tumore. Jedoch bleibt der Einfluss ionisierender Strahlung auf die Eph-Familie von RTKs ungeklärt. Die Response der Tumorgewebe auf ionisierende Strahlung ist hoch abhängig von der Tumorumgebung. Die Mechanismen der Wirkung ionisierender Strahlung auf Tumorendothelzellen und die induzierte Interaktion zwischen Tumorzellen und Tumorumgebung sind bisher wenig bekannt.

Das Ziel der vorliegenden Arbeit ist die Wirkung ionisierender Strahlung auf die Eph-Familie in der Lungenkarzinomzelle A549 und Endothelzellen in vitro und in vivo zu analysieren. Außerdem soll die funktionelle Interaktion zwischen Tumorzellen und Endothelzellen, besonders die Rolle der Eph-Familie in der Tumor-Umgebung-Kommunikation auf ionisierende Strahlung untersucht werden. Eine Blockade des angiogenetischen Prozesses oder ein gezieltes Töten von angiogenetischen Zellen ist eine potenziell vielversprechende Krebstherapie. Das Hauptziel der Arbeit ist, die Mechanismen und die Wirkung der Kombination von neuen Eph Inhibitor und ionisierender Strahlung bei Krebstherapie zu erläutern.

In der vorliegenden Arbeit wird ein kritischer Überblick über klinische und biologische Aspekte der angiogenetischen Faktoren bzw. die Biologie der Eph/ephrin Familie beim Lungenkarzinom dargestellt.

Des Weiteren wurden in dieser Arbeit die Mechanismen der Eph Familie in funktioneller Interaktion zwischen Tumor- und Endothelzellen untersucht. Die experimentellen Ergebnisse könnten darauf hinweisen, dass ionisierende Strahlung mit der angewendeten Dosis die Expression unterschiedlicher Mitglieder von Eph/ephrin in Tumorzellen induziert und damit die Tumorangiogenese stimuliert werden konnte. Dabei konnte eine transkriptionelle Aktivierung von EphA2 und seinem Liganden ephrinA1, aber nicht EphB4/ephrinB2 in Lungenadenokarzinomzellen *in vitro* gezeigt werden, während die analysierten Faktoren bei bestrahlten Endothelzellen nicht induziert werden konnten. Mit Hilfe der Immunfluoreszenz-Untersuchungen wurde eine stärkere Membranfärbung der EphA2 in bestrahlten Xenotransplantaten von Lungentumorzelle A549 gefunden. Immunohistochemische Analysen zeigten stärkere Expression der EphA2 und ephrinA1 in bestrahlten A549-Xenotransplantaten im Vergleich mit den nicht bestrahlten Geweben. Weiterhin wurde mit Hilfe des WTT-Assay festgestellt, dass die Inhibition der EphA2 durch lösliche EphA2-Fc Rezeptorchimäre keine Wirkung auf Viabilität der Lungenadenokarzinomzellen *in vitro* nach ionisierender Strahlung hat. Im Gegenteil konnte erhöhte Migration der Endothelzellen in Kokultivierung mit bestrahlten A549-Zellen *in vitro* festgestellt werden. Eine Blockade des EphA2-Rezeptors könnte die Tumor-induzierte Migration der Endothelzellen signifikant vermindern. Die vorliegende Arbeit zeigte erstmals die mögliche Beteiligung verschiedener Mitglieder von Eph/ephrin an der induzierten Tumorangiogenese durch ionisierende Strahlung sowohl *in vitro* als auch *in vivo*. Die vorliegenden Daten könnten auf einen neuen Mechanismus hinweisen, dass die Tumorzellen wahrscheinlich ihr vaskuläres System vor der zytotoxischen Wirkung ionisierender Strahlung schützen können, um so durch die Verwendung der EphA2-Inhibitoren in Kombination mit Strahlentherapie die Behandlung des Lungenadenokarzinoms zu rationalisieren.

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## **10. APPENDIX**

### **10.1. ACADEMIC TEACHERS**

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## 10.2 DECLARATION

Emmanouil Fokas  
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Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Humanmedizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel

*“The effect of irradiation on the Eph family of receptor tyrosine kinases in human lung adenocarcinoma”*

am Zentrum für Radiologie der Philipps-Universität Marburg, Klinik für Strahlentherapie, unter Leitung von Frau Prof. Dr. med R. Engenhardt-Cabillic ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Ich habe bisher weder an einem in- und ausländischem medizinischem Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit oder eine andere Arbeit als Dissertation vorgelegt.

Marburg, März 2008

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