TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

*SARJA - SER. D OSA - TOM. 791*

MEDICA - ODONTOLOGICA

# ErbB Ligands in Angiogenesis

by

Erika Iivanainen

TURUN YLIOPISTO
Turku 2008

From the Department of Medical Biochemistry and Molecular Biology, and MediCity Research Laboratory, University of Turku, and Turku Graduate School of Biomedical Sciences, Turku, Finland

***Supervised by***

Professor Klaus Elenius, MD, PhD
Department of Medical Biochemistry and Molecular Biology and
MediCity Research Laboratory
University of Turku
Turku, Finland

***Reviewed by***

Professor Lena Claesson-Welsh, PhD
Department of Genetics and Pathology
Rudbeck Laboratory
Uppsala, Sweden

*and*

Dr. Mikko Savontaus, MD, PhD
Turku Centre for Biotechnology
University of Turku
Turku, Finland

***Opponent***

Professor Taina Pihlajaniemi, MD, PhD
Department of Medical Biochemistry and Molecular Biology and
Collagen Research Unit
University of Oulu
Oulu, Finland

ISBN 978-951-29-3474-4 (PRINT)
ISBN 978-951-29-3475-1 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2007

*To my parents*

**Erika Iivanainen**

**ErbB ligands in angiogenesis**

Department of Medical Biochemistry and Molecular Biology, MediCity Research Laboratory, and Turku Graduate School of Biomedical Sciences (TuBS), University of Turku, Turku, Finland
Annales Universitatis Turkuensis
Painosalama Oy, Turku, Finland 2007

# ABSTRACT

The formation of new blood vessels, *i.e.* angiogenesis, is an important phenomenon during normal development and wound repair, as well as during various pathological processes, such as tumor growth and metastasis. Specific growth factors regulate angiogenesis by modulating the different cellular functions of endothelial cells (EC), and periendothelial cells, such as pericytes (PC) and smooth muscle cells (SMC), which interact with ECs in a paracrine manner.

ErbB receptors form a subgroup of transmembrane receptor tyrosine kinases that interact with growth factors of the epidermal growth factor (EGF) family. ErbB receptors regulate behaviour of a variety of normal as well as tumor cell types. Cancer drugs that target epidermal growth factor receptor (EGFR, ErbB1) or ErbB2 receptor have been approved for clinical use. It has been speculated that part of the antitumor activity of ErbB inhibitor compounds result from an antiangiogenic mechanism.

The results presented here indicate a role for endothelial-derived EGF-like growth factors heparin binding EGF-like growth factor (HB-EGF) and neuregulin-1 (NRG-1) in the paracrine regulation of angiogenesis. HB-EGF, EGFR and ErbB2 are shown to mediate interaction between ECs and SMCs *in vitro*, and gefitinib, an inhibitor of EGFR kinase activity, suppresses recruitment of PCs/SMCs *in vivo*. NRG-1 is shown to regulate EC functions *in vitro* and angiogenesis *in vivo* by indirect mechanisms involving vascular endothelial growth factor-A (VEGF-A) and VEGF receptor-2 (VEGFR-2). Furthermore, EGFR activity is demonstrated to regulate recruitment of bone marrow-derived perivascular cells during tumor neovascularization *in vivo*.

These results indicate that ErbB signaling is involved in the cellular processes of new blood vessel formation. This study gives new information about the role of ErbB ligands and receptors in angiogenesis and vasculogenesis and about the mechanisms by which ErbB inhibitor drugs such as gefitinib affect tumor growth.

**Key words**: angiogenesis, EGFR, ErbB, endothelial cell, HB-EGF, NRG-1, smooth muscle cell, vasculogenesis

**Erika Iivanainen**

**ErbB ligandit verisuonten uudismuodostuksessa**

Lääketieteellinen biokemia ja molekyylibiologia, MediCity tutkimuslaboratorio ja Turun biolääketieteellinen tutkijakoulu (TuBS), Turun yliopisto, Turku
Annales Universitatis Turkuensis
Painosalama Oy, Turku 2007

# TIIVISTELMÄ

Verisuonten uudismuodostusta eli angiogeneesiä tarvitaan mm. alkion kehityksen ja kudosvaurion paranemisen yhteydessä, sekä useissa patologisissa tiloissa kuten syövän kasvussa ja metastasoinnissa. Angiogeneesiä säätelevät erilaiset spesifit kasvutekijät, jotka vaikuttavat endoteelisolujen sekä endoteelisolujen kanssa parakriinisessa vuorovaikutuksessa olevien perisyyttien ja sileiden lihassolujen toimintaan.

ErbB-reseptorit muodostavat reseptorityrosiinikinaasiperheen, jonka ligandeja ovat epidermaalisen kasvutekijän (EGF) kaltaiset kasvutekijät. ErbB-reseptorit säätelevät sekä monien normaalien että tuumorisolujen toimintaa. Kliinisessä käytössä on syöpälääkkeitä, joiden vaikutus kohdistuu EGF-reseptoriin (EGFR, ErbB1) tai ErbB2-reseptoriin. Näiden syöpälääkkeiden on ajateltu vaikuttavan tuumorin kasvuun osin angiogeneesin säätelyn kautta.

Tulostemme perusteella ErbB-ligandit, hepariinia sitova EGF:n kaltainen kasvutekijä (HB-EGF) ja neureguliini-1 (NRG-1) osallistuvat angiogeneesin parakriiniseen säätelyyn. HB-EGF:n, EGFR:n ja ErbB2:n osoitettiin säätelevän endoteelisolujen ja sileiden lihassolujen välistä vuorovaikutusta *in vitro*, ja gefitinibi, EGFR:n pienimolekulaarinen estäjä, vähensi sileiden lihassolujen kerääntymistä verisuoniin *in vivo*. NRG-1 puolestaan sääteli endoteelisolujen toimintoja *in vitro* ja angiogeneesiä *in vivo* epäsuorasti vaskulaarisen endoteelikasvutekijä-A:n (VEGF-A) ja VEGF-reseptori 2:n (VEGFR-2) välityksellä. Lisäksi hiirikokeissa, joissa käytettiin gefitinibiä, havaittiin EGFR:n osallistuvan luuydinperäisten solujen kulkeutumiseen syöpäkasvaimen verisuonten ympärille *in vivo*.

Tulosten mukaan ErbB-reseptorien kautta tapahtuva signalointi säätelee solutasolla uusien verisuonten muodostumista. Tutkimuksen avulla saatiin uutta tietoa ErbB-ligandien ja reseptorien merkityksestä angiogeneesissä sekä mekanismeista, joiden kautta EGFR:iin kohdistuvat syöpälääkkeet, kuten gefitinibi, vaikuttavat tuumorin kasvuun.

**Avaintermit:** angiogeneesi, endoteelisolu, EGFR, ErbB, HB-EGF, NRG-1, sileä lihassolu, vaskulogeneesi

# TABLE OF CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| ADAM | A disintegrin and a metalloproteinase |
| ADCC | Antibody-dependent cellular cytotoxicity |
| Ang | Angiopoietin |
| ATP | Adenosine triphosphate |
| AR | Amphiregulin |
| BM | Bone marrow |
| BTC | Betacellulin |
| CAM | Chorioallantoic membrane |
| CEP | Circulating endothelial progenitor cell |
| CM | Conditioned medium |
| DT | Diphteria toxin |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EGFP | Enhanced green fluorescence protein |
| EGFR | Epidermal growth factor receptor |
| EPC | Endothelial progenitor cell |
| EPG | Epigen |
| EPR | Epiregulin |
| FDA | Food and drug administration, USA |
| FGF | Fibroblast growth factor |
| Flk-1 | Fetal liver kinase-1 (mouse VEGFR-2) |
| Flt-1 | Fms-like tyrosine kinase-1 (VEGFR-1) |
| Flt-4 | Fms-like tyrosine kinase-4 (VEGFR-3) |
| HB-EGF | Heparin binding EGF-like growth factor |
| HER | Human epidermal growth factor receptor |
| HIF | Hypoxia-inducible factor |
| HNSCC | Squamous cell carcinoma of the head and neck |
| ICD | Intracellular domain |
| IL | Interleukin |
| kD | Kilodalton |
| KDR | Kinase-insert domain containing receptor (human VEGFR-2) |
| mAb | Monoclonal antibody |
| MAPK | Mitogen-activated protein kinase |
| MMP | Matrix metalloproteinase |
| MVD | Microvascular density |
| NG2 | Neuron-glial antigen 2 |

| | |
|---|---|
| NRG | Neuregulin |
| NRP | Neuropilin |
| NSCLC | Non-small cell lung cancer |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PC | Pericyte |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PDGFR | Platelet-derived growth factor receptor |
| PECAM-1 | Platelet/endothelial cell adhesion molecule-1 |
| PI-3K | Phosphatidyl inositol 3-kinase |
| PKC | Protein kinase C |
| PMA | Phorbol 13-myristate 12-acetate |
| PlGF | Placental growth factor |
| RTK | Receptor tyrosine kinase |
| siRNA | Small-interfering RNA |
| SMA | Smooth muscle actin |
| SMC | Smooth muscle cell |
| TGF-α | Transforming growth factor-α |
| TGF-β | Transforming growth factor-β |
| Tie | Tyrosine kinase with Ig and EGF homology domains |
| TK | Tyrosine kinase |
| TKI | Tyrosine kinase inhibitor |
| TSH | Thyroid stimulating hormone |
| TSP-1 | Trombospondin-1 |
| VE | Vascular endothelial |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |
| vWF | von Willebrand factor, factor VII-related antigen |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-III)

**I** Iivanainen E[1]., Nelimarkka L[1]., Elenius V., Heikkinen SM., Junttila TT., Sihombing L., Sundvall M., Määttä J., Laine VJ., Ylä-Herttuala S., Higashiyama S., Alitalo K., Elenius K. (2003) Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor. FASEB J. 17: 1609-1621.
[1] These authors contributed equally to this work

**II** Iivanainen E., Paatero I., Heikkinen SM., Junttila TT., Cao R., Klint P., Jaakkola PM., Cao Y., Elenius K. (2007) Intra- and extracellular signaling by endothelial neuregulin-1. Exp Cell Res. 313: 2896-2909.

**III** Iivanainen E., Lauttia S., Zhang N., Tvorogov D., Kulmala, J., Grenman R., Salven P., and Elenius K. Effects of EGFR inhibition by gefitinib on tumor angiogenesis and vasculogenesis. Submitted.

# INTRODUCTION

Angiogenesis is necessary for normal development, reproduction and tissue repair as well as for many pathological conditions, such as tumor growth and metastasis. The observations that tumor growth needs angiogenesis (Folkman, 1971) and the finding of major growth factors that mediate angiogenesis (Ferrara et al., 1992) has extended the understanding of tumor growth and opened new therapeutic methods to treat neoplastic diseases.

Angiogenesis is characterized by the migration, proliferation and differentiation of endothelial cells (ECs) as well as the recruitment of vascular pericytes (PCs)/smooth muscle cells (SMCs) around ECs. The interaction between vascular ECs and SMCs is necessary for the formation of mature vascular structures. All these angiogenic functions are regulated by specific angiogenic growth factors (Conway et al., 2001).

Historically vasculogenesis has been thought to be a mechanism that occurs only during embryonic development. However, several recent studies have suggested that vasculogenesis can also occur after embryogenesis in adult organisms. Postnatal vasculogenesis is characterized by the mobilization of bone marrow (BM)-derived progenitor cells to hypoxic sites and incorporation into growing vessels (Rafii and Lyden, 2003).

The four transmembrane members of the ErbB receptor tyrosine kinase family are receptors for growth factors of the EGF family. Although the significance of ErbB signaling for the behaviour of tumor cells has been intensively studied (Yarden and Sliwkowski, 2001), relatively little is known about their role in tumor neovascularization. ErbB receptor research has led to the development of targeted cancer drugs, such as the therapeutic anti-EGFR (ErbB1) and -ErbB2 antibodies cetuximab and trastuzumab, and the EGFR kinase inhibitors gefitinib and erlotinib. Moreover, all these drugs have been shown to suppress tumor angiogenesis.

The aim of this study was to characterize the significance of ErbB ligands and receptors in angiogenesis. Novel information was obtained about the molecular and cellular mechanisms by which the ErbB signaling system regulates angiogenesis and postnatal vasculogenesis.

# REVIEW OF THE LITERATURE

## 1. FORMATION OF NEW BLOOD VESSELS

### 1.1. Vasculogenesis, angiogenesis and postnatal vasculogenesis

New blood vessels are required to supply oxygen and nutrients to the growing tissues during embryogenesis and adult life. Vascularization of tissues is accomplished by two processes: vasculogenesis and angiogenesis. During embryogenesis first blood vessels are formed by vasculogenesis where mesoderm-derived primitive precursor cells (hemangioblasts) differentiate into endothelial precursor cells (angioblasts) extra-embryonically in blood islands of the yolk sac. The periphery of the blood islands consists of angioblasts, whereas the central cells of a blood island are hematopoietic precursor cells. A hemangioblast is a common precursor cell for hematopoietic precursor cells and angioblasts. In response to growth factors, angioblasts in the blood islands start to proliferate and differentiate into endothelial cells and lumen formation occurs. Blood islands make connections and together with capillaries formed by intraembryonic angioblasts form the primary vascular plexus (Risau and Flamme, 1995; Conway et al., 2001).

After the formation of the primary vascular plexus, new blood vessels are formed by angiogenesis where local pre-existing vessels form capillary sprouts or become divided by periendothelial cells (intussuspection) or by transendothelial cells (bridging). The vasculature is nearly quiescent in the normal adult mammal. Physiological angiogenesis occurs mainly in the cycling ovary and in the placenta during the female reproductive cycle and pregnancy, respectively. Outside of the female reproductive system, angiogenesis in adults occurs mainly in pathological situations such as in cancer, psoriasis, arthritis, atherosclerosis and ocular neovascularization as well as during wound healing (Folkman, 1995; Risau, 1997; Carmeliet, 2000).

Until recently, vasculogenesis was thought to be a mechanism that is restricted to the formation of new blood vessels during embryonic development. However, several lines of recent evidence suggest that BM-derived cells capable of differentiating into ECs or PCs/SMCs contribute to the formation of new blood vessels also in adult organisms, a process known as postnatal vasculogenesis (Rafii et al., 2002; Asahara and Kawamoto, 2004; Lamagna and Bergers, 2006).

### 1.2. Cellular mechanisms of angiogenesis and postnatal vasculogenesis

Blood vessels are composed of endothelial cells (ECs), which form the inner lining of the vessel wall and periendothelial cells – referred to as pericytes, vascular smooth muscle cells, mural cells or Rouget cells. These two cell types are separated by the vascular basement membrane (Fig.1) (Hirschi and D'Amore, 1996). Both ECs and PCs are suggested to contribute to the formation of basement membrane (Mandarino et al., 1993).

### *1.2.1. Endothelial cells*

In response to an appropriate stimulus, normally quiescent vasculature can become activated and start to form new blood vessels. During the first steps of angiogenesis ECs need to loosen inter-endothelial cell contacts and detach periendothelial cell support, to destabilize mature vessels. Vascular permeability increases and the blood clotting protein fibrinogen leaks out of the vascular bed and forms a fibrin gel. For invasion into the underlying interstitium, ECs need to degrade and invade through insoluble fibrin, the underlying basement membrane, as well as the interstitial matrix. During vessel growth, proliferating endothelial cells migrate to new sites and assemble as solid cords that acquire a lumen (Carmeliet, 2000; Iivanainen et al., 2003).

Although ECs share several common characteristics, they also display considerable heterogeneity. Differences have been observed in their morphology, function and gene expression profile. Morphological diversity includes changes in size, shape and thickness. For instance, ECs on microvessels are flattened and elongated whereas the ECs of larger vessels are thicker and polygonal (Thorin and Shreeve, 1998). The functional heterogeneity of ECs includes differences in the regulation of vasoconstriction and vasodilatation, blood coagulation and anticoagulation, fibrinolysis, leukocyte homing, acute inflammation and wound healing. ECs also have different organ-specific functions, such as the involvement of brain ECs in the maintenance of the blood brain barrier. Gene expression differences have been observed between different populations of ECs; for instance between the ECs of arteries and veins, large and small vessels as well as normal and tumor vessels (Cleaver and Melton, 2003). Both environmental and genetic factors influence EC specialization (Conway and Carmeliet, 2004).

ECs can be characterized by specific cell surface marker proteins. Many of these proteins are also expressed by hematopoietic precursor cells or mature blood cells (Garlanda and Dejana, 1997). For instance, PECAM-1 (platelet/endothelial cell adhesion molecule-1, also referred to as CD31), a glycoprotein of the immunoglobulin superfamily, is expressed on the surface of some hematopoietic precursor cells, circulating platelets, subsets of leukocytes and in the intercellular junctions of ECs (Baumann et al., 2004). The expression of the marker protein can also vary between different sized vessels and between artery and vein. Factor VII-related antigen (vWF) is a plasma glycoprotein expressed more in the ECs of larger vessels and in venous ECs compared with microvessels and arterial ECs (Yamamoto et al., 1998). Vascular endothelial cadherin (VE-Cadherin) is an EC specific adhesion molecule expressed by most EC populations in large and small vessels, arteries and veins. It is localised at the cell-cell junctions of ECs. VE-Cadherin differs from other EC markers in that it is not expressed in blood cells or in hematopoietic precursor cells (Breier et al., 1996).

### *1.2.2. Pericytes and vascular smooth muscle cells*

Periendothelial cells, pericytes and vascular smooth muscle cells, are required to stabilize newly formed blood vessels and to give vessel both physical and chemical support. Without the support of periendothelial cells, vessels become leaky, hyperdilated, dysfunctional and regressive (Fig. 1) (Benjamin et al., 1998; Bergers and Song, 2005).

Periendothelial cells have different characteristics according to their location. In the microvasculature (capillaries) periendothelial cells only partially cover the vessels and are called pericytes. The periendothelial cells of large vessels (arteries and veins) are single or multilayered and generally called smooth muscle cells. Intermediate sized vessels, venules and arterioles, have periendothelial cells that have the characteristics of both PCs and SMCs. PCs and SMCs also differ in their location relative to the endothelium, their morphology and their expression of cell surface markers. PCs appear to be embedded in the basement membrane and have direct contact with ECs, whereas the basement membrane separates ECs and SMCs in smaller vessels, and basement membrane with a layer of mesenchymal cells and extracellular matrix, in larger vessels (Gerhardt and Betsholtz, 2003; Armulik et al., 2005).

Functional differences also distinguish PCs and SMCs. PCs may directly regulate EC-SMC communication, whereas SMCs are thought to contribute to vascular tone and contraction (Armulik et al., 2005). In addition, PCs have been suggested to have an active role during angiogenesis based on findings that they are found at angiogenic sprouts (Morikawa et al., 2002) and are a source of vascular endothelial growth factor-A (VEGF-A) (Darland et al., 2003). PCs also play a role in the regulation of capillary diameter and endothelial differentiation (Hellstrom et al., 2001).

PCs and SMCs can develop from various cell types depending on their location in the embryo. These include mesenchymal stem cells, neural crest cells and epicardial cells. For instance PCs/SMCs in the blood vessels of forebrain and cardiac outflow tract may derive from neural crest cells, and PCs/SMCs in the coronary vessels of the heart may derive from epicardial cells (Hungerford and Little, 1999). The relationship between endothelial and periendothelial cell lineages has also been addressed. ECs and PCs share a common progenitor cell in the embryonic mesoderm, the VEGFR-2-positive angioblast. This progenitor cell has been shown to differentiate into EC upon stimulation by VEGF-A and into a PC upon stimulation by platelet-derived growth factor-BB (PDGF-BB) (Yamashita et al., 2000). In addition, it has been suggested that ECs can transdifferentiate into SMCs (DeRuiter et al., 1997).

There is no general periendothelial cell-specific marker because of the diverse characteristics, functions and locations of these cells in various organs. Cellular markers used to identify periendothelial cells include alpha smooth muscle actin (α-SMA), NG2-proteoglycan, PDGF receptor-β (PDGFR-β) and desmin (Bergers and Song, 2005). α-SMA is an isoform of the cytoskeletal protein actin family normally restricted to smooth-muscle cell lineage cells. It is supposed to be a marker of differentiated PCs. α-SMA expression has been detected in the periendothelial cells of most intermediate sized vessels, but not in capillaries. Therefore, all PCs cannot be identified on the basis of α-SMA. In contrast, desmin, another contractile filament, as well as NG2, a chondroitin sulfate proteoglycan and PDGFR-β, a tyrosine kinase receptor, are also expressed in the immature periendothelial cells of developing microvasculature (Ozerdem et al., 2001; McDonald and Choyke, 2003; Bergers and Song, 2005).

### *1.2.3. Endothelial-pericyte/smooth muscle cell interaction*

During angiogenesis, the newly formed endothelial tubes recruit PCs/SMCs by secreting growth factors. PCs communicate with ECs by direct physical cell-cell contact as well as by paracrine signaling pathways (Bergers and Song, 2005). They influence endothelial permeability, proliferation, survival, migration, and maturation as well as stabilize the vessel structure (Morikawa et al., 2002; von Tell et al., 2006). Furthermore, PC/SMC loss has been associated with the onset of neovascularization (Orlidge and D'Amore, 1987). For instance in hemangiomas, where excessive EC proliferation leads to the vascular abnormalities, only few, if any, PCs/SMCs are observed (Feldman et al., 1978; (Orlidge and D'Amore, 1987).

The interaction between ECs and PCs/SMCs is essential for the formation of functional, mature blood vessels (Fig. 1). In genetic mouse models, failure in the interaction between ECs and periendothelial cells leads to severe cardiovascular defects and lethality (Lindahl et al., 1998). Observations of the abnormal EC-SMC interaction have also been made in various human pathological conditions including tumor angiogenesis (Armulik et al., 2005).

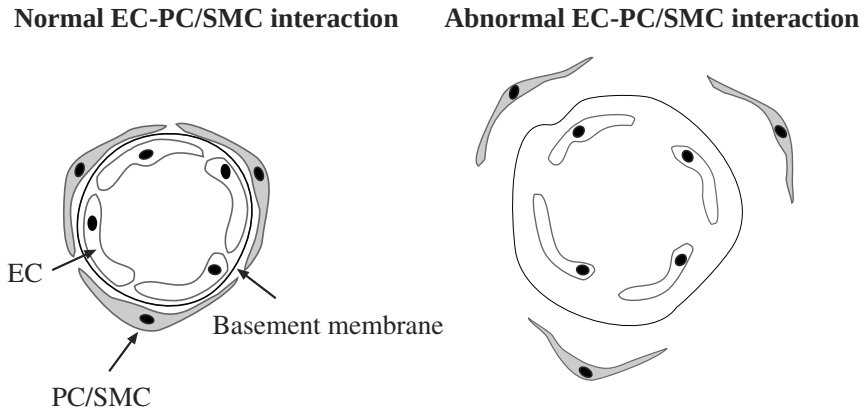


**Figure 1.** Blood vessel structure in normal and abnormal endothelial-pericyte/smooth muscle cell interaction. Blood vessels consist of endothelial cells (EC) and pericytes (PC)/smooth muscle cells (SMC) separated by vascular basement membrane. EC-SMC interaction is important for the development of normal vasculature. Abnormal EC-SMC interaction leads to leaky, hyperdilated and dysfunctional blood vessels.

### *1.2.4. Bone marrow-derived progenitor cells*

Postnatal vasculogenesis is characterized by the mobilization of BM-derived progenitor cells to hypoxic sites and incorporation into growing vessels. BM-derived ECs have been shown to contribute to postnatal vasculogenesis in a variety of animal models of angiogenesis including models of wound healing, limb ischemia, postmyocardial infarction, atherosclerosis and cancer. During postnatal vasculogenesis, a population of BM-derived cells, endothelial progenitor cells (EPC), have been shown to be mobilized to circulation generating circulating EPC (CEP). These cells are supposed to incorporate to the vessel wall and promote both physiological and

pathological neovascularization (Fig. 2) (Rafii and Lyden, 2003; Asahara and Kawamoto, 2004). The extent of BM-derived cells contributing to the vessels of ischemic tissue has been variable in different studies from almost no contribution to 90% (Crosby et al., 2000; Jackson et al., 2001; Kocher et al., 2001; Lyden et al., 2001; De Palma et al., 2003; Garcia-Barros et al., 2003; Urbich et al., 2003; Gothert et al., 2004; Ziegelhoeffer et al., 2004). This discrepancy has been suggested to arise among other things due to different experimental models such as different model of ischemia or the efficiency of bone marrow cell engraftment (Urbich and Dimmeler, 2004). EPCs have been suggested to originate from hematopoietic, mesenchymal as well as side population of bone marrow stem cells (Doyle et al., 2006).

Recent data have suggested that BM-derived progenitor cells can also differentiate to the periendothelial cells expressing PC markers, such as NG2, SMA or desmin, as well as to myofibroblasts, the stromal cells having features of both muscle cells and fibroblasts (Fig. 2) (Direkze et al., 2004; Rajantie et al., 2004; Song et al., 2005; Lamagna and Bergers, 2006). The origin of BM-derived pericyte progenitor cells, hematopoietic or mesenchymal, is unclear (Lamagna and Bergers, 2006).

There have also been reports of the presence of BM-derived cells localized perivascularly to the vasculature of ischemic or tumor tissues. These cells have been indicated not to express EC markers or PC/SMC markers (Fig. 2). These cells are suggested to include hematopoietic cells as well as mesenchymal cells and are supposed to promote vascular growth in a paracrine manner secreting angiogenic growth factors, such as VEGF-A and fibroblast growth factor-2 (FGF-2), or give the vessels physical support (Fig. 2) (Ziegelhoeffer et al., 2004; De Palma et al., 2005; Kopp et al., 2006).

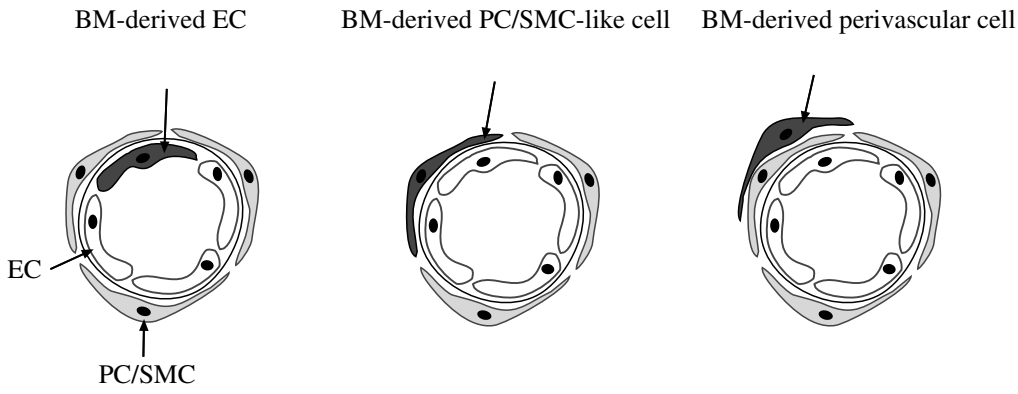


**Figure 2.** Contribution of bone marrow (BM)-derived cells to neovascularization. BM-derived cells have been suggested to differentiate into vascular endothelial cells (EC), pericytes (PC) or smooth muscle cell (SMC)-like cells, and perivascular cells not expressing PC/SMC or EC markers.

Recruitment of BM-derived EC precursors has been reported to be necessary for tumor growth and angiogenesis in mouse (Lyden et al., 2001). On the other hand, also the extent to which EPCs contribute to ECs of tumor vasculature has been variable from non-existent (De Palma et al., 2003; Gothert et al., 2004; Rajantie et al., 2004; De

Palma et al., 2005; Shinde Patil et al., 2005) to abundant (Lyden et al., 2001; Garcia-Barros et al., 2003). Different experimental models and the difficulties with distinguishing periendothelial cells and ECs may explain this discrepancy (De Palma et al., 2005). Furthermore, also hematopoietic BM-derived perivascular cells have been demonstrated to play important role in tumor neovascularization and tumor growth (De Palma et al., 2005).

Altogether, the emerging evidence of the crucial role of BM-derived cells in the formation of new blood vessels has led to the suggestion that hematopoietic stem cells or EPCs could be a novel target for the treatment of angiogenesis-dependent diseases, such as cancer (Rafii et al., 2002).

### 1.3. Receptor tyrosine kinases in angiogenesis and postnatal vasculogenesis

Signaling cascades mediated by specific growth factors regulate the functions of vascular cells. Several of these growth factors bind to and modulate the activity of receptor tyrosine kinases (RTKs). RTKs are transmembrane proteins that have intrinsic tyrosine kinase activity. They regulate various cellular functions such as cell proliferation, differentiation, migration and survival (Gschwind et al., 2004). The best characterized angiogenesis-related growth factor families include the VEGF, angiopoietin (Ang) and PDGF families (Fig. 3) (Conway et al., 2001).

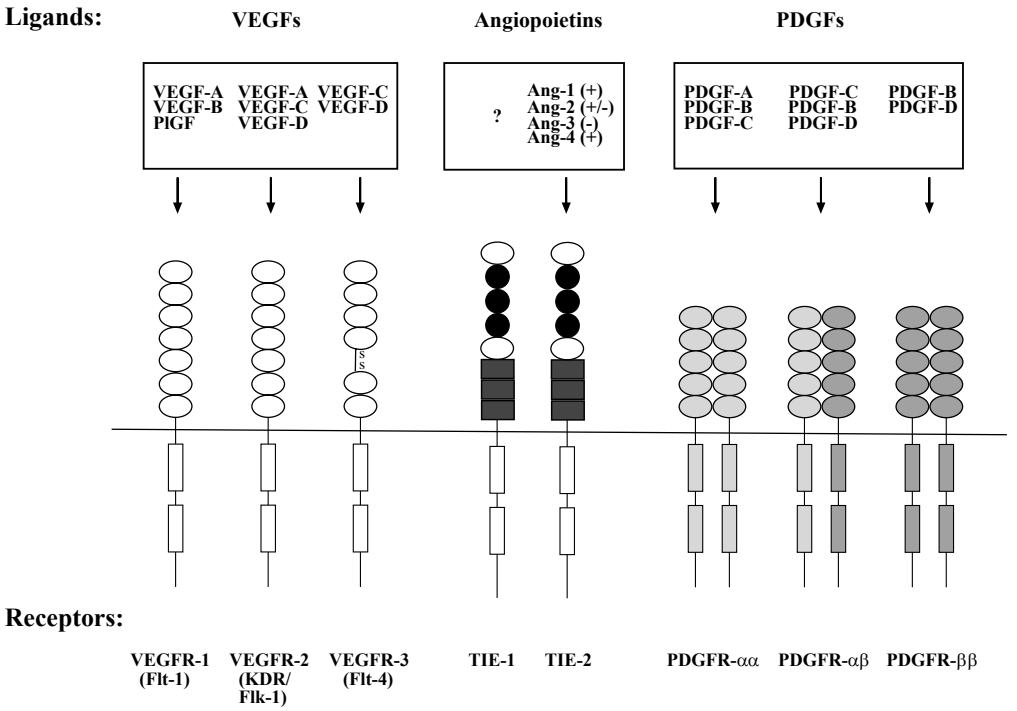


**Figure 3.** Schematic representation of three families of vascular growth factors and their receptors. "+"or "-" indicates whether the particular angiopoietin activates or inhibits the Tie-2 receptor. Modified from review articles by Betsholtz et al. (2001), Jones et al. (2001), and Li and Eriksson (2003).

In addition to growth factors, other molecular signals are needed for vessel formation. For example, the interaction between endothelial cells and extracellular matrices is important for the formation of new blood vessels. The recognition of the extracellular matrix, by ECs occurs mostly via integrins and cell surface heparan sulfate proteoglycans. Invasion of ECs through ECM requires matrix-degrading enzymes, such as matrix metalloproteinases (MMPs) (Iivanainen et al., 2003).

### *1.3.1. Vascular endothelial growth factors (VEGFs) and VEGF receptors*

The VEGF family of growth factors consists of several members including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). These growth factors bind to their cognate transmembrane tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1, KDR) and VEGFR-3 (Flt-4) (Ferrara et al., 2003; Alitalo et al., 2005). VEGF-A exerts its biological effect binding to the VEGFR-1 and VEGFR-2, VEGF-B and PlGF bind to the VEGFR-1. VEGF-C and VEGF-D signal through VEGFR-3. Human VEGF-C and VEGF-D can also signal through VEGFR-2 after proteolytic processing, but they bind with lower activity to VEGFR-2 than VEGFR-3 (Olsson et al., 2006) (Fig. 3). In addition to RTKs, VEGF-A interacts with coreceptors, the neuropilins (NRPs) (Soker et al., 1998).

VEGF receptors are mainly expressed on the surface of blood ECs (VEGFR-1, VEGFR-2) or lymphatic ECs (VEGFR-2, VEGFR-3) and a subset of hematopoietic stem cells (VEGFR-1, VEGFR-2) (Ferrara et al., 2003). In addition, VEGFR-2-positive BM-derived endothelial progenitor cells have been shown to incorporate into blood vessels, and VEGFR-1-positive BM-derived hematopoietic precursor cells have been shown to co-mobilize with them and decorate tumor blood vessels (Lyden et al., 2001).

VEGF-A (also called VEGF) was originally identified as a vascular permeability factor secreted by tumor cells. It is the first characterized vascular-specific growth factor (Senger et al., 1983). Alternative exon splicing results in the formation of at least six different molecular isoforms of the single VEGF-A gene. The predominant isoform of VEGF-A is VEGF165. Native VEGF-A is a basic, heparin-binding, homodimeric glycoprotein. It is bound to the cell surface and the extracellular matrix or is secreted in a diffusible form. VEGF-A overexpression has been demonstrated in a variety of solid tumors (Ferrara et al., 2003).

Oxygen pressure is an important regulator of a VEGF-A expression (Shweiki et al., 1992). In tissues with low oxygen pressure, hypoxia-inducible factor-1 (HIF-1) transcription factor has a central role in the stimulation of VEGF-A transcription (Forsythe et al., 1996). A number of growth factors, such as EGF, TGF-$\alpha$, TGF-$\beta$ and PDGF also contribute to the regulation of VEGF-A expression (Finkenzeller et al., 1992; Goldman et al., 1993; Frank et al., 1995; O-charoenrat et al., 2000). In addition, cytokines, such as interleukin-6 (IL6) and hormones, such as thyroid stimulating hormone (TSH) regulate VEGF-A expression (Cohen et al., 1996; Soh et al., 1996).

VEGF-A has been demonstrated to induce angiogenesis in a variety of angiogenic *in vivo* models. During the initial steps of angiogenesis VEGF-A induces vascular permeability and promotes endothelial proliferation, migration and tube formation

(Fig. 4) (Ferrara et al., 2003). It functions as a survival factor for ECs inducing the activation of anti-apoptotic factors by these cells (Gerber et al., 1998).

VEGF-A has also been shown to be involved in postnatal vasculogenesis. It has been shown to regulate the mobilization of BM-derived endothelial progenitor cells to peripheral blood (Asahara et al., 1999; Hattori et al., 2001). During tumor neovascularization it stimulates the incorporation of BM-derived cells to endothelium of tumor blood vessels. It also regulates the recruitment of perivascularly located BM-derived cells not incorporated within endothelium to the selected target organs such as heart and liver (Grunewald et al., 2006; Li et al., 2006).

Vasculature has been suggested to be VEGF-A-dependent until PCs cover new blood vessels (Benjamin et al., 1998). Differentiated PCs have been shown to secrete VEGF-A that may act as a survival and stabilizing factor for underlying ECs (Darland et al., 2003). These findings suggest that VEGF-A produced by PCs or SMCs may act in a juxtracrine/paracrine manner and stimulate vessel maturation and stabilization. In addition, VEGF-A has been shown to accelerate PC recruitment (Benjamin et al., 1998). Altogether, VEGF-A seems to have numerous functions during angiogenesis, such as initiation of angiogenesis, maintenance of the viability of immature vessels and recruitment of PCs (Benjamin et al., 1998).

From other isoforms of the VEGF family, VEGF-B, VEGF-D and PlGF, but not VEGF-C have been demonstrated to stimulate angiogenesis *in vivo* (Byzova et al., 2002; Pipp et al., 2003; Rissanen et al., 2003; Silvestre et al., 2003). VEGF-D has also been shown to stimulate PC coverage of blood vessels (Rissanen et al., 2003). According to *in vitro* and *in vivo* studies, VEGF-C and VEGF-D are mainly involved in the formation of lymphatic vessels (Tammela et al., 2005). Besides VEGF-A, PlGF has been shown to stimulate postnatal vasculogenesis in tumor tissue (Li et al., 2006).

Gene targeting studies have demonstrated that VEGF-A is necessary for the formation of blood vessels, both during vasculogenesis and angiogenesis. Even the inactivation of a single allele of *VEGF-A* gene (VEGF-A +/-) is embryonically lethal. This phenotype indicates an important role for VEGF-A in stages of early vascular development including differentiation of blood islands (vasculogenesis), vessel sprouting (angiogenesis), lumen formation and the formation of large vessels (Carmeliet et al., 1996; Ferrara et al., 1996).

Knockout studies have supported the role of VEGF-C in lymphangiogenesis. Homozygous mice with disrupted *VEGF-C* genes die prenatally due to accumulation of fluid in tissues. The formation of lymphatic vessels is impaired due to a defect in lymphatic EC sprouting. However, angiogenesis occurs normally in *VEGF-C* -/- mice (Karkkainen et al., 2004). The vascular phenotypes of other VEGFs are more discrete and may be established in pathological conditions but not during development. Mice lacking the *VEGF-B* gene are viable and fertile but have dysfunctional coronary vasculature (Bellomo et al., 2000). Mice lacking the *VEGF-D* gene are healthy and fertile and have a functional lymphatic and vascular system suggesting that VEGF-D does not have an important role in the embryonic development of lymphatic vessels (Baldwin et al., 2005). However, the finding that mice overexpressing VEGF-D

demonstrate development of a hyperplastic lymphatic vessel network similar to mice overexpressing VEGF-C supports the role for VEGF-D in lymphangiogenesis (Jeltsch et al., 1997; Veikkola et al., 2001). Mice lacking the *PlGF* gene are viable and fertile. They have a subtle remodelling defect of the retinal vessels during embryogenesis and reduced pathological postnatal angiogenesis during ischemia, inflammation, wound healing and cancer (Carmeliet et al., 2001).

Data from the targeting of VEGFRs have also confirmed the significance of the VEGF signaling system in the formation of blood and lymphatic vessels. For example, mice lacking *VEGFR-1* gene die during embryogenesis and have disorganized vasculature and abnormally large vascular structures in the yolk sac and within the embryo. VEGFR-1 has been reported to be essential for the organization of embryonic vasculature, but not for endothelial differentiation or continued proliferation (Fong et al., 1995). In contrast, targeting of *VEGFR-2* gene leads to embryonic lethality due to an early defect in the development of hematopoietic and endothelial cells. Yolk sac blood islands are absent and there is a complete absence of organized blood vessels in embryos and yolk sacs indicating that VEGFR-2 is required very early in the development of the endothelial lineage (Shalaby et al., 1995). Mice lacking *VEGFR-3* gene also die during embryogenesis and have underdeveloped vasculature. Large vessels are abnormally organized with defective lumens. This leads to fluid accumulation in the pericardial cavity and cardiovascular failure (Dumont et al., 1998). In addition, analysis of *VEGFR-1* TK domain-deficient mice has demonstrated a role for VEGFR-1 in the recruitment of monocytes/macrophages to the tumors as well as in the stimulation of tumor vascularization (Hiratsuka et al., 2001).

### *1.3.2. Angiopoietins and Tie receptors*

In addition to VEGFs, the angiopoietin growth factor family is relatively specific for the endothelium. There are four members of the angiopoietin family, Ang-1, Ang-2, Ang-3 and Ang-4. The angiopoietins best characterized for their role in angiogenesis are Ang-1 and Ang-2. All angiopoietins bind primarily to the Tie-2 receptor expressed mostly on ECs and cells of the hematopoietic lineage (Jones et al., 2001). Ang-1 appears to act as a naturally occurring agonist for Tie-2, whereas Ang-2 can stimulate or inhibit Tie-2 receptor under different conditions. *In vivo* Ang-2 acts as a Tie-2 antagonist in vascular ECs, whereas in lymphatic vessels it acts as a Tie-2 agonist (Fig. 3) (Davis et al., 1996; Maisonpierre et al., 1997; Teichert-Kuliszewska et al., 2001; Gale et al., 2002). It has been unclear which ligands bind and activate Tie-1, which is also expressed in ECs and some hematopoietic cell lineages. Ang-1 has been shown to activate Tie-1 but direct binding to the extracellular domain has not been demonstrated. In addition, Tie-1 has been shown to signal as a heterodimer with Tie-2 (Saharinen et al., 2005).

Unlike VEGF-A, Ang-1 does not participate in the early events of blood vessel formation, such as EC migration or tube formation. It plays a role in the later stages of vessel formation promoting angiogenic remodelling as well as vessel maturation and stabilization (Fig. 4) (Suri et al., 1996). While Ang-1 is expressed widely in the adult, Ang-2 is expressed only at the sites where angiogenesis occurs such as the ovary, uterus, and placenta. It has been suggested that Ang-2 blocks the stabilizing signal

provided by Ang-1. This may lead either to vessel regression or new vessel sprouting depending on the presence of VEGF-A (Hanahan, 1997; Maisonpierre et al., 1997).

During tumor neovascularization, tumors have been shown to recruit Tie-2 expressing monocytes near the vasculature. These cells do not express EC or PC/SMC markers and are suggested to regulate angiogenesis by paracrine manner (De Palma et al., 2005). In addition, Ang-1 has been shown to mobilize BM-derived endothelial progenitor cells (Hattori et al., 2001).

Mice lacking *Ang-1* or *Tie-2* genes die during midgestation. The mice have defects in the association between the endocardium and myocardium as well as in interaction between ECs and PCs/SMCs (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). These knockout results and the finding that Tie-2 is expressed mostly in the ECs have led to the hypothesis that mesenchymal cell-derived Ang-1 binds to the Tie-2 receptor on ECs and activates the secretion of signaling molecules from ECs that in turn stimulates the recruitment of PCs or SMCs to the growing vessels. PDGF-B as well as HB-EGF has been suggested to occur as signal molecules from ECs to PCs/SMCs (Folkman and D'Amore, 1996). Mice lacking the *Ang-2* gene die postnatally and have defects in postnatal angiogenetic remodelling in the eye as well as in development of lymphatic vasculature (Gale et al., 2002). Targeted disruption of *Tie-1* gene in turn leads to embryonic lethality during late embryogenesis and perinatally because of leaky vessels and defects in microvessel integrity (Puri et al., 1995; Sato et al., 1995).

### *1.3.3. Platelet-derived growth factors (PDGFs) and PDGF receptors*

PDGF was originally identified in a search for serum factors that induce arterial SMC proliferation (Ross et al., 1974). The PDGF family consists of four ligands, PDGF-A, -B, -C and -D. All these ligands form disulfide linked homo- and heterodimers and bind dimeric complexes of PDGFR-α and PDGFR-β receptor tyrosine kinases. PDGF-A A binds PDGFR-αα; PDGF-B binds PDGFR-αα, PDGFR-ββ, and PDGFR-αβ; PDGF-C binds PDGFR-αα and PDGFR-αβ; while PDGF-D binds PDGFR-ββ and PDGFR-αβ (Fig. 3) (Betsholtz et al., 2001; Li and Eriksson, 2003).

All PDGF ligands (PDGF-A, PDGF-B, PDGF-C and PDGF-D) have been reported to stimulate angiogenesis *in vivo* (Risau et al., 1992; Oikawa et al., 1994; Cao et al., 2002; Li et al., 2003). Both PDGF-receptors PDGFR-α and PDGFR-β are expressed by ECs as well as by PCs/SMCs but PDGFR-β predominates in PCs/SMCs (Resink et al., 1990; Beitz et al., 1991; Holmgren et al., 1991; Marx et al., 1994; Lindahl et al., 1997; Hellstrom et al., 1999; Hellstrom et al., 2001; Li et al., 2005).

PDGFs have been shown to regulate EC as well as SMC functions. Specifically, PDGF-B stimulates proliferation and migration and PDGF-C stimulates migration of ECs, while PDGF-A does not have effects on ECs *in vitro* (Marx et al., 1994; Li et al., 2005). PDGF-B is an important regulator of PC/SMC migration and proliferation but also PDGF-A, PDGF-C and PDGF-D have been demonstrated to stimulate the proliferation of SMCs (Resink et al., 1990; Lindahl et al., 1997; Hellstrom et al., 2001; Uutela et al., 2001). In addition, PDGF-B has been suggested to induce intratumoral lymphangiogenesis and promote lymphatic metastases (Cao et al., 2004).

PDGF-C has also been suggested to contribute to postnatal vasculogenesis by inducing BM-derived cell differentiation into ECs and SMCs during ischemic conditions (Li et al., 2005). Moreover, PDGFR-β positive BM-derived cells have been shown to differentiate into PCs during tumor vasculogenesis (Song et al., 2005).

Altogether, these results suggest that the effects of PDGFs on *in vivo* angiogenesis can be mediated directly via ECs or by more indirect mechanism involving effects on SMCs or BM-derived precursor cells.

Genetic mouse models have demonstrated an important role for PDGF-B and PDGFR-β in the recruitment of periendothelial cells to newly formed blood vessels (Fig. 4). Mice lacking *PDGF-B* or *PDGFR-β* gene die perinatally with extensive hemorrhage and vessels lacking or incompletely covered by periendothelial cells (Leveen et al., 1994; Soriano, 1994; Lindahl et al., 1997; Hellstrom et al., 1999). Analysis of EC restricted *PDGF-B* knockouts has demostrated that during embryonic development, vascular endothelial cells secrete PDGF-B that functions as a chemoattractant for SMCs or PC progenitors expressing PDGFR-β (Bjarnegard et al., 2004). However, in mice lacking *PDGF-B* or *PDGFR-β* genes, SMCs and PCs are only affected in a subset of vessels, suggesting that there may also be other factors that regulate periendothelial cell recruitment (Lindahl et al., 1997; Hellstrom et al., 1999). Mice lacking other PDGF-family members do not have as obvious vascular phenotypes as *PDGF-B*- and *PDGFR-β*-null mice (Betsholtz et al., 2001).

The role of PDGFR-β in EC-SMC interaction is also demonstrated in mouse tumor models where inhibition of PDGFR-β together with VEGFR-2 (with SU6668) has been shown to block tumor angiogenesis through defect in EC-SMC interaction (Shaheen et al., 2001b; Bergers et al., 2003; Erber et al., 2004).

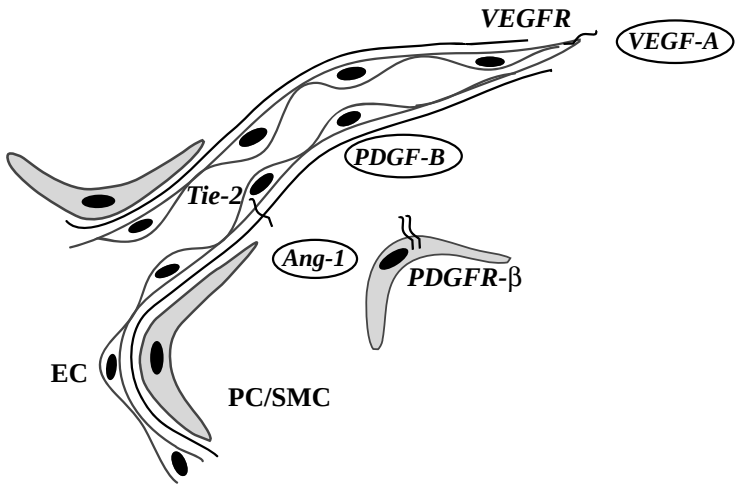


**Figure 4.** Molecular regulation of blood vessel formation by VEGF-A, Ang-1 and PDGF-B. VEGF-A stimulates endothelial cell (EC) proliferation, migration and differentiation at the initial steps of angiogenesis. Ang1/Tie2 signaling is required for vessel stabilization by smooth muscle cells (SMCs). PDGF-B and PDGFR-β are involved in the recruitment of pericytes (PCs) or SMCs by ECs.

# 2. TUMOR ANGIOGENESIS AND VASCULOGENESIS

## 2.1. Significance of angiogenesis and vasculogenesis for tumor growth

Since Judah Folkman suggested in 1971 that tumor growth is dependent on angiogenesis and that inhibition of angiogenesis could be used as a method to treat human cancer (Folkman, 1971), the understanding of tumor angiogenesis has been enhanced exceedingly. The growth of any tumor mass to a size greater than 2-3 $mm^3$ has been suggested to require angiogenesis (Folkman, 1995). The first antiangiogenic drug brought to clinic, bevacizumab (Avastin), has proved that inhibition of angiogenesis is suitable method to inhibit tumor growth (Ferrara et al., 2005).

Recently, postnatal vasculogenesis has also been shown to be involved in the formation of tumor blood vessels. Tumor growth has been shown to lead to the mobilization of circulating endothelial progenitor cells and treatment of tumor-bearing mice with antiangiogenic agents results in the reduction of the mobilization and recruitment of these cells (Rafii et al., 2002).

## 2.2. Angiogenic switch

It has been supposed that the initiation of angiogenesis, the angiogenic switch, needs a shift in the relative balance between angiogenic and anti-angiogenic factors. The common activators of tumor angiogenesis include receptor tyrosine kinase ligands such as VEGF-A, fibroblast growth factor-2 (FGF-2), PDGF-B, and epidermal growth factor (EGF) (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003). Some hormone metabolites, modulators of apoptosis and various antiangiogenic peptides may funtion as endogenous inhibitors of angiogenesis (Nyberg et al., 2005). Several endogenous inhibitors of angiogenesis such as angiostatin, endostatin, tumstatin and canstatin are molecules derived from larger naturally occurring matrix proteins that are not themselves antiangiogenic (Bergers and Benjamin, 2003). Endostatin, a 20 kDa internal fragment of C-terminus of collagen XVIII, has the broadest anti-cancer activity of the endogenous angiogenic inhibitors (Heljasvaara et al., 2005; Folkman, 2006).

The angiogenic switch can occur at any stage of tumor growth. The nature of the tumor and its microenvironment determine at which stage in the tumor progression angiogenic processes are initiated. Different factors such as oncogenes, tumor suppressor genes or hypoxia may affect the relative balance between activators and inhibitors of angiogenesis (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003).

## 2.3. Structure and function of tumor blood vessels

Tumor blood vessels have multiple structural and functional abnormalities. In contrast to normal blood vessels, tumor vasculature is disorganized and vessels are dilated and leaky (Carmeliet, 2000; McDonald and Baluk, 2002; Mollica et al., 2003). The basement membrane of tumor blood vessels is loosely associated with vascular cells, variably thick, has multiple redundant layers and makes projections into the perivascular stroma (Baluk et al., 2003). Endothelial cells have an abnormal shape, grow on top of each other and may project into the lumen (Hashizume et al., 2000). In addition, PCs are abnormally

loosely associated with ECs and make cytoplasmic processes into the tumor tissue. These features are suggested to lead to tumor vessel leakiness (Morikawa et al., 2002).

Structural changes in tumor blood vessels have been suggested to result from altered balance of angiogenic molecules, such as VEGF-A and angiopoietins, in tumor microenvironment. These changes may also make tumor blood vessels more vulnerable compared to normal blood vessels during antiangiogenic therapy (Carmeliet, 2000; Jain and Munn, 2000).

### 2.4. Genetic changes in tumor ECs

Vascular cells in tumor blood vessels have been considered genetically stable and less prone to develop drug resistance. However, it was recently demonstrated that tumor-associated ECs have cytogenetic abnormalities, such as aneuploidy and abnormal multiple centrosomes. This chromosome instability may have an effect on the development of resistance also for anti-angiogenic drugs (Hida et al., 2004).

Gene expression studies have demonstrated, that tumor ECs are also distinct from ECs in normal tissues at the molecular level. It has been shown that a number of the genes that are elevated in tumor ECs compared to normal ECs encode for extracellular matrix proteins but most of them do not have established functions. Most of the genes that are differentially expressed in tumor ECs compared to normal ECs are also expressed in angiogenic vessels during corpus luteum formation and wound healing supporting the hypothesis that the same signals may function during physiological and pathological angiogenesis (St Croix et al., 2000).

## 3. ERBB RECEPTORS AND LIGANDS

The epidermal growth factor receptor (EGFR) family of RTKs includes four members: EGFR (HER1 or ErbB1) (Ullrich et al., 1984), ErbB2 (Neu or HER2) (Schechter et al., 1984; Coussens et al., 1985), ErbB3 (HER3) (Kraus et al., 1989; Plowman et al., 1990) and ErbB4 (HER4) (Plowman et al., 1993) (Fig. 5). These receptors are called ErbB because of the close similarity of EGFR with an avian eryhtroblastic leukemia viral oncogene *v-erb-B* (Downward et al., 1984). The HER abbreviation comes from the words "human EGF receptor". HER2/neu acronym derives from the sequence homology of HER2 with c-neu oncogene that can cause neuroblastoma in rats (Coussens et al., 1985).

### 3.1. Receptor structure

All ErbB receptors are ~ 170-180 kDa transmembrane proteins that consist of a glycosylated and disulfide-bonded extracellular domain, a single hydrophobic transmembrane domain, and an intracellular domain with a tyrosine kinase and multiple phosphorylation sites. The extracellular region of each ErbB receptors can be divided into four domains: I-IV. Domains I and III participate in ligand binding and cysteine rich domains II and IV in receptor dimerization (Hynes and Lane, 2005).

### 3.2. Ligands

Activation of ErbB receptors is regulated by interaction with cognate ligands. ErbB receptors interact in a selective and specific manner with eleven members of the EGF-like peptide growth factor family: epidermal growth factor (EGF) (Cohen, 1962; Savage and Cohen, 1972; Gray et al., 1983), transforming growth factor-α (TGF-α) (Derynck et al., 1984), amphiregulin (AR) (Shoyab et al., 1989), epigen (EPG) (Strachan et al., 2001), heparin binding EGF-like growth factor (HB-EGF) (Higashiyama et al., 1991), betacellulin (BTC) (Sasada et al., 1993), epiregulin (EPR) (Toyoda et al., 1995), and neuregulins 1-4 (NRG 1-4) (Holmes et al., 1992; Peles et al., 1992; Falls et al., 1993; Marchionni et al., 1993; Carraway et al., 1997; Zhang et al., 1997; Harari et al., 1999). Moreover, different splice variants multiply the number of ErbB ligands. For instance at least 15 different isoforms are produced from the NRG-1 gene as a result of alternative splicing (Falls, 2003).

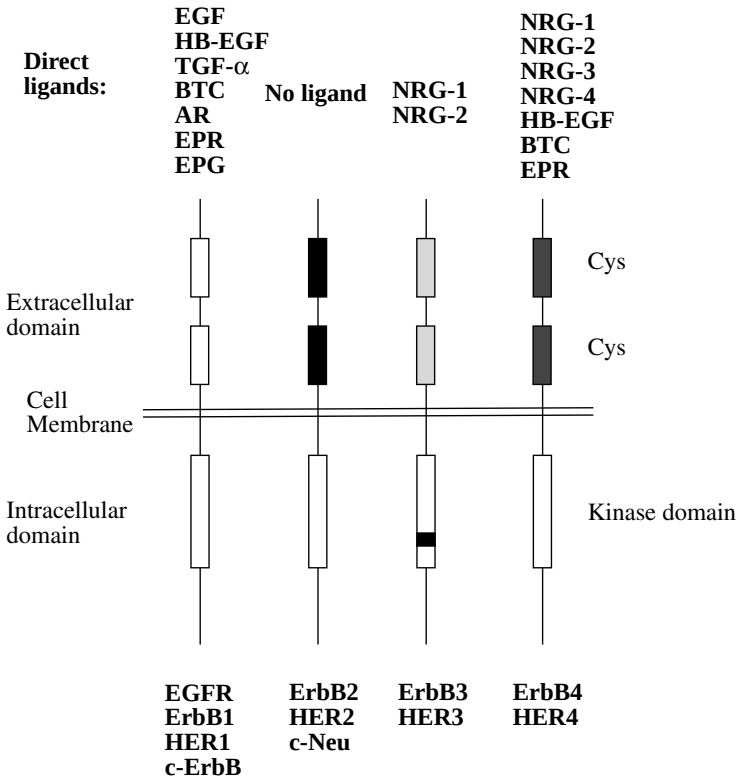


**Figure 5.** ErbB receptor binding specificities for EGF-like growth factors. Black rectangle in kinase domain of ErbB3: kinase domain is non-functional.

Each ErbB ligand contains an EGF-like domain that determines its binding specificity. According to ErbB receptor binding, ErbB ligands can be divided into three groups. The first includes EGF, TGF-α, AR and EPG, which are specific ligands for EGFR. The second includes HB-EGF, EPR, and BTC, which have dual specificity for EGFR

and ErbB4. The third group consists of NRGs, which can bind either to ErbB3 and ErbB4 (NRG-1 and NRG-2) or only to ErbB4 (NRG-3 and NRG-4) (Fig. 5) (Yarden and Sliwkowski, 2001). In addition to binding ErbB receptors, ErbB ligands can bind to different co-receptors. For instance HB-EGF binds to the cell surface heparan sulfate proteoglycans that modulate its ErbB receptor binding and activity (Higashiyama et al., 1993).

ErbB ligands are produced as transmembrane precursors and processed by proteolysis, referred to as ectodomain shedding, leading to the release of soluble growth factors (Fig. 6). Members of the ADAM (a disintegrin and metalloproteinase) family and matrix metalloproteinases (MMPs) are involved in ectodomain shedding of ErbB ligands (Sanderson et al., 2006).

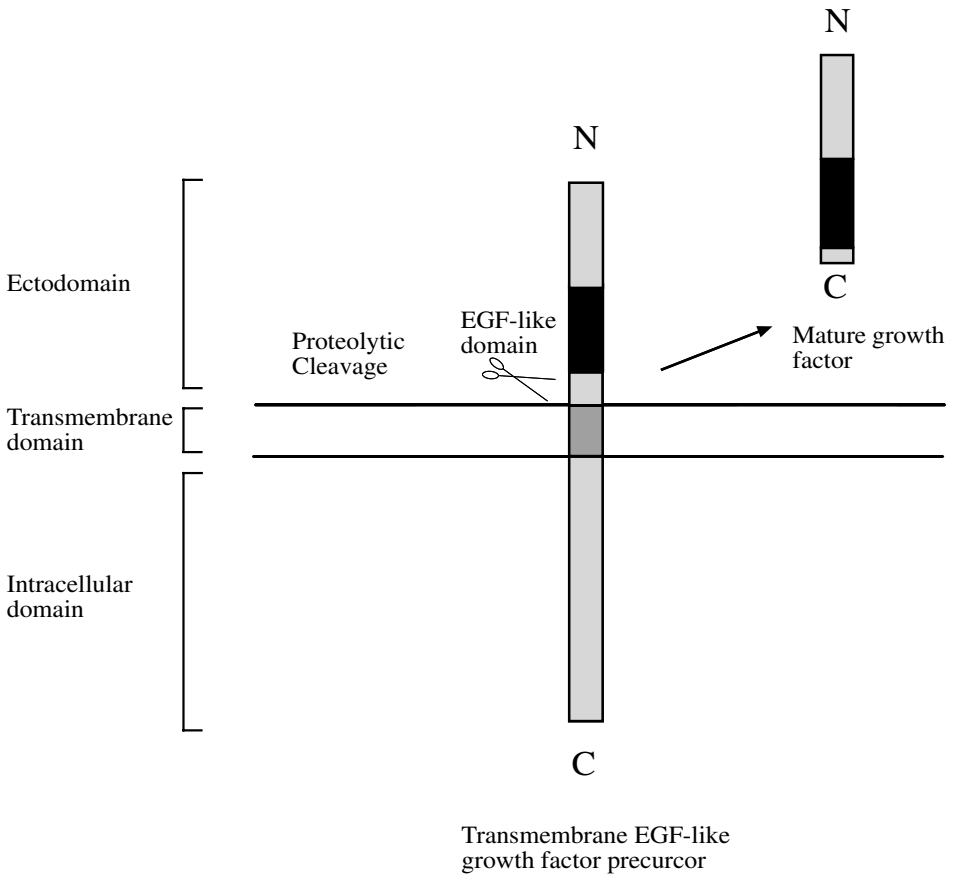


**Figure 6.** Schematic structure of EGF-like growth factors. The transmembrane precursor consists of an N-terminal ectodomain containing the EGF-like domain, transmembrane domain and C-terminal intracellular domain. The mature ectodomain is released by proteolytic cleavage at the extracellular juxtamembrane region of the precursor protein. Modified from review article by Sanderson et al. (2006).

ErbB ligands can also signal as cell membrane-attached precursor forms. During this juxtracrine signaling, adjacent cells are stimulated by membrane-bound growth factor in a cell-cell contact. Juxtracrine signaling has been documented for TGF-α, HB-EGF and type III isoforms of NRG-1 (Anklesaria et al., 1990; Higashiyama et al., 1995; Falls, 2003).

It is also possible that membrane anchored ErbB ligands function as receptors. For example, NRG-1 is capable of reverse signaling, in which receptor binding activates proteolysis of NRG-1 and generation of a soluble intracellular domain with signaling activity (Bao et al., 2003). Furthermore, transmembrane form of HB-EGF is a unique receptor for diphteria toxin (DT), and a DT mutant (CRM 197) can be used to inhibit human HB-EGF (Naglich et al., 1992).

### 3.3. Signal transduction

Ligand binding to the ectodomain of the ErbB receptors leads to conformational changes and receptor dimerization. ErbB receptors can form both homo and heterodimers. The ErbB3 receptor does not have an active tyrosine kinase and thus has to form a heterodimer with other ErbB receptors (Citri and Yarden, 2006).

Domain II of the ErbB ectodomain has a dimerization arm that is exposed after ligand binding and promotes receptor-receptor dimerization. When the ligand is not bound to the receptor, the dimerization arm from domain II interacts with domain IV and the receptor is in the `tethered´ conformation and unable to dimerize. The structure of ErbB2 resembles the ligand-activated structure of other receptors. This may explain why no soluble ligand has been found for ErbB2 that prefers to function as a co-receptor for the other three receptors (Hynes and Lane, 2005).

Receptor dimerization results in activation of the intrinsic kinase domain and phosphorylation of specific tyrosine residues in the intracellular domain. Recruitment of signaling molecules to the phosphorylated tyrosine residues leads to the activation of downstream signaling components. The mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase (PI-3K) pathways are common signaling cascades downstream of ErbBs (Olayioye et al., 2000).

### 3.4. Functions in development

The biological significance of ErbB signaling during normal development has been demonstrated by different gene targeting studies in mice. These studies have shown important role for ErbB signaling in several developmental processes such as cardiovascular, neural, mammary gland and epithelial development (Casalini et al., 2004).

*EGFR* gene targeted mice have different phenotypes according to the genetic background of the mouse. All *EGFR* -/- phenotypes are lethal. The embryos die either in peri-implantation due to inner cell mass degeneration, at mid gestation due to defects in placenta or postnatally suffering from abnormalities in a broad range of organs, including eyelid, skin, kidney, brain, liver, lung, and gastrointestinal tract due to defective epithelial or neural development (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995).

### *3.4.1. Functions in cardiovascular development*

Gene targeting studies have indicated that the ErbB signaling system is essential for cardiovascular development. Inactivation of *ErbB2, ErbB4* and *NRG-1* genes leads to mid-embryonic lethality, as a result of cardiac dysfunction associated with the failure to develop myocardial trabeculae, projections of the heart ventricles that maintain blood flow (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). NRG-1 expression on endocardial endothelial cells and ErbB2 and ErbB4 on cardiomyocytes suggest that the paracrine signaling between endocardium-derived NRG-1 and the ErbB2/ErbB4 heterodimer expressed in the myocardium is necessary for heart development (Marchionni, 1995).

The trabecular defects of mice with targeted *NRG-1*, *ErbB2* or *ErbB4* genes are similar to those described in mice with targeted *Ang-1* and *Tie-2* genes. The expression of Ang-1 in the heart myocardium and Tie-2 in the heart endocardium compared to NRG-1 expression in the heart endocardium and ErbB2/ErbB4 expression in the heart myocardium suggests that they may have reciprocal interactions during heart development (Suri et al., 1996).

While NRG-1, ErbB2 and ErbB4 function in trabecular formation, HB-EGF, EGFR and ErbB3 have major roles in cardiac valve development. Mice lacking *EGFR* gene as well as mice expressing a non-functional EGFR mutant exhibit cardiac valve enlargement (Chen et al., 2000). Most of the mice lacking *HB-EGF* die in the first postnatal week and demonstrate enlarged cardiac valves resembling the heart defects in *EGFR* -/- mice (Iwamoto et al., 2003; Jackson et al., 2003). Thus, it has been suggested that HB-EGF signals through EGFR during heart valve development (Iwamoto and Mekada, 2006). Mice with targeted *ErbB3* gene die during embryogenesis and have defective cardiac cushion formation leading to cardiac valve underdevelopment and dysfunction (Erickson et al., 1997).

In addition to valve defects, *HB-EGF* gene targeted mice demonstrate cardiomyocyte hypertrophy, ventricular chamber enlargement, and reduced cardiac function (Iwamoto et al., 2003). The cardiac phenotype of the mice with conditional mutation of the *ErbB2* gene in ventricular cardiomyocytes demonstrate dilated cardiomyopathy resembling the cardiac phenotype of mice with targeted *HB-EGF* gene (Ozcelik et al., 2002). Thus, HB-EGF-ErbB2 signaling has been suggested to be involved in the regulation of the maintenance of mature heart functions (Iwamoto and Mekada, 2006).

### *3.4.2. Functions in neural development*

ErbB signaling also regulates neural development. *EGFR* gene targeted mice develop progressive neurodegeneration in the central nervous system (Sibilia et al., 1998). Deletion of *NRG-1*, *ErbB2* or *ErbB3* gene leads to defects in the migration of neural crest cells, the cell population that generates *e.g.* the majority of the peripheral nervous system. Also the number of neural crest cell-derived Schwann cells is reduced, which is suggested to lead degeneration of motor and sensory

neurons in *ErbB3* targeted mice (Erickson et al., 1997; Riethmacher et al., 1997; Britsch et al., 1998). Deletion of *ErbB4* gene results in defective hindbrain axon guidance and cranial neural crest cell migration in the central nervous system (Gassmann et al., 1995; Golding et al., 2000). Interestingly, recent findings have demonstrated that same molecules that guide axonal growth, such as semaphorins and netrins, may also involve in the blood vessel guidance, suggesting that blood vessels and nerves share common molecular mechanisms to reach their targets (Autiero et al., 2005).

### 3.5. Tumor biology

The ErbB family includes well-characterized human oncogenes. EGFR was the first RTK directly associated with human cancer (Gschwind et al., 2004). Aberrant activation of the ErbB signaling system in cancer may lead to the abnormal proliferation, migration or invasion of neoplastic cells as well as stimulation of angiogenesis. ErbB signaling can be activated via paracrine stimulation by EGF-like ligands secreted from adjacent stromal cells or via autocrine stimulation by increased expression of EGF-like ligands or ErbB receptors in tumor cells. Alternatively, constitutive receptor activation of a mutated ErbB receptor can stimulate tumor growth (Marmor et al., 2004; Hynes and Lane, 2005).

In particular, the activation of EGFR or ErbB2 has been associated with the tumorigenesis. Amplification or increased transcription of *EGFR* gene leading to overexpression has been detected in human cancers such as colorectal and pancreatic cancer, squamous cell carcinoma of the head and neck (HNSCC) and non-small cell lung cancer (NSCLC) (Salomon et al., 1995). Mutations with deletion of exons from the extracellular domain of EGFR (EGFRvIII), leading to uncontrolled constitutive kinase activity and increased transforming potential, have been found in gliomas (Ekstrand et al., 1992; Boerner et al., 2003). Small somatic mutations that enhance EGFR activity have been reported in the intracellular kinase domain of EGFR in lung cancer patients. These mutations sensitize the tumors to EGFR tyrosine kinase inhibitor therapy (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004).

*ErbB2* gene amplification is a common mechanism leading to receptor overexpression in several cancers such as breast, ovarian, gastric and bladder carcinomas. ErbB2 gene amplification associates with poor patient prognosis in breast cancer (Slamon et al., 1987; Slamon et al., 1989; Sauter et al., 1993; Tanner et al., 2005).

# 4. ERBB SIGNALING SYSTEM IN BLOOD VESSEL FORMATION

Although the significance of ErbB signaling for the behaviour of tumor cells has been intensively studied, relatively little is known about their roles in different cell types contributing to angiogenesis. Gene targeting studies have revealed the essential roles of ErbB signaling in heart development. *In vitro* and *in vivo* studies have also suggested important roles for these molecules in blood vessel formation.

## 4.1. ErbB receptors in blood vessel formation

### *4.1.1. ErbB receptor expression and activation in ECs*

There are variable reports about the expression pattern of ErbB receptors in vascular endothelial cells. EGFR expression has been shown frequently in ECs *in vitro* (Schreiber et al., 1986; Baker et al., 2002; Hirata et al., 2002; Sini et al., 2005) and *in vivo* (Real et al., 1986; Baker et al., 2002; Sini et al., 2005) but also *in vitro* ErbB2, ErbB3 and ErbB4 expression has been reported (Russell et al., 1999; Kim et al., 2003a; Sini et al., 2005). Based on electron microscopy analysis of the immature capillaries of granuloma tissue, EGFR receptor localizes into coated pits, intracellular vesicles and lysosome-like structures in ECs (Wakui et al., 1990; Wakui, 1992).

EGFR ligands such as EGF, HB-EGF and BTC, as well as ErbB3/ErbB4 ligand NRG-1 have been shown to stimulate ErbB phosphorylation in ECs (Russell et al., 1999; Baker et al., 2002; Hirata et al., 2002; Kim et al., 2003a; Sini et al., 2005; Baker et al., 2006). In contrast, it has also been reported that ECs are not responsive to NRG-1 (Sini et al., 2005). Furthermore, EGFR ligands have been shown to stimulate tyrosine phosphorylation of EGFR but not ErbB2, and it has been suggested that in ECs EGFR homodimers have a central role in the signal transduction of EGFR ligands (Sini et al., 2005). In addition, EGFR inhibitor (gefitinib) has been shown to inhibit EGF-stimulated phosphorylation of EGFR in ECs (Hirata et al., 2002).

Growth factor exposure has been shown to regulate ErbB expression and activation in ECs during *in vivo* angiogenesis. In a mouse corneal pocket assay, EGFR expression is induced in ECs during EGF- or VEGF-A-stimulated angiogenesis. In addition, EGF but not VEGF-A stimulates endothelial EGFR phosphorylation in neovasculature (Hirata et al., 2004). It has also been reported that ECs in the vasculature of EGF/TGF-α expressing bladder, pancreatic and renal cancers express activated EGFR, whereas ECs in the blood vessels of EGF/TGF-α- negative renal cancer are negative for EGFR (Baker et al., 2002).

The tumor blood vessel ECs in a variety of tumor types such as prostata, melanoma and renal cell carcinoma are known to express EGFR (Kim et al., 2003b; Sini et al., 2005; Amin et al., 2006; Baker et al., 2006). The expression of ErbB receptors in tumor ECs compared to normal ECs has also been analyzed. It has been reported that tumor ECs differ from normal ECs in their ErbB expression. Analysis of tumor blood-vessel

associated ECs isolated from xenografts of melanoma grown in nude mice, has indicated that tumor ECs express EGFR, ErbB2 and ErbB4, whereas normal ECs, isolated from mouse skin, express ErbB2, ErbB3 and ErbB4. As a result of these receptor expression patterns, tumor ECs respond to EGF stimulation and EGFR inhibition, whereas normal ECs do not, but instead are responsive to NRG-1 (Amin et al., 2006). In addition, it has been reported that ECs in the vasculature of bone tumors express activated EGFR, but ECs in uninvolved bone or muscle tumors do not. EGFR/ErbB2 inhibitor (PKI 166) has been shown to reduce endothelial EGFR phosphorylation and induce EC apoptosis in these bone tumor blood vessels (Kim et al., 2003b).

### *4.1.2. ErbB receptor expression and activation in SMCs*

Relatively less is known about the expression of the ErbB receptors in non-endothelial cell types associated with blood vessels, such as PCs or SMCs. There are reports suggesting, that SMCs similar to ECs, express EGFR *in vitro* (Saltis et al., 1995; Tang et al., 2000; Kim et al., 2003a; Shin et al., 2003; Mifune et al., 2004; Ying et al., 2007; Zhou et al., 2007) as well as *in vivo* (Nakata et al., 1996; Shin et al., 2003). Based on electron microscopy analysis of the immature capillaries of granuloma tissue EGFR localizes in the PC cell membrane and at the tips of the PC cytoplasmic projections where they interdigitate with ECs as well as in lysososome-like structures of PCs (Wakui, 1992). In addition to EGFR, ErbB2, ErbB3 and ErbB4 expression has been reported in SMCs *in vitro* (Kim et al., 2003a; Shin et al., 2003).

Also the activation of ErbB receptors has been demonstrated in normal SMCs in culture. EGF and BTC have been reported to stimulate the phosphorylation of all ErbB receptors in SMCs (Shin et al., 2003). It has also reported that AR induces phosphorylation of EGFR, but not ErbB2, ErbB3 or ErbB4 in SMCs (Shin et al., 2003). In addition, EGFR inhibitors such as AG-1478 or PD-168393 inhibit BTC or EGF stimulated EGFR phosphorylation in SMCs (Mifune et al., 2004; Ying et al., 2007). However, the expression or activation of ErbB receptors in tumor blood vessel PCs or SMCs has not been analyzed in detail.

### *4.1.3. ErbB receptors in indirect regulation of blood vessel formation*

Interaction between tumor cells and their microenvironment is necessary for the tumor growth. ErbB receptor activation in tumor cells can result in increased expression of angiogenic factors by tumor cells. This effect of ErbBs not mediated directly via ErbB signaling at EC surface, is here discussed as indirect regulation of angiogenesis (Fig. 7).

*In vivo* studies with tumor xenografts have shown that tumors with ErbB overexpression demonstrate increased expression of angiogenic factors, such as VEGF-A (Yen et al., 2002). Inhibition of ErbB activation by anti-EGFR antibody (C225; cetuximab), EGFR tyrosine kinase inhibitor (gefitinib), EGFR/ErbB2 inhibitor (PKI 166) or ErbB2 neutralizing antibody (4D5, trastuzumab) in various tumor types such as human pancreatic, colon, epidermoid, bladder, breast, ovarian or gastric carcinomas reduces the expression of angiogenic factors, such as VEGF-A, IL8, FGF-2 and TGF-α and decreases

microvascular density (Ciardiello et al., 1996; Petit et al., 1997; Perrotte et al., 1999; Bruns et al., 2000a; Bruns et al., 2000b; Ciardiello et al., 2000; Ciardiello et al., 2001). Moreover, inhibition of ErbB2 expression by small interfering RNA (siRNA) in ovarian and breast cancer cell lines induces expression of the anti-angiogenic factor trombospondin-1 (TSP-1) but reduces the expression of VEGF-A (Yang et al., 2004).

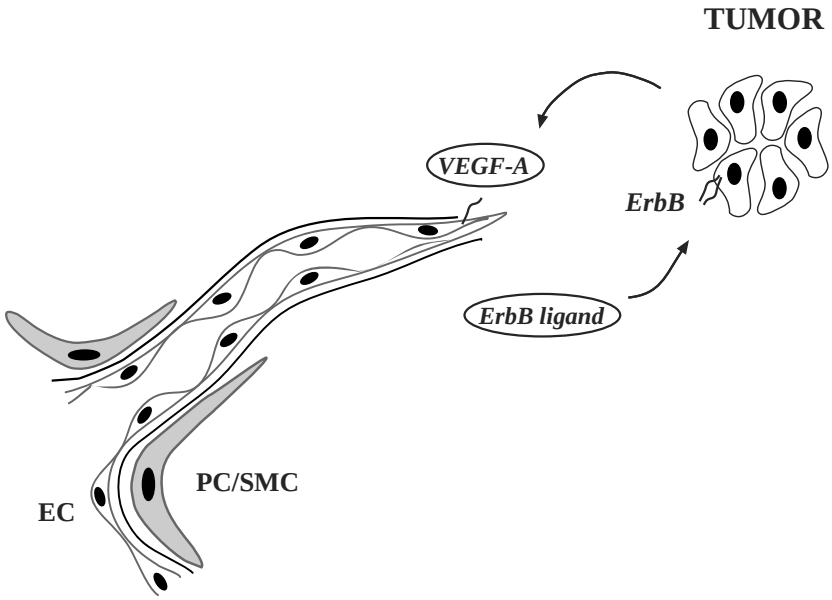


**Figure 7**. The indirect regulation of angiogenesis involves upregulation of angiogenic growth factor, such as VEGF-A from non-vascular cells, such as tumor cells.

## 4.2. ErbB ligands in blood vessel formation

### *4.2.1. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α)*

The effect of EGF on blood vessels was established in 1973 when Savage and Cohen reported that administration of EGF to the mouse cornea makes blood vessels more prominent (Savage and Cohen, 1973). The angiogenic potential of EGF and TGF-α has been compared in hamster cheek pouch assay, and TGF-α has been reported to be a more potent mediator of angiogenesis than EGF (Schreiber et al., 1986).

The direct effect of EGF and TGF-α on EC functions has been demonstrated with different EC lines *in vitro*. EGF has been shown to stimulate EC functions such as proliferation, migration and tube formation of micro- and macrovascular ECs from different species, such as human, bovine and murine (McAuslan et al., 1985; Mawatari et al., 1989; Mawatari et al., 1991; Matsuda et al., 1992; Ono et al., 1992; Sato et al., 1993; Hirata et al., 2002; Kim et al., 2003a; Sini et al., 2005). TGF-α has been shown to be equally potent mitogen for ECs *in vitro* as EGF (Schreiber et al., 1986). It has also been shown to stimulate tube formation of ECs (Okamura et al., 1992; Ono et al.,

1992; Sato et al., 1993; Sini et al., 2005). These results suggest that EGF and TGF-α can exert their angiogenic activity directly through the ErbB receptors on the EC surface. However, it is likely that angiogenic blood vessels *in vivo* are exposed to both ErbB family ligands and VEGF-A. Supporting the role of EGF in the regulation of angiogenesis, EGF and VEGF-A have been shown to co-operate in the regulation of EC proliferation (Sini et al., 2005).

EGF and TGF-α have been shown to regulate angiogenesis also indirectly by stimulating expression of other angiogenic factors. They have been reported to affect on the expression of angiogenic growth factors, such as VEGF-A or IL-8 in several different tumor cell lines including glioma, HNSCC, vulvar, epidermoid, gastric and hepatocellular carcinoma *in vitro* (Goldman et al., 1993; O-charoenrat et al., 2000; Hirata et al., 2002; Akagi et al., 2003; Ueda et al., 2006). In addition TGF-α upregulates VEGF-C and downregulates VEGF-D expression in HNSCC *in vitro* (O-charoenrat et al., 2000). EGF has also been demonstrated to stimulate VEGF-A expression in ECs (Hirata et al., 2002) and SMCs (Sini et al., 2005). In addition, TGF-α has been suggested to regulate cutaneous angiogenesis involved in psoriasis and wound healing by stimulating VEGF-A expression from keratinocytes (Detmar et al., 1994).

### *4.2.2. Heparin-binding EGF-like growth factor (HB-EGF) and betacellulin (BTC)*

HB-EGF was originally identified as a protein secreted by human macrophages (Higashiyama et al., 1991). In addition, it has found to be expressed in vascular ECs as well as SMCs (Yoshizumi et al., 1992; Dluz et al., 1993). Furthermore, HB-EGF has been shown to be expressed *in vivo* in the ECs and SMCs of coronary arteries (Nakata et al., 1996).

HB-EGF has been shown to be angiogenic *in vivo* in a rabbit cornea assay as well as a mouse subcutaneous angiogenesis assay (Abramovitch et al., 1998). In addition, the angiogenic potential of HB-EGF has been demonstrated in a xenograft study, in which human bladder carcinoma cell line expressing soluble HB-EGF enhanced tumor angiogenesis compared to control cells (Ongusaha et al., 2004). HB-EGF is a potent chemotactic and mitogenic factor for SMCs (Higashiyama et al., 1991; Higashiyama et al., 1993). SMC and EC coculture studies have demonstrated that HB-EGF activates SMCs to produce VEGF-A that in turn stimulates EC proliferation (Abramovitch et al., 1998). HB-EGF may also regulate EC functions directly. HB-EGF has been shown to stimulate EGFR phosphorylation in ECs (Sini et al., 2005) but it does not stimulate the proliferation of ECs (Higashiyama et al., 1991). Instead, it has been reported to stimulate tube formation and migration of ECs (Ushiro et al., 1996; Abramovitch et al., 1998).

BTC has also been shown to be expressed in SMCs *in vivo*. BTC expression has been detected in vascular SMCs and the macrophages of human atherosclerotic coronary arteries (Tamura et al., 2001; Shin et al., 2003).

The effect of BTC on *in vivo* angiogenesis has been shown in the matrigel plug assay (Kim et al., 2003a). BTC was originally found to stimulate the proliferation of vascular SMCs but not ECs (Shing et al., 1993). In addition it stimulates EGFR, ErbB2, ErbB3

and ErbB4 phosphorylation in SMCs (Shin et al., 2003; Mifune et al., 2004), as well as proliferation and migration of SMCs (Shing et al., 1993; Mifune et al., 2004). Later *in vitro* studies have suggested that BTC can also stimulate EC DNA synthesis, migration, survival and tube formation, as well as, ErbB2, ErbB3 and ErbB4 tyrosine phosphorylation in micro- and macrovascular ECs (Kim et al., 2003a). In addition, BTC upregulates the expression of VEGF-A and VEGF-B and downregulates the expression of VEGF-D in HNSCCs *in vitro* (O-charoenrat et al., 2000).

### *4.2.3. Neuregulins*

Based on the important role of NRG-1 in heart development, it was thought to regulate also the blood vessel formation (Russell et al., 1999). Endothelial expression of NRG-1 has been reported in cardiac microvascular ECs (Cote et al., 2005). In addition, SMCs have been shown to express NRG-1 (Kato et al., 2003). Recently, also a splice variant of NRG-4 has shown to be expressed in ECs of human prostate cancer blood vessels (Hayes et al., 2007).

NRG-1 has been shown to stimulate angiogenesis *in vivo* in a rat corneal angiogenesis assay (Russell et al., 1999), in chick chorioallantoic membrane assay (CAM) (Yen et al., 2000) and in a murine angiogenesis assay (Bagheri-Yarmand et al., 2000). The effect of NRG-1 on *in vivo* angiogenesis can be blocked by an anti-VEGF antibody, which suggests that VEGF-A mediates the angiogenic effect of NRG-1 (Bagheri-Yarmand et al., 2000).

As evidence of a direct effect of NRG-1 on ECs, it has been shown to stimulate EC proliferation and tube formation and ErbB2 tyrosine phosphorylation in ECs, through ErbB2 heterodimerization with ErbB3 or ErbB4 (Russell et al., 1999). Furthermore, it has been shown that NRG-1 can stimulate micro- and macrovascular EC migration and EC tube formation, which can be blocked by an anti-ErbB2 antibody (trastuzumab) (Bagheri-Yarmand et al., 2000). Also an inhibitory effect of NRG-1 on vascular cells has been reported. It has been shown to inhibit tumor EC proliferation (Amin et al., 2006), as well as SMC proliferation and migration (Clement et al., 2007).

NRG-2 has also been suggested to have an inhibitory effect on angiogenesis. It has been shown to block EC proliferation *in vitro* and CAM angiogenesis *in vivo* (Nakano et al., 2004).

NRG-1 may also involve in the indirect regulation of angiogenesis. It has been shown to stimulate the expression of VEGF-A in breast and lung cancer cell lines but not in normal human mammal or bronchial primary cells. Conditioned media from NRG-1-treated cancer cells, but not from normal cells, stimulate EC proliferation and this effect is inhibited by a VEGF-A neutralizing antibody (Ab293A) (Yen et al., 2000). In addition, NRG-1 has been reported to stimulate VEGF-A expression from human epithelial breast cancer cells and HNSCCs. This effect can be inhibited by anti-ErbB2 antibody (trastuzumab) (Bagheri-Yarmand et al., 2000; O-charoenrat et al., 2000).

Expression of ErbB ligands in vascular ECs and SMCs, their effect on angiogenesis, as well as their role in development have been summarized in Table 1.

**Table 1.** ErbB ligands in angiogenesis. The effect is stimulatory if inhibition has not been mentioned.

| Growth factor | Binding to ErbBs | Expression in EC/SMC | Effect on *in vitro* angiogenesis | Effect on *in vivo* angiogenesis | Null mouse phenotype | References |
|---|---|---|---|---|---|---|
| **EGF** | EGFR | EC (1)<br>SMC (1) | **EC** DNA synthesis (2)<br>**EC** survival (2)<br>**EC** migration (3)<br>**EC** proliferation (4)<br>**EC** tube formation (5)<br>**SMC** migration (6) | Hamster cheek pouch (7)<br>Mouse cornea (8)<br>Rabbit cornea (9) | Viable, no overt phenotype (10) | 1. (Wakui et al., 1990)<br>2. (Kim et al., 2003a)<br>3. (McAuslan et al., 1985)<br>4. (Gospodarowicz and Bialecki, 1979)<br>5. (Mawatari et al., 1989)<br>6. (Abramovitch et al., 1998)<br>7. (Schreiber et al., 1986)<br>8. (Hirata et al., 2002)<br>9. (Gospodarowicz et al., 1979)<br>10. (Luetteke et al., 1999) |
| **TGF-α** | EGFR | EC (1) | **EC** proliferation (2)<br>**EC** tube formation (3)<br>**SMC** migration (4) | Hamster cheek pouch (2) | Viable, abnormal skin, wavy hair, curly whiskers, corneal inflammation, defect in eyelid closure (5-7) | 1. (Dunn et al., 2000)<br>2. (Schreiber et al., 1986)<br>3. (Okamura et al., 1992)<br>4. (Abramovitch et al., 1998)<br>5. (Mann et al., 1993)<br>6. (Luetteke et al., 1993)<br>7. (Luetteke et al., 1999) |
| **AR** | EGFR | SMC (1) | **SMC** proliferation (1) | - | Viable, defect in mammary glands ductal development (2) | 1. (Kato et al., 2003)<br>2. (Luetteke et al., 1999) |
| **EPG** | EGFR | - | - | - | - | - |
| **HB-EGF** | EGFR<br>ErbB4 | EC (1)<br>SMC (2) | **EC** migration (3)<br>**EC** tube formation (4)<br>**SMC** proliferation (5)<br>**SMC** migration (6) | Rabbit cornea (3)<br>Mouse subcutaneous assay (3) | Perinatal or postnatal lethality, heart failure with enlarged ventricular chambers and cardiac valves, defects in lung and eyelid development, defect in wound healing (7-10) | 1. (Arkonac et al., 1998)<br>2. (Dluz et al., 1993)<br>3. (Abramovitch et al., 1998)<br>4. (Ushiro et al., 1996)<br>5. (Higashiyama et al., 1991)<br>6. (Higashiyama et al., 1993)<br>7. (Iwamoto et al., 2003)<br>8. (Jackson et al., 2003)<br>9. (Mine et al., 2005)<br>10. (Shirakata et al., 2005) |
| **EPR** | EGFR<br>ErbB4 | SMC (1) | **SMC** proliferation (1)<br>**SMC** differentiation (2) | - | Viable, chronic dermatitis, defective innate immunity (3) | 1. (Taylor et al., 1999)<br>2. (Takahashi et al., 2003)<br>3. (Shirasawa et al., 2004) |
| **BTC** | EGFR<br>ErbB4 | SMC (1) | **EC** DNA synthesis (2)<br>**EC** migration (2)<br>**EC** tube formation (2)<br>**EC** survival (2)<br>**SMC** proliferation (1)<br>**SMC** migration (3) | Matrigel plug assay (2) | Viable, no overt phenotype (4) | 1. (Tamura et al., 2001)<br>2. (Kim et al., 2003a)<br>3. (Mifune et al., 2004)<br>4. (Jackson et al., 2003) |
| **NRG-1** | ErbB3<br>ErbB4 | EC (1)<br>SMC (2) | **EC** migration (3)<br>**EC** tube formation (3)<br>**EC** proliferation (inhibition) (4)<br>**SMC** proliferation (inhibition) (5) | Rat cornea (3) CAM (6)<br>Murine angiogenesis assay (7) | Embryonic lethality, defect in peripheral neural and cardiac trabeculae development (8) | 1. (Cote et al., 2005)<br>2. (Kato et al., 2003)<br>3. (Russell et al., 1999)<br>4. (Amin et al., 2006)<br>5. (Clement et al., 2007)<br>6. (Yen et al., 2000)<br>7. (Bagheri-Yarmand et al., 2000)<br>8. (Meyer and Birchmeier, 1995) |
| **NRG-2** | ErbB3<br>ErbB4 | - | **EC** proliferation (inhibition) (1) | CAM (inhibitory) (1) | Viable, early growth retardation, reduced reproductive capacity (2) | 1. (Nakano et al., 2004)<br>2. (Britto et al., 2004) |
| **NRG-3** | ErbB4 | - | - | - | - | - |
| **NRG-4** | ErbB4 | EC (splice variant) (1) | - | - | - | 1. (Hayes et al., 2007) |

EC= endothelial cell, SMC= smooth muscle cell, CAM= chorioallantoic membrane angiogenesis assay

## 5. ERBB RECEPTOR TARGETED CANCER DRUGS IN THE REGULATION OF TUMOR ANGIOGENESIS

ErbB receptor research has led to the development of targeted cancer drugs including anti-ErbB monoclonal antibodies (mAbs) directed against the receptor extracellular domain and low molecular weight tyrosine-kinase inhibitors (TKIs) directed against the intracellular tyrosine kinase domain (Fig. 8). Cancer drugs that target EGFR (erlotinib, gefitinib, cetuximab) or ErbB2 (trastuzumab) or both EGFR and ErbB2 (lapatinib) receptors have been approved for clinical use.

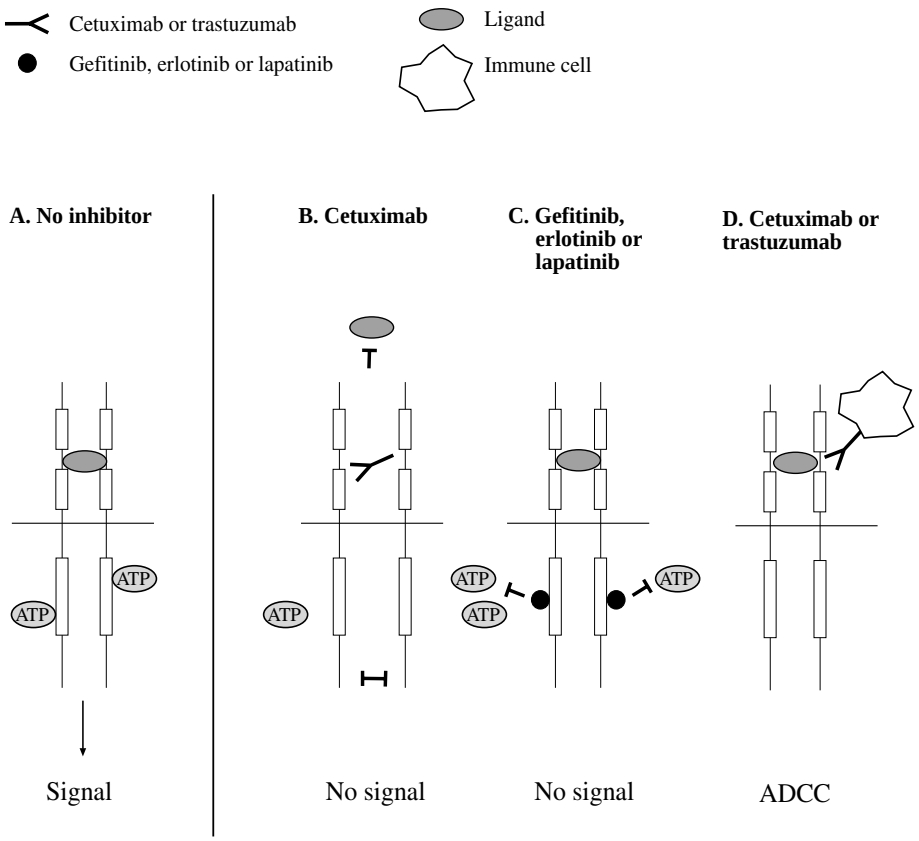


**Figure 8**. Possible functional mechanisms of ErbB receptor targeted cancer therapeutics. A. Ligand binding to the ErbB receptor leads to receptor dimerization and phosphorylation of specific tyrosine residues in the intracellular domain. B. Cetuximab inhibits ligand binding and receptor dimerization. C. Gefitinib, erlotinib and lapatinib inhibit ATP binding within the tyrosine kinase domain of ErbB receptor. D. Cetuximab and trastuzumab activate antibody-dependent cellular cytotoxicity (ADCC). Modified from review article by Elenius (2006).

Part of the antitumor activity of ErbB inhibitor compounds has been speculated to result from an antiangiogenic mechanism. The cellular mechanisms responsible for the possible antiangiogenic effect have not been fully described. Indirect inhibition of

angiogenesis via downregulation of VEGF-A and/or other proangiogenic growth factors has been supposed to contribute to the ErbB targeted drugs' effects on tumor growth (Kumar and Yarmand-Bagheri, 2001; Dutta and Maity, 2007). The direct effect of EGFR targeted therapies on ECs has also been suggested based on their effect on ECs *in vitro* (Baker et al., 2002; Hirata et al., 2002).

During tumorigenesis the genetically unstable tumor cells acquire multiple mutations, which contribute to drug resistance. Targeting of only one alteration may not be sufficient to eliminate an invasive tumor. Therefore, it has been suggested that combination therapies may turn out to be more effective than use of single agents in cancer treatment. For example, it has been suggested that inhibition of EGFR signaling in tumor cells would make them more dependent on angiogenesis and vulnerable to antiangiogenic therapy (Rak et al., 2002; Viloria-Petit and Kerbel, 2004).

## 5.1. Antibodies

### *5.1.1. Cetuximab (Erbitux)*

Cetuximab is a chimeric human/murine monoclonal antibody that binds to the extracellular domain of EGFR. It inhibits ligand binding to the EGFR and blocks tyrosine kinase phosphorylation and promotes receptor internalization (Fig. 8) (Sato et al., 1983; Gill et al., 1984; Sunada et al., 1986; Goldstein et al., 1995). Cetuximab has been shown to inhibit tumor growth in variety of tumor xenografts, including epidermoid, prostate, colon and renal cell carcinoma (Ciardiello and Tortora, 2001). Cetuximab has been approved by the U.S. Food and Drug Administration (FDA) for treatment of patients with advanced colorectal cancer and advanced squamous cell carcinoma of the head and neck (www.fda.gov). The observation that the inhibitory effect of cetuximab in tumor xenografts is often more pronounced than what is seen *in vitro* in cell culture has led to the suggestion that additional anticancer mechanisms such as anti-angiogenic effects may be involved *in vivo* (Harari, 2004).

Cetuximab has been shown to inhibit the tube formation of ECs *in vitro* (Huang et al., 2002b). In addition, there are several reports about the indirect inhibition of angiogenesis by cetuximab in tumor xenografts. Usually cetuximab has been shown to reduce tumor growth and angiogenesis together with decrease of expression of known angiogenic growth factors, such as VEGF-A, IL8 or FGF-2 (Petit et al., 1997; Perrotte et al., 1999; Bruns et al., 2000a; Karashima et al., 2002; Morelli et al., 2006). In addition, cetuximab has been shown to stimulate EC apoptosis in tumor xenograft blood vessels *in vivo* (Bruns et al., 2000a; Inoue et al., 2000; Karashima et al., 2002). The effects of cetuximab on *in vivo* angiogenesis in these analysis represent an indirect effect through the suppression of angiogenic growth factor expression in cancer cells since cetuximab does not bind mouse EGFR (Goldstein et al., 1995).

Elevated VEGF-A expression and blood vessel density has been observed to correlate with acquired resistance of human epidermoid carcinoma xenografts to anti-EGFR-antibodies, including cetuximab. Resistance for anti- EGFR- antibody treatment may arise, at least in part, by selection of tumor cell populations that have increased

angiogenic capacity (Viloria-Petit et al., 2001; Ciardiello et al., 2004). It has been suggested that the combination of a blood vessel targeted drug, such as VEGF-A inhibitor, with an EGFR inhibitor could be beneficial in the tumor treatment (Tabernero, 2007). Supporting the use of combination therapy, combining cetuximab with a VEGFR-2 inhibitor (ZD6474) or anti-VEGFR-2 antibody (DC101) resulted in improved antiangiogenic effect in tumor xenografts (Shaheen et al., 2001a; Morelli et al., 2006).

#### *5.1.2. Trastuzumab (Herceptin)*

Trastuzumab (Herceptin) was the first RTK targeted anti-cancer drug that was brought into clinical use. Trastuzumab is a humanized version of the mouse monoclonal antibody 4D5 (Fendly et al., 1990). It is targeted against the extracellular domain of the ErbB2 receptor (Fig. 8). Since 1998 trastuzumab has been used for treatment of ErbB2-positive metastatic breast cancer. In 2006 it was approved for adjuvant use for high-risk early breast cancer (www.fda.gov). Trastuzumab has been shown to inhibit proliferation of tumor cells, such as breast cancer cells overexpressing ErbB2 both *in vitro* and *in vivo* (Hudziak et al., 1989; Baselga et al., 1998). The mechanism how trastuzumab function is not fully understood. Its proposed mechanisms of action include stimulation of receptor internalization, inhibition of ErbB2 cleavage and antibody-dependent cellular cytotoxicity (ADCC). One of the possible mechanisms by which trastuzumab prevents tumor growth has been suggested to be the inhibition of angiogenesis (Hudis, 2007).

Trastuzumab has been demonstrated to directly inhibit NRG-1-stimulated EC migration and tube formation *in vitro* (Bagheri-Yarmand et al., 2000). Furthermore, trastuzumab has been reported to induce normalization and regression of the breast tumor vasculature. It reduces vessel diameter, volume and permeability, but not vessel length (Izumi et al., 2002). An indirect anti-angiogenic effect has been suggested to be caused by downregulation of angiogenic factors, including VEGF-A, TGF-α, Ang-1, plasminogen-activator inhibitor-1 (PAI-1), IL8 and upregulation of antiangiogenic factor trombospondin-1 (TSP-1) (Izumi et al., 2002; Wen et al., 2006). Trastuzumab has also been reported to reduce recruitment of vascular SMCs in ErbB2 expressing Wilms' tumor xenografts (Yokoi et al., 2003).

### 5.2. Small molecular tyrosine kinase inhibitors

#### *5.2.1. Gefitinib (Iressa)*

Gefitinib is an orally active small molecular tyrosine kinase inhibitor that blocks ATP binding within the tyrosine kinase domain of the EGFR and subsequent signal transduction from activated EGFR (Fig. 8) (Herbst et al., 2004). It inhibits the growth of several tumor cell lines such as prostate, ovarian, breast, colon, lung and head and neck carcinomas *in vitro* as well as tumor growth of a variety of human xenografts, including colon, vulvar, ovarian, breast, lung and prostate tumor (Ciardiello et al., 2000; Sirotnak et al., 2000; Ciardiello and Tortora, 2001). In Asia Pacific region gefitinib is used in the treatment of pretreated advanced NSCLC (www.astrazeneca.com).

In addition to effects on tumor cells, gefitinib has been reported to have antiangiogenic properties. It has been shown to block EGF-stimulated phosphorylation of EGFR and downstream signaling in ECs *in vitro* (Hirata et al., 2002; Hirata et al., 2004). In addition, it inhibits EC proliferation, migration and tube formation (Hirata et al., 2002; Huang et al., 2002a; Hirata et al., 2004; Sini et al., 2005).

The effect of gefitinib on *in vivo* angiogenesis has been analyzed in different angiogenesis models. It has been shown to inhibit EGF-stimulated angiogenesis in a mouse corneal angiogenesis assay (Hirata et al., 2002; Hirata et al., 2004; Ono and Kuwano, 2006), inhibit human squamous cell carcinoma vascularization in matrigel plugs (Huang et al., 2002a), as well as the growth and vascularization of colon cancer in tumor xenografts (Ciardiello et al., 2001).

As an example of the efficiency of combination therapy, gefitinib combined with tumor selective vascular targeting agent (ZD6126) on tumor angiogenesis was more effective in the reduction of tumor growth and microvascular density (Raben et al., 2004). The combination of two EGFR targeted cancer therapies, gefitinib and cetuximab has also been studied. The combination was more efficient in the reduction of tumor size and microvascular density in squamous vulvar carcinoma xenografts than either agent alone (Matar et al., 2004).

It has been suggested that microvascular damage is a key mechanism in tumor response to low dose range radiation (Garcia-Barros et al., 2003). Gefitinib has been shown to improve the effects of radiation therapy *in vitro* and *in vivo* in several tumor cell lines and tumor xenografts, respectively (Bianco et al., 2002; Huang et al., 2002a; Raben et al., 2002; Solomon et al., 2003). Interestingly, combination of gefitinib and radiation has been shown to inhibit angiogenesis to a greater extent compared to treatment with gefitinib or radiation alone in NSCLC xenografts (Raben et al., 2002).

### *5.2.2. Erlotinib (Tarceva)*

Erlotinib is another orally active small molecular weight inhibitor that blocks EGFR kinase activity (Fig. 8). Erlotinib has an effect on the growth of tumor cells such as head and neck in *in vitro* and *in vivo* studies (Moyer et al., 1997; Pollack et al., 1999). It has been approved for treatment of locally advanced or metastatic NSCLC and metastatic pancreatic cancer (www.fda.gov).

The effect of erlotinib on angiogenesis has not been extensively investigated. Erlotinib has been shown to decrease VEGF-A expression in HNSCC cells and breast cancer cells (Pore et al., 2006; Emlet et al., 2007) and inhibit angiogenesis in peripheral nerve sheat tumor xenografts (Mahller et al., 2007).

# AIMS OF THE PRESENT STUDY

The specific aims of the study:

I. To characterize the significance of ErbB signaling system in the paracrine interaction between endothelial and vascular smooth muscle cells.

II. To characterize the role of neuregulin-1 in angiogenesis.

III. To characterize the effect of EGFR inhibition on tumor vasculogenesis and angiogenesis *in vivo*.

# MATERIALS AND METHODS

Materials and methods are described in more detail in original publications (I-III).

## Growth factors, chemicals and inhibitors (I-III)

**Table 2.** Growth factors, chemicals and inhibitors

| Reagent | Application | Concentration | Source | Used in |
|---|---|---|---|---|
| **Growth factors** | | | | |
| EGF | ErbB stimulation | 50 ng/mL | R&D | I |
| BTC | ErbB stimulation | 50 ng/mL | R&D | I |
| NRG-1β1 | ErbB stimulation | 20-100 ng/mL<br>100 ng/disk (CAM assay)<br>160 ng/pellet (mouse corneal assay) | R&D | I, II |
| NRG-1 | ErbB stimulation | 10-100 ng/mL | Neomarkers | II |
| NRG-2 | ErbB stimulation | 50 ng/mL | Dr. S. Higashiyama | I |
| HB-EGF | ErbB stimulation | 20-50 ng/mL | R&D and Dr. J. Abraham | I |
| Ang-1-Fc fusion protein | Tie-2 stimulation | Concentrated medium from Ang-1-Fc transfected cells | | I |
| Ang-1 adenoviral protein | Tie-2 stimulation | 400 MOI exposition to an adenovirus encoding Ang-1 | Drs. K. Alitalo and S. Ylä-Herttuala | I |
| Ang-2-Fc fusion protein | Tie-2 stimulation | Concentrated medium from Ang-2-Fc transfected cells | | I |
| Ang-2 | Tie-2 stimulation | 100-1000 ng/mL | R&D | I |
| FGF-2 | FGFR stimulation | 3-100 ng/mL | PeproTech Inc. | I, II |
| VEGF-A | VEGFR stimulation | 160 ng/pellet (mouse corneal assay) | R&D | II |
| **Chemicals regulating NRG-1 cleavage** | | | | |
| ErbB4 IgG | NRG-1 cleavage activation | 10 μg/mL | Genentech | II |
| PMA | PKC activation | 100 ng/mL | Sigma Aldrich | II |
| GSI IX | Gamma-secretase inhibition | 20 μM | Calbiochem | II |
| Compoud E | Gamma-secretase inhibition | 50 nM | Axxora Platform | II |
| **ErbB receptor inhibition** | | | | |
| Gefitinib | EGFR inhibition | *In vitro*: 1-10 μM<br>*In vivo*: 100 mg/kg | Astra Zeneca | III |
| PD 153035 | EGFR inhibition | 1 μM | Calbiochem | I |
| Trastuzumab | ErbB2 inhibition | 10 μg/mL | Roche | I |
| **ErbB ligand neutralization** | | | | |
| CRM197 | HB-EGF neutralization | 100 μg/mL | Sigma | I |
| α-HB-EGF | HB-EGF neutralization | 1 μg/mL | R&D | I |
| **VEGFR-2 and VEGF inhibition** | | | | |
| SU1498 | VEGFR-2 inhibition | 3.9 μg/disk (CAM-assay) | Calbiochem | II |
| Bevacizumab | VEGF-A neutralization | 1 μg/mL | Genentech | II |

## Antibodies (I-III)

**Table 3.** Primary antibodies used in immunological applications

| Primary antibody | Antigen | Source | Application | Used in |
|---|---|---|---|---|
| **Antibodies for detection of ErbB receptors and ligands** | | | | |
| Anti-EGFR | EGFR | Santa Cruz | WB | I, II |
| C-18 | ErbB2 | Santa Cruz | WB | I, II |
| C-17 | ErbB3 | Santa Cruz | WB | I, II |
| C-18 | ErbB4 | Santa Cruz | WB | I, II |
| C-18 | HB-EGF | Santa Cruz | WB | I |
| M-18 | HB-EGF | Santa Cruz | WB | I |
| Neutralizing anti-HB-EGF | HB-EGF | R&D | WB | I |
| HB-EGF immune serum | HB-EGF | Dr. R. Adam | IHC | I |
| Neuregulin Ab-1 | NRG-1 | NeoMarkers | WB, IHC | II |
| F-20 | NRG-1 | Santa Cruz | WB | II |
| C-20 | NRG-1 | Santa Cruz | ICC | II |
| **Antibodies for detection of phosphorylation** | | | | |
| 4G10 | Phospho Tyr | Upstate Biotech Inc. | WB | I |
| Phospho anti-ErbB1 | Tyr 1068 | Cell Signaling Tech. | WB | I |
| Phospho anti-ErbB2 | Tyr 1248 | Cell Signaling Tech. | WB | I |
| **Antibodies for detection of ECs or PCs/SMCs** | | | | |
| Anti-CD34, My10 | CD34 | BD Biosciences | IHC | I, II |
| Anti-CD31, PECAM | CD31 | BD Biosciences | IHC | III |
| Anti-α-smooth muscle actin | α-SMA | Sigma | IHC | I |
| Anti-α-smooth muscle actin, Cy3 conjugate | α-SMA | Sigma | IHC | III |
| Anti-NG2 | NG-2 | Chemicon | IHC | III |
| **Other antibodies** | | | | |
| HIF-1α | HIF-1α | BD | WB | II |
| Anti-Actin | Actin | Santa Cruz | WB | II |
| Anti-Fc | Fc | Zymed | WB | I |
| Anti-Lamin B | Lamin B | Santa Cruz | WB | II |

Abbreviations; IHC=Immunohistochemistry, ICC=Immunocytochemistry, WB=Western blotting

## Cell lines (I-III)

**Table 4.** Cell lines

| Cell Line | Description | Species | Source or reference | Used in |
|---|---|---|---|---|
| **Endothelial cells** | | | | |
| HUVEC | Human umbilical vein endothelial cell | Human | Isolated from umbilical cord | I, II, III |
| EA.HY 926 | HUVEC and A549 hybridoma | Human | (Emeis and Edgell, 1988) | II |
| BEC | Blood endothelial cell | Human | Dr. K. Alitalo | II |
| LEC | Lymphatic endothelial cell | Human | Dr. K. Alitalo | II |
| BCE | Bovine capillary endothelial cell | Bovine | Dr. M. Klagsbrun or isolated from bovine adrenal cortex | I, II |
| BAEC | Bovine aortic endothelial cell | Bovine | Isolated from bovine aorta | I, II |
| IBEC | Immortalized mouse brain endothelial cell | Murine | Dr. L. Claesson-Welsh | II |
| **Smooth muscle cells** | | | | |
| HASMC | Human aortic smooth muscle cell | Human | ATCC, Promocell | I, II |
| BASMC | Bovine aortic smooth muscle cell | Bovine | Isolated from bovine aorta | I |

**Other cells**

| | | | | |
|---|---|---|---|---|
| HEK293 | Human embryonic kidney epithelial cell | Human | Dr. M. Scheinin | I |
| HaCaT | Skin keratinocyte | Human | Dr. J. Heino | II |
| Tepa | Skin fibroblast | Human | Dr. V-M. Kähäri | II |
| HBL-100 | Breast epithelial cell | Human | Dr. P. Härkönen | II |
| T-47D | Breast cancer cell | Human | ATCC | I, II, III |
| HT-29 | Colorectal cancer cell | Human | Dr. J. Eriksson | III |
| S115 | Breast cancer cell | Murine | Dr. P. Härkönen | III |
| B-16 | Melanoma cell | Murine | Dr. P. Salven | III |
| 32D | Myeloid cell | Murine | (Pierce et al., 1988) | I, II |
| NIH 3T3 | Fibroblast | Murine | (Zhang et al., 1996) | I, II |

## Methods (I-III)

**Table 5.** Methods

***In vitro***

| Method | Used and described in |
|---|---|
| Adenoviral infection of cell cultures | I |
| Boyden chamber migration assay | I, II |
| Cell culture | I, II, III |
| Clonogenic growth assay | III |
| Immunofluorescence microscopy of cells | II |
| MTS proliferation assay | II |
| Northern blotting | I |
| Production of Ang-1 and Ang-2 recombinant protein | I |
| Quantitative real-time RT-PCR | I, II, III |
| RT-PCR | I, II |
| Transfection of cells | I |
| Tube formation assay | II |
| Western blotting | I, II, III |

***In vivo***

| | |
|---|---|
| Chorioallantoic membrane (CAM) assay | II |
| Immunofluorescence microscopy of mouse tumor tissues | III |
| Immunohistochemistry of patient tissues | I, II |
| Mouse corneal micropocket assay | II |
| Mouse tumor angiogenesis model: tumor cell transplantation | III |
| Mouse tumor vasculogenesis model: bone marrow transplantation | III |

**Other methods**

| | |
|---|---|
| Statistical analysis | III |
| Vessel quantification | II, III |

# RESULTS

## 1. The expression of ErbB ligands and receptors in vascular endothelial cells and smooth muscle cells (I, II)

The role of ErbB receptors in tumor growth has been intensively studied but relatively little is known about the role of these receptors in the regulation of angiogenesis or postnatal vasculogenesis, processes also known to contribute to tumor growth and metastasis.

To investigate the role of ErbB ligands and receptors in angiogenesis, different methods were set up to isolate ECs and SMCs from vessels of different sizes and from different species. The primary endothelial cell types used in this study were human umbilical vein ECs (HUVECs) (I, Figs. 1, 2, 5, 6 and 7; II, Figs. 1, 2, 3, 4, 6 and 7; III, Fig. 1), blood vascular ECs (BEC) (II, Fig. 1), lymphatic vascular ECs (LEC) (II, Fig. 1), ECs generated by fusion of HUVECs with cancer cells (EAHY) (II, Fig. 3), bovine capillary ECs (BCE) (I, Figs. 1, 2 and 5; II, Fig. 3), bovine aortic ECs (BAEC) (II, Fig. 3) and immortalized brain ECs from mouse (IBEC) (II, Figs. 3 and 4). The primary SMC lines used in this study included human aortic SMCs (HASMCs) (I, Figs. 1, 3, 4 and 5), and bovine aortic SMCs (BASMCs) (I, Figs. 1, 3, 4 and 5). BEC, BCE, LEC and IBEC cells represent microvascular cell types. Other cell types were obtained from larger vessels such as veins or aorta.

To characterize the role of ErbB signaling in angiogenesis, ErbB ligand expression was analyzed in primary ECs from different sources (HUVEC, BEC, LEC). HB-EGF and NRG-1 were expressed in HUVECs at mRNA level when analyzed by RT-PCR (I, Fig. 2A) or by real-time RT-PCR (II, Fig. 1A). Microvascular BECs and lymphatic LECs expressed mostly HB-EGF (II, Fig. 1A). No specific expression was detected for TGF-α, EPR, AR, BTC, NRG-1, NRG-2, NRG-3, or NRG-4 (I, Fig. 2A; II, Fig. 1A).

The expression of HB-EGF and NRG-1 in ECs was also confirmed at the protein level. The expression of HB-EGF in HUVECs was analyzed with four different HB-EGF antibodies raised against either intracellular or extracellular domain of HB-EGF. All antibodies recognized the 46-48 kDa HB-EGF doublet band (I, Fig. 2B). The secretion of soluble HB-EGF was confirmed by ELISA analysis of HUVEC conditioned medium (CM) with two different HB-EGF antibodies (I, data not shown). Furthermore, BCEs also expressed HB-EGF protein (I, Fig. 2B). The expression of ~120 kDa NRG-1 precursor protein was shown by Western blot analyses using antibodies recognizing either the extra- or intracellular domain of the NRG-1 precursor (II, Fig. 2A, data not shown).

The NRG-1 gene can produce a number of structurally and functionally different isoforms by alternative splicing (Falls, 2003). The RT-PCR analysis, together with Western analysis demonstrated that isoforms expressed by HUVECs included Type I isoforms α2a, β1a, β2a, as well as Type III isoform α2a (II, Fig. 1B).

The expression of HB-EGF and NRG-1 in ECs was also confirmed *in vivo* by immunohistochemical staining. Immunostaining of 10-wk-old human fetus demonstrated HB-EGF expression in ECs in the majority of developing vascular structures including arterial, venous and capillary vessels (I, Fig. 8). Furthermore, NRG-1 was shown to colocalize with ECs in the capillaries and venules of breast cancer tissue (II, Fig. 1C).

ErbB ligand expression was also characterized in SMCs. Real time RT-PCR analysis demonstrated that HASMCs did not express high levels of any of the analyzed ErbB ligands (II, Fig. 1A). In immunohistochemical analysis of a 10-wk-old human fetus, SMCs of blood vessels were slightly positive for HB-EGF (I, Fig. 8).

To characterize whether ECs and SMCs make receptors for ErbB ligands, the ErbB receptor pattern was analyzed in the primary ECs of human, bovine and murine origin (HUVEC, BEC, LEC, EA.HY, BAEC, BCE and IBEC) as well as SMCs of human and bovine origin (HASMC and BASMC). This analysis demonstrated that both ECs and SMCs expressed EGFR and ErbB2, but not ErbB3 or ErbB4 in both mRNA and protein levels (I, Fig. 3; II, Figs. 3A and B). In HUVECs the ErbB mRNA expression relative to β-actin expression was 0.14% and 0.34% for EGFR and ErbB2, respectively (II, Fig. 3A). The signal for ErbB3 and ErbB4 was close to the background. In HASMCs the ErbB mRNA expression relative to β-actin expression was 3.9%, 1.2%, 0.02%, and 0.05% for EGFR, ErbB2, ErbB3 and ErbB4, respectively (I, page 1613).

## 2. The role of endothelial cell-derived ErbB ligands in angiogenesis

### 2.1. Regulation of endothelial-smooth muscle cell interaction by HB-EGF (I)

The paracrine interplay between ECs and SMCs is essential for normal development and maintenance of the cardiovascular system. ErbB receptors and their ligands regulate the interplay between the endocardium and myocardium in the heart (Marchionni, 1995). The expression of HB-EGF in ECs and its receptors in SMCs led to the hypothesis that these molecules could also have role in the interaction between vascular ECs and SMCs.

In order to characterize the role of ErbB ligands in the paracrine interaction between ECs and non-endothelial cells, CM were collected from ECs and SMCs. The capacity of these media to stimulate EC or SMC migration was measured using a Boyden chamber migration assays. Paracrine signaling between ECs and SMCs was indicated as the migratory responses of human and bovine aortic SMCs (HASMC and BASMC) to factors secreted by primary ECs but not vice versa (I, Fig. 1).

To analyze the role of HB-EGF in EC-SMC interaction we used different methods to block HB-EGF signaling in the recruitment of SMCs by ECs. Neutralizing HB-EGF with CRM197, a mutated non-toxic analogue of DT, or a neutralizing anti-HB-EGF antibody or inhibiting EGFR with tyrosine kinase inhibitor (PD 153035) or ErbB2 with an inhibitory antibody for ErbB2 (trastuzumab) suppressed EC-CM-stimulated migration of SMCs in the Boyden chamber migration assays (I, Figs. 5A-C). Thus,

endothelial-derived HB-EGF mediated SMC migration by activating EGFR and ErbB2 on the surface of SMCs. HB-EGF neutralization or EGFR and ErbB2 inhibition also blocked migration of SMCs stimulated by recombinant HB-EGF (I, Figs. 5D and 5E). Furthermore, NRG-1 did not stimulate the migration of SMCs, consistent with the lack of NRG-1 receptors in SMCs (I, Fig. 5D).

Ang-1, a factor known to be required for proper EC-SMC interaction *in vivo*, up-regulated HB-EGF expression in ECs both by mRNA and protein levels (I, Figs. 6B, 6C and 7A). On the contrary, Ang-2 downregulated rather than stimulated HB-EGF mRNA expression (I, Figs. 6B and C). Furthermore, CM from Ang-1-treated HUVECs stimulated SMC migration in an HB-EGF dependent manner (I, Fig. 7B). These results suggest that Ang-1 mediates SMC migration partly stimulating expression of HB-EGF in ECs.

To characterize the role of HB-EGF in angiogenesis *in vivo*, human embryos were analyzed by immunohistochemistry. In this analysis, strong HB-EGF staining colocalized with ECs of vessels containing SMA-positive PCs or SMCs. However, ECs from hyaloid vessels in the developing eye expressed less, if any, HB-EGF. Interestingly, these vessels were also not associated with any detectable SMA-positivity (I, Figs. 8I-L). These findings together with the knowledge of later regression of the hyaloid vessels from the developing vitreous (Zhu et al., 2000) support the role of HB-EGF in EC-SMC interaction during *in vivo* angiogenesis. Furthermore, blood ECs (BECs) expressed more HB-EGF than the lymphatic ECs (LECs) (II, Fig. 1A). This is consistent with the relative lack of supporting SMCs in lymphatic vessels (Alitalo et al., 2005) and the suggested role of HB-EGF in SMC recruitment.

### 2.2. Regulation of angiogenesis by NRG-1 (II)

The formation of new blood vessels is regulated by specific growth factors that modulate different cellular functions of ECs, as well as other cell types such as SMCs that interact with ECs in a paracrine manner (Carmeliet, 2000). Since observed lack of NRG-1 receptors in SMCs, NRG-1 was unlikely to mediate EC-SMC interaction and its function in angiogenesis was suggested to differ from HB-EGF.

To characterize the role of NRG-1 in angiogenesis, its effect on neovascularization *in vivo* was analyzed. NRG-1 was shown to stimulate *in vivo* angiogenesis in the mouse corneal assay as well as the chick CAM assay (II, Figs. 5 and 7D). In addition, hypoxia was shown to regulate NRG-1 expression in HUVECs, supporting a role of NRG-1 in angiogenesis (II, Fig. 2A).

During angiogenesis ECs respond to growth factors by proliferating, migrating and differentiating and finally forming new blood vessel sprouts (Conway et al., 2001). *In vivo* growth factors can stimulate ECs either directly through the receptors on EC surface or indirectly through the stimulation of angiogenic growth factor secretion from other non-endothelial cells, such as tumor cells or cells of the immune system (Folkman and Shing, 1992).

To analyze the effect of NRG-1 on EC functions *in vitro,* migration, proliferation and differentiation assays were performed. NRG-1 did not directly regulate EC functions, consistent with the lack of NRG-1 receptors in ECs (II, Figs. 3A, B and 7A-C).

To determine whether NRG-1 stimulates angiogenesis through paracrine induction of VEGF family members, the effect of NRG-1 stimulation on the expression of VEGF mRNAs was analyzed in different cell lines. Real-time RT-PCR analysis demonstrated that NRG-1 stimulated VEGF-A mRNA expression in epithelial cells such as keratinocytes (HaCat) and breast epithelial cells (HBL-100) detected by real time RT-PCR (II, Fig. 6A, data not shown). The finding was confirmed with a VEGF-A ELISA assay, in which VEGF-A protein expression was stimulated 3-fold in HaCats and 1.4-fold in HBL-100 cells after NRG-stimulation (II, page 2905). VEGF-B and VEGF-C mRNA expression was rather inhibited than stimulated by NRG-1 and VEGF-D expression was not detected in HaCats (II, Figs. 6B-D).

To investigate whether VEGF-A was necessary for the regulation of EC functions by NRG-1, anti-VEGF-A antibody (bevacizumab) was tested in HaCat CM stimulated EC migration and proliferation assays. CM from NRG-1 treated HaCat cells stimulated EC migration and proliferation, both of which were blocked by anti-VEGF-A antibody (II, Figs. 7A-C). To analyze whether VEGF system was involved in NRG-1 stimulated angiogenesis *in vivo*, VEGFR-2 inhibitor (SU1498) was tested in CAM assay. SU1498 inhibited NRG-1-stimulated formation of new capillaries (II, Fig. 7D). Together these results suggest that extracellular domain of NRG-1 stimulates angiogenesis by an indirect mechanism by up-regulation of VEGF-A in non-vascular cell types.

Many of the NRG isoforms are synthesized as membrane-anchored precursors from which soluble mature growth factors can be shed. In addition, transmembrane NRG-1 may participate in signal transduction inside of the cell by releasing a soluble intracellular domain (ICD) upon stimulation of the NRG-1 precursor with an ErbB ectodomain (Bao et al., 2003). To further characterize the role of EC-derived NRG-1 in angiogenesis, we analyzed its processing in HUVECs. Western blot analysis of HUVECs demonstrated that 120 kDa transmembrane NRG-1 was processed into extracellular and intracellular fragments. Treatment of HUVECs with PMA, which is known to stimulate protein shedding, as well as, with γ-secretase inhibitor, which inhibits the cleavage of the intracellular domain, stimulated the accumulation of ~60 kDa carboxyterminal fragment. In addition, ~60 kDa extracellular domain was detectable in concentrated HUVEC CM with NRG-1 antibody against the extracellular domain of NRG-1 (II, Fig. 2A, data not shown). Furthermore, the treatment of ECs with a fusion protein ErbB4-IgG, stimulated the localization of the intracellular domain of NRG-1 into the nucleus and inhibited serum-stimulated EC migration (II, Figs. 2B-D). These findings suggest that NRG-1 ICD is a functional signaling molecule in ECs.

Altogether, these results indicate that NRG-1 may regulate EC functions and angiogenesis both indirectly via the extracellular domain, as well as directly via the intracellular domain.

## 3. The effect of EGFR inhibition on angiogenesis and postnatal vasculogenesis *in vivo* (III)

We demonstrated that HB-EGF/EGFR signaling was important for the recruitment of SMCs by ECs *in vitro* (I). To analyze the significance of this observation *in vivo*, the effect of an EGFR TKI gefitinib was tested using a syngeneic mouse model with B-16 melanoma cell tumor.

B16 melanoma cells did not express EGFR receptor at mRNA or at protein levels, when analyzed by real-time RT-PCR or Western blotting, respectively (III, Figs. 1A and B). B16 cells were also relatively resistant to gefitinib treatment in clonogenic growth assay. The IC50 of B16 cells for gefitinib (5.1 μM) was relatively high compared to the IC50 of gefitinib-sensitive head and neck cancer cell line UT-SCC19A (0.18 μM) or IC50 of HUVECs (1.6 μM) (III, Fig. 1C and page 9). The lack of EGFR expression in B16 melanoma cells and the relative resistance of B16 cells to EGFR inhibition by gefitinib suggested that these cells could be used to study the significance of EGFR signaling in tumor stroma including vascular structures without direct effects on tumor cells.

To analyze the effect of EGFR inhibition on tumor angiogenesis and vasculogenesis B16 tumor cells were injected to the flank of syngeneic C57BL/6J mice with bone marrows reconstituted with GFP-positive cells. Gefitinib or buffer control treatment was started before tumors reached the size of 100 $mm^3$. Daily per-oral gefitinib treatment for 7 days led to the moderate, but statistically non-significant reduction in tumor growth (III, Fig. 2). Tumor samples were collected after 7 days treatment and prepared to frozen tissue sections for immunofluorescence staining. Tissue sections were stained with anti-CD31, anti-SMA or anti-NG2 antibodies and analyzed by confocal microscopy.

In this analysis gefitinib slightly stimulated microvascular density (MVD) when CD31-positive vascular structures were scored from areas with most intensive blood vessel density, so called vascular hot spots (III, Figs. 3B and C). The number of tumor vessels with PCs or SMCs that stained positive for SMA was similar in gefitinib and control treated groups (III, Fig. 4). However, the number of CD-31 positive tumor vessels smaller than 50μm diameter, covered with NG2-positive PCs/SMCs was reduced in gefitinib-treated mice (III, Figs. 5A and E). Also the ratio of NG2-positive PC/SMCs over CD31-positive ECs in these tumor vessels was reduced demonstrating reduced PC coverage (III, Figs. 5F and G).

In order to analyze the role of EGFR in the recruitment of BM-derived cell to the tumor vasculature, the C57BL/6J mice were irradiated and their bone marrow was reconstituted by GFP-positive bone marrow from C57BL/6-TgN(ACTbEGFP)1Osb/J mice. B16 melanoma cells were injected to the mice with reconstituted bone marrow and the localization of GFP-positive bone marrow-derived cells in the vasculature was analyzed by confocal microscopy of tumor sections.

Most of the GFP-positive cells were located either intraluminally or perivascularly (Fig. 6A). The number of GFP-positive BM-derived perivascular cells was significantly decreased in the gefitinib-treated mice (Figs. 6B and C). These cells were closely associated with tumor blood vessels but were not positive for CD31, NG2 or SMA. (III, Fig. 6B).

These results suggest a role for EGFR signaling in the recruitment of PCs or SMCs to the growing vasculature *in vivo*, as well as in the recruitment of perivascular BM-derived cells during tumor neovascularization.

# DISCUSSION

In the studies presented here, different approaches were used to analyze the role of ErbB signaling in the development of new blood vessels. Studies I and II were mainly *in vitro* studies that characterized the role of EC-derived HB-EGF (I) and NRG-1 (II) in angiogenesis. Study III was an *in vivo* study, in which the role of EGFR signaling was analyzed in angiogenesis and postnatal vasculogenesis during tumor growth.

## 1. The expression of ErbB ligands in vasculature

The vascular system consists of two principal cell types, ECs and PCs/SMCs (Hirschi and D'Amore, 1996). During this study we analyzed the expression of ErbB ligands in vascular and lymphatic ECs as well as vascular SMCs *in vitro*. The expression of EC-specific ErbB ligands was also analyzed in different vascular structures *in vivo*.

The analysis of ErbB ligand expression in HUVECs demonstrated that they expressed HB-EGF and NRG-1. These results were consistent with previous findings that have shown HB-EGF mRNA and protein, as well as, NRG-1 protein expression in ECs (Yoshizumi et al., 1992; Arkonac et al., 1998; Zhao et al., 1998; Cote et al., 2005). Neither real-time RT-PCR nor classical RT-PCR analyses showed significant expression of other ErbB ligands including EGF, TGF-α, AR, BTC, EPR, NRG-2, NRG-3 or NRG-4 in blood vessel ECs (I, II). However, at least EGF and TGF-α expression have previously been detected by immunohistochemistry in the endothelium of tumor, granulation or uterine tissues (Wakui et al., 1990; Dunn et al., 2000; Andronowska et al., 2006). This difference between *in vitro* and *in vivo* expression may be explained by the effect of other factors *in vivo* to the stimulation of EGF and TGF-α expression in blood vessel endothelium during angiogenic processes. In addition, ECs have been shown to express the B3 isoform of NRG-4 (Hayes et al., 2007). This isoform would not be detected with the primers and probe used for our RT-PCR analyses.

Microvascular BECs demonstrated relatively less NRG-1 expression when compared to HB-EGF expression than vein-derived HUVECs, which expressed similar quantities of both ligands (II). This may mean that the functional significance of the two ligands differs in different vascular beds. The immunohistochemical analysis of breast cancer blood vessels also demonstrated NRG-1 staining in venule-like structures and capillaries in the vicinity of tumor cells (II). However, no comparative analysis of NRG-1 expression in different vascular structures has been reported.

HB-EGF was the only ErbB ligand expressed by lymphatic ECs (II). To our knowledge, no detailed analysis of the role of HB-EGF in lymphangiogenesis is currently available. The lack of NRG-1 expression in lymphatic ECs and the inability of NRG-1 to stimulate the expression of lymphangiogenic growth factors, VEGF-C

and VEGF-D in epithelial cells, suggest that NRG-1 does not have a role in lymphangiogenesis (II).

The analysis of ErbB ligand mRNA expression in SMCs demonstrated moderate expression of AR, HB-EGF and NRG-1 although at reduced levels when compared to ECs. Other laboratories have demonstrated that SMCs express several ErbB ligands such as EGF, AR, EPR, HB-EGF, BTC, and NRG-1 (Table 1). This difference may be explained by the use of different cell lines and methods for the detection of ErbB ligand expression.

In immunohistochemical analysis of developing vascular structures, we detected only slightly positive staining for HB-EGF in the SMCs of blood vessels, which had clear endothelial HB-EGF staining (I). In real-time RT-PCR analysis SMCs expressed only 0.4% of the levels at HB-EGF m-RNA expressed by ECs (II). Similar results were obtained by Western blot analysis in which HB-EGF was barely detectable in SMCs under conditions that gave a clearly detectable HB-EGF band for ECs (I). These results suggest that the primary vascular source for HB-EGF is the endothelium and support the role of HB-EGF in directional chemotaxis of SMCs toward ECs.

Taken together, these data represent to our knowledge the first analysis of the whole ErbB ligand pattern in vascular ECs, lymphatic ECs and SMCs. The results suggest that ErbB ligands may have various functions during blood vessel formation.

## 2. The expression of ErbB receptors in vasculature

The ErbB expression in ECs as well as SMCs has been inconsistent in the different studies. Most studies have demonstrated EGFR expression in ECs and SMCs, but also the expression of ErbB2, ErbB3 and ErbB4 has been reported (Schreiber et al., 1986; Russell et al., 1999; Baker et al., 2002; Hirata et al., 2002; Kim et al., 2003a; Shin et al., 2003; Sini et al., 2005).

Our analysis of ErbB expression showed EGFR and ErbB2 but not ErbB3 or ErbB4 expression in several EC as well as SMC lines both at mRNA and protein levels (I, II). The differencies in the ErbB expression patterns observed by different laboratories may be explained by the source of ECs or SMCs, including different species (human, murine or bovine), different tissues or different sizes of the vessels (capillary, artery, vein or aorta) from which cells are isolated. For example, Notch and Eph expression has been shown to differ between venous and arterial ECs (Torres-Vazquez et al., 2003). Culture conditions, cell passages and cell freezing may also influence different protein expression.

## 3. The direct endothelial effects by ErbB ligands

Several studies have demonstrated the stimulation of *in vivo* angiogenesis by recombinant ErbB ligands, such as EGF, TGF-α, BTC, AR, HB-EGF or NRG-1 (Table 1). Direct regulation of ECs by ErbB ligands, such as EGF, TGF-α, BTC, HB-EGF and NRG-1, has been demonstrated *in vitro* as the ability of ErbB ligands to stimulate EC proliferation, migration or differentiation (Table 1).

The expression of EGFR in ECs supports the results of the direct regulation of angiogenesis by EGFR ligands, such as EGF and TGF-α (Tang et al., 2000; Mifune et al., 2004; Sini et al., 2005). Our results have also demonstrated stimulation of EC proliferation by EGFR ligands EGF, TGF-α and BTC as well as stimulation of tube formation by HB-EGF (unpublished results and II, Fig. 4C).

NRG-1, a ligand for ErbB3 and ErbB4, has been shown to stimulate angiogenesis *in vivo* as well as regulate EC functions directly *in vitro* (Russell et al., 1999). We were also able to demonstrate the stimulation of angiogenesis by NRG-1 in two different angiogenesis assays, in CAM assay and a mouse corneal angiogenesis assay, but we did not observe stimulation of EC proliferation, migration or tube formation by recombinant NRG-1 *in vitro* (II). This was consistent with the lack of NRG-1 receptor expression in ECs used in our analyses. The different expression patterns of ErbB receptors in analyzed ECs may explain the different *in vitro* results. The use of different isoforms of recombinant NRG-1 proteins may also have an effect on the results (NRG-1β3 or NRG-1β1) but also different results have been obtained when the same isoforms have been used (Bagheri-Yarmand et al., 2000; Yen et al., 2000). Altogether, these results suggest that NRG-1 regulates angiogenesis and that the mechanisms may involve both direct as well as indirect effects on ECs.

## 4. ErbB ligands in the interaction between ECs and SMCs

The interaction between ECs and SMCs is necessary for the formation of functional blood vessels. Our results suggested that HB-EGF is the major EGF-like ligand expressed by ECs that participates in the paracrine signaling between ECs and SMCs (I). The role of HB-EGF in the interaction between ECs and SMCs was supported by the previous findings of HB-EGF expression in ECs and the function of HB-EGF as a chemoattractant for SMCs (Raab and Klagsbrun, 1997). Some indirect implications had been made about the role of HB-EGF in EC/SMC interaction (Folkman and D'Amore, 1996; Abramovitch et al., 1998; Arkonac et al., 1998), but the role of HB-EGF in the recruitment of SMCs by ECs had not been experimentally addressed before.

In addition to HB-EGF, PDGF-B has been shown to be an important regulator of SMC recruitment both *in vitro* and *in vivo* (Fig. 9). It has been shown to regulate interaction between ECs and SMCs also during embryogenesis. Mice lacking *PDGF-B* or *PDGFR-β* genes demonstrate site-specific reduction of PCs and SMCs around blood vessels. Differential loss of PCs has been observed depending on the tissue examined. For

instance in the brain, but not in most other tissues, complete loss of PCs is observed. Variety has also been observed in the requirement of PDGF-B for SMC proliferation and migration in different types of vessels. For instance, in veins PDGF-B does not seem to play a role in the SMC recruitment (Hellstrom et al., 1999). Heterogenous expression of PDGF-B is also detected in tumor blood vessels (Abramsson et al., 2002). These findings suggest that PDGF-B is an important mediator of PC and SMC recruitment, but also other factors may participate in the interaction between ECs and SMCs. In accordance, our comparison of HB-EGF and PDGF-B staining in tumor and normal tissue blood vessels demonstrated that there are blood vessels, which are positive for both PDGF-B and HB-EGF, as well as vessels that express one but not the other, suggesting that the functions of the two ligands are not redundant (unpublished results).

Our results suggested that Ang-1 mediates SMC migration partly stimulating expression of HB-EGF in ECs (I; Fig. 9). Tie-2 is mostly EC-specific, whereas its ligand Ang-1 is expressed by perivascular and mural cells. It has been suggested that Ang-1 binding to Tie-2 activates a signal from ECs that recruits PCs/SMCs around ECs. PDGF-B and HB-EGF have been proposed to be involved in the Ang-1-regulated recruitment of PCs/SMCs by ECs (Folkman and D'Amore, 1996).

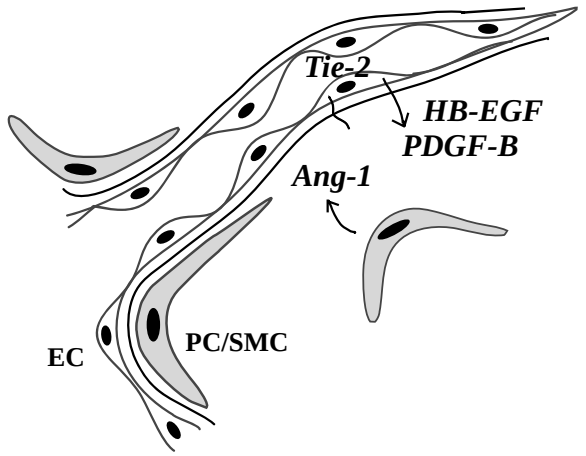


**Figure 9.** Recruitment of pericytes (PCs)/smooth muscle cells (SMCs) by endothelial cells (ECs). Ang-1, PDGF-B and HB-EGF are involved in the recruitment of PCs/SMCs by ECs.

The expression of Tie-2 has recently been detected also in vascular SMCs, and Ang-1 in assistant with VEGF-A has been shown to regulate the migration of SMCs directly. However, the expression of Tie-2 in SMCs is much lower than in ECs (Tian et al., 2002; Metheny-Barlow et al., 2004) and the suggested source of Ang-1 has been mesenchymal cells (Davis et al., 1996) supporting the recruitment of PCs/SMCs by growth factor from Tie-2 expressing ECs.

The analysis of mice lacking *HB-EGF* gene has not revealed a vascular phenotype to this day (Iwamoto et al., 2003; Jackson et al., 2003; Mine et al., 2005; Shirakata et al., 2005;

Nanba et al., 2006). This does not exclude a role for HB-EGF in the recruitment of SMCs. The detailed analysis of the vasculature of HB-EGF targeted mice has not been reported. In addition, HB-EGF may have an important role in post-natal pathological angiogenesis, for example during tumor growth. A similar example is found in the VEGF family. Mice lacking *PlGF* gene do not have a vascular defect during embryogenesis, but demonstrate defective pathological angiogenesis after birth (Carmeliet et al., 2001).

## 5. The indirect regulation of angiogenesis by ErbB ligands

In addition to the direct effects on ECs, ErbB ligands have been shown to mediate angiogenesis by regulating the expression of known angiogenic growth factors, such as VEGF-A from non-vascular cell types (Goldman et al., 1993; O-charoenrat et al., 2000; Hirata et al., 2002; Akagi et al., 2003; Ueda et al., 2006).

The expression of NRG-1 in ECs and its effect on *in vivo* angiogenesis led us to further address the mechanism by which it regulates angiogenesis. The lack of *in vitro* effects of recombinant NRG-1 on cultured ECs indicated that it has to regulate angiogenesis by an indirect mechanism. NRG-1 was demonstrated to stimulate expression of the major angiogenic growth factor, VEGF-A, in epithelial cells *in vitro,* as well as regulate EC functions *in vitro* and angiogenesis *in vivo* in a VEGF-A and VEGFR-2 dependent manner. These results suggest that NRG-1 regulates angiogenesis by mechanisms involving VEGF-A up-regulation in non-vascular cell types. In addition the stimulation of NRG-1 expression by hypoxia supported its role in the stimulation of angiogenesis (II).

Our findings about the indirect regulation of EC functions and angiogenesis by NRG-1 confirm the findings of other groups (Xiong et al., 2001; Bagheri-Yarmand et al., 2000). Furthermore, it has been demonstrated that NRG-1 stimulates VEGF-A secretion in breast and lung cancer cell lines, but not normal mammary or bronchial epithelial cell lines (Yen et al., 2000). However, our results suggested that NRG-1 stimulates the expression of VEGF-A also in normal epithelial cells of the skin and breast at both mRNA and protein levels *in vitro* (II). Our results about stimulation of angiogenesis by NRG-1 in a VEGF system dependent manner in tissues where tumor cells are not present (chorioallantoic membrane and cornea) (II) are consistent with previous results by others where in the murine model of angiogenesis NRG-1-stimulated angiogenesis is blocked by an anti-VEGF-A antibody (Bagheri-Yarmand et al., 2000).

## 6. The role of EGFR in tumor angiogenesis and vasculogenesis

At the time we initiated *in vivo* mouse studies with EGFR inhibitor others had addressed the effect of EGFR inhibition on tumor angiogenesis (Petit et al., 1997; Perrotte et al., 1999; Ciardiello et al., 2001; Huang et al., 2002a; Morelli et al., 2006). However, the mechanism by which EGFR inhibition blocked tumor neovascularization was not completely characterized and possible effects on postnatal vasculogenesis had not been addressed in detail.

EGFR inhibition by tyrosine kinase inhibitors gefitinib and erlotinib has been shown to suppress angiogenesis *in vivo* in different angiogenic models and tumor xenografts (Ciardiello et al., 2001; Huang et al., 2002a; Mahller et al., 2007). According to the *in vitro* effect of EGFR inhibition on EC functions, part of the anti-angiogenenic effect of EGFR inhibition has been attributed to be direct effect on the endothelium (Hirata et al., 2002; Huang et al., 2002a; Hirata et al., 2004; Sini et al., 2005). However, to our knowledge the effect of EGFR inhibition on the recruitment of PCs or SMCs during tumor neovascularization *in vivo* has not been demonstrated before.

The findings that EGFR inhibition by gefitinib reduced NG-2 positive vessels smaller than 50 μm diameter and that the NG-2 positive PC to EC ratio was reduced in these vessels suggested that EGFR inhibition partly blocks the recruitment of PCs or SMCs (III). This supports the previously detected role of EGFR ligand HB-EGF in the recruitment of SMCs by ECs *in vitro* (I). We could not detect a difference in SMA positive vessels between gefitinib treated and control mice (III). Since NG2 is expressed widely in PCs and SMCs (Ozerdem et al., 2001; Ozerdem et al., 2002; Ozerdem and Stallcup, 2003), whereas SMA is supposed to be a marker of more differentiated PCs/SMCs, it might be possible that all PCs of angiogenic microvasculature are not detected using SMA as a marker of PCs (Nehls and Drenckhahn, 1991; McDonald and Choyke, 2003). The common use of SMA as a marker for PCs and SMCs might also explain why the effect of gefitinib on PC/SMC recruitment has not been previously observed.

Our finding that EGFR inhibition stimulated MVD was not expected, in the light of previous findings, which demonstrated the inhibition of angiogenesis by EGFR targeting drugs (III; review of literature 5.2.1, 5.2.2). Since tumor cells used in this study did not express EGFR and were relatively resistant to EGFR inhibition *in vitro*, we did not expect to detect indirect effects on angiogenesis of blocking EGFR signaling in tumor cells. In addition, the gefitinib treatment was terminated relatively early (after 7 days) when no significant effect on mean tumor volume was observed. Moreover, our use of a syngeneic tumor model differs from the commonly used tumor xenografts that utilize immunodeficient mice. Furthermore, PC loss has been suggested to stimulate angiogenesis possibly through the destabilization of blood vessels (Hanahan, 1997). Since gefitinib inhibited PC/SMC recruitment this may also explain the increase of MVD after gefitinib treatment (III).

Postnatal vasculogenesis has been shown to be an active process during tumor neovascularization. However, the mechanisms that regulate recruitment of BM-derived cells to vasculature are not fully characterized. It has been proposed that BM-derived cells can incorporate to the vasculature either as a part of the endothelium or pericyte layer. Perivascular BM-derived cells, which do not express either EC or PC markers, have also been observed (Lamagna and Bergers, 2006). The importance of BM-derived cells for the formation of tumor blood vessels has been previously demonstrated. Inhibition of postnatal vasculogenesis has been suggested to provide a novel approach to block tumor growth and angiogenesis (Lyden et al., 2001).

*In vivo* analysis of postnatal vasculogenesis using genetically reconstituted mice with GFP-positive bone marrow carrying B16 melanoma tumor demonstrated that EGFR inhibition by gefitinib decreased the number of BM-derived cells in the close vicinity of blood vessels (III). The identity of these perivascular BM-derived cells is unclear. It has been proposed that they might be fibroblasts, undifferentiated PCs, macrophages or other cells of the immune system that support blood vessel growth by providing growth factors (Rajantie et al., 2004; Ziegelhoeffer et al., 2004). However, only few GFP-positive cells expressing CD31, NG2 or SMA were detected (III).

The mechanism by which EGFR signaling regulates the recruitment of BM-derived cells is not known. EGFR receptor and HB-EGF have been shown to be involved in the regulation of BM-derived mesenchymal stem cell functions. It has been reported that EGFR is expressed on the surface of BM-derived mesenchymal stem cells and HB-EGF regulates proliferation and differentiation of these cells in an EGFR-dependent manner (Krampera et al., 2005). In addition, gefitinib has been shown to inhibit EGFR activation and proliferation of mesenchymal stem cell like cells *in vitro* (Normanno et al., 2005).

## 7. Potential of ErbB inhibition as an antiangiogenic therapy

EGFR and ErbB2 have been well demonstrated to promote growth of different malignancies. Cancer drugs that target these receptors, such as cetuximab, trastuzumab and gefitinib have been shown to suppress tumor growth as well as tumor angiogenesis (Petit et al., 1997; Ciardiello et al., 2001; Izumi et al., 2002). The observations about the expression of EGFR and ErbB2 receptors in the vascular cells and their role in the recruitment of SMCs by ECs, suggest that available ErbB inhibitor drugs could be used to target directly ECs, SMCs or EC-SMC interaction.

It has been suggested that anti-angiogenic cancer treatments directed against different molecular and/or cell targets might increase the efficacy of and decrease the resistance to cancer therapy (Carmeliet, 2005). For example, broad-spectrum receptor TKIs which inhibit both EC and tumor cell functions such as Sutent (Sunitinib; SU11248), an inhibitor of PDGFRs and VEGFRs, are effective as cancer monotherapy for certain tumors (Carmeliet, 2005). A more potent antitumor and anti-angiogenic activity has also been achieved by concomitant inhibition of ErbB and VEGFR-2 activities (Shaheen et al., 2001a). Furthermore, TKIs that target EGFR and VEGFR-2 (vandetanib; Zactima; ZD6474) or EGFR, ErbB2 and VEGFR-2 (AEE788), show antitumor and antiangiogenic activity and are currently investigated in clinical trials (Traxler et al., 2004; Herbst et al., 2007).

Therapeutic antibodies or inhibitors that bind ErbB ligands are not currently available in clinic. The role of HB-EGF and NRG-1 (I, II) in angiogenesis supports the use of neutralizing HB-EGF or NRG-1 antibodies or other compounds such as non-toxic DT derivatives in the tumor treatment. For instance, monoclonal antibody against VEGF-A

i.e. bevacizumab (Avastin), is an anti-angiogenic drug used in the treatment of metastatic colon cancer and NSCLC in clinic (www.fda.com).

BM-derived cells that contribute to postnatal vasculogenesis have been suggested to act as novel targets for anti-angiogenic therapy (Lyden et al., 2001). Observation that EGFR-inhibition regulates the recruitment of BM-derived cells to perivascular space, suggest a role for ErbB signaling in the regulation of tumor vasculogenesis. However, further investigation is needed to elucidate the relevance of targeting BM-derived perivascular cells by cancer drugs such as EGFR inhibitors.

Taken together, our results suggest that ErbB signaling plays an important role in the regulation of angiogenesis *in vitro* and *in vivo* and that ErbB targeted therapeutics could be also used in the inhibition of tumor neovascularization.

# CONCLUSION

The present study focused on characterizing the role of endothelial cell-derived ErbB ligands, i.e. HB-EGF and NRG-1 in angiogenesis as well as the role of EGFR in tumor neovascularization. Based on the presented results the following conclusions can be made:

1. Of EGF-like ligands ECs express primarily HB-EGF and NRG-1.
2. ECs and SMCs express EGFR and ErbB2, but not ErbB3 or ErbB4.
3. HB-EGF and EGFR are involved in the recruitment of SMCs by ECs.
4. Angiopoietin regulates the HB-EGF-mediated recruitment of SMCs.
5. NRG-1 regulates angiogenesis by an indirect mechanism involving paracrine upregulation of VEGF-A in non-vascular cell types.
6. EGFR is involved in the recruitment of perivascular bone marrow-derived cells during tumor neovascularization.

# ACKNOWLEDGEMENTS

This work was carried out at the Department of Medical Biochemistry and Molecular Biology and MediCity Research Laboratory, University of Turku during the years 1999-2007. I wish to thank Professors Jukka Finne, Risto Penttinen and Klaus Elenius at the Department of Medical Biochemistry, and Professor Sirpa Jalkanen at MediCity Research Laboratory for opportunity to work in these facilities.

I wish to express my sincere gratitude and respect to my supervisor Professor Klaus Elenius, whose personal contribution has been crucial during this study.

Turku Graduate School of Biomedical Sciences (TuBS) and its director Professor Olli Lassila are acknowledged for educational and financial support. I also wish to thank members of my supervisory committee, Professor Jyrki Heino and Docent Hannu Järveläinen for their comments and good advices.

Professor Lena Claesson-Welsh and Dr. Mikko Savontaus are acknowledged for reviewing the manuscript and for their valuable remarks.

I wish to thank my co-authors Drs. Kari Alitalo, Renhai Cao, Yihai Cao, Varpu Elenius, Reidar Grenman, Satu-Maria Heikkinen, Shigeki Higashiyama, Panu Jaakkola, Teemu Junttila, Peter Klint, Jarmo Kulmala, Jukka Laine, Susanna Lauttia, Jorma Määttä, Lassi Nelimarkka, Ilkka Paatero, Petri Salven, Laura Sihombing, Maria Sundvall, Denis Tvorogov, Seppo Ylä-Herttuala and Na Zhang for collaboration and invaluable help during this study.

All the present and former members of the laboratory: Varpu Elenius, Satu-Maria Heikkinen, Maija Hollmén, Teemu Junttila, Taina Korpela, Anna Korhonen, Kari Kurppa, Jorma Määttä, Lassi Nelimarkka, Ilkka Paatero, Erno Peltola, Liisa Peri, Minna Santanen, Mika Savisalo, Laura Sihombing, Maria Sundvall, Maria Tuominen, Jenni Tukkimäki, Denis Tvorogov, Ville Veikkolainen and Sami Virtanen are acknowledged for their help and good company.

Katri Aarniemi, Svetlana Egutkina, Ion Iagar, Pia Kuivas, Marja Nykänen, Liisa Peltonen, Marita Potila, Pirjo Rantala, Raili Salonen, Mika Savisalo, Satu Suvisaari, Jenni Tukkimäki and Maria Tuominen are acknowledged for excellent technical assistance. I also wish to thank Rakel Mattson, Helena Katainen and Elina Wiik for valuable secretial help.

I am deeply grateful to my family and friends for their support and love.

Finally, I want to thank Jarkko for understanding, love and care.

This study was financially supported by the grants from: Emil Aaltonen Foundation, Finnish Cancer Organization, The Finnish Cultural Foundation, Finnish Medical Society Duodecim, Ida Montin Foundation, University of Turku/ Faculty of Medicine,

Turku University Foundation and the stipend funds of the University of Turku. These are all acknowledged.

Turku, December 2007

Erika Iivanainen

# REFERENCE


Abramovitch, R., Neeman, M., Reich, R., Stein, I., Keshet, E., Abraham, J., Solomon, A. and Marikovsky, M. (1998). Intercellular communication between vascular smooth muscle and endothelial cells mediated by heparin-binding epidermal growth factor-like growth factor and vascular endothelial growth factor. *FEBS Lett* 425, 441-7.

Abramsson, A., Berlin, O., Papayan, H., Paulin, D., Shani, M. and Betsholtz, C. (2002). Analysis of mural cell recruitment to tumor vessels. *Circulation* 105, 112-7.

Akagi, M., Kawaguchi, M., Liu, W., McCarty, M. F., Takeda, A., Fan, F., Stoeltzing, O., Parikh, A. A., Jung, Y. D., Bucana, C. D. et al. (2003). Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. *Br J Cancer* 88, 796-802.

Alitalo, K., Tammela, T. and Petrova, T. V. (2005). Lymphangiogenesis in development and human disease. *Nature* 438, 946-53.

Amin, D. N., Hida, K., Bielenberg, D. R. and Klagsbrun, M. (2006). Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. *Cancer Res* 66, 2173-80.

Andronowska, A., Postek, A. and Doboszynska, T. (2006). Epidermal growth factor and epidermal growth factor receptor immunoreactivity in the endothelial cells of the uterine artery and its branches during different stages of the estrous cycle in the pig. *Pol J Vet Sci* 9, 165-70.

Anklesaria, P., Teixido, J., Laiho, M., Pierce, J. H., Greenberger, J. S. and Massague, J. (1990). Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor alpha to epidermal growth factor receptors promotes cell proliferation. *Proc Natl Acad Sci U S A* 87, 3289-93.

Arkonac, B. M., Foster, L. C., Sibinga, N. E., Patterson, C., Lai, K., Tsai, J. C., Lee, M. E., Perrella, M. A. and Haber, E. (1998). Vascular endothelial growth factor induces heparin-binding epidermal growth factor-like growth factor in vascular endothelial cells. *J Biol Chem* 273, 4400-5.

Armulik, A., Abramsson, A. and Betsholtz, C. (2005). Endothelial/pericyte interactions. *Circ Res* 97, 512-23.

Asahara, T. and Kawamoto, A. (2004). Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* 287, C572-9.

Asahara, T., Takahashi, T., Masuda, H., Kalka, C., Chen, D., Iwaguro, H., Inai, Y., Silver, M. and Isner, J. M. (1999). VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *Embo J* 18, 3964-72.

Autiero, M., De Smet, F., Claes, F. and Carmeliet, P. (2005). Role of neural guidance signals in blood vessel navigation. *Cardiovasc Res* 65, 629-38.

Bagheri-Yarmand, R., Vadlamudi, R. K., Wang, R. A., Mendelsohn, J. and Kumar, R. (2000). Vascular endothelial growth factor up-regulation via p21-activated kinase-1 signaling regulates heregulin-beta1-mediated angiogenesis. *J Biol Chem* 275, 39451-7.

Baker, C. H., Kedar, D., McCarty, M. F., Tsan, R., Weber, K. L., Bucana, C. D. and Fidler, I. J. (2002). Blockade of epidermal growth factor receptor signaling on tumor cells and tumor-associated endothelial cells for therapy of human carcinomas. *Am J Pathol* 161, 929-38.

Baker, C. H., Pino, M. S. and Fidler, I. J. (2006). Phosphorylated epidermal growth factor receptor on tumor-associated endothelial cells in human renal cell carcinoma is a primary target for therapy by tyrosine kinase inhibitors. *Neoplasia* 8, 470-6.

Baldwin, M. E., Halford, M. M., Roufail, S., Williams, R. A., Hibbs, M. L., Grail, D., Kubo, H., Stacker, S. A. and Achen, M. G. (2005). Vascular endothelial growth factor D is dispensable for development of the lymphatic system. *Mol Cell Biol* 25, 2441-9.

Baluk, P., Morikawa, S., Haskell, A., Mancuso, M. and McDonald, D. M. (2003). Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. *Am J Pathol* 163, 1801-15.

Bao, J., Wolpowitz, D., Role, L. W. and Talmage, D. A. (2003). Back signaling by the Nrg-1 intracellular domain. *J Cell Biol* 161, 1133-41.

Baselga, J., Norton, L., Albanell, J., Kim, Y. M. and Mendelsohn, J. (1998). Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res* 58, 2825-31.

Baumann, C. I., Bailey, A. S., Li, W., Ferkowicz, M. J., Yoder, M. C. and Fleming, W. H. (2004). PECAM-1 is expressed on hematopoietic stem cells throughout ontogeny and identifies a population of erythroid progenitors. *Blood* 104, 1010-6.

Beitz, J. G., Kim, I. S., Calabresi, P. and Frackelton, A. R., Jr. (1991). Human microvascular endothelial cells express receptors for platelet-derived growth factor. *Proc Natl Acad Sci U S A* 88, 2021-5.

Bellomo, D., Headrick, J. P., Silins, G. U., Paterson, C. A., Thomas, P. S., Gartside, M., Mould, A., Cahill, M. M., Tonks, I. D., Grimmond, S. M. et al. (2000). Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ Res* 86, E29-35.

Benjamin, L. E., Hemo, I. and Keshet, E. (1998). A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* 125, 1591-8.

Bergers, G. and Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 3, 401-10.

Bergers, G. and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol* 7, 452-64.

Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E. and Hanahan, D. (2003). Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest* 111, 1287-95.

Betsholtz, C., Karlsson, L. and Lindahl, P. (2001). Developmental roles of platelet-derived growth factors. *Bioessays* 23, 494-507.

Bianco, C., Tortora, G., Bianco, R., Caputo, R., Veneziani, B. M., Caputo, R., Damiano, V., Troiani, T., Fontanini, G., Raben, D. et al. (2002). Enhancement of antitumor activity of ionizing radiation by combined treatment with the selective epidermal growth factor receptor-tyrosine kinase inhibitor ZD1839 (Iressa). *Clin Cancer Res* 8, 3250-8.

Bjarnegard, M., Enge, M., Norlin, J., Gustafsdottir, S., Fredriksson, S., Abramsson, A., Takemoto, M., Gustafsson, E., Fassler, R. and Betsholtz, C. (2004). Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. *Development* 131, 1847-57.

Boerner, J. L., Danielsen, A. and Maihle, N. J. (2003). Ligand-independent oncogenic signaling by the epidermal growth factor receptor: v-ErbB as a paradigm. *Exp Cell Res* 284, 111-21.

Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnurch, H., Gotsch, U., Vestweber, D., Risau, W. and Dejana, E. (1996). Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* 87, 630-41.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C. and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev* 12, 1825-36.

Britto, J. M., Lukehurst, S., Weller, R., Fraser, C., Qiu, Y., Hertzog, P. and Busfield, S. J. (2004). Generation and characterization of neuregulin-2-deficient mice. *Mol Cell Biol* 24, 8221-6.

Bruns, C. J., Harbison, M. T., Davis, D. W., Portera, C. A., Tsan, R., McConkey, D. J., Evans, D. B., Abbruzzese, J. L., Hicklin, D. J. and Radinsky, R. (2000a). Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. *Clin Cancer Res* 6, 1936-48.

Bruns, C. J., Solorzano, C. C., Harbison, M. T., Ozawa, S., Tsan, R., Fan, D., Abbruzzese, J., Traxler, P., Buchdunger, E., Radinsky, R. et al. (2000b). Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 60, 2926-35.

Byzova, T. V., Goldman, C. K., Jankau, J., Chen, J., Cabrera, G., Achen, M. G., Stacker, S. A., Carnevale, K. A., Siemionow, M., Deitcher, S. R. et al. (2002). Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns in vivo. *Blood* 99, 4434-42.

Cao, R., Bjorndahl, M. A., Religa, P., Clasper, S., Garvin, S., Galter, D., Meister, B., Ikomi, F., Tritsaris, K., Dissing, S. et al. (2004). PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell* 6, 333-45.

Cao, R., Brakenhielm, E., Li, X., Pietras, K., Widenfalk, J., Ostman, A., Eriksson, U. and Cao, Y. (2002). Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-alphaalpha and -alphabeta receptors. *Faseb J* 16, 1575-83.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6, 389-95.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. *Nature* 438, 932-6.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C. et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380, 435-9.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H. et al. (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 7, 575-83.

Carraway, K. L., 3rd, Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M. and Lai, C. (1997). Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature* 387, 512-6.

Casalini, P., Iorio, M. V., Galmozzi, E. and Menard, S. (2004). Role of HER receptors family in development and differentiation. *J Cell Physiol* 200, 343-50.

Chen, B., Bronson, R. T., Klaman, L. D., Hampton, T. G., Wang, J. F., Green, P. J., Magnuson, T., Douglas, P. S., Morgan, J. P. and Neel, B. G. (2000). Mice mutant for Egfr and Shp2 have defective cardiac semilunar valvulogenesis. *Nat Genet* 24, 296-9.

Ciardiello, F., Bianco, R., Caputo, R., Caputo, R., Damiano, V., Troiani, T., Melisi, D., De Vita, F., De Placido, S., Bianco, A. R. et al. (2004). Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. *Clin Cancer Res* 10, 784-93.

Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Fontanini, G., Cuccato, S., De Placido, S., Bianco, A. R. and Tortora, G. (2001). Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 7, 1459-65.

Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomatico, G., De Placido, S., Bianco, A. R. and Tortora, G. (2000). Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 6, 2053-63.

Ciardiello, F., Damiano, V., Bianco, R., Bianco, C., Fontanini, G., De Laurentiis, M., De Placido, S., Mendelsohn, J., Bianco, A. R. and Tortora, G. (1996). Antitumor activity of combined blockade of epidermal growth factor receptor and protein kinase A. *J Natl Cancer Inst* 88, 1770-6.

Ciardiello, F. and Tortora, G. (2001). A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res* 7, 2958-70.

Citri, A. and Yarden, Y. (2006). EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* 7, 505-16.

Cleaver, O. and Melton, D. A. (2003). Endothelial signaling during development. *Nat Med* 9, 661-8.

Clement, C. M., Thomas, L. K., Mou, Y., Croslan, D. R., Gibbons, G. H. and Ford, B. D. (2007). Neuregulin-1 attenuates neointimal formation following vascular injury and inhibits the proliferation of vascular smooth muscle cells. *J Vasc Res* 44, 303-12.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* 237, 1555-62.

Cohen, T., Nahari, D., Cerem, L. W., Neufeld, G. and Levi, B. Z. (1996). Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* 271, 736-41.

Conway, E. M. and Carmeliet, P. (2004). The diversity of endothelial cells: a challenge for therapeutic angiogenesis. *Genome Biol* 5, 207.

Conway, E. M., Collen, D. and Carmeliet, P. (2001). Molecular mechanisms of blood vessel growth. *Cardiovasc Res* 49, 507-21.

Cote, G. M., Miller, T. A., Lebrasseur, N. K., Kuramochi, Y. and Sawyer, D. B. (2005). Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. *Exp Cell Res* 311, 135-46.

Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U. et al. (1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 230, 1132-9.

Crosby, J. R., Kaminski, W. E., Schatteman, G., Martin, P. J., Raines, E. W., Seifert, R. A. and Bowen-Pope, D. F. (2000). Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circ Res* 87, 728-30.

Darland, D. C., Massingham, L. J., Smith, S. R., Piek, E., Saint-Geniez, M. and D'Amore, P. A. (2003). Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Dev Biol* 264, 275-88.

Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C. et al. (1996). Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 87, 1161-9.

De Palma, M., Venneri, M. A., Galli, R., Sergi Sergi, L., Politi, L. S., Sampaolesi, M. and Naldini, L. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 8, 211-26.

De Palma, M., Venneri, M. A., Roca, C. and Naldini, L. (2003). Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med* 9, 789-95.

DeRuiter, M. C., Poelmann, R. E., VanMunsteren, J. C., Mironov, V., Markwald, R. R. and Gittenberger-de Groot, A. C. (1997). Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. *Circ Res* 80, 444-51.

Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y. and Goeddel, D. V. (1984). Human transforming growth factor-alpha: precursor structure and expression in E. coli. *Cell* 38, 287-97.

Detmar, M., Brown, L. F., Claffey, K. P., Yeo, K. T., Kocher, O., Jackman, R. W., Berse, B. and Dvorak, H. F. (1994). Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med* 180, 1141-6.

Direkze, N. C., Hodivala-Dilke, K., Jeffery, R., Hunt, T., Poulsom, R., Oukrif, D., Alison, M. R. and Wright, N. A. (2004). Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Res* 64, 8492-5.

Dluz, S. M., Higashiyama, S., Damm, D., Abraham, J. A. and Klagsbrun, M. (1993). Heparin-binding epidermal growth factor-like growth factor expression in cultured fetal human vascular smooth muscle cells. Induction of mRNA levels and secretion of active mitogen. *J Biol Chem* 268, 18330-4.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M. D. (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature* 307, 521-7.

Doyle, B., Metharom, P. and Caplice, N. M. (2006). Endothelial progenitor cells. *Endothelium* 13, 403-10.

Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A. and Breitman, M. L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev* 8, 1897-909.

Dumont, D. J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M. and Alitalo, K. (1998). Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 282, 946-9.

Dunn, I. F., Heese, O. and Black, P. M. (2000). Growth factors in glioma angiogenesis: FGFs, PDGF, EGF, and TGFs. *J Neurooncol* 50, 121-37.

Dutta, P. R. and Maity, A. (2007). Cellular responses to EGFR inhibitors and their relevance to cancer therapy. *Cancer Lett* 254, 165-77.

Ekstrand, A. J., Sugawa, N., James, C. D. and Collins, V. P. (1992). Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proc Natl Acad Sci U S A* 89, 4309-13.

Elenius, K. (2006). Epidermaalisen kasvutekijän reseptorin estäjät syövän hoidossa. *Duodecim* 122, 1331-8.

Emeis, J. J. and Edgell, C. J. (1988). Fibrinolytic properties of a human endothelial hybrid cell line (Ea.hy 926). *Blood* 71, 1669-75.

Emlet, D. R., Brown, K. A., Kociban, D. L., Pollice, A. A., Smith, C. A., Ong, B. B. and Shackney, S. E. (2007). Response to trastuzumab, erlotinib, and bevacizumab, alone and in combination, is correlated with the level of human epidermal growth factor receptor-2 expression in human breast cancer cell lines. *Mol Cancer Ther* 6, 2664-74.

Erber, R., Thurnher, A., Katsen, A. D., Groth, G., Kerger, H., Hammes, H. P., Menger, M. D., Ullrich, A. and Vajkoczy, P. (2004). Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *Faseb J* 18, 338-40.

Erickson, S. L., O'Shea, K. S., Ghaboosi, N., Loverro, L., Frantz, G., Bauer, M., Lu, L. H. and Moore, M. W. (1997). ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development* 124, 4999-5011.

Falls, D. L. (2003). Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res* 284, 14-30.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S. and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell* 72, 801-15.

Feldman, P. S., Shneidman, D. and Kaplan, C. (1978). Ultrastructure of infantile hemangioendothelioma of the liver. *Cancer* 42, 521-7.

Fendly, B. M., Winget, M., Hudziak, R. M., Lipari, M. T., Napier, M. A. and Ullrich, A. (1990). Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. *Cancer Res* 50, 1550-8.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380, 439-42.

Ferrara, N., Gerber, H. P. and LeCouter, J. (2003). The biology of VEGF and its receptors. *Nat Med* 9, 669-76.

Ferrara, N., Hillan, K. J. and Novotny, W. (2005). Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun* 333, 328-35.

Ferrara, N., Houck, K., Jakeman, L. and Leung, D. W. (1992). Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 13, 18-32.

Finkenzeller, G., Marme, D., Weich, H. A. and Hug, H. (1992). Platelet-derived growth factor-induced transcription of the vascular endothelial growth factor gene is mediated by protein kinase C. *Cancer Res* 52, 4821-3.

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285, 1182-6.

Folkman, J. (1995). Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N Engl J Med* 333, 1757-63.

Folkman, J. (2006). Antiangiogenesis in cancer therapy--endostatin and its mechanisms of action. *Exp Cell Res* 312, 594-607.

Folkman, J. and D'Amore, P. A. (1996). Blood vessel formation: what is its molecular basis? *Cell* 87, 1153-5.

Folkman, J. and Shing, Y. (1992). Angiogenesis. *J Biol Chem* 267, 10931-4.

Fong, G. H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376, 66-70.

Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D. and Semenza, G. L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* 16, 4604-13.

Frank, S., Hubner, G., Breier, G., Longaker, M. T., Greenhalgh, D. G. and Werner, S. (1995). Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. *J Biol Chem* 270, 12607-13.

Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D. et al. (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell* 3, 411-23.

Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., Fuks, Z. and Kolesnick, R. (2003). Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 300, 1155-9.

Garlanda, C. and Dejana, E. (1997). Heterogeneity of endothelial cells. Specific markers. *Arterioscler Thromb Vasc Biol* 17, 1193-202.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* 378, 390-4.

Gerber, H. P., Dixit, V. and Ferrara, N. (1998). Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 273, 13313-6.

Gerhardt, H. and Betsholtz, C. (2003). Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res* 314, 15-23.

Gill, G. N., Kawamoto, T., Cochet, C., Le, A., Sato, J. D., Masui, H., McLeod, C. and Mendelsohn, J. (1984). Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* 259, 7755-60.

Golding, J. P., Trainor, P., Krumlauf, R. and Gassmann, M. (2000). Defects in pathfinding by cranial neural crest cells in mice lacking the neuregulin receptor ErbB4. *Nat Cell Biol* 2, 103-9.

Goldman, C. K., Kim, J., Wong, W. L., King, V., Brock, T. and Gillespie, G. Y. (1993). Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell* 4, 121-33.

Goldstein, N. I., Prewett, M., Zuklys, K., Rockwell, P. and Mendelsohn, J. (1995). Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin Cancer Res* 1, 1311-8.

Gospodarowicz, D. and Bialecki, H. (1979). Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine, and human origin. *Endocrinology* 104, 757-64.

Gospodarowicz, D., Bialecki, H. and Thakral, T. K. (1979). The angiogenic activity of the fibroblast and epidermal growth factor. *Exp Eye Res* 28, 501-14.

Gothert, J. R., Gustin, S. E., van Eekelen, J. A., Schmidt, U., Hall, M. A., Jane, S. M., Green, A. R., Gottgens, B., Izon, D. J. and Begley, C. G. (2004). Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* 104, 1769-77.

Gray, A., Dull, T. J. and Ullrich, A. (1983). Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor. *Nature* 303, 722-5.

Grunewald, M., Avraham, I., Dor, Y., Bachar-Lustig, E., Itin, A., Jung, S., Chimenti, S., Landsman, L., Abramovitch, R. and Keshet, E. (2006). VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 124, 175-89.

Gschwind, A., Fischer, O. M. and Ullrich, A. (2004). The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* 4, 361-70.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. *Science* 277, 48-50.

Hanahan, D. and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353-64.

Harari, D., Tzahar, E., Romano, J., Shelly, M., Pierce, J. H., Andrews, G. C. and Yarden, Y. (1999). Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene* 18, 2681-9.

Harari, P. M. (2004). Epidermal growth factor receptor inhibition strategies in oncology. *Endocr Relat Cancer* 11, 689-708.

Hashizume, H., Baluk, P., Morikawa, S., McLean, J. W., Thurston, G., Roberge, S., Jain, R. K. and McDonald, D. M. (2000). Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol* 156, 1363-80.

Hattori, K., Dias, S., Heissig, B., Hackett, N. R., Lyden, D., Tateno, M., Hicklin, D. J., Zhu, Z., Witte, L., Crystal, R. G. et al. (2001). Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* 193, 1005-14.

Hayes, N. V., Newsam, R. J., Baines, A. J. and Gullick, W. J. (2007). Characterization of the cell membrane-associated products of the Neuregulin 4 gene. *Oncogene*.

Heljasvaara, R., Nyberg, P., Luostarinen, J., Parikka, M., Heikkila, P., Rehn, M., Sorsa, T., Salo, T. and Pihlajaniemi, T. (2005). Generation of biologically active endostatin fragments from human collagen XVIII by distinct matrix metalloproteases. *Exp Cell Res* 307, 292-304.

Hellstrom, M., Gerhardt, H., Kalen, M., Li, X., Eriksson, U., Wolburg, H. and Betsholtz, C. (2001). Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J Cell Biol* 153, 543-53.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A. and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 126, 3047-55.

Herbst, R. S., Fukuoka, M. and Baselga, J. (2004). Gefitinib--a novel targeted approach to treating cancer. *Nat Rev Cancer* 4, 956-65.

Herbst, R. S., Heymach, J. V., O'Reilly, M. S., Onn, A. and Ryan, A. J. (2007). Vandetanib (ZD6474): an orally available receptor tyrosine kinase inhibitor that selectively targets pathways critical for tumor growth and angiogenesis. *Expert Opin Investig Drugs* 16, 239-49.

Hida, K., Hida, Y., Amin, D. N., Flint, A. F., Panigrahy, D., Morton, C. C. and Klagsbrun, M. (2004). Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res* 64, 8249-55.

Higashiyama, S., Abraham, J. A. and Klagsbrun, M. (1993). Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J Cell Biol* 122, 933-40.

Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C. and Klagsbrun, M. (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* 251, 936-9.

Higashiyama, S., Iwamoto, R., Goishi, K., Raab, G., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). The membrane protein CD9/DRAP 27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. *J Cell Biol* 128, 929-38.

Hirata, A., Ogawa, S., Kometani, T., Kuwano, T., Naito, S., Kuwano, M. and Ono, M. (2002). ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 62, 2554-60.

Hirata, A., Uehara, H., Izumi, K., Naito, S., Kuwano, M. and Ono, M. (2004). Direct inhibition of EGF receptor activation in vascular endothelial cells by gefitinib ('Iressa', ZD1839). *Cancer Sci* 95, 614-8.

Hiratsuka, S., Maru, Y., Okada, A., Seiki, M., Noda, T. and Shibuya, M. (2001). Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. *Cancer Res* 61, 1207-13.

Hirschi, K. K. and D'Amore, P. A. (1996). Pericytes in the microvasculature. *Cardiovasc Res* 32, 687-98.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D. et al. (1992). Identification of heregulin, a specific activator of p185erbB2. *Science* 256, 1205-10.

Holmgren, L., Glaser, A., Pfeifer-Ohlsson, S. and Ohlsson, R. (1991). Angiogenesis during human extraembryonic development involves the spatiotemporal control of PDGF ligand and receptor gene expression. *Development* 113, 749-54.

Huang, S. M., Li, J., Armstrong, E. A. and Harari, P. M. (2002a). Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). *Cancer Res* 62, 4300-6.

Huang, S. M., Li, J. and Harari, P. M. (2002b). Molecular inhibition of angiogenesis and metastatic potential in human squamous cell carcinomas after epidermal growth factor receptor blockade. *Mol Cancer Ther* 1, 507-14.

Hudis, C. A. (2007). Trastuzumab--mechanism of action and use in clinical practice. *N Engl J Med* 357, 39-51.

Hudziak, R. M., Lewis, G. D., Winget, M., Fendly, B. M., Shepard, H. M. and Ullrich, A. (1989). p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol* 9, 1165-72.

Hungerford, J. E. and Little, C. D. (1999). Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. *J Vasc Res* 36, 2-27.

Hynes, N. E. and Lane, H. A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 5, 341-54.

Iivanainen, E., Kahari, V. M., Heino, J. and Elenius, K. (2003). Endothelial cell-matrix interactions. *Microsc Res Tech* 60, 13-22.

Inoue, K., Slaton, J. W., Perrotte, P., Davis, D. W., Bruns, C. J., Hicklin, D. J., McConkey, D. J., Sweeney, P., Radinsky, R. and Dinney, C. P. (2000). Paclitaxel enhances the effects of the anti-epidermal growth factor receptor monoclonal antibody ImClone C225 in mice with metastatic human bladder transitional cell carcinoma. *Clin Cancer Res* 6, 4874-84.

Iwamoto, R. and Mekada, E. (2006). ErbB and HB-EGF signaling in heart development and function. *Cell Struct Funct* 31, 1-14.

Iwamoto, R., Yamazaki, S., Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Raab, G. et al. (2003). Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc Natl Acad Sci U S A* 100, 3221-6.

Izumi, Y., Xu, L., di Tomaso, E., Fukumura, D. and Jain, R. K. (2002). Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature* 416, 279-80.

Jackson, K. A., Majka, S. M., Wang, H., Pocius, J., Hartley, C. J., Majesky, M. W., Entman, M. L., Michael, L. H., Hirschi, K. K. and Goodell, M. A. (2001). Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest* 107, 1395-402.

Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C. and Lee, D. C. (2003). Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *Embo J* 22, 2704-16.

Jain, R. K. and Munn, L. L. (2000). Leaky vessels? Call Ang1! *Nat Med* 6, 131-2.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K. and Alitalo, K. (1997). Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* 276, 1423-5.

Jones, N., Iljin, K., Dumont, D. J. and Alitalo, K. (2001). Tie receptors: new modulators of angiogenic and lymphangiogenic responses. *Nat Rev Mol Cell Biol* 2, 257-67.

Karashima, T., Sweeney, P., Slaton, J. W., Kim, S. J., Kedar, D., Izawa, J. I., Fan, Z., Pettaway, C., Hicklin, D. J., Shuin, T. et al. (2002). Inhibition of angiogenesis by the antiepidermal growth factor receptor antibody ImClone C225 in androgen-independent prostate cancer growing orthotopically in nude mice. *Clin Cancer Res* 8, 1253-64.

Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H. et al. (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 5, 74-80.

Kato, M., Inazu, T., Kawai, Y., Masamura, K., Yoshida, M., Tanaka, N., Miyamoto, K. and Miyamori, I. (2003). Amphiregulin is a potent mitogen for the vascular smooth muscle cell line, A7r5. *Biochem Biophys Res Commun* 301, 1109-15.

Kim, H. S., Shin, H. S., Kwak, H. J., Cho, C. H., Lee, C. O. and Koh, G. Y. (2003a). Betacellulin induces angiogenesis through activation of mitogen-activated protein kinase and phosphatidylinositol 3'-kinase in endothelial cell. *Faseb J* 17, 318-20.

Kim, S. J., Uehara, H., Karashima, T., Shepherd, D. L., Killion, J. J. and Fidler, I. J. (2003b). Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. *Clin Cancer Res* 9, 1200-10.

Kocher, A. A., Schuster, M. D., Szabolcs, M. J., Takuma, S., Burkhoff, D., Wang, J., Homma, S., Edwards, N. M. and Itescu, S. (2001). Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 7, 430-6.

Kopp, H. G., Ramos, C. A. and Rafii, S. (2006). Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. *Curr Opin Hematol* 13, 175-81.

Krampera, M., Pasini, A., Rigo, A., Scupoli, M. T., Tecchio, C., Malpeli, G., Scarpa, A., Dazzi, F., Pizzolo, G. and Vinante, F. (2005). HB-EGF/HER-1 signaling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multilineage differentiation. *Blood* 106, 59-66.

Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. and Aaronson, S. A. (1989). Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci U S A* 86, 9193-7.

Kumar, R. and Yarmand-Bagheri, R. (2001). The role of HER2 in angiogenesis. *Semin Oncol* 28, 27-32.

Lamagna, C. and Bergers, G. (2006). The bone marrow constitutes a reservoir of pericyte progenitors. *J Leukoc Biol* 80, 677-81.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* 378, 394-8.

Leveen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E. and Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev* 8, 1875-87.

Li, B., Sharpe, E. E., Maupin, A. B., Teleron, A. A., Pyle, A. L., Carmeliet, P. and Young, P. P. (2006). VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *Faseb J* 20, 1495-7.

Li, H., Fredriksson, L., Li, X. and Eriksson, U. (2003). PDGF-D is a potent transforming and angiogenic growth factor. *Oncogene* 22, 1501-10.

Li, X. and Eriksson, U. (2003). Novel PDGF family members: PDGF-C and PDGF-D. *Cytokine Growth Factor Rev* 14, 91-8.

Li, X., Tjwa, M., Moons, L., Fons, P., Noel, A., Ny, A., Zhou, J. M., Lennartsson, J., Li, H., Luttun, A. et al. (2005). Revascularization of ischemic tissues by PDGF-CC via effects on endothelial cells and their progenitors. *J Clin Invest* 115, 118-27.

Lindahl, P., Hellstrom, M., Kalen, M. and Betsholtz, C. (1998). Endothelial-perivascular cell signaling in vascular development: lessons from knockout mice. *Curr Opin Lipidol* 9, 407-11.

Lindahl, P., Johansson, B. R., Leveen, P. and Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 277, 242-5.

Luetteke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A. and Lee, D. C. (1999). Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 126, 2739-50.

Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O. and Lee, D. C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* 73, 263-78.

Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L. et al. (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 7, 1194-201.

Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., Harris, P. L., Haserlat, S. M., Supko, J. G., Haluska, F. G. et al. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350, 2129-39.

Mahller, Y. Y., Vaikunth, S. S., Currier, M. A., Miller, S. J., Ripberger, M. C., Hsu, Y. H., Mehrian-Shai, R., Collins, M. H., Crombleholme, T. M., Ratner, N. et al. (2007). Oncolytic HSV and erlotinib inhibit tumor growth and angiogenesis in a novel malignant peripheral nerve sheath tumor xenograft model. *Mol Ther* 15, 279-86.

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

Mandarino, L. J., Sundarraj, N., Finlayson, J. and Hassell, H. R. (1993). Regulation of fibronectin and laminin synthesis by retinal capillary endothelial cells and pericytes in vitro. *Exp Eye Res* 57, 609-21.

Mann, G. B., Fowler, K. J., Gabriel, A., Nice, E. C., Williams, R. L. and Dunn, A. R. (1993). Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 73, 249-61.

Marchionni, M. A. (1995). Cell-cell signalling. neu tack on neuregulin. *Nature* 378, 334-5.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K. et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* 362, 312-8.

Marmor, M. D., Skaria, K. B. and Yarden, Y. (2004). Signal transduction and oncogenesis by ErbB/HER receptors. *Int J Radiat Oncol Biol Phys* 58, 903-13.

Marx, M., Perlmutter, R. A. and Madri, J. A. (1994). Modulation of platelet-derived growth factor receptor expression in microvascular endothelial cells during in vitro angiogenesis. *J Clin Invest* 93, 131-9.

Matar, P., Rojo, F., Cassia, R., Moreno-Bueno, G., Di Cosimo, S., Tabernero, J., Guzman, M., Rodriguez, S., Arribas, J., Palacios, J. et al. (2004). Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefitinib (ZD1839) and the monoclonal antibody cetuximab (IMC-C225): superiority over single-agent receptor targeting. *Clin Cancer Res* 10, 6487-501.

Matsuda, T., Okamura, K., Sato, Y., Morimoto, A., Ono, M., Kohno, K. and Kuwano, M. (1992). Decreased response to epidermal growth factor during cellular senescence in cultured human microvascular endothelial cells. *J Cell Physiol* 150, 510-6.

Mawatari, M., Kohno, K., Mizoguchi, H., Matsuda, T., Asoh, K., Van Damme, J., Welgus, H. G. and Kuwano, M. (1989). Effects of tumor necrosis factor and epidermal growth factor on cell morphology, cell surface receptors, and the production of tissue inhibitor of metalloproteinases and IL-6 in human microvascular endothelial cells. *J Immunol* 143, 1619-27.

Mawatari, M., Okamura, K., Matsuda, T., Hamanaka, R., Mizoguchi, H., Higashio, K., Kohno, K. and Kuwano, M. (1991). Tumor necrosis factor and epidermal growth factor modulate migration of human microvascular endothelial cells and production of tissue-type plasminogen activator and its inhibitor. *Exp Cell Res* 192, 574-80.

McAuslan, B. R., Bender, V., Reilly, W. and Moss, B. A. (1985). New functions of epidermal growth factor: stimulation of capillary endothelial cell migration and matrix dependent proliferation. *Cell Biol Int Rep* 9, 175-82.

McDonald, D. M. and Baluk, P. (2002). Significance of blood vessel leakiness in cancer. *Cancer Res* 62, 5381-5.

McDonald, D. M. and Choyke, P. L. (2003). Imaging of angiogenesis: from microscope to clinic. *Nat Med* 9, 713-25.

Metheny-Barlow, L. J., Tian, S., Hayes, A. J. and Li, L. Y. (2004). Direct chemotactic action of angiopoietin-1 on mesenchymal cells in the presence of VEGF. *Microvasc Res* 68, 221-30.

Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* 378, 386-90.

Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z. and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* 376, 337-41.

Mifune, M., Ohtsu, H., Suzuki, H., Frank, G. D., Inagami, T., Utsunomiya, H., Dempsey, P. J. and Eguchi, S. (2004). Signal transduction of betacellulin in growth and migration of vascular smooth muscle cells. *Am J Physiol Cell Physiol* 287, C807-13.

Mine, N., Iwamoto, R. and Mekada, E. (2005). HB-EGF promotes epithelial cell migration in eyelid development. *Development* 132, 4317-26.

Mollica, F., Jain, R. K. and Netti, P. A. (2003). A model for temporal heterogeneities of tumor blood flow. *Microvasc Res* 65, 56-60.

Morelli, M. P., Cascone, T., Troiani, T., Tuccillo, C., Bianco, R., Normanno, N., Romano, M., Veneziani, B. M., Fontanini, G., Eckhardt, S. G. et al. (2006). Anti-tumor activity of the combination of cetuximab, an anti-EGFR blocking monoclonal antibody and ZD6474, an inhibitor of VEGFR and EGFR tyrosine kinases. *J Cell Physiol* 208, 344-53.

Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R. K. and McDonald, D. M. (2002). Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol* 160, 985-1000.

Moyer, J. D., Barbacci, E. G., Iwata, K. K., Arnold, L., Boman, B., Cunningham, A., DiOrio, C., Doty, J., Morin, M. J., Moyer, M. P. et al. (1997). Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 57, 4838-48.

Naglich, J. G., Metherall, J. E., Russell, D. W. and Eidels, L. (1992). Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* 69, 1051-61.

Nakano, N., Higashiyama, S., Ohmoto, H., Ishiguro, H., Taniguchi, N. and Wada, Y. (2004). The N-terminal region of NTAK/neuregulin-2 isoforms has an inhibitory activity on angiogenesis. *J Biol Chem* 279, 11465-70.

Nakata, A., Miyagawa, J., Yamashita, S., Nishida, M., Tamura, R., Yamamori, K., Nakamura, T., Nozaki, S., Kameda-Takemura, K., Kawata, S. et al. (1996). Localization of heparin-binding epidermal growth factor-like growth factor in human coronary arteries. Possible roles of HB-EGF in the formation of coronary atherosclerosis. *Circulation* 94, 2778-86.

Nanba, D., Kinugasa, Y., Morimoto, C., Koizumi, M., Yamamura, H., Takahashi, K., Takakura, N., Mekada, E., Hashimoto, K. and Higashiyama, S. (2006). Loss of HB-EGF in smooth muscle or endothelial cell lineages causes heart malformation. *Biochem Biophys Res Commun* 350, 315-21.

Nehls, V. and Drenckhahn, D. (1991). Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. *J Cell Biol* 113, 147-54.

Normanno, N., De Luca, A., Aldinucci, D., Maiello, M. R., Mancino, M., D'Antonio, A., De Filippi, R. and Pinto, A. (2005). Gefitinib inhibits the ability of human bone marrow stromal cells to induce osteoclast differentiation: implications for the pathogenesis and treatment of bone metastasis. *Endocr Relat Cancer* 12, 471-82.

Nyberg, P., Xie, L. and Kalluri, R. (2005). Endogenous inhibitors of angiogenesis. *Cancer Res* 65, 3967-79.

O-charoenrat, P., Rhys-Evans, P., Modjtahedi, H. and Eccles, S. A. (2000). Vascular endothelial growth factor family members are differentially regulated by c-erbB signaling in head and neck squamous carcinoma cells. *Clin Exp Metastasis* 18, 155-61.

Oikawa, T., Onozawa, C., Sakaguchi, M., Morita, I. and Murota, S. (1994). Three isoforms of platelet-derived growth factors all have the capability to induce angiogenesis in vivo. *Biol Pharm Bull* 17, 1686-8.

Okamura, K., Morimoto, A., Hamanaka, R., Ono, M., Kohno, K., Uchida, Y. and Kuwano, M. (1992). A model system for tumor angiogenesis: involvement of transforming growth factor-alpha in tube formation of human microvascular endothelial cells induced by esophageal cancer cells. *Biochem Biophys Res Commun* 186, 1471-9.

Olayioye, M. A., Neve, R. M., Lane, H. A. and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *Embo J* 19, 3159-67.

Olsson, A. K., Dimberg, A., Kreuger, J. and Claesson-Welsh, L. (2006). VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 7, 359-71.

Ongusaha, P. P., Kwak, J. C., Zwible, A. J., Macip, S., Higashiyama, S., Taniguchi, N., Fang, L. and Lee, S. W. (2004). HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res* 64, 5283-90.

Ono, M. and Kuwano, M. (2006). Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. *Clin Cancer Res* 12, 7242-51.

Ono, M., Okamura, K., Nakayama, Y., Tomita, M., Sato, Y., Komatsu, Y. and Kuwano, M. (1992). Induction of human microvascular endothelial tubular morphogenesis by human keratinocytes: involvement of transforming growth factor-alpha. *Biochem Biophys Res Commun* 189, 601-9.

Orlidge, A. and D'Amore, P. A. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol* 105, 1455-62.

Ozcelik, C., Erdmann, B., Pilz, B., Wettschureck, N., Britsch, S., Hubner, N., Chien, K. R., Birchmeier, C. and Garratt, A. N. (2002). Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci U S A* 99, 8880-5.

Ozerdem, U., Grako, K. A., Dahlin-Huppe, K., Monosov, E. and Stallcup, W. B. (2001). NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. *Dev Dyn* 222, 218-27.

Ozerdem, U., Monosov, E. and Stallcup, W. B. (2002). NG2 proteoglycan expression by pericytes in pathological microvasculature. *Microvasc Res* 63, 129-34.

Ozerdem, U. and Stallcup, W. B. (2003). Early contribution of pericytes to angiogenic sprouting and tube formation. *Angiogenesis* 6, 241-9.

Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J. et al. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304, 1497-500.

Pao, W., Miller, V., Zakowski, M., Doherty, J., Politi, K., Sarkaria, I., Singh, B., Heelan, R., Rusch, V., Fulton, L. et al. (2004). EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 101, 13306-11.

Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Levy, R. B. and Yarden, Y. (1992). Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* 69, 205-16.

Perrotte, P., Matsumoto, T., Inoue, K., Kuniyasu, H., Eve, B. Y., Hicklin, D. J., Radinsky, R. and Dinney, C. P. (1999). Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 5, 257-65.

Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B. and Kerbel, R. S. (1997). Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 151, 1523-30.

Pierce, J. H., Ruggiero, M., Fleming, T. P., Di Fiore, P. P., Greenberger, J. S., Varticovski, L., Schlessinger, J., Rovera, G. and Aaronson, S. A. (1988). Signal transduction through the EGF receptor transfected in IL-3-dependent hematopoietic cells. *Science* 239, 628-31.

Pipp, F., Heil, M., Issbrucker, K., Ziegelhoeffer, T., Martin, S., van den Heuvel, J., Weich, H., Fernandez, B., Golomb, G., Carmeliet, P. et al. (2003). VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. *Circ Res* 92, 378-85.

Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G. and Shoyab, M. (1993). Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci U S A* 90, 1746-50.

Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. and Shoyab, M. (1990). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc Natl Acad Sci U S A* 87, 4905-9.

Pollack, V. A., Savage, D. M., Baker, D. A., Tsaparikos, K. E., Sloan, D. E., Moyer, J. D., Barbacci, E. G., Pustilnik, L. R., Smolarek, T. A., Davis, J. A. et al. (1999). Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition in situ and antitumor effects in athymic mice. *J Pharmacol Exp Ther* 291, 739-48.

Pore, N., Gupta, A. K., Cerniglia, G. J., Jiang, Z., Bernhard, E. J., Evans, S. M., Koch, C. J., Hahn, S. M. and Maity, A. (2006). Nelfinavir down-regulates hypoxia-inducible factor 1alpha and VEGF expression and increases tumor oxygenation: implications for radiotherapy. *Cancer Res* 66, 9252-9.

Puri, M. C., Rossant, J., Alitalo, K., Bernstein, A. and Partanen, J. (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *Embo J* 14, 5884-91.

Raab, G. and Klagsbrun, M. (1997). Heparin-binding EGF-like growth factor. *Biochim Biophys Acta* 1333, F179-99.

Raben, D., Bianco, C., Damiano, V., Bianco, R., Melisi, D., Mignogna, C., D'Armiento, F. P., Cionini, L., Bianco, A. R., Tortora, G. et al. (2004). Antitumor activity of ZD6126, a novel vascular-targeting agent, is enhanced when combined with ZD1839, an epidermal growth factor receptor tyrosine kinase inhibitor, and potentiates the effects of radiation in a human non-small cell lung cancer xenograft model. *Mol Cancer Ther* 3, 977-83.

Raben, D., Helfrich, B. A., Chan, D., Johnson, G. and Bunn, P. A., Jr. (2002). ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. *Semin Oncol* 29, 37-46.

Rafii, S. and Lyden, D. (2003). Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 9, 702-12.

Rafii, S., Lyden, D., Benezra, R., Hattori, K. and Heissig, B. (2002). Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer* 2, 826-35.

Rajantie, I., Ilmonen, M., Alminaite, A., Ozerdem, U., Alitalo, K. and Salven, P. (2004). Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. *Blood* 104, 2084-6.

Rak, J., Yu, J. L., Kerbel, R. S. and Coomber, B. L. (2002). What do oncogenic mutations have to do with angiogenesis/vascular dependence of tumors? *Cancer Res* 62, 1931-4.

Real, F. X., Rettig, W. J., Chesa, P. G., Melamed, M. R., Old, L. J. and Mendelsohn, J. (1986). Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. *Cancer Res* 46, 4726-31.

Resink, T. J., Scott-Burden, T., Hahn, A. W., Rouge, M., Hosang, M., Powell, J. S. and Buhler, F. R. (1990). Specific growth stimulation of cultured smooth muscle cells from spontaneously hypertensive rats by platelet-derived growth factor A-chain homodimer. *Cell Regul* 1, 821-31.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* 389, 725-30.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* 386, 671-4.

Risau, W., Drexler, H., Mironov, V., Smits, A., Siegbahn, A., Funa, K. and Heldin, C. H. (1992). Platelet-derived growth factor is angiogenic in vivo. *Growth Factors* 7, 261-6.

Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annu Rev Cell Dev Biol* 11, 73-91.

Rissanen, T. T., Markkanen, J. E., Gruchala, M., Heikura, T., Puranen, A., Kettunen, M. I., Kholova, I., Kauppinen, R. A., Achen, M. G., Stacker, S. A. et al. (2003). VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ Res* 92, 1098-106.

Ross, R., Glomset, J., Kariya, B. and Harker, L. (1974). A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci U S A* 71, 1207-10.

Russell, K. S., Stern, D. F., Polverini, P. J. and Bender, J. R. (1999). Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis. *Am J Physiol* 277, H2205-11.

Saharinen, P., Kerkela, K., Ekman, N., Marron, M., Brindle, N., Lee, G. M., Augustin, H., Koh, G. Y. and Alitalo, K. (2005). Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. *J Cell Biol* 169, 239-43.

Salomon, D. S., Brandt, R., Ciardiello, F. and Normanno, N. (1995). Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 19, 183-232.

Saltis, J., Thomas, A. C., Agrotis, A., Campbell, J. H., Campbell, G. R. and Bobik, A. (1995). Expression of growth factor receptors in arterial smooth muscle cells. Dependency on cell phenotype and serum factors. *Atherosclerosis* 118, 77-87.

Sanderson, M. P., Dempsey, P. J. and Dunbar, A. J. (2006). Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. *Growth Factors* 24, 121-36.

Sasada, R., Ono, Y., Taniyama, Y., Shing, Y., Folkman, J. and Igarashi, K. (1993). Cloning and expression of cDNA encoding human betacellulin, a new member of the EGF family. *Biochem Biophys Res Commun* 190, 1173-9.

Sato, J. D., Kawamoto, T., Le, A. D., Mendelsohn, J., Polikoff, J. and Sato, G. H. (1983). Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* 1, 511-29.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376, 70-4.

Sato, Y., Okamura, K., Morimoto, A., Hamanaka, R., Hamaguchi, K., Shimada, T., Ono, M., Kohno, K., Sakata, T. and Kuwano, M. (1993). Indispensable role of tissue-type plasminogen activator in growth factor-dependent tube formation of human microvascular endothelial cells in vitro. *Exp Cell Res* 204, 223-9.

Sauter, G., Moch, H., Moore, D., Carroll, P., Kerschmann, R., Chew, K., Mihatsch, M. J., Gudat, F. and Waldman, F. (1993). Heterogeneity of erbB-2 gene amplification in bladder cancer. *Cancer Res* 53, 2199-203.

Savage, C. R., Jr. and Cohen, S. (1972). Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. *J Biol Chem* 247, 7609-11.

Savage, C. R., Jr. and Cohen, S. (1973). Proliferation of corneal epithelium induced by epidermal growth factor. *Exp Eye Res* 15, 361-6.

Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I. and Weinberg, R. A. (1984). The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature* 312, 513-6.

Schreiber, A. B., Winkler, M. E. and Derynck, R. (1986). Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. *Science* 232, 1250-3.

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S. and Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219, 983-5.

Shaheen, R. M., Ahmad, S. A., Liu, W., Reinmuth, N., Jung, Y. D., Tseng, W. W., Drazan, K. E., Bucana, C. D., Hicklin, D. J. and Ellis, L. M. (2001a). Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. *Br J Cancer* 85, 584-9.

Shaheen, R. M., Tseng, W. W., Davis, D. W., Liu, W., Reinmuth, N., Vellagas, R., Wieczorek, A. A., Ogura, Y., McConkey, D. J., Drazan, K. E. et al. (2001b). Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms. *Cancer Res* 61, 1464-8.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L. and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376, 62-6.

Shin, H. S., Lee, H. J., Nishida, M., Lee, M. S., Tamura, R., Yamashita, S., Matsuzawa, Y., Lee, I. K. and Koh, G. Y. (2003). Betacellulin and amphiregulin induce upregulation of cyclin D1 and DNA synthesis activity through differential signaling pathways in vascular smooth muscle cells. *Circ Res* 93, 302-10.

Shinde Patil, V. R., Friedrich, E. B., Wolley, A. E., Gerszten, R. E., Allport, J. R. and Weissleder, R. (2005). Bone marrow-derived lin(-)c-kit(+)Sca-1+ stem cells do not contribute to vasculogenesis in Lewis lung carcinoma. *Neoplasia* 7, 234-40.

Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K. and Folkman, J. (1993). Betacellulin: a mitogen from pancreatic beta cell tumors. *Science* 259, 1604-7.

Shirakata, Y., Kimura, R., Nanba, D., Iwamoto, R., Tokumaru, S., Morimoto, C., Yokota, K., Nakamura, M., Sayama, K., Mekada, E. et al. (2005). Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. *J Cell Sci* 118, 2363-70.

Shirasawa, S., Sugiyama, S., Baba, I., Inokuchi, J., Sekine, S., Ogino, K., Kawamura, Y., Dohi, T., Fujimoto, M. and Sasazuki, T. (2004). Dermatitis due to epiregulin deficiency and a critical role of epiregulin in immune-related responses of keratinocyte and macrophage. *Proc Natl Acad Sci U S A* 101, 13921-6.

Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. G. and Todaro, G. J. (1989). Structure and function of human amphiregulin: a member of the epidermal growth factor family. *Science* 243, 1074-6.

Shweiki, D., Itin, A., Soffer, D. and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359, 843-5.

Sibilia, M., Steinbach, J. P., Stingl, L., Aguzzi, A. and Wagner, E. F. (1998). A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. *Embo J* 17, 719-31.

Sibilia, M. and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* 269, 234-8.

Silvestre, J. S., Tamarat, R., Ebrahimian, T. G., Le-Roux, A., Clergue, M., Emmanuel, F., Duriez, M., Schwartz, B., Branellec, D. and Levy, B. I. (2003). Vascular endothelial growth factor-B promotes in vivo angiogenesis. *Circ Res* 93, 114-23.

Sini, P., Wyder, L., Schnell, C., O'Reilly, T., Littlewood, A., Brandt, R., Hynes, N. E. and Wood, J. (2005). The antitumor and antiangiogenic activity of vascular endothelial growth factor receptor inhibition is potentiated by ErbB1 blockade. *Clin Cancer Res* 11, 4521-32.

Sirotnak, F. M., Zakowski, M. F., Miller, V. A., Scher, H. I. and Kris, M. G. (2000). Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 6, 4885-92.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235, 177-82.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244, 707-12.

Soh, E. Y., Sobhi, S. A., Wong, M. G., Meng, Y. G., Siperstein, A. E., Clark, O. H. and Duh, Q. Y. (1996). Thyroid-stimulating hormone promotes the secretion of vascular endothelial growth factor in thyroid cancer cell lines. *Surgery* 120, 944-7.

Soker, S., Takashima, S., Miao, H. Q., Neufeld, G. and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 92, 735-45.

Solomon, B., Hagekyriakou, J., Trivett, M. K., Stacker, S. A., McArthur, G. A. and Cullinane, C. (2003). EGFR blockade with ZD1839 ("Iressa") potentiates the antitumor effects of single and multiple fractions of ionizing radiation in human A431 squamous cell carcinoma. Epidermal growth factor receptor. *Int J Radiat Oncol Biol Phys* 55, 713-23.

Song, S., Ewald, A. J., Stallcup, W., Werb, Z. and Bergers, G. (2005). PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nat Cell Biol* 7, 870-9.

Soriano, P. (1994). Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev* 8, 1888-96.

St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B. et al. (2000). Genes expressed in human tumor endothelium. *Science* 289, 1197-202.

Strachan, L., Murison, J. G., Prestidge, R. L., Sleeman, M. A., Watson, J. D. and Kumble, K. D. (2001). Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily. *J Biol Chem* 276, 18265-71.

Sunada, H., Magun, B. E., Mendelsohn, J. and MacLeod, C. L. (1986). Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. *Proc Natl Acad Sci U S A* 83, 3825-9.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N. and Yancopoulos, G. D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87, 1171-80.

Tabernero, J. (2007). The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. *Mol Cancer Res* 5, 203-20.

Takahashi, M., Hayashi, K., Yoshida, K., Ohkawa, Y., Komurasaki, T., Kitabatake, A., Ogawa, A., Nishida, W., Yano, M., Monden, M. et al. (2003). Epiregulin as a major autocrine/paracrine factor released from ERK- and p38MAPK-activated vascular smooth muscle cells. *Circulation* 108, 2524-9.

Tammela, T., Enholm, B., Alitalo, K. and Paavonen, K. (2005). The biology of vascular endothelial growth factors. *Cardiovasc Res* 65, 550-63.

Tamura, R., Miyagawa, J., Nishida, M., Kihara, S., Sasada, R., Igarashi, K., Nakata, A., Yamamori, K., Kameda-Takemura, K., Yamashita, S. et al. (2001). Immunohistochemical localization of Betacellulin, a member of epidermal growth factor family, in atherosclerotic plaques of human aorta. *Atherosclerosis* 155, 413-23.

Tang, C. K., Gong, X. Q., Moscatello, D. K., Wong, A. J. and Lippman, M. E. (2000). Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer. *Cancer Res* 60, 3081-7.

Tanner, M., Hollmen, M., Junttila, T. T., Kapanen, A. I., Tommola, S., Soini, Y., Helin, H., Salo, J., Joensuu, H., Sihvo, E. et al. (2005). Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. *Ann Oncol* 16, 273-8.

Taylor, D. S., Cheng, X., Pawlowski, J. E., Wallace, A. R., Ferrer, P. and Molloy, C. J. (1999). Epiregulin is a potent vascular smooth muscle cell-derived mitogen induced by angiotensin II, endothelin-1, and thrombin. *Proc Natl Acad Sci U S A* 96, 1633-8.

Teichert-Kuliszewska, K., Maisonpierre, P. C., Jones, N., Campbell, A. I., Master, Z., Bendeck, M. P., Alitalo, K., Dumont, D. J., Yancopoulos, G. D. and Stewart, D. J. (2001). Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. *Cardiovasc Res* 49, 659-70.

Thorin, E. and Shreeve, S. M. (1998). Heterogeneity of vascular endothelial cells in normal and disease states. *Pharmacol Ther* 78, 155-66.

Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C. et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* 269, 230-4.

Tian, S., Hayes, A. J., Metheny-Barlow, L. J. and Li, L. Y. (2002). Stabilization of breast cancer xenograft tumour neovasculature by angiopoietin-1. *Br J Cancer* 86, 645-51.

Torres-Vazquez, J., Kamei, M. and Weinstein, B. M. (2003). Molecular distinction between arteries and veins. *Cell Tissue Res* 314, 43-59.

Toyoda, H., Komurasaki, T., Ikeda, Y., Yoshimoto, M. and Morimoto, S. (1995). Molecular cloning of mouse epiregulin, a novel epidermal growth factor-related protein, expressed in the early stage of development. *FEBS Lett* 377, 403-7.

Traxler, P., Allegrini, P. R., Brandt, R., Brueggen, J., Cozens, R., Fabbro, D., Grosios, K., Lane, H. A., McSheehy, P., Mestan, J. et al. (2004). AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. *Cancer Res* 64, 4931-41.

Ueda, S., Basaki, Y., Yoshie, M., Ogawa, K., Sakisaka, S., Kuwano, M. and Ono, M. (2006). PTEN/Akt signaling through epidermal growth factor receptor is prerequisite for angiogenesis by hepatocellular carcinoma cells that is susceptible to inhibition by gefitinib. *Cancer Res* 66, 5346-53.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J. et al. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309, 418-25.

Urbich, C. and Dimmeler, S. (2004). Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 95, 343-53.

Urbich, C., Heeschen, C., Aicher, A., Dernbach, E., Zeiher, A. M. and Dimmeler, S. (2003). Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation* 108, 2511-6.

Ushiro, S., Ono, M., Izumi, H., Kohno, K., Taniguchi, N., Higashiyama, S. and Kuwano, M. (1996). Heparin-binding epidermal growth factor-like growth factor: p91 activation induction of plasminogen activator/inhibitor, and tubular morphogenesis in human microvascular endothelial cells. *Jpn J Cancer Res* 87, 68-77.

Uutela, M., Lauren, J., Bergsten, E., Li, X., Horelli-Kuitunen, N., Eriksson, U. and Alitalo, K. (2001). Chromosomal location, exon structure, and vascular expression patterns of the human PDGFC and PDGFC genes. *Circulation* 103, 2242-7.

Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T. V., Kubo, H., Thurston, G., McDonald, D. M., Achen, M. G. et al. (2001). Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *Embo J* 20, 1223-31.

Viloria-Petit, A., Crombet, T., Jothy, S., Hicklin, D., Bohlen, P., Schlaeppi, J. M., Rak, J. and Kerbel, R. S. (2001). Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. *Cancer Res* 61, 5090-101.

Viloria-Petit, A. M. and Kerbel, R. S. (2004). Acquired resistance to EGFR inhibitors: mechanisms and prevention strategies. *Int J Radiat Oncol Biol Phys* 58, 914-26.

von Tell, D., Armulik, A. and Betsholtz, C. (2006). Pericytes and vascular stability. *Exp Cell Res* 312, 623-9.

Wakui, S. (1992). Epidermal growth factor receptor at endothelial cell and pericyte interdigitation in human granulation tissue. *Microvasc Res* 44, 255-62.

Wakui, S., Furusato, M., Tanaka, M., Allsbrook, W. C., Jr., Kano, Y. and Ushigome, S. (1990). Endothelium and pericyte interdigitation: pathway for epidermal growth factor? *Microvasc Res* 40, 285-91.

Wen, X. F., Yang, G., Mao, W., Thornton, A., Liu, J., Bast, R. C., Jr. and Le, X. F. (2006). HER2 signaling modulates the equilibrium between pro- and antiangiogenic factors via distinct pathways: implications for HER2-targeted antibody therapy. *Oncogene* 25, 6986-96.

Xiong, S., Grijalva, R., Zhang, L., Nguyen, N. T., Pisters, P. W., Pollock, R. E. and Yu, D. (2001). Up-regulation of vascular endothelial growth factor in breast cancer cells by the heregulin-beta1-activated p38 signaling pathway enhances endothelial cell migration. *Cancer Res* 61, 1727-32.

Yamamoto, K., de Waard, V., Fearns, C. and Loskutoff, D. J. (1998). Tissue distribution and regulation of murine von Willebrand factor gene expression in vivo. *Blood* 92, 2791-801.

Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K. and Nishikawa, S. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 408, 92-6.

Yang, G., Cai, K. Q., Thompson-Lanza, J. A., Bast, R. C., Jr. and Liu, J. (2004). Inhibition of breast and ovarian tumor growth through multiple signaling pathways by using retrovirus-mediated small interfering RNA against Her-2/neu gene expression. *J Biol Chem* 279, 4339-45.

Yarden, Y. and Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2, 127-37.

Yen, L., Benlimame, N., Nie, Z. R., Xiao, D., Wang, T., Al Moustafa, A. E., Esumi, H., Milanini, J., Hynes, N. E., Pages, G. et al. (2002). Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol Biol Cell* 13, 4029-44.

Yen, L., You, X. L., Al Moustafa, A. E., Batist, G., Hynes, N. E., Mader, S., Meloche, S. and Alaoui-Jamali, M. A. (2000). Heregulin selectively upregulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. *Oncogene* 19, 3460-9.

Ying, W. Z., Zhang, H. G. and Sanders, P. W. (2007). EGF receptor activity modulates apoptosis induced by inhibition of the proteasome of vascular smooth muscle cells. *J Am Soc Nephrol* 18, 131-42.

Yokoi, A., McCrudden, K. W., Huang, J., Kim, E. S., Soffer, S. Z., Frischer, J. S., Serur, A., New, T., Yuan, J., Mansukhani, M. et al. (2003). Human epidermal growth factor receptor signaling contributes to tumor growth via angiogenesis in her2/neu-expressing experimental Wilms' tumor. *J Pediatr Surg* 38, 1569-73.

Yoshizumi, M., Kourembanas, S., Temizer, D. H., Cambria, R. P., Quertermous, T. and Lee, M. E. (1992). Tumor necrosis factor increases transcription of the heparin-binding epidermal growth factor-like growth factor gene in vascular endothelial cells. *J Biol Chem* 267, 9467-9.

Zhang, D., Sliwkowski, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J. and Godowski, P. J. (1997). Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. *Proc Natl Acad Sci U S A* 94, 9562-7.

Zhang, K., Sun, J., Liu, N., Wen, D., Chang, D., Thomason, A. and Yoshinaga, S. K. (1996). Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. *J Biol Chem* 271, 3884-90.

Zhao, Y. Y., Sawyer, D. R., Baliga, R. R., Opel, D. J., Han, X., Marchionni, M. A. and Kelly, R. A. (1998). Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J Biol Chem* 273, 10261-9.

Zhou, W., Ibe, B. O. and Raj, J. U. (2007). Platelet-activating factor induces ovine fetal pulmonary venous smooth muscle cell proliferation: role of epidermal growth factor receptor transactivation. *Am J Physiol Heart Circ Physiol* 292, H2773-81.

Zhu, M., Madigan, M. C., van Driel, D., Maslim, J., Billson, F. A., Provis, J. M. and Penfold, P. L. (2000). The human hyaloid system: cell death and vascular regression. *Exp Eye Res* 70, 767-76.

Ziegelhoeffer, T., Fernandez, B., Kostin, S., Heil, M., Voswinckel, R., Helisch, A. and Schaper, W. (2004). Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ Res* 94, 230-8.

# ORIGINAL PUBLICATIONS