Gene expression profiling of tumor angiogenesis

Citation for published version (APA):

van Beijnum, J. R. (2006). *Gene expression profiling of tumor angiogenesis*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.20060427jb>

Document status and date:

Published: 01/01/2006

DOI:

[10.26481/dis.20060427jb](https://doi.org/10.26481/dis.20060427jb)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

A vertical strip on the left side of the page shows a microscopic image of tumor tissue, characterized by irregular, dark, and dense cellular structures.

Gene expression profiling of tumor angiogenesis

© J.R. van Beijnum, Maastricht 2006

ISBN-10: 90-9020568-3

ISBN-13: 978-90-9020568-7

Printed by Grafisch bedrijf Ponsen en Looijen B.V.

Special thanks to Toon Hezemans – Tejo Haas Productions

A vertical strip on the left side of the page shows a microscopic image of tissue, likely a histological section, with various cellular structures and patterns.

Gene expression profiling of tumor angiogenesis

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 27 april 2006 om 14.00 uur

door

Judith Rosina van Beijnum

geboren op 11 december 1977 te Nijmegen

Promotor:

Prof. dr. A.W. Griffioen

Beoordelingscommissie:

Prof. dr. J. Waltenberger (Voorzitter)

Prof. dr. R. Bicknell (Birmingham University, United Kingdom)

Prof. dr. W.A. Buurman

Dr. M.F.B.G. Gebbink (Universiteit Utrecht)

Prof. dr. B.G. Wouters

Financial support by the Dr. Ir. Van de Laar Stichting for the publication of this thesis is gratefully acknowledged.

>> Abbreviations

3D	Three-dimensional
Ab	Antibody
AGE	Advanced glycation end product(s)
BCE	Bovine capillary endothelial cell(s)
BSA	Bovine serum albumin
bFGF	Basic fibroblast growth factor
BPI	Bactericidal/permeability-increasing protein
CAM	Chick chorioallantoic membrane
CDC	Cell division cycle
cDNA	Complementary DNA
CM	Conditioned medium
DNA	Deoxyribonucleic acid
EC	Endothelial cell(s)
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EST	Expressed sequence tag
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
GAG	General angiogenesis gene
HDMEC	Human dermal microvascular endothelial cell(s)
HGF	Hepatocyte growth factor
HIF-1 α	Hypoxia inducible factor 1 α
HMEC	Human microvascular endothelial cell(s)
HMGB1	High-mobility group box 1
HMVEC	Human microvascular endothelial cell(s)
HUVEC	Human umbilical vein endothelial cell(s)
ICAM1	Intercellular adhesion molecule 1
IGFBP7	Insulin-like growth factor binding protein 7
IL	Interleukin
MMP	Matrix metalloproteinase
NEC	Normal endothelial cell(s)
NF κ B	Nuclear factor κ B
NGR	Asparagine-glycine-arginine
NRP	Neuropilin
ODN	Oligodeoxynucleotide
PAK	p21 activated kinase
PBS	Phosphate buffered saline
PF	Paraformaldehyde
PF4	Platelet-factor 4
PKC	Protein kinase C

PLEC	Placenta endothelial cell(s)
PMA	Phorbol myristate acetate
RAGE	Receptor for advanced glycation end products
RGD	Arginine-glycine-aspartic acid
RNA	Ribonucleic acid
RNAi	RNA interference
ROK	Rho activated kinase
SAGE	Serial analysis of gene expression
ShRNA	Small hairpin RNA
SiRNA	Small interfering RNA
SPARC	Secreted protein, acidic, rich in cysteine
SSH	Suppression subtractive hybridization
TAG	Tumor angiogenesis gene
TEC	Tumor endothelial cell(s)
TEM	Tumor endothelial marker
TIE	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TNF α	Tumor necrosis factor α
TSP1	Thrombospondin 1
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor

>> Contents

Chapter 1	General introduction	11
Chapter 2	<i>In silico</i> analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles	33
Chapter 3	Gene expression of tumor angiogenesis dissected; specific targeting of tumor endothelium	61
Chapter 4	Interference with vimentin expression affects the angiogenic potential of tumor endothelial cells	83
Chapter 5	High-mobility group box 1 (HMGB1) positively contributes to tumor angiogenesis	103
Chapter 6	Towards high-throughput functional target discovery in angiogenesis research	119
Chapter 7	General discussion	139
Summary		152
Samenvatting		156
Dankwoord		160
Curriculum Vitae		164

Chapter 1

General introduction

Based on:

Femke Hillen, Veerle Melotte, Judy R. van Beijnum, Arjan W. Griffioen.

Endothelial cell biology. In: Staton CA, Lewis C, Bicknell R, editors.

Angiogenesis assays: a critical appraisal of current techniques. London, United Kindom: John Wiley and Sons. In press.

>> Angiogenesis and tumor angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing ones, is pivotal to a variety of physiological as well as pathological processes. Most tissues are critically dependent on an adequate supply of oxygen and nutrients and removal of waste products via the vasculature. Normal tissue growth, for example during prenatal development, wound healing and during the female reproductive cycle is characterized by new vessel formation. Angiogenesis is also induced during pathological conditions including cancer progression, arthritis, atherosclerosis, endometriosis and diabetic retinopathy [1].

In normal tissues, a balance is maintained between the effects of anti-angiogenic factors and those of pro-angiogenic factors, and endothelial cells (EC) are generally quiescent displaying little or no proliferation. Disturbance of this balance in favor of pro-angiogenic factors is referred to as the 'angiogenic switch' [2]. Angiogenesis can be switched on in response to various stimuli, including hypoxia, metabolic stress and cellular signals. The limited diffusion of oxygen and nutrients is about 100-200 μm [3], therefore, initial tumors can not grow beyond a size of approximately 2 mm^3 without the attraction of novel blood vessels.

Once angiogenesis is initiated, a sequence of events is set in motion. Under the influence of pro-angiogenic factors, EC lining existing blood vessels become activated. Before they can migrate in the direction of the stimulus, the underlying basement membrane is degraded by matrix metalloproteinases (MMPs), which are produced by EC, tumor cells, fibroblasts and inflammatory cells [1]. To extend towards the tissue in need of vascular supply, the EC proliferate and form a tube-like structure that is stabilized by mural cells such as pericytes during the maturation phase (Figure 1.1). In tumors however, continuous angiogenic stimulation results in the formation of a highly distorted vasculature that frequently lacks pericyte coverage, shows excessive branching and is uneven in diameter. Blood flow is chaotic and variable which may help sustain hypoxia and hence continued angiogenic stimulation in certain regions of the tumor [3]. Furthermore, tumor blood vessels are leaky due to widened interendothelial junctions, the presence of a discontinuous basement membrane and protrusions of EC into the vascular lumen [3,4].

>> Regulation of angiogenesis

Pro-angiogenic factors

A vast number of secreted pro-angiogenic factors and their receptors have been described over the last decades (for recent reviews see [5-8]) and novel insights in human biology continue to extend the number of positive contributors to angiogenesis. One of the best-known pro-angiogenic factors is vascular endothelial growth factor (VEGF), first discovered in 1983 as vascular permeability factor by Harold Dvorak [9].

VEGF is a survival factor for endothelial cells but also stimulates EC to secrete MMPs to allow invasion into the surrounding matrix [10]. VEGF can be released from tumor cells under the influence of hypoxia inducible factor-1 α (HIF-1 α), an oxygen responsive transcription factor. In addition, mutations in tumor-suppressor genes (e.g. p53) and oncogenes (e.g. Ras and ErbB-2) in tumor cells may also stimulate VEGF release via induction of HIF-1 α [11-13]. The biological effects of the VEGF family are mediated via cell surface VEGF receptors Flt-1 (VEGFR-1), KDR (VEGFR-2) and Flt-4 (VEGFR-3), the latter being mainly present on lymphatic endothelial cells [10]. To date, VEGF-A is the best characterized molecule of the VEGF family. It interacts with both Flt-1 and KDR but these receptors have different effects. KDR is the major mediator of the mitogenic and angiogenic effects of VEGF-A. Flt-1 is involved in the induction of MMPs but may also function as a decoy receptor [14,15].

Basic fibroblast growth factor (bFGF) is another important pro-angiogenic factor that stimulates EC proliferation and survival. bFGF signaling occurs through four high affinity tyrosine kinase transmembrane receptors (FGFR1-4). It is produced by many cells, among which macrophages and tumor cells, and is subsequently secreted into the extracellular matrix (ECM). bFGF binds to heparan sulfate proteoglycans,

Figure 1.1: Schematic representation of tumor angiogenesis.

A) In response to hypoxia, tumor cells secrete angiogenic factors that activate endothelial cells in a nearby vessel. **B)** Angiogenesis is initiated when activated endothelial cells degrade the underlying matrix and start to migrate towards the angiogenic stimuli. **C)** Proliferation of endothelial cells allows the formation of a tube structure in the direction of the tumor. **D)** A newly formed vascular network enables the tumor to continue growth and may in addition be used as a metastatic route.

located on the surface of many cells and in the ECM, and can be released by matrix degrading enzymes. Several intracellular signaling pathways are activated by bFGF-FGFR interaction, including the Ras pathway and the PI3K pathway [7,10].

Angiopoietins and their TIE (tyrosine kinase with immunoglobulin-like and EGF-like domains) receptors are also critical regulators of angiogenesis. Angiopoietin-1 (Ang-1) interacts with TIE2 and inhibits EC apoptosis and stimulates endothelial sprouting [8,16]. Ang-1 is further required for remodeling and maturation of immature vasculature as it stimulates the recruitment of pericytes. Ang-2 is involved in postnatal angiogenic and vascular remodeling events and in detaching smooth muscle cells and loosening underlying matrix, thereby allowing EC to migrate. Ang-2, in concert with VEGF is also angiogenic, although in the absence of VEGF, Ang-2 may actually induce vessel regression [17,18].

Guidance molecules

Recent studies have showed some analogies between molecular regulation of neural development and angiogenesis (reviewed by Klagsbrun [6] and Suchting [19]). Neuropilins (NRPs) are receptors for the repulsive axon guidance factors class 3 Semaphorins (Sema3). Sema3A is a ligand for NRP-1 whereas Sema3F is a ligand for NRP-2 [20,21]. In addition, NRPs function as receptors for specific VEGF splice variants [22]. Semaphorins and VEGF compete for binding the NRPs, hence they inhibit VEGF-mediated angiogenesis [23]. Semaphorins also have a direct inhibitory effect on EC migration, protrusion of lamellipodia and sprouting [23], possibly through influencing endothelial integrins [24]. Ephrin ligands and Eph receptors have also been recognized for their role in stimulating angiogenesis [25], after initially being implicated in repulsive neuronal guidance [26]. Both the Eph receptors and their ephrin ligands are membrane-bound and play important roles in arterio-venous differentiation and in positioning EC [6,25]. Eph-ephrin signaling cross-signals with the VEGF-VEGFR axis, for example, blockade of the EphA receptor inhibits VEGF induced angiogenesis [27-30]. More recently, secreted Slit proteins and their roundabout receptors (Robo) were demonstrated to play a role in angiogenesis next to their roles in axon pathfinding [6,19,28,31]. Whether in angiogenesis they are either attractant or repulsive is still under debate. Slit2 promoted endothelial tube formation and acted as a chemoattractant *in vitro*. Blocking its receptor Robo1 reduced tumor angiogenesis in a mouse tumor model [32]. Another report however showed that exposure of EC to Slit2 inhibited migration [33]. Soluble Robo4 was shown to inhibit EC migration and proliferation [33,34], but its binding to any of the Slit proteins is still under debate. Finally, netrins, bi-functional guidance cues in neuronal development, have been implicated in regulating angiogenesis. Netrins can interact with two types of receptors, deleted in colorectal cancer (DCC) and the uncoordinated-5 (Unc5) family [35]. Endothelial tip cells express Unc5b, and treatment with Netrin-1 resulted in tip cell filopodia retraction [36], suggesting negative regulation of angiogenesis by this

ligand/receptor pair. However, others have reported Netrin-1 to be a pro-angiogenic factor [37].

Many parallels between neuronal guidance and angiogenesis have been uncovered that help organize network formation by a combination of attractive and repulsive cues. However, the precise action of different guidance molecules in tumor angiogenesis has not been elucidated yet and contradicting results have been obtained. Nevertheless, these findings expand our knowledge on the molecular regulation of angiogenesis [6,19].

Anti-angiogenic factors

Several endogenous anti-angiogenic factors have been discovered that counterbalance the actions of pro-angiogenic factors. In 1980, interferon- α was shown to inhibit EC migration [38] and has been used since 1988 to treat primarily hemangiomas and angiolymphomas [39]. Interferon- α inhibits the production of bFGF, the angiogenic factor these tumors are highly dependent on [40]. Thrombospondin-1 (TSP1) is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions [41,42]. Binding of TSP1 to CD36 activates apoptosis by inducing p38 and jun N-terminal kinase, and subsequently the cell-surface expression of Fas ligand that triggers the caspase cascade [43,44]. Platelet factor-4 (PF4) has also been mentioned as endogenous angiogenesis inhibitor [1,45]. PF4 can directly interact with pro-angiogenic growth factors such as bFGF and VEGF [45]. In addition, PF4 derived molecules also have anti-angiogenic action [46,47].

Bactericidal/permeability-increasing protein (BPI), produced by neutrophils, has antibacterial effects, but also shows similarities to PF4. BPI inhibits EC growth, induces EC apoptosis and inhibits angiogenesis *in vitro* and *in vivo* [48]. Though the receptor for BPI is unknown, it is likely that its effects are mediated by binding heparan sulphates in the ECM [48].

Interestingly, many endogenous angiogenesis inhibitors are cleavage products from larger proteins. For example, endostatin is a fragment of collagen XVIII, angiostatin is a fragment of plasminogen and tumstatin is a fragment of collagen IV. The collagen fragments endostatin and tumstatin are formed by MMP cleavage of the respective collagens and may function as negative feedback molecules during vascular remodeling. Their precise mechanism of action has not yet been fully elucidated but several studies point to interactions with endothelial integrins that mediate their effects [49].

>> Angiogenesis inhibition

The hypothesis that tumor growth is angiogenesis-dependent was postulated over three decades ago [50], and triggered a wide interest in inhibiting this process as a novel therapeutic strategy to treat cancer. Though angiogenesis does not initiate tumor

growth, it promotes tumor progression to clinical significance and offers a major route for metastatic spreading of tumor cells to other locations in the body. Different anti-angiogenic and vascular targeting agents have been developed over the last decades and this recently resulted in the FDA approval of AvastinTM, an anti-VEGF antibody for the treatment of metastatic colorectal cancer [51].

Anti-angiogenesis as cancer treatment

Therapeutic inhibition of tumor angiogenesis is considered to have a number of major advantages over the current conventional therapies (surgery, radiation and chemotherapy). One blood vessel provides metabolic support to a surrounding area of tumor cells, hence, eradication of only a limited number of endothelial cells will result in the death of a multitude of tumor cells. Another major advantage is that EC are generally accessible via the blood stream. This makes the delivery of anti-angiogenic agents relatively easier as compared to chemotherapeutic drugs targeting the tumor cells (that first need to diffuse into the tumor mass), especially when large molecules such as antibodies are used as therapeutics, as with AvastinTM. One of the major disadvantages of chemotherapy is its deleterious effect on non-malignant cells causing severe side effects. Angiogenesis is limited in adult life, hence interference in this process is thought to result in much less severe side effects. Nevertheless, impaired physiological angiogenic responses have been observed in patients treated with AvastinTM [52]. Finally, unlike tumor cells that frequently contain genomic mutations, EC in a tumor are the offspring of normal endothelial cells and are considered to be genetically stable, diminishing the chance of acquired drug resistance associated with clonal selection of mutations [53]. Though in essence this may be correct, in clinical practice resistance to anti-angiogenic therapy is noticeable [54,55]. EC heterogeneity, between individuals as well as within a tumor as a consequence of microenvironmental actions, may confer selective sensitivity to certain anti-angiogenic agents. Also, genetic drift may modulate the angiogenic profile of a tumor, i.e. the types and relative amounts of different pro-angiogenic factors it secretes, which may drastically limit the effect of an inhibitor directed at only one type of angiogenic factor [14,56]. Furthermore, tumor cells may become increasingly hypoxia resistant [57], hence less dependent on angiogenesis. Since tumors have also been shown to use alternative routes to vascularization, e.g. vessel cooption, vascular mimicry and recruitment of bone marrow derived cells [58], their sensitivity to angiogenesis inhibition is not always guaranteed. In addition, two recent studies have shown that tumor EC can harbor genetic abnormalities [59,60].

Angiogenesis inhibitors

The realization that cancer could be treated and prevented by targeting its blood supply created an explosion of interest in finding inhibitors of angiogenesis. In addition to endogenous angiogenesis inhibitors (described above), numerous compounds have been developed and tested, some of which are highlighted below.

VEGF is the most frequently targeted molecule for anti-angiogenesis. In addition to the anti-VEGF antibody Avastin™ that scavenges VEGF, a chimaeric soluble VEGF receptor (VEGF-Trap) is also able to reduce local VEGF concentrations [61]. Furthermore, many compounds have been developed that target the tyrosine kinase activity of VEGF receptors, for example PTK787 [62] and SU11248 [63], currently in clinical trials. Inhibitors of MMPs are also under investigation [64-66].

Anginex is a designed cytokine-like peptide that was selected from a library of synthetic peptides with homology to endogenous anti-angiogenic proteins [67]. It is a potent inhibitor of EC proliferation and migration *in vitro* [67], and inhibits tumor growth in mice by targeting tumor EC [46,68]. Anginex synergizes with conventional chemotherapeutics, other angiogenesis inhibitors and radiation therapy to inhibit tumor growth [69,70]. More recently, partial mimetics of anginex were shown to be even more potent inhibitors of tumor growth in mice models [71]. Furthermore, recombinant anginex has been successfully produced to exploit its tumor endothelium targeting capacities for gene therapy purposes and imaging [72].

Combretastatin A-4 phosphate (combeAp) is a tubulin-polymerization interfering compound that is selectively toxic to EC. In addition, it inhibits endothelial migration and tube formation and induces apoptosis. Finally, it inhibits tumor growth in rats and mice [73]. CombreAp inhibits blood flow in tumors, leading to a rapid vascular shutdown and subsequently hypoxia and central necrosis [74].

Fumagillin, or TNP-470, inhibits EC proliferation without inducing apoptosis. It inhibits cell movement, but has only minor effects on tube formation [75]. It has been extensively tested in different tumor models and shown to be very effective. However, neurotoxicity limits its clinical utility [76]. Conjugated to a polymer to form caplostatin, fumagillin is no longer neurotoxic yet still effective in inhibiting tumor growth [77].

Though the primary aim of anti-angiogenic agents is the eradication of tumor blood vessels, many of them also transiently normalize tumor vasculature, thereby allowing a better penetration of cytotoxic drugs or sensitization of the tumor to radiotherapy [78]. Furthermore, not only classical anti-angiogenic agents can reduce the tumor vasculature. It has been proposed that chemotherapeutic compounds can be administered in an anti-angiogenic schedule, i.e. continued, low-dose administration. EC are killed by the compound and consequently tumor growth is inhibited. In contrast, administration of a maximum tolerated dose comprises the risk of tumor cells acquiring drug resistance and renewed outgrowth of the tumor [14,79]. In the future, the therapeutic potential of angiogenesis inhibitors might be further enhanced by optimization of dosing and combining different agents.

Angiogenesis inhibition and vascular targeting

Two general strategies can be employed to attack the tumor's blood supply. Either the formation of novel blood vessels can be inhibited, the narrow definition of angiogenesis inhibition, but it is also possible to destroy existing tumor vessels, frequently referred to as vascular targeting. However, depending on the mode of action

of the agent, there is considerable overlap between these two applications. For example, VEGF inhibition both prevents angiogenesis but as it also acts as a survival factor for EC, it will also result in the death of existing EC. Nevertheless, for the design of effective angiogenesis inhibitors or vascular targeting agents for cancer treatment, thorough knowledge of EC biology is a prerequisite.

>> Endothelial cell heterogeneity

The endothelium exhibits a remarkable diversity of cellular properties that are adapted to the needs of the underlying tissue, which is related to the microenvironmental factors acting in each different organ and the specialized function for the endothelium therein [80-82]. Even within one organ, EC display heterogeneity.

EC heterogeneity may be explained by variations in the microenvironment ('nurture') as well as by genetic predisposition ('nature') [83]. Interactions between EC and their precise microenvironments are thought to play an important role in determining the phenotype of the EC. Depending on the tissue or organ, different soluble mediators may be present that act on the EC and EC may be in contact with different cell types or matrix components. In addition, hemodynamic factors may also govern changes in endothelium [84].

Microenvironment

EC heterogeneity may be exploited therapeutically. As described below and in other chapters of this thesis, EC display tissue specific features that are mediated by the microenvironment. Expression of specific markers on EC of for example tumors may be targeted in a therapeutic setting to accomplish eradication of these cells without major side effects arising from targeting EC in healthy tissues or organs. The pronounced influence of the microenvironment on EC phenotype has major implications for the study of specific EC subpopulations derived from particular organs or tissues. EC isolated from specific locations in the body that are subsequently cultured may lose some of their tissue-specific characteristics, or acquire new phenotypic properties in response to the new environment *in vitro* (reviewed by Aird [82]). Merely culturing EC might already induce changes. For example, blood-brain barrier EC that specifically express P-glycoprotein lose this expression *in vitro*. When cultured in the presence of brain matrix or astrocytes, the expression is restored, though matrix derived from other organs is not capable of stimulating this expression [85].

The identification of organ- and disease-specific vascular markers has been compromised by difficulties in isolating pure populations of EC and subsequent culture. The isolation of capillary EC from tissues faces challenges related to the relatively limited percentage of EC present in tissues and the impossibility for cannulation of the small vessels, unlike larger vessels. Diverse methods have been used to isolate EC

from tissues, mainly based on the expression of endothelial specific markers, by using fluorescence activated cell sorting (FACS) [86] or by cell sorting using magnetic beads [87] following enzymatic tissue digestion (Figure 1.2).

Vascular address system

Peptide libraries displayed on the surface of phage have also been used to identify vascular markers [88]. This method relies on binding of the displayed peptide with vascular cell surface molecules in organs and tissues. Bound phage can be isolated from the tissues and the coding sequence of the peptide analyzed. This approach provides information on the ligand that interacts with the surface markers, though it does not elucidate the identity of the marker itself.

In vivo phage display offers an unbiased approach to identify surface markers on EC. Random peptides displayed on phage were injected in mice and recovered from different organs and malignancies where they interacted with endothelial surface molecules. Peptides specific for brain vasculature have been identified as well as peptides that home to lung, pancreas, skin, intestine, uterus, adrenal gland, retina and prostate [89], and tumor vasculature [90,91]. This approach has provided clues of a vascular address system that can be used for organ-specific targeting of normal blood vessels or disease related targeting of blood vessels, such as in tumors [90,91]. The famous RGD sequence (arginine-glycine-aspartic acid) targets α_V integrins in tumor vessels [92] and the NGR sequence (asparagine-glycine-arginine) was shown to target aminopeptidase N or CD13 [93]. Differences exist between EC present in tumors compared to EC in healthy tissue counterparts. Additional heterogeneity may exist

Figure 1.2: Isolation of endothelial cells from tissues.

A) Solid tissues (e.g. tumors) consist of diverse cells, including tumor cells, endothelial cells, stromal cells and inflammatory cells. **B)** The tissue mass is enzymatically digested to create a single-cell suspension. **C)** Antibodies specific for endothelial cells are allowed to bind. **D)** Magnetic particles with specific reactivity for the antibodies are added to the cell suspension and coat the endothelial cells. A magnet is used to pull the magnetic bead covered cells out from the total cell suspension.

between different tumors and even within tumors [90]. Furthermore, stage-specific homing of phage-displayed peptides to tumor vasculature has been reported [94]. However, the identification of the molecule targeted by the peptide displayed on phage remains a challenging task.

>> Gene expression in endothelial cells

Endothelial cells in a particular microenvironment interact with various types of cells, such as fibroblasts, pericytes, immune cells, tumor cells or organ cells. In addition, EC can interact with various ECM components and are exposed to different growth factors and cytokines released by its neighbors [95]. As a result, EC lining different vessels in different tissues exhibit morphological and functional specializations that are reflected in their gene expression profiles [96,97].

Arteries and veins

Differences in gene expression have been observed between arterial and venous endothelium. These differences are established even before circulation begins [98]. Venous-specific genes, identified by large scale gene expression analysis of EC of diverse origin include EphB4 and NRP-2, and a number of genes critically involved in left-right asymmetry such as *smoothed*, *lefty-1*, *lefty-2* and growth differentiation factor [96]. Interestingly, defects in left-right symmetry are frequently accompanied by vascular malformations [99]. Among the arterial endothelial specific genes are the cell surface proteins Notch4, EVA1, CD44 and Ephrin-B1, metabolic enzymes such as alcohol dehydrogenase A1 and endothelial lipase, keratin 7 and the basic helix-loop-helix transcription factor Hey2 that is induced by Notch signaling [96].

Large vessels and capillaries

Clear differential expression is also evident between large vessel derived endothelium and microvascular endothelium [96]. Genes involved in biosynthesis and remodeling of ECM, such as fibronectin, osteonectin and collagens Va1 and Va2 are overexpressed in large vessel endothelium. Basement membrane proteins such as collagens IVa1 and IVa2, and their interaction partners such as integrins $\alpha 4$, $\alpha 1$, $\alpha 9$ and $\beta 4$ are overexpressed in microvascular EC. In addition, large vessel EC express genes associated with neuronal guidance such as those discussed above. In contrast microvascular EC express receptors for paracrine signals of neuroglial cells such as interleukin (IL)-1 receptor, IL-6 receptor, platelet-derived growth factor receptor (PDGFR) and endothelin receptor-1. Furthermore, microvascular EC express secreted factors that promote survival and differentiation of neuroglial cells [96]. In addition, EC display organ-specific gene expression. For example, osteoglycin, secreted frizzled related protein (sFRP) and phospholipase A2, group X1IA (PLA2G12) were specific for

lung endothelium, whereas galanin and the calcitonin receptor emerged as uterus specific. Skin endothelium expressed many genes involved in cholesterol biosynthesis, such as stearoyl-CoA desaturase (SCD), fatty acid desaturase (FADS2), and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1), pointing towards active roles played by the endothelium in the organ physiology [96].

>> Gene expression profiling for target identification

Large-scale gene expression profiling has become feasible with the development of techniques such as serial analysis of gene expression (SAGE) [100], suppression subtractive hybridization (SSH) [101] and cDNA microarrays [102], and in the past decade vast amounts of data on gene expression in healthy and diseased tissues has been generated. In the early years, microarray technology was limited to the analysis of known genes that could be spotted on the arrays but since the recent completion of the human genome project virtually every gene that is expressed in the human body can be sampled. Refined techniques such as oligonucleotide arrays allow very high throughput gene expression profiling to be performed with very high sequence selectivity. In contrast to cDNA and oligonucleotide arrays, SSH and SAGE do not depend on known sequences. SSH involves the creation of cDNA libraries with genes overexpressed in one cell population compared to another [101]. Subsequent screening of these cDNA repertoires enables the identification of differentially expressed sequences. SAGE relies on the creation of sequence tags representative of a unique mRNA that are concatamerized and subsequently sequenced. The number of unique tags derived from cells in one condition is counted and compared to that of cells in another condition [100].

Analysis of gene expression can be used to determine molecular changes associated with particular phenotypes or diseases. The data can provide insight in the molecular mechanisms and pathways that govern disease. Importantly, differentially expressed genes may be used as targets for therapeutic intervention. For example, certain breast cancers overexpress ErbB-2, which is targeted by HerceptinTM [103]. The phenotypic differences of tumor EC as compared to EC in normal tissues argue for differences in gene expression that may be exploited therapeutically to attack the tumor's vasculature. Crucial to this is that the targeted gene is to a certain extent specific for the diseased tissue to prevent side effects associated with interference in non-diseased tissue.

Therapeutic targets

Some aspects need to be considered to determine whether a differentially expressed gene may be a putative therapeutic target. Surface expressed or secreted proteins are most easily accessible and can be used for targeted therapy using antibodies, small molecules with or without conjugation to cytotoxic molecules. Such

targets can also be exploited for imaging purposes (e.g. for disease monitoring and diagnosis) by conjugation to detector molecules (Figure 1.3).

Differential expression of a gene can have either a causative or an associative relation to the disease or condition. Interfering with the function of a gene that has a causative contribution to the disease phenotype, for example involved in migration of cells, is most effective, as the whole process will be inhibited. Blocking differentially expressed genes that have a function in a process that does not directly contribute to the disease phenotype is not likely to give significant therapeutic effect.

Different agents can be designed to therapeutically target differentially expressed proteins. Antibodies can be used that block surface expressed proteins and hence inhibit cell function or induce antibody-mediated cytotoxicity. They can also be conjugated to bioactive molecules such as toxins or cytokines to accomplish biological effect. Other binding molecules (e.g. peptide ligands, aptamers) are also suitable. However, these approaches generally rely on targeting a surface expressed or secreted protein [104]. Gene vectors may be used to silence expression of intracellular targets and interrupt crucial cell processes, for example progression through the cell cycle, but also need to be delivered at the proper location. This can be achieved by changing the tropism of a viral vector to redirect it to the target cell [105], or by placing the transgene under control of a target cell specific promoter (transcriptional targeting) [106] (Figure 1.3).

Though the identification of differentially expressed genes involved in disease processes has been boosted by now readily available technologies, the validation of a gene as putative therapeutic target is still a time-consuming and labor-intensive process. Target identification can be performed in a high-throughput fashion, but target validation is mainly being performed on a gene-to-gene basis. *In vitro* methods can be increased in throughput [107], but *in vivo* validation remains laborious.

>> Identification of tumor endothelial targets

The identification of putative targets for therapeutic intervention in tumor angiogenesis or for vascular targeting holds promise for the future design of agents that combine the specificity of targeted therapy with the effectivity of attacking the tumor vasculature. The first proof that targeted anti-vascular therapy can eradicate solid tumors was provided by Burrows and Thorpe in 1993 [108]. They used a neuroblastoma cell line expressing interferon- γ that induced major histocompatibility class (MHC) class II expression on the tumor vasculature in a xenograft model. MHC class II on the tumor vasculature was targeted with an antibody coupled to ricin. The ricin caused cell death of the tumor endothelium and subsequent eradication of the tumor [108]. Unfortunately, the development of therapy targeted at the tumor vasculature in humans has been hampered by a limited availability of suitable targets so far identified. Different studies have attempted to identify tumor endothelial targets

Figure 1.3: Targeting of tumor endothelium

A) Different binding molecules can be designed that can be administered intravenously. **B)** The binding of these targeting agents to the tumor vasculature depends on the presence of the appropriate target molecules on the tumor endothelium. The agents can act directly, or can be conjugated to bioactive molecules for local destruction of the tumor endothelium (**C**), or conjugated to compounds that enable imaging of the tumor vasculature (**D**).

for anticancer therapy by using *in vitro* models that mimic the conditions in tumor and normal tissues respectively. However, as the microenvironment has a major influence on gene expression in EC [80-82], targets identified by *in vitro* models may not be readily applicable *in vivo*. To date, some tumor endothelial markers have been identified, but (pre-)clinical data is still limited.

Tumor endothelial markers

Using bioinformatics approaches, roundabout-4 (Robo4) and endothelial-specific protein disulfide isomerase (EndoPDI) were identified as endothelial-specific genes [109,110]. Subsequent *in situ* hybridization showed predominant expression of Robo4 on the tumor vasculature [31]. Fibronectin containing the ED-B domain is undetectable in healthy tissues, but is abundantly present in the tumors and localizes to the vasculature [111]. The human antibody L19 was shown to localize to the tumor vasculature in animal models and in humans [112,113]. SAGE analysis of tumor EC identified a number of tumor endothelial markers (TEMs) [87]. One of these was later proven to be identical to endosialin, which is predominantly expressed by pericytes associated with tumor vessels [114]. Another TEM was shown to be the anthrax-toxin

receptor. Binding of the toxin to the receptor elicits cell death, which may explain the antitumor activity of the toxin [115].

A few *in vivo* approaches have so far been exploited to identify tumor endothelial targets. Terminal perfusion of tumor-bearing rats with silica particles was performed to coat the luminal membrane of the endothelium for subsequent isolation using subcellular fractionation. Differentially expressed proteins were then identified by a combination of two-dimensional gel electrophoresis and mass-spectrometry. Several proteins were shown to be upregulated on the tumor endothelium, including annexin A1, aminopeptidase N and NRP-1. Antibody-mediated targeting of annexin A1 was used for scintigraphic detection of tumors in rats, but also effectively eradicated the tumors [116]. A similar perfusion approach was performed using a biotin ester to label accessible endothelial molecules [117]. Biotinylated proteins were isolated using streptavidin and directly processed for identification by mass-spectrometry. Several tissue- and tumor-specific endothelial proteins were identified, including an acetyl-CoA carboxylase 265 fragment that was present on the endothelium of both F9 teratocarcinoma and RENCA renal carcinoma in mice [117]. Though these data are certainly promising, the quest for more and better targets for angiogenesis inhibition and vascular targeting needs to continue to have broader applicability and clinical effectivity.

>> Outline of this thesis

The aim of the work described in this thesis is the identification and validation of novel candidate target genes for therapeutic inhibition in tumor angiogenesis. For the design of novel and better anti-angiogenic and vascular targeting agents, it is crucial to have thorough knowledge of the differences in gene and protein expression in tumor EC versus normal EC. Various large-scale gene expression profiling surveys have been conducted in EC. Diverse *in vitro* models have been used to study the effects of angiogenic growth factors such as bFGF and VEGF on gene expression in EC [118-122]. Furthermore, gene expression changes in tube-forming EC *in vitro* have been studied [123-127]. In chapter 2, different studies describing gene expression changes in both growth factor stimulated EC *in vitro* and in tube-forming EC *in vitro* are compared. Furthermore, data obtained from SAGE analysis of isolated EC from different tumors are compared [87,128,129].

Since angiogenesis is not limited to pathological conditions such as cancer, merely comparing gene expression profiles in tumor EC with that of normal EC will also result in the identification of molecules that are involved in physiological angiogenesis. Therapeutic targeting of such molecules could lead to unwanted side effects associated with impaired physiological angiogenesis. Based on the hypothesis that differences exist between angiogenic EC in tumors as compared to tissues with ongoing physiological angiogenesis, we performed a gene expression analysis of not

only tumor and normal, resting EC, but also of placenta EC representative of physiological angiogenesis (chapter 3). Differential expression of a putative target between tumor angiogenesis and physiological angiogenesis creates a therapeutic window in which tumor angiogenesis might be affected without inducing side effects associated with physiological angiogenesis. Candidate genes were identified that are overexpressed in tumor EC compared to both normal, resting EC and physiologically activated placenta EC. The contribution of several cell-surface or secreted candidate genes to the process of angiogenesis was analyzed by different *in vitro* and *in vivo* assays. Vimentin, a well-known mesenchymal marker and type III intermediate filament protein was targeted using an antibody and inhibited tumor growth and angiogenesis. Further analysis showed that inhibition of vimentin expression in EC impairs migration and sprouting (chapter 4). Inhibition of high-mobility group box 1 (HMGB1), previously described as an inflammatory mediator, also resulted in impaired migration and sprouting of EC. This was accompanied by a decrease in MMP expression (chapter 5).

As the validation of putative therapeutic targets is a labor-intensive and time-consuming effort, high-throughput methods are being developed that facilitate the process. First, validation assays can be miniaturized, allowing the parallel analysis of multiple putative targets. Second, functional screenings can be applied that directly isolate or identify a gene or protein that functionally contributes to a particular phenotype (chapter 6). These developments will allow for more straightforward target identification and validation of disease targets, thereby reducing time from the laboratory to the clinic.

>> References

- [1] Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* 2000;52 (2):237-68.
- [2] Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86 (3):353-64.
- [3] Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407 (6801):249-57.
- [4] Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol* 2000;156 (4):1363-80.
- [5] Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004;25 (4):581-611.
- [6] Klagsbrun M, Eichmann A. A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. *Cytokine Growth Factor Rev* 2005;16 (4-5):535-48.
- [7] Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev* 2005;16 (2):159-78.
- [8] Tait CR, Jones PF. Angiopoietins in tumours: the angiogenic switch. *J Pathol* 2004;204 (1):1-10.
- [9] Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219 (4587):983-5.
- [10] Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol Sci* 2001;22 (4):201-7.
- [11] Rak J, Filmus J, Finkenzeller G, Grugel S, Marme D, Kerbel RS. Oncogenes as inducers of tumor angiogenesis. *Cancer Metastasis Rev* 1995;14 (4):263-77.
- [12] Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol* 2001;21 (12):3995-4004.
- [13] Jones A, Fujiyama C, Blanche C, Moore JW, Fuggle S, Cranston D, Bicknell R, Harris AL. Relation of vascular endothelial growth factor production to expression and regulation of hypoxia-inducible factor-1 alpha and hypoxia-inducible factor-2 alpha in human bladder tumors and cell lines. *Clin Cancer Res* 2001;7 (5):1263-72.
- [14] Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature* 2005;438 (7070):967-74.
- [15] Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 1994;269 (43):26988-95.
- [16] Davis S, Yancopoulos GD. The angiopoietins: Yin and Yang in angiogenesis. *Curr Top Microbiol Immunol* 1999;237:173-85.
- [17] Oshima Y, Oshima S, Nambu H, Kachi S, Takahashi K, Umeda N, Shen J, Dong A, Apte RS, Duh E, et al. Different effects of angiopoietin-2 in different vascular beds: new vessels are most sensitive. *Faseb J* 2005;19 (8):963-5.
- [18] Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 1997;277 (5322):55-60.
- [19] Suchting S, Bicknell R, Eichmann A. Neuronal clues to vascular guidance. *Exp Cell Res* 2005.
- [20] He Z, Tessier-Lavigne M. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell* 1997;90 (4):739-51.
- [21] Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. Neuropilin is a semaphorin III receptor. *Cell* 1997;90 (4):753-62.
- [22] Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;92 (6):735-45.
- [23] Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M. Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. *J Cell Biol* 1999;146 (1):233-42.

- [24] Serini G, Valdembrì D, Zanivan S, Morterra G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M, et al. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* 2003;424 (6947):391-7.
- [25] Herault M, Schaffner F, Augustin HG. Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. *Exp Cell Res* 2005.
- [26] Orioli D, Klein R. The Eph receptor family: axonal guidance by contact repulsion. *Trends Genet* 1997;13 (9):354-9.
- [27] Chen J, Hicks D, Brantley-Sieders D, Cheng N, McCollum GW, Qi-Werdich X, Penn J. Inhibition of retinal neovascularization by soluble EphA2 receptor. *Exp Eye Res* 2005.
- [28] Bicknell R, Harris AL. Novel angiogenic signaling pathways and vascular targets. *Annu Rev Pharmacol Toxicol* 2004;44:219-38.
- [29] Kim I, Ryu YS, Kwak HJ, Ahn SY, Oh JL, Yancopoulos GD, Gale NW, Koh GY. EphB ligand, ephrinB2, suppresses the VEGF- and angiopoietin 1-induced Ras/mitogen-activated protein kinase pathway in venous endothelial cells. *Faseb J* 2002;16 (9):1126-8.
- [30] Vihanto MM, Plock J, Ermi D, Frey BM, Frey FJ, Huynh-Do U. Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin. *Faseb J* 2005;19 (12):1689-91.
- [31] Huminiecki L, Gorn M, Suchting S, Poulsom R, Bicknell R. Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics* 2002;79 (4):547-52.
- [32] Wang B, Xiao Y, Ding BB, Zhang N, Yuan X, Gui L, Qian KX, Duan S, Chen Z, Rao Y, et al. Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. *Cancer Cell* 2003;4 (1):19-29.
- [33] Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, Chien CB, Wu JY, Urness LD, Li DY. Robo4 is a vascular-specific receptor that inhibits endothelial migration. *Dev Biol* 2003;261 (1):251-67.
- [34] Suchting S, Heal P, Tahtis K, Stewart LM, Bicknell R. Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. *Faseb J* 2005;19 (1):121-3.
- [35] Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, Stein E. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell* 1999;97 (7):927-41.
- [36] Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Breant C, Claes F, De Smet F, Thomas JL, et al. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* 2004;432 (7014):179-86.
- [37] Park KW, Crouse D, Lee M, Karnik SK, Sorensen LK, Murphy KJ, Kuo CJ, Li DY. The axonal attractant Netrin-1 is an angiogenic factor. *Proc Natl Acad Sci U S A* 2004;101 (46):16210-5.
- [38] Brouty-Boye D, Zetter BR. Inhibition of cell motility by interferon. *Science* 1980;208 (4443):516-8.
- [39] Marler JJ, Rubin JB, Trede NS, Connors S, Grier H, Upton J, Mulliken JB, Folkman J. Successful antiangiogenic therapy of giant cell angioblastoma with interferon alfa 2b: report of 2 cases. *Pediatrics* 2002;109 (2):E37.
- [40] Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, Fidler IJ. Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci U S A* 1995;92 (10):4562-6.
- [41] Tarabozetti G, Roberts D, Liotta LA, Giavazzi R. Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. *J Cell Biol* 1990;111 (2):765-72.
- [42] Sheibani N, Newman PJ, Frazier WA. Thrombospondin-1, a natural inhibitor of angiogenesis, regulates platelet-endothelial cell adhesion molecule-1 expression and endothelial cell morphogenesis. *Mol Biol Cell* 1997;8 (7):1329-41.
- [43] Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med* 2000;6 (1):41-8.
- [44] Armstrong LC, Bornstein P. Thrombospondins 1 and 2 function as inhibitors of angiogenesis. *Matrix Biol* 2003;22 (1):63-71.
- [45] Bikfalvi A. Platelet factor 4: an inhibitor of angiogenesis. *Semin Thromb Hemost* 2004;30 (3):379-85.

- [46] van der Schaft DW, Dings RP, de Lussanet QG, van Eijk LI, Nap AW, Beets-Tan RG, Bouma-Ter Steege JC, Wagstaff J, Mayo KH, Griffioen AW. The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *Faseb J* 2002;16 (14):1991-3.
- [47] Bikfalvi A, Gimenez-Gallego G. The control of angiogenesis and tumor invasion by platelet factor-4 and platelet factor-4-derived molecules. *Semin Thromb Hemost* 2004;30 (1):137-44.
- [48] van der Schaft DW, Toebes EA, Haseman JR, Mayo KH, Griffioen AW. Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood* 2000;96 (1):176-81.
- [49] Sudhakar A, Sugimoto H, Yang C, Lively J, Zeisberg M, Kalluri R. Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. *Proc Natl Acad Sci U S A* 2003;100 (8):4766-71.
- [50] Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;285 (21):1182-6.
- [51] Ferrara N. VEGF as a therapeutic target in cancer. *Oncology* 2005;69 Suppl 3:11-6.
- [52] Scappaticci FA, Fehrenbacher L, Cartwright T, Hainsworth JD, Heim W, Berlin J, Kabbinavar F, Novotny W, Sarkar S, Hurwitz H. Surgical wound healing complications in metastatic colorectal cancer patients treated with bevacizumab. *J Surg Oncol* 2005;91 (3):173-80.
- [53] Kerbel RS. Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. *Bioessays* 1991;13 (1):31-6.
- [54] Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005;438 (7070):932-6.
- [55] Miller KD, Sweeney CJ, Sledge GW, Jr. Can tumor angiogenesis be inhibited without resistance? *Exs* 2005 (94):95-112.
- [56] Cahill DP, Kinzler KW, Vogelstein B, Lengauer C. Genetic instability and darwinian selection in tumours. *Trends Cell Biol* 1999;9 (12):M57-60.
- [57] Wouters BG, van den Beucken T, Magagnin MG, Lambin P, Koumenis C. Targeting hypoxia tolerance in cancer. *Drug Resist Updat* 2004;7 (1):25-40.
- [58] Auguste P, Lemiere S, Larrieu-Lahargue F, Bikfalvi A. Molecular mechanisms of tumor vascularization. *Crit Rev Oncol Hematol* 2005;54 (1):53-61.
- [59] Streubel B, Chott A, Huber D, Exner M, Jager U, Wagner O, Schwarzwinger I. Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. *N Engl J Med* 2004;351 (3):250-9.
- [60] Hida K, Hida Y, Amin DN, Flint AF, Panigrahy D, Morton CC, Klagsbrun M. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res* 2004;64 (22):8249-55.
- [61] Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A* 2002;99 (17):11393-8.
- [62] Thomas AL, Morgan B, Horsfield MA, Higginson A, Kay A, Lee L, Masson E, Puccio-Pick M, Laurent D, Steward WP. Phase I study of the safety, tolerability, pharmacokinetics, and pharmacodynamics of PTK787/ZK 222584 administered twice daily in patients with advanced cancer. *J Clin Oncol* 2005;23 (18):4162-71.
- [63] Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, et al. Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J Clin Oncol* 2006;24 (1):16-24.
- [64] Gingras D, Boivin D, Deckers C, Gendron S, Barthomeuf C, Beliveau R. Neovastat--a novel antiangiogenic drug for cancer therapy. *Anticancer Drugs* 2003;14 (2):91-6.
- [65] Maquoi E, Sounni NE, Devy L, Olivier F, Franken F, Krell HW, Grams F, Foidart JM, Noel A. Anti-invasive, antitumoral, and antiangiogenic efficacy of a pyrimidine-2,4,6-trione derivative, an orally active and selective matrix metalloproteinases inhibitor. *Clin Cancer Res* 2004;10 (12 Pt 1):4038-47.
- [66] Jones PH, Christodoulos K, Dobbs N, Thavasu P, Balkwill F, Blann AD, Caine GJ, Kumar S, Kakkar AJ, Gompertz N, et al. Combination antiangiogenesis therapy with marimastat, captopril and fragmin in patients with advanced cancer. *Br J Cancer* 2004;91 (1):30-6.

- [67] Griffioen AW, van der Schaft DW, Barendsz-Janson AF, Cox A, Struijker Boudier HA, Hillen HF, Mayo KH. Anginex, a designed peptide that inhibits angiogenesis. *Biochem J* 2001;354 (Pt 2):233-42.
- [68] Dings RP, van der Schaft DW, Hargittai B, Haseman J, Griffioen AW, Mayo KH. Anti-tumor activity of the novel angiogenesis inhibitor anginex. *Cancer Lett* 2003;194 (1):55-66.
- [69] Dings RP, Williams BW, Song CW, Griffioen AW, Mayo KH, Griffin RJ. Anginex synergizes with radiation therapy to inhibit tumor growth by radiosensitizing endothelial cells. *Int J Cancer* 2005;115 (2):312-9.
- [70] Dings RP, Yokoyama Y, Ramakrishnan S, Griffioen AW, Mayo KH. The designed angiostatic peptide anginex synergistically improves chemotherapy and antiangiogenesis therapy with angiostatin. *Cancer Res* 2003;63 (2):382-5.
- [71] Mayo KH, Dings RP, Flader C, Nesmelova I, Hargittai B, van der Schaft DW, van Eijk LI, Walek D, Haseman J, Hoye TR, et al. Design of a partial peptide mimetic of anginex with antiangiogenic and anticancer activity. *J Biol Chem* 2003;278 (46):45746-52.
- [72] Brandwijk RJ, Nesmelova I, Dings RP, Mayo KH, Thijssen VL, Griffioen AW. Cloning an artificial gene encoding angiostatic anginex: From designed peptide to functional recombinant protein. *Biochem Biophys Res Commun* 2005;333 (4):1261-8.
- [73] Ahmed B, Van Eijk LI, Bouma-Ter Steege JC, Van Der Schaft DW, Van Esch AM, Joosten-Achjanie SR, Lambin P, Landuyt W, Griffioen AW. Vascular targeting effect of combretastatin A-4 phosphate dominates the inherent angiogenesis inhibitory activity. *Int J Cancer* 2003;105 (1):20-5.
- [74] El-Emir E, Boxer GM, Petrie IA, Boden RW, Dearling JL, Begent RH, Pedley RB. Tumour parameters affected by combretastatin A-4 phosphate therapy in a human colorectal xenograft model in nude mice. *Eur J Cancer* 2005;41 (5):799-806.
- [75] Hayot C, Farnelle S, De Decker R, Decaestecker C, Darro F, Kiss R, Van Damme M. In vitro pharmacological characterizations of the anti-angiogenic and anti-tumor cell migration properties mediated by microtubule-affecting drugs, with special emphasis on the organization of the actin cytoskeleton. *Int J Oncol* 2002;21 (2):417-25.
- [76] Folkman J. Angiogenesis. *Annu Rev Med* 2006;57:1-18.
- [77] Satchi-Fainaro R, Mamluk R, Wang L, Short SM, Nagy JA, Feng D, Dvorak AM, Dvorak HF, Puder M, Mukhopadhyay D, et al. Inhibition of vessel permeability by TNP-470 and its polymer conjugate, caplostatin. *Cancer Cell* 2005;7 (3):251-61.
- [78] Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;307 (5706):58-62.
- [79] Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* 2004;4 (6):423-36.
- [80] Zetter BR. Endothelial heterogeneity: Influence of vessel size, organ localization, and species specificity on the properties of cultured endothelial cells: CRC Press, Florida, 1988.
- [81] McCarthy SA, Kuzu I, Gatter KC, Bicknell R. Heterogeneity of the endothelial cell and its role in organ preference of tumour metastasis. *Trends Pharmacol Sci* 1991;12 (12):462-7.
- [82] Aird WC. Endothelial cell heterogeneity. *Crit Care Med* 2003;31 (4 Suppl):S221-30.
- [83] Aird WC. Endothelial cell heterogeneity: a case for nature and nurture. *Blood* 2004;103 (11):3994-5.
- [84] Hillen F, Melotte V, van Beijnum JR, Griffioen AW. Endothelial cell biology. In: Staton CA, Lewis C, Bicknell R, editors. *Angiogenesis assays: a critical appraisal of current techniques*. London: John Wiley and Sons, *In press*.
- [85] Tatsuta T, Naito M, Mikami K, Tsuruo T. Enhanced expression by the brain matrix of P-glycoprotein in brain capillary endothelial cells. *Cell Growth Differ* 1994;5 (10):1145-52.
- [86] Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM. Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. *Am J Physiol Heart Circ Physiol* 2003;285 (5):H1917-38.
- [87] St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, et al. Genes expressed in human tumor endothelium. *Science* 2000;289 (5482):1197-202.
- [88] Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. *Nature* 1996;380 (6572):364-6.

- [89] Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R, Ruoslahti E. Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. *J Clin Invest* 1998;102 (2):430-7.
- [90] Pasqualini R, Arap W, McDonald DM. Probing the structural and molecular diversity of tumor vasculature. *Trends Mol Med* 2002;8 (12):563-71.
- [91] Ruoslahti E, Rajotte D. An address system in the vasculature of normal tissues and tumors. *Annu Rev Immunol* 2000;18:813-27.
- [92] Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol* 1997;15 (6):542-6.
- [93] Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res* 2000;60 (3):722-7.
- [94] Joyce JA, Laakkonen P, Bernasconi M, Bergers G, Ruoslahti E, Hanahan D. Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis. *Cancer Cell* 2003;4 (5):393-403.
- [95] Jung YD, Ahmad SA, Liu W, Reinmuth N, Parikh A, Stoeltzing O, Fan F, Ellis LM. The role of the microenvironment and intercellular cross-talk in tumor angiogenesis. *Semin Cancer Biol* 2002;12 (2):105-12.
- [96] Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A* 2003;100 (19):10623-8.
- [97] Ho M, Yang E, Matcuk G, Deng D, Sampas N, Tsalenko A, Tabibiazar R, Zhang Y, Chen M, Talbi S, et al. Identification of endothelial cell genes by combined database mining and microarray analysis. *Physiol Genomics* 2003;13 (3):249-62.
- [98] Lawson ND, Weinstein BM. Arteries and veins: making a difference with zebrafish. *Nat Rev Genet* 2002;3 (9):674-82.
- [99] Meno C, Shimono A, Saijoh Y, Yashiro K, Mochida K, Ohishi S, Noji S, Kondoh H, Hamada H. lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. *Cell* 1998;94 (3):287-97.
- [100] Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995;270 (5235):484-7.
- [101] Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci U S A* 1996;93 (12):6025-30.
- [102] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995;270 (5235):467-70.
- [103] Goldenberg MM. Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. *Clin Ther* 1999;21 (2):309-18.
- [104] Neri D, Bicknell R. Tumour vascular targeting. *Nat Rev Cancer* 2005;5 (6):436-46.
- [105] Work LM, Buning H, Hunt E, Nicklin SA, Denby L, Britton N, Leike K, Odenthal M, Drebber U, Hallek M, et al. Vascular bed-targeted in vivo gene delivery using tropism-modified adeno-associated viruses. *Mol Ther* 2005.
- [106] Nicklin SA, Reynolds PN, Brosnan MJ, White SJ, Curiel DT, Dominiczak AF, Baker AH. Analysis of cell-specific promoters for viral gene therapy targeted at the vascular endothelium. *Hypertension* 2001;38 (1):65-70.
- [107] van Beijnum JR, Eijgelaar WJ, Griffioen AW. Towards high-throughput functional target discovery in angiogenesis research. *Trends Mol Med* 2005.
- [108] Burrows FJ, Thorpe PE. Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature. *Proc Natl Acad Sci U S A* 1993;90 (19):8996-9000.
- [109] Sullivan DC, Huminiecki L, Moore JW, Boyle JJ, Poulsom R, Creamer D, Barker J, Bicknell R. EndoPDI, a novel protein-disulfide isomerase-like protein that is preferentially expressed in endothelial cells acts as a stress survival factor. *J Biol Chem* 2003;278 (47):47079-88.
- [110] Huminiecki L, Bicknell R. In silico cloning of novel endothelial-specific genes. *Genome Res* 2000;10 (11):1796-806.

- [111] Zardi L, Carnemolla B, Siri A, Petersen TE, Paoletta G, Sebastio G, Baralle FE. Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *Embo J* 1987;6 (8):2337-42.
- [112] Santimaria M, Moscatelli G, Viale GL, Giovannoni L, Neri G, Viti F, Leprini A, Borsi L, Castellani P, Zardi L, et al. Immunoscintigraphic detection of the ED-B domain of fibronectin, a marker of angiogenesis, in patients with cancer. *Clin Cancer Res* 2003;9 (2):571-9.
- [113] Borsi L, Balza E, Bestagno M, Castellani P, Carnemolla B, Biro A, Leprini A, Sepulveda J, Burrone O, Neri D, et al. Selective targeting of tumoral vasculature: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. *Int J Cancer* 2002;102 (1):75-85.
- [114] MacFadyen JR, Haworth O, Roberston D, Hardie D, Webster MT, Morris HR, Panico M, Sutton-Smith M, Dell A, van der Geer P, et al. Endosialin (TEM1, CD248) is a marker of stromal fibroblasts and is not selectively expressed on tumour endothelium. *FEBS Lett* 2005;579 (12):2569-75.
- [115] Duesbery NS, Resau J, Webb CP, Koochekpour S, Koo HM, Leppla SH, Vande Woude GF. Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. *Proc Natl Acad Sci U S A* 2001;98 (7):4089-94.
- [116] Oh P, Li Y, Yu J, Durr E, Krasinska KM, Carver LA, Testa JE, Schnitzer JE. Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature* 2004;429 (6992):629-35.
- [117] Rybak JN, Ettore A, Kaissling B, Giavazzi R, Neri D, Elia G. In vivo protein biotinylation for identification of organ-specific antigens accessible from the vasculature. *Nat Methods* 2005;2 (4):291-8.
- [118] Wang JL, Liu YH, Lee MC, Nguyen TM, Lee C, Kim A, Nguyen M. Identification of tumor angiogenesis-related genes by subtractive hybridization. *Microvasc Res* 2000;59 (3):394-7.
- [119] Zhang H-T, Gorn M, Smith K, Graham AP, Lau KKW, Bicknell R. Transcriptional profiling of human microvascular endothelial cells in the proliferative and quiescent state using cDNA arrays. *Angiogenesis* 1999;3:211-9.
- [120] Abe M, Sato Y. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. *Angiogenesis* 2001;4 (4):289-98.
- [121] Jih YJ, Lien WH, Tsai WC, Yang GW, Li C, Wu LW. Distinct regulation of genes by bFGF and VEGF-A in endothelial cells. *Angiogenesis* 2001;4 (4):313-21.
- [122] Gerritsen ME, Tomlinson JE, Zlot C, Ziman M, Hwang S. Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells. *Br J Pharmacol* 2003;140 (4):595-610.
- [123] Aitkenhead M, Wang SJ, Nakatsu MN, Mestas J, Heard C, Hughes CC. Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: induction of ESM-1, (beta)ig-h3, and NrCAM. *Microvasc Res* 2002;63 (2):159-71.
- [124] Bell SE, Mavila A, Salazar R, Bayless KJ, Kanagala S, Maxwell SA, Davis GE. Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling. *J Cell Sci* 2001;114 (Pt 15):2755-73.
- [125] Gerritsen ME, Soriano R, Yang S, Zlot C, Ingle G, Toy K, Williams PM. Branching out: a molecular fingerprint of endothelial differentiation into tube-like structures generated by affymetrix oligonucleotide arrays. *Microcirculation* 2003;10 (1):63-81.
- [126] Kahn J, Mehraban F, Ingle G, Xin X, Bryant JE, Vehar G, Schoenfeld J, Grimaldi CJ, Peale F, Draksharapu A, et al. Gene expression profiling in an in vitro model of angiogenesis. *Am J Pathol* 2000;156 (6):1887-900.
- [127] Wary KK, Thakker GD, Humtsoe JO, Yang J. Analysis of VEGF-responsive Genes Involved in the activation of endothelial cells. *Mol Cancer* 2003;2 (1):25.
- [128] Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, et al. Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Res* 2004;64 (21):7857-66.
- [129] Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Walter-Yohrling J, et al. Vascular gene expression in nonneoplastic and malignant brain. *Am J Pathol* 2004;165 (2):601-8.

A vertical strip on the left side of the page shows a microscopic image of tissue, likely showing cellular structures and possibly some staining.

Chapter 2

In silico analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles

Judy R. van Beijnum & Arjan W. Griffioen

Biochim Biophys Acta. 2005 Jul 25;1755(2):121-34

>> Abstract

In vitro models have been extensively used to map gene expression in EC but few studies have used cells from *in vivo* sources directly. Here, we compare different gene expression surveys on both cultured and fresh tissue derived EC, and it emerges that gene expression profiles can be paralleled with the angiogenic stage of the cells. EC stimulated with different growth factors in monolayer cultures exhibit gene expression profiles indicative of an active proliferative state, whereas gene expression in tube forming cells *in vitro* involves genes implicated in cell adhesion processes. Genes overexpressed in tumor EC are biased towards ECM remodeling, a late event in angiogenesis. The elucidation of gene expression profiles under these different conditions will contribute to a better understanding of the molecular mechanisms during angiogenesis in both pathological and physiological circumstances and will have implications for the development of angiogenesis interfering treatment strategies.

>> Introduction

Endothelial cell biology and angiogenesis

Endothelial cells (EC) are among the most quiescent cells of the body since their turnover rate can be up to hundreds of days. However, during the growth of new capillaries from pre-existing blood vessels (angiogenesis), activated EC can proliferate as rapidly as bone marrow cells [1], highlighting their capacity to readily respond to microenvironmental changes. Angiogenesis is in essence a physiological phenomenon and is a hallmark of processes such as wound healing, embryonic development, tissue remodeling and the female reproductive cycle, in which tissue growth is essential. However, angiogenesis also occurs during pathological conditions such as diabetic retinopathy, rheumatoid arthritis and cancer. The role of angiogenesis in the progression of cancer has gained particular interest once it became clear that the growth, invasion and metastasis of tumors are dependent on the formation of blood vessels [2]. In addition to providing a metastatic route, the vasculature provides the indispensable metabolic support for the tumor. Without adequate blood supply, the size of tumors is limited due to deprivation of oxygen and nutrients in the tumor cell mass. In response to these limitations tumors start to produce angiogenic factors that activate EC to initiate sprouting from nearby pre-existing vessels [2]. The genetic change of tumor cells, leading to the formation of tumor vasculature, is referred to as the angiogenic switch [1,3]. In the tumor microenvironment, both anti-angiogenic and pro-angiogenic factors are expressed, and angiogenesis is induced once the balance tips over in favor of the latter. The expression of pro-angiogenic factors can be induced by hypoxia, and also by oncogene activation or tumor-suppressor gene mutations; the relative contribution of these events is likely to be determined by the specific type and location of the tumor [3,4]. A variety of endothelial growth factors have been described, among which vascular endothelial growth factors (VEGFs) [5], fibroblast growth factors (FGFs) [6], hepatocyte growth factor (HGF) [7] and the angiopoietins [8].

Once angiogenesis is induced, a sequence of events is set in motion. Angiogenic sprouting comprises (i) local degradation of the basement membrane and ECM surrounding the capillaries, (ii) migration of EC in the direction of the tumor mass, (iii) proliferation of EC, (iv) differentiation and (v) subsequently the formation of tube-like structures, in which attraction of pericytes and smooth muscle cells and deposition of new ECM occurs. During the final stages, the ECM is remodeled, and sprouts will fuse with each other to create a circulation [4,9].

Tumor progression is highly dependent on the development of a tumor vasculature by angiogenic sprouting. The precise requirements of tumors, however, may vary among different tumor types and during tumor progression. Hence, tumor phenotype heterogeneity dictates tumor vasculature heterogeneity [4]. It has been recognized that tumor vessels are irregularly shaped, tortuous and often leaky. Under normal conditions, vessel density is tightly controlled and newly formed vessels rapidly mature, but the aberrant balance of angiogenic factors produced in malignancies result in a

chaotic tumor vasculature [4]. Tumor vessels fail to become quiescent; therefore, tumors have been described as 'wounds that never heal' [10].

Angiogenesis inhibition

Given the observation that most tumors are angiogenesis dependent, both for metabolic support and for metastatic dissemination, inhibition of angiogenesis is thought to be a promising strategy for the treatment of solid tumors [11], and likely hematological malignancies as well [12,13]. The concept of angiogenesis inhibition for cancer treatment has gained much attention over the past years and a number of different strategies have been employed, based on knowledge of EC biology and behavior during angiogenesis. To this purpose, synthetic agents have been developed that act on EC, a number of endogenous proteins and peptides with anti-angiogenic actions have been discovered, but also the use of antibodies to interfere with the angiogenesis process has been explored.

In addition to interfering with the unwanted *de novo* formation of blood vessels, targeting existing tumor blood vessels for destruction resulting in tumor regression (vascular targeting), may also hold promise. EC express genes that are not or to a lower extent expressed by other cell types [14,15] and this may offer the possibility of using gene products that are specific for a certain subset of EC for targeting purposes. It has been shown that EC exhibit differences in gene and protein expression, relating to their microenvironmental origin and activation status [16,17].

Tumor blood vessels express specific markers that are not present on normal blood vessels and these markers can be present on the EC themselves, on pericytes and on the ECM [18]. By *in vivo* phage display selections, it was demonstrated that EC express 'address molecules' that differ according to their location in the body and their specific signature [19,20]. A phage-displayed peptide that specifically bound to tumor vasculature contained an RGD sequence (arginine-glycine-aspartic acid), known to bind integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ [20]. Another tumor vasculature specific peptide, containing the NGR sequence (asparagine-glycine-arginine), was shown to bind aminopeptidase N, also known as CD13 [21]. Although this protein is also expressed in several epithelial tissues and macrophages, the cyclic NGR peptide does not bind to these cells, indicating a specific form of aminopeptidase N is present on angiogenically activated EC [22]. Other markers that are upregulated on tumor EC include prostate-specific membrane antigen (PSMA) [23], CD44 [16], endoglin [24] and the oncofetal ED-B domain of fibronectin [25], whereas CD34 and intercellular adhesion molecule 1 (ICAM1) are suppressed [17,26].

Probably the most crucial element in both anti-angiogenic and vascular targeting approaches is the identification of specific target molecules. Since angiogenesis is not limited to pathological conditions, careful evaluation of the putative targets is warranted to prevent side effects associated with impaired physiological angiogenesis. Gene expression studies can be very helpful tools in elucidating the transcriptional differences between EC of different sources and to identify tissue or tumor endothelial

specific markers. Furthermore, gene expression analysis in this context will lead to a better understanding of EC biology during (tumor) angiogenesis. Different models have been developed to study angiogenesis, but the temporal and spatial complex actions of all factors exerting effect on EC *in vivo* may not be accurately reflected *in vitro*.

Here we will overview different studies that used large-scale gene expression profiling of EC in different stages of angiogenic activation *in vitro* and *in vivo*. Gene expression changes associated with the different stages of angiogenesis that emerge from these studies are discussed and related to their potential use in the development of angiogenesis inhibitors or vascular targeting agents.

>> Gene expression profiling in angiogenesis research

Expression profiling techniques

Large-scale gene expression profiling techniques are widely used to detect changes in transcript expression levels and provide the tools to study molecular events in biological processes. For the comparison of two or more cell types, a number of techniques have been developed and employed. Serial analysis of gene expression (SAGE) provides a quantitative analysis of large numbers of transcripts, based on sequence analysis of short specific, concatenated tags. Multiple direct comparisons are possible by assessing the frequencies at which transcripts are present in the libraries [27]. Another technique, suppression subtractive hybridization (SSH) combines an effective cDNA subtraction between two samples with the requirement of only limited amounts of starting material and is, like SAGE, independent of previously cloned genes and existing sequence information [28]. SSH can be used in combination with cDNA arrays to screen for differentially expressed genes [29]. cDNA can be spotted on nylon filters for hybridization with radioactively labeled probes, usually referred to as macroarrays due to their relatively low spot density and their large size. Sequential hybridization of different samples to identical arrays allows for comparison of gene expression in the different probes.

Differential display provides a systematic and unbiased procedure to detect differences in mRNA species [30] and GeneCalling™ is a modification of this technique. Double-stranded cDNA is digested with pairs of restriction enzymes, amplified with specific linkers and resolved on polyacrylamide gel. Based on fragment length and the combination of restriction enzymes used, transcripts are identified using sophisticated database searches [31].

The advent of microarray technology has boosted gene expression profiling research [32]. High-density robotic printing of thousands of cDNAs on glass slides and subsequent two-color fluorescent hybridization and detection allows for a quantitative comparison of genes in complex probe mixtures [32]. Oligonucleotide arrays are a variation on the theme of microarrays. Complex probes of one experimental sample at the time are hybridized and signal intensities are measured by scanning [33]. A

limitation of the latter techniques is that they rely fully on known sequences, but ongoing efforts to characterize and annotate the human genome are likely to enable screening the majority of expressed transcripts in the near future. Furthermore, data management and analysis are key to success when using these large-scale gene expression profiling techniques [34]. Clustering analysis can help identify coordinately regulated genes that are likely to be functionally related in the applied experimental conditions [35]. All of these techniques have their own advantages and disadvantages related to scale, effort, cost and availability of sample material. Furthermore, techniques can be combined to enhance sensitivity or throughput.

In silico profiling of EC

The availability of public gene expression data sets has opened additional doors for gene expression profiling studies. By using different bioinformatics tools to query existing databases, it is possible to identify genes that are expressed in a particular cell type [14,15] or to overlap data sets to identify genes that are co-regulated under different circumstances [36]. *In silico* comparison of existing data sets may also be helpful in the elucidation of gene expression on tumor endothelium. Available databases of gene expression profiles in EC can be overlapped with expression profiles of tumors to identify commonly regulated genes [36,37]. A minor drawback of this approach might be that only limited gene expression data of EC from *in vivo* sources is available to date. Also, formats need to be interchangeable for easy data transfer and data analysis.

In this review, we analyze published data sets of genes induced in growth factor stimulated and tube forming EC *in vitro*, as well as from EC isolated from tumor tissues. Gene expression profiles of EC treated with bFGF, VEGF, HGF, epidermal growth factor (EGF) and different combinations thereof are reviewed and common pathways identified that play key roles in the initial activation of EC. Differentiation of EC in tube-like structures can be accomplished by providing three-dimensional (3D) support, leading to the induction of genes associated with this process. Finally, genes identified to be overexpressed on tumor EC of different types of tumors are discussed. This method allows filtering out interesting candidate genes amenable for interference in angiogenic events in different stages of progression, and can provide tools to define therapeutic agents to perform these tasks.

Gene expression associated with cytokine and growth factor stimulation of EC in vitro

Gene expression profiling has been applied to EC treated with different pro-angiogenic cytokines in various models to elucidate genes involved in angiogenesis. SSH in combination with differential screening was used to study transcriptional changes in human umbilical vein EC (HUVEC) cultured for 4 hours in the presence of tumor conditioned medium (CM) from the melanoma cell line C8161 and the breast cancer cell line MDA-MB231 that contain high levels of angiogenic stimulators [38]. Twenty genes were confirmed to be upregulated, some of which had previously been

associated with angiogenesis, e.g. E-selectin [39] and fibronectin [40]. Cytokines associated with angiogenesis that were identified in this study included monocyte-chemoattractant protein-1 (MCP-1) [41] and interleukin-8 [42]. One of the most interesting candidate genes for follow-up was endomucin. Endomucin is a highly glycosylated transmembrane protein expressed predominantly on vascular EC [43] and high endothelial venules, where it can function as a ligand for L-selectin [44]. Its expression seems to correlate with EC proliferation as it is increased upon stimulation with bFGF, tumor necrosis factor α (TNF α) and under the influence of tumor CM [43]. Due to its elevated expression in angiogenically activated EC and its cell surface localization, endomucin might be a putative tumor endothelium targeting candidate. However, it remains to be determined whether a sufficiently large therapeutic window can be created for therapies not to interfere with endomucin expressed on non-tumor EC. Furthermore, although the authors state that this *in vitro* model provides an accurate stimulation of the tumor environment, one can question whether 4 hours of stimulation with tumor-derived factors is sufficient for EC to adopt a tumor endothelial phenotype since tumor initiation and progression is a relatively slow process.

By using HUVEC cultured for three days in the presence of bFGF, VEGF and supernatant of two colorectal tumor cell lines, Caco-2 and LS174T, we created SSH repertoires that were used for macroarray screening. Twenty different genes were identified that showed upregulation in both tumor derived EC when compared to normal EC (isolated from fresh tissues) and in HUVEC cultured in the above mentioned conditions compared to quiescent HUVEC [45]. Functional annotation of the identified transcripts showed that most genes were indicative of a high proliferative status of the EC, since more than one third of these genes are involved in protein turnover and metabolism such as ribosomal proteins L21 and S27, and HSP90. Furthermore, a number of genes have previously been associated with angiogenesis, such as thymosin β 4, matrix metalloproteinase (MMP) 10 and integrin β 1. Thymosin β 4 is implicated in EC migration, proliferation and differentiation by interfering with actin polymerization [46]. Exogenously applied, it acts as a chemoattractant for EC, stimulates migration, increases MMP production [47] and induces tube formation and sprouting [46,48]. The most interesting aspect of thymosin β 4 is therefore that it affects EC biology both as endogenous and exogenous peptide, creating ample opportunities for modulating EC function.

A comparison of quiescent versus proliferating EC stimulated with EGF by cDNA array revealed a large proportion of genes associated with cell cycle regulation to be upregulated in proliferating human dermal microvascular EC (HDMEC) [49]. Various cyclins were induced, among which G1/S specific cyclins D1 and D2 and the mitosis specific cyclins B1 and G. Increased expression of cyclin D1 in response to VEGF and bFGF has later been reported [50]. Furthermore, cyclin D1 expression has been shown to be downregulated in EC by the anti-angiogenic agent TNP-470 [51] and the endogenous angiogenesis inhibitor endostatin [50]. Endostatin induces cell cycle arrest in G1, associated with an inhibition of hyperphosphorylation of Rb protein and a

downregulation of cyclin D1 promoter activity [50]. However, forced overexpression of cyclin D1 in EC overrides the effect of endostatin [50]. The study by Zhang et al. [49] also confirmed the upregulation of the known angiogenesis stimulators interleukin-8, TIE-1 and TIE-2 in activated EC. Furthermore, it stressed the involvement of different integrin subunits (α V, α 5, α 6 and β 1) in EC adhesion and tumor angiogenesis [52,53]. As discussed earlier, integrins pose interesting candidate molecules for interference in angiogenesis [54,55]. Connective tissue growth factor (CTGF) was also upregulated in HDMEC in response to EGF and belongs to the CCN family of matricellular proteins. Several members of this family are positively involved in angiogenesis and their activities are mediated by integrins and by the induction of MMP expression [56,57]. CTGF (CCN2) and CYR61 (CCN1) are transcriptionally activated in EC by bFGF and VEGF and are capable of promoting EC growth, migration, adhesion and survival. Furthermore, these proteins can act in a paracrine or autocrine fashion by upregulating the endothelial growth factors VEGF and bFGF [58]. CCN proteins have been recognized to be important regulators in angiogenesis during wound healing, placenta formation, fibrosis and tumor growth [58], hence attempts to interfere in the complex actions of these proteins with the aim of inhibiting tumor angiogenesis may turn out to be difficult.

Microarray analysis was used to identify genes induced by VEGF at different time points in HUVEC [59]. Over a period of 24 hours, 139 genes showed induction by VEGF, of which 53 were induced within the first two hours of VEGF stimulation. From this analysis it was apparent that a number of genes show peak expression at early time points and reduce their expression to basal levels within 12 hours after stimulation with VEGF. Among the genes upregulated within 30 minutes were 3 transcription factors (early growth response (EGR) 1, EGR2 and EGR3) and 10 genes associated with cellular support, involved in metabolism, protein turnover and protein transport. Five known positive regulators of angiogenesis were identified: cyclooxygenase-2 (COX-2), heparin-binding epidermal growth factor-like growth factor (HBEGF), EGR1, CYR61 and angiopoietin-2. Induced by cytokines, COX-2 catalyzes the production of prostaglandins from arachidonic acid [60]. COX-2 metabolites promote Rac1 and CDC42 activation resulting in integrin α V β 3-mediated cell spreading and migration [61]. Furthermore, COX-2 inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs), have a protective role against the development of cancer [62], and this effect is partly due to diminished tumor angiogenesis via the inhibition of α V β 3-mediated Rac1 activation [52,63]. HBEGF has been associated with the induction of tube-like structures of EC in type I collagen [64] and increased tumorigenicity associated with increased angiogenic potential [65]. EGR1 is a key transcription factor in EC and mediates the VEGF induced upregulation of tissue factor, platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β), as well as the transcription of membrane type 1 MMP (MMP14), facilitating the invasion of EC into the surrounding matrix [66]. Growth factor induced EGR1 transcriptional activity is inhibited

by the NGFI-A binding protein 2 (NAB2) corepressor as well as by NSAIDs, the latter further explaining the observed protective role of these drugs [67].

A survey of genes regulated by VEGF, bFGF and the combination of both revealed only a partial overlap in expression profiles of HUVEC [68]. Interestingly, the combination of bFGF and VEGF induced a number of VEGF receptors, whereas the administration of VEGF alone failed to do so. Various cyclins and cyclin-dependent kinases were induced by the different treatments [68]. Furthermore, EGR1 was identified to be induced by the combination of bFGF and VEGF, in contrast to the finding of Abe et al. [59] who identified this transcription factor to be upregulated by treatment with VEGF alone. It is interesting to note that bFGF treatment regulated the expression of the oncogenic transcription factors *myc*, *jun* and *fos* [68]. Induction of oncogenes was also observed in bFGF transformed EC [69], suggesting a specific trait of bFGF action.

Relatively little overlap was also observed when the expression profiles of HUVEC treated with HGF, with VEGF, and with a combination of both were compared [70]. In addition, few genes were coordinately regulated after 4 hours and 24 hours of VEGF treatment. The importance of the above-mentioned key angiogenesis transcription factor EGR1 is underscored by the fact that both combination treatments (bFGF+VEGF and HGF+VEGF) induced its expression, evident already 1 hour and 4 hours following growth factor treatment, respectively [68,70].

The above described studies used cultured EC stimulated with different growth factors as models for angiogenesis. It appears, however, that only a few genes are commonly regulated during growth factor stimulation of EC (Table 2.1). Components of the mitogen-activated protein kinase (MAPK) signaling pathway (*jun*, *myc*, MAP2K3) and cytokine signaling components (Flt1, IL-8, IL6ST, CCL2, TEK) were distinctly present, as were different cell cycle mediators (MCM2, CDC20, CCND2). Different effects on gene expression profiles by different growth factors and their combination was also evident in the studies performed by Gerritsen et al. [70] and Jih et al. [68], indicating largely separate signaling pathways are mediating the responses. Furthermore, different time points were analyzed which can have considerable effect on the identification of early acting genes and transcription factors that activate secondary genes. The gene expression platforms and data analysis in the above discussed studies differ considerably, creating additional bias. Further contributing to the observed differences can be the diversity of the EC used in the above described studies. EC of macrovascular origin, such as HUVEC [38,59,68,70], have recently been shown to differ substantially from microvascular EC, such as HDMEC [49], by large scale gene expression profiling [71]. These differences are supposed to support local specialization of the vascular beds and consequently contribute to inherent differences in expression patterns.

Table 2.1: Growth factor regulation of common players in *in vitro* models of angiogenesis

Function	Description	Gene	UniGene ID	Growth factor regulation		References
Cell cycle regulators and apoptosis related proteins						
	MCM2 minichromosome maintenance deficient 2, mitotin (<i>S. cerevisiae</i>)	MCM2	Hs.477481	VEGF	VEGF+HGF	[59,70]
	CDC20 cell division cycle 20 homolog (<i>S. cerevisiae</i>)	CDC20	Hs.524947	VEGF	VEGF+HGF	[68,70]
	Cyclin D2	CCND2	Hs.376071	EGF	bFGF+VEGF	[49,68]
	DNA-damage-inducible transcript 3	DDIT3	Hs.370771	EGF	bFGF+VEGF	[49,68]
	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	Hs.505777	VEGF	bFGF+VEGF	[59,68]
	Caspase 3	CASP3	Hs.141125	VEGF	EGF	[49,68]
Extracellular matrix proteins						
	Serine (or cysteine) proteinase inhibitor, clade B, member 2	SERPINB2	Hs.514913	VEGF	VEGF+HGF	[59,68]
Growth factors, cytokines and hormones						
	Angiopoietin 2	ANGPT2	Hs.549021	VEGF	VEGF+HGF	[59,70]
	Chemokine (C-C motif) ligand 2	CCL2	Hs.303649	CM	bFGF+VEGF	[38,68]
	Interleukin 8	IL8	Hs.624	VEGF	EGF CM	[38,49,68]
	Diphtheria toxin receptor (HB-EGF-like growth factor)	HBEGF	Hs.799	VEGF		[59,68]
Metabolism						
	Prostaglandin-endoperoxide synthase 2 (COX-2)	PTGS2	Hs.196384	CM	VEGF	[38,59]
	Superoxide dismutase 2, mitochondrial	SOD2	Hs.487046	CM	bFGF+VEGF	[38,68]
Protein turnover, modification and transport						
	Alpha-2-macroglobulin	A2M	Hs.212838	VEGF	VEGF+HGF	[59,70]
Cell surface proteins, antigens, receptors and adhesion molecules						
	Low density lipoprotein receptor	LDLR	Hs.213289	VEGF	VEGF+HGF	[59,70]
	AXL receptor tyrosine kinase	AXL	Hs.466791	CM	bFGF+VEGF	[38,68]
	Integrin α V	ITGAV	Hs.436873	EGF	VEGF	[49,68]
	Integrin β 3	ITGB3	Hs.218040	EGF	bFGF	[49,68]
	TEK tyrosine kinase, endothelial	TEK	Hs.89640	EGF	bFGF+VEGF	[49,68]
	Tyrosine kinase with Ig-like and EGF-like domains 1	TIE	Hs.788024	EGF	bFGF+VEGF	[49,68]
	Thrombomodulin	THBD	Hs.2030	VEGF	VEGF+HGF	[59,68,70]
	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	IL6ST	Hs.532082	VEGF	VEGF+HGF	[68,70]
	Fms-related tyrosine kinase 1 (VEGF receptor)	FLT1	Hs.507621	bFGF+VEGF	VEGF+HGF	[68,70]
	Integrin α 6	ITGA6	Hs.133397	bFGF+VEGF EGF	VEGF+HGF	[49,68,70]
Signal transduction proteins						
	Mitogen-activated protein kinase kinase 3	MAP2K3	Hs.514012	bFGF	VEGF+HGF	[68,70]
Transcription factors and chromatin associated proteins						
	Early growth response 1	EGR1	Hs.326035	bFGF+VEGF VEGF	VEGF+HGF	[59,68,70]
	V-jun sarcoma virus 17 oncogene homolog (avian)	JUN	Hs.525704	bFGF	EGF	[49,68]

When reviewing the functional classification rather than the identity of the genes reported to be upregulated in the six studies discussed, it emerges that cell cycle related genes make up the larger part of regulated genes, followed by genes

associated with metabolism (Figure 2.1A). Such a 'cell cycle signature' of upregulated genes can be related to the transition from quiescent to proliferative EC under the influence of these growth factors, an early event in angiogenesis.

Gene expression associated with tube formation of EC in vitro

EC grown in collagen type 1 gels form tube structures *in vitro*. During this differentiation process, changes in gene expression are evident. A time course of gene expression during capillary morphogenesis in 3D collagen matrices was studied using microarrays to compare expression profiles of HUVEC at 0, 8, 24 and 48 hours [72]. Extracellular matrix components comprised the most abundant functional class of genes upregulated during capillary formation in this model. Most pronounced was the upregulation of collagen IV α 1 and laminin γ 1, both involved in basement membrane synthesis, assembly and ECM remodeling. Very interesting was the finding of a novel capillary morphogenesis gene, CMG-2, that encodes for a 45kDa transmembrane protein and that showed binding to collagen type IV and laminin but not to fibronectin and osteopontin [72]. Integrin β 1, which can dimerize with upregulated integrins α 1 and α 2, also shows affinity for collagen type IV and laminin [73], stressing again the importance of extracellular matrix remodeling events during capillary formation. Also, a number of signaling pathways involved in capillary formation emerged from the data. JAK/STAT pathway signaling molecules were induced, among which gp130, leukemia inhibitory factor, janus kinase-1 (JAK-1) and its inhibitor suppressor of cytokine signaling-1 (SOCS-1). Sprouty, a negative regulator of bFGF signaling was upregulated. HUVEC transfected with a mouse sprouty4 showed a decrease in cell migration [74]. Inhibitor of differentiation genes (Id1, Id2 and Id3) were downregulated, as were ephrin A1 and the Eph receptors B1 and B4. This is intriguing since these proteins have been implicated in the promotion of (tumor) angiogenesis [75]. However, they might play a temporal role during the different stages of angiogenesis [76,77]. Id proteins are implicated in the control of cell cycle progression and cell differentiation [78]. Heterodimers of basic helix-loop-helix (bHLH) transcription factors normally bind E-boxes in the promoters of differentiation genes, and Id proteins prevent transcription of these genes by forming non-functional heterodimers with bHLH proteins. Upregulation of Id3 can therefore prevent differentiation of EC. Furthermore, Id proteins are upregulated in tumor EC and induce the expression of integrin α V β 3 that binds MMP2, required for EC migration, indicating that Id proteins might be involved in the switch from quiescent to angiogenic EC. Loss of Id1 and Id3 leads to a decreased VEGF production and impaired tumor angiogenesis, suggesting that blocking Id protein expression might be an approach to block angiogenesis [78]. Indeed, knockdown of Id3 by RNA interference (RNAi) was able to overcome VEGF induced proliferation, migration and tube formation in HUVEC [79]. Id proteins are also required for neurogenesis [78] and it has recently been appreciated that many genes involved in the development of neuronal tissues are also implicated in angiogenesis, such as the neuropilins, ephrins and roundabout family [80-83]. Other developmental genes,

among which genes involved in the Jagged/Notch pathway, have also been implicated in particular aspects of angiogenesis, such as adhesion, proliferation and migration [84-86]. The activation of Notch upon ligand binding leads to the transcription of its target genes, including the enhancer of split E(spl) bHLH transcription factors [80,87].

It is interesting to note that neuronal cell adhesion molecule (NRCAM), a protein involved in neuronal development, emerges as upregulated during endothelial tube formation, further extending the analogies observed between neurogenesis and angiogenesis [88,89]. NRCAM acts in concert with integrin $\beta 1$ in mediating neuronal adhesion and neurite outgrowth [90]. NRCAM and integrin αV were indeed heavily upregulated in tube forming capillary EC in collagen matrices when compared to proliferating EC *in vitro*, hence NRCAM is likely to play a role in cell-cell communication and migration during angiogenesis [88,89]. Furthermore, NRCAM was extensively expressed in brain but also in placenta, a highly vascularized and angiogenic tissue [88].

In a similar study, HUVEC were cultured in 3D type I collagen gel, in the presence or absence of VEGF [91]. SSH in combination with differential screening identified 31 genes that were upregulated under the influence of VEGF in this culture model, including 4 different MMPs (MMP1, MMP2, MMP3 and MMP9) and the lysosomal cysteine protease cathepsin B, all of which are associated with invasion in the collagen gel. In addition, known EC adhesion molecules, including VE-cadherin, vascular cell adhesion molecule-1 (VCAM1) and integrin $\alpha 2$, were upregulated in response to VEGF. Interesting novel candidate genes involved in angiogenesis include epiregulin, a member of the EGF family of mitogens and an activator of COX-2 [92,93], and VCIP, a transmembrane phosphatase with an RGD motif that mediates cell-cell interactions [94,95].

Using GeneCallingTM, Kahn et al. [96] aimed to identify genes upregulated in HUVEC cultured in 3D collagen gels, stimulated with phorbol myristate acetate (PMA), VEGF and bFGF. Genes of different classes were identified, but most prominently upregulated were those associated with extracellular matrix remodeling and adhesion. Of these, integrin $\alpha 2$, collagen IV, MMPs and cathepsin B have already been discussed. This study was later used to identify genes both upregulated in *in vitro* models of EC differentiation and associated with tumor vasculature by an *in silico* comparison [36]. In total, 128 genes were identified to be upregulated in *in vitro* models of angiogenesis as well as in tumor samples compared to normal tissue controls. From these, all genes that showed expression in epithelial tumor cell lines were subtracted yielding a list of 24 genes. The most prominently regulated genes included stanniocalcin-1 and OX40 ligand, previously unrecognized for a role in angiogenesis. Stanniocalcin-1 (STC1) was identified to be upregulated during tube formation of EC [72,91,96] and during stimulation of EC with a combination of HGF and VEGF [70]. STC1 is a secreted glycoprotein whose fish homolog regulates calcium and phosphate uptake and excretion. An angio-inhibitory role of STC1 was demonstrated by the inhibition of migration of EC in response to HGF in the presence of recombinant STC1.

Furthermore, STC1 expression might act as a stabilizing factor contributing to the maturation of newly formed vessels [97]. OX40 ligand or gp34 is a membrane-bound TNF superfamily member thought to play a role in T-cell costimulatory responses and T-cell interactions with endothelium [98,99]. OX40 binding to gp34 leads to the induction of fos and jun expression and the subsequent downstream activation of CCL5/RANTES [100].

A comprehensive overview of gene expression in endothelial tube formation *in vitro* in different experimental models investigated by means of oligonucleotide arrays has recently been published [89] and discusses relevant genes in light of their functional classification. Here, it also emerges that genes related to metabolism, extracellular matrix remodeling and cell surface molecules including adhesion molecules comprise an important group of regulated genes in endothelial tube formation.

A number of genes have been identified (Table 2.2), mainly adhesion molecules (NRCAM, VCAM1, ICAM1), integrins (integrin α V and α 2) and matrix remodeling mediators (MMP1, MMP9, laminins α 4 and γ 1) that show induction in response to different combinations of growth factors in the reported tube formation models. However, when all genes reported in the above described studies [72,88,89,91,96] are subject to functional classification, it appears that cell-cell contact, cell-matrix interactions and matrix turnover related genes represent the larger part of genes regulated in these models of tube formation in three dimensional culture (Figure 2.1B). Furthermore, a clear shift in global gene expression is observed in these models representing the intermediate stages of angiogenesis (Figure 2.1B) when compared to the early stages of angiogenesis (Figure 2.1A).

Gene expression associated with angiogenic EC in vivo

To date, only a very limited number of studies have attempted to characterize the gene expression profile of tumor EC. The major challenge in doing so is to obtain a sufficiently pure population of cells. The difficulty lies in the fact that only a few percent of the tissue mass consists of EC, and that the EC are embedded in extracellular matrix components and surrounded by pericytes. Ideally, the isolated cells should be used in downstream applications immediately, omitting any subculturing steps or time consuming isolation procedures that can introduce changes in gene expression [101,102]. Antibody-based procedures in combination with magnetic bead separation have been used to isolate EC from tissues [101]. In addition high-speed fluorescence activated cell sorting can be applied to isolate EC from tissue sources [102].

The first study published to tackle these technical challenges used SAGE technology to identify markers in EC isolated with magnetic beads [101]. Both a negative selection using antibody-coupled beads directed against epithelial cells, leukocytes, monocytes and macrophages and a positive selection using P1H12 antibody-coupled beads ensured the selection of a highly pure population of EC from both colon tumor and normal colon tissue. SAGE tags were categorized as pan-

Table 2.2: Regulation of common players in *in vitro* models of 3D collagen tube formation

Function	Description	Gene	UniGene ID	Growth factor regulation		References
Cell cycle regulators and apoptosis related proteins						
BCL2-related protein A1	BCL2A1	Hs.227817	PMA+bFGF+VEGF+HGF	PMA+bFGF+VEGF	[89,96]	
Cytoskeleton proteins						
Gelsolin	GSN	Hs.522373	PMA+bFGF+VEGF+HGF	PMA	[72,89]	
Myosin IC	MYO1C	Hs.289226	PMA+bFGF+VEGF	PMA	[72,96]	
Extracellular matrix proteins						
A disintegrin and metalloproteinase domain 10	ADAM10	Hs.172028	PMA+bFGF+VEGF+HGF	bFGF+VEGF	[88,89]	
Collagen IV α 1	COL4A1	Hs.17441	PMA+bFGF+VEGF	PMA	[72,96]	
Cathepsin B	CTSB	Hs.520898	PMA+bFGF+VEGF	VEGF	[91,96]	
Laminin α 4	LAMA4	Hs.213861	PMA+bFGF+VEGF+HGF	PMA	[72,89]	
Laminin γ 1	LAMC1	Hs.497039	PMA+bFGF+VEGF+HGF	PMA	[72,89]	
Matrix metalloproteinase 1	MMP1	Hs.83169	PMA+bFGF+VEGF	VEGF	[91,96]	
Matrix metalloproteinase 9	MMP9	Hs.297413	PMA+bFGF+VEGF	VEGF	[91,96]	
Nidogen 2	NID2	Hs.369840	PMA+bFGF+VEGF+HGF	PMA+bFGF+VEGF	[89,96]	
Tissue factor pathway inhibitor 2	TFPI2	Hs.438231	PMA+bFGF+VEGF+HGF PMA	PMA+bFGF+VEGF	[72,89, 96]	
Growth factors, cytokines and hormones						
Placental growth factor	PGF	Hs.252820	PMA+bFGF+VEGF	PMA	[72,96]	
Stanniocalcin 1	STC1	Hs.25590	PMA+bFGF+VEGF	VEGF PMA	[72,91,96]	
Transforming growth factor β 1	TGFB1	Hs.1103	PMA+bFGF+VEGF	bFGF+VEGF	[88,96]	
Metabolism						
HMG-Coenzyme A reductase	HMGCR	Hs.11899	PMA+bFGF+VEGF+HGF	PMA	[72,89]	
Protein turnover, modification and transport						
Peptidylglycine α -amidating monooxygenase	PAM	Hs.369430	PMA+bFGF+VEGF+HGF	PMA+bFGF+VEGF	[89,96]	
Cell surface proteins, antigens, receptors and adhesion molecules						
Ectonucleoside triphosphate diphosphohydrolase 1	ENTPD1	Hs.523173	PMA+bFGF+VEGF+HGF	PMA	[72,89]	
Chemokine (C-X-C motif) receptor 4	CXCR4	Hs.421986	PMA+bFGF+VEGF+HGF	PMA+bFGF+VEGF	[89,96]	
Dipeptidylpeptidase 4	DPP4	Hs.368912	PMA+bFGF+VEGF+HGF	PMA	[72,89]	
Fms-related tyrosine kinase 1	FLT1	Hs.507621	PMA	VEGF	[72,89,91]	
Intercellular adhesion molecule 1 (CD54)	ICAM1	Hs.515126	PMA+bFGF+VEGF+HGF	PMA	[72,89]	
Interleukin 6 signal transducer	IL6ST	Hs.532082	PMA+bFGF+VEGF	PMA	[72,96]	
Integrin α 2 (CD49B)	ITGA2	Hs.482077	PMA+bFGF+VEGF+HGF PMA+bFGF+VEGF	PMA VEGF	[72,89,91,96]	
Integrin α V (CD51)	ITGAV	Hs.436873	PMA+bFGF+VEGF+HGF PMA	bFGF+VEGF	[72,88,89]	
Neuronal cell adhesion molecule	NRCAM	Hs.21422	PMA+bFGF+VEGF+HGF	bFGF+VEGF	[88,89]	
Podocalyxin-like	PODXL	Hs.16426	PMA+bFGF+VEGF	PMA	[72,96]	
Vascular cell adhesion molecule 1	VCAM1	Hs.109225	PMA+bFGF+VEGF+HGF	VEGF	[89,91]	

endothelial markers when expression was high in normal EC, tumor EC and in HUVEC and human microvascular EC (HMVEC). Tumor endothelial markers (TEMs) showed high expression in tumor-derived EC but not in normal EC or in HUVEC, HMVEC, or cell lines. A substantial number of previously uncharacterized genes have been classified as TEMs in this study. This can be partly attributed to the technique used, SAGE, which is not dependent on previously documented or cloned genes. Also, as EC represent only a small fraction of the total cell population in tissues, libraries constructed from these tissues will only reveal endothelial genes that are very highly expressed [101]. Interestingly, TEMs showed a strong bias towards genes functioning in extracellular matrix turnover such as collagens $\text{I}\alpha 1$, $\text{I}\alpha 2$, $\text{III}\alpha 1$, $\text{IV}\alpha 1$, $\text{VI}\alpha 3$ and $\text{XII}\alpha 1$, and matrix metalloproteinases MMP11 and MMP2, stressing the importance of ECM remodeling during angiogenesis *in vivo*. When expressed as a percentage of tags, 82% of the TEMs coded for proteins that function in ECM remodeling and cell migration [101]. Furthermore, many TEMs were involved in adhesion and cytoskeletal remodeling, already discussed to be involved in tube formation. Genes thought to play a role in the initiation of angiogenesis, e.g. nuclear signaling molecules, and genes involved in cell-cycle regulation, metabolism and proliferation were almost completely absent [101].

Very recently, SAGE profiles were published representing malignant brain endothelium and invasive breast carcinoma endothelium [103,104]. Two normal brain EC samples were compared with three glioma-derived EC preparations. The majority of glioma endothelial markers (GEMs) function in extracellular matrix remodeling or are expressed as surface molecules involved in cell-cell interactions and adhesion. Collagens $\text{I}\alpha 1$, $\text{IV}\alpha 1$, $\text{IV}\alpha 2$, and $\text{VI}\alpha 1$ were shown to be overexpressed in glioma EC, as were a number of MMPs [104]. A similar profile is apparent in breast tumor EC compared to normal breast EC [103]. Combining the information in these three SAGE data sets, it is readily apparent that genes involved in extracellular matrix remodeling and cell-cell or cell-matrix contact represent the majority of upregulated genes during tumor angiogenesis in different types of tumors (Figure 2.1C; Table 2.3).

From the available SAGE data sets, a number of genes can be identified that are overexpressed on the endothelium of different tumors. Colon tumor and glioma EC overexpressed collagens $\text{I}\alpha 1$, $\text{III}\alpha 1$, $\text{IV}\alpha 1$ and CD164/sialomucin-like [101,104]. Collagen $\text{IV}\alpha 2$, MMP9, SerpinE, SPARC and an expressed sequence tag (EST) were coordinately upregulated in both glioma EC and breast tumor EC when compared to their respective normal counterparts [103,104]. Interestingly, almost no overlap in tumor endothelial genes is seen between colon and breast tumors, though the functional classes of upregulated molecules do indicate similarities in gene expression (Table 2.3). Most of these genes are associated with the basement membrane (BM), a specialized form of extracellular matrix. It has been recognized that BM components such as collagen IV, laminin, nidogen and SPARC are key regulators of angiogenesis. Type IV collagen provides the scaffold for BM assembly and is together with collagens type XV and type XVIII associated with vasculature. EC are generally quiescent when

bound to the BM suggesting this matrix provides growth arresting cues [105]. During angiogenesis, MMP9 is produced that mobilizes sequestered growth factors and degrades collagen type IV, thereby disrupting BM organization and exposing cryptic domains of partially degraded collagens. These domains are proangiogenic, and further stimulate EC proliferation [106]. When BM degradation reaches completion, MMP9 resistant collagen fragments remain that are generally anti-angiogenic, such as arrestin and tumstatin. The anti-angiogenic activity of these endogenous inhibitors generated by proteolytic cleavage of collagens is mediated by binding to integrins, mainly type $\beta 1$ [105].

SPARC expression has previously been associated with EC in various tissues [107,108]. It is involved in tissue remodeling and proteolytic peptides produced by MMPs in the extracellular matrix can have differential effects on angiogenesis [109]. This interplay between several overexpressed genes in tumor EC may hold promise for the design and application of therapeutic modalities. One major concern that arises from the above described studies is the uncertainty that the genes that were identified are truly markers of tumor angiogenesis rather than physiological angiogenesis. Indeed, most of the TEMs described by St Croix et al. were also expressed during angiogenesis of corpus luteum formation and wound healing [101]. Furthermore, in all published SAGE studies, a relatively small number of samples was used: 2 invasive breast tumor specimen were compared to 1 normal breast sample [103], EC isolated from 1 colon tumor were compared to EC from 1 normal colon [101] and EC from 2 normal temporal lobotomies were compared to 3 grade III or grade IV gliomas [104].

Table 2.3: Tumor endothelial markers in brain, breast and colon identified by SAGE

Function	Description	Gene	UniGene ID	Expression Madden et al. [104]	StCroix et al. [101]	Parker et al. [103]
Extracellular matrix proteins						
	Secreted protein, acidic, cysteine-rich	SPARC	Hs.111779	Brain	Colon	Breast
	Collagen $\alpha 1$	COL1A1	Hs.172928	Brain	Colon	
	Collagen III $\alpha 1$	COL3A1	Hs.443625	Brain	Colon	
	Collagen IV $\alpha 1$	COL4A1	Hs.17441	Brain	Colon	
	Collagen IV $\alpha 2$	COL4A2	Hs.508716	Brain		Breast
	Matrix metalloproteinase 1	MMP1	Hs.83169	Brain	Colon	
	Matrix metalloproteinase 9	MMP9	Hs.297413	Brain		Breast
	CD164 antigen, sialomucin	CD164	Hs.195727	Brain	Colon	
	Serine (or cysteine) proteinase inhibitor, clade E, member 1	SERPINE1	Hs.414795	Brain		Breast
Cell surface proteins, antigens, receptors and adhesion molecules						
	Thy-1 cell surface antigen	THY1	HS.134643	Brain	Colon	
	Plexin domain containing 1	PLXDC1	HS.125036		Colon	Breast
Unknown						
	Homo sapiens clone FLC1492 PRO3121 mRNA	EST		Brain		Breast

The expression of extracellular matrix remodeling related genes and those involved cytoskeleton and cell interactions seems a predominant theme in EC *in vivo* (Figure 2.1C). The identification of novel genes and genes previously not thought to be involved in angiogenesis by using unbiased gene expression profiling techniques will shed new light on the process of angiogenesis and the possible interference therein for therapeutic purposes. Since angiogenesis is not limited to pathological conditions, careful evaluation of putative markers for their tissue specificity is warranted if these are to be used in targeting undesired angiogenesis.

>> EC diversity

Microenvironmental interactions in angiogenesis

EC in a particular microenvironment interact with various types of cells, such as fibroblasts, pericytes, immune cells, tumor cells or organ cells. In addition, EC can interact with various ECM components and are exposed to different growth factors and cytokines released by its neighbors [110]. As a result, EC lining different vessels exhibit morphological and functional specializations. EC derived from arteries, veins and tissues clearly differed in global gene expression profiles, related to various processes such as ECM remodeling and coagulation cascades [14,71]. A gene expression comparison of cerebral EC (HCEC), part of the blood-brain barrier, with HUVEC revealed that HCEC are characterized by the expression of genes associated with neuroprotection and growth support. Marked differences in growth factor protein release between HCEC and HUVEC in culture were observed that supported the gene expression data [111]. It has been demonstrated that tumor EC isolated from renal cell carcinomas (RCC) retain their persistently activated phenotype in culture. Initially it was shown that RCC tumor EC downregulate adhesion molecules such as ICAM1, ICAM2 and CD34 [112]. Later it was shown that RCC tumor EC were resistant to serum-withdrawal induced apoptosis, a higher proliferation rate and more pronounced tube formation in comparison to HMVEC [113]. Thus, it seems that the original microenvironment programs the endothelial phenotype and that these properties persist (at least to some extent) in both *in vitro* behavior and gene expression.

Tumor angiogenesis models

One of the advantages of using cell culture models that mimic an *in vivo* situation *in vitro* is the virtually unlimited supply of cells that can be obtained for these purposes. Furthermore, assay conditions can be carefully monitored and can be kept constant ensuring a certain degree of reproducibility. However, in cell culture systems, cells are no longer in their natural environment and might respond aberrantly to certain stimuli, giving a false representation of the *in vivo* situation [114]. Also, in primary cell cultures such as HUVEC, the culture process itself can induce changes in these cells [102,115].

Figure 2.1: Functional classification of annotated genes associated with (tumor-) angiogenesis. **A**) Genes upregulated by growth factor stimulation of EC *in vitro* were classified according to their functions (N=464) [38,45,49,59,68,70]. **B**) Genes associated with capillary tube formation of EC *in vitro* (N=385) [72,88,89,91,96]. **C**) Putative tumor endothelial markers overexpressed in EC isolated from tumor tissue and compared to non-tumor EC (N=69) [101,103,104]. Gene function was associated with signal transduction (A), transcription (B), cell cycle regulation or apoptosis (C), cytoskeleton (D), extracellular matrix remodeling (E), growth factor, hormone or cytokine action (F), metabolism (G), protein turnover, modification and transport (H), cell surface molecules, antigens, receptors and adhesion molecules (I), or unknown function (J).

When regarding angiogenesis in cancer, tumor EC have generally resided in a tumor microenvironment for months to years, whereas culture systems only cover a time period of days. This discrepancy between *in vitro* and *in vivo* conditions might translate in an incomplete or hampered mimic of *in vivo* conditions. Thus, though *in vitro* approaches might provide useful information in certain settings, the results require cautious interpretation.

Several attempts have been made to overcome these limitations. Co-culture of EC with tumor cells allows studying the interaction between the different cell types. Increased proliferation and network formation of HUVEC was apparent in co-culture with U87 glioma cells. Interestingly, global gene expression profiles of EC in co-culture concordantly shifted towards more genes involved in ECM remodeling and cytoskeletal changes [116]. Depending on the model used, direct interactions, or bidirectional cross-talk over a membrane can be analyzed.

Endothelial precursor cells (EPC), derived from CD34+/AC133+ bone marrow progenitor cells were evaluated as a model for tumor EC in comparison to widely used microvascular EC (HMVEC). Only partial overlap (~ 30%) in gene expression patterns of HMVEC and EPC was evident, pointing towards intrinsic differences between these cells. Hence, it was postulated that EPC could be a better model for tumor EC than HMVEC or HUVEC. However, the expression of 565 EPC gene tags overlapped with

tumor EC, whereas 503 HMVEC gene tags overlapped with tumor EC [117], suggesting that EPC provide only a marginally better model for tumor EC.

Although apparently similarly regulated, the exact differences, if existing, between angiogenic EC under physiological circumstances and angiogenically activated EC in tumors have not fully been elucidated. Different models have been developed to study angiogenesis *in vitro*, however, it may be difficult to accurately mimic the temporal and spatial complex actions of all factors exerting effect on EC *in vivo*. Therefore, extrapolation of data generated by *in vitro* experiments to the *in vivo* situation might be limited.

EC heterogeneity and therapeutic interference

EC diversity observed in tissues, large and small vessels, arteries and veins, and tumors constitutes a challenge for the development of therapeutic strategies for the interference with angiogenesis. From the above described SAGE analysis it was apparent that there was only very limited overlap in genes regulated concordantly in breast and colorectal cancer EC, but that both colon tumor EC and breast tumor EC associated genes were overexpressed in glioma EC. This indicates tumor EC heterogeneity between different tumors and holds implications for the design of future targeted therapies. Also, this heterogeneity may lead to differences in responsiveness to current (anti-angiogenic) therapies. In addition, EC heterogeneity may exist within a tumor [110].

The markedly different global gene expression profiles in growth factor stimulated EC, tube forming EC in 3D collagen and EC isolated from tumors (Figure 2.1), stresses the influence of environmental factors on phenotype and gene expression. Therefore, for both the identification of putative therapeutic targets and for the evaluation of novel therapeutics, careful evaluation of the model systems used is warranted.

>> Implications and future directions

Different studies have attempted to find novel targets for angiogenesis interfering therapies by elucidating gene expression patterns of EC. A number of investigators stimulated EC in culture with different cytokines and growth factors [38,49,59,68,70] whereas others used tube formation models *in vitro* [72,88,89,91,96]. Only very few attempts have been made to profile EC from *in vivo* sources, mainly due to cumbersome isolation techniques that are required to isolate sufficiently pure populations of EC from tissues [101,103,104]. These different approaches, cell sources and cytokines applied, have led to the identification of different subsets of genes regulated under these circumstances. In general, genes involved in ECM or BM remodeling, e.g. collagen IV α 1, MMP1 and MMP9, showed upregulation in both endothelial tube formation *in vitro* and in tumor endothelium *in vivo*. Also, different integrins were identified in the diverse EC sources described here. Some of these

molecules have been explored for therapeutic interference in cancer. Different inhibitors of MMP activity have been developed for anticancer therapy [118] but they were not very successful in clinical trials [119]. Inhibiting EC adhesion to ECM components by blocking integrin $\alpha V\beta 3$ induces apoptosis in EC and tumor regression [120]. Currently, two αV integrin inhibiting agents, monoclonal antibody Vitaxin and cyclic RGD-like peptide cilengitide, are about to enter the market (www.medimmune.com, www.merck.de). Furthermore, different proteolytic fragments of BM collagens have proven anti-angiogenic activity [121]. Gene expression profiles emerging from the different models described, although not exhaustive, are best viewed in the context of the distribution of the identified genes over a number of functional classes. When the functional classification of the reported genes associated with *in vitro* growth factor and cytokine stimulation of EC growing in monolayers is regarded, it is apparent that cell cycle related genes make up the larger part of regulated genes; followed by genes associated with metabolism (Figure 2.1A). Genes associated with tube formation are those involved in cell-cell and cell-matrix contact, extracellular matrix turnover and signaling (Figure 2.1B). The involvement of extracellular matrix remodeling proteins is even more evident in EC isolated from fresh tissues (Figure 2.1C).

The observation that very few genes overlap in the above mentioned types of studies might reflect the different activation of the used EC sources. Stimuli applied *in vitro* might be incomplete for a perfect mimic of the tumor microenvironment. It is possible that the concentrations of cytokines and growth factors used are not representative for the *in vivo* situation. *In vitro* activation of EC with only one angiogenic growth factor may even overstimulate one pathway and silence others. Likewise, the presence of other specific types of cells such as pericytes, stromal or epithelial cells *in vivo* influences the phenotype and hence the gene expression profile of the endothelium by a plethora of different signals. Furthermore, one might speculate what the influence of time is; tumor EC have generally been exposed to their tumor environment for months to years whereas most *in vitro* studies cover only a limited period of time. It has been proposed that tumor EC have further progressed into the sequence of angiogenic events, whereas cultured EC are at the early stages of angiogenesis. This could explain the gradual increase (from confluent monolayers of cultured cells, via *in vitro* stimulated tube forming cells to tumor-derived EC) of the proportion of expressed genes that are associated with extracellular matrix remodeling, a late event in angiogenesis.

Novel genes and genes not previously associated with angiogenesis identified by gene expression profiling studies will provide more insight in the molecular events that direct angiogenesis and may hold clues for the development of novel therapeutic moieties. Tumor endothelium specific markers that are expressed on the cell may be targeted for vessel destruction by, for example, antibodies conjugated to cytotoxic drugs. Gene products that play a crucial role in the active sprouting of capillaries from

existing vessels are putative candidates for therapeutic interference in the angiogenesis process.

The successes in isolating pure fractions of EC has provided the opportunity to study gene expression in EC from *in vivo* sources directly, without the introduction of artifacts by culturing steps. Also, bioinformatics approaches to identify novel endothelial markers [15,36,37,122] and to functionally annotate them [123] are promising. Furthermore, public availability of gene expression data in uniform formats provides a tool for surveying gene expression profiles over a large number of experiments, to identify common denominators of certain processes and signaling pathways [124-127]. Taken together, these progresses in understanding angiogenic EC biology, and the concomitant identification of molecules pivotal to this process, might eventually result in the development of novel, potent and specific angiogenesis modulators for therapeutic purposes.

>> References

- [1] Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992;3 (2):65-71.
- [2] Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;285 (21):1182-6.
- [3] Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86 (3):353-64.
- [4] Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003;3 (6):401-10.
- [5] Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 1992;13 (1):18-32.
- [6] Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science* 1984;223 (4642):1296-9.
- [7] Zhang YW, Su Y, Volpert OV, Vande Woude GF. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. *Proc Natl Acad Sci U S A* 2003;100 (22):12718-23.
- [8] Davis S, Yancopoulos GD. The angiopoietins: Yin and Yang in angiogenesis. *Curr Top Microbiol Immunol* 1999;237:173-85.
- [9] Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 2000;6 (4):389-95.
- [10] Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986;315 (26):1650-9.
- [11] Folkman J, Ingber D. Inhibition of angiogenesis. *Semin Cancer Biol* 1992;3 (2):89-96.
- [12] Ribatti D, Vacca A, Dammacco F, English D. Angiogenesis and anti-angiogenesis in hematological malignancies. *J Hematother Stem Cell Res* 2003;12 (1):11-22.
- [13] Perez-Atayde AR, Sallan SE, Tedrow U, Connors S, Allred E, Folkman J. Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia. *Am J Pathol* 1997;150 (3):815-21.
- [14] Ho M, Yang E, Matcuk G, Deng D, Sampas N, Tsalenko A, Tabibiazar R, Zhang Y, Chen M, Talbi S, et al. Identification of endothelial cell genes by combined database mining and microarray analysis. *Physiol Genomics* 2003;13 (3):249-62.
- [15] Humnietz L, Bicknell R. In silico cloning of novel endothelial-specific genes. *Genome Res* 2000;10 (11):1796-806.
- [16] Griffioen AW, Coenen MJ, Damen CA, Hellwig SM, van Weering DH, Vooyes W, Blijham GH, Groenewegen G. CD44 is involved in tumor angiogenesis; an activation antigen on human endothelial cells. *Blood* 1997;90 (3):1150-9.
- [17] Hellwig SM, Damen CA, van Adrichem NP, Blijham GH, Groenewegen G, Griffioen AW. Endothelial CD34 is suppressed in human malignancies: role of angiogenic factors. *Cancer Lett* 1997;120 (2):203-11.
- [18] Ruoslahti E. Specialization of tumour vasculature. *Nat Rev Cancer* 2002;2 (2):83-90.
- [19] Ruoslahti E, Rajotte D. An address system in the vasculature of normal tissues and tumors. *Annu Rev Immunol* 2000;18:813-27.
- [20] Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. *Nature* 1996;380 (6572):364-6.
- [21] Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res* 2000;60 (3):722-7.
- [22] Curnis F, Arrighi G, Sacchi A, Fischetti L, Arap W, Pasqualini R, Corti A. Differential binding of drugs containing the NGR motif to CD13 isoforms in tumor vessels, epithelia, and myeloid cells. *Cancer Res* 2002;62 (3):867-74.
- [23] Chang SS, Reuter VE, Heston WD, Bander NH, Grauer LS, Gaudin PB. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res* 1999;59 (13):3192-8.

- [24] Burrows FJ, Derbyshire EJ, Tazzari PL, Amlot P, Gazdar AF, King SW, Letarte M, Vitetta ES, Thorpe PE. Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy. *Clin Cancer Res* 1995;1 (12):1623-34.
- [25] Castellani P, Viale G, Dorcaratto A, Nicolo G, Kaczmarek J, Querze G, Zardi L. The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int J Cancer* 1994;59 (5):612-8.
- [26] Griffioen AW, Damen CA, Martinotti S, Blijham GH, Groenewegen G. Endothelial intercellular adhesion molecule-1 expression is suppressed in human malignancies: the role of angiogenic factors. *Cancer Res* 1996;56 (5):1111-17.
- [27] Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995;270 (5235):484-7.
- [28] Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci U S A* 1996;93 (12):6025-30.
- [29] Yang GP, Ross DT, Kuang WW, Brown PO, Weigel RJ. Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. *Nucleic Acids Res* 1999;27 (6):1517-23.
- [30] Liang P, Pardee AB. Differential display. A general protocol. *Mol Biotechnol* 1998;10 (3):261-7.
- [31] Shimkets RA, Lowe DG, Tai JT, Sehl P, Jin H, Yang R, Predki PF, Rothberg BE, Murtha MT, Roth ME, et al. Gene expression analysis by transcript profiling coupled to a gene database query. *Nat Biotechnol* 1999;17 (8):798-803.
- [32] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995;270 (5235):467-70.
- [33] Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ. High density synthetic oligonucleotide arrays. *Nat Genet* 1999;21 (1 Suppl):20-4.
- [34] Wu TD. Analysing gene expression data from DNA microarrays to identify candidate genes. *J Pathol* 2001;195 (1):53-65.
- [35] Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95 (25):14863-8.
- [36] Gerritsen ME, Soriano R, Yang S, Ingle G, Zlot C, Toy K, Winer J, Draksharapu A, Peale F, Wu TD, et al. In silico data filtering to identify new angiogenesis targets from a large in vitro gene profiling data set. *Physiol Genomics* 2002;10 (1):13-20.
- [37] Gerritsen ME, Peale FV, Jr., Wu T. Gene expression profiling in silico: relative expression of candidate angiogenesis associated genes in renal cell carcinomas. *Exp Nephrol* 2002;10 (2):114-9.
- [38] Wang JL, Liu YH, Lee MC, Nguyen TM, Lee C, Kim A, Nguyen M. Identification of tumor angiogenesis-related genes by subtractive hybridization. *Microvasc Res* 2000;59 (3):394-7.
- [39] Bischoff J, Brasel C, Kraling B, Vranovska K. E-selectin is upregulated in proliferating endothelial cells in vitro. *Microcirculation* 1997;4 (2):279-87.
- [40] Tonnesen MG, Jenkins D, Jr., Siegal SL, Lee LA, Huff JC, Clark RA. Expression of fibronectin, laminin, and factor VIII-related antigen during development of the human cutaneous microvasculature. *J Invest Dermatol* 1985;85 (6):564-8.
- [41] Wempe F, Lindner V, Augustin HG. Basic fibroblast growth factor (bFGF) regulates the expression of the CC chemokine monocyte chemoattractant protein-1 (MCP-1) in autocrine-activated endothelial cells. *Arterioscler Thromb Vasc Biol* 1997;17 (11):2471-8.
- [42] Miller LJ, Kurtzman SH, Wang Y, Anderson KH, Lindquist RR, Kreutzer DL. Expression of interleukin-8 receptors on tumor cells and vascular endothelial cells in human breast cancer tissue. *Anticancer Res* 1998;18 (1A):77-81.
- [43] Liu C, Shao ZM, Zhang L, Beatty P, Sartippour M, Lane T, Livingston E, Nguyen M. Human endomucin is an endothelial marker. *Biochem Biophys Res Commun* 2001;288 (1):129-36.
- [44] Kanda H, Tanaka T, Matsumoto M, Umemoto E, Ebisuno Y, Kinoshita M, Noda M, Kannagi R, Hirata T, Murai T, et al. Endomucin, a sialomucin expressed in high endothelial venules, supports L-selectin-mediated rolling. *Int Immunol* 2004;16 (9):1265-74.
- [45] van Beijnum JR, Dings RPM, van der Linden E, Zwaans BMM, Ramaekers FCS, Mayo KH, Griffioen AW. Gene expression tumor angiogenesis dissected; specific targeting of tumor endothelium. Submitted.

- [46] Philp D, Huff T, Gho YS, Hannappel E, Kleinman HK. The actin binding site on thymosin beta4 promotes angiogenesis. *Faseb J* 2003;17 (14):2103-5.
- [47] Malinda KM, Goldstein AL, Kleinman HK. Thymosin beta 4 stimulates directional migration of human umbilical vein endothelial cells. *Faseb J* 1997;11 (6):474-81.
- [48] Cha HJ, Jeong MJ, Kleinman HK. Role of thymosin beta4 in tumor metastasis and angiogenesis. *J Natl Cancer Inst* 2003;95 (22):1674-80.
- [49] Zhang H-T, Gorn M, Smith K, Graham AP, Lau KKW, Bicknell R. Transcriptional profiling of human microvascular endothelial cells in the proliferative and quiescent state using cDNA arrays. *Angiogenesis* 1999;3:211-9.
- [50] Hanai J, Dhanabal M, Karumanchi SA, Albanese C, Waterman M, Chan B, Ramchandran R, Pestell R, Sukhatme VP. Endostatin causes G1 arrest of endothelial cells through inhibition of cyclin D1. *J Biol Chem* 2002;277 (19):16464-9.
- [51] Hori A, Ikeyama S, Sudo K. Suppression of cyclin D1 mRNA expression by the angiogenesis inhibitor TNP-470 (AGM-1470) in vascular endothelial cells. *Biochem Biophys Res Commun* 1994;204 (3):1067-73.
- [52] Dormond O, Ruegg C. Regulation of endothelial cell integrin function and angiogenesis by COX-2, cAMP and Protein Kinase A. *Thromb Haemost* 2003;90 (4):577-85.
- [53] Ruegg C, Mariotti A. Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. *Cell Mol Life Sci* 2003;60 (6):1135-57.
- [54] Ruegg C, Dormond O, Foletti A. Suppression of tumor angiogenesis through the inhibition of integrin function and signaling in endothelial cells: which side to target? *Endothelium* 2002;9 (3):151-60.
- [55] Gutheil JC, Campbell TN, Pierce PR, Watkins JD, Huse WD, Bodkin DJ, Cheresch DA. Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin alphavbeta3. *Clin Cancer Res* 2000;6 (8):3056-61.
- [56] Chen N, Leu SJ, Todorovic V, Lam SC, Lau LF. Identification of a novel integrin alphavbeta3 binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells. *J Biol Chem* 2004;279 (42):44166-76.
- [57] Brigstock DR. The CCN family: a new stimulus package. *J Endocrinol* 2003;178 (2):169-75.
- [58] Brigstock DR. Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 2002;5 (3):153-65.
- [59] Abe M, Sato Y. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. *Angiogenesis* 2001;4 (4):289-98.
- [60] Kuwano T, Nakao S, Yamamoto H, Tsuneyoshi M, Yamamoto T, Kuwano M, Ono M. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *Faseb J* 2004;18 (2):300-10.
- [61] Dormond O, Foletti A, Paroz C, Ruegg C. NSAIDs inhibit alpha V beta 3 integrin-mediated and Cdc42/Rac-dependent endothelial-cell spreading, migration and angiogenesis. *Nat Med* 2001;7 (9):1041-7.
- [62] Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* 1999;18 (55):7908-16.
- [63] Gately S. The contributions of cyclooxygenase-2 to tumor angiogenesis. *Cancer Metastasis Rev* 2000;19 (1-2):19-27.
- [64] Ushiro S, Ono M, Izumi H, Kohno K, Taniguchi N, Higashiyama S, Kuwano M. 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* 1996;87 (1):68-77.
- [65] Ongusaha PP, Kwak JC, Zwible AJ, Macip S, Higashiyama S, Taniguchi N, Fang L, Lee SW. HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res* 2004;64 (15):5283-90.
- [66] Haas TL, Stitelman D, Davis SJ, Apte SS, Madri JA. Egr-1 mediates extracellular matrix-driven transcription of membrane type 1 matrix metalloproteinase in endothelium. *J Biol Chem* 1999;274 (32):22679-85.
- [67] Torrance CJ, Jackson PE, Montgomery E, Kinzler KW, Vogelstein B, Wissner A, Nunes M, Frost P, Discafani CM. Combinatorial chemoprevention of intestinal neoplasia. *Nat Med* 2000;6 (9):1024-8.
- [68] Jih YJ, Lien WH, Tsai WC, Yang GW, Li C, Wu LW. Distinct regulation of genes by bFGF and VEGF-A in endothelial cells. *Angiogenesis* 2001;4 (4):313-21.

- [69] Dell'Era P, Coco L, Ronca R, Sennino B, Presta M. Gene expression profile in fibroblast growth factor 2-transformed endothelial cells. *Oncogene* 2002;21 (15):2433-40.
- [70] Gerritsen ME, Tomlinson JE, Zlot C, Ziman M, Hwang S. Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells. *Br J Pharmacol* 2003;140 (4):595-610.
- [71] Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A* 2003;100 (19):10623-8.
- [72] Bell SE, Mavila A, Salazar R, Bayless KJ, Kanagala S, Maxwell SA, Davis GE. Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling. *J Cell Sci* 2001;114 (Pt 15):2755-73.
- [73] Senger DR, Claffey KP, Benes JE, Perruzzi CA, Sergiou AP, Detmar M. Angiogenesis promoted by vascular endothelial growth factor: regulation through $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. *Proc Natl Acad Sci U S A* 1997;94 (25):13612-7.
- [74] Lee SH, Schloss DJ, Jarvis L, Krasnow MA, Swain JL. Inhibition of angiogenesis by a mouse sprouty protein. *J Biol Chem* 2001;276 (6):4128-33.
- [75] Sullivan DC, Bicknell R. New molecular pathways in angiogenesis. *Br J Cancer* 2003;89 (2):228-31.
- [76] Oike Y, Ito Y, Hamada K, Zhang XQ, Miyata K, Arai F, Inada T, Araki K, Nakagata N, Takeya M, et al. Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood* 2002;100 (4):1326-33.
- [77] Norton JD. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* 2000;113 (Pt 22):3897-905.
- [78] Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, et al. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* 1999;401 (6754):670-7.
- [79] Sakurai D, Tsuchiya N, Yamaguchi A, Okaji Y, Tsuno NH, Kobata T, Takahashi K, Tokunaga K. Crucial role of inhibitor of DNA binding/differentiation in the vascular endothelial growth factor-induced activation and angiogenic processes of human endothelial cells. *J Immunol* 2004;173 (9):5801-9.
- [80] Bicknell R, Harris AL. Novel angiogenic signaling pathways and vascular targets. *Annu Rev Pharmacol Toxicol* 2004;44:219-38.
- [81] Parikh AA, Fan F, Liu WB, Ahmad SA, Stoeltzing O, Reinmuth N, Bielenberg D, Bucana CD, Klagsbrun M, Ellis LM. Neuropilin-1 in human colon cancer: expression, regulation, and role in induction of angiogenesis. *Am J Pathol* 2004;164 (6):2139-51.
- [82] Shima DT, Mailhos C. Vascular developmental biology: getting nervous. *Curr Opin Genet Dev* 2000;10 (5):536-42.
- [83] Suchting S, Heal P, Tahtis K, Stewart LM, Bicknell R. Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. *Faseb J* 2005;19 (1):121-3.
- [84] Zimrin AB, Pepper MS, McMahon GA, Nguyen F, Montesano R, Maciag T. An antisense oligonucleotide to the notch ligand jagged enhances fibroblast growth factor-induced angiogenesis in vitro. *J Biol Chem* 1996;271 (51):32499-502.
- [85] Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol* 2003;23 (1):14-25.
- [86] Lindner V, Booth C, Prudovsky I, Small D, Maciag T, Liaw L. Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction. *Am J Pathol* 2001;159 (3):875-83.
- [87] Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. *Arterioscler Thromb Vasc Biol* 2003;23 (4):543-53.
- [88] Aitkenhead M, Wang SJ, Nakatsu MN, Mestas J, Heard C, Hughes CC. Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: induction of ESM-1, β 1-galactosidase, and NrCAM. *Microvasc Res* 2002;63 (2):159-71.
- [89] Gerritsen ME, Soriano R, Yang S, Zlot C, Ingle G, Toy K, Williams PM. Branching out: a molecular fingerprint of endothelial differentiation into tube-like structures generated by affymetrix oligonucleotide arrays. *Microcirculation* 2003;10 (1):63-81.

- [90] Treubert U, Brummendorf T. Functional cooperation of beta1-integrins and members of the Ig superfamily in neurite outgrowth induction. *J Neurosci* 1998;18 (5):1795-805.
- [91] Wary KK, Thakker GD, Humtsoe JO, Yang J. Analysis of VEGF-responsive Genes Involved in the activation of endothelial cells. *Mol Cancer* 2003;2 (1):25.
- [92] Sasaki E, Pai R, Halter F, Komurasaki T, Arakawa T, Kobayashi K, Kuroki T, Tarnawski AS. Induction of cyclooxygenase-2 in a rat gastric epithelial cell line by epiregulin and basic fibroblast growth factor. *J Clin Gastroenterol* 1998;27 Suppl 1:S21-7.
- [93] Toyoda H, Komurasaki T, Uchida D, Morimoto S. Distribution of mRNA for human epiregulin, a differentially expressed member of the epidermal growth factor family. *Biochem J* 1997;326 (Pt 1):69-75.
- [94] Kai M, Wada I, Imai S, Sakane F, Kanoh H. Cloning and characterization of two human isozymes of Mg²⁺-independent phosphatidic acid phosphatase. *J Biol Chem* 1997;272 (39):24572-8.
- [95] Humtsoe JO, Feng S, Thakker GD, Yang J, Hong J, Wary KK. Regulation of cell-cell interactions by phosphatidic acid phosphatase 2b/VCIP. *Embo J* 2003;22 (7):1539-54.
- [96] Kahn J, Mehraban F, Ingle G, Xin X, Bryant JE, Vehar G, Schoenfeld J, Grimaldi CJ, Peale F, Draksharapu A, et al. Gene expression profiling in an in vitro model of angiogenesis. *Am J Pathol* 2000;156 (6):1887-900.
- [97] Zlot C, Ingle G, Hongo J, Yang S, Sheng Z, Schwall R, Paoni N, Wang F, Peale FV, Jr., Gerritsen ME. Stanniocalcin 1 is an autocrine modulator of endothelial angiogenic responses to hepatocyte growth factor. *J Biol Chem* 2003.
- [98] Arch RH, Thompson CB. 4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kappaB. *Mol Cell Biol* 1998;18 (1):558-65.
- [99] Imura A, Hori T, Imada K, Ishikawa T, Tanaka Y, Maeda M, Imamura S, Uchiyama T. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *J Exp Med* 1996;183 (5):2185-95.
- [100] Matsumura Y, Hori T, Kawamata S, Imura A, Uchiyama T. Intracellular signaling of gp34, the OX40 ligand: induction of c-jun and c-fos mRNA expression through gp34 upon binding of its receptor, OX40. *J Immunol* 1999;163 (6):3007-11.
- [101] St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, et al. Genes expressed in human tumor endothelium. *Science* 2000;289 (5482):1197-202.
- [102] Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM. Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. *Am J Physiol Heart Circ Physiol* 2003;285 (5):H1917-38.
- [103] Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, et al. Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Res* 2004;64 (21):7857-66.
- [104] Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Waller-Yohrling J, et al. Vascular gene expression in nonneoplastic and malignant brain. *Am J Pathol* 2004;165 (2):601-8.
- [105] Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer* 2003;3 (6):422-33.
- [106] Xu J, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC. Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol* 2001;154 (5):1069-79.
- [107] Porter PL, Sage EH, Lane TF, Funk SE, Gown AM. Distribution of SPARC in normal and neoplastic human tissue. *J Histochem Cytochem* 1995;43 (8):791-800.
- [108] Van Beijnum JR, Moerkerk PT, Gerbers AJ, De Bruine AP, Arends JW, Hoogenboom HR, Hufton SE. Target validation for genomics using peptide-specific phage antibodies: a study of five gene products overexpressed in colorectal cancer. *Int J Cancer* 2002;101 (2):118-27.
- [109] Sage EH, Reed M, Funk SE, Truong T, Steadele M, Puolakkainen P, Maurice DH, Bassuk JA. Cleavage of the matricellular protein SPARC by matrix metalloproteinase 3 produces polypeptides that influence angiogenesis. *J Biol Chem* 2003;278 (39):37849-57.

- [110] Jung YD, Ahmad SA, Liu W, Reinmuth N, Parikh A, Stoeltzing O, Fan F, Ellis LM. The role of the microenvironment and intercellular cross-talk in tumor angiogenesis. *Semin Cancer Biol* 2002;12 (2):105-12.
- [111] Kallmann BA, Wagner S, Hummel V, Buttmann M, Bayas A, Tonn JC, Rieckmann P. Characteristic gene expression profile of primary human cerebral endothelial cells. *Faseb J* 2002;16 (6):589-91.
- [112] Griffioen AW. Phenotype of the tumor vasculature; cell adhesion as a target for tumor therapy. *The Cancer Journal* 1997;10 (5):249-55.
- [113] Bussolati B, Deambrosio I, Russo S, Deregibus MC, Camussi G. Altered angiogenesis and survival in human tumor-derived endothelial cells. *Faseb J* 2003;17 (9):1159-61.
- [114] Egginton S, Gerritsen M. Lumen formation: in vivo versus in vitro observations. *Microcirculation* 2003;10 (1):45-61.
- [115] Aird WC, Glueck SB, Dzau VJ, Pratt RE. Separating the wheat from the chaff: focus on "in silico data filtering to identify new angiogenesis targets from a large in vitro gene profile data set". *Physiol Genomics* 2002;10 (1):1-3.
- [116] Khodarev NN, Yu J, Labay E, Darga T, Brown CK, Mauceri HJ, Yassari R, Gupta N, Weichselbaum RR. Tumour-endothelium interactions in co-culture: coordinated changes of gene expression profiles and phenotypic properties of endothelial cells. *J Cell Sci* 2003;116 (Pt 6):1013-22.
- [117] Bagley RG, Walter-Yohrling J, Cao X, Weber W, Simons B, Cook BP, Chartrand SD, Wang C, Madden SL, Teicher BA. Endothelial precursor cells as a model of tumor endothelium: characterization and comparison with mature endothelial cells. *Cancer Res* 2003;63 (18):5866-73.
- [118] Rudek MA, Venitz J, Figg WD. Matrix metalloproteinase inhibitors: do they have a place in anticancer therapy? *Pharmacotherapy* 2002;22 (6):705-20.
- [119] Overall CM, Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer* 2002;2 (9):657-72.
- [120] Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresch DA. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994;79 (7):1157-64.
- [121] Sund M, Xie L, Kalluri R. The contribution of vascular basement membranes and extracellular matrix to the mechanics of tumor angiogenesis. *Apmis* 2004;112 (7-8):450-62.
- [122] Huminiecki L, Gorn M, Suchting S, Poulsom R, Bicknell R. Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics* 2002;79 (4):547-52.
- [123] Novatchkova M, Eisenhaber F. Can molecular mechanisms of biological processes be extracted from expression profiles? Case study: endothelial contribution to tumor-induced angiogenesis. *Bioessays* 2001;23 (12):1159-75.
- [124] Dudoit S, Gentleman RC, Quackenbush J. Open source software for the analysis of microarray data. *Biotechniques* 2003;Suppl:45-51.
- [125] Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat Genet* 2001;29 (4):365-71.
- [126] Brazma A, Parkinson H, Sarkans U, Shojatalab M, Vilo J, Abeygunawardena N, Holloway E, Kapushesky M, Kemmeren P, Lara GG, et al. ArrayExpress--a public repository for microarray gene expression data at the EBI. *Nucleic Acids Res* 2003;31 (1):68-71.
- [127] Stoeckert CJ, Jr., Causton HC, Ball CA. Microarray databases: standards and ontologies. *Nat Genet* 2002;32 Suppl:469-73.

Chapter 3

Gene expression of tumor angiogenesis dissected; specific targeting of tumor endothelium

Judy R. van Beijnum, Ruud P.M. Dings, Edith van der Linden, Bernadette M.M. Zwaans, Frans C.S. Ramaekers, Kevin H. Mayo & Arjan W. Griffioen

Submitted

>> Abstract

Crucial to designing angiostatic and vascular targeting agents is the identification of target molecules. Since angiogenesis is not limited to pathologies, careful evaluation of putative therapeutic targets is warranted to prevent side effects associated with impaired physiological angiogenesis. To identify tumor-specific angiogenesis markers, we compared transcriptional profiles of angiogenic endothelial cells isolated from both malignant and non-malignant tissues with that of resting endothelial cells. We identified 17 genes that show specific overexpression in tumor endothelium but not in angiogenic endothelium of normal tissues, creating a therapeutic window for tumor vasculature specific targeting. Antibody targeting of four cell-surface expressed or secreted products (vimentin, CD59, HMGB1 and IGFBP7) inhibited angiogenesis *in vitro* and *in vivo*. Finally, targeting endothelial vimentin in a mouse tumor model significantly inhibited tumor growth and reduced microvessel density. Our results demonstrate the utility of the identification and subsequent targeting of specific tumor endothelial markers for anticancer therapy.

>> Introduction

Tumor progression and the development of distant metastases require the presence of an extensive vasculature [1,2]. Active angiogenesis is a hallmark of most malignancies and inhibition of this process is considered to be a promising strategy for the treatment of tumors. In order to develop the most specific and effective anti-angiogenic therapies for treating cancer, it is of importance to have a fundamental understanding of the molecular differences between tumor endothelial cells (EC) and their normal counterparts. Since angiogenesis is not limited to pathological conditions, careful evaluation of the putative targets is warranted to prevent side effects associated with impaired normal physiological angiogenesis. Gene expression profiling techniques are widely used to detect changes in transcript expression levels to study molecular events in biological processes and to identify tissue or tumor endothelium specific markers. Different cell culture models have been developed to study angiogenesis, but the temporal and spatial complex actions of all factors exerting effect on EC *in vivo* may not be accurately reflected in cultured EC [3]. Gene expression analysis of tumor EC can be problematic because these cells are embedded in complex tissues and comprise only a small fraction of the cells present in a tumor.

To date, only a limited number of studies have characterized the gene expression profile of freshly isolated tumor EC [4-6]. SAGE tag repertoires were generated from EC isolated from both tumor and normal tissues and were compared to identify differentially expressed genes for the discovery of tumor endothelial markers. In these studies, however, gene expression associated with physiological processes was never directly taken into account. In addition, proof of successful targeting of tumor endothelial markers *in vivo* for cancer therapy has as yet never been provided.

In the present study we used suppression subtractive hybridization (SSH) to compare gene expression profiles of isolated EC from colon carcinoma tissues, non-malignant angiogenic placental tissues, and non-angiogenic normal tissues. We identified 17 tumor angiogenesis genes (TAGs) overexpressed in tumor endothelium as compared to both angiogenic and non-angiogenic endothelium. Four of these genes, encoding surface expressed or secreted proteins, i.e. vimentin, CD59, high-mobility group box 1 (HMGB1) and insulin-like growth factor binding protein 7 (IGFBP7), were selected for further investigation. Antibodies targeting these proteins inhibited angiogenesis both in *in vitro* and *in vivo* assays. We demonstrate the success of our approach by targeting one of these markers, i.e. vimentin, in a mouse tumor model, resulting in markedly inhibited tumor growth, accompanied with a reduced microvessel density. These results validate the used approach of target finding and suggest the applicability of the identified TAGs.

>> Materials and methods

Isolation of endothelial cells from fresh tissues

Colorectal carcinoma tissues (Dukes C, n=5) and distant normal colon tissues of the same patient (n=5) were obtained directly after surgery. Fresh placental tissues (n=5) were obtained from the department of Obstetrics (University Hospital Maastricht). EC were isolated by previously described methods [4], with minor modifications. Tissues were minced with surgical blades and digested for 30' with 1 mg/ml collagenase (Life Technologies) and 2.5 U/ml dispase (Life Technologies) at 37°C during continuous agitation. DNase I (Sigma) was added to a final concentration of 100 µg/ml and the cell suspension was incubated for another 30' prior to Ficoll Paque density gradient centrifugation (Amersham Biosciences). EC were immunolabeled with anti-CD31 (clones JC/70A, DAKO; and EN4, Monosan) and anti-CD34 antibodies (clone Qbend10, Novocastra) and isolated by positive selection using goat anti-mouse IgG coated paramagnetic beads (Dyna). The purity of the isolated EC fraction was assessed by immunofluorescence staining for endothelium specific von Willebrand Factor (rabbit polyclonal antibody, DAKO), and was estimated to be over 97%.

Cell culture

HUVEC were isolated and cultured as previously described [7] using standard cell culture materials (Greiner Bio-One). HUVEC were activated in RPMI-1640 (Life Technologies) supplemented with 20% human serum, 10% filter-sterile conditioned medium of the colorectal tumor cell line LS174T, 10% filter-sterile conditioned medium of the Caco-2 colorectal tumor cell line, 2 mM L-glutamine (Life Technologies), 50 ng/ml streptomycin (MP Biomedicals), 50 U/ml penicillin (MP Biomedicals), 1 ng/ml bFGF (Reliatech) and 10 ng/ml VEGF (Reliatech) until 80% confluence was reached. Quiescent EC were prepared by culturing in culture medium supplemented with low amounts (2%) of serum.

RNA isolation and cDNA synthesis

RNA was isolated using RNeasy Mini reagents (Qiagen) according to the manufacturer's instructions. RNA samples were pooled per 5 EC fractions and SMART[™] cDNA (BD Biosciences) was synthesized from the RNA and amplified for use in SSH.

Suppression subtractive hybridization (SSH)

SSH was performed with the PCR-Select[™] cDNA subtraction kit (BD Biosciences) according to the manufacturers' instructions. Subtractions were performed to create cDNA repertoires enriched for genes overexpressed in tumor EC (TEC) and for genes differentially expressed in activated and quiescent HUVEC. Subtracted cDNA repertoires were T/A cloned in pCR2.1 (Invitrogen) and introduced in TOP10 cells,

according to the manufacturers' instructions. Individual colonies were picked and grown overnight at 37°C in 2xTY bacterial medium (BD Biosciences) supplemented with 10 µg/ml ampicillin (Roche Applied Science).

Differential screening

Inserts were amplified using the adaptor specific primers Nested 1 and Nested 2R (BD Biosciences) using HotGoldstar Taq polymerase (Eurogentec). PCR products were spotted in duplicate onto nylon membranes (Eurogentec) and hybridized to ³³P-dCTP (Amersham) labeled cDNA probes derived from colon tumor EC (TEC), normal colon EC (NEC), placenta EC (PLEC), activated and quiescent HUVEC. Membranes were exposed to phosphor screens (Kodak) and images were acquired using the Personal FX phosphorimager (Bio-Rad) at a resolution of 50 µm and analyzed as Tiff files using Quantity One software (Bio-Rad). All experiments were performed two times.

Data was processed in MS Excel to identify differentially expressed transcripts. Pair-wise comparisons were performed between duplicate filters hybridized with different probes. Duplicate spots showed excellent concordance ($R^2 > 0.99$, data not shown) and were averaged. Average spot intensities were included in the analysis when expression was at least 2.5 times background in any experiment. Spot intensities were normalized for total intensity of the filters under comparison. Gene expression ratios were calculated using the average normalized intensities for each spotted insert cDNA. Hierarchical clustering analysis was performed with Cluster 3.0 [8] and visualized using TreeView (Michael Eisen, University of California at Berkeley, CA).

Sequencing and database searching

Inserts of selected clones were sequenced using BigDye™ Terminator Cycle Sequencing mix (Applied Biosystems) and analyzed on a 3100 Genetic Analyzer (Applied Biosystems; Genome Center Maastricht). Homology searches were performed using NCBI nucleotide-nucleotide Blast (blastn) algorithm on the combined GenBank/EMBL/DDBJ non-redundant (nr) and expressed sequence tags (EST) databases (<http://www.ncbi.nlm.nih.gov/blast/>). Functional annotation of the genes was determined using public GeneOntology databases.

Real-time quantitative PCR

SYBR green assays were performed using 10 ng cDNA template per reaction, consisting of 1x SYBR Green Master Mix (Applied Biosystems) and 200 µM of each primer (Sigma Genosys) (Table 3.1). Reactions were run and analyzed on the ABI7700, performed in triplicate and analyzed using SDS software (Applied Biosystems). All experiments were normalized for cyclophilin A transcript expression to account for variations in template input as previously described [9].

Table 3.1: Amplification primers for TAGs

TAG	Gene ID	Accession No	Forward 5'-3'	Reverse 5'-3'	Length
TAG-1	ARPC2	NM_152862.1	GCAACTGAAGGCTGGAACA	TGAAGAGGCGCAACATTTAA	62
TAG-3	CD59	NM_203330	AGTGGGTGAATGTGGTTATGGCC	CTGGCACTGCTCAGGATGTCTTC	85
TAG-4	CDK2AP1	NM_004642.2	GCTGGCCATCATTGAAGAGCT	TCTCCATGGCACTCTTGCTCC	71
TAG-5	Col1A1	NM_000088.2	GAGATCGAGATCCGCGC	TGCAGCCATCGACAGTGAC	64
TAG-7	Col4A1	NM_001845.2	GGCACCCCATCTGTTGATCAC	GGTAAAGAATTTTGGTCCCAGAA	94
TAG-21	HMGB1	NM_002128	TCTAAGAAGTGCTCAGAGAGGTG	TTCATTCTCTTTCATAACGGG	104
TAG-23	EST	BC041913	CAAAATTCAGTGGCAAGCGGA	GGTGTGCCCTTTAATGCAGCTT	105
TAG-25	HEYL	NM_014571	TCTGAGCTGCCCTTCACCAC	ACGTGCCCTTCACATATGAGCCAG	122
TAG-27	EST	NM_017994	ATTTTGTGCCGAGAAGGTGGC	AGCAGGCAAGGATTATGGTTCTC	93
TAG-28	HSIRPR	X02160	TTCTCAAGGGTGCGAGCTCATC	TCCTCCCTTGGCCACCAATG	138
TAG-29	IGFBP7	NM_001553	AAGGGGTCACTATGGAGTTCAAA	GGCACTCATATTCTCCAGCATCT	153
TAG-30	LDHB	NM_002300	TGGGCTATTGGATTAAGTGTGGC	TTGACACGGGATGAATCCTGG	76
TAG-31	PPAP2B	CV337080	TGGGGAGAATCACATTGGGTC	ATGGCTTCAGAGCTGGTCATGG	111
TAG-32	PHC3	AJ320486	TGCGAGATGAATTCAGAGCACAG	TTGATGCGTGACAGATCTTCAG	123
TAG-33	SPARC	NM_003118	GCACCACCCGCTTTTTTC	GATCCTTGTCGATATCCTTCTG	108
TAG-38	HSPC014	AF077200	CATTCAGGGTCTATTTGCTCCG	GAAGACGCTGAACCTGCTGC	71
TAG-39	VIM	X56134	ACACACTCAGTGCAGCAATATAT	GGAGTGTCGGTTGTTAAGAACTA	152

Immunohistochemistry

Colon carcinoma and normal colon tissue sections were stained with mouse anti-human CD31 (clone JC70/A, DAKO), mouse anti-human vimentin (clone V9, DAKO), mouse anti-human CD59 (clone MEM-43, Chemicon), rabbit anti-human HMGB1 (kind gift of Dr. R.G. Roeder, The Rockefeller University, New York, NY), and rabbit anti-human IGFBP7 (kind gift of Dr. R. Rosenfeld, Oregon Health and Sciences University, Portland, OR). Primary antibodies were diluted in phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA) and detected with peroxidase conjugated rabbit anti-mouse IgG (DAKO) or goat anti-rabbit IgG (DAKO). Color was developed using DAB according to standard protocols.

FACS analysis

Single cell suspensions of fresh colorectal carcinoma and normal colon tissues were obtained as described above and fixed in 1% paraformaldehyde. EC were stained with a PE-labeled anti-CD31 antibody (Serotec) and separated from other cells by cell sorting (BD FACSaria; BD Biosciences). CD31 positive cells were subsequently stained using the following antibodies, diluted in PBS, 0.5% BSA: rabbit anti-vimentin (kind gift of Dr. F.C.S. Ramaekers, Maastricht University, Maastricht, The Netherlands), rabbit anti-IGFBP7 and rabbit anti-HMGB1 followed by biotinylated swine anti-rabbit IgG (DAKO) and streptavidin-FITC (DAKO).

In vitro sprouting

Sprouting of EC was studied in the tube formation assay. Cytodex-3 beads were overgrown with bovine capillary EC (BCE) and transferred to a 3D gel, as described previously [7]. Antibodies dialyzed to PBS were added to the collagen gel and culture medium at the described concentrations. A non-relevant control anti-c-myc antibody was included as control (9E10; Roche). Beads with cells were incubated for 48 hours, after which photographs were taken. Five concentric rings were projected over the photographs, and the number of intersections of rings and sprouting EC was determined digitally and used as a measure of *in vitro* tube formation.

Chick chorioallantoic membrane (CAM) assay

Fertilized white leghorn chicken eggs were used to monitor vessel development in the CAM as described previously [7]. Antibodies were dialyzed to 0.9% NaCl and administered at the indicated concentrations in a volume of 65 μ l for four consecutive days. On day 14, the CAMs were photographed. Quantification of microvessels was performed as described for the sprouting assay.

Mouse tumor model

Female athymic Swiss nu/nu mice were used and randomly split in four groups. All experiments were approved by the University of Minnesota Research Animal Resources ethical committee. Mice (n=6 per group) were inoculated with 10^6 LS174T colorectal carcinoma cells in 100 μ l RPMI subcutaneously in the right flank. Four days post-inoculation, when established and palpable tumors were present, treatment was started. Mice were treated by i.p. injections every third day with purified RV202 anti-vimentin antibody (10 mg/kg and 1 mg/kg; MUBio Products), isotype matched IIB5 anti-BrdU control antibody (10 mg/kg; MUBio) or saline alone. Tumor volume was determined daily by measuring the diameters of tumors using calipers and calculated as follows: $\text{width}^2 \times \text{length} \times 0.52$. Mice were weighed daily to assess possible toxic effects of the treatment. Cryosections (5 μ m) of excised tumors were stained for CD31 and microvessel density was evaluated as described previously [10]. Antibody targeted to the tissue *in vivo* was detected using biotinylated secondary goat anti-mouse antibodies (DAKO), and visualized with streptavidin-FITC.

Statistics

Results are expressed as mean $\pm$ SEM. Comparisons of values in the *in vitro* bioassays were assessed using the Student's t-test. For statistical analysis of the tumor growth curves, two-way ANOVA analysis was performed.

>> Results

Identification of tumor angiogenesis genes by SSH

We performed suppression subtractive hybridization (SSH) in combination with cDNA array screening to identify novel tumor specific EC markers in an unbiased manner. We isolated tumor EC (TEC) from a series of colon carcinoma tissues and patient-matched normal EC (NEC) from adjacent normal colon tissues, as well as from placental tissues (PLEC). These isolations were >97% pure and therefore perfectly suitable for gene expression analysis as has been demonstrated before [11]. RNA was isolated and used to create subtraction repertoires of genes overexpressed in TEC. In addition, HUVEC were stimulated *in vitro* with tumor-conditioned medium and used to create additional subtraction repertoires. Subtracted cDNA libraries comprising a total of 2746 cDNA sequences were arrayed and screened for differential expression in TEC, NEC, PLEC and HUVEC. Merely comparing gene expression differences between TEC and NEC may identify genes involved in angiogenesis in general, in addition to genes that represent a 'tumor signature' of the EC. To distinguish between these two groups, a comparison with PLEC - representing angiogenic EC - was included. Those genes that are overexpressed in TEC compared to both NEC and PLEC were considered to be tumor angiogenesis specific (Figure 3.1A).

By comparing expression profiles of TEC with NEC, PLEC, and HUVEC, we categorized the TEC overexpressed genes in three different subgroups. Firstly, we identified genes specific for tumor endothelium (tumor angiogenesis genes, TAGs). Forty-one transcripts classified as TAGs (Figure 3.1B, Table 3.2) and showed overexpression in TEC vs. NEC as well as in TEC vs. PLEC. Secondly, 85 transcripts were found to be upregulated in TEC vs. NEC, as well as in PLEC vs. NEC (general angiogenesis genes group A, GAG/A, Figure 3.1B, Table 3.2). Thirdly, we identified 24 upregulated transcripts in activated HUVEC vs. quiescent HUVEC, as well as in TEC vs. NEC, which we named GAG/B markers (Figure 3.1B, Table 3.2).

Table 3.2: Characteristics of EC gene expression identified by differential screening of SSH repertoires

Classification of tumor angiogenesis genes ^a		Spots ^e	Gene IDs ^f
TAG ^a	TEC > NEC and TEC > PLEC	41	17
GAG/A ^b	TEC > NEC and PLEC > NEC	85	46
GAG/B ^c	TEC > NEC and HUVEC+ > HUVEC-	24	20

^a Tumor angiogenesis associated genes

^b General angiogenesis genes (*in vivo* markers)

^c General angiogenesis genes (*in vitro* and *in vivo* markers)

^d TEC: Tumor endothelial cell; NEC: Normal endothelial cell; PLEC: Placental endothelial cell; HUVEC+: tumor-conditioned HUVEC; HUVEC-: quiescent HUVEC

^e Number of spots that showed at least 2-fold difference in expression in the indicated comparisons

^f Number of different genes representing differentially expressed spots

Sequence analysis revealed that the 41 TAG transcripts represented 17 different genes (Table 3.3). Five of these have previously been identified by SAGE analysis as overexpressed on tumor derived EC, validating our approach [4-6]. The highly abundant collagen $\text{I}\alpha 1$ and collagen $\text{IV}\alpha 1$ have been reported to be overexpressed on the endothelium of different tumors, including colon carcinoma, breast carcinoma and glioma [4-6], pointing towards the possible existence of pan-tumor endothelium specific transcripts. HEYL is a basic helix-loop-helix transcription factor that has recently been reported as upregulated in breast tumor vasculature [5]. SPARC and IGFBP7 have been classified as pan-endothelial markers and showed overexpression in EC isolated from colon carcinoma as compared to EC derived from normal colon [4].

Figure 3.1: Endothelial gene expression analysis.

A) Interrelationship between EC of different sources and their gene expression signatures. EC from a malignant and pro-angiogenic environment (TEC) are compared with EC from organ matched and patient matched non-malignant sources (NEC), and with non-malignant pro-angiogenic microenvironment (placenta) derived EC (PLEC) to identify the subset of genes that show expression induced by the tumor microenvironment specifically (tumor EC 'signature' markers). **B)** Venn diagram represents of subtraction repertoire screening. Four pair-wise comparisons were performed by cDNA array screening of SSH repertoires: tumor conditioned (HUVEC+) vs. quiescent HUVEC (HUVEC-), colorectal carcinoma EC vs. normal colon EC (TEC vs. NEC), colorectal carcinoma EC vs. placental EC (TEC vs. PLEC) and placental EC vs. normal colon EC (PLEC vs. NEC). Included are clones that showed at least a 2-fold difference in expression. The shaded area represents the TAGs (overexpressed in TEC vs. NEC and in TEC vs. PLEC). **C)** TAG markers are strongly biased towards genes associated with extracellular matrix remodeling. **D)** GAG/A markers (overexpressed in both TEC and PLEC vs. NEC) show a diverse functional profile. **E)** GAG/B markers (overexpressed in TEC and in activated HUVEC) are biased to protein turnover and transcriptional activity.

We identified two more TAG transcripts with proposed roles in angiogenesis. PPAP2B has been described as a gene that is upregulated during VEGF-stimulated tube formation of EC *in vitro* [12]. Very recently, the cytokine HMGB1 was recognized for its role in promoting angiogenesis *in vitro* [13]. The 10 remaining TAG markers have so far not been reported to functionally contribute to (tumor) angiogenesis, though some are described as being expressed in EC, e.g. vimentin and CD59 [14,15].

Gene expression of tumor EC resembles the profile of physiological angiogenesis

Further gene expression analysis revealed that the majority of TEC overexpressed transcripts [(18+67)/142 $\approx$ 60%] are also associated with angiogenesis under physiological conditions *in vivo*, and are therefore not specific for tumor angiogenesis (Figure 3.1B). These 85 GAG/A transcripts represent 46 different genes (Table 3.4), including genes that have been associated with angiogenesis such as MMPs [16], integrin β 1 [17] and endothelial cell specific molecule-1 [18].

Table 3.3: Tumor angiogenesis genes (TAGs)

TAG ^a	Gene ID ^b		Accession No ^c	Function ^d	No of spots ^e	Library origin ^f	
Genes previously associated with angiogenesis							
TAG-5	Collagen I α 1	Col1A1	NM_000088.2	Extracellular matrix	4		x
TAG-7	Collagen IV α 1	Col4A1	NM_001845.2	Extracellular matrix	16	x	x
TAG-21	High mobility group box -1	HMGB1	NM_002128.3	Extracellular matrix	1		x
TAG-25	Hairy/enhancer of split with YRPW motif	HEYL	NM_014571	Transcription	1		x
TAG-29	Insulin-like growth factor binding protein 7	IGFBP7	NM_001553	Extracellular matrix	1	x	
TAG-31	Phosphatidic acid phosphatase type 2B	PPAP2B	CV337080	Metabolism, cell maintenance	1		x
TAG-33	Secreted protein acidic, rich in cysteine	SPARC	NM_003118.1	Extracellular matrix	5		x
Genes with no previous association to angiogenesis							
TAG-1	Actin related protein 2/3	ARPC2	NM_152862.1	Cytoskeleton	2		x
TAG-3	CD59 antigen p18-20	CD59	NM_000611	Cell surface	1		x
TAG-4	CDK2-associated protein 1	CDK2AP1	NM_004642.2	Apoptosis, Cell cycle	1		x
TAG-23	IMAGE 5299642	EST	BC041913	Unknown	1	x	
TAG-27	IMAGE 4332094	EST	NM_017994.1	Unknown	1		x
TAG-28	Insulin receptor precursor	HSIRPR	X02160	Cell surface	1		x
TAG-30	Lactate dehydrogenase B	LDHB	NM_002300	Metabolism, cell maintenance	1	x	
TAG-32	Polyhomeotic like 3	PHC3	AJ320486	Unknown	1		x
TAG-38	Voltage gated K channel β subunit 4.1	HSPC014	AF077200	Unknown	1	x	
TAG-39	Vimentin	VIM	X56134	Cytoskeleton	3	x	x

^a TAG: Tumor angiogenesis gene

^c GenBank accession number

^e Number of spots that represented the TAG

^b Sequence identity

^d Functional classification of the reported TAG

^f SSH cDNA repertoire origin of the spots

In vitro endothelial cell activation has limited value for studying tumor angiogenesis

From our gene expression analysis, it is obvious that only a limited number of genes upregulated in TEC vs. NEC overlapped with genes overexpressed in tumor-conditioned HUVEC *in vitro* (GAG/B; Figure 3.1B, Table 3.4). Most of the GAG/B markers overlap with genes associated with physiological angiogenesis (GAG/A; Figure 3.1B, Table 3.4). In addition, hierarchical clustering analysis suggests that the overall expression pattern in the HUVEC model relates most to that emerging from physiological angiogenesis (i.e. the comparison between PLEC and NEC; Figure 3.2).

TAG markers are functionally classified as associated with late events of angiogenesis

We assigned a functional annotation to every gene, and analyzed the distribution of genes within different functional classes. TAG markers are predominantly biased towards genes associated with cytoskeletal and extracellular matrix remodeling, indicative of late events in the process of tumor angiogenesis. Genes associated with protein turnover and transcription, indicative of early events in the angiogenesis cascade, are underrepresented within TAG (Figure 3.1C). In contrast, the latter genes are present in the GAG/A class (Figure 3.1D). Similarly, GAG/B molecules are mainly involved in these early stages in the angiogenesis process (Figure 3.1E).

Figure 3.2: Hierarchical clustering of differential gene expression patterns in TEC, NEC, PLEC and HUVEC.

Hierarchical clustering analysis of expression ratios of the entire SSH repertoire. The dendrogram visualizes the results of hierarchical clustering analysis based on similarities in gene expression patterns of the different comparisons indicated to the right of the clustered image maps. Expression ratios are color-coded as indicated on the far right and shown for the indicated comparisons. Bars at the bottom indicate clustered regions containing the genes that confer a tumor 'signature' to EC (TAGs). The dendrogram at the left is an indicator of overall correlation between the comparisons shown in the rows. Note that the differential gene expression during physiological angiogenesis (PLEC vs. NEC) is most closely related to the differential gene expression in activated vs. quiescent HUVEC (H+ vs. H-).

Table 3.4: General angiogenesis genes (GAG/A and GAG/B)

Gene ID ^a		Accession No ^b	Class ^c	Function ^d	No of spots ^e	Library origin ^f HUVEC TEC
A kinase (PRKA) anchor protein 13 (AKAP13)	AKAP13	NM_007200	A	Signaling	1	x
Cathepsin B	CTSB	NM_147783.1	A	Extracellular matrix	11	x
Caveolin 1, caveolae protein	CAV1	NM_001753	A	Receptor	2	x
CD86 antigen	CD86	NM_175862	A	Surface antigen	1	x
cDNA FLJ32199	EST	AK056761	A	Unknown	1	x
Chemokine (C-C motif) ligand 2	CCL2	NM_002982	A	Signaling	1	x
dCMP deaminase	DCTD	NM_001921.1	A	Metabolism, cell maintenance	1	x
Defender against cell death 1	DAD1	NM_001344	A	Apoptosis, Cell cycle	1	x
EST10870 HUVEC	EST	AA296386	A	Unknown	2	x
Eukaryotic translation initiation factor 4A, isoform 1	EIF4A1	NM_001416	A	Protein turnover	1	x
FK506 binding protein 1A	FKBP1A	NM_000801	A	Signaling	1	x
FLJ37490	EST	AK094809.1	A	Unknown	2	x
Heme binding protein 1	HEBP1	NM_015987.2	A	Metabolism, cell maintenance	1	x
Hypothetical protein MGC 7036	MGC7036	NM_145058	A	Unknown	1	x
IMAGE 2028956	EST	AI793182	A	Unknown	1	x
IMAGE 2096486	EST	AI422919	A	Unknown	1	x
IMAGE 2816112	EST	AW269823	A	Unknown	1	x
Integrin beta 1	ITGB1	NM_002211	A	Receptor	1	x
Isoprenylcysteine carboxyl methyltransferase	ICMT	NM_170705.1	A	Metabolism, cell maintenance	1	x
Lung cancer oncogene 5	HLC5	AY117690.1	A	Unknown	1	x
Major histocompatibility complex, class II, DR alpha	HLA-DRA	BC032350	A	Surface antigen	24	x
Matrix metalloproteinase 1, interstitial collagenase	MMP1	NM_002421	A	Extracellular matrix	1	x
Matrix metalloproteinase 3, stromelysin 1	MMP3	NM_002422	A	Extracellular matrix	1	x
Pituitary tumor-transforming 1 interacting protein	PTTG1IP	NM_004339.2	A	Signaling	1	x
Rad51 associated protein	RAD51AP1	NM_006479	A	Metabolism, cell maintenance	1	x
Ribosomal protein L22	RPL22	NM_000983	A	Protein turnover	1	x
Tubulin, alpha 3	TUBA3	BC050637	A	Cytoskeleton	1	x
v-rat simian leukemia viral oncogene homolog B	RALB	BC018163	A	Signaling	1	x
Androgen-induced 1	AIG-1	BC025278	A,B	Unknown	1	x
ATP synthase H ⁺ transporting complex, subunit c	ATP5G1	NM_001575	A,B	Metabolism, cell maintenance	1	x
Diazepam binding inhibitor	DBI	M15887	A,B	Signaling	1	x
Ectonucleoside triphosphate diphosphohydrolase 1	ENTPD1	BC047664	A,B	Receptor	1	x
Endothelial cell-specific molecule 1	ESM1	NM_007036	A,B	Receptor	1	x
Eukaryotic translation elongation factor 1 epsilon 1	EEF1E1	NM_004280.2	A,B	Protein turnover	2	x
Heterogeneous nuclear ribonucleoprotein C C1/C2)	HNRPC	BC003394	A,B	Protein turnover	1	x
HSP90 alpha	HSPCA	NM_005348	A,B	Metabolism, cell maintenance	1	x
IMAGE 757234	EST	BX115183	A,B	Unknown	1	x
IMAGE 4182080, CARD8 homologue	CARD8	BC046136	A,B	Apoptosis, Cell cycle	1	x

KIAA0349, FLJ33555, Ubr1 homologue	KIAA0349	AB002347	A,B	Protein turnover	1	x
Matrix metalloproteinase 10, stromelysin 2	MMP10	NM_002425	A,B	Extracellular matrix	2	x
Mitochondrial ribosomal protein S27	MRPS27	BC011818	A,B	Protein turnover	1	x
Split hand/foot malformation (ectrodactyly) type 1	SHFM1	NM_006304	A,B	Apoptosis, Cell cycle	1	x
SRY (sex-determining region Y)-box 4	SOX4	NM_003107	A,B	Transcription	2	x
Thymosin, beta 4, X-linked	TMSB4X	NM_021109	A,B	Cytoskeleton	1	x
Ubiquitin conjugating enzyme E2L3	UBE2L3	NM_003347	A,B	Protein turnover	1	x
Zinc finger motif enhancer binding protein 2	ZNF644	NM_032186	A,B	Transcription	1	x
Platelet/endothelial cell adhesion molecule 1	PECAM1	NM_000442.2	B	Surface antigen	1	x
Ribosomal protein L21	RPL21	NM_000982.2	B	Protein turnover	2	x

^a Sequence identity

^b GenBank accession number

^c GAG class

^d Functional classification of the reported GAG

^e Number of spots that represented the GAG

^f SSH cDNA repertoire origin of the spots

Validation of TAGs

Overexpression of the 17 TAGs was confirmed using real-time quantitative PCR as a second independent validation technique. For 16 different genes (94%) overexpression in TEC vs. NEC was confirmed, also for 16 genes (94%) overexpression in TEC vs. PLEC was confirmed (Figure 3.3A). Taken together, 15 out of 17 (88%) genes were validated by RTQ-PCR to be TAG markers.

For subsequent studies of TAG markers at the protein level, we selected 4 different membrane associated or secreted molecules that may be feasible drug targets: (i) CD59 (TAG-3), a GPI membrane-anchored inhibitor of complement activation [19]; (ii) HMGB1 (TAG-21), a secreted cytokine as well as a non-histone DNA binding protein [20,21]; (iii) insulin-like growth factor binding protein 7 (IGFBP7, TAG-29), a secreted molecule with growth factor modulating function [22]; and (iv) vimentin (TAG-39), an intermediate filament protein that was recently demonstrated in macrophages to be actively secreted [23]. Immunohistochemical analysis in colorectal carcinoma and normal colon epithelium indicated that all four proteins were overexpressed on the tumor vasculature. While vimentin, IGFBP7 and CD59 were predominantly expressed in the endothelial compartment, HMGB1 was found to be expressed in stromal and epithelial cells as well (Figure 3.3B). Vimentin expression was detected in EC of both tumor and normal colon tissue, though heavily overexpressed on tumor endothelium. IGFBP7 expression in normal colon tissue was hardly detected, whereas tumor blood vessels showed abundant expression of IGFBP7. CD59 expression was mainly localized to vasculature, in particular to the luminal cell membrane (Figure 3.3B). This is in line with its reported expression as membrane protein with a role in protecting EC from complement-mediated lysis by binding complement proteins C8 and C9 to prevent the formation of the membrane attack complex [19]. HMGB1 staining was detected in EC, as cytoplasmic protein, but

also in epithelial cells, where the localization was predominantly nuclear. In addition, diffuse stromal staining was observed. Protein expression was much more abundant in colorectal tumor tissue compared to normal colon tissue, predominantly in the stromal compartment, consistent with a secretion product (Figure 3.3B) [24].

Since immunohistochemistry is a qualitative rather than a quantitative technique, we set out to determine the expression of these TAGs on freshly isolated EC of tumor and normal tissues by flowcytometry. We were able to quantitatively confirm the overexpression of vimentin, IGFBP7 and HMGB1 protein on colon tumor endothelium compared to normal colon endothelium (Figure 3.3C). Antibody species cross-reactivity prevented CD59 protein detection on freshly isolated EC by this method. The expression of CD31 did not differ between TEC and NEC (Figure 3.3C). These observations further support the value of these TAG proteins as tumor EC markers.

Figure 3.3: Expression validation of TAGs.

A) Transcriptional validation of TAG markers by quantitative real-time PCR. Expression ratios (mean $\pm$ SEM) in TEC vs. NEC (black bars) and TEC vs. PLEC (grey bars) normalized for cyclophilin A are shown. **B)** Immunohistochemical staining of CD31/CD34 and different TAGs in colorectal tumor and normal colon tissue sections. Scale bar represents 50 μ m. **C)** Protein expression levels (mean fluorescence intensity, $\pm$ SEM) of HMGB1, IGFBP7 and vimentin on tumor EC as compared to normal EC, assessed by flow cytometry.

Interference with TAG proteins inhibits angiogenesis *in vitro* and *in vivo*

To investigate whether the overexpression of the selected TAG markers is causally related to the process of angiogenesis, *in vitro* bioassays were performed. Antibodies directed against CD59, HMGB1, IGFBP7 and vimentin were tested for their effect on endothelial tube formation in an *in vitro* collagen gel based sprout formation assay. Antibodies directed against the latter three showed significant inhibitory effects on sprout formation *in vitro* whereas antibodies directed against CD59 had a limited effect. The control antibody did not show any effect (Figure 3.4A). These observations suggest that the targeted proteins are actively involved in the process of capillary tube formation *in vitro*.

To investigate whether these TAGs are involved in angiogenesis *in vivo*, the antibodies were tested in the chorioallantoic membrane (CAM) of the chick embryo. All four antibodies were checked for reactivity with CAM endothelium by immunohistochemistry. Three of the antibodies (directed against CD59, HMGB1 and vimentin) positively stained CAM blood vessels and were therefore usable in the CAM assay (data not shown). Similar to the sprout formation assay, antibodies against

Figure 3.4: Inhibition of *in vitro* and *in vivo* angiogenesis by targeting of TAG proteins.

A) Sprout formation (mean±SEM) of BCE in a 3D-collagen gel is inhibited by the addition of antibodies (10 µg/ml) directed against different cell surface and secreted TAGs. **B)** Angiogenesis in the CAM is inhibited by treatment with antibodies (10µg/ml) directed against the indicated TAGs. Control antibody used is 9E10 anti-myc antibody.

CD59, HMGB1 and vimentin inhibited angiogenesis by 27%, 45% and 40%, respectively, while a control antibody did not show any activity (Figure 3.4B). These results strongly suggest a role for these molecules in the process of angiogenesis and together with the overexpression on tumor endothelium support their potential for use in targeting of tumor vasculature as therapy against cancer.

In a subsequent experiment we provided evidence for the validity of our approach to identify endothelial targets that can be used for the treatment of cancer. Athymic mice bearing human LS174T colon carcinoma tumors were treated with the mouse-reactive RV202 mouse anti-vimentin antibody. This treatment resulted in a significant dose-dependent inhibition of tumor growth (Figure 3.5A). Since LS174T tumor cells do not express vimentin (Figure 3.5B, panels a and b), this effect was the result of targeting endothelial vimentin. The latter was supported by the reduced microvessel density (MVD) in the RV202 treated tumors (Figure 3.5B, panels e and f), as compared to MVD in mice treated with control antibody or saline (Figure 3.5B, panels c and d; Figure 3.5C). Furthermore, we showed the binding of injected RV202 antibody to the tumor vasculature (Figure 3.5E), suggesting that activity is mediated through EC vimentin. Treatment with anti-vimentin antibody did not induce toxicity, as determined by normal weight gain (Figure 3.5D) and behavior.

>> Discussion

In the present study, we describe the identification of tumor angiogenesis genes (TAGs) that are overexpressed in tumor EC, also when compared to physiologically activated placental EC. From the series of 17 TAGs we identified several different genes that encoded membrane-bound or secreted proteins. Four of these were selected to investigate their role in angiogenesis and to serve as tumor endothelial target for therapeutic applications. We demonstrated that all four proteins are necessary in the process of angiogenesis and provided proof that TAGs can be used for intervention in angiogenesis and tumor growth using antibodies as a treatment opportunity. A crucial element in designing anti-angiogenic and vascular targeting approaches is the identification of specific target molecules. Some tumor endothelial cell associated markers have been identified [4-6,25,26], but translation to the clinic remains an obstacle. Since angiogenesis is not limited to pathological conditions, careful evaluation of putative targets is warranted to prevent side effects associated with impaired physiological angiogenesis. The followed subtraction strategy provided us with genes that are overexpressed on tumor endothelium but this does not exclude the possibility of expression on other endothelial cells as well. We demonstrated in a mouse tumor model that overexpression on tumor EC provides a therapeutic window that can be used to treat cancer. Alternatively, the used approach also does not exclude the identification of markers that are expressed by tumor cells. It is interesting to speculate that this may even be considered an advantage for therapeutic

application. Finally, it may be argued that a target can be expressed by other (vascular) cells (e.g. pericytes). Although, the latter scenario applies for this situation too, we demonstrate in the mouse model that injected (anti-vimentin) antibody definitely targets the tumor EC (Figure 3.5E).

Vimentin is an extensively studied intermediate filament protein (reviewed by Hendrix et al. [27]) which has also been described as a target gene of HIF-1 α , a major

Figure 3.5: Inhibition of tumor growth by targeting tumor endothelial vimentin.

A) Tumor growth curves of LS174T human colon carcinoma tumor xenografts in nude mice, treated with vehicle, IIB5 anti-BrdU isotype matched control antibody (10 mg/kg) or RV202 anti-vimentin antibody (10 mg/kg and 1 mg/kg). Antibodies were administered every 3 days i.p. for a period of 12 days. A dose-dependent inhibition of tumor growth is evident in mice treated with anti-vimentin antibody (1 mg/kg, ** $p < 0.0001$; 10 mg/kg, ** $p < 0.0001$), whereas treatment with the isotype control antibody did not show inhibition of tumor growth (IIB5 10 mg/kg, $p = 0.661$). **B**) Immunohistochemical staining of LS174T tumor xenografts in mice with CD31 (a) and anti-vimentin antibody RV202 (b) show that vimentin expression is restricted to the endothelium. Microvessel staining with phycoerythrin-labeled CD31 antibody in control mice (c), isotype control antibody treated mice (d), RV202 (1 mg/kg/treatment) (e) and RV202 (10 mg/kg/treatment) (f). **C**) Quantification of microvessel density was assessed digitally (** $p < 0.001$, Student's t-test). **D**) Body weight of mice during treatment, indicating absence of toxicity. **E**) Detection of treatment antibodies targeted to the tumor endothelium. Mouse antibodies were detected (green fluorescence) in mice treated with saline (a), isotype control antibody (b), RV202 (1 mg/kg/treatment) (c) and RV202 (10 mg/kg/treatment) (d). Endothelial cells are stained with phycoerythrin-labeled anti-CD31 antibody in red. Yellow color indicates co-localization. Scale bar = 25 μ m.

inducer of VEGF [28]. In different tumor cell lines, vimentin expression has been linked to increased invasiveness [29,30]. We have shown quantitative data on the overexpression of vimentin on EC in colon tumor samples compared to normal colon samples, both at the transcriptional level (Figure 3.3A) and the protein level (Figures 3.3B and 3.3C). These results suggest a contribution of this protein to the tumor endothelial phenotype. We present evidence that targeting of vimentin by means of antibodies clearly inhibits angiogenesis both *in vitro* and *in vivo*. In a mouse tumor model, an almost full abrogation of tumor growth was observed. Due to the absence of vimentin on tumor cells in this model (Figure 3.5B, panel b), and the demonstration of homing of injected antibody to the tumor EC (Figure 3.5E, panels c and d), this was suggested to be caused by a direct effect on tumor angiogenesis. The latter was supported by the marked inhibition of microvessel density. An interesting and as yet not fully elucidated aspect of vimentin is its presumed extracellular localization that makes it targetable using antibodies. Vimentin is an abundant intermediate filament protein in cells of mesenchymal origin. However, it has been shown to be actively secreted in macrophages and to be functionally involved in phagocytosis of bacteria [23]. One might speculate that in tumor endothelium vimentin is associated with integrins [31,32] that mediate its externalization. This cell biological aspect of vimentin awaits further study.

Although HMGB1 was originally identified as a non-histone DNA binding molecule [20], more recently focus has shifted towards its role as a secreted cytokine. As an extracellular protein, it has been involved in the regulation of cell migration [33], tumorigenesis [34], cell activation [21], inflammation [35] and sprouting of EC [13]. Given its role in positive feedback mechanisms in endothelial cell activation, cell migration and invasion, targeting HMGB1 as a means of anti-angiogenic therapy is promising. Here we demonstrated that an antibody directed against HMGB1 was effective at inhibiting endothelial cell sprouting *in vitro* as well as angiogenesis *in vivo*.

CD59 is a GPI anchored membrane protein and an inhibitor of complement activation [19]. Cells overexpressing CD59 show resistance to antibody-mediated immunotherapy when complement activation is compromised [19]. Neutralizing CD59 function can improve tumor immunotherapy [36]. Therefore, theoretically, anti-CD59 antibodies may have dual efficacy *in vivo*, both by inducing complement upon antibody binding and by inhibiting CD59 as a complement inhibitory protein. Complement activation does not apply *in vitro*, which may explain our result that antibodies directed against CD59 were not readily effective in our *in vitro* assays. It may well be that targeting of CD59 *in vivo* is more successful [37]. Indeed, in the *in vivo* CAM assay we observed a small but significant effect of anti-CD59 antibodies on vessel formation.

IGFBP7 is a secreted protein that accumulates in the basement membrane, where it can bind collagens type II, IV and V, heparan sulfates and different cytokines [22,38]. By binding these collagens, it supports the organization of EC into tube-like structures. In addition, IGFBP7 induces prostacyclin secretion from EC, a potent vasodilator that may mediate metastatic spread of tumors [22]. In summary, there is ample evidence

for an important function of IGFBP7 in tumor blood vessels. Indeed, we showed that overexpression of IGFBP7 in tumor endothelium was evident both at the transcriptional level and at the protein level. In addition, targeting IGFBP7 with an antibody clearly inhibited endothelial sprouting *in vitro*, possibly caused by inhibition of the interaction between IGFBP7 and collagens present in the three-dimensional culture matrix.

The extensive bias towards genes functioning in extracellular matrix remodeling among the TAG markers (Figure 3.1C) was also evident in published SAGE data sets of isolated tumor EC that were only compared to normal EC. Genes supposedly playing a role in the initiation of angiogenesis are only rarely identified in gene expression profiling of EC derived from tumors [3-6]. In contrast, gene expression profiling of growth factor stimulated EC and of three-dimensional EC cultures show biases to early events such as cell turnover and cell adhesion, respectively (reviewed by van Beijnum et al. [3]), and can be paralleled with the functional gene distribution in GAG/B and GAG/A, respectively (Figures 3.1D and 3.1E). These differences in global gene expression profiles in EC cultured *in vitro* as compared with isolated EC are likely influenced by differences in microenvironment and duration of stimuli *in vitro* vs. *in vivo*. Therefore, extrapolation of data generated by *in vitro* experiments to the *in vivo* situation may be limited for it appears difficult to accurately mimic *in vitro* the complex temporal and spatial actions present *in vivo*, emphasizing the importance of approaches that make use of more relevant cell sources such as tissue derived cells.

The data presented here demonstrate the presence of genes that show differential expression in tumor angiogenesis compared to physiological angiogenesis which may be sufficient to create a therapeutic window for cancer treatment without inducing significant side effects associated with interference in physiological angiogenesis. The successful targeting of tumor endothelium with antibodies directed at one such TAG, vimentin, and the subsequent inhibition of tumor growth underscores the power of our approach and holds promise for the therapeutic development of the remaining TAGs.

>> Acknowledgements

We thank Susan Joosten and Mat Rousch for technical assistance with endothelial cell selections. This work was supported by a grant from the Research Institute for Growth and Development (GROW), Maastricht University. The study has been financially supported by the sixth EU Framework Programme (Integrated Project 'Angiotargeting'; contract No. 504743) in the area of 'Life sciences, genomics and biotechnology for health'.

>> References

- [1] Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* 2000;52 (2):237-68.
- [2] Folkman J. Tumor angiogenesis. In: Holland JF, Frei B, Bast RC Jr., Kufe DW, Pollock RE, Weichselbaum RR, editor. *Cancer Medicine*, 5th Edition. Ontario, Canada: B.C. Decker Inc., 2000. pp. 132-52.
- [3] van Beijnum JR, Griffioen AW. In silico analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles. *Biochim Biophys Acta* 2005;1755 (2):121-34.
- [4] St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, et al. Genes expressed in human tumor endothelium. *Science* 2000;289 (5482):1197-202.
- [5] Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, et al. Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Res* 2004;64 (21):7857-66.
- [6] Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Walter-Yohrling J, et al. Vascular gene expression in nonneoplastic and malignant brain. *Am J Pathol* 2004;165 (2):601-8.
- [7] van der Schaft DW, Toebes EA, Haseman JR, Mayo KH, Griffioen AW. Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood* 2000;96 (1):176-81.
- [8] de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics* 2004;20 (9):1453-4.
- [9] Thijssen VL, Brandwijk RJ, Dings RP, Griffioen AW. Angiogenesis gene expression profiling in xenograft models to study cellular interactions. *Exp Cell Res* 2004;299 (2):286-93.
- [10] Dings RP, Yokoyama Y, Ramakrishnan S, Griffioen AW, Mayo KH. The designed angiostatic peptide angioex synergistically improves chemotherapy and antiangiogenesis therapy with angiostatin. *Cancer Res* 2003;63 (2):382-5.
- [11] Szaniszló P, Wang N, Sinha M, Reece LM, Van Hook JW, Luxon BA, Leary JF. Getting the right cells to the array: Gene expression microarray analysis of cell mixtures and sorted cells. *Cytometry A* 2004;59 (2):191-202.
- [12] Humtsoe JO, Feng S, Thakker GD, Yang J, Hong J, Wary KK. Regulation of cell-cell interactions by phosphatidic acid phosphatase 2b/VCIP. *Embo J* 2003;22 (7):1539-54.
- [13] Schlueter C, Weber H, Meyer B, Rogalla P, Roser K, Hauke S, Bullerdiek J. Angiogenetic signaling through hypoxia: HMB1: an angiogenetic switch molecule. *Am J Pathol* 2005;166 (4):1259-63.
- [14] Brooimans RA, Van der Ark AA, Tomita M, Van Es LA, Daha MR. CD59 expressed by human endothelial cells functions as a protective molecule against complement-mediated lysis. *Eur J Immunol* 1992;22 (3):791-7.
- [15] Obermeyer N, Janson N, Bergmann J, Buck F, Ito WD. Proteome analysis of migrating versus nonmigrating rat heart endothelial cells reveals distinct expression patterns. *Endothelium* 2003;10 (3):167-78.
- [16] Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol* 2001;21 (7):1104-17.
- [17] Senger DR, Perruzzi CA, Streit M, Kotliansky VE, de Fougerolles AR, Detmar M. The alpha(1)beta(1) and alpha(2)beta(1) integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. *Am J Pathol* 2002;160 (1):195-204.
- [18] Aitkenhead M, Wang SJ, Nakatsu MN, Mestas J, Heard C, Hughes CC. Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: induction of ESM-1, (beta)1g-h3, and NrCAM. *Microvasc Res* 2002;63 (2):159-71.
- [19] Gelderman KA, Tomlinson S, Ross GD, Gorter A. Complement function in mAb-mediated cancer immunotherapy. *Trends Immunol* 2004;25 (3):158-64.
- [20] Goodwin GH, Sanders C, Johns EW. A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. *Eur J Biochem* 1973;38 (1):14-9.
- [21] Treutiger CJ, Mullins GE, Johansson AS, Rouhiainen A, Rauvala HM, Erlandsson-Harris H, Andersson U, Yang H, Tracey KJ, Andersson J, et al. High mobility group 1 B-box mediates activation of human endothelium. *J Intern Med* 2003;254 (4):375-85.
- [22] Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K, Miyazaki K. Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. *Proc Natl Acad Sci U S A* 1996;93 (16):8384-9.
- [23] Mor-Vaknin N, Punturieri A, Sitwala K, Markovitz DM. Vimentin is secreted by activated macrophages. *Nat Cell Biol* 2003;5 (1):59-63.

- [24] Huttunen HJ, Rauvala H. Amphoterin as an extracellular regulator of cell motility: from discovery to disease. *J Intern Med* 2004;255 (3):351-66.
- [25] Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. *Nature* 1996;380 (6572):364-6.
- [26] Ruoslahti E, Rajotte D. An address system in the vasculature of normal tissues and tumors. *Annu Rev Immunol* 2000;18:813-27.
- [27] Hendrix MJ, Seftor EA, Chu YW, Trevor KT, Seftor RE. Role of intermediate filaments in migration, invasion and metastasis. *Cancer Metastasis Rev* 1996;15 (4):507-25.
- [28] Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res* 2003;63 (5):1138-43.
- [29] Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, Foidart JM. Transactivation of vimentin by beta-catenin in human breast cancer cells. *Cancer Res* 2003;63 (10):2658-64.
- [30] Singh S, Sadacharan S, Su S, Belldgrun A, Persad S, Singh G. Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer. *Cancer Res* 2003;63 (9):2306-11.
- [31] Maniotis AJ, Chen CS, Ingber DE. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc Natl Acad Sci U S A* 1997;94 (3):849-54.
- [32] Gonzales M, Weksler B, Tsuruta D, Goldman RD, Yoon KJ, Hopkinson SB, Flitney FW, Jones JC. Structure and function of a vimentin-associated matrix adhesion in endothelial cells. *Mol Biol Cell* 2001;12 (1):85-100.
- [33] Fages C, Nolo R, Huttunen HJ, Eskelinen E, Rauvala H. Regulation of cell migration by amphoterin. *J Cell Sci* 2000;113 (Pt 4):611-20.
- [34] Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature* 2000;405 (6784):354-60.
- [35] Fluza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, Suffredini AF. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 2003;101 (7):2652-60.
- [36] Golay J, Lazzari M, Facchinetti V, Bernasconi S, Borleri G, Barbui T, Rambaldi A, Introna M. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 2001;98 (12):3383-9.
- [37] Durrant LG, Spendlove I. Immunization against tumor cell surface complement-regulatory proteins. *Curr Opin Investig Drugs* 2001;2 (7):959-66.
- [38] Nagakubo D, Murai T, Tanaka T, Usui T, Matsumoto M, Sekiguchi K, Miyasaka M. A high endothelial venule secretory protein, mac25/angiomodulin, interacts with multiple high endothelial venule-associated molecules including chemokines. *J Immunol* 2003;171 (2):553-61.

Chapter 4

Interference with vimentin expression affects the angiogenic potential of tumor endothelial cells

Judy R. van Beijnum, Edith van der Linden, Bernadette M.M. Zwaans, Marjan Harmsma, Frans C.S. Ramaekers & Arjan W. Griffioen

>> Abstract

We have previously shown the specific overexpression of vimentin in tumor endothelial cells and the fact that targeting vimentin with antibodies inhibits tumor growth and angiogenesis. Here, we further examine the role of vimentin in endothelial cells. Flowcytometry of non-permeabilized endothelial cells suggests that a cell-surface associated pool of vimentin exists. Surface associated vimentin is inversely related to cell density and regulated by phosphorylation. We found that targeting surface associated vimentin with antibodies and downregulation by RNA interference impairs the ability of endothelial cells to migrate and to form capillary sprouts, while growth and cell viability were unaffected. Finally, antibodies directed against vimentin are capable of reducing vascular density in a model of tumor-induced angiogenesis on the chick chorioallantoic membrane. In conclusion, we provide novel insights in the role of vimentin in angiogenesis and provide proof of its feasibility as targeting molecule for the treatment of cancer.

>> Introduction

Angiogenesis is a hallmark of diverse pathologies such as cancer, rheumatoid arthritis and diabetic retinopathy [1,2]. Inhibiting the process is considered to be a promising treatment strategy. The identification of gene products specifically expressed on tumor endothelial cells amenable for targeting may aid in the development of novel molecular imaging approaches and therapeutic modalities for combating cancer.

We have recently identified a series of genes (tumor angiogenesis genes or TAGs) upregulated in tumor endothelial cells (TEC) compared to resting, normal endothelial cells (NEC) as well as physiologically activated placenta endothelial cells (PLEC) by suppression subtractive hybridization (SSH). We identified vimentin as a gene that was overexpressed in TEC compared to both NEC and PLEC, and hence defined it as a tumor angiogenesis gene (TAG) [3].

Vimentin is the major type III intermediate filament protein of mesenchymal cells, including endothelial cells (EC). It is an important structural protein involved in cytoskeletal organization and mechanical transduction of extracellular signals to the nucleus [4]. Expression of vimentin has been associated with different types of cancer. It is used as a tumor marker for the diagnosis of melanoma [5]. In addition, co-expression of cytokeratin and vimentin in epithelial tumors, epithelial-mesenchymal transition (EMT), is indicative of increased aggressiveness [6,7]. Recently, vimentin has been detected as a secreted protein in activated macrophages [8] and as a surface protein on T-lymphocytes [9,10] and neutrophils [11], which may point to a possible more elaborate role for vimentin in angiogenesis than thus far appreciated.

We further investigated the role of vimentin in different aspects of angiogenesis by targeting the protein with antibodies and by modulation of transcript levels with RNA interference and expression constructs. We show that cell survival and proliferation is dependent on the presence of vimentin and that migration and capillary sprouting of EC is mediated by extracellular vimentin. In addition, we show that targeting vimentin with different antibodies inhibits angiogenesis *in vitro* as well as *in vivo*. In summary, we provide evidence for an active role for vimentin in angiogenesis and show that interference with the function and expression of the protein affects the angiogenic potential of EC.

>> Materials and Methods

Cell culture and flowcytometry

Native HUVEC were obtained by perfusion of umbilical veins with trypsin, allowed to adhere 2 hours to gelatin-coated tissue flasks and harvested by trypsinization. HUVEC, EVL2 [12] and human microvascular EC (HMEC) were cultured as previously described [13]. Where indicated, okadaic acid (Merck) was added for 16 hours.

For flowcytometry, cells were fixed in 1% paraformaldehyde (Merck) for 20 minutes at room temperature or in 70% ethanol on ice for 20 minutes. Cells were incubated in 25 μ l reactions with primary antibodies (V9 [DAKO], RV202 [MUBio Products], CD31-PE [Serotec], rabbit polyclonal anti-vimentin [MUBio Products]), appropriately diluted in PBS/0.1% BSA/0.01% sodium azide. Primary antibodies were detected using biotinylated rabbit-anti-mouse IgG (DAKO) or swine-anti-rabbit IgG (DAKO) in combination with streptavidin-FITC (DAKO). Flowcytometry was performed on a FACS Calibur (BD Biosciences) and data were analyzed using CellQuest™ software.

Fresh normal colon and colon tumor tissues were obtained from excision surgery at the department of Pathology (University Hospital Maastricht). Tissues were minced with surgical blades, digested with 1 mg/ml collagenase (Life Technologies), 2.5 U/ml dispase (Life Technologies) and 100 μ g/ml DNase I (Sigma) at 37°C with continuous agitation prior to Ficoll Paque gradient density centrifugation (Amersham). EC were stained with a PE-labeled anti-CD31 antibody (Serotec) and separated from other cells by cell sorting (BD FACSAria).

Real-time quantitative PCR

EC were isolated from fresh colon tumor tissues, patient-matched normal colon tissues and placental tissues as previously described [3]. SYBR green assays were performed using 10ng cDNA template per reaction, consisting of 1x SYBR Green Master Mix (Applied Biosystems) and 200 μ M of each primer (Sigma Genosys,) as previously described [14]. Primers used for amplification of the vimentin transcript were 5'-ACACACTCAGTGCAGCAATATAT-3' and 5'-GGAGTGTCTGGTTGTTAAGAACTA-3'.

Vimentin expression constructs and transfection of endothelial cells

Expression constructs containing vimentin cDNA in the sense and antisense orientation were a kind gift of Dr Scott Su (Hamilton Regional Cancer Centre, Canada) [15]. Vimentin sense cDNA was excised from pCMV-Sport6 using *KpnI*/*NotI* (New England Biolabs) and religated in pcDNA3.1+-hygro (Life Technologies). Vimentin antisense cDNA was recloned to pcDNA3.1+-hygro using *HindIII*/*EcoRI* (NEB).

EVLC2 cells were transfected using Nucleofector technology (Amaxa). Briefly, 5×10^5 cells were harvested and resuspended in 100 μ l Nucleofector solution R. The cell suspension was mixed with 1 μ g DNA and transferred to an electroporation cuvette. Following transfection using program T20, 500 μ l filtered HUVEC medium was added and cells were transferred to 2 wells of a gelatin coated 24-wells cell culture plate. Successfully transfected cells were selected based on hygromycin resistance using 25 μ g/ml hygromycin (Life Technologies).

Proliferation assay

5×10^3 cells were seeded in 96-well cell culture plates coated with 0.2% gelatin (Merck) and allowed to adhere for 2 hours. Antibodies, dialyzed to PBS to remove

traces of azide, were added to the culture medium in the indicated concentrations. Cells were cultured for 72 hours. During the last 6 hours of the assay, the culture was pulsed with 0.5 μCi [methyl- ^3H]-thymidine (Amersham) per well. Activity was measured using liquid scintillation counting (Wallac LSC; PerkinElmer) [13].

Migration assay

5×10^3 cells were seeded in individual wells of gelatin coated 96-well cell culture plates and grown to confluence. Using a blunt pipette tip, a cross-shaped wound of approximately 750 μm wide was made in the well. Cells were washed with PBS, and fresh medium was added. Where appropriate, dialyzed antibody was added to the medium in the indicated concentrations. Wound width was measured in 4 predefined locations per well at T= 0, 2, 4, 6 and 8 hours [13].

Cell cycle analysis

Cells, including floating cells, were harvested and fixed in 70% ethanol. Cells were resuspended in DNA extraction buffer (45 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.5 mM citric acid, 0.1% Triton-X100, pH7.4) (Merck) and incubated at 37°C for 20 minutes. Propidium iodide (Merck) was then added to a final concentration of 2 $\mu\text{g/ml}$. Based on DNA content and scatter, cells were classified as dividing (G2/M phase), resting (G1/G0), apoptotic or necrotic [16].

In vitro angiogenesis assay

Sprouting and tube formation of EC were studied with the use of cytodex-3 beads overgrown with BCE in a 3D gel as previously described [16]. Where appropriate, antibodies dialyzed to PBS were added to the collagen gel and overlay medium in the described concentrations.

Cells were incubated 48 hrs, after which photographs were taken of the beads. Five concentric rings were projected over the photographs, and the number of intersections of rings and sprouting EC was determined and used as a measure of *in vitro* tube formation.

RNA interference

Small interfering RNA (siRNA) duplexes were obtained from Eurogentec, one targeting vimentin and one negative control. Cells were transfected using JetSi-ENDO (Eurogentec) according to the manufacturers' instructions. Briefly, 2500 HUVEC were seeded in a gelatin coated 96-well cell culture plate and allowed to adhere overnight. Medium was replaced with DMEM (Life Technologies) containing L-glutamine (Life Technologies). SiRNA-JetSi-ENDO complexes were made by first combining 0.2 μl JetSi-ENDO with 10 μl RPMI 1640 (Life Technologies) per well; this was incubated 20 minutes at room temperature to generate mix A. SiRNA duplexes were added to 10 μl RPMI-1640 to form mix B. Mix A was added to mix B and incubated at room

temperature for 30 minutes. Complex AB (20 μ l) was added dropwise to the cells and incubated 4 hours. Transfection medium was then replaced with normal medium and cells were grown for 72 hours prior to assaying. BCE were first grown on the cytodex beads as described above, treated with siRNA duplexes and grown for 48 hours prior to being embedded in the collagen gel.

Immunocytochemistry

HUVEC were cultured on gelatin coated glass coverslips in 24-well cell culture plates. Cells were fixed with 3% formaldehyde and incubated with anti-vimentin antibody clones V9 (DAKO) and RV202 (Mubio Products). Primary antibodies were visualized using FITC conjugated rabbit-anti-mouse IgG. Cells were subsequently permeabilized to stain nuclei with DAPI (Life Technologies) and actin filaments with phalloidin-TRITC (Life Technologies). Images were obtained using a Zeiss Axioscope fluorescence microscope (Zeiss).

Chick chorioallantoic membrane (CAM) assay

Fertilized eggs of Lohman-selected White Leghorns were incubated as previously described [13]. At day 10, a silicone ring was placed on the chorioallantoic membrane (CAM) to apply antibody treatment and transplant LS174T spheroids.

LS174T colorectal tumor cells were cultured in DMEM supplemented with 10% FCS and L-glutamine. Cells were seeded in non-adherent cell culture plates (Greiner Bio-One) at a density of 10^5 cells/ml for 10 days to allow spheroids to form. A small surface area (approximately 3x3mm) in the silicone ring placed on the CAM was denudated using lens paper and uniformly looking spheroids of 0.5-1 mm in diameter were applied on the CAM. Antibodies were dialyzed to 0.9% NaCl and administered in the indicated concentrations in a volume of 65 μ l for four consecutive days. At day 14, the CAMs were photographed. Five concentric rings were projected on the image. The number of intersections of rings and blood vessels was determined and used as a measure of vessel density.

Sections of the CAMs were excised, formalin-fixed and paraffin embedded. Tissue sections (5 μ m) were pretreated with xylene and rehydrated in a series of ethanol. Endogenous peroxidase activity was blocked using H₂O₂, and sections were boiled in citrate buffer (10mM, pH6.0) for antigen retrieval. Sections were incubated with RV202 and primary antibodies were detected with biotinylated rabbit anti-mouse (DAKO) in combination with streptavidin-HRP (DAKO). Antibody-binding was detected using DAB (Sigma).

Statistical analysis

Results are expressed as mean $\pm$ SEM and statistical significance was assessed using Student's t-test. Experiments were repeated at least three times. Representative examples are shown where applicable.

>> Results

Vimentin expression is induced in activated endothelial cells

Different studies have attempted to profile the gene expression changes associated with tumor angiogenesis *in vivo* (reviewed by van Beijnum et al. [17]). Major drawback of these studies was that they merely compared gene expression in tumor and normal EC, and did not take into account the gene expression changes associated with physiological angiogenesis. We approached this issue by simultaneously comparing gene expression profiles of tumor endothelial cells (TEC), isolated from fresh colon tumor tissues, with the gene expression profiles of normal, resting endothelial cells (NEC) isolated from fresh normal colon tissues, and with that of angiogenically activated placenta endothelial cells (PLEC) [3]. We performed SSH in combination with cDNA array screening and identified vimentin as a transcript that was overexpressed in colon TEC compared to NEC and angiogenic PLEC [3].

We validated the expression differences in these different endothelial cell sources by real-time quantitative PCR. Vimentin is significantly overexpressed in TEC as compared to both PLEC and NEC (Figure 4.1A). Vimentin expression on colon tumor and normal colon EC was assessed on CD31 positive cells sorted from single cell suspensions by FACS to determine whether the observed changes in mRNA levels

Figure 4.1: Vimentin expression in endothelial cells of different origins.

A) Vimentin mRNA expression was assessed by real-time quantitative PCR and normalized to cyclophilin A transcript levels. Endothelial cells (EC) were isolated from fresh colon tumor tissues (TEC), patient-matched normal colon tissues (NEC) and placental tissues (PLEC) using antibody-coated paramagnetic beads as previously described [3]. Vimentin mRNA expression is significantly higher in TEC compared to angiogenically activated PLEC and resting NEC. **B)** Vimentin protein expression on EC *in vivo* was assessed by flowcytometry of sorted EC from fresh colon tumor tissues and patient-matched normal colon tissues. CD31 expression did not differ between tumor EC and normal EC, whereas vimentin expression on tumor EC is significantly increased. **C)** Direct isolates of umbilical vein EC (native) were compared with HUVEC cultured in the presence of angiogenic additional growth factors (activated). Total vimentin expression was assessed by flowcytometry and shown to relate to the activation status of the EC.

were correlated to protein levels. Indeed, colon tumor EC expressed significantly more vimentin protein than normal colon EC whereas CD31 expression did not differ (Figure 4.1B), strongly suggesting tumor-driven induction of vimentin on EC *in vivo*. As HUVEC are frequently used in angiogenesis research, we investigated the expression of vimentin in cultured and native, directly isolated, HUVEC by flowcytometry. The expression of vimentin was significantly higher in HUVEC that had been cultured for several days when compared to HUVEC directly isolated from umbilical cords (Figure 4.1C), suggesting that increased vimentin expression is also associated with activation of EC *in vitro*.

To determine whether vimentin would be a relevant targeting candidate molecule on EC, we determined its accessibility for antibodies using different fixation methods and flowcytometry. Cells were either fixed with 1% paraformaldehyde to detect surface associated protein [18], or fixed with permeabilizing 70% ethanol to detect total cellular vimentin. We used two monoclonal antibodies directed against vimentin, clones V9 and RV202, and these showed comparable results on HUVEC (Figure 4.2A), EVLC2 and HMEC (data not shown). Flowcytometry shows that all EC abundantly express vimentin (Figure 4.2A, left panel), but that a percentage of the EC also has detectable vimentin when fixed with paraformaldehyde (Figure 4.2A, right panel), suggesting membrane-associated vimentin is present on EC.

We further investigated the expression of vimentin on EC grown in monolayers. In contrast to permeabilized, ethanol fixed cells that all displayed abundant vimentin intermediate filament staining throughout the cytoplasm (Figure 4.2B, panel a), in non-permeabilized formaldehyde fixed EC we observed membranous staining in a

Figure 4.2: Vimentin expression is detected on the surface of endothelial cells and is inversely associated with cell density.

A) EC were either fixed with 1% paraformaldehyde or with 70% ethanol. Vimentin expression was assessed by flowcytometry using two different anti-vimentin monoclonal antibody clones, V9 and RV202. A clear difference in immunoreactivity is observed. **B)** Immunofluorescence of HUVEC growing in monolayer. Abundant vimentin expression is detected in permeabilized cells (panel a), whereas a subpopulation of formaldehyde fixed cells showed membranous (panel b) as well as patchy staining of vimentin at the cell periphery (panel c). Scale bar = 10µm. **C)** Surface associated vimentin expression on EC is associated with cell density. Confluent, contact-inhibited EC express considerably less vimentin on their surface, measured by flowcytometry of 1% paraformaldehyde fixed cells, as compared to non-confluent, exponentially growing EC (left panels). Total cellular vimentin detected in 70% ethanol fixed cells by flowcytometry did not differ between confluent and non-confluent cells (right panels). **D)** Effect of okadaic acid treatment on vimentin expression. HUVEC were treated with different concentrations of the phosphatase inhibitor okadaic acid and cell surface associated vimentin was assessed by flowcytometry of paraformaldehyde fixed cells and increases with increasing okadaic acid concentrations (left y-axis), in contrast to 70% ethanol fixed cells (right y-axis). PF: 1% paraformaldehyde fixation; EtOH: 70% ethanol fixation.

subpopulation of cells (Figure 4.2B, panel b). In addition, a patchy staining was observed at the edges of the cells (Figure 4.2B, panel c). The observation that vimentin expression was increased in activated EC made us investigate whether surface associated vimentin expression would be related to cell density. EC grown in culture become contact inhibited once cell density has reached its maximum. Indeed, as shown by flowcytometry, vimentin antibody immunoreactivity of paraformaldehyde fixed cells was markedly increased in non-confluent, exponentially growing EC. In contrast, no difference in vimentin expression was detected in permeabilized cells (Figure 4.2C). Vimentin can be phosphorylated on serine by diverse protein kinases [19-21], and in macrophages this has been associated with increased secretion [8]. We tested whether surface accessible vimentin was dependent on protein phosphorylation. Okadaic acid is a phosphatase type 2A inhibitor and treatment of EC with okadaic acid increased the amount of vimentin detected by flowcytometry on paraformaldehyde fixed cells, but did not affect the total amount of vimentin (Figure 4.2D), suggesting surface associated expression might be related to vimentin phosphorylation status.

Knockdown of vimentin expression primarily inhibits migration and sprouting

To test the influence of vimentin expression levels in EC on their angiogenic properties *in vitro*, we stably transfected EVLC2 cells with expression vectors containing vimentin cDNA in either the sense or the antisense orientation [15] to increase or reduce vimentin expression, respectively (Figure 4.3A). Growth curves of the transfected cells indicated that EVLC2 harboring the antisense construct were not viable. Also, ectopically expressed vimentin did not stimulate the growth of the EC compared to non-transfected control or empty vector transfected cells (Figure 4.3B). This indicated that vimentin is essential for EC survival and growth, but that induced overexpression does not further contribute to these processes.

Figure 4.3: Transfection of full-length vimentin does not affect endothelial cell growth but antisense expression of vimentin is lethal.

A) EVLC2 cells were stably transfected with expression vectors expressing full-length vimentin cDNA in either the sense or antisense orientation under control of a CMV promoter. Expression of full-length vimentin constructs increased the expression of

vimentin as assessed by flowcytometry. **B)** Cell proliferation of transfected EVLC2 was assessed. Cell growth was to a limited extent affected by transfection of the vector. EC transfected with the anti-sense construct were not viable.

To circumvent problems associated with lethality after complete knockdown of vimentin by the antisense expression construct, we employed RNA interference to transiently knockdown vimentin expression in HUVEC. Different concentrations of siRNA duplexes were tested for their effect on vimentin protein expression, as effective knockdown is dependent on protein abundance and turnover dynamics, as well as on cell type [22]. Vimentin siRNA duplexes or control siRNA duplexes were introduced in HUVEC and vimentin expression was assessed by flowcytometry after 72 hrs on both ethanol fixed and paraformaldehyde fixed cells. Total vimentin expression was only reduced by 10% using 50nM siRNA duplex (Figure 4.4A), whereas vimentin detected on paraformaldehyde fixed cells was reduced by almost 60% (Figure 4.2B), suggesting that the surface associated vimentin fraction is primarily affected upon partial protein knockdown by RNA interference. Using higher concentrations of siRNA duplexes, both total cellular vimentin (Figure 4.4A) and surface associated vimentin (Figure 4.4B) were downregulated by approximately 50%. Proliferation of EC was significantly inhibited upon 50% reduction of total vimentin levels (Figure 4.4C). In addition, total cell death in HUVEC was significantly increased (Figure 4.4D). Treatment of HUVEC with 50 nM vimentin siRNA duplex, resulting in downregulation of the surface detectable but to a minimal extent total vimentin, did not affect the cell death or proliferation (Figures 4.4C and D), suggesting that a certain level of vimentin is required for EC viability. In contrast, a reduction in surface associated vimentin by 50 nM vimentin siRNA duplex already significantly decreased EC migration (Figure 4.4E), indicating that surface associated vimentin is critically involved in this process. Finally, capillary sprouting was also inhibited by 50nM vimentin siRNA duplex (Figure 4.4F). Taken together, these data point to an important role for surface associated vimentin in EC migration and capillary tube formation, but also indicate that threshold levels of vimentin expression are required for EC viability and survival.

Targeting vimentin inhibits angiogenesis in vitro and in vivo

The accessibility of vimentin for antibodies in non-permeabilized cells allows targeting of this protein in *in vitro* and in (pre-)clinical settings. We therefore investigated the effect of the presence of anti-vimentin antibodies on EC migration and sprouting. Both clone V9 (Figure 4.5A) and clone RV202 (Figure 4.5B) significantly inhibited HUVEC migration. A control IgG did not affect EC migration. The presence of V9 in the culture medium did not affect HUVEC proliferation as assessed by ³H-thymidine incorporation (Figure 4.5C). We further investigated the role of extracellular vimentin on angiogenesis in an *in vitro* angiogenesis model. Sprouting of BCE in a collagen gel was significantly inhibited by the presence of antibodies directed at vimentin (Figure 4.5D).

Figure 4.4: Inhibition of vimentin expression by RNA interference affects the angiogenic potential of endothelial cells.

HUVEC were transiently transfected with siRNA duplexes directed against vimentin. **A)** Vimentin expression was decreased with increasing vimentin siRNA duplex concentrations as determined by flowcytometry of ethanol (EtOH) fixed cells. **B)** Surface expression of vimentin on paraformaldehyde (PF) fixed cells was significantly decreased in response to low dose siRNA treatment. **C)** Downregulation of total vimentin but not surface associated vimentin leads to a decrease in the fraction of dividing cells (black bars). No effects of identical concentrations of non-relevant siRNA duplexes were observed (grey bars). **D)** Downregulation of total vimentin by siRNA (black bars) but not of surface associated vimentin leads to endothelial cell death. No significant effects of identical concentrations of non-relevant siRNA duplexes were observed (grey bars). **E)** Downregulation of vimentin surface expression by siRNA inhibits the capacity of EC to migrate into a wounded area. **F)** Capillary sprouting of EC was markedly inhibited by downregulation of surface associated vimentin. Control samples in A), B) and F) were treated with RPMI only. Non-relevant control duplex had no inhibitory effect on vimentin expression or on EC sprouting (data not shown).

To provide an *in vivo* model of tumor angiogenesis, human LS174T colon carcinoma cell line spheroids were transplanted onto the CAM. This resulted in a markedly induced vasculature with a distorted morphology (Figure 4.6A), whereas the vasculature in the CAM distant from the tumor appears normal (Figure 4.6B). Immunostaining of sections of CAMs with anti-vimentin antibodies shows specific staining of vessels in the membrane, as compared to a control antibody (Figures 4.6E and 4.6D, respectively). Anti-vimentin antibodies were capable of reducing LS174T spheroid induced vasculature (Figure 4.6C). CAMs treated with the antibody still show morphologically abnormal vasculature (Figure 4.6C, inset) but the vascular density is

reduced to approximately normal levels (Figure 4.6F). These data indicate that targeting vimentin by means of extracellularly applied antibodies reduced the angiogenic potential of EC *in vitro* and the tumor induced angiogenic response *in vivo*.

>> Discussion

Vimentin is the major intermediate filament protein of mesenchymal cells and its expression can be regulated by HIF-1 α [23], β -catenin/TCF [24], VEGF [25] and Twist [26]. Co-expression of vimentin and keratin (EMT) in diverse types of tumor cells has been associated with increased malignancy as a result of increased motility and invasiveness [6,7]. Vimentin expression was shown to be elevated in tumor EC compared to resting EC as well as physiologically activated EC, which suggests a functional contribution of this protein that is advantageous to tumor progression and/or survival. Downregulation of vimentin by an antisense expression construct proved lethal in EC, though upregulation by expression vectors did not affect cell growth (Figure 4.3B).

Figure 4.5: Targeting surface accessible vimentin with antibodies inhibits endothelial migration and sprouting.

A, B) Two different antibodies reactive with vimentin were added to wounded cultures of HUVEC. Migration into the wounded area was significantly inhibited by both V9 (A) and RV202 (B). **C)** Antibodies reactive with vimentin added to the culture did not affect endothelial cell viability and proliferation. **D)** Capillary sprouting in a 3D collagen matrix was dose-dependently inhibited by both anti-vimentin monoclonal antibodies.

Aggressive tumor cell lines tend to express more vimentin than non-aggressive tumor cell lines [15,27,28]. However, transfection of vimentin in non-aggressive, hormone-responsive LNCaP prostate cancer cells alone was not sufficient to increase invasiveness *in vitro*. In contrast, downregulation of vimentin by transfection of aggressive, hormone-refractory CL1 prostate cancer cells with a vimentin antisense expression construct inhibited migration and invasion *in vitro* [15]. In a model of EMT in breast carcinoma cells it was shown that vimentin is specifically induced in migrating epithelial cells and that vimentin expression levels were correlated with migration speed [29]. Again, transfection of these cells with a vimentin expression construct did not increase migration, whereas downregulation of vimentin reduced the migration speed of the cells [29].

Figure 4.6: Targeting vimentin with antibodies inhibits tumor angiogenesis in an *in vivo* model of the chick chorioallantoic membrane.

A) LS174T human colon tumor cell spheroids were grown on the chick CAM and markedly induced vasculature with a disorganized appearance. **B)** Untreated sections of the CAM distant from the growing tumor cells show a normal vascular density and morphology. **C)** Treatment of the CAMs with RV202 reduced the extent of the tumor-induced vasculature. Morphological examination of these vessels shows thin and tortuous vessels that are composed of loops (inset). **D, E)** Immunohistochemical staining of paraffin embedded tissue sections of CAMs show no staining in the control (D) whereas RV202 specifically immunostained vessels in the chick CAM (E). **F)** Treatment of LS174T tumors growing on CAMs significantly reduced vascular density to normal levels.

Our data indicate that EC express cell surface associated vimentin, as shown by both flowcytometry and immunofluorescence. Downregulation of this fraction by siRNA interfered with EC migration and capillary sprout formation but did not affect cell growth and survival. Marked downregulation of total cellular vimentin compromised cell proliferation and survival and was finally lethal, suggesting a threshold level of vimentin is required in EC. Thus, cell surface associated vimentin plays an important role in EC migration and sprouting, but does not influence EC viability. The presence of surface accessible vimentin on tumor EC makes this protein a putative targeting molecule. We have previously shown the success of targeting endothelial vimentin in a mouse tumor model [3]. Here, we showed that antibody mediated targeting of vimentin inhibited EC migration and tube formation but not proliferation *in vitro* and tumor-induced angiogenesis on the CAM *in vivo*. Thus, these results support a crucial role for cell surface accessible endothelial vimentin in angiogenesis. Indeed, recently it is becoming more and more apparent that vimentin expression is not restricted to the cytoplasm. The presence of vimentin reactive auto-antibodies in autoimmune diseases [30-32] and following transplantation [33] also suggests that certain pools of vimentin are accessible to the immune system, hence are present in the extracellular milieu. In fact, several immune effector cells have been shown to express vimentin at the cell surface, but its physiological significance is as yet unknown [9,11]. Interestingly, vimentin is actively secreted from activated macrophages, and involved in the process of phagocytosis of bacteria [8].

Surface associated vimentin is reduced upon contact inhibition of cells, and increased in the presence of phosphatase inhibitors, pointing towards a phosphorylation dependent process by which vimentin is presented on the cell surface. Phosphorylation of vimentin leads to reorganization of the intermediate filaments. Vimentin can be phosphorylated on serine residues by different kinases, e.g. p21 activated kinase (PAK), Rho kinase (ROK) α , protein kinase A (PKA), protein kinase C (PKC), and cell division cycle (CDC) 2 kinase [19-21,34]. CDC42 and Rac1 have been described to play a major role in EC migration via modulation of integrin $\alpha V\beta 3$ [35]. A downstream target of Rac1 is PAK, which promotes the formation of lamellipodia [19,20], a process that hence may involve vimentin phosphorylation. Interactions between vimentin and different integrins and its presence in focal adhesions have been demonstrated in different studies [36-38], and mediate the transduction of mechanical signals to the nucleus. In addition, ROK α increases the number of focal adhesion structures where integrins associate with the supporting extracellular matrix [19]. Vimentin filaments are wrapped around $\alpha V\beta 3$ integrins, in association with plectin, in so-called vimentin associated matrix adhesions (VMAs) in EC [37,38]. These VMAs were assembled in migrating cells and induced by growth factors, and partially disassembled in contact-inhibited cells [37]. Vimentin also interacts with $\alpha 2\beta 1$ integrins, again more pronounced in spread cells than in confluent cultures [36]. PKC ϵ -mediated phosphorylation of vimentin is involved in the recycling integrin $\beta 1$. During this process integrin $\beta 1$ is present in endosomal vesicles along with PKC ϵ and vimentin [34]. The

importance of vimentin organization by kinases was demonstrated by the observation that overexpression of vimentin in PKC ϵ -/- cells did not increase cell motility, in contrast to wild-type cells [34]. Recently, vimentin was shown to be involved in transendothelial migration of peripheral blood mononuclear cells (PBMC), and vimentin of both cell types was required for stabilization of the interaction [39]. Furthermore, lymphocytes of vim-/- mice had markedly reduced integrin β 1 levels and vim-/- endothelial cells expressed reduced levels of ICAM1 and VCAM1, suggesting the involvement of vimentin in positioning adhesion molecules for interactions [39]. One might speculate that vimentin may become cell surface exposed in EC as a consequence of modulating the expression and/or position of integrins and that this is dependent on phosphorylation by specific protein kinases. The reorganization of vimentin filaments and the redistribution of adhesion molecules contribute to an increased angiogenic potential. The N-terminal domain of vimentin can interact with negatively charged phospholipid bilayers [40], providing a putative anchor for the protein on the cell surface. However, the precise externalization of vimentin and its role in cell motility and angiogenesis awaits further study.

In conclusion, we have shown that vimentin expression is increased in angiogenically activated EC and that interference with surface associated vimentin fraction results in inhibition of endothelial migration and tube formation, as well as with tumor-induced angiogenesis *in vivo*. This surface accessibility and its functional contribution to tumor angiogenesis make it a valuable target for antiangiogenic anticancer therapeutics.

>> References

- [1] Folkman J. Tumor angiogenesis. In: Holland JF FEI, Bast RC Jr., Kufe DW, Pollock RE, Weichselbaum RR, editor. *Cancer Medicine*, 5th Edition. Ontario, Canada: B.C. Decker Inc., 2000. pp. 132-52.
- [2] Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* 2000;52 (2):237-68.
- [3] van Beijnum JR, Dings RPM, van der Linden E, Zwaans BMM, Ramaekers FCS, Mayo KH, Griffioen AW. Gene expression tumor angiogenesis dissected; specific targeting of tumor endothelium. Submitted.
- [4] Maniotis AJ, Chen CS, Ingber DE. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc Natl Acad Sci U S A* 1997;94 (3):849-54.
- [5] Huszar M, Halkin H, Herczeg E, Bubis J, Geiger B. Use of antibodies to intermediate filaments in the diagnosis of metastatic melanotic malignant melanoma. *Hum Pathol* 1983;14 (11):1006-8.
- [6] Hendrix MJ, Seftor EA, Chu YW, Seftor RE, Nagle RB, McDaniel KM, Leong SP, Yohem KH, Leibovitz AM, Meyskens FL, Jr., et al. Coexpression of vimentin and keratins by human melanoma tumor cells: correlation with invasive and metastatic potential. *J Natl Cancer Inst* 1992;84 (3):165-74.
- [7] Ramaekers FC, Haag D, Kant A, Moesker O, Jap PH, Voolijs GP. Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. *Proc Natl Acad Sci U S A* 1983;80 (9):2618-22.
- [8] Mor-Vaknin N, Punturieri A, Sitwala K, Markovitz DM. Vimentin is secreted by activated macrophages. *Nat Cell Biol* 2003;5 (1):59-63.
- [9] Boillard E, Bourgoin SG, Bernatchez C, Surette ME. Identification of an autoantigen on the surface of apoptotic human T cells as a new protein interacting with inflammatory group IIA phospholipase A2. *Blood* 2003;102 (8):2901-9.
- [10] Huet D, Bagot M, Loyaux D, Capdevielle J, Conraux L, Ferrara P, Bensussan A, Marie-Cardine A. SC5 mAb represents a unique tool for the detection of extracellular vimentin as a specific marker of Sezary cells. *J Immunol* 2006;176 (1):652-9.
- [11] Moisan E, Girard D. Cell surface expression of intermediate filament proteins vimentin and lamin B1 in human neutrophil spontaneous apoptosis. *J Leukoc Biol* 2005.
- [12] van Leeuwen EB, Veenstra R, van Wijk R, Molema G, Hoekstra A, Rutgers MH, van der Meer J. Characterization of immortalized human umbilical and iliac vein endothelial cell lines after transfection with SV40 large T-antigen. *Blood Coagul Fibrinolysis* 2000;11 (1):15-25.
- [13] van der Schaft DW, Dings RP, de Lussanet QG, van Eijk LI, Nap AW, Beets-Tan RG, Bouma-Ter Steege JC, Wagstaff J, Mayo KH, Griffioen AW. The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *Faseb J* 2002;16 (14):1991-3.
- [14] Thijssen VL, Brandwijk RJ, Dings RP, Griffioen AW. Angiogenesis gene expression profiling in xenograft models to study cellular interactions. *Exp Cell Res* 2004;299 (2):286-93.
- [15] Singh S, Sadacharan S, Su S, Belldgrun A, Persad S, Singh G. Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer. *Cancer Res* 2003;63 (9):2306-11.
- [16] van der Schaft DW, Toebe EA, Haseman JR, Mayo KH, Griffioen AW. Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood* 2000;96 (1):176-81.
- [17] van Beijnum JR, Griffioen AW. In silico analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles. *Biochim Biophys Acta* 2005;1755 (2):121-34.
- [18] Rouhiainen A, Kuja-Panula J, Wilkman E, Pakkanen J, Stenfors J, Tuominen RK, Lepantalo M, Carpen O, Parkkinen J, Rauvala H. Regulation of monocyte migration by amphoterin (HMGB1). *Blood* 2004;104 (4):1174-82.
- [19] Chan W, Kozma R, Yasui Y, Inagaki M, Leung T, Manser E, Lim L. Vimentin intermediate filament reorganization by Cdc42: involvement of PAK and p70 S6 kinase. *Eur J Cell Biol* 2002;81 (12):692-701.
- [20] Goto H, Tanabe K, Manser E, Lim L, Yasui Y, Inagaki M. Phosphorylation and reorganization of vimentin by p21-activated kinase (PAK). *Genes Cells* 2002;7 (2):91-7.
- [21] Ermakova S, Choi BY, Choi HS, Kang BS, Bode AM, Dong Z. The intermediate filament protein vimentin is a new target for epigallocatechin gallate. *J Biol Chem* 2005;280 (17):16882-90.
- [22] Chatterjee-Kishore M, Miller CP. Exploring the sounds of silence: RNAi-mediated gene silencing for target identification and validation. *Drug Discov Today* 2005;10 (22):1559-65.
- [23] Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res* 2003;63 (5):1138-43.

- [24] Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, Foidart JM. Transactivation of vimentin by beta-catenin in human breast cancer cells. *Cancer Res* 2003;63 (10):2658-64.
- [25] Yang AD, Camp ER, Fan F, Shen L, Gray MJ, Liu W, Somcio R, Bauer TW, Wu Y, Hicklin DJ, et al. Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells. *Cancer Res* 2006;66 (1):46-51.
- [26] Mironchik Y, Winnard PT, Jr., Vesuna F, Kato Y, Wildes F, Pathak AP, Kominsky S, Artemov D, Bhujwala Z, Van Diest P, et al. Twist overexpression induces in vivo angiogenesis and correlates with chromosomal instability in breast cancer. *Cancer Res* 2005;65 (23):10801-9.
- [27] Sommers CL, Walker-Jones D, Heckford SE, Worland P, Valverius E, Clark R, McCormick F, Stampfer M, Abularach S, Gelmann EP. Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. *Cancer Res* 1989;49 (15):4258-63.
- [28] Thompson EW, Paik S, Brunner N, Sommers CL, Zugmaier G, Clarke R, Shima TB, Torri J, Donahue S, Lippman ME, et al. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *J Cell Physiol* 1992;150 (3):534-44.
- [29] Gilles C, Polette M, Zahm JM, Tournier JM, Volders L, Foidart JM, Birembaut P. Vimentin contributes to human mammary epithelial cell migration. *J Cell Sci* 1999;112 (Pt 24):4615-25.
- [30] Vossenaar ER, Despres N, Lapointe E, van der Heijden A, Lora M, Senshu T, van Venrooij WJ, Menard HA. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res Ther* 2004;6 (2):R142-50.
- [31] von Mikecz A, Konstantinov K, Buchwald DS, Gerace L, Tan EM. High frequency of autoantibodies to insoluble cellular antigens in patients with chronic fatigue syndrome. *Arthritis Rheum* 1997;40 (2):295-305.
- [32] Franch A, Castellote C, Vila JL, Vilario S, Castell M. Anticytoskeletal autoantibody development in adjuvant arthritis. *J Rheumatol* 1994;21 (3):489-97.
- [33] Jonker M, Danskin A, Haanstra K, Wubben J, Kondova I, Kuhn EM, Rose M. The autoimmune response to vimentin after renal transplantation in nonhuman primates is immunosuppression dependent. *Transplantation* 2005;80 (3):385-93.
- [34] Ivaska J, Vuoriluoto K, Huovinen T, Izawa I, Inagaki M, Parker PJ. PKCepsilon-mediated phosphorylation of vimentin controls integrin recycling and motility. *Embo J* 2005;24 (22):3834-45.
- [35] Dormond O, Foletti A, Paroz C, Ruegg C. NSAIDs inhibit alpha V beta 3 integrin-mediated and Cdc42/Rac-dependent endothelial-cell spreading, migration and angiogenesis. *Nat Med* 2001;7 (9):1041-7.
- [36] Kreis S, Schonfeld HJ, Melchior C, Steiner B, Kieffer N. The intermediate filament protein vimentin binds specifically to a recombinant integrin alpha2/beta1 cytoplasmic tail complex and co-localizes with native alpha2/beta1 in endothelial cell focal adhesions. *Exp Cell Res* 2005;305 (1):110-21.
- [37] Gonzales M, Weksler B, Tsuruta D, Goldman RD, Yoon KJ, Hopkinson SB, Flitney FW, Jones JC. Structure and function of a vimentin-associated matrix adhesion in endothelial cells. *Mol Biol Cell* 2001;12 (1):85-100.
- [38] Tsuruta D, Jones JC. The vimentin cytoskeleton regulates focal contact size and adhesion of endothelial cells subjected to shear stress. *J Cell Sci* 2003;116 (Pt 24):4977-84.
- [39] Nieminen M, Henttinen T, Merinen M, Marttila-Ichihara F, Eriksson JE, Jalkanen S. Vimentin function in lymphocyte adhesion and transcellular migration. *Nat Cell Biol* 2006.
- [40] Perides G, Harter C, Traub P. Electrostatic and hydrophobic interactions of the intermediate filament protein vimentin and its amino terminus with lipid bilayers. *J Biol Chem* 1987;262 (28):13742-9.

A vertical strip on the left side of the page shows a microscopic image of tissue, likely a tumor, with a complex, irregular structure and varying shades of gray.

Chapter 5

High-mobility group box 1 (HMGB1) positively contributes to tumor angiogenesis

Judy R. van Beijnum, Edith van der Linden & Arjan W. Griffioen

>> Abstract

High-mobility group box 1 (HMGB1) was originally identified as a chromatin-binding molecule, though it has later been recognized as a cytokine. We identified HMGB1 to be overexpressed in tumor endothelial cells. By modulating expression and function we demonstrate that HMGB1 is involved in diverse aspects of the angiogenic process. Overexpression of HMGB1 in endothelial cells increased migration and MMP9 expression, whereas downregulation of HMGB1 by antisense cDNA resulted in an impaired response to angiogenic growth factors and decreased angiogenic sprouting. Furthermore, antibodies directed against HMGB1 inhibited endothelial migration and sprouting *in vitro* but did not impact growth factor induced proliferation. In the chick chorioallantoic membrane assay anti-HMGB1 antibodies were found to inhibit tumor-induced angiogenesis. The present results indicate a significant role in tumor angiogenesis and may identify HMGB1 as a potential target for anti-angiogenesis or vascular targeting anti-tumor therapy strategies.

>> Introduction

Angiogenesis is pivotal to processes where new tissue growth is required. In addition to the development and progression of cancer, new blood vessel growth is also involved in the development of other pathologies, including atherosclerosis and diabetic retinopathy. The concept of inhibiting angiogenesis as a treatment strategy for cancer has gained widespread interest over the last decades. To develop specific anti-angiogenic or vascular targeting agents, detailed insight in the phenotype of tumor endothelial cells (TEC) is required. We have recently conducted a gene expression survey to identify genes overexpressed in TEC compared to both resting, normal endothelial cells (NEC) and angiogenic placenta endothelial cells (PLEC), and identified high-mobility group box 1 (HMGB1) as a tumor angiogenesis gene (TAG) [1].

HMGB1 was first discovered as a chromatin binding protein [2]. It interacts with DNA and bends it, to facilitate interaction with diverse transcription factors, such as p53 [3]. Years later, it was identified as a membrane-bound protein that facilitates the outgrowth of neurites [4]. More recently, HMGB1 has been recognized as a late mediator of endotoxin lethality in mice [5] and many studies have focussed on its role as an inflammatory cytokine since. The primary receptor for HMGB1 is the Ig superfamily member called receptor for advanced glycation end products (RAGE), though it can also bind toll-like receptor (TLR) 2 and TLR4 [6]. Advanced glycation end products (AGE) are formed during periods of hyperglycemia in diabetic individuals and AGE-RAGE interactions contribute to vascular complications such as retinopathy and nephropathy in diabetes [7,8]. HMGB-RAGE signaling has been implicated in inflammation and cancer progression (reviewed by Bierhaus et al. [9]). HMGB1 is abundantly present in many cancers and blocking HMGB1-RAGE interactions by using anti-HMGB1, anti-RAGE and signaling-deficient RAGE molecules resulted in decreased tumor growth in a glioma model [10]. Here, we identify HMGB1 as an active player in tumor angiogenesis. HMGB1 expression is induced in TEC and tumor-conditioned HUVEC. Furthermore, interference with HMGB1 expression and function affects migration and sprouting of endothelial cells, and the proliferative response to angiogenic factors. Finally, we show that tumor angiogenesis *in vivo* is inhibited by anti-HMGB1 antibodies. The role of HMGB1 in the progression of cancer is hereby extended from a previously predominant role in tumor cells themselves towards an additional role for endothelial HMGB1.

>> Materials and methods

Cell culture

HUVEC were obtained by perfusion of umbilical veins with trypsin. EC were cultured in RPMI-1640 (Life Technologies) supplemented with 20% heat-inactivated human serum, glutamine and penicillin/streptomycin (Life Technologies) in gelatin

coated tissue flasks. Where indicated, 1 ng/ml bFGF (Reliatech) and 10 ng/ml VEGF (Reliatech) were added. Tumor-conditioned HUVEC (HUVEC+) were supplemented with 10% conditioned medium of Caco-2 and LS174T in addition to bFGF and VEGF. HUVEC were cultured under quiescent conditions by reducing the serum concentration to 2%. Colon tumor cell lines were cultured in DMEM (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum.

Real-time quantitative PCR

Cultured cells were harvested by trypsinization. EC were isolated from fresh colon tumor tissues, patient-matched normal colon tissues and placental tissues as previously described [1]. RNA was isolated using RNeasy-mini spin columns (Qiagen) according to the manufacturers' instructions. cDNA was reverse transcribed using MMLV-RT (Bio-Rad) according to the manufacturers' instructions. SYBR green assays were performed using 10ng cDNA template per reaction, consisting of 1x SYBR Green Master Mix (Bio-Rad) and 200 μ M of each primer (Sigma Genosys) as previously described [11]. Primers used for amplification of the HMGB1 transcript were 5'-TCTAAGAAGTGCTCAGAGAGGTG -3' and 5'- TTCATTTCTCTTTCATAACGGG-3', for MMP2 5'-AGATGCCTGGAATGCCAT-3' and 5'-GGTTCTCCAGCTTAGGTAAT-3', and for MMP9 5'-TACTGTGCCTTTGAGTCCG-3' and 5'-TTGTGCGCGATAAGGAAG-3'.

Flowcytometry

EC were cultured as described. Cells were fixed in 70% ethanol on ice for 30 minutes and incubated in 25 μ l reactions with primary antibodies [mouse anti-HMGB1 (Sigma)] appropriately diluted in PBS/0.1% BSA/0.01% sodium azide/0.05% triton X-100. Primary antibodies were detected using biotinylated rabbit-anti-mouse IgG (DAKO) in combination with streptavidin-FITC (DAKO).

Fresh normal colon and colon tumor tissues were obtained from excision surgery at the department of Pathology (University Hospital Maastricht). Single-cell suspensions were prepared as described previously [1]. Endothelial cells were stained with a PE-labeled anti-CD31 antibody (Serotec) and separated from other cells by cell sorting (BD FACSaria; BD Biosciences). Sorted cells were incubated with rabbit anti-HMGB1 (kind gift of R.G. Roeder, Rockefeller University, NY) and subsequently with biotinylated swine anti-rabbit IgG (DAKO) in combination with streptavidin-FITC (DAKO). Cells were analyzed on a flowcytometer (FACS Calibur; BD Biosciences) and acquired data was analyzed using CellQuestTM software.

Immunofluorescence

EVLC2 were grown in gelatin coated 96-well cell culture plates. Cells were fixed on ice with 70% ethanol and permeabilized with 0.05% triton X-100 in PBS. Cells were incubated with mouse anti-HMGB1 (Sigma) and subsequently with biotin conjugated rabbit anti-mouse IgG (DAKO). Fluorescent label was provided by streptavidin-FITC.

Images were acquired under an inverted microscope equipped with a fluorescence unit (Leica).

Constructs and transfection of endothelial cells

HMGB1 open reading frame was amplified from HUVEC cDNA (using 5'-GGGCACTGGGCGACTCTGTG-3' and 5'-ACTGCGCTAGAACCACTTATTC-3') and inserted in pCDNA3-Hygro (Life Technologies) using *HindIII* and *XhoI* restriction enzymes (New England Biolabs).

EVLC2 cells, SV40 large T-antigen transformed HUVEC [12] were transfected using Nucleofector technology (Amaxa). Briefly, 5×10^5 cells were harvested and resuspended in 100 μ l Nucleofector solution R. The cell suspension was mixed with 1 μ g DNA and transferred to an electroporation cuvette. Following transfection using program T20, 500 μ l filtered HUVEC medium was added and cells were transferred to 2 wells of a gelatin coated 24-wells cell culture plate. Successfully transfected cells were selected based on hygromycin resistance using 25 μ g/ml hygromycin (Life Technologies). Cell growth was monitored by seeding 5×10^3 cells per well in gelatin-coated 96-well plates and counting the number of cells per field in 4 locations of the wells.

Antisense oligodeoxynucleotides (ODNs)

Where indicated, 5 μ M antisense ODNs were added to the culture for 72 hours. Phosphorothioate-modified oligonucleotides were designed using Antisense Design from IDT SciTools (<http://www.idtdna.com/SciTools/SciTools.aspx>) according to previously published guidelines [13]. The following ODNs were used: 5'-AGGATCTCCTTTGCCCAT-3' (HMGB1 ODN) and 5'-TCCCTAGTGACTCTTCC-3' (control ODN) (Eurogentec).

Migration assay

5×10^3 cells were seeded in individual wells of gelatin coated 96-well cell culture plates and grown to confluence. Using a blunt pipette tip, a cross-shaped wound of approximately 750 μ m wide was made in the well. Cells were washed with PBS, and fresh medium was added. Where appropriate, antibody dialyzed to PBS (to remove traces of azide) was added to the medium in the indicated concentrations. Wound width was measured at 4 predefined locations per well at T= 0, 2, 4, 6 and 8 hours.

Proliferation assay

5×10^3 cells were seeded in 96-well gelatin-coated cell culture plates and allowed to adhere for 2 hours. Antibodies, dialyzed to PBS, were added to the culture medium in the indicated concentrations. Cells were cultured for 72 hours. During the last 6 hours of the assay, the culture was pulsed with 0.5 μ Ci [methyl- 3 H]-thymidine (Amersham) per well. Activity was measured using liquid scintillation counting (Wallac LSC; PerkinElmer) [14].

In vitro angiogenesis assay

Sprouting and tube formation of EC were studied with the use of cytodex-3 beads overgrown with BCE in a 3D gel as previously described [14]. Where appropriate, antibodies dialyzed to PBS were added to the collagen gel and overlay medium in the described concentrations.

Cells were incubated 48 hours, after which photographs were taken of the beads. Five concentric rings were projected over the photographs, and the number of intersections of rings and sprouting endothelial cells was determined and used as a measure of *in vitro* tube formation.

Alternatively, a tumor blood vessel was prepared free from its surrounding tumor mass and sections of approximately 2 mm in length were embedded in collagen as described above. Tissue sections were incubated for 7 days in the presence of bFGF and VEGF to induce sprout formation.

Chick chorioallantoic membrane (CAM) assay

Fertilized eggs of Lohman-selected White Leghorns were incubated for 3 days at 37°C, 55% relative air humidity, while being rotated every hour. At day 3, a rectangular window (1x1.5 cm) was made in the shell. Albumin (2 ml) was withdrawn through the blunt end of the egg. The window was covered with transparent tape to prevent dehydration, and the eggs were further incubated without rotation until day 10. At day 10, a silicone ring was placed on the chorioallantoic membrane (CAM) to apply antibody treatment and transplant LS174T spheroids.

LS174T were seeded in non-adherent cell culture plates (Greiner Bio-One) at a density of 10^5 cells/ml for 10 days to allow spheroids to form. A small surface area (approximately 3x3mm) in the silicone ring placed on the CAM was denudated using lens paper and uniformly looking spheroids of 0.5-1 mm in diameter were applied on the CAM. Antibodies were dialyzed to 0.9% NaCl and administered in a volume of 65 μ l for four consecutive days. At day 14, the CAMs were photographed. Five concentric rings were projected on the image. The number of intersections of rings and blood vessels was determined and used as a measure of vessel density.

Pieces of the CAMs were excised, formalin-fixed and paraffin embedded. Tissue sections (5 μ m) were pretreated with xylene and rehydrated in a series of ethanol. Endogenous peroxidase activity was blocked using H₂O₂, and sections were boiled in citrate buffer (10mM, pH6.0) for antigen retrieval. Sections were incubated with mouse anti-HMGB1 (Sigma) and primary antibodies were detected with biotinylated rabbit anti-mouse (DAKO) in combination with streptavidin-HRP (DAKO). Antibody binding was detected using DAB (Sigma).

>> Results

HMGB1 expression in endothelial cells

Suppression subtractive hybridization (SSH) in combination with cDNA array screening identified HMGB1 to be overexpressed in colon tumor endothelial cells (TEC) as compared to both activated placenta endothelial cells (PLEC) and resting endothelial cells derived from normal colon (NEC) [1]. Differential gene expression levels were validated by means of quantitative real-time PCR on mRNA derived from HUVEC cultured under activating or silencing conditions and from TEC, NEC and PLEC. As shown in figure 5.1, mRNA expression of HMGB1 is augmented in activated HUVEC (Figure 5.1A) and in TEC as compared to NEC and PLEC (Figure 5.1B). Furthermore, we observed considerable heterogeneity in HMGB1 expression among different colon tumor cell lines, as well between different endothelial cells.

Figure 5.1: HMGB1 expression in endothelium.

A) Real-time quantitative PCR (RTQ-PCR) on tumor-conditioned HUVEC (HUVEC+) and quiescent HUVEC (HUVEC-). Expression levels were normalized against cyclophilin A transcript expression and expressed as percentage of expression in HUVEC+. **B)** RTQ-PCR on endothelial cells isolated from colon tumors (TEC), normal colon (NEC) and placenta tissues (PLEC). Expression levels were normalized against cyclophilin A transcript expression and expressed as a percentage of the expression in TEC. **C)** RTQ-PCR analysis of HMGB1 expression in diverse colon tumor cell lines (SW480, HT29, LS174T and Caco-2) and different types of endothelial cells (HUVEC, HMEC, EVLC2). Expression levels were normalized against cyclophilin A transcript expression and expressed relative to HMGB1 expression levels in SW480. **D)** HMGB1 protein expression levels in CD31 positive colon tumor endothelial cells and patient-matched normal colon endothelial cells assessed by flowcytometry. Expression levels of CD31 did not differ between TEC and NEC (data not shown). **E)** HMGB1 protein expression levels in HMEC and EVLC2 assessed by flowcytometry. A low to moderate expression of HMGB1 is detected in all cells. **F)** Immunofluorescence analysis of HMGB1 expression in EVLC2 cells. Scale bar = 10µm.

HMEC, endothelial cells of microvascular origin, expressed more HMGB1 than HUVEC and EVLC2, both of macrovascular origin (Figure 5.1C). We subsequently verified that mRNA levels represented protein levels *in vivo*. FACS analysis on CD31 positive cells was performed on single-cell suspensions from colon tumor and normal colon tissues. HMGB1 expression was considerably higher in TEC than in NEC (Figure 5.1D), whereas CD31 expression did not differ (data not shown). Thus, HMGB1 is upregulated in activated and tumor endothelial cells. Flowcytometry also confirmed low levels of HMGB1 protein expression in cultured endothelial cells (Figure 5.1E). Protein localization was assessed using immunofluorescence of cells grown in monolayers. We observed nuclear staining but also cytoplasmic staining (Figure 5.1F), that has previously been associated with secretion of the protein [15].

Figure 5.2: Effects of HMGB1 sense and antisense expression constructs.

A) EVLC2 cells were stably transfected with expression constructs harboring the open reading frame of HMGB1 either in the sense (HMGB1-S) or in the antisense (HMGB1-AS) orientation. Flowcytometry of transfected cells demonstrates increased and reduced HMGB1 protein levels in these cells respectively as compared to mock transfected cells. **B)** Cell growth of both HMGB1-S and HMGB1-AS is impaired compared to control cells, but do not differ from each other. **C)** HMGB1-AS expressing cells have an impaired ability to respond to angiogenic growth factors as shown by a decreased proliferation rate. Basal proliferation is not affected. ****** $p < 0.01$ towards EVLC2 cells. **D)** Overexpression of HMGB1 increases the capacity of EVLC2 cells to migrate into a wounded area. Reduced HMGB1 expression only minimally reduces migration. **E)** RTQ-PCR analysis of HMGB1-S and HMGB1-AS transfected cells. HMGB1 mRNA expression is clearly reduced in HMGB1-AS transfected cells compared to HMGB1-S transfected cells (left). MMP2 expression is equal in both HMGB1-S and HMGB1-AS transfected cells (middle). MMP9 expression is induced in HMGB1-S transfected cells (right).

Modulation of HMGB1 expression affects angiogenesis

To address the significance of these findings, we employed different techniques to modulate the expression levels of HMGB1 in EC to determine the exact roles of HMGB1 in different aspects of angiogenesis. The open reading frame of HMGB1 was cloned in either the sense (HMGB1-S) or the antisense (HMGB1-AS) orientation in an expression vector to achieve up- or downregulation of the protein, respectively. Expression levels of HMGB1 protein were assessed by flowcytometry following stable transfection of EVLC2 cells (Figure 5.2A). Both HMGB1-S and HMGB1-AS transfected EVLC2 demonstrated impaired growth characteristics as compared to control cells, suggesting altered HMGB1 expression affects EC biology (Figure 5.2B). However, in contrast to HMGB1-S expressing cells, HMGB1-AS expressing cells were not capable of increasing their proliferative rate upon stimulation with angiogenic factors (Figure 5.2C). HMGB1 also affected the migrational capacity of EVLC2 in a wounding assay. Cells overexpressing HMGB1 showed faster closure of a wounded monolayer, but downregulation of HMGB1 levels had only minor inhibitory effects on EC migration (Figure 5.2D). HMGB1 mRNA has previously been reported to be upregulated in migrating cells, and downregulated upon contact inhibition [16], linking HMGB1 expression levels with the migrational capacity of cells. Migration and invasion are highly dependent on the degradation of the surrounding matrix by MMPs. Membrane-bound HMGB1 can, via tissue plasminogen activator (tPA), activate MMPs that facilitate matrix degradation [17], and AGE-RAGE signaling induces MMP2 and MMP9 mRNA expression [18]. We thus investigated the expression of MMP2 and MMP9 in transfected cells. No differences in MMP2 mRNA levels were detected between HMGB1-S and HMGB1-AS, but MMP9 expression was induced approximately 3-fold in HMGB1-S (Figure 5.2E).

Figure 5.3: Effects of HMGB1 antisense ODNs on the angiogenic properties of EC.

A) EC were treated with 5 μ M antisense ODN for 72 hours. Expression of HMGB1 was specifically downregulated by HMGB1 ODNs but not by control ODNs. The expression of CD31 was not affected. **B)** HMGB1 antisense oligodeoxynucleotides (ODNs) inhibited the migration of EC into a wounded area, though this did not differ significantly from the non-relevant control ODN. **C)** HMGB1 ODNs significantly inhibited sprouting of EC. The control ODN only marginally inhibited EC sprouting. **D)** HMGB1 ODNs inhibited the proliferation of EC, whereas a control ODN did not.

An alternative strategy for protein knockdown that does not necessarily require cell transfection is the use of ODNs [19,20]. Indeed, a FITC-labeled non-relevant ODN added to the culture was readily taken up by EC (data not shown). Using a HMGB1-specific ODN (Figure 5.3A), we observed inhibition of EC migration (Figure 5.3B) and sprouting (Figure 5.3C), though in some cases a considerable effect was observed in the presence of a non-specific control ODN, indicative of off-target effects. Growth factor induced proliferation was inhibited in HMGB1 ODN treated EC (Figure 5.3D), which is in concordance with the data obtained using the expression constructs.

Antibody targeting of HMGB1 inhibits endothelial cell migration and sprouting

EC can secrete HMGB1 when stimulated with LPS and $\text{TNF}\alpha$, which is preceded by cytoplasmic expression of the protein [15]. Membrane-bound HMGB1 has been involved in the outgrowth of neurites and anti-HMGB1 antibodies inhibited this process [17,21]. Though we could not detect HMGB1 protein on intact cells (data not shown) the detection of HMGB1 in the cytoplasm of EC made us investigate whether anti-HMGB1 antibodies would affect the angiogenic capacity of endothelial cells. Anti-HMGB1 antibodies did not affect growth factor induced proliferation of endothelial cells (Figure 5.4A), but did inhibit migration (Figure 5.4B). In addition, we tested the effect on tube formation of BCE in a matrix of collagen. Sprouting of BCE was clearly inhibited by antibodies directed against HMGB1 (Figure 5.4C and D). In an *ex vivo* approach, tumor blood vessels were prepared free from their surrounding cell mass and 2 mm sections were embedded in collagen. Small vessel sprouts were induced by bFGF and VEGF in the controls, but treatment with anti-HMGB1 antibodies inhibited the sprouting (Figure 5.4E). This inhibitory effect of antibodies implicates an extracellular role for HMGB1 in endothelial migration and sprout formation [16].

Anti-tumor treatment using anti-HMGB1 antibodies

The effect of targeting HMGB1 using antibodies was tested in an *in vivo* tumor angiogenesis model using the CAM. LS174T tumor cells spheroids were transplanted onto the CAM of a developing chick embryo and anti-HMGB1 antibodies were applied. Anti-HMGB1 antibodies are immunoreactive with blood vessels in the CAM as well as with stromal and epithelial cells (Figure 5.5A and B). Administration of anti-HMGB1 antibodies resulted in a marked reduction of blood vessel density induced by the tumor cells (Figure 5.5C-F). These data point to an important role for HMGB1 in tumor angiogenesis, and its potential as target for therapeutic inhibition of tumor angiogenesis.

Figure 5.4: Inhibition of endothelial migration and sprouting by anti-HMGB1 antibodies.

A) Growth factor induced proliferation of HUVEC is not affected by the presence of 10µg/ml anti-HMGB1 antibodies in the culture medium. **B)** Migration of HUVEC into a wounded area is inhibited by the presence of anti-HMGB1 antibodies (10µg/ml). **C, D)** Sprouting of BCE embedded in a 3D collagen matrix is inhibited by the presence of anti-HMGB1 antibodies (10µg/ml) (C). Both the number of sprouts formed and the distance they extend from the cell-covered beads is

inhibited by the presence of the antibodies (D). **E)** Growth factor induced endothelial sprout formation of tumor vessels *ex vivo* is inhibited by anti-HMGB1 antibodies.

>> Discussion

We previously identified HMGB1 as overexpressed in TEC [1]. Here we document the role of HMGB1 in different aspects of angiogenesis and demonstrate a predominant role for the protein in migration and sprouting of EC.

HMGB1 is a player in several feedback loops, both positive and negative, upon interaction with its receptor RAGE. HMGB1 mediated RAGE activation results in increased RAGE expression, thereby amplifying RAGE related signaling events [22]. RAGE activation of EC leads to phosphorylation and inactivation of IκB, resulting in nuclear translocation of nuclear factor κB (NFκB), stimulating the production of several cytokines, such as TNFα, IL1β and IL-8, and the upregulation of adhesion molecule expression, including VCAM1, ICAM1 and E-selectin [23,24]. TNFα stimulates secretion of HMGB1 from EC [15]. RAGE activation also results in the activation of CDC42 and Rac, and the phosphorylation of MAPK, ERK1/2, jun N-terminal kinase (JNK) and p38 [24]. Furthermore, membrane-bound HMGB1 can interact with plasminogen and tPA, resulting in the local generation of plasmin which is subsequently involved in breakdown of HMGB1, but also in the activation of MMP2 and

Figure 5.5: Targeting HMGB1 inhibits tumor-induced angiogenesis *in vivo*.

LS174T tumor cells were transplanted onto the CAM were treated with anti-HMGB1 antibody (10 µg/ml). **A-B)** HMGB1 antibodies were immunoreactive with the CAMs and stained endothelial cells, but also stromal and epithelial compartments (A, negative control; B, anti-HMGB1 antibody). **C-F)** Treatment with anti-HMGB1 antibodies reduced the tumor-induced vessel density (** p < 0.001 towards tumor and normal) (C). LS174T tumor cells induce increased vascular density and irregular morphology (D) as compared to normal untreated CAMs (E). Tumor induced vessel density was reduced by treatment with anti-HMGB1 antibodies (F).

MMP9 [17]. In addition, increased tPA secretion has been shown upon HUVEC stimulation with HMGB1 [24].

Secreted HMGB1 can interact with RAGE. Though we could not directly demonstrate the secretion of HMGB1, we detected the protein in the cytoplasm of EC, which was previously shown to precede externalization [15]. However, HMGB1 lacks a classical secretion signal [25,26]. Acetylation of HMGB1 prevents its re-entry to the nucleus resulting in cytoplasmic accumulation [25]. In tumors, necrosis of tumor cells results in the abundant presence of HMGB1 in the microenvironment. Indeed, in addition to endothelial localization, we observed diffuse stromal expression of HMGB1 in colon tumor tissues [1], as well as in diverse colon tumor cell lines.

Activation of RAGE by AGE on endothelial cells increases VEGF production [27,28], though so far, this has not been demonstrated for HMGB1. Recently, it was

shown that recombinant HMGB1, albeit at high concentrations, could induce tube formation of HUVEC, with its effect approaching that of VEGF [29]. Therefore, secreted HMGB1 might induce upregulation of VEGF via RAGE, which then facilitates endothelial tube formation and migration. Though EC may not be the major contributors of VEGF production in tumors, *in vitro* effects of HMGB1 might be caused by increased endothelial VEGF production resulting from HMGB1-RAGE interactions. HMGB1-AS expressing cells indeed failed to respond to activation by bFGF and VEGF. Anti-HMGB1 antibodies however exerted no effect. Stimulation of EC with recombinant HMGB1 and subsequent measurement of VEGF mRNA and protein levels in the absence or presence of RAGE blocking antibodies might clarify this issue.

Capillary sprouting of endothelial cells embedded in collagen requires local degradation of matrix components. AGE-RAGE signaling induces MMP expression [18], and membrane-bound HMGB1 can activate MMPs [17]. Furthermore, HMGB1 was detected in the filopodia of migrating neurites [4], suggesting a direct contribution to migration of cells. Taken together, these data suggest a role for HMGB1 in endothelial cell migration. Indeed, anti-HMGB1 antibodies inhibited EC migration and sprout formation and overexpression of HMGB1 increased migration and MMP9 expression of EC. However, whether the expression level of HMGB1 in EC correlates with MMP activity and whether the latter can be modified by exogenous HMGB1 needs to be addressed. The differential effects observed on migration and sprouting of EC as compared to growth factor activation after antibody treatment possibly suggest that these processes are mediated by different actions of HMGB1. HMGB1-mediated sprouting and migration might be regulated by both a RAGE-induced upregulation of VEGF and an induction of MMP activity, whereas a proliferative response might not depend on MMP induction.

In the CAM assay, we observed a significant inhibition of tumor-induced vessel density after treatment with anti-HMGB1 antibodies. However, we cannot exclude a possible additional effect on tumor cell derived HMGB1. Blocking HMGB1-RAGE interactions also inhibited tumor growth and invasion in C6 gliomas, LLC and Ha-ras +/+ spontaneous papillomas [10]. Unfortunately, in this report the authors did not address the vascularization of these tumors after treatment, rendering it speculative whether the observed effects were mediated by reduced angiogenesis.

In conclusion, we have demonstrated a role for HMGB1 in different aspects of angiogenesis and that inhibiting HMGB1 results in impaired (tumor) angiogenesis. Though we cannot exclude a possible transcription mediating role of HMGB1, we propose a role for HMGB1 in autocrine and paracrine regulation of VEGF and MMP production in tumor and endothelial cells that contributes to tumor growth and angiogenesis.

>> References

- [1] van Beijnum JR, Dings RPM, van der Linden E, Zwaans BMM, Ramaekers FCS, Mayo KH, Griffioen AW. Gene expression tumor angiogenesis dissected; specific targeting of tumor endothelium. Submitted.
- [2] Yu SS, Li HJ, Goodwin GH, Johns EW. Interaction of non-histone chromosomal proteins HMG1 and HMG2 with DNA. *Eur J Biochem* 1977;78 (2):497-502.
- [3] McKinney K, Prives C. Efficient specific DNA binding by p53 requires both its central and C-terminal domains as revealed by studies with high-mobility group 1 protein. *Mol Cell Biol* 2002;22 (19):6797-808.
- [4] Merenmies J, Pihlaskari R, Laitinen J, Wartiovaara J, Rauvala H. 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. *J Biol Chem* 1991;266 (25):16722-9.
- [5] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999;285 (5425):248-51.
- [6] Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, Sohn JW, Yamada S, Maruyama I, Banerjee A, et al. High Mobility Group Box 1 protein (HMGB1) interacts with multiple Toll like receptors. *Am J Physiol Cell Physiol* 2005.
- [7] Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. *Circ Res* 2004;95 (3):233-8.
- [8] Myint KM, Yamamoto Y, Sakurai S, Harashima A, Watanabe T, Li H, Takeuchi A, Yoshimura K, Yonekura H, Yamamoto H. Blockade of diabetic vascular injury by controlling of AGE-RAGE system. *Curr Drug Targets* 2005;6 (4):447-52.
- [9] Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM, Nawroth PP. Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med* 2005.
- [10] Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature* 2000;405 (6784):354-60.
- [11] Thijssen VL, Brandwijk RJ, Dings RP, Griffioen AW. Angiogenesis gene expression profiling in xenograft models to study cellular interactions. *Exp Cell Res* 2004;299 (2):286-93.
- [12] van Leeuwen EB, Veenstra R, van Wijk R, Molema G, Hoekstra A, Rutgers MH, van der Meer J. Characterization of immortalized human umbilical and iliac vein endothelial cell lines after transfection with SV40 large T-antigen. *Blood Coagul Fibrinolysis* 2000;11 (1):15-25.
- [13] Matveeva OV, Tsodikov AD, Giddings M, Freier SM, Wyatt JR, Spiridonov AN, Shabalina SA, Gesteland RF, Atkins JF. Identification of sequence motifs in oligonucleotides whose presence is correlated with antisense activity. *Nucleic Acids Res* 2000;28 (15):2862-5.
- [14] van der Schaft DW, Dings RP, de Lussanet QG, van Eijk LI, Nap AW, Beets-Tan RG, Bouma-Ter Steege JC, Wagstaff J, Mayo KH, Griffioen AW. The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *Faseb J* 2002;16 (14):1991-3.
- [15] Mullins GE, Sunden-Cullberg J, Johansson AS, Rouhiainen A, Erlandsson-Harris H, Yang H, Tracey KJ, Rauvala H, Palmblad J, Andersson J, et al. Activation of human umbilical vein endothelial cells leads to relocation and release of high-mobility group box chromosomal protein 1. *Scand J Immunol* 2004;60 (6):566-73.
- [16] Fages C, Nolo R, Huttunen HJ, Eskelinen E, Rauvala H. Regulation of cell migration by amphoterin. *J Cell Sci* 2000;113 (Pt 4):611-20.
- [17] Parkkinen J, Rauvala H. Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin. Enhancement of t-PA-catalyzed plasminogen activation by amphoterin. *J Biol Chem* 1991;266 (25):16730-5.
- [18] Ishiguro H, Nakaigawa N, Miyoshi Y, Fujinami K, Kubota Y, Uemura H. Receptor for advanced glycation end products (RAGE) and its ligand, amphoterin are overexpressed and associated with prostate cancer development. *Prostate* 2005;64 (1):92-100.
- [19] Gleave ME, Monia BP. Antisense therapy for cancer. *Nat Rev Cancer* 2005;5 (6):468-79.
- [20] Shi F, Nomden A, Oberle V, Engberts JB, Hoekstra D. Efficient cationic lipid-mediated delivery of antisense oligonucleotides into eukaryotic cells: down-regulation of the corticotropin-releasing factor receptor. *Nucleic Acids Res* 2001;29 (10):2079-87.
- [21] Parkkinen J, Raulo E, Merenmies J, Nolo R, Kajander EO, Baumann M, Rauvala H. Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation. *J Biol Chem* 1993;268 (26):19726-38.
- [22] Huttunen HJ, Rauvala H. Amphoterin as an extracellular regulator of cell motility: from discovery to disease. *J Intern Med* 2004;255 (3):351-66.

- [23] Treutiger CJ, Mullins GE, Johansson AS, Rouhiainen A, Rauvala HM, Erlandsson-Harris H, Andersson U, Yang H, Tracey KJ, Andersson J, et al. High mobility group 1 B-box mediates activation of human endothelium. *J Intern Med* 2003;254 (4):375-85.
- [24] Fiuza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, Suffredini AF. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 2003;101 (7):2652-60.
- [25] Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *Embo J* 2003;22 (20):5551-60.
- [26] Erlandsson Harris H, Andersson U. Mini-review: The nuclear protein HMGB1 as a proinflammatory mediator. *Eur J Immunol* 2004;34 (6):1503-12.
- [27] Okamoto T, Yamagishi S, Inagaki Y, Amano S, Koga K, Abe R, Takeuchi M, Ohno S, Yoshimura A, Makita Z. Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin. *Faseb J* 2002;16 (14):1928-30.
- [28] Yamagishi S, Yonekura H, Yamamoto Y, Katsuno K, Sato F, Mita I, Ooka H, Satozawa N, Kawakami T, Nomura M, et al. Advanced glycation end products-driven angiogenesis in vitro. Induction of the growth and tube formation of human microvascular endothelial cells through autocrine vascular endothelial growth factor. *J Biol Chem* 1997;272 (13):8723-30.
- [29] Schlueter C, Weber H, Meyer B, Rogalla P, Roser K, Hauke S, Bullerdiek J. Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule. *Am J Pathol* 2005;166 (4):1259-63.

Chapter 6

Towards high-throughput functional target discovery in angiogenesis research

Judy R. van Beijnum, Wouter J. Eijgelaar & Arjan W. Griffioen

Trends Mol Med. 2006 Jan;12(1):44-52.

>> Abstract

Angiogenesis is a hallmark of malignancies and other proliferative diseases and inhibition of this process is considered to be a promising treatment strategy. Classical gene expression analyses performed in the past decade have generated vast lists of genes associated with disease but have so far yielded only limited novel therapeutic targets for clinical applications. Recently, the focus has shifted from target identification based on gene expression analysis, to identification of genes based on the function of the encoded protein. Disease target genes can now be identified in a high-throughput fashion based on functional properties directly related to the disease phenotype. This new approach significantly shortens the time span for the development of therapeutic applications from the laboratory bench to the hospital bedside.

>> Identification of targets for angiogenesis modulation

Angiogenesis, the formation of novel blood vessels from pre-existing blood vessels, is a hallmark of numerous pathologies such as cancer, rheumatoid arthritis, endometriosis, atherosclerosis and diabetic retinopathy [1,2]. In normal tissues, blood vessels and capillaries are generally in a resting state, maintained by a fine-tuned balance of pro-angiogenic and anti-angiogenic factors present in the microenvironment. However, different stimuli can disturb this balance in favor of pro-angiogenic factors. These factors activate endothelial cells (EC) that subsequently degrade surrounding matrix components, migrate in the direction of the stimulus, proliferate and form a new capillary. Inhibition of angiogenesis, particularly in cancer, is a promising treatment strategy, and considerable effort has focused on the identification of markers on tumor EC [3-6] that might be targeted for therapeutic intervention. The possibility of using a gene as a target for therapeutic intervention depends on the role of that gene, or the protein it encodes, in a biological mechanism or a disease pathway. To identify gene function, diverse assays can be employed to mimic angiogenesis related processes *in vitro* such as capillary tube formation in three-dimensional matrices (e.g. Matrigel™) or migration of EC towards a stimulus or into a wounded area [7]. Other assays, such as mouse tumor models or the chick chorioallantoic membrane assay (CAM) can be employed to study gene function in tumor angiogenesis *in vivo* [8]. From a clinical perspective, targeting a gene product that has a causal relationship with the disease phenotype is probably the most effective therapeutic intervention, because it enables control of the whole biological process.

High-throughput gene expression profiling analysis, boosted by the invention of cDNA microarrays [9] and the publication of the human genome sequence [10], has yielded a vast number of putative disease target genes, but has not yet produced clinically applicable targeted therapeutics. Lists of differentially expressed genes associated with known conditions do not reveal functional information or direct (causative) relevance to a given disease. The characterization and functional validation of these targets *in vitro* and in pre-clinical models is a time- and labor-intensive process. In addition, a relatively large proportion of these candidate genes will be of limited use because they do not directly contribute to disease development or progression. Thus, strategies are required that distinguish causative and associative changes in gene expression. Recently, the focus has shifted from the identification of disease target genes based on differential expression levels in diseased versus non-diseased tissues, to the identification of candidate target genes based on function, creating a direct link between gene expression levels and phenotype. These novel technologies enable a direct approach for elucidating clinically useful therapeutic targets, thereby reducing time, cost and effort for the development of therapeutics from the laboratory bench to the hospital bedside.

In this review, we highlight techniques used for the validation and functional identification of novel therapeutic targets. First, we discuss validation strategies of

candidate targets identified by gene expression profiling studies through modulation of protein expression levels and the effect on cellular properties (from gene to function). Second, we illustrate the functional identification of novel targets using gain-of-function and loss-of-function expression screens (from function to gene), and discuss their applications in angiogenesis research.

Glossary

Antisense oligodeoxynucleotide (ODN): A single-stranded, DNA-like molecule of 17-22 nucleotides in length that is complementary to its target mRNA and blocks protein translation by forming DNA-RNA duplexes.

Array: A systematically arranged repertoire of DNA, cells or tissues. These arrays can be arranged in microtiter plates, or printed on glass slides or chips (microarray).

cDNA library: A collection of cDNA sequences in separate vectors.

Expression library: A collection of cDNA sequences incorporated in expression vectors for expression of transgenes in target cells.

Expression profiling: The study of expression levels of genes or proteins in different tissues.

Expression vector (construct): A circular DNA molecule containing a frequently transcribed promoter with a downstream gene to express. It is used to introduce and express a specific gene (transgene) in a target cell.

Functional genomics: The research field that aims to identify the function of genes.

Gain-of-function assay: The identification of gene function by inducing overexpression of the gene in the studied cell type.

Loss-of-function assay: The identification of gene function by inducing knockdown or knockout of the gene in the studied cell type.

Promoter: A DNA sequence that controls transcription of the downstream gene.

Reporter gene: A gene co-expressed on an expression vector that induces an irrelevant but detectable phenotype [e.g. green fluorescent protein (GFP)].

RNA interference (RNAi): The mechanism by which protein expression is specifically inhibited by interference in RNA translation into a protein, using a natural cellular defense mechanism against double-stranded (ds)RNA.

Selection marker: A gene co-expressed on an expression vector that enables successfully transfected cells to be selected out of untransfected cells (e.g. a gene that encodes a protein that confers resistance to cytostatic agents).

shRNA: Small inverted repeats (hairpins), also known as palindromes (19-29 nucleotides) linked by a loop of 6-9 bases, that can be cleaved by Dicer to form siRNA duplexes, which are then shunted into the RNAi pathway.

siRNA: A short, double stranded RNA fragment complementary to a specific target mRNA that induces RNA interference.

Target validation: Demonstration of the role of a particular molecular target in a pathogenic or physiologic process.

Transfection/transduction (transient or stable): Introduction of DNA or RNA into eukaryotic cells. Transfection typically involves opening transient 'holes' or gates in cells to enable the entry of extracellular molecules such as supercoiled plasmid DNA and siRNA. Transfection can be transient (the inserted DNA is lost over several generations) or stable (the inserted DNA is incorporated into the genome and is passed on to daughter cells). If viral particles are used, transfection is often referred to as transduction.

Transgene: Exogenous gene expressed in a cell from an expression construct

>> Target validation: From gene to function

Gene and protein expression profiling techniques have been applied to study molecular events in angiogenesis [6]. Differentially expressed molecules might serve as targets for therapeutic intervention. Validating the relevance of a target for therapeutic applications requires the establishment of a causative relationship between protein expression and/or function and the disease phenotype. This can be achieved by either blocking protein function or by modulating protein expression levels. In this review, we describe several techniques to achieve knockout or knockdown of gene expression, of which RNA interference (RNAi) and antisense oligodeoxynucleotides (ODNs) are most widely used. Alternatively, cDNA expression constructs can be introduced in the cells to achieve either up- or downregulation of the target protein.

RNA interference

Knockdown of protein expression offers a powerful means for target validation as protein levels can be directly correlated with the phenotype. RNAi is based on the introduction into cells of small, double stranded RNA molecules [small interfering RNAs (siRNAs)] that target the corresponding endogenous mRNA for destruction, thereby downregulating expression levels of the encoded protein (Figure 6.1A) [11-13]. The exploitation of RNAi has revolutionized functional genomics research, because it is an extremely effective tool for specific gene silencing. siRNAs may be chemically synthesized, produced by expression constructs [14-17] or generated by restriction digestion of long double-stranded RNA molecules produced *in vitro* (Box 1) [18-21]. This technique is useful for identifying the function of genes in a specific biological process. RNAi has been used extensively to target angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). In diverse murine models, administration of VEGF siRNAs inhibits tumor growth and angiogenesis [22]. In addition, siRNAs can be targeted selectively to different VEGF splice variants and isoforms [22,23]. EC transformed by bFGF overexpress polymerase δ p12 subunit (p12) in a gene expression survey [24]. Subsequent knockdown of p12 by RNAi abrogates bFGF induced proliferation of EC, suggesting an important role for p12 in the response of EC to bFGF [25].

SiRNAs might be directly applicable as therapeutic agents and circumvent the need for the design of small-molecule inhibitors of protein activity. *In vivo* delivery of siRNAs for therapeutic purposes is still in its infancy, but the potential of this type of therapy has been recognized. However, the uptake of siRNAs *in vivo* is not always very effective, and thus requires high doses of siRNAs to achieve an acceptable therapeutic effect. In addition, systemic delivery of siRNA is needed for proper therapeutic implementation because many target organs can not be reached by local administration. To increase selectivity and avoid nonspecificity, it is of major importance the siRNAs target only the cells of interest. Recently, siRNAs that target VEGF were conjugated to antibody-protamine fusion proteins for specific delivery into

tumor cells, resulting in inhibited tumor growth in mice [26]. In this study, nanoparticles displaying the RGD (arginine-glycine-aspartic acid) peptide were used for targeted delivery of siRNAs to tumor neovasculature for the knockdown of VEGF receptor 2 [26]. Both tumor growth and angiogenesis were inhibited following intravenous administration of the particles to mice with N2A neuroblastoma tumors [26]. These approaches are promising for the future exploration of various viral and non-viral vector systems for the targeted delivery of siRNA *in vivo* [27,28].

Thus RNAi is a promising and versatile tool for target validation studies and an upcoming tool for therapeutic intervention. This is further strengthened by the recent advances in systemic, targeted delivery of siRNAs. Major drawbacks of RNAi include the limited success rate of target site selection and the high costs of siRNA synthesis; however, several technical advances have partially overcome these limitations (Box 1).

Box 1. RNA interference

In recent years, an RNA based gene silencing mechanism, originally based on a cellular response to double stranded RNA (dsRNA) derived from replicating viruses or integrated transposons, has been elucidated. These long intracellular dsRNAs are transformed into short dsRNAs called short interfering RNAs (siRNAs) by the activation of a family member of RNase III ribonucleases termed Dicer. The siRNAs are incorporated into the RNA-induced silencing complex (RISC), which recognizes and degrades the homologous target mRNA, preventing protein translation [11-13].

RNAi in its current applications is based on the introduction of exogenous chemically synthesized siRNAs of 18-22nt in length to achieve knockdown of the target protein for functional studies. The problem is the design of the siRNA sequences, which depends on published sequence information. Only approximately one in four siRNAs effectively silences gene expression. siRNA transfection is transient, lasting 3-7 days, depending on the rate of cell division and intracellular degradation; therefore, it is a useful tool only in assays based on short periods of incubation. However, for analysis of long-term effects of silencing (e.g. in cell differentiation assays or when studying proteins with a low turnover rate) a more sustained silencing response is needed. To overcome these limitations, expression vectors have been created that can be stably transfected in cells to drive transcription of small hairpin RNA (shRNA) molecules [14,16], or complementary 19-21 nucleotides RNA sequences [15,17] that have almost the same properties as classic siRNAs. Vector modifications can include the addition of tissue-specific promoters [57], tetON-tetOFF systems [58,59], and Cre-loxP regulation [60] to further improve tissue specificity and regulation of target protein expression levels.

Because the design of the siRNAs is crucial for success, methods that render *in silico* predictions of the targeted sequences superfluous have been pursued extensively. Alternative methods, based on restriction enzyme digestion of double stranded DNA [e.g. restriction enzyme-generated siRNA (REGS) [19] and siRNA production by enzymatic engineering of DNA (SPEED)] [18] or RNA [e.g. endonuclease prepared siRNA (esiRNA)] [20,21] have been described recently. These techniques enable efficient generation of siRNAs that cover the complete target mRNA, but have the intrinsic risk of targeting homologous, non-target sequences resulting in unwanted side effects.

Antisense oligonucleotides

An alternative to RNAi-mediated downregulation of protein expression is the use of antisense ODNs which are small single-stranded, DNA-like molecules (Box 2). Binding of antisense ODNs to their complementary target mRNA results in inhibition of protein synthesis or destruction of the mRNA by RNase H, resulting in reduced levels of the target protein (Figure 6.1B) [29].

The high level of expression of VEGF in many types of tumors and other vascular abnormalities has made it a frequently exploited target for anti-angiogenic therapy. Microarray analysis of VEGF treated EC [30] shows that Down syndrome candidate region 1 (DSCR1) is induced within 30 minutes after exposure to VEGF. DSCR1 was subsequently downregulated in EC using antisense ODNs to examine the effects in diverse angiogenesis assays [31]. The antisense ODN inhibited VEGF-induced transwell migration of HUVEC, which suggests a role for DSCR1 in migration. In a recent study, an orthotopic transplantation model of pancreatic cancer was used to study the effect of VEGF inhibition by an antisense ODN [32]; this inhibition increased survival and reduced tumor growth and microvessel density [32].

Box 2. Antisense oligodeoxynucleotides

Antisense oligodeoxynucleotides (ODNs) are small, single-stranded DNA-like molecules. The application of these antisense molecules for inhibition of gene expression has been explored since the mid-1980s, but the advent of siRNA technology has partially overshadowed it. Antisense ODNs bind to the complementary target mRNA, creating a DNA-RNA hybrid. The working mechanism of antisense ODNs to inhibit protein translation is mediated by: (i) triggering RNase H; (ii) interfering with splicing; (iii) blocking the sequences needed for translation by ribosomes; or (iv) inhibiting the ribosome itself [33]. Similar to siRNAs, the knockdown efficiency and specificity are dependent on the design of the ODN, but several software packages and guidelines are available; these rely on algorithms that predict secondary and tertiary structures of RNA or on identifying particular ODN sequence motifs of proven efficacy [61,62]. Different oligonucleotide analogs and chemical modifications aim to improve stability, affinity and recruitment of RNase H, and to reduce toxicity. Frequently used ODNs are phosphorothioate ODNs [63], peptide nucleic acid (PNA) [64], locked nucleic acid (LNA) [65] and morpholinos [66]. They differ somewhat in their mode of action, ability to recruit RNase H, and efficiency of intracellular delivery [29,33].

Although the use of ODNs represents a versatile tool in gene expression research, there are general limitations to the application of this technology. Being charged molecules, ODNs are not readily absorbed in many cell types and are prone to endonuclease and exonuclease digestion. ODNs enter cells primarily via endocytosis but can become trapped in the endosomal-lysosomal compartment. Chemical modifications of the ODNs, the use of classical transfection reagents or the use of specific carrier molecules might overcome these limitations [29]. Another general drawback associated with the use of antisense ODNs is that their effect is transient. These characteristics should be considered carefully when designing target validation experiments using antisense ODNs.

In addition to being a valuable tool in target validation studies, antisense ODNs have also entered clinical trials as therapeutic agents for the treatment of diverse conditions and were shown to efficiently suppress target gene expression [33]. From a clinical point of view, the efficient uptake of antisense ODNs by EC might favor these molecules over siRNAs for therapeutic protein knockdown.

Expression constructs

A straightforward route for studying gene function is to upregulate or overexpress of a gene using expression constructs and to screen for phenotypic changes (Box 3). The mRNA of interest is first cloned in an expression vector and is then introduced into the cells to induce expression of the target protein (Figure 6.1C). Numerous different modifications of these vectors are possible, enabling customization of this technique to almost every experimental setup. However, various cell types such as hematopoietic cells or primary cells (including EC) are very difficult to transfect [34-36]; to overcome this limitation, viral vectors are used to transfect these cells [37,38].

Gene expression analysis of EC derived from invasive breast carcinoma showed that HEYL, a transcriptional mediator of Notch signaling [39], is upregulated compared with HEYL in EC isolated from normal breast tissues [4]. To functionally validate HEYL, EC were transduced with adenoviral vectors expressing HEYL and assayed for different aspects of the angiogenesis process such as proliferation, migration, tube formation and apoptosis. Proliferation, but not migration or tube formation, was stimulated by overexpression of HEYL. Also, HEYL protected against apoptosis in serum-starved cells [4].

Box 3. Expression vectors as tools in functional genomics

Increased expression of a target protein (gain-of-function) can be achieved by introduction of expression cassettes that are incorporated into vectors and contain the target gene open reading frame preceded by a promoter known to be actively transcribed in the studied cell type. The same strategy can be exploited for loss-of-function studies (e.g. by transcription of vector encoded shRNA or antisense cDNA that can interact with the target mRNA to prevent protein translation, resulting in reduced protein levels). Expression systems can be customized according to the users' definitions, but commercially available vectors already cover a wide range of possibilities. Depending on the aims of the study, systems that differ in, for example, the type of promoter sequences, the presence of reporter genes or selection markers, the efficiency and mode of transfer into cells or the presence of regulatory sequences that enable modulation of expression level are available. Various cell types, such as hematopoietic cells or primary cells are difficult to transfect by conventional methods such as electroporation or the use of cationic lipids, although some progress has been made [34-36]. The use of viral vectors (e.g. lentivirus, adenovirus or retrovirus) provides an alternative to traditional methods because these have a higher transduction efficiency [37,38]. In addition to functional analysis of putative target proteins, expression constructs can be incorporated into various delivery vehicles for application in gene therapy.

The use of expression constructs in cells is a relatively straightforward approach to study phenotypic changes induced by increased expression of the target protein. In addition to functional analysis of putative target proteins, expression constructs can be incorporated in various delivery vehicles for application in gene therapy.

Gene expression profiling studies have identified numerous genes that are associated with angiogenesis *in vitro* and *in vivo*, but these genes can only be fully exploited for therapeutic benefit if there is a causal relationship between the expression of the encoded protein and angiogenic properties of EC. Different tools are available to study the function of a particular protein and to validate its potential usefulness as a therapeutic target. Both overexpression of a protein driven by expression vectors and knockdown of protein expression by antisense ODNs or RNAi offer the opportunity to determine the effect of altered protein expression on cell phenotype whereby protein function can be deduced. However, alterations of the expression levels of a target gene may not always lead to a change in phenotype because of functional redundancy in signaling mechanisms. In addition, when transfection efficiency is limited, the induced phenotype change in individual cells might be obscured by the phenotype of background of untransfected cells.

Figure 6.1: Classical target validation techniques.

A) RNA interference. Double-stranded RNA molecules are incorporated in the RNA induced silencer complex (RISC) forming an active siRNA complex. This complex recognizes the complementary target mRNA that is subsequently cleaved, resulting in downregulated mRNA levels. **B)** Antisense oligodeoxynucleotides (ODNs). Antisense ODNs base-pair with their complementary target mRNA and can subsequently block the ribosome or induce mRNA degradation by RNase H, resulting in inhibition of protein synthesis. **C)** cDNA expression vectors. Expression cassettes containing the cDNA of interest (shaded box) is optionally flanked by sequences that facilitate the localization or detection of the encoded protein (striped boxes). Under the control of a promoter sequence these cassettes can be introduced in target cells to express the cDNA, causing overexpression of the encoded protein. Additionally, transfected cells can be selected for using vector-encoded antibiotic resistance proteins or fluorescent proteins (checkered box).

>> Functional target discovery: From function to gene

The wealth of gene expression data generated to date has presented the scientific community with the formidable challenge of functionally annotating the individual genes in a multitude of processes and diseases. Recently, diverse high-throughput methods have been shown to functionally annotate putative target molecules, overcoming these limitations. Advances in gene library generation, transfection methods, miniaturization of assays and screening techniques have provided the opportunity for target validation to keep up with the pace of target identification at a genomics scale. In addition, it is now feasible to perform genome-scale screens directly for phenotypic properties, thereby shifting focus from target identification based on expression patterns to function based target discovery. Here, we describe and discuss high-throughput methods that aim to identify candidate target genes based on the cellular phenotypes they induce. These approaches screen for functional properties induced by altering the expression levels of target proteins and therefore require no prior knowledge of the encoding sequence. Thus, genes having a causal role rather than an associative role in a particular disease can be identified directly.

High-throughput library screening

Transfection of cells with conventional expression constructs can be miniaturized to enable high-throughput, parallel assessment of the consequences of transgene expression. Arrayed expression libraries present a powerful, high-throughput tool for the analysis of gene function on a genome-wide scale using cell-based functional assays. Individually arrayed expression constructs are used for parallel transfections of cells in microtiter plates (Figure 6.2A). The arrayed format enables screening of the library in various cell assays for the expressed sequences that induce a particular phenotype, providing a direct linkage between phenotype and gene expression. Target cells can be directly transfected with the expression constructs and assayed for phenotypic properties (Figure 6.2B). Alternatively, transfection of non-target cells, resulting in the induction of secreted factors, and the subsequent transfer of conditioned medium to the target cell can be used to screen for biological effects of the secreted factor (Figure 6.2C).

Pro-angiogenic factors are secreted proteins that can act in a paracrine or autocrine fashion. To identify novel secreted pro-angiogenic factors, HeLa cells were first infected with adenoviral expression vectors, and conditioned medium of these cells was then transferred to HUVEC embedded in collagen. Capillary tube formation of HUVEC induced by the secreted factors present in the conditioned medium was measured to determine possible pro-angiogenic effects [40]. Three different genes were reported to induce capillary tube formation: a truncated variant of VEGF, an inflammatory/transcription activator (CYR61) and an interferon-regulatory factor [40]. In a similar experiment, proliferation of EC mediated by secreted proteins was measured and a previously unknown factor as potent as VEGF was identified

(<http://www.xantos.de/index.php?page=Cancer>). These findings illustrate the potential of using large-scale screens to assign functional clues to as unknown genes or assign novel functions to known genes. A general drawback of screening libraries for secreted factors with predefined activities is the predicted low hit rate. Only a minor proportion of the genes present in the transfected libraries encode secreted proteins and only a limited number of these will mediate the desired effect. In a similar way, libraries that express siRNA constructs can be used for loss-of-function screens [41] (<http://www.galadeno.com/technology/sirna.html>; <http://www.xantos.de/index.php?page=cDNA+Screening+Tools>).

Expression library screening generates a wealth of functional data in an unbiased manner without *a priori* known sequence information and enables the identification of putative therapeutic targets based on the effect of alteration of the target protein level. New modulators of angiogenesis that might be targets in a therapeutic setting have been identified. In contrast to cell microarrays (described below), assays based on tissue culture in wells are compatible with both adherent cells and cells that grow in

Figure 6.2: Arrayed expression library screening.

A) Expression constructs are arrayed to individual wells of microtiter plates (arrayed library) and used for parallel transfection of cells in individual wells. **B)** Transfected cells can be directly subjected to phenotype analysis (e.g. to assess proliferation), linking the expressed sequence to the observed functional properties of the cells. **C)** Alternatively, transfected cells can produce soluble, secreted factors (stars and boxes) that might act in a paracrine fashion on other cells. The conditioned medium of the transfected cells, containing these factors, is applied to other cells that are subsequently screened for changes in phenotype as described.

suspension. Key to success using this library screening technique is the design of cell-based assays and data acquisition methods that are compatible with its high-throughput nature.

Transfected cell arrays

Further miniaturization of gene function analysis has become possible by the development of transfected cell arrays or cell (micro)arrays [42-47]. This technique is a promising new application that combines the high throughput of arrayed nucleic acid on glass slides and the functional readouts of cell-based assays. Oligonucleotides or expression libraries are printed, together with a transfection reagent, on glass slides. Cells are plated on the transfection layer, incorporate the DNA or RNA (referred to as reverse transfection), creating localized spots of transfected cells in a layer of untransfected cells. Genes whose expression induces the cellular phenotype of interest can then be identified (Figure 6.3A). After transfection any phenotype analysis that is compatible with the small amount of cells growing on a glass slide can be performed. Using this technology, genes involved in apoptosis induction, cell adhesion and tyrosine kinase signaling [43], proteasome function [46] and NF κ B signaling [44] were identified. However, the small amounts of nucleic acid that are reverse transfected pose some technical challenges. Transfection efficiency has to be relatively high to achieve a significant knockdown that will enable phenotypic differences to be detected in a background of untransfected cells. In addition, cell adhesion has to be maintained and the spread of the nucleic acid-lipid complexes has to be restricted. The advantage of a miniaturized microarray format compared with microtiter plate-based techniques is particularly pronounced for cells that originate from limiting sources, such as primary cells. However, primary cells are hard to transfect and difficult to handle. The same limitations also apply to other cell types (e.g. EC). Overcoming these technical challenges would provide new opportunities in angiogenesis research, where (primary) EC are frequently used. Recently, Yoshikawa et al. [45] developed an optimized protocol for efficient on-chip siRNA transfection of mesenchymal stem cells, suggesting that transfected cell arrays will become a more widely applicable tool.

Both gain-of-function assays using conventional expression vectors and loss-of-function assays using small-hairpin RNA (shRNA) expression vectors can be performed. However, this technology is limited to adherent cells and two-dimensional growth, and assays are restricted to relatively static features. For example, capillary tube formation of EC requires the presence of a three-dimensional matrix, which might prove difficult to adapt to this platform. Similarly, assaying migration of EC is not compatible with the array technology because readout relies on strictly localized transfected cells on the slide. However, this array technology can identify genes that, for example, induce proliferation or apoptosis in EC, or mediate the effect of anti-angiogenic compounds, thereby providing insight in basic EC biology and putative therapeutic targets.

Figure 6.3: Functional target identification.

A) Transfected cell arrays. Nucleic acids are mixed with transfection reagents and spotted on glass slides to create the arrays. Cells are then seeded on the glass slides in cell-culture dishes. By reverse transfection, cells growing on top of a spot take up the nucleic acid. This creates an array of transfected cells within a background of untransfected cells. Finally, the effect of the internalized expression vector on the cell phenotype is measured [e.g. by detection of specific molecules (left), by assessing cell viability (middle) or by monitoring cell morphology (right)]. **B)** Genetic screens. High-complexity expression libraries (either cDNA for gain-of-function screens or shRNA for loss-of-function screens), usually of viral nature, are introduced into a population of cells. A selection procedure is applied to retrieve those cells from the population in which the expression construct induces the phenotype of interest. The responsible sequence can be retrieved from the selected cells. Subsequent selection rounds using the sequences retrieved from the first round can increase stringency and reduce the identification of false-positives.

Genetic screens

In genetic screens, complex expression libraries are introduced in a pool of cells that is then screened for individual cDNAs that confer a selectable phenotype. Cells that exhibit the trait of interest are thereby separated from the pool of cells and the inserted cDNA responsible is subsequently retrieved (Figure 6.3B). Selection can be based on, for example, cell survival, compound resistance, reporter gene expression or induction of particular proteins. The power of this approach lies in its unbiased nature because only genes directly relevant to the phenotype under study are selected and identified, providing immediate functional information. One major drawback of this approach is that induction or knockdown of a protein resulting in a lethal phenotype can not be selected. To identify genes that rescue EC from p53 mediated growth arrest, a retroviral cDNA library was introduced in Large-T endothelial cells (LTEC) that express the temperature sensitive large-T antigen (tsLT). These cells proliferate when grown at 32°C, owing to the binding to and inactivation of pRB and p53 by tsLT. At 39°C, tsLT becomes inactive and releases pRB and p53, and the cells enter growth arrest. Only those gene products that circumvent this p53 action permit cell growth at 39°C, thus

enabling selection of these cells. A novel low-density lipoprotein (LDL) receptor and a Ras-like GTPase are responsible for rescuing cellular growth arrest in LTEC. Overexpression of this Ras-like GTPase induces capillary tube formation in MatrigelTM *in vitro* in HMEC and *in vivo* in mice, but its precise mechanism is unknown [48]. The development of siRNA expression vectors enables loss-of-function screening, using the same format as the gain-of-function assays.

Candidate gene identification directly based on function significantly reduces efforts relating to its validation as a putative therapeutic target and clinical development. Moreover, these approaches do not need sequence information *a priori* and are therefore relatively unbiased. Depending on the resources available, the choice can be made between screening individual genes in a well-based or array platform and applying genetic screens to functionally identify genes of interest. The highly parallel nature of library screening in microtiter plates and transfected cell arrays is advantageous because some genes may be involved in one process but not in another. Such genes may be missed in genetic screens when sequentially different phenotypic manifestations are selected. Recent advances in high content image analysis [49,50] facilitate phenotypic analyses to be performed at a genome-wide scale, and will positively impact both the quantity and the quality of the data generated by these high-throughput applications.

>> Functional genomics in angiogenesis research

Recent advances in high-throughput functional genomics will have a major impact on our future understanding of both pathological and physiological angiogenesis. This shift in focus towards functional identification of putative therapeutic targets enables more efficient clinical translation. Most techniques described in this review rely on efficient transfection of cells. This can be a complicating factor when studying some cell types, such as EC. Recent developments in high-throughput electroporation [51], more efficient methods of transfection of primary (endothelial) cells [34,35], the use of viral vectors [38,52,53], and extensive optimizations for reverse transfection [45,54] might help to overcome these limitations. High-throughput assays for EC migration [55,56] and capillary tube formation [40] meet the requirements for relevant angiogenesis assays and enable the identification of mediators of these processes in arrayed settings. By contrast, genetic screens focus more on cell viability or induction of specific surface proteins whereby cells can be selected for further characterization of the gene of interest.

>> Concluding remarks

Functional validation tools, originally developed for the analysis of individual genes, have become useful for high-throughput functional identification with almost no limitations. In conclusion, the use of high-throughput gene expression analysis, which merely generates associative data rather than causative data, is being caught up by novel methods that enable direct functional linkage between the gene expression level and the phenotype. Although technical challenges remain, recent progresses have been made in the generation of libraries, and high-content data analysis will enable a broader scientific community to use these tools and to generate a more complete understanding of cellular processes and associated gene expression changes in a wide range of cell systems and diseases (Box 4).

Box 4. Outstanding questions

- ♦ Will functional target discovery prove superior to classical target identification by gene expression profiling?
 - ♦ What (combination of) assays should be used preferably to functionally identify modulators of angiogenesis?
 - ♦ How can we miniaturize assays but retain their relevance to the *in vivo* angiogenesis process?
 - ♦ How can we overcome limitations associated with the low transfection efficiency of EC to enable more efficient functional identification of tumor endothelial cell targets?
 - ♦ How can we integrate the wealth of functional data generated by high-throughput methods and translate this into the development of novel angiogenesis inhibiting therapeutics?
 - ♦ How can we further streamline the process from functional target discovery to clinical use?
-

>> Acknowledgements

This work was supported by a grant from the Research Institute for Growth and Development (GROW), Maastricht University. The study has been financially supported by the sixth EU Framework Programme (Integrated Project 'Angiotargeting; contract No. 504743) in the area of 'Life sciences, genomics and biotechnology for health'.

>> References

- [1] Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* 2000;52 (2):237-68.
- [2] Folkman J. Tumor angiogenesis. In: Holland JF FEI, Bast RC Jr., Kufe DW, Pollock RE, Weichselbaum RR, editor. *Cancer Medicine*, 5th Edition. Ontario, Canada: B.C. Decker Inc., 2000. pp. 132-52.
- [3] Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Walter-Yohrling J, et al. Vascular gene expression in nonneoplastic and malignant brain. *Am J Pathol* 2004;165 (2):601-8.
- [4] Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, et al. Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Res* 2004;64 (21):7857-66.
- [5] St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, et al. Genes expressed in human tumor endothelium. *Science* 2000;289 (5482):1197-202.
- [6] van Beijnum JR, Griffioen AW. In silico analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles. *Biochim Biophys Acta* 2005;1755 (2):121-34.
- [7] Auerbach R, Lewis R, Shinnars B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. *Clin Chem* 2003;49 (1):32-40.
- [8] Hasan J, Shnyder SD, Bibby M, Double JA, Bicknel R, Jayson GC. Quantitative angiogenesis assays in vivo—a review. *Angiogenesis* 2004;7 (1):1-16.
- [9] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995;270 (5235):467-70.
- [10] Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, et al. The sequence of the human genome. *Science* 2001;291 (5507):1304-51.
- [11] Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411 (6836):494-8.
- [12] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391 (6669):806-11.
- [13] Plasterk RH. RNA silencing: the genome's immune system. *Science* 2002;296 (5571):1263-5.
- [14] Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;296 (5567):550-3.
- [15] Miyagishi M, Taira K. U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* 2002;20 (5):497-500.
- [16] Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002;16 (8):948-58.
- [17] Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, Rossi J. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* 2002;20 (5):500-5.
- [18] Luo B, Heard AD, Lodish HF. Small interfering RNA production by enzymatic engineering of DNA (SPEED). *Proc Natl Acad Sci U S A* 2004;101 (15):5494-9.
- [19] Sen G, Wehrman TS, Myers JW, Blau HM. Restriction enzyme-generated siRNA (REGS) vectors and libraries. *Nat Genet* 2004;36 (2):183-9.
- [20] Yang D, Buchholz F, Huang Z, Goga A, Chen CY, Brodsky FM, Bishop JM. Short RNA duplexes produced by hydrolysis with *Escherichia coli* RNase III mediate effective RNA interference in mammalian cells. *Proc Natl Acad Sci U S A* 2002;99 (15):9942-7.
- [21] Kittler R, Putz G, Pelletier L, Poser I, Heninger AK, Drechsel D, Fischer S, Konstantinova I, Habermann B, Grabner H, et al. An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* 2004;432 (7020):1036-40.
- [22] Lu PY, Xie FY, Woodle MC. Modulation of angiogenesis with siRNA inhibitors for novel therapeutics. *Trends Mol Med* 2005;11 (3):104-13.
- [23] Chen Z, Varney ML, Backora MW, Cowan K, Solheim JC, Talmadge JE, Singh RK. Down-regulation of vascular endothelial cell growth factor-C expression using small interfering RNA vectors in mammary tumors inhibits tumor lymphangiogenesis and spontaneous metastasis and enhances survival. *Cancer Res* 2005;65 (19):9004-11.
- [24] Dell'Era P, Coco L, Ronca R, Sennino B, Presta M. Gene expression profile in fibroblast growth factor 2-transformed endothelial cells. *Oncogene* 2002;21 (15):2433-40.
- [25] Dell'Era P, Nicoli S, Peri G, Nieddu M, Ennas MG, Presta M. FGF2-induced upregulation of DNA polymerase-delta p12 subunit in endothelial cells. *Oncogene* 2005;24 (6):1117-21.

- [26] Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang Q, Storm G, Molema G, Lu PY, Scaria PV, Woodle MC. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res* 2004;32 (19):e149.
- [27] Lu PY, Xie F, Woodle MC. In vivo application of RNA interference: from functional genomics to therapeutics. *Adv Genet* 2005;54:117-42.
- [28] Sioud M. siRNA Delivery In Vivo. *Methods Mol Biol* 2005;309:237-50.
- [29] Shi F, Hoekstra D. Effective intracellular delivery of oligonucleotides in order to make sense of antisense. *J Control Release* 2004;97 (2):189-209.
- [30] Abe M, Sato Y. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. *Angiogenesis* 2001;4 (4):289-98.
- [31] Iizuka M, Abe M, Shiiba K, Sasaki I, Sato Y. Down syndrome candidate region 1, a downstream target of VEGF, participates in endothelial cell migration and angiogenesis. *J Vasc Res* 2004;41 (4):334-44.
- [32] Hotz HG, Hines OJ, Masood R, Hotz B, Foitzik T, Buhr HJ, Gill PS, Reber HA. VEGF antisense therapy inhibits tumor growth and improves survival in experimental pancreatic cancer. *Surgery* 2005;137 (2):192-9.
- [33] Gleave ME, Monia BP. Antisense therapy for cancer. *Nat Rev Cancer* 2005;5 (6):468-79.
- [34] Zaric V, Weltin D, Erbacher P, Remy JS, Behr JP, Stephan D. Effective polyethylenimine-mediated gene transfer into human endothelial cells. *J Gene Med* 2004;6 (2):176-84.
- [35] Hernandez JL, Coll T, Ciudad CJ. A highly efficient electroporation method for the transfection of endothelial cells. *Angiogenesis* 2004;7 (3):235-41.
- [36] Iversen N, Birkenes B, Torsdalen K, Djurovic S. Electroporation by nucleofector is the best nonviral transfection technique in human endothelial and smooth muscle cells. *Genet Vaccines Ther* 2005;3 (1):2.
- [37] Horwood NJ, Smith C, Andreaskos E, Quattrocchi E, Brennan FM, Feldmann M, Foxwell BM. High-efficiency gene transfer into nontransformed cells: utility for studying gene regulation and analysis of potential therapeutic targets. *Arthritis Res* 2002;4 Suppl 3:S215-25.
- [38] Li M, Rossi JJ. Lentiviral Vector Delivery of siRNA and shRNA Encoding Genes into Cultured and Primary Hematopoietic Cells. *Methods Mol Biol* 2005;309:261-72.
- [39] Leimeister C, Schumacher N, Steidl C, Gessler M. Analysis of HeyL expression in wild-type and Notch pathway mutant mouse embryos. *Mech Dev* 2000;98 (1-2):175-8.
- [40] Michiels F, van Es H, van Rompaey L, Merchiers P, Francken B, Pittois K, van der Schueren J, Brys R, Vandersmissen J, Beirincx F, et al. Arrayed adenoviral expression libraries for functional screening. *Nat Biotechnol* 2002;20 (11):1154-7.
- [41] Arts GJ, Langemeijer E, Tissingh R, Ma L, Pavliska H, Dokic K, Dooijes R, Mesic E, Clasen R, Michiels F, et al. Adenoviral vectors expressing siRNAs for discovery and validation of gene function. *Genome Res* 2003;13 (10):2325-32.
- [42] Wheeler DB, Bailey SN, Guertin DA, Carpenter AE, Higgins CO, Sabatini DM. RNAi living-cell microarrays for loss-of-function screens in *Drosophila melanogaster* cells. *Nat Methods* 2004;1 (2):127-32.
- [43] Ziauddin J, Sabatini DM. Microarrays of cells expressing defined cDNAs. *Nature* 2001;411 (6833):107-10.
- [44] Zheng L, Liu J, Batalov S, Zhou D, Orth A, Ding S, Schultz PG. An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. *Proc Natl Acad Sci U S A* 2004;101 (1):135-40.
- [45] Yoshikawa T, Uchimura E, Kishi M, Funeriu DP, Miyake M, Miyake J. Transfection microarray of human mesenchymal stem cells and on-chip siRNA gene knockdown. *J Control Release* 2004;96 (2):227-32.
- [46] Silva JM, Mizuno H, Brady A, Lucito R, Hannon GJ. RNA interference microarrays: high-throughput loss-of-function genetics in mammalian cells. *Proc Natl Acad Sci U S A* 2004;101 (17):6548-52.
- [47] Mousses S, Caplen NJ, Cornelison R, Weaver D, Basik M, Hautaniemi S, Elkahoul AG, Lotufo RA, Choudary A, Dougherty ER, et al. RNAi microarray analysis in cultured mammalian cells. *Genome Res* 2003;13 (10):2341-7.
- [48] Abstracts of the 15th Endothelial Cell Research Symposium. Maastricht, the Netherlands, 23 November 2004. *Angiogenesis* 2004. pp. 165-89.
- [49] Harada JN, Bower KE, Orth AP, Callaway S, Nelson CG, Laris C, Hogenesch JB, Vogt PK, Chanda SK. Identification of novel mammalian growth regulatory factors by genome-scale quantitative image analysis. *Genome Res* 2005;15 (8):1136-44.
- [50] Conrad C, Erle E, Warnat P, Daigle N, Lorch T, Ellenberg J, Pepperkok R, Ellis R. Automatic identification of subcellular phenotypes on human cell arrays. *Genome Res* 2004;14 (6):1130-6.
- [51] Clayton J. RNA interference: the silent treatment. *Nature* 2004;431 (7008):599-605.
- [52] Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* 2002;2 (3):243-7.
- [53] Mizuguchi H, Hayakawa T. Targeted adenovirus vectors. *Hum Gene Ther* 2004;15 (11):1034-44.

- [54] Palmer E, Freeman T. Cell-based microarrays: current progress, future prospects. *Pharmacogenomics* 2005;6 (5):527-34.
- [55] Mastuyugin V, McWhinnie E, Labow M, Buxton F. A quantitative high-throughput endothelial cell migration assay. *J Biomol Screen* 2004;9 (8):712-8.
- [56] Yarrow JC, Perlman ZE, Westwood NJ, Mitchison TJ. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC Biotechnol* 2004;4:21.
- [57] Song J, Pang S, Lu Y, Yokoyama KK, Zheng JY, Chiu R. Gene silencing in androgen-responsive prostate cancer cells from the tissue-specific prostate-specific antigen promoter. *Cancer Res* 2004;64 (21):7661-3.
- [58] van de Wetering M, Oving I, Muncan V, Pon Fong MT, Brantjes H, van Leenen D, Holstege FC, Brummelkamp TR, Agami R, Clevers H. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Rep* 2003;4 (6):609-15.
- [59] Matsukura S, Jones PA, Takai D. Establishment of conditional vectors for hairpin siRNA knockdowns. *Nucleic Acids Res* 2003;31 (15):e777.
- [60] Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, Van Parijs L, Jaenisch R, Jacks T. Cre-lox-regulated conditional RNA interference from transgenes. *Proc Natl Acad Sci U S A* 2004;101 (28):10380-5.
- [61] Matveeva OV, Mathews DH, Tsodikov AD, Shabalina SA, Gesteland RF, Atkins JF, Freier SM. Thermodynamic criteria for high hit rate antisense oligonucleotide design. *Nucleic Acids Res* 2003;31 (17):4989-94.
- [62] Giddings MC, Shah AA, Freier S, Atkins JF, Gesteland RF, Matveeva OV. Artificial neural network prediction of antisense oligodeoxynucleotide activity. *Nucleic Acids Res* 2002;30 (19):4295-304.
- [63] Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim Biophys Acta* 1999;1489 (1):69-84.
- [64] Ray A, Norden B. Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. *Faseb J* 2000;14 (9):1041-60.
- [65] Jepsen JS, Wengel J. LNA-antisense rivals siRNA for gene silencing. *Curr Opin Drug Discov Devel* 2004;7 (2):188-94.
- [66] Schmajuk G, Sierakowska H, Kole R. Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. *J Biol Chem* 1999;274 (31):21783-9.

Chapter 7

General discussion

>> Gene expression in endothelial cells

The inhibition of new vessel formation in tumors and the destruction of existing tumor vasculature is thought to present a powerful means of cancer treatment. A thorough understanding of the functional and phenotypic characteristics of tumor endothelial cells is pivotal to designing novel therapeutics.

In this thesis we describe the identification of candidate target genes for angiostatic tumor therapy by investigating gene expression changes in tumor endothelium as compared to normal and angiogenic endothelium. It has been recognized that tumor endothelial cells differ from normal, resting endothelial cells, and that these characteristics are highly influenced by microenvironmental clues (chapter 1). The gene expression response of endothelial cells to different angiogenic factors *in vitro* has been extensively studied. As described in chapter 2, different growth factors applied to endothelial cells *in vitro* evoke different gene expression responses. Part of these differences can be intrinsic to the different growth factors applied; other biases may be related to the gene expression profiling platform and particular culture conditions that were used. Nevertheless, the functional signature of growth factor stimulated endothelial cells is indicative of active proliferation and turnover. Similarly, only a limited number of genes is concordantly regulated in different tube formation assays, though a clear functional bias towards adhesion molecules and matrix remodeling proteins is observed. However, the extrapolation of these data to the *in vivo* situation is limited. Therefore it may be desirable to use endothelial cells isolated directly from the tissues of interest. Based on the expression of specific markers, endothelial cells can be purified by using immunomagnetic or flowcytometric separation techniques [1]. So far, few studies have so far reported on gene expression profiles of endothelial cells isolated from tumor tissues [2-4]. Comparison of upregulated genes in these different tumors revealed a particularly strong bias towards extracellular matrix proteins, especially collagens, though only SPARC was upregulated in all tumor types examined. In conclusion, comparison of *in vitro* models with the *in vivo* situation indicates clear differences in gene expression patterns.

We observed similar differences when we compared genes overexpressed in tumor endothelial cells with genes upregulated in activated HUVEC (chapter 3), as only 24 transcripts of a total of 265 (9%) were concordantly overexpressed in the two comparisons. It has been shown that endothelial cells isolated from fresh tissues retain their phenotype in culture for some time [5-8] though over time their phenotype may change as a consequence of the altered microenvironment in culture [9,10]. Recent work has demonstrated that isolated tumor endothelial cells maintained in culture differed considerably from normal endothelial cells [11,12]. Thus, cultured tumor endothelial cells may be considered an alternative source for expression profiling studies, circumventing problems associated with limited material available directly after isolation. We used suppression subtractive hybridization (SSH) to profile gene expression in diverse angiogenic and non-angiogenic endothelial cells. Major strengths

of SSH are its independence of known sequences and the generation of non-redundant repertoires, enabling the discovery of rare and previously unknown transcripts, for example, certain tissue-specific splice variants. Comparing gene expression in tumor angiogenesis with physiological angiogenesis in the placenta revealed considerable overlap (85/142) referred to as GAG/A transcripts. This is not surprising, since many angiogenic factors play a role in both physiological and pathological angiogenesis. Moreover, it emphasizes the importance of our approach to focus on TAGs, which are genes that are overexpressed in tumor endothelial cells vs. both normal endothelial cells and placenta endothelial cells. This way, the chance of interfering with physiological angiogenesis when targeting a TAG is minimized.

Using our method, we identified SPARC, collagen I α 1 and collagen IV α 1 that have previously been confirmed as tumor endothelial markers in brain, colon and breast tumors by SAGE [2-4]. However, the latter studies used only a very limited number of tumors and normal tissues for their analysis, and therefore have a considerable risk of representing patient-related features rather than general ones. We pooled the RNA of endothelial cells isolated from 5 different individuals, and used normal tissue of the same patients to minimize the introduction of such artifacts. Therefore, we are confident that the TAGs we identified are representative for the endothelium of most colorectal tumors. However, different types of tumors may display different TAGs – though some TAGs appear to be pan-tumor endothelium specific – and it would be valuable to further investigate this to enable the development of therapeutics applicable to diverse types of cancer.

>> Target validation

Though gene expression studies provide valuable insights in the biology of the tissue under investigation, they do not discriminate between genes that are causally related to the phenotype, i.e. normalization of the changed expression level or blocking the aberrantly expressed protein results in reversal of the phenotype, and genes that are merely changed in expression to meet altered metabolic demands. Because the characterization and functional validation is a time-consuming and laborious process, it is critical to prioritize putative therapeutic targets. Based on gene ontology databases and literature searches, some functional clues about a gene can be elucidated to determine its potential as a therapeutically targetable molecule. For vascular targeting strategies, extracellular localization of the targeted protein is required, though for classical anti-angiogenic strategies, molecules that function in cell-cycle progression or apoptosis regulation may also be valuable. From the set of 17 TAGs, we chose four genes with a reported extracellular localization: vimentin, CD59, HMGB1, and IGFBP7 (chapter 3). Using antibodies as targeting molecules we tested whether interference with protein function had impact on endothelial cells *in vitro* as well as *in vivo*. In chapter 3, we show that antibodies directed at these proteins interfered with tube

formation of bovine endothelial cells. Furthermore, antibodies directed against HMGB1, CD59 and vimentin also inhibited blood vessel formation *in vivo* in the CAM assay. This latter model is actually a model for developmental angiogenesis and might not be considered appropriate to investigate tumor angiogenesis. However, different genes playing a role in developmental processes have been shown to be re-expressed during tumorigenesis and tumor angiogenesis [14], e.g. the ED-B domain of fibronectin [15]. In subsequent experiments, colon tumor cells were grown on top of the CAMs to provide a better model and antibodies directed against HMGB1 and vimentin were capable of inhibiting the tumor induced vasculature (chapters 4 and 5). Our findings that antibodies directed against vimentin were capable of inhibiting tumor vascularization and growth provides proof that the molecules we identified might be used for targeting tumor angiogenesis. Furthermore, the observation that anti-vimentin antibodies target to the tumor vasculature provides the opportunity to image the tumor vasculature for diagnosis and/or disease monitoring [16,17], or to deliver therapeutics [18,19].

Vimentin

The successful inhibition of angiogenesis by targeting vimentin with antibodies was somewhat surprising, as the protein has been extensively documented as an intracellular cytoskeletal protein. However, recent publications have questioned the exclusive intracellular localization of vimentin. In macrophages, secreted vimentin was shown to be actively involved in phagocytosis of bacteria [20]. Vimentin tail domain, detected by antibody clone V9, was detected on the surface of neutrophils undergoing apoptosis, though at present it is not clear whether the detected vimentin is representative of the total protein or of a caspase generated cleavage fragment [21]. In contrast, on apoptotic T-lymphocytes both the rod and the tail domain of vimentin could be detected on the surface using different antibodies [22]. Furthermore, vimentin was identified as the ligand for SC5 mAb and located on the extracellular side of the plasma membrane of activated normal T-lymphocytes and malignant Sézary syndrome cells [23]. Thus, vimentin expression on the surface of EC might be feasible, and might contribute to tumor angiogenesis.

In chapter 4 we further characterized the function of vimentin in different aspects of angiogenesis. Two different antibodies reactive with vimentin were capable of inhibiting endothelial cell migration and tube formation. This suggests the presence of vimentin on the cell surface or in the extracellular environment, as antibodies added to the culture medium can only interfere with proteins that are accessible from the extracellular space. Surface associated expression was further substantiated by the observations that non-permeabilized cells show immunoreactivity with the vimentin antibodies in flowcytometry and in immunofluorescence studies. This surface associated pool of vimentin appears more dynamic than the total cellular pool of vimentin as it was more abundant in expansively growing cells than in contact-inhibited cells, showed polarization in the direction of migration and was increased after treatment with the phosphatase 2A inhibitor okadaic acid. Interestingly,

epigallocatechin gallate (EGCG), a green tea compound that has proven anti-angiogenic activity [24,25], was recently shown to bind vimentin [26]. EGCG can specifically block the phosphorylation of vimentin, via inhibition of CDC2 kinase activity, suggesting that phosphorylation of vimentin plays an important role in angiogenesis. Taken together, we have shown increased vimentin expression on the surface of tumor endothelial cells and demonstrated that interference with vimentin function impairs the angiogenic potential of endothelial cells.

Based on our observations, one might expect severe vascular defects or even embryonic lethality in *vim*^{-/-} mice. Interestingly, vimentin knockout mice are viable and show no obvious phenotype [27]. Only detailed examination revealed vascular defects [27-29] and impaired wound healing due to delayed migration of fibroblasts [30]. Furthermore, transplantation of *vim*^{-/-} teratocarcinoma cells in *vim*^{+/+} recipient mice did not show any differences as compared to *vim*^{+/+} teratocarcinomas transplanted in *vim*^{+/+} mice [31]. Though the endothelial cells of *vim*^{-/-} recipient mice do not express vimentin, compensatory mechanisms may be responsible for survival and tumor angiogenesis. Whether fibroblasts and other vimentin expressing cells also display vimentin on the cell surface, and whether this may give rise to adverse effects in a therapeutic setting requires further investigation.

It was recently postulated that PAL-E antibody, used for decades to distinguish blood and lymphatic vessels, recognizes a secreted vimentin dimer [32]. This PAL-E reactive protein was immunoprecipitated from HMEC and was recognized by different anti-vimentin antibodies. Hence, we hypothesized that this secreted form of vimentin might be the surface accessible fraction, which we successfully targeted with anti-vimentin antibodies in a therapeutic anti-angiogenesis setting. Indeed, *in vitro* migration and tube formation assays in the presence of PAL-E suggested an inhibitory effect of the antibody on the angiogenic properties of EC (data not shown). However, others have argued the antigen reactive with PAL-E antibody to be plasmalemmal vesicle 1 (PV-1), a homodimeric transmembrane protein associated with caveolae [33]. Transfection of vimentin and PAL-E antigen negative CHO cells with PV-1 resulted in immunoreactivity with PAL-E. Also, clearance of lysates with V9 antibody did not reduce PAL-E reactivity, suggesting vimentin is not the antigen recognized by PAL-E. However, both studies lack the final proof of the identity of PAL-E antigen. First of all, V9 should have been used by Xu et al. [32] to immunoprecipitate a protein reactive with PAL-E in western blot, to confirm the immunoreactivity of immunoprecipitated PAL-E antigen with anti-vimentin antibodies. Second, downregulation of vimentin and PV-1 expression in PAL-E reactive endothelial cells by using RNA interference should reveal which gene is responsible for PAL-E reactivity. Thirdly, vimentin could be expressed in cells that are not reactive with PAL-E to determine whether it induces reactivity, though cell-type related protein modification factors may not allow PAL-E antigen to form in non microvascular endothelial cells. Our own observations point to a role for PAL-E antigen in EC migration and tube formation similar to that of vimentin, but we can not be conclusive about the identity of the antigen. Thus, whether PAL-E

targets a post-translationally modified form of vimentin or the human homologue of PV-1 (or possibly another protein) is still under debate.

HMGB1

HMGB1 is another protein that was initially regarded to be expressed intracellularly as a chromatin binding molecule [34], but later was shown to be expressed extracellularly as well [35,36]. As described in chapter 5, antibodies directed against HMGB1 were effective in inhibiting endothelial cell migration and tube formation as well as blood vessel formation in the CAM assay, though we could not directly assess whether it was expressed on the surface of endothelial cells or whether it was secreted. Mullins et al. [37] reported that secretion of HMGB1 is preceded by relocalization of the protein from the nucleus to the cytoplasm after stimulation of endothelial cells with $\text{TNF}\alpha$ and LPS. Using immunofluorescence, we observed cytoplasmic staining for HMGB1 in a subset of endothelial cells cultured *in vitro*, suggesting the protein may be secreted.

HMGB1 exerts its extracellular effects by interacting with the receptor for advanced glycation end products (RAGE) (recently reviewed by Bierhaus et al. [38]). Another ligand for RAGE, AGE, induces VEGF production in endothelial cells [39,40]. Hence HMGB1-RAGE signaling may also induce VEGF production, though this has not been proven directly. Though EC may not be the major contributors of VEGF production in tumors, *in vitro* effects of HMGB1 may be caused by increased VEGF production resulting from HMGB1-RAGE interactions. Stimulation of endothelial cells with recombinant HMGB1 in the presence or absence of RAGE blocking antibodies and measurement of VEGF levels might clarify this issue. Blocking HMGB1-RAGE interactions inhibited tumor growth and invasion in C6 gliomas, LLC and Ha-ras +/+ spontaneous papillomas [41]. Unfortunately, the authors did not address the vascular density of treated tumors to determine whether some of the effects were mediated by angiogenesis inhibition, as we observed a predominant endothelial localization of HMGB1 protein in colon tumors by immunohistochemistry. At this moment, our data and that of others point to a role for HMGB1 in tumor angiogenesis, but whether it will prove to be a feasible therapeutic target requires further investigation.

The therapeutic potential of the remainder of the TAGs has not been elucidated yet. Based on the successful targeting of vimentin and HMGB1 in (tumor) angiogenesis *in vitro* as well as *in vivo*, we are confident that more TAGs will prove at least equally good therapeutic targets. The overexpression of collagens $\text{I}\alpha 1$ and $\text{IV}\alpha 1$ on the endothelial cells of other types of cancer [2-4] suggests these molecules might be interesting candidate genes for future investigation. A further challenge lies in the characterization of the genes with as yet an unknown function. It is interesting to note that TAG-38 (HSPC014) has previously been identified as overexpressed in an SSH analysis of colon tumor tissue vs. normal colon tissue [42]. Whether the encoded protein is present on both tumor and endothelial cells, is not known. A search for

common transcription factors regulating the expression of TAGs by TransFac (<http://www.gene-regulation.com/pub/databases.html#transfac>) revealed that of only a few TAGs the transcription factors were known. Hence, no conclusive statement can be made about possible transcription factors that are responsible for the observed gene regulation. Ingenuity pathway analysis (<http://www.ingenuity.com>) revealed that the dominant pathways that TAGs and GAGs are involved in are cancer, invasion of cells, apoptosis of tumor cells, cellular growth and proliferation, and cell movement (Table 7.1). Surprisingly, vimentin is not classified as gene playing a role in cancer and cell movement, though extensive literature is available describing these functions [30,43-45], and the same is true for HMGB1 [36,46,47]. Though very useful tools for the analysis of large datasets, such database analysis apparently need to be interpreted with caution as they have their limitations.

Table 7.1: Pathway analysis of TAGs and GAGs

Cell growth & proliferation	Cancer	Cell motility	Invasion of tumor cells	Cell death & apoptosis
AKAP13	AKAP13			
ATP5G1				CARD8
CAV1	CAV1	CAV1	CAV1	CAV1
CCL2	CCL2	CCL2	CCL2	CCL2
	CD59	CD59		CD59
CD86	CD86	CD86		
CDK2AP1				
COL1A1	COL1A1			
CTSB	CTSB	CTSB	CTSB	CTSB
EIF4A1				
FKBP1A				FKBP1A
HEYL				FOSL2
HNRPC				HEYL
				HNRPC
ICMT	ICMT	HSPCA		
IGFBP7				IGFBP7
INSR	INSR			INSR
ITGB1	ITGB1	ITGB1	ITGB1	ITGB1
	MMP1	MMP1	MMP1	MMP1
MMP3	MMP3	MMP3	MMP3	MMP3
PECAM1	PECAM1	PECAM1		PECAM1
PSMF1				
	RALB			
RASGRF2				
	RPL22			RNASE1
SHFM1				
SPARC		SPARC	SPARC	SPARC
		TMSB4X		
			VIM	

>> Technological advances

The recent development of small interfering RNA molecules for knockdown of protein expression allows studying protein function without using small molecules or antibodies that block the protein directly. This is especially useful when genes are investigated for which no antibodies or small molecule inhibitors are available yet. Generally, the technique requires the introduction of small interfering RNA molecules into the cell, either as duplex RNA molecules or incorporated in vector systems, by transfection. Primary cells, e.g. EC can however be difficult to transfect, resulting in low transgene expression, which may obscure the effects of protein knockdown, and may be difficult to apply *in vivo*. As described in chapter 6, the cumbersome process of target validation following gene expression profiling analysis has resulted in the development of novel high-throughput technologies for functional target validation and identification. Several techniques employing RNA interference or expression vectors are now available to identify candidate target genes based on the cellular phenotype they induce, enabling a more direct approach for identifying clinically useful therapeutic targets and reducing target validation efforts. Implementing such technologies in angiogenesis research could contribute considerably to a more efficient identification of putative therapeutic candidate molecules.

Bearing vascular targeting applications in mind, direct analysis of proteins expressed on tumor endothelial cells poses an alternative to gene expression profiling studies. These approaches may also take into account post-translational modifications of proteins. Peptide- and antibody phage display approaches have yielded some tumor endothelial targets directly accessible from the blood stream [48,49]. Recently, direct analysis of endothelial membrane fractions has been used to identify differentially expressed proteins on the luminal surface of tumor endothelial cells using peptide mass-spectrometry [50,51]. Major advantages of these proteomics approaches over genomics approaches are the ability to directly focus on extracellularly expressed proteins and to take into account post-translational protein modifications. A drawback of these techniques is unfortunately their currently limited applicability to the human situation.

>> Conclusions and outlook

We have successfully used SSH to profile changes in gene expression in endothelial cells of different origins in order to identify genes associated specifically with tumor angiogenesis. We identified both known and novel markers of tumor angiogenesis (TAGs). We subsequently assessed the functional contribution of different TAGs to various aspects of angiogenesis *in vitro* and *in vivo*. We show that antibody targeting of one of the TAGs, i.e. vimentin, effectively reduces tumor size and tumor vascular density, underscoring the feasibility of our approach.

Inhibition of angiogenesis is thought to contribute considerably to the treatment of cancer in the near future, though it is envisaged that this will mainly be in combination with other therapeutic modalities, such as chemotherapeutics. Inhibition of new vessel formation can help prevent the outgrowth of metastasis to clinically relevant tumors and prevent renewed growth of tumors that have acquired drug resistance. Clinical medicine will benefit most from truly novel therapeutics that have been rationally designed.

The approval of anti-VEGF antibodies merely illustrates the interest in vascular targeting and angiogenesis inhibition as anticancer strategy, but these drugs rely basically on the same idea as classical chemotherapeutics, i.e. a larger dependence of the diseased cell than the healthy cell on the factor or process being blocked. Hence, side effects of anti-VEGF antibodies as well as other angiogenic growth factor inhibition are imminent. In fact, besides its vascular activity, VEGF is trophic for nerve cells, lung epithelium and cardiac muscle, pointing out that when anti-VEGF therapeutics are overdosed this may give rise to neurodegeneration, respiratory distress and cardiac failure [52,53]. The use of anti-VEGF therapeutics should be carefully considered in patients at risk for stroke or other ischaemic heart diseases.

The deprivation of angiogenic growth factors is also prone to the development of drug resistance as tumors may escape the angiogenesis inhibition by shifting to the production of other growth factors. Agents that counteract the endothelial phenotype induced by the presence of the tumor can accomplish a prolonged suppression of angiogenesis. For example, endostatin was shown to downregulate several pathways that are induced during angiogenesis, resetting the activated endothelial cell to a quiescent one [54]. Profiling the response of tumor cells and endothelial cells to anginex revealed a considerable induction of angiopoietin-3/4, TIE1 and TIE2, and a decrease in VEGF-B and VEGF-D expression in non-tumor tissue (vasculature and stroma) [55]. The identification of the endothelial receptors that mediate these effects may further facilitate the development of novel angiogenesis inhibitors.

In conclusion, the identification of molecular targets amenable for interference with tumor endothelial cell function provides a more detailed insight in the molecular regulation of tumor angiogenesis and enables the development of novel diagnostic tools and improved strategies for the treatment of cancer by therapeutic interference in angiogenesis.

>> References

- [1] Hillen F, Melotte V, van Beijnum JR, Griffioen AW. Endothelial cell biology. In: Staton CA, Lewis C, Bicknell R, editors. *Angiogenesis assays: a critical appraisal of current techniques*. London: John Wiley and Sons, *In press*.
- [2] Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Walter-Yohrling J, Rouleau C, Akmaev VR, Wang CJ, Cao X, St Martin TB, Roberts BL, Teicher BA, Klinger KW, Stan RV, Lucey B, Carson-Walter EB, Larterra J, Walter KA. Vascular gene expression in nonneoplastic and malignant brain. *Am J Pathol* 2004;165 (2):601-8.
- [3] Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, Nacht M, Teicher BA, Klinger KW, Sukumar S, Madden SL. Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Res* 2004;64 (21):7857-66.
- [4] St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW. Genes expressed in human tumor endothelium. *Science* 2000;289 (5482):1197-202.
- [5] Griffioen AW. Phenotype of the tumor vasculature; cell adhesion as a target for tumor therapy. *The Cancer Journal* 1997;10 (5):249-55.
- [6] Folkman J, Haudenschild CC, Zetter BR. Long-term culture of capillary endothelial cells. *Proc Natl Acad Sci U S A* 1979;76 (10):5217-21.
- [7] Bussolati B, Deambrosio I, Russo S, Derigibus MC, Camussi G. Altered angiogenesis and survival in human tumor-derived endothelial cells. *Faseb J* 2003;17 (9):1159-61.
- [8] Kallmann BA, Wagner S, Hummel V, Buttman M, Bayas A, Tonn JC, Rieckmann P. Characteristic gene expression profile of primary human cerebral endothelial cells. *Faseb J* 2002;16 (6):589-91.
- [9] Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM. Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. *Am J Physiol Heart Circ Physiol* 2003;285 (5):H1917-38.
- [10] Oxhorn BC, Hirzel DJ, Buxton IL. Isolation and characterization of large numbers of endothelial cells for studies of cell signaling. *Microvasc Res* 2002;64 (2):302-15.
- [11] Bian XW, Jiang XF, Chen JH, Bai JS, Dai C, Wang QL, Lu JY, Zhao W, Xin R, Liu MY, Shi JQ, Wang JM. Increased angiogenic capabilities of endothelial cells from microvessels of malignant human gliomas. *Int Immunopharmacol* 2006;6 (1):90-9.
- [12] Grange C, Bussolati B, Bruno S, Fonsato V, Sapino A, Camussi G. Isolation and characterization of human breast tumor-derived endothelial cells. *Oncol Rep* 2006;15 (2):381-6.
- [13] Szaniszló P, Wang N, Sinha M, Reece LM, Van Hook JW, Luxon BA, Leary JF. Getting the right cells to the array: Gene expression microarray analysis of cell mixtures and sorted cells. *Cytometry A* 2004;59 (2):191-202.
- [14] Coggin JH, Jr. The implications of embryonic gene expression in neoplasia. *Crit Rev Oncol Hematol* 1986;5 (1):37-55.
- [15] Kaczmarek J, Castellani P, Nicolo G, Spina B, Allemanni G, Zardi L. Distribution of oncofetal fibronectin isoforms in normal, hyperplastic and neoplastic human breast tissues. *Int J Cancer* 1994;59 (1):11-6.
- [16] Brack SS, Dinkelborg LM, Neri D. Molecular targeting of angiogenesis for imaging and therapy. *Eur J Nucl Med Mol Imaging* 2004;31 (9):1327-41.
- [17] Mulder WJ, Strijkers GJ, Griffioen AW, van Bloois L, Molema G, Storm G, Koning GA, Nicolay K. A liposomal system for contrast-enhanced magnetic resonance imaging of molecular targets. *Bioconjug Chem* 2004;15 (4):799-806.
- [18] Fabbrini M, Trachsel E, Soldani P, Bindi S, Alessi P, Bracci L, Kosmehl H, Zardi L, Neri D, Neri P. Selective occlusion of tumor blood vessels by targeted delivery of an antibody-photosensitizer conjugate. *Int J Cancer* 2005.
- [19] Neri D, Bicknell R. Tumour vascular targeting. *Nat Rev Cancer* 2005;5 (6):436-46.
- [20] Mor-Vaknin N, Puntieri A, Sitwala K, Markovitz DM. Vimentin is secreted by activated macrophages. *Nat Cell Biol* 2003;5 (1):59-63.
- [21] Moisan E, Girard D. Cell surface expression of intermediate filament proteins vimentin and lamin B1 in human neutrophil spontaneous apoptosis. *J Leukoc Biol* 2005.
- [22] Boilard E, Bourgoin SG, Bernatchez C, Surette ME. Identification of an autoantigen on the surface of apoptotic human T cells as a new protein interacting with inflammatory group IIA phospholipase A2. *Blood* 2003;102 (8):2901-9.
- [23] Huet D, Bagot M, Loyaux D, Capdevielle J, Conraux L, Ferrara P, Bensussan A, Marie-Cardine A. SC5 mAb Represents a Unique Tool for the Detection of Extracellular Vimentin as a Specific Marker of Sezary Cells. *J Immunol* 2006;176 (1):652-9.

- [24] Tang FY, Nguyen N, Meydani M. Green tea catechins inhibit VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule. *Int J Cancer* 2003;106 (6):871-8.
- [25] Fassina G, Vene R, Morini M, Minghelli S, Benelli R, Noonan DM, Albini A. Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. *Clin Cancer Res* 2004;10 (14):4865-73.
- [26] Ermakova S, Choi BY, Choi HS, Kang BS, Bode AM, Dong Z. The intermediate filament protein vimentin is a new target for epigallocatechin gallate. *J Biol Chem* 2005;280 (17):16882-90.
- [27] Colucci-Guyon E, Portier MM, Dunia I, Paulin D, Pournin S, Babinet C. Mice lacking vimentin develop and reproduce without an obvious phenotype. *Cell* 1994;79 (4):679-94.
- [28] Henrion D, Terzi F, Matrougui K, Duriez M, Boulanger CM, Colucci-Guyon E, Babinet C, Briand P, Friedlander G, Poitevin P, Levy BI. Impaired flow-induced dilation in mesenteric resistance arteries from mice lacking vimentin. *J Clin Invest* 1997;100 (11):2909-14.
- [29] Terzi F, Henrion D, Colucci-Guyon E, Federici P, Babinet C, Levy BI, Briand P, Friedlander G. Reduction of renal mass is lethal in mice lacking vimentin. Role of endothelin-nitric oxide imbalance. *J Clin Invest* 1997;100 (6):1520-8.
- [30] Eckes B, Dogic D, Colucci-Guyon E, Wang N, Maniotis A, Ingber D, Merckling A, Langa F, Aumailley M, Delouvee A, Kotliansky V, Babinet C, Krieg T. Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts. *J Cell Sci* 1998;111 (Pt 13):1897-907.
- [31] Langa F, Kress C, Colucci-Guyon E, Khun H, Vandormael-Pournin S, Huerre M, Babinet C. Teratocarcinomas induced by embryonic stem (ES) cells lacking vimentin: an approach to study the role of vimentin in tumorigenesis. *J Cell Sci* 2000;113 Pt 19:3463-72.
- [32] Xu B, DeWaal RM, Mor-Vaknin N, Hibbard C, Markovitz DM, Kahn ML. The Endothelial Cell-Specific Antibody PAL-E Identifies a Secreted Form of Vimentin in the Blood Vasculature. *Mol Cell Biol* 2004;24 (20):9198-206.
- [33] Niemela H, Elima K, Henttinen T, Ijala H, Salmi M, Jalkanen S. Molecular identification of PAL-E, a widely used endothelial-cell marker. *Blood* 2005;106 (10):3405-9.
- [34] Yu SS, Li HJ, Goodwin GH, Johns EW. Interaction of non-histone chromosomal proteins HMG1 and HMG2 with DNA. *Eur J Biochem* 1977;78 (2):497-502.
- [35] Parkkinen J, Rauvala H. Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphotericin. Enhancement of t-PA-catalyzed plasminogen activation by amphotericin. *J Biol Chem* 1991;266 (25):16730-5.
- [36] Merenmies J, Pihlaskari R, Laitinen J, Wartiovaara J, Rauvala H. 30-kDa heparin-binding protein of brain (amphotericin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. *J Biol Chem* 1991;266 (25):16722-9.
- [37] Mullins GE, Sunden-Cullberg J, Johansson AS, Rouhiainen A, Erlandsson-Harris H, Yang H, Tracey KJ, Rauvala H, Palmblad J, Andersson J, Treutiger CJ. Activation of human umbilical vein endothelial cells leads to relocation and release of high-mobility group box chromosomal protein 1. *Scand J Immunol* 2004;60 (6):566-73.
- [38] Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM, Nawroth PP. Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med* 2005.
- [39] Okamoto T, Yamagishi S, Inagaki Y, Amano S, Koga K, Abe R, Takeuchi M, Ohno S, Yoshimura A, Makita Z. Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin. *Faseb J* 2002;16 (14):1928-30.
- [40] Yamagishi S, Yonekura H, Yamamoto Y, Katsuno K, Sato F, Mita I, Ooka H, Satozawa N, Kawakami T, Nomura M, Yamamoto H. Advanced glycation end products-driven angiogenesis in vitro. Induction of the growth and tube formation of human microvascular endothelial cells through autocrine vascular endothelial growth factor. *J Biol Chem* 1997;272 (13):8723-30.
- [41] Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM. Blockade of RAGE-amphotericin signalling suppresses tumour growth and metastases. *Nature* 2000;405 (6784):354-60.
- [42] Van Beijnum JR, Moerkerk PT, Gerbers AJ, De Bruine AP, Arends JW, Hoogenboom HR, Hufton SE. Target validation for genomics using peptide-specific phage antibodies: a study of five gene products overexpressed in colorectal cancer. *Int J Cancer* 2002;101 (2):118-27.
- [43] Ramaekers FC, Haag D, Kant A, Moesker O, Jap PH, Vooijs GP. Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. *Proc Natl Acad Sci U S A* 1983;80 (9):2618-22.
- [44] Hendrix MJ, Seftor EA, Chu YW, Trevor KT, Seftor RE. Role of intermediate filaments in migration, invasion and metastasis. *Cancer Metastasis Rev* 1996;15 (4):507-25.
- [45] Gilles C, Polette M, Zahm JM, Tournier JM, Volders L, Foidart JM, Birembaut P. Vimentin contributes to human mammary epithelial cell migration. *J Cell Sci* 1999;112 (Pt 24):4615-25.
- [46] Fages C, Nolo R, Huttunen HJ, Eskelinen E, Rauvala H. Regulation of cell migration by amphotericin. *J Cell Sci* 2000;113 (Pt 4):611-20.

- [47] Huttunen HJ, Rauvala H. Amphoterin as an extracellular regulator of cell motility: from discovery to disease. *J Intern Med* 2004;255 (3):351-66.
- [48] Borsi L, Balza E, Bestagno M, Castellani P, Carnemolla B, Biro A, Leprini A, Sepulveda J, Burrone O, Neri D, Zardi L. Selective targeting of tumoral vasculature: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. *Int J Cancer* 2002;102 (1):75-85.
- [49] Ruoslahti E, Rajotte D. An address system in the vasculature of normal tissues and tumors. *Annu Rev Immunol* 2000;18:813-27.
- [50] Rybak JN, Ettore A, Kaissling B, Giavazzi R, Neri D, Elia G. In vivo protein biotinylation for identification of organ-specific antigens accessible from the vasculature. *Nat Methods* 2005;2 (4):291-8.
- [51] Oh P, Li Y, Yu J, Durr E, Krasinska KM, Carver LA, Testa JE, Schnitzer JE. Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature* 2004;429 (6992):629-35.
- [52] Greenberg DA, Jin K. From angiogenesis to neuropathology. *Nature* 2005;438 (7070):954-9.
- [53] Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005;438 (7070):932-6.
- [54] Abdollahi A, Hahnfeldt P, Maercker C, Grone HJ, Debus J, Ansorge W, Folkman J, Hlatky L, Huber PE. Endostatin's antiangiogenic signaling network. *Mol Cell* 2004;13 (5):649-63.
- [55] Thijssen VL, Brandwijk RJ, Dings RP, Griffioen AW. Angiogenesis gene expression profiling in xenograft models to study cellular interactions. *Exp Cell Res* 2004;299 (2):286-93.

Summary

Angiogenesis, the formation of new blood vessels from pre-existing ones, is pivotal to a variety of physiological as well as pathological processes. Angiogenesis can be switched on in response to various stimuli, including hypoxia, metabolic stress and cellular signals. The hypothesis that tumor growth is angiogenesis-dependent was postulated over three decades ago and triggered a wide interest in inhibiting this process as a novel therapeutic strategy to treat cancer. Therapeutic inhibition of tumor angiogenesis is considered to have a number of major advantages over the current conventional therapies. Endothelial cells are easily accessible via the blood stream, are genetically stable and eradication of only a limited number of endothelial cells will result in the death of a multitude of tumor cells. Two general strategies can be employed to attack the tumor's blood supply. Either the formation of novel blood vessels can be inhibited - the narrow definition of angiogenesis inhibition - but it is also possible to destroy existing tumor vessels - frequently referred to as vascular targeting. However, depending on the mode of action of the agent, there is considerable overlap between these two applications. Nevertheless, for the design of effective angiogenesis inhibitors or vascular targeting agents for cancer treatment, thorough knowledge of endothelial cell biology is a prerequisite.

Analysis of gene expression can be used to determine molecular changes associated with particular phenotypes or diseases. The data can provide insight in the molecular mechanisms and pathways that govern disease. Importantly, differentially expressed genes may be used as targets for therapeutic intervention. The identification of putative targets for therapeutic intervention in tumor angiogenesis or for vascular targeting promises the combined specificity of targeted therapy and the efficacy of attacking the tumor vasculature.

In chapter 2 we analyzed published data of gene expression profiles of endothelial cells stimulated with growth factors *in vitro*, tube forming endothelial cells *in vitro* and isolated endothelial cells *in vivo*. Though different experimental setups were used, several genes were generally upregulated after growth factor stimulation *in vitro*. Moreover, these genes were biased towards those involved in cell-cycle regulation and metabolism. This 'cell-cycle signature' can be ascribed to the transition from quiescent to activated proliferative endothelium under the influence of these growth factors. Endothelial cells differentiating into tube-like structures exhibit a gene expression profile indicative of cell-cell and cell-matrix interactions, evidenced by upregulation of adhesion molecules (e.g. ICAM1, VCAM1, NRCAM), integrins (ITGA2, ITGAV) and matrix turnover proteins (MMP1, MMP9, CTSB). Finally, genes identified by SAGE to be overexpressed in different tumor endothelial cells were evaluated. Again, genes involved in matrix remodeling and deposition are highly upregulated, such as different types of collagens and SPARC, representative of later stages of angiogenesis.

In chapter 3 we describe the analysis of gene expression in endothelial cells of malignant as well as non-malignant angiogenic tissues and compare this with gene expression in resting endothelial cells. By these means, genes that are commonly upregulated during angiogenesis are filtered out to yield only those that mark events

associated with tumor angiogenesis. These tumor angiogenesis genes (TAGs) might be useful targets for imaging, diagnosis and therapy. When examining the functional classification of the TAGs, it emerges that there is a considerable bias towards genes associated with extracellular matrix remodeling. General angiogenesis genes (GAGs) can be divided into two groups: (i) those that are overexpressed in tumor endothelium (compared to normal endothelium) and simultaneously in placenta endothelium (compared to normal endothelium) (GAG/A), and (ii) genes that are overexpressed in tumor endothelium and simultaneously in activated HUVEC (GAG/B). The functional profile of GAG/A genes shows a bias to cell-adhesion whereas the GAG/B genes are biased towards cell and protein turnover. In conclusion, tumor endothelial cells are a heterogeneous population, containing cells in different stages of the angiogenic cascade. Furthermore, *in vitro* models of tumor conditioned endothelial cells are only of limited value in representing the *in vivo* events during tumor angiogenesis. Also, many genes are induced during both physiological angiogenesis and tumor angiogenesis. When translating putative candidate genes to a clinical setting, care must be taken not to interfere with physiological angiogenesis. Finally, we show in chapter 3 that targeting TAG-39, vimentin, with antibodies in a mouse tumor model is capable of inhibiting tumor growth and tumor vessel density.

In chapter 4 the contribution of vimentin to different aspects of the angiogenesis process is further characterized. Vimentin can be detected on non-permeabilized cells, suggesting cell-surface expression of the protein on endothelial cells. Downregulation of total vimentin by different knockdown strategies suggests that a threshold level of vimentin is necessary for survival of endothelial cells, whereas downregulation of surface-associated vimentin results in impaired migration and tube formation. This surface-associated pool of vimentin is increased in exponentially growing cells and might be regulated by phosphorylation as treatment with the phosphatase inhibitor okadaic acid results in increased vimentin immunoreactivity of non-permeabilized cells, but not of permeabilized cells. Our data suggest that surface-exposed vimentin contributes positively to cell migration and tube formation, and that interference with the expression or the function of this pool negatively impacts the angiogenic potential of endothelial cells.

In chapter 5 the contribution of TAG-21 (HMGB1) to angiogenesis is addressed. Inhibition of HMGB1 protein expression inhibited cell migration and MMP9 expression, suggesting an active involvement of HMGB1 in this process. Targeting HMGB1 *in vitro* with antibodies inhibited migration and tube formation of endothelial cells, supporting these results. *In vivo*, targeting HMGB1 with antibodies inhibited vessel formation on the CAM grafted with tumor cells. Taken together, HMGB1 positively contributes to angiogenesis, and targeting the protein has an anti-angiogenic effect. However, its role in inflammatory processes requires caution when translating this to a therapeutic anticancer setting.

Chapter 6 provides an outlook to the implementation of high-throughput target validation technologies and functional identification of therapeutically targetable

molecules in angiogenesis. Major drawback of gene expression profiling techniques is that they generate vast lists of potential disease target genes, but these do not reveal functional information or direct (causative) relevance to the disease. Hence, validation of these putative targets is a time-consuming and labor-intensive process. Recent technical advances in high-throughput analysis of gene function *in vitro*, including library generation, transfection methods, miniaturization of assays and screening techniques have brought about the opportunity for target validation to keep up with the pace of target identification at a genomics scale. Furthermore, novel methods have been developed to identify candidate target genes based on the phenotype they induce in an unbiased screening approach. Examples of these techniques are transfected cell arrays and expression library screening *in vitro* that allow functional annotation of genes in a high-throughput setting. Genetic screens, either gain-of-function or loss-of function, can be applied to directly identify candidate genes that confer a selectable phenotype to the cells.

In conclusion, we have shown that tumor endothelial cells exhibit a gene expression profile that distinguishes them from cultured endothelial cells as well as from endothelial cells in non-tumor angiogenic tissue. Functional analysis indicated these genes are heavily biased towards extracellular matrix remodeling. We identified both genes previously acknowledged for a role in angiogenesis and genes not previously associated with angiogenesis. *In vitro* angiogenesis assays demonstrate the essential contribution of HMGB1 and vimentin to endothelial cell migration and tube formation. *In vivo*, therapeutic targeting of tumor vascular vimentin reduced tumor growth and vessel density, demonstrating the efficacy of our approach. Hence, the tumor angiogenesis genes (TAGs) we identified are promising candidate targets for therapeutic anticancer treatment.

Samenvatting

Angiogenese, de vorming van nieuwe bloedvaten uit bestaande vaten, is vereist voor een aantal fysiologische alsook pathologische processen. Het angiogeneseproces kan worden geïnitieerd in reactie op verschillende stimuli, waaronder hypoxie, metabole stress en cellulaire signalen. De hypothese dat tumorgroei angiogenese-afhankelijk is, werd meer dan dertig jaar geleden voor het eerst geuit en heeft bij velen interesse gewekt in het concept dat angiogenese-inhibitie een nieuwe manier is om kanker te behandelen.

Angiogenese-inhibitie als antikanker therapie heeft een aantal voordelen: de endotheelcellen die de binnenkant van een bloedvat bekleden, zijn gemakkelijk bereikbaar via het bloed, zijn genetisch stabiel, en het doden van een beperkt aantal endotheelcellen heeft de dood van een meervoud aan tumorcellen tot gevolg. Twee algemene strategieën kunnen worden gebruikt om de bloedvatvoorziening van een tumor aan te vallen. De vorming van nieuwe bloedvaten kan worden geremd – angiogenese-inhibitie – maar het is ook mogelijk om bestaande vaten te vernietigen – meestal vasculaire targetting genoemd. Afhankelijk van de werking van een stof kan deze zorgen voor zowel angiogenese-inhibitie als vasculaire targetting, waardoor er redelijk veel overlap is tussen deze termen. Hoe dan ook, voor het ontwerpen van therapeutica met deze eigenschappen is een specifieke kennis van de endotheelcelbiologie vereist.

Genexpressie-analyses kunnen worden gebruikt om de moleculaire veranderingen te bepalen die gepaard gaan met een ziekte of een ander proces. Hiermee worden de moleculaire mechanismen waarvan de ziekte afhankelijk is duidelijk. De identificatie van targetmoleculen die geschikt zijn voor therapeutische interferentie belooft een mogelijke therapie die zowel zeer specifiek werkt, alsook zeer effectief is.

In hoofdstuk 2 hebben we gepubliceerde genexpressiedata geanalyseerd van groeifactor-gestimuleerde endotheelcellen, van buisvormende endotheelcellen, en van uit weefsels geïsoleerde endotheelcellen. Hoewel in deze studies verschillende experimentele methoden werden gebruikt, is er een aantal genen aan te wijzen die onder invloed van angiogene groeifactoren verhoogd tot expressie komen. Veel van de groeifactor-gereguleerde genen zijn betrokken bij regulatie van de celcyclus en bij metabole processen, wat waarschijnlijk te maken heeft met de overgang van rustend endotheel naar geactiveerd endotheel onder invloed van angiogene factoren. Het genexpressieprofiel van buisvormende endotheelcellen wijst op cel-cel en cel-matrix interacties, aangezien vele adhesiemoleculen (bv. ICAM1, VCAM1 en NRCAM), integrines (ITGA2, ITGAV) en matrix-afbrekende eiwitten (bv. MMP1, MMP9, CTSB) verhoogd tot expressie komen. In endotheelcellen die waren geïsoleerd uit tumoren kwamen ook met name die genen die betrokken zijn bij de afzetting van matrixmoleculen en de herstructurering van matrix, zoals SPARC en verschillende collagenen, verhoogd tot expressie.

In hoofdstuk 3 beschrijven we het genexpressieprofiel van endotheelcellen geïsoleerd uit zowel maligne als niet-maligne angiogene weefsels en vergelijken dit met het genexpressieprofiel van normale, rustende endotheelcellen. Op deze manier

kunnen de genen die betrokken zijn bij fysiologische angiogenese worden uitgefilterd en blijven juist de genen die betrokken zijn bij tumorangiogenese over. Deze tumorangiogenese genen (TAGs) zouden bruikbare targets kunnen zijn voor beeldvormende technieken om angiogenese zichtbaar te maken, alsook voor diagnostische doeleinden en voor therapeutische doeleinden. Wanneer we kijken naar de functionele classificatie van deze TAGs, valt op dat vele betrokken zijn bij de herstructurering van de extracellulaire matrix. Genen die ook betrokken zijn bij fysiologische angiogenese (GAGs) kunnen in twee groepen worden onderverdeeld: (i) genen die verhoogd tot expressie komen in tumorendothel (vergeleken met normaal endothel) en tegelijkertijd in placenta-endothel (vergeleken met normaal endothel) (GAG/A), en (ii) genen die verhoogd tot expressie komen in tumorendothel (vergeleken met normaal endothel) en tegelijkertijd ook in geactiveerde HUVEC (GAG/B). Een groot deel van de GAG/A genen is betrokken bij celadhesie, terwijl GAG/B genen voornamelijk betrokken zijn bij celdeling en eiwitproductie. Tumorendothelcellen vormen dus een heterogene populatie, bestaande uit cellen die zich in verschillende fasen van het angiogenese proces bevinden. Verder is gebleken dat endothelcellen gekweekt in aanwezigheid van geconditioneerd medium van tumorcellen weinig overlap in genexpressieprofiel vertonen met endothelcellen geïsoleerd uit tumoren. Dus, *in vitro* modellen zijn maar in een beperkte mate representatief voor de *in vivo* situatie. Ook zijn veel genen bij zowel fysiologische angiogenese als tumorangiogenese betrokken. Bij de implementatie van mogelijke kandidaatgenen in een klinische toepassing voor de behandeling van kanker, moet worden voorkomen dat fysiologische angiogenese wordt geremd. Ten slotte tonen we in hoofdstuk 3 aan dat antilichamen gericht tegen TAG-39, vimentine, tumorangiogenese en tumorgroei in een muismodel remmen.

In hoofdstuk 4 hebben we de bijdrage van vimentine in verschillende aspecten van angiogenese bekeken. Het feit dat we vimentine kunnen detecteren op niet-gepermeabiliseerde cellen duidt erop dat vimentine ook op het oppervlak van endothelcellen tot expressie komt. Remming van vimentine-expressie laat zien dat endothelcellen een minimale hoeveelheid vimentine nodig hebben om te overleven en dat remming van celoppervlak gerelateerde vimentine-expressie de migratie en buisvormende capaciteit van endothelcellen remt. Vimentine-expressie gerelateerd aan de oppervlakte van de cel is verhoogd in exponentieel groeiende cellen en wordt mogelijk gereguleerd door fosforylering, aangezien de fosfataseremmer okadaic acid zorgt voor een verminderde immuunreactiviteit van niet-gepermeabiliseerde cellen, maar niet van gepermeabiliseerde cellen. Dit alles duidt erop dat celoppervlak-gerelateerd vimentine een bijdrage levert aan endothelcel migratie en buisvorming, en dat het remmen van de functie of de expressie van deze vimentinefractie een negatieve invloed heeft op angiogene processen.

In hoofdstuk 5 beschrijven we de bijdrage van TAG-21, HMGB1, aan het angiogeneseprocess. Remming van HMGB1 expressie remt endothelcelmigratie en MMP9 expressie, wat duidt op een actieve rol van HMGB1 in angiogenese.

Antilichamen gericht tegen HMGB1 remmen migratie en buisvorming van endotheelcellen *in vitro*, en de bloedvatvorming onder invloed van tumorcellen op het chorionmembraan van het kippenei *in vivo*. Samengevat levert HMGB1 een positieve bijdrage aan angiogenese, en heeft remming van het eiwit een anti-angiogeen effect. Aangezien het eiwit ook een rol speelt in ontstekingsreacties, dient eventuele implementatie in een klinische setting met grote voorzichtigheid te gebeuren.

Hoofdstuk 6 beschouwt de implementatie van geavanceerde target-validatietechnologieën en de functionele identificatie van therapeutische targets voor toepassing in angiogenese onderzoek. Genexpressiestudies resulteren in lange lijsten met genen die veranderd tot expressie komen tijdens ziekte, maar geven geen directe functionele informatie over het gen of informatie over een causale bijdrage aan het ziektebeeld. Validatie van deze genen voor hun mogelijke bruikbaarheid voor therapeutische doeleinden is zeer tijdrovend en arbeidsintensief. Nieuwe, geavanceerde technologieën om de functie van genen te bestuderen, zoals het produceren van genenbanken, transfectiemethoden, miniaturisatie van experimenten en screeningsmethoden, stellen ons nu in staat het target-validatieproces in de pas te laten lopen met het target-identificatieproces. Nieuwe methoden zijn ontwikkeld om kandidaatgenen te identificeren, gebaseerd op het fenotype dat ze induceren. Voorbeelden van deze technieken zijn arrays van getransfecteerde cellen en het screenen van expressie-genenbanken om de functie van genen te analyseren. Genetische screens, zowel voor toename als voor verlies van functie, kunnen worden gebruikt om kandidaatgenen te identificeren die een selecteerbaar fenotype induceren.

In dit proefschrift tonen we aan dat tumorendotheelcellen een genexpressieprofiel hebben dat ze onderscheidt van gekweekte endotheelcellen alsook van endotheelcellen in niet-maligne angiogene weefsels. Veel van deze genen zijn betrokken bij de herstructurering van de extracellulaire matrix. Van een aantal van deze genen was hun betrokkenheid bij angiogenese al vastgesteld, maar van een aantal nog niet. Onze experimenten tonen aan dat vimentine en HMGB1 een rol spelen in de migratie en buisvorming van endotheelcellen. Het remmen van vasculair vimentine remt tumorgroei en angiogenese in een muismodel en demonstreert de toepasbaarheid van onze studie. De tumorangiogenese genen (TAGs) die wij hebben geïdentificeerd zijn veelbelovende kandidaat genen voor de ontwikkeling van nieuwe therapieën voor de behandeling van kanker.

Dankwoord

ZOEF....he wat was die windvlaag? Oh, een Judy...

Waarschijnlijk een bekend fenomeen voor eenieder die mij wel eens op het lab is tegengekomen, nou ja, die ik ooit ben voorbijgevlogen. Ik zal dus maar beginnen met iedereen te bedanken die op tijd is opzij gesprongen, al dan niet gewaarschuwd door het geluid van mijn schoenen en/of een plotselinge verandering in luchtdruk.

Nu is het echter even tijd om stil te staan bij iedereen die ik te vaak en te snel voorbij ben gerend. Ik neem jullie een aantal jaren mee terug in de tijd. Mijn moeder voorzag dat ik professor in de weet-niet-kunde zou worden. Nou, doctor ben ik nu, en misschien word ik nog ooit professor in de weet-iets-kunde. Mijn vader legde mij aan de hand van de wetten van Mendel uit waarom rode koeien zwarte kalfjes konden krijgen, en daarmee is mijn interesse in genetica en later genexpressie gewekt. Ben ik nu van de melkboer of een hooimeid, of allebei?

Mijn eerste stapjes zette ik tientallen jaren geleden welliswaar bij mijn ouders thuis in de kamer, maar mijn eerste stapjes in het onderzoek bij Pathologie. Hard werken ging hier gepaard met de nodige (on)gein, niet in het minst door de aanwezigheid van mijn toenmalige labgenoten (ja helaas, de verleden tijd van labgenieten), waaronder Marco, Guido, Helen, Natasja en Ricardo. Na deze 'fage' periode brak een nieuw tijdperk aan met angiogenese als sleutelwoord, gekenmerkt door zowel oude als nieuwe gezichten. Arjan gaf me de gelegenheid bestaande en nieuwe ideeën met elkaar te verenigen. Hoewel ik al heel wat kneepjes van Ricardo en Peter had geleerd, was cellen kweken toch wel even wat nieuws. Nestor Daisy (is hier een vrouwelijk woord voor?) liet zien hoe je uit een bloederige navelstreng een florerende endotheelcelpopulatie haalt. Susan nam het vervolgens op zich om deze cellen uit al even bloederige placenta's, tumoren en darmen te halen, een kunstvorm die later door Edith met verve is uitgebouwd. Ook wist Edith altijd 'fac(t)s' van fictie te scheiden op de flowcytometer. Voor DNA drama's en de juiste vector was Victor de aangewezen persoon. Carolien heeft tijdens haar korte stageperiode de ski's en sno's uitgezocht, maar helaas liggen deze data nog ondergesneeuwd. Bernadette en Wouter wisten naast waardevolle data ook heerlijke appeltaarten te produceren.

Natuurlijk heb ik niet alles alleen te danken aan mijn collega's. Een goede buur is je beste vriend. Met Thijs, Femke en Mokie vaak genoten van alle gezellige barbekoeis, vino's, appeltaarten, tapas, nog meer vino's, witte haren en nagels in mijn schouders; zeer geschikt om na een dagje rondvliegen op het lab de zinnen te verzetten. Laten we er nog veel van genieten! Bram en Koannie, van huisgenoten tot vrienden tot burens; Sietske en Ivo, van collega's tot vrienden tot burens; en tot paranimfen. De beste burens kun je ook kiezen! De Roserije is er niet op achteruitgegaan de afgelopen jaren, het bierpeil in onze koelkast daarentegen des te meer. Gelukkig weet Peet dat altijd in goede banen te leiden en wordt de koelkast gevuld, Truus gevoerd en veel gesmuld. Liefde gaat toch ook wel een beetje door de maag. Huisje, boompje, Hummertje; we hebben het bijna voor elkaar.

Een goede collega is ook je beste vriend. Vele heerlijke, en af en toe ook minder heerlijke recepten zijn de afgelopen jaren de revue gepasseerd. Wanneer koken nog

eens onderdeel wordt van een sollicitatie zitten Chiel, Nicole, Rob, Ricardo, Caroline en Louise in ieder geval gebakken. Afgewisseld met lab-praat over sequencen, weekendjes met twister en liplezen, de nodige biertjes en de nieuwste wasmiddelen tips (hoezo we worden oud ?!), komen we toch maar de woensdagavonden door. De pannekoeken (pannenkoeken?) van Barry zullen ons trouwens ook nog lang heugen, evenals de breezer-salade, het breezer-ontbijt, de breezer-lunch en breezer-apres-swim.

Terug naar het heden... Voor jullie ligt een boekje dat ondanks mijn haat-liefde verhouding met Microsoft toch tot strand is gekomen. Meermalen kwam het stoom uit mijn oren, doch niet uit mijn computer. Misschien heeft dit te maken met de paradox dat multi-tasken een vrouweigenschap is en Bill Gates een man is? Gelukkig is het koffieapparaat geduldig en geeft MSN voldoende slachtoffers om virtueel op af te reageren <<buzzzz>>. Ik ga er vanuit dat mijn kamergenoten wel weten dat alle scheldkanonnades nooit bedoeld zijn geweest om hen onder vuur te nemen. Tja, soms is mijn stressbestendigheid nu eenmaal rechtevenredig met de crashbestendigheid van mijn computer.

Zonder Els ook was de Nederlandse samenvatting verdwaald geraakt in de jungle van uitgaven van het groene boekje en veranderende spellingsregels. En ondanks of misschien wel dankzij zijn zeebenen was Jan altijd een rots in de branding. Wanneer optimisme en vertrouwen nog eens in pilvorm verkrijgbaar worden kunnen we hier op de afdeling wel inpakken.

Op naar de toekomst... een paar opmerkingen. Allereerst wil ik mijn mede-AIOs, Veerle, Debby, Ricardo, Karolien en Femke heel veel succes wensen met het schrijven van hun dankwoord. Tenslotte, eenieder die aan dit onderzoek, of op een andere manier aan de totstandkoming van dit proefschrift hebben bijgedragen: gracias, dankjewel, danke, merci, takk, grazie, obrigado, σας ευχαριστούμε, thanx! Laten we er een leuk feest van maken!

Tot op dat moment had orkaan Judy nog niet veel schade aangericht.

Curriculum Vitae

1.1.17

Judy van Beijnum

Judith Rosina van Beijnum was born on December 11th 1977 in Nijmegen, The Netherlands. Following primary school in Horssen, she completed her secondary education at the Pax Christi College in Druten in 1995. In August 1995 she started at Maastricht University with studies in Health Sciences. Between 1995 and 1999, she was also actively involved in diverse committees of student association s.v. KoKo, which aimed to introduce first-year students to the city of Maastricht and its social life.

She decided to specialize in Biological Health Sciences and in 1999, she was introduced into the world of research. Her first internship was under supervision of dr. Simon Hufton and dr. Hennie Hoogenboom, during which she validated potential colorectal cancer antigens. This internship was extended with a project in which she isolated and characterized anti-peptide phage displayed antibodies that target colorectal cancer antigens. In May 2000 she obtained her MSc degree and started as PhD student at the department of Pathology, in the group CESAME, continuing the work initiated during her internship.

After the collapse of CESAME in May 2001, she initiated a new PhD project at the Angiogenesis Laboratory under supervision of Prof. dr. Arjan Griffioen. Since then, she has studied gene expression in endothelial cells under different angiogenic and non-angiogenic conditions to identify novel targets for the treatment and imaging of human tumors. As of April 2006, she will continue these studies as a post-doc in the Angiogenesis Laboratory.

>> Publications

Van Beijnum JR, Dings RPM, Van der Linden E, Zwaans BMM, Ramaekers FCS, Mayo KH, Griffioen AW. Gene expression of tumor angiogenesis dissected; specific targeting of tumor endothelium. *Submitted*

Mulder WJ, Castermans, KC, Van Beijnum JR, Oude Egbrink MGA, Chin PTK, Storm G, Strijkers GJ, Griffioen AW, Nicolay K. Multimodality molecular imaging of tumor angiogenesis using quantum dots. *Submitted*

Derksen PWB, Liu X, Saradin F, Evers B, Van Beijnum JR, Griffioen AW, Van der Gulden H, Zevenhoven J, Peterse H, Cardiff R, Vink J, Krimpenfort P, Berns A, Jonkers J. Somatic inactivation of E-cadherin and p53 leads to metastatic lobular breast cancer. *Submitted*

Hillen F, Melotte V, Van Beijnum JR, Griffioen AW. Endothelial cell biology. In: Staton C, Lewis C, Bicknell R, editors. Angiogenesis assays: a critical appraisal of current techniques. London: John Wiley and Sons, *In press*.

Van Beijnum JR, Eijgelaar WJ, Griffioen AW. Towards high-throughput functional target discovery in angiogenesis research. *Trends Mol Med*. 2006 Jan;12(1):44-52

Van Beijnum JR, Griffioen AW. *In silico* analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles. *Biochim Biophys Acta*. 2005 Jul 25;1755(2):121-34.

Van Beijnum JR, Fan TP, Griffioen AW. *Euroconference Angiogenesis II*. Angiogenesis. 2003;6(3):159-64.

Van Beijnum JR, Griffioen AW. Transcriptional profiling of angiogenically activated endothelial cells: gene expression reflects the angiogenic stage. *Applied Genomics and Proteomics* 2003;2(4):207-223.

Van Beijnum JR, Moerkerk PT, Gerbers AJ, De Bruine AP, Arends JW, Hoogenboom HR, Hufton SE. Target validation for genomics using peptide-specific phage antibodies: a study of five gene products overexpressed in colorectal cancer. *Int J Cancer*. 2002 Sep 10;101(2):118-27.

>> Posters and abstracts

15th Endothelial cell research symposium, Maastricht, 2004

Van Beijnum JR, Van der Linden E, Griffioen AW. The transcriptional profile of tumor endothelial cells indicates distinct stages of angiogenesis; implications for anticancer therapy

Published in:

Abstracts of the 15th Endothelial Cell Research Symposium Maastricht, The Netherlands. Angiogenesis. 2004;7(2):165-187.

AACR Annual Meeting, Orlando FL, 2004

Van Beijnum JR, Van der Linden E, Griffioen AW. The transcriptional profile of tumor endothelial cells indicates distinct stages of angiogenesis; implications for anticancer therapy

Euroconference Angiogenesis II, Paris, 2003

Van Beijnum JR, Van der Linden E, Joosten S, Wagstaff J, Griffioen AW. Angiomic profiling of tumor endothelium

2nd International symposium on genetic anticancer agents, Valencia, 2001

Van Beijnum JR, Moerkerk PT, Gerbers AJ, De Bruine AP, Arends JW, Hoogenboom HR, Hufton SE. Gene expression profiling of colorectal cancer and the use of antibody phage display to isolate specific targeting molecules

Financial support for the publication of this thesis is gratefully acknowledged:

Bio-Rad
Greiner Bio-One
BD Biosciences