Targeting CD44v6, a co-receptor for Met and VEGFR-2 in endothelial cells, inhibits tumour angiogenesis

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ABSTRACT

Members of the transmembrane glycoprotein family CD44 containing variant exon v6 sequences have been shown to act as co-receptors for the receptor tyrosine kinase (RTK) Met in epithelial cells (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007).

This work demonstrates that the co-receptor function of CD44v6 can be expanded to endothelial cells (ECs) and to another RTK, VEGFR-2. As VEGFR-2 is the most prominent RTK in angiogenesis, these findings indicate that also CD44v6 is strongly involved in this process.

Both in the case of VEGFR-2 and Met, CD44v6 is not only required for receptor activation, a function performed by the CD44v6 ectodomain, but also plays a role in signal transduction. The intracellular domain of CD44v6 recruits ERM (ezrin, radixin, moesin) proteins bound to the actin cytoskeleton, a prerequisite for activation of further components of the signalling cascades.

Interestingly a small peptide and an antibody directed against CD44v6 block the co-receptor function of CD44v6. Both tools strongly inhibit activation of Met or VEGFR-2 by their respective ligands in ECs. Consequently they interfere with physiologic EC behaviour such as ligand-induced migration, sprouting and tube formation. Treatment with the CD44v6 antibody and the CD44v6 peptide also interfered with tumour vascularisation in two orthotopic tumour models. Both the antibody and the peptide significantly reduced the microvessel density in a mammary carcinoma system. More drastic effects were seen in the case of human pancreatic carcinoma cells injected in the tail of the pancreas of nude mice. There, not only the tumour size was decreased, but also the vessel density and the vessel size were drastically reduced.

In conclusion blocking of CD44v6 is a very attractive approach for inhibition of pathologic angiogenesis as demonstrated in tumour progression. Targeting one single protein, CD44v6, results in the inhibition of two RTKs. Therefore the CD44v6 peptide might be a promising tool to use in an antiangiogenic therapy.

ZUSAMMENFASSUNG

Die gezielte Blockierung von CD44v6, einem Ko-Rezeptor für Met und VEGFR-2, reduziert Angiogenese in Tumoren

Isoformen der Transmembran-Glykoproteinfamilie CD44, die die Sequenz des varianten Exons v6 enthalten, können als Ko-Rezeptoren für die Rezeptortyrosinkinase Met in Epithelzellen dienen (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007).

Diese Arbeit zeigt, dass die Funktion von CD44v6 als Ko-Rezeptor auch auf Endothelzellen (ECs, endothelial cells) und auf eine andere Rezeptortyrosinkinase, nämlich VEGFR-2, übertragen werden kann. Da der VEGFR-2 einer der wichtigsten Rezeptoren in der Angiogenese ist, zeigen diese Ergebnisse, dass auch CD44v6 im Prozess der Angiogenese eine bedeutende Rolle spielt.

Sowohl für VEGFR-2 als auch für Met wurde gezeigt, dass CD44v6 nicht nur für die Rezeptoraktivierung benötigt wird, eine Aufgabe, die die Ektodomäne von CD44 übernimmt, sondern auch für die intrazelluläre Signalweiterleitung. Die zytoplasmatische Domäne von CD44v6 kann ERM-Proteine (Ezrin, Radixin, Moesin) binden, welche wiederum an das Aktinzytoskelett gekoppelt sind. Dies ist eine Voraussetzung für die Aktivierung weiterer Bestandteile der Signalkaskade.

Interessanterweise kann die Ko-Rezeptorfunktion von CD44v6 sowohl durch ein kleines Peptid als auch durch einen Antikörper, die beide gegen CD44v6 gerichtet sind, blockiert werden. Sie verhindern die Aktivierung von Met und VEGFR-2 durch die entsprechenden Liganden HGF und VEGF-A. Als Folge davon beeinträchtigen sie das physiologische Verhalten der Endothelzellen, wie zum Beispiel die ligand-gerichtete Migration, das Aussprießen aus einem Zellverbund sowie die Bildung von gefäßähnlichen Strukturen. Die Behandlung mit dem CD44v6 Antikörper oder dem CD44v6 Peptid reduziert auch die Vaskularisierung von Tumoren in zwei orthotopen Tumor-Modellen in Mäusen. Sowohl der Antikörper als auch das Peptid reduzierten die Gefäßdichte in einem Tumor der Brust signifikant. Deutlich stärkere Effekte konnten in einem Pankreas-Karzinom-Modell erzielt werden. Hier waren nicht nur die Gefäßdichte, sondern auch die Gefäßgröße und das Tumorvolumen deutlich reduziert.

Zusammenfassend zeigt diese Arbeit, dass die Blockierung von CD44v6 ein interessanter Ansatz ist, um pathologische Angiogenese wie zum Beispiel bei der Tumorentwicklung zu hemmen. Die gezielte Ausschaltung eines einzigen Proteins, CD44v6, führt zur Inaktivierung von zwei Rezeptortyrosinkinasen. Deshalb ist das CD44v6 Peptid ein vielversprechendes Mittel, das in einer antiangiogenen Therapie eingesetzt werden könnte.

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ABBREVIATIONS

AER	apical ectodermal ridge
Arp2/3	actin related protein 2/3
C	Celsius
CAM	cellular adhesion molecule
CD44	cluster of differentiation 44
CD44s	CD44 standard
cDNA	complementary DNA
CEP	circulating endothelial precursors
CO_2	Carbon dioxide
Crk	CT10 regulator of kinase
Da	Dalton
DEP	density-enriched phosphatase
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	desoxy ribonucleic acid
dNTP	desoxy nucleotide triphosphate
DTT	dithiothreitol
E	embryonic day of development
EC	endothelial cell
ECL	enhanced chemiluminescence
ECGM	endothelial cell growth medium
ECGM MV	endothelial cell growth medium microvascular
ECM	extracellular matrix
EDTA	ethylendioxide tetra acetic acid
e.g.	for example
EGF	epidermal growth factor
Erk	extracellular signal-regulated kinase
ERM	ezrin, radixin and moesin
F-actin	filamentous actin
FCS	foetal calf serum
FGF	fibroblast growth factor
FL	foetal liver
Flt	fsm-like tyrosine kinase receptor; mouse foetal liver kinase
fpVCT	flat-panel volume computed tomography
g	gram
Gab1	Grb2-associated binding protein 1
GAG	glycosaminoglycans
GEF	guanine nucleotide exchange factor
GF	growth factor
GM-CSF	granulocyte-macrophage colony stimulation factor
Grb2	growth factor receptor bound protein 2
HA	hyaluronan
HAOEC	human aortic endothelial cell
HCMEC	human cardiac microvascular endothelial cell
HGF	hepatocyte growth factor
HOS	human osteosarcoma cells
HRE	Hypoxia response element

HRP	horseradish peroxidase
HS	heparan sulphate
HSPG	heparan sulphated proteoglycan
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IGF	insuline-like growth factor
IHC	immunohistochemistry
IL	interleukin
INF	interferon
InlB	InternalinB
k	kilo
KDR	Kinase insert domain containing receptor
LTBMC	long-term bone marrow culture
µ	micro
MBD	Met binding domain
Met	mesenchymal-epithelial transition factor
mm	millimetre
MMP	matrix metalloproteinase
mRNA	Messenger ribonucleic acid
n	nano
NRP	neuropilin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pep	peptide
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PlGF	placenta growth factor
PMSF	phenylmethylsulfonyl-fluoride
PSI	plexin, semaphorin and integrin
Psp	phagocyte glycoprotein
PTB	phosphotyrosine binding
RNA	ribonucleic acid
RNase	ribonuclease
Rnasin	Rnase inhibitor
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction
sCD44	soluble CD44
Sck	Shc related adaptor protein
SDS	sodiumdodecylsulphate
SF	scatter factor
SH2	Src-homology 2
Shb	Src homology 2 domain containing adaptor protein B
Shc	Src-homology 2 containing protein
SOS	son-of-sevenless
Src	short for sarcoma: family of proto-oncogenic tyrosine kinases
sv	snake venom
TEMED	tetramethylethylenediamine

TGF	transforming growth factor
TIMP	tissue inhibitors of MMP
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TPR	translocated promoter region
TSG	tumour necrosis factor stimulated gene
TSP	thrombospondin
U	unit
uPA	urokinase-like plasminogen activator
v	variant
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VLS	vascular leak syndrome
VPF	vascular permeability factor

INTRODUCTION

1 Angiogenesis under physiologic and pathologic conditions

Every living cell in an organism needs oxygen, nutrients and other metabolites to survive. In addition, it has to remove carbon dioxide and metabolic waste like urea and uric acid. In some small organisms including e.g. flatworms these functions are fulfilled by simple diffusion. In bigger animals diffusion is not sufficient for an adequate supply with oxygen and nutrients. Therefore most invertebrates and all vertebrates developed a circulatory system to handle these important functions during evolution. The circulatory system consists of two components, the cardiovascular and the lymphatic system.

In the closed cardiovascular system blood circulates through the body driven by the heart pump. It reaches the tissue capillaries via arteries and arterioles. The arterial pressure induces extravasation of plasma and nutrients that will invade the surrounding tissues. Oxygen will then diffuse to places with lower partial pressure. In return, CO_2 and the interstitial fluid containing waste products enter the capillaries and are removed via venules and veins.

The open lymphatic system is responsible for the transport of the retained interstitial fluid from the periphery back to the cardiovascular circulatory system. Lymphatic capillaries are blind ending and very permeable, the fluid can easily enter this network and is transported slowly via the thoracic and lymphatic duct to the subclavian vein into the blood system. Additionally the lymphatic system transports antigen-presenting cells and participates in immune responses.

The establishment of the complex network of blood vessels within an organism is the result of two processes: vasculogenesis and angiogenesis. Vasculogenesis occurs during embryonic development. Precursors of endothelial cells (EC), so called angioblasts that emerge from the mesoderm, express some typical EC markers but do not form any lumen. They differentiate into blood islands, which fuse to a primitive vasculature and form a primary vascular plexus (reviewed in (Risau and Flamme 1995)). This is followed by angiogenesis, the formation of new blood vessels that develop from the existing vasculature. New blood vessels are formed by sprouting and branching from the primary vascular plexus (sprouting angiogenesis) or by splitting vessels in a way that the ECs grow into the lumen forming transcapillary pillars (non-sprouting angiogenesis) in order to establish an adequate vasculature for developing and growing organs (Risau and Flamme 1995; Patan et al. 1996).

1.1 Steps of angiogenesis

Angiogenesis is split in different steps that are regulated by activating as well as inhibiting molecules. One of the first events is vasodilatation combined with an increased vascular permeability of pre-existing vessels. Plasma proteins extravasate and generate the optimal microenvironment for EC migration. Pericytes are removed from the branching vessels, and the basement membrane and the extracellular matrix (ECM) are locally degraded and remodelled by specific proteases such as matrix metalloproteinases. Then sprouting occurs. The ECs loosen their contacts to neighbouring cells and to the ECM. Attracted by chemotactic angiogenic stimuli they start to migrate and proliferate. Upon arrest, the ECs adhere to each other and form a lumen. Further proliferation takes place in order to enlarge the vessel diameter. The vessels mature by recruiting mural cells that stabilise the new vasculature (reviewed in (Risau 1997; Papetti and Herman 2002; Bergers and Benjamin 2003)).

1.2 Pathological angiogenesis

In adults the vasculature is usually quiescent and angiogenesis happens primarily in the female ovarian cycle, where the blood vessels supplying the endometrium are established periodically. Also wound healing processes following tissue injury depend on angiogenesis as the newly formed tissue needs to be provided with oxygen.

However uncontrolled angiogenesis is the reason for a number of diseases. During age-related macular degeneration, neovascular glaucoma and diabetic retinopathy, an excessive network of blood vessels grows beneath the retina in the eye and can cause blindness. Overexpression of angiogenic growth factors contributes to the establishment of these diseases. An elevated level of VEGF-A (vascular endothelial growth factor A) is detectable in the retina as well as the vitreous of eyes during proliferative diabetic retinopathy (Adamis et al. 1994). The local treatment of patients suffering from age-related macular degeneration with drugs antagonising VEGF-A like Ranibizumab (Lucentis), which is the antigen-detecting fragment of the humanised monoclonal antibody Bevacizumab (Avastin), decreases disease progression and increases the life quality (Rosenfeld 2006; Rosenfeld et al. 2006; Gillies and Wong 2007).

Chronic inflammatory diseases are another example where angiogenesis is involved in pathologic processes. They include rheumatoid arthritis as well as psoriasis and in both hyper-vascularisation of a specific tissue is part of the clinical picture (summarised in (Polverini 1995)). In psoriasis the exceeded vascularisation is traced back to an increased amount of the

proangiogenic polypeptide interleukin-8 (IL-8) and a reduced level of the angiogenesis inhibitor thrombospondin (Nickoloff et al. 1994).

1.3 Tumour-induced angiogenensis

The best-investigated pathologic process of blood vessel formation is angiogenesis in tumour growth and tumour progression. Like healthy tissues, a tumour needs to be properly supplied with oxygen and nutrients and has to get rid of waste products. Close proximity to blood vessels fulfils these requirements (Papetti and Herman 2002). A discrete step in tumour development is the induction of a tumour vasculature, called the angiogenic switch. The tumour modifies the balance of pro- and antiangiogenic signals emphasising the activating one's and thereby triggers EC sprouting, migration and proliferation.

Tumour-induced angiogenesis is similar to physiologic angiogenesis. Lack of oxygen induces an imbalance of pro- and antiangiogenic factors, existing endothelium starts to sprout, migrate and proliferate. But tumour-induced vessels are often structurally abnormal. They are not strictly organised in arterioles, venules and capillaries but combine the characteristics of all of them. Frequently they are dilated or convoluted, leaky and haemorrhagic as they have many fenestrae and transcellular holes. Some tumour vessels can even be useless as they lead to dead ends or the blood flows alternately in both directions (reviewed in (Bergers and Benjamin 2003)). Furthermore reduced contact with functional pericytes can cause decreased vessels stability (Benjamin et al. 1999; Morikawa et al. 2002) and even tumour cells can be integrated into the vessel walls (Chang et al. 2000).

In addition tumours make use of another mechanism to induce blood vessel formation. Circulating endothelial precursors (CEP) derived from bone marrow are suggested to participate in formation of new tumour blood vessels, a process that reminds "neovasculogenesis". They have angioblast-like characteristics though they are present in the adult circulation (Rafii 2000). When recruitment of these precursors is impaired, tumour angiogenesis and tumour growth are reduced (Lyden et al. 2001).

Tumour-induced blood vessels do not only supply the growing tumour with substances, they even offer a possibility for tumour progression. The newly formed vessels can serve as entry sites into the circulatory system for metastasising tumour cells (Liotta et al. 1974). Once in the blood vessels, they are distributed throughout the organism and are able to give rise to metastases.

1.4 Regulation of angiogenesis

A complex interplay between numerous activators and inhibitors controls exactly where angiogenesis takes place (see Figure 1). Angiogenic regulators include soluble mediators that are able to activate their respective receptor on ECs, membrane-bound proteins that influence cell-cell or cell-extracellular matrix interactions and substances that are able to change the environment like matrix metalloproteinases (reviewed in (Papetti and Herman 2002)). This work concentrates on the function of two soluble mediators, vascular endothelial growth factor A (VEGF-A) and hepatocyte growth factor (HGF).

Angiogenesis activators	**Angiogenesis inhibitors**
vascular endothelial growth factors (VEGFs)	angiostatin
fibroblast growth factors (FGFs)	thrombospondin-1 (TSP-1)
hepatocyte growth factor (HGF)	tissue inhibitors of MMPs (TIMPs)
platelet-derived growth factor (PDGF)	protamine
placenta growth factor (PlGF)	interferon-α (INF-α)
angiopoietins	soluble receptors for VEGF
angiotropin	soluble receptors for FGF
angiogenin	
insulin-like growth factor I (IGF-I)	
epidermal growth factor (EGF)	
transforming growth factors (TGFs)	
tumour necrosis factor-α (TNF-α)	
hypoxia	
nitric oxide	
matrix metalloproteinases (MMPs)	
prostaglandin	
nicotinamide	

Figure 1: Selection of important activators and inhibitors of angiogenensis. The function of those angiogenesis modulators is reviewed in (Papetti and Herman 2002)

2 VEGF

VEGFs belong to the VEGF/PDGF (Platelet-derived growth factor) supergene family. The VEGF proteins include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, svVEGF (snake venom VEGF) and PlGF (Placenta growth factor). All of them are ligands for receptor tyrosine kinases that are primarily expressed on ECs and are involved in angiogenic or lymphangiogenic processes. The predominantly expressed and best-characterised VEGF is VEGF-A.

VEGF-A is the most prominent activator of angiogenesis. It was discovered independently by two groups as a peptide with a unique specificity for vascular ECs (Ferrara and Henzel 1989; Plouet et al. 1989). It turned out that VEGF is identical to the vascular permeability factor (VPF)

that induces vascular leakage, which allows tissue infiltration of plasma proteins (Senger et al. 1983; Keck et al. 1989).

2.1 Structure of VEGF-A

The VEGF-A gene is organised in eight exons (Tischer et al. 1991). Alternative splicing of exons 6 and 7 gives rise to at least nine different isoforms which range in length from 121 to 206 amino acid residues (VEGF-A121 to VEGF-A206) (Tischer et al. 1991) (see Figure 2). Till now it is not clear how the splicing is regulated, but the VEGF producing cells preferentially express VEGF-A121, VEGF-A165 and VEGF-A189.

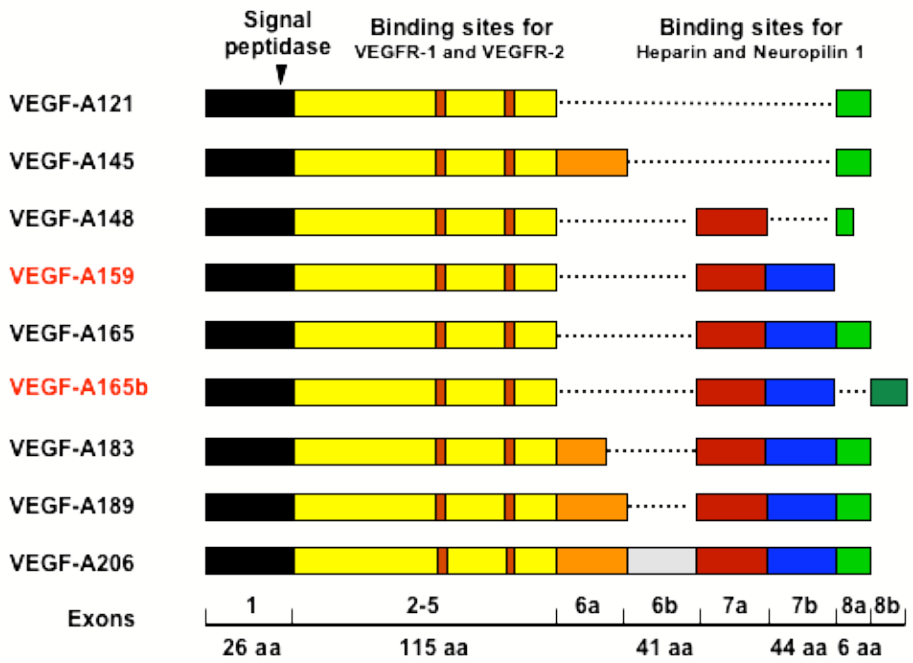

Figure 2: Schematic presentation of VEGF-A isoforms. Exons 6 and 7 are subject to alternative splicing. The VEGF-A isoforms written in red show inhibitory effects.

VEGF-A isoforms contain eight cysteine residues that are conserved also in other VEGF proteins and PDGF (platelet-derived growth factor) (Keck et al. 1989). Two of them form an intrachain disulfide-bonded knot motif (McDonald and Hendrickson 1993). In order to become biologically active they are organised as homodimers. Two monomers associate via hydrophobic interactions and this aggregation is then stabilised by two interchain disulfide bonds between the cysteines 51 and 61 (Potgens et al. 1994). VEGF-A dimers are ligands for the receptor tyrosine kinases (RTKs) vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2). Three acidic residues in exon 3 of VEGF-A (Asp63,

Glu64, Glu67) are required for binding to VEGFR-1 whereas three basic residues in exon 4 (Arg82, Lys84, His86) are essential for binding to VEGFR-2 (Keyt et al. 1996).

VEGF-A165 is the predominant VEGF-A isoform. The basic 46 kDa protein is able to bind heparin via a motif containing 15 basic amino acids encoded by exon 7 (Ferrara and Henzel 1989). About 50-70 % of the secreted VEGF-A165 remains associated to the cell surface or the ECM probably via heparan sulphate proteoglycan (HSPG) interactions (Houck et al. 1992).

Another isoform of VEGF-A is VEGF-A165b. It differs from VEGF-A165 by the carboxyterminal amino acid residues encoded by exon 8. It acts as an inhibitor because it can bind to the VEGFR-2 with a similar affinity than VEGF-A165 but is not able to activate downstream signalling pathways (Woolard et al. 2004).

VEGF-A121 is a weakly acidic protein that lacks the amino acids encoded by exons 6 and 7. VEGF-A121 is not able to bind heparin because the heparin binding motives are missing. Therefore it is secreted and not bound to the ECM or to the cell surface (Houck et al. 1992).

The two isoforms VEGF-A189 and VEGF-A206 show strong heparin binding and are completely sequestered in the ECM or to a lesser extend at the cell membrane (Houck et al. 1992). Both isoforms are not able to stimulate EC mitogenesis. VEGFA-189 is able to bind VEGFR-1, but it needs to be cleaved and released to bind and activate VEGFR-2.

VEGF-A145 binds heparin with a similar affinity than VEGF-A165. After secretion it is partly bound to the ECM via heparin and structures independent of heparin or heparan sulphate (HS) (Poltorak et al. 1997).

VEGF-A isoforms that are bound to the ECM via heparin or heparan sulphate constitute a reservoir of these growth factors. They are biologically inactive in the bound form, but they can activate their receptors when they are released. A slow release of bound VEGF-A can take place in presence of heparin, heparan sulphate and heparinases, whereas they are set free rapidly by the proteolytic enzyme plasmin (Houck et al. 1992; Plouet et al. 1997). Interestingly plasminogen activators (that convert enzymatic inactive plasminogen into the proteolytic enzyme plasmin) are expressed by ECs following stimulation of either VEGF-A or bFGF. Consequently activation of ECs with the angiogenic factors VEGF-A or bFGF results in a increased concentration of plasminogen and the local release of stored ECM-bound VEGF-A isoforms that enhance the angiogenic stimulus (Moscatelli et al. 1986; Pepper et al. 1991).

2.2 Physiologic functions of VEGF-A

VEGF-A is essential for embryonic development. Its importance can be deduced from animals where the loss of just one VEGF-A allele results in embryonic lethality. These animals die at an

age of embryonic day 11 to 12 due to a deficiency in EC development and a lack of vessel formation (Carmeliet et al. 1996; Ferrara et al. 1996). VEGF-A contributes both to vasculogenesis and angiogenesis. On the one hand it directly affects the formation of blood vessels because it stimulates EC proliferation, migration, sprouting as well as tube formation. On the other hand it has an indirect impact on angiogenesis as it induces the expression of proteases such as interstitial collagenase, uPA (urokinase-like plaminogen activator) and tPA (tissue plasminogen activator) (Pepper et al. 1991; Unemori et al. 1992). These proteases release the ECs from their environment, a prerequisite for migration and invasion. VEGF-A induces vascular permeability and leakage, so that plasma proteins are able to infiltrate the surrounding tissues (Senger et al. 1983; Keck et al. 1989). In addition it is a key survival factor for ECs (Alon et al. 1995; Benjamin and Keshet 1997) as it induces the expression of the anti-apoptotic proteins Bcl-2 and A1.

VEGF-A can act in a paracrine or autocrine way as it is expressed by a number of cells including ECs, macrophages and activated T cells. Elevated levels of VEGF-A were also found in the majority of human tumours (Ferrara and Davis-Smyth 1997). VEGF-A gene expression is tightly regulated. Hypoxia is a strong activator of VEGF-A expression (Levy et al. 1995; Liu et al. 1995). On the one hand HIF-1 (hypoxia-induced factor 1), which is stabilised by low oxygen concentrations, binds to the hypoxia response element (HRE) upstream of the VEGF-A gene and enhances VEGF-A expression. On the other hand the hypoxia-induced stability factor (HuR) increases the half-life of VEGF-A mRNA (Levy et al. 1998). In addition many cytokines and growth factors upregulate VEGF-A mRNA, e.g. EGF (epidermal growth factor), TGF-β (transforming growth factor β), IL-1α (interleukin 1 α), IL-6 (interleukin 6) and IGF-1 (insulin-like growth factor 1) (Brogi et al. 1994; Pertovaara et al. 1994; Li et al. 1995; Cohen et al. 1996; Warren et al. 1996) and transformation can also induce VEGF-A expression, e.g. mutations of p53 (Kieser et al. 1994; Mukhopadhyay et al. 1995) or ras (Grugel et al. 1995; Rak et al. 1995) lead to VEGF-A upregulation.

3 Receptors for VEGF

The three main receptors for VEGFs, VEGFR-1, VEGFR-2 and VEGFR-3, are RTKs that get activated upon ligand binding. Ligand induced dimerisation of the receptors causes structural changes of the transmembrane and the juxtamembrane domains and leads to autophosphorylation of the kinase domains. Further phosphorylation of several tyrosine residues forms a platform for adapter molecules that promote signalling.

VEGFR-1 and VEGFR-2 are mainly localised on the surface of vascular ECs (Ferrara and Davis-Smyth 1997). Additionally they were identified on bone marrow-derived cells (Kabrun et al. 1997) like macrophages and monocytes (Sawano et al. 2001) and vascular smooth muscle cells (Ishida et al. 2001). Their structure is very similar. Seven immunoglobulin-like domains form the extracellular region, of which the 2nd (VEGFR-1) or the 2nd and 3rd (VEGFR-2) are the binding sites for VEGF-A165 (Davis-Smyth et al. 1996; Wiesmann et al. 1997; Davis-Smyth et al. 1998; Fuh et al. 1998). A single transmembrane region is followed by the intracellular tyrosine sequence that is interrupted by a kinase insert domain (Shibuya et al. 1990; Terman et al. 1991; Pajusola et al. 1992). VEGFR-3 is primarily expressed on lymphatic ECs and can become activated by VEGF-C and VEGF-D. It is an important regulator for lymphangiogenesis.
A second group of receptors for VEGFs are two neuropilin (NRP) proteins. NRP-1 was originally identified as a semaphorin receptor involved in neuronal guidance. Later it was found that NRP-1 binds VEGF-A165, VEGF-B, PlGF-2 and some VEGF-E variants. A second NRP protein (NPR-2) was identified because of its sequence homology with NRP-1. It binds VEGF-A145, VEGF-A165 and VEGF-C. Their function in VEGF signalling is described in chapter 3.3.

3.1 VEGFR-1

VEGFR-1 (also Flt-1: fms-like tyrosine kinase receptor; mouse fetal liver kinase 1) acts as a receptor for the three ligands VEGF-A (de Vries et al. 1992), VEGF-B (Olofsson et al. 1998) and PlGF (Park et al. 1994). It is the receptor with the highest affinity for VEGF-A, but its activation results only in weak tyrosine phosphorylation and weak mitogenic signalling (de Vries et al. 1992; Waltenberger et al. 1994). Therefore it was proposed that VEGFR-1 might mainly act as a decoy receptor with inhibitory effects (Park et al. 1994). An alternatively spliced soluble VEGFR-1 also acts as an inhibitor of VEGF-A activity, binding VEGF-A and reducing activation of VEGFR-2 (Kendall and Thomas 1993). This inhibitory function of VEGFR-1 is absent in VEGFR-1 knockout mice where an excessive proliferation of angioblasts and an overgrowth of unorganised ECs lead to death between embryonic day 8.5 and 9.5 (Fong et al. 1995; Fong et al. 1999).
Besides its negative function VEGFR-1 appears also to promote a positive effect as it is involved in VEGF-A165-induced migration, where the receptor mediates reorganisation of the actin cytoskeleton via the p38 MAPK pathway (Kanno et al. 2000).

3.2 VEGFR-2

VEGFR-2 (also KDR: kinase insert domain containing receptor; Flk-1 in mice) is a receptor for VEGF-A, VEGF-E, svVEGF and processed forms of VEGF-C and VEGF-D. It acts as the major mediator of the mitogenic, proliferative, angiogenic, prosurvival and permeability-enhancing effects of VEGF-A. It has an essential role in embryonic development as demonstrated by the lethality of the knockout mice between embryonic day 8.5 and 9.5 due to a lack of vasculogenesis and an impaired development of blood islands and organised blood vessels (Shalaby et al. 1995; Shalaby et al. 1997).

Upon ligand-induced VEGFR-2 dimerisation, several intracellular tyrosine residues are autophosphorylated. Tyr1175 is the major VEGF-A165-dependent autophosphorylation site in the carboxyterminal domain of VEGFR-2 that creates a binding site for cellular adaptor proteins like Sck (Shc related adaptor protein) (Warner et al. 2000), Shb (Src homology 2 domain containing adaptor protein B) (Holmqvist et al. 2004), p85 of PI3K (phosphoinositide 3 kinase) (Dayanir et al. 2001) and for PLC-γ (phospholipase C γ) (Takahashi et al. 2001). Further signal transduction involves different signalling pathways like PLC-γ-PKC-raf-MEK-Erk, p38-MAPK, PI3K-PKB/Akt that alone or in combination result in vascular permeability, migration, proliferation and survival of ECs (Guo et al. 1995; Rousseau et al. 1997; Gerber et al. 1998a; Gerber et al. 1998b; Eliceiri et al. 1999; Takahashi et al. 1999; Meadows et al. 2001).

3.3 Modulation of VEGFR-2 activation

VEGFR-2 collaborates with several cell adhesion molecules (CAMs) that are able to modify receptor activation and cellular responses. For example the association of VEGFR-2 with VE-cadherin at cell confluency inhibits receptor phosphorylation presumably by recruitment of the density-enriched phosphatase-1 (DEP-1) and thereby prevents further EC growth (Lampugnani et al. 2003; Lampugnani et al. 2006). On the other hand the complex of VE-cadherin, VEGFR-2 and PI3K induced by VEGF-A appears to be required for survival of ECs (Carmeliet et al. 1999). Furthermore VEGF-A165 requires heparin-like molecules at or in proximity to the cell membrane that increase binding to its receptors (Gitay-Goren et al. 1992).

Also neuropilins can act as co-receptors for VEGFR-2. Expression of NRP-1 in addition to VEGFR-2 on ECs increases the mitogenic activity induced by VEGF-A165 (Soker et al. 1998). A complex containing VEGFR-2 and NRP-1 is required for full bioactivity of the VEGF-A/VEGFR-2 system (Soker et al. 1998; Whitaker et al. 2001; Soker et al. 2002). VEGFR-2 and

NRP-1 do not directly interact (Fuh et al. 2000), but the ligand VEGF-A165 seems to bridge the receptors by binding to both simultaneously (Soker et al. 2002).

4 HGF and its receptor Met

The VEGFRs are mainly expressed on ECs and have been associated with angiogenesis quite early. Met however is expressed in a variety of tissues, predominantly on epithelial cells. Its functions in development have been studied extensively, but its participation in angiogenesis was only recognised in the early 1990ies (Bussolino et al. 1992; Grant et al. 1993).

Hepatocyte growth factor/scatter factor (HGF/SF; here abbreviated as HGF) belongs to the plasminogen-related growth factors (PRGFs) and is primarily secreted by mesenchymal cells. It was discovered as a mitogen inducing scattering and local motility of epithelial cells (Stoker et al. 1987). Independently HGF was characterised as a potent mitogen for hepatocytes (Nakamura et al. 1989; Zarnegar and Michalopoulos 1989). Finally it has been shown that both are identical molecules (Naldini et al. 1991). HGF functions by activating the receptor tyrosine kinase Met (mesenchymal-epithelial transition factor), which is primarily expressed on epithelial cells (Chan et al. 1988; Di Renzo et al. 1991). As HGF is secreted by mesenchymal cells in proximity to Met expressing cells, receptor activation is mainly paracrine (Sonnenberg et al. 1993). Met is a proto-oncogene that was first identified as the oncogene TPR-Met, where the intracellular kinase domain of Met is connected to the gene product of the TPR (translocated promoter region) gene by chromosomal translocation. This chromosomal rearrangement was induced with the chemical carcinogen N-methyl-N'-nitrosoguanidin in human osteosarcoma cells (HOS) (Cooper et al. 1984; Park et al. 1986). As the TPR part of that protein codes for two protein-protein interaction sequences, TPR-Met forms a dimer and is constitutively active (see structure and activation of Met in introduction chapter 4.2).

Met signalling induced by HGF is essential for embryonic development as knockout mice lacking either HGF or Met die during embryonic development at E14.5 due to a placental defect (Bladt et al. 1995; Uehara et al. 1995). Additionally the development of other organs is affected. Reduced cell number and abnormal cell morphology cause a reduced liver size (Schmidt et al. 1995), and defects in skeletal muscle development is due to impaired migration of myogenic precursor cells (Bladt et al. 1995). Also the development of motoneurons is hampered (Ebens et al. 1996).

4.1 Structure of HGF

HGF is a heterodimeric protein that is transcribed as a single chain precursor and proteolytically processed by serine proteases to the biological active heterodimer. The two resulting subunits α (69 kDa) and β (34 kDa) are linked by a disulfide bond (Miyazawa et al. 1989; Nakamura et al. 1989; Naka et al. 1992). The α-subunit contains four kringle domains (K1-K4), of which K1, K2 and K4 are required for receptor activation (Hartmann et al. 1992; Holmes et al. 2007). The β-subunit contains a domain homologous to serine proteases but without enzymatic activity. This domain, the K1 domain and the N-terminal part of the α-subunit form the binding site for the Met receptor (Lokker et al. 1992; Holmes et al. 2007).

4.2 Structure of Met

Similar to its ligand HGF, Met is a heterodimeric protein that is processed by proteolytic cleavage of one precursor protein. The resulting α-subunit (50 kDa) is located extracellular and is connected to the β-subunit via a disulfide bond. The α-subunit together with the first 212 amino acids of the β-subunit is homologous to the semaphorin-like sema domain. The β-subunit (145 kDa) is membrane spanning and the extracellular part contains an additional sema domain, a cysteine rich plexin, semaphorin and integrin (PSI) domain as well as four immunoglobulin-like domains (Ig1-4). The tyrosine kinase domain and the carboxyterminal part essential for downstream signalling are localised in the intracellular region (Giordano et al. 1989).
Binding of HGF to the α-subunit and the first 212 amino acids of the β-subunit allows receptor dimerisation, followed by autophosphorylation of three tyrosine residues in the kinase domain (Bottaro et al. 1991; Gherardi et al. 2003). Phosphorylation of further tyrosine residues is the prerequisite for intracellular signalling.

4.3 Biological function of HGF/Met

Activation of Met by its ligand HGF is the trigger for different cellular responses. Met signalling leads to proliferation of several different cell types like endothelial cells, hepatocytes, keratinocytes, melanocytes and renal cells. Also migration and scattering, suggesting the loss of cell junctions, as well as invasive growth into the ECM are induced by HGF/Met signalling (reviewed in (Bardelli and Comoglio 1997; Birchmeier and Gherardi 1998)). All these cellular responses following Met signalling are necessary during development and wound healing processes. However they also take place in tumour progression. Therefore it is not surprising that deregulation of HGF and Met activation or upregulation of both proteins are found in numerous

human tumours (reviewed in (Bardelli et al. 1997; Birchmeier et al. 2003)). In addition overexpression of Met or HGF often correlates with poor prognosis indicating a role in the metastatic process. Tumour cells make use of different mechanisms to increase Met activation. For example, osteosarcomas express both the ligand HGF and the receptor Met, so that Met can be activated in an autocrine manner (Fukuda et al. 1998). Many carcinomas do overexpress Met, so they are hyperresponsive to normal HGF levels. If the receptors are expressed at very high levels, oligomerisation can lead to ligand-independent constitutive receptor activation. Mutations and structural changes in the receptor are another way to achieve ligand-independent signalling (reviewed in (Hanahan and Weinberg 2000; Birchmeier et al. 2003)).

The evidence that Met is important for tumour progression is given by several experimental approaches. Animal models reveal that invasiveness and metastatic properties of cancer cell lines are increased by overexpression of HGF and/or Met (Giordano et al. 1993; Rong et al. 1994). In line with this observation the tumourigenic potential of tumour cells is reduced when HGF or Met are downregulated (Abounader et al. 2002).

In addition to their direct function on tumour cells, Met and HGF contribute to tumour progression via a second mechanism: they are involved in angiogenesis. Using several animal models the effect of HGF on angiogenesis was proven *in vivo* (Bussolino et al. 1992; Grant et al. 1993; Rosen et al. 1993; Rosen and Goldberg 1997). HGF is a potent proangiogenic factor that activates Met on ECs and thereby contributes to the formation of new blood vessels. HGF promotes angiogenesis in two ways. On the one hand it directly activates the receptor Met on ECs to support EC's growth and motility. On the other hand HGF can stimulate and boost angiogenesis indirectly. Human vascular smooth muscle cells and keratinocytes respond to HGF activation with increased expression of VEGF-A and VEGFR-2 expression is upregulated in ECs after HGF treatment (Van Belle et al. 1998; Wojta et al. 1999). In addition, the angiogenesis inhibitor thrombospondin-1 can be downregulated following HGF induction in tumour cells (Zhang et al. 2003).

4.4 HGF-induced signalling

HGF-induced dimerisation of Met results in autophosphorylation of three tyrosines within the kinase domain, Tyr1230, Tyr1234 and Tyr1235. Additionally the tyrosine residues Tyr1349 and Tyr1256 in the carboxyterminal part of Met are phosphorylated and serve as a docking site for several adaptor proteins (Ponzetto et al. 1994). These interactions between adaptor proteins and Met predominantly occur via Src-homology-2 (SH2) domains, phosphotyrosine binding (PTB) domains or Met binding domains (MBD).

Proteins that directly bind activated Met include Grb2 (growth factor receptor-bound protein 2) (Ponzetto et al. 1996), Gab1 (Grb2-associated binding protein 1) (Weidner et al. 1996), PI3K (phosphoinoditide 3-kinase) (Graziani et al. 1991; Ponzetto et al. 1994) and Shc (SH2-containing protein) (Pelicci et al. 1995). Among these, Gab1 plays a central role for Met signalling as it is a large multi-adaptor protein that provides a docking site for further signalling molecules that thereby indirectly interact with Met (Sachs et al. 1996; Maroun et al. 1999), e.g. the proteins Crk (CT10 regulator of kinase) (Garcia-Guzman et al. 1999; Sakkab et al. 2000), PLC (phospholipase C), Shp2 (a protein tyrosine phosphatase), PI3K and Shc. The important role of Gab1 for Met signalling can be deduced from Gab1 knockout mice that show placental, liver and muscle defects that are similar to the defects observed in HGF or Met knockout mice (Sachs et al. 1996; Sachs et al. 2000). In addition Gab1 itself can bind Met indirectly via Grb2 that also acts as an adapter between Met and other molecules. Grb2 binds also Shc and the Ras guanine nucleotide exchange factor SOS (son-of-sevenless) (Graziani et al. 1993). Further molecules involved in Met signalling are Src (Rahimi et al. 1998) and Stat3 (signal transducer and activator of transcription 3) (Boccaccio et al. 1998; Zhang et al. 2002).

In summary Met signalling is very complex. The recruitment of all these signalling molecules to phosphorylated Met cause the activation of several distinct but interacting signalling pathways including Ras-MAPK, PI3K, Src, Stat3, Akt and JNK (Royal et al. 1997; Campbell et al. 1998; Schaeper et al. 2000; Gu and Neel 2003). Activation of one pathway alone or a combination of several pathways together then results in the HGF-induced cellular responses described in chapter 4.3.

4.5 Modulators of Met signalling

Although Met signalling induced by HGF is already very complex due to the involvement of numerous adapter molecules and signalling pathways, it can be further modulated and influenced by additional transmembrane proteins.

One of the modulators is α6β4 integrin. It is a cell surface adhesion molecule that can bind laminins and is usually involved in interactions with the ECM. It is upregulated in invasive carcinomas (Rabinovitz and Mercurio 1996), and in carcinoma cells it is shown that Met is able to form a complex with α6β4 integrin. After activation with HGF the cytoplasmic domains of Met and α6β4 are tyrosine phosphorylated and both serve as docking sites for signalling molecules like Shc and PI3K (Trusolino et al. 2001). This synergistic cooperation results in activation of the Ras/MAPK pathway and in signal enhancement.

An HGF independent interacting partner for Met is plexinB1. Plexins are receptors for semaphorins and are involved in the control of axon guidance. Interestingly Met, plexins and semaphorins share a similar structure, the sema domain. In cells where plexinB1 and Met are co-expressed, activation of plexinB1 with its ligand semaphorin 4D (Sema4D) leads to tyrosine phosphorylation of Met and results in cellular responses similar to HGF stimulation. (Giordano et al. 2002; Basile et al. 2005). In ECs the activation of Met via Sema4D and plexinB1 induces angiogenesis (Conrotto et al. 2005).

On ECs HGF-induced activation of Met and downstream signalling depends on the presence of neuropilins (NRPs). These proteins were first discovered as semaphorin receptors involved in axonal guidance. Later they were identified as co-receptors for VEGFRs (see introduction chapter 3). When NRP-1 and NRP-2 are downregulated, HGF-induced EC migration and proliferation is impaired (Sulpice et al. 2007).

The best-characterised regulator of Met activation appears to be an isoform of the transmembrane protein family CD44, CD44v6 (see introduction chapter 5.5).

5 CD44

Proteins of the CD44 family form a polymorphic group of transmembrane glycoproteins that are widely expressed on vertebrate cells. Their molecular weights range from 80 to 230 kDa. They were first discovered in 1980 as an antigen that is recognised by a monoclonal antibody raised against human leukocytes (Dalchau et al. 1980). Further studies revealed that these proteins are identical with proteins called Hermes (Goldstein et al. 1989), HUTCH-I (Gallatin et al. 1989), ECM-III (class III extracellular matrix receptor) (Carter and Wayner 1988), HCAM (Goldstein and Butcher 1990) and Pgp-1 (phagocyte glycoprotein 1) (Hughes et al. 1983) (summarised in (Haynes et al. 1989)). The heterogeneity of CD44 proteins is due to alternative splicing of 10 variable exons and additional post-translational modifications, e.g. glycosylation and binding of GAG (glycosaminoglycans).

5.1 CD44 gene structure

A single gene that is localised on chromosome 11 in humans (Goodfellow et al. 1982) and chromosome 2 in mice (Colombatti et al. 1982) encodes all CD44 proteins. The CD44 gene is highly conserved between species and it is organised into 20 exons (Figure 3), where exons 1-17 code for the extracellular part of the proteins, exon 18 encodes the hydrophobic transmembrane domain including 3 intracellular amino acids and exon 19 and 20 give rise to the intracellular

part of CD44. Exons 6-15 are the variant exons (also called variant exons v1-v10) that can be inserted into the protein in different combinations (Screaton et al. 1992; Screaton et al. 1993; Tölg et al. 1993). Also exons 19 and 20 are spliced alternatively. Insertion of exon 19 generates a small cytoplasmic domain of 5 amino acids due to an alternative stop codon, whereas inclusion of exon 20 generates a cytoplasmic tail of 73 amino acids. Inclusion of exon 19 is a rare event, most CD44 isoforms contain exon 20 (Goldstein et al. 1989; Goldstein and Butcher 1990; Thorne et al. 2004). In humans the variant exon v1 (exon 6) is not expressed because it contains a stop codon (Screaton et al. 1993; Tölg et al. 1993), and exon v3 contains a second splice acceptor site and therefore exists in two variants.

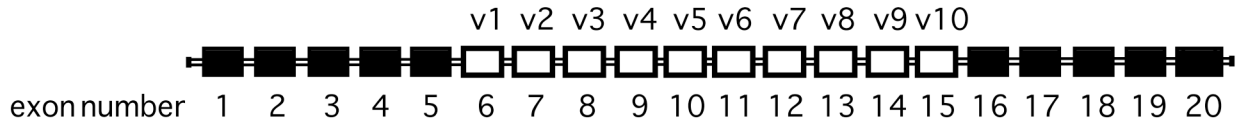

Figure 3: Schematic presentation of the CD44 gene. Exons 5-15 are subject to alternative splicing and are also known as variant (v) exons v1-v10.

5.2 CD44 protein structure

The smallest CD44 isoform is CD44 standard (CD44s), where all the variant exons are excluded. It is expressed in vertebrates in nearly all cells. The heterogeneity of CD44 molecules is achieved by insertion of different combinations of the variant exons 6-15 (respective variant exons v1-v10) into the extracellular stem region of CD44s between exons 5 and 16 (Screaton et al. 1992) and by posttranslational modifications. The largest CD44 isoform found in human keratinocytes is CD44v2-v10 that contains all variant exons (Bloor et al. 2001).

The extracellular domain

The first five exons are highly conserved amongst mammalian species (~85 %). The aminoterminal part (exon 1) is followed by a link-homology hyaluronan-binding domain (exon 2 and 3), that is similar to a domain found in cartilage link protein, proteoglycan core protein (Deak et al. 1986; Neame et al. 1986), aggrecan and tumour necrosis factor stimulated gene 6 (TSG-6) (Kohda et al. 1996; Bajorath et al. 1998) and that has been shown to be the binding site for hyaluronan (HA) (Thorne et al. 2004). Six cysteine residues form intramolecular disulfide bonds (Goldstein et al. 1989) and are involved in correct folding and HA binding (Banerji et al. 1998).

Exons 4, 5, 16 and 17 encode the stem region that is close to the transmembrane domain. In CD44s this region is rather small, but it can be enlarged by insertion of sequences encoded by the variant exons between exons 5 and 16 (Screaton et al. 1992; Tölg et al. 1993). This stem region exhibits a cleavage site for metalloproteases and serine proteases. Cleavage gives rise to a soluble CD44 (sCD44). This mechanism might be used to downregulate CD44 on the cell surface, and sCD44 competes with cell-bound CD44 for ligand binding (Bazil and Strominger 1994; Katoh et al. 1994).

The extracellular part of CD44 provides a number of sites for posttranslational modifications, both in the constant part as well as in the variant part. Among them are N-linked and O-linked glycosilations and GAG modifications, e.g. chondroitin sulphation (Brown et al. 1991; Sleeman et al. 1997; Greenfield et al. 1999). The only site for modifications by heparan sulphate is localised in the variant exon v3 containing the consensus Ser-Gly-X-Gly motif. This modification enables heparin-binding proteins to attach to CD44 (Bennett et al. 1995; Sherman et al. 1998; Jones et al. 2000). All these posttranslational modifications are cell type specific and can modify CD44 function (English et al. 1998).

The transmembrane domain

The single-spanning transmembrane domain of CD44 is encoded by exon 18 and consists of hydrophobic amino acids and a cysteine. Mutation studies showed that this cysteine is required for HA binding, possibly by clustering several CD44 molecules (Liu and Sy 1996). The transmembrane region can be posttranslational modified by palmitoylation that enhances the interaction of ankyrin with the cytoplasmic part of CD44 (see below) (Bourguignon et al. 1991).

The intracellular domain

The cytoplasmic part of CD44 encoded by exon 20 interacts with different proteins. Ankyrin was the first protein that was found to interact with the intracellular domain. This interaction is involved in hyaluronan-dependent cell adhesion and motility (Lokeshwar et al. 1994). Two stretches of basic amino acids close to the transmembrane domain act as a binding site for the proteins ezrin, moesin and radixin (ERM) (Legg and Isacke 1998). These proteins are able to bind to various membrane proteins via their N-terminal domain while the C terminus attaches to filamentous actin (F-actin) (Turunen et al. 1994). They serve as linkers that connect the actin cytoskeleton with the cell membrane. Merlin, a tumour suppressor protein structurally related to ERMs, also binds to this binding site. It has no actin binding domain and therefore cannot link the cytoskeleton to CD44. Recently it was shown that a HA-induced complex including CD44,

N-WASP (neural Wiskitt-Aldrich syndrome protein) and Arp2/3 (actin related protein 2/3) induces actin polymerisation (Bourguignon et al. 2007). Additionally other proteins such as the Rho-family of GTPases and members of the Src family of non-receptor tyrosine kinases form complexes with the cytoplasmic part of CD44 and link CD44 to downstream signalling pathways. It is not clear if their interaction is functionally relevant (Ponta et al. 2003; Thorne et al. 2004).

The intracellular part of CD44 contains conserved serine residues. In resting cells, Ser325 is phosphorylated and is important in HA mediated cell migration (Peck and Isacke 1998). Activation of the cells by HA leads to a change in phosphorylation, Ser325 gets dephosphorylated, while Ser291 gets phosphorylated by PKC (protein kinase C). This impairs the binding of ERM proteins in a regulated way (Legg et al. 2002). Ankyrin binding to CD44 is also dependent on serine phosphorylation, which is modulated by Rho kinase (Bourguignon et al. 1999).

5.3 Functions of CD44 in physiologic and pathologic conditions

Mainly interference studies with antibodies recognising all or only specific CD44 isoforms demonstrate that CD44 is involved in processes such as adhesion, proliferation and migration. More specifically these studies revealed that CD44 is involved in physiological processes like limb development, haematopoiesis, lymphocyte activation and homing, immune functions and axon guidance, as well as in pathological processes such as inflammation, wound healing and tumourigenesis.

CD44 and embryonic development

CD44 isoforms take over an important role in limb development. In the apical ectodermal ridge (AER) of rats the predominantly expressed isoform of CD44 is CD44v3-10. It colocalises temporally and spatially with FGF-8 on the surface of AER cells throughout early limb development. Via heparan sulphate residues at variant exon v3, CD44 can bind FGF-8 (fibroblast growth factor 8) and FGF-4 and present these growth factors to their receptors in the underlying mesenchymal cells in order to induce a proliferative signal and correct limb outgrowth (Wainwright 1998).

Also in the heart and in the somites CD44 is highly expressed during embryonic development, suggesting a role in HA-mediated morphogenesis and organogenesis (Wheatley et al. 1993).

Furthermore CD44 is involved in the maturation of T cells. The migration of foetal liver (FL) cells to the thymus and their development to mature T cells requires CD44 (Kawakami et al. 1999).

CD44 isoforms play a role during embryonic development. An antibody directed against CD44v6 hampered embryonic development until day 16-18 of gestation in pregnant rats, and the use of an antibody recognising all CD44 isoforms leads to a delay in birth and an increased number of intrauterine abortions (Zoller et al. 1997).

CD44 and haematopoiesis

CD44 is required for the development of haematopoietic progenitor cells for myelopoiesis and lymphopoiesis. The participation of CD44 in haematopoiesis was characterised in long-term bone marrow cultures (LTBMCs). An antibody recognising all CD44 isoforms blocked the development of progenitor cells and the generation of lymphoid and myeloid cells (Miyake et al. 1990; Khaldoyanidi et al. 1996). Interesting results were obtained with antibodies directed against the variant isoforms CD44v4 and CD44v6. The antibodies induce bone marrow macrophages to produce granulocyte-macrophage colony stimulating factor (GM-CSF) (both antibodies) and interleukin 6 (IL-6) (CD44v6 antibody), thus stimulating myelopoiesis (both antibodies) and lymphopoiesis (CD44v6 antibody) (Rossbach et al. 1996; Khaldoyanidi et al. 2002).

CD44 and lymphocyte homing and activation

Lymphocyte homing is the return of lymphocytes from the blood vasculature back to lymphatic tissues. CD44 is involved in this process (Shimizu and Shaw 1991). Specifically the homing to the thymus depends on CD44, as bone-marrow cells treated with CD44 antibody were not able to reach the thymus (O'Neill 1989), whereas homing to lymph nodes was not impaired (Camp et al. 1993).

The extravasation of lymphocytes from the blood vessels into surrounding tissue also depends on CD44. The so-called lymphocyte rolling is impaired by treatment with antibodies against CD44, and also by addition of HA (DeGrendele et al. 1996).

Lymphocytes need CD44 as well for maturation, as T and B lymphocytes require CD44 isoforms containing variant exon v6 to get activated (Huet et al. 1989; Arch et al. 1992).

CD44 in inflammation and autoimmune diseases

CD44 and its ligand HA are involved in several inflammatory processes and autoimmune diseases. Leukocytes and parenchymal cells increase their CD44 expression during inflammation (Foster et al. 1998). CD44- and HA-dependent interactions of cells with the ECM seem to be an important part of inflammatory processes, since loss of those interactions leads to an impaired inflammation. An antibody that blocks binding of HA to CD44 reduces inflammation in a mouse model of rheumatoid arthritis, whereas an antibody that increases HA binding to CD44 increases inflammation (Mikecz et al. 1999). Additionally the symptoms of collagen type-II induced rheumatoid arthritis can be reduced with CD44 antibodies (Zeidler et al. 1995).

Antibodies to CD44 also prevent the passage of T cells through the blood-brain barrier in a mouse model of multiple sclerosis and allergic encephalomyelitis, and thereby impair the formation of lesions in the brain (Brocke et al. 1999).

CD44 is also involved in skin inflammation and inflammatory colitis (bowel disease), where it elongates the survival of lymphocytes (Camp et al. 1993; Wittig et al. 2000).

Concerning the endothelium, the infiltration of cytotoxic T cells that leads to interleukin 2 (IL-2) induced vascular leak syndrome (VLS) and EC injury is reduced in CD44 knockout animals (Rafi-Janajreh et al. 1999). Furthermore cultured microvascular ECs increase the expression of HA after incubation with inflammatory cytokines such as IL-1β or TNF-α (Mohamadzadeh et al. 1998).

CD44 and tumourigenesis

Numerous studies show that the tumourigenic and metastatic behaviour of several cancer cells correlates with expression of CD44 isoforms, especially with the expression of variant isoforms. One of the most striking evidence came from one study where the variant isoform CD44v4-v7 was introduced into non-metastasising rat pancreatic cancer cells and rendered these cells metastatic (Günthert et al. 1991). Metastasis could then be blocked with antibodies directed against CD44v6 (Günthert et al. 1991; Seiter et al. 1993). The expression of variant isoforms of CD44 often correlates with poor prognosis, e.g. the expression of CD44v6 in colorectal carcinoma (Sleeman et al. 1995; Naor et al. 1997; Wielenga et al. 1998) or the expression of CD44 isoforms containing variant exons v6, v7 and v8 in cervical cancer (Kainz et al. 1995), suggesting a role of CD44 in metastasis formation. Also CD44s can be involved in tumour progression. Indeed a CD44 negative Burkitt lymphoma cell line only became metastatic after transfection with CD44s.

However, there are cases where the presence of CD44 isoforms does not influence tumour progression. Interestingly the introduction of CD44v8-v10 did not affect tumour development (Sy et al. 1991; Sy et al. 1992). The same has been shown for mouse melanoma cells, the presence of CD44s was required for tumour growth (Bartolazzi et al. 1994).
Surprisingly, cases where CD44 was claimed to act as a "tumour repressor" were also reported. Indeed, CD44s has even been shown to reduce the malignancy of tumours. Overexpression of the smallest CD44 isoform repressed metastasis formation of a prostate carcinoma cell line (Gao et al. 1997), and tumour growth of transformed fibroblasts was drastically reduced when the cells were transfected with a construct encoding CD44s (Schmits et al. 1997).

5.4 Deletion of CD44 in mice

Targeted deletion of a single gene in animals is a powerful tool to investigate the function of the respective protein *in vivo*. In the case of CD44 two different transgenic animals are available. On the one hand there exist classical total knockout mice in which the CD44 gene is disrupted right from the beginning of development. On the other hand, another knockout strategy was used where CD44 was suppressed specifically in the skin at a later time point, namely at embryonic day E11.5. Interestingly the different mice developed quite different phenotypes.

CD44 total knockout

CD44 knockout animals were generated by germline deletion of different sequences of the CD44 gene (Schmits et al. 1997; Protin et al. 1999). Despite the numerous functions of CD44 during development and in adulthood, the CD44 null mice are born in the normal Mendelian ratio, they are viable, fertile and develop normally. They show no alterations in size or tissue architecture but have some abnormalities in the haematopoietic system. Indeed, the tissue distribution of myeloid progenitor cells between bone marrow and spleen is altered (Schmits et al. 1997). In addition, lymphocyte homing to the thymus and lymph nodes is impaired, whereas the lymphocyte development seems not to be affected (Protin et al. 1999). Pregnant mice show some defects after birth, e.g. the maintenance of lactation is impaired and uterine involution is accelerated (Yu et al. 2002).
Additional phenotypes of the CD44 knockout mice were found when the animals were challenged. For example the infection with *Cryptosporidium parvum*, a parasite that settles in the intestinal tract and causes diarrhoea, resulted in increased formation of granuloma compared to wild type mice. This suggests defects in T cell function (Schmits et al. 1997). Another study showed that induced lung injury was followed by accumulation of apoptotic neutrophils and pro-

inflammatory fragments of hyaluronan leading to death in knockout animals. Death of these animals could be prevented by injection of CD44 positive leukocytes, suggesting a role of CD44 in anti-inflammatory processes (Teder et al. 2002).
An insight in the role of CD44 for tumour progression is given in studies where CD44 null mice were crossed with animals containing point mutations in tumour suppressor genes, e.g. $p53^{+/tm1}$ (targeted mutation 1 of p53 gene). Wild type animals containing these point mutations develop malign, metastasising tumours, whereas the CD44 knockout mice develop benign sarcomas. The presence of CD44 does not influence tumour incidence, but in these animal models it is required for metastasis (Weber et al. 2002).

CD44 selectively suppressed in keratinocytes

An alternative approach undertaken to investigate the biological function of CD44 is the generation of transgenic mice expressing an antisense CD44 cDNA. The complete CD44 antisense sequence was cloned downstream of the bovine keratin-5 promoter (Kaya et al. 1997), in order to knock down CD44 in all layers of the epidermis and outer root sheath of hair follicles. In contrast to the absence of phenotype in the skin of animals with germline CD44 gene disruptions, these transgenic animals developed a thick skin with reduced elasticity. The HA distribution was altered, as there was a loss of HA in the interkeratinocyte spaces but an accumulation in the superficial dermis. All this together lead to a 4-7 day delay in wound healing as compared to wild type animals. Delays in hair regrowth (5 days) were caused by reduced keratinocyte proliferation in the hair bulb.

These contradictory data obtained with CD44 inactivation in the classical knockout and in the transgenic animals suggest that the loss of CD44 at different times in development has different consequences. Therefore one hypothesis is that the loss of CD44 in early development can be compensated by other proteins that might be differentially expressed, whereas this compensation is not possible at later times, e.g. when the keratin-5 promoter gets activated.
This compensatory mechanism is supported by an additional study investigating limb bud development. In wild type mice the mesenchymal cells of the limb bud required heparan sulphated isoforms of CD44 to present FGF to its receptor. CD44 knockout animals seem to utilise other HSPGs for this growth factor presentation (Wainwright 1998).
In addition, studies in which $CD44^{-/-}$ mice were crossed with $Met^{+/-}$ mice showed a role of CD44 in embryogenesis and collaboration between Met and CD44 *in vivo*. Around 70 % of the animals with just one Met allele in the CD44 null background died at birth due to a breathing defect

generated by impaired synaptic transmission in the respiratory rhythm-generating network and alterations in the phrenic nerve (Matzke et al. 2007). In these $CD44^{-/-}$ $Met^{+/-}$ mice, the potential substitute protein must be present but seems not as effective as CD44.

Analysis of Met activation in cells devoid of CD44v6 lead to the most striking evidence that at least one other protein compensates for CD44v6. Met can get activated by its ligand HGF in the human hepatocellular carcinoma cell line HepG2. In this cell system another transmembrane molecule, ICAM-1, is able to take over the functions of CD44 leading to Met activation and signalling, and inhibition of ICAM-1 results in impaired Met activation (Olaku 2008). Furthermore liver regeneration after partial hepatectomy, a process driven by Met and HGF, also requires CD44v6 in wild type mice, whereas ICAM-1 substitutes for CD44 in CD44 null animals (Olaku, unpublished data).

5.5 Molecular mechanisms of CD44 function

The findings that CD44 is involved in several different processes like haematopoiesis, immune system functions, inflammation and tumourigenesis suggests that CD44 might contribute to several mechanisms that lead to signalling.

CD44 – a ligand binding receptor

CD44 interacts with soluble extracellular components as well as with the ECM. The best-characterised interacting partner of CD44 is HA (Aruffo et al. 1990). HA is a hydrophilic linear polysaccharide consisting of repeats of D-glucuronic acid and N-acetyl-D-glucosamine with a high molecular mass. HA binds to the extracellular link domain in the constant region of CD44 (He et al. 1992) and to a domain near the transmembrane region (Peach et al. 1993) and this interaction results in cell migration.

The binding affinity of CD44 to HA can be modulated from inside the cells by changes in glycosylation of the extracellular domains (Skelton et al. 1998) or phosphorylation of specific intracellular serine residues (Naor et al. 1997). These modulations might be important for migration in HA rich matrices as well as for leukocyte rolling. In addition different variant CD44 isoforms show different affinities to HA, whereby CD44v4-v7 binds HA stronger than CD44s (Sleeman et al. 1996; Sleeman et al. 1997).

Interestingly CD44 is also involved in HA metabolism: it mediates HA uptake via endocytosis (Culty et al. 1992; Hua et al. 1993). This is physiologically relevant as expression of antisense CD44 in the skin (see introduction chapter 5.4) leads to abnormal accumulation of HA in the skin (Kaya et al. 1997).

A more passive role of CD44 is its participation in cell-cell contacts. Indeed HA can act as a bridge between cells expressing CD44.

CD44 – a binding partner for enzymes and growth factors

The extracellular part of CD44 binds several enzymes and growth factors and acts as a platform where crucial interactions take place. For example the matrix metalloproteinase 9 (MMP9) binds to CD44 on tumour cells and subsequently degrades collagen IV in the ECM mediating tumour cell invasion (Yu and Stamenkovic 1999). In addition MMP9 bound to CD44 cleaves the inactive proform of TGF-β, leading to tumour angiogenesis induced by active TGF-β (Yu and Stamenkovic 2000).

Heparan sulphated CD44v3 isoforms can recruit MMP7 and the proform of heparin-binding epidermal growth factor (HB-EGF) to the cell surface of epithelial cells. MMP7 converts the HB-EGF into its active form by proteolytic cleavage, and active HB-EGF activates its receptor ErbB4, thereby increasing cell survival and tissue remodelling (Yu et al. 2002).

As described above also CD44v3 HSPG isoforms on cells of the apical ectodermal ridge (AER) can bind FGF and present the growth factor to its receptor expressed on underlying mesenchymal cells. This process is necessary for limb bud development (Sherman et al. 1998).

CD44 – a co-receptor for receptor tyrosine kinases

Numerous growth factors interact with target cells via RTKs. However, only the presence of the growth factors is not sufficient for receptor activation. Several RTKs require a co-receptor in addition to their ligand in order to get activated and to promote signalling (Orian-Rousseau and Ponta 2008).

This co-receptor function can be fulfilled by CD44 isoforms. They have been shown to act as co-receptors for different RTKs, e.g. for members of the ErbB receptor tyrosine kinase family. In that case CD44s seems to be required for the receptor heterodimerisation of ErbB2 and ErbB3 after induction with the ligand neuregulin in primary Schwann cells, a prerequisite for Schwann cell differentiation, survival and development of the peripheral nervous system (Meyer and Birchmeier 1995; Sherman et al. 2000). The collaboration between CD44 and ErbB2 has been shown to be necessary for activation of ovarian tumour cells (Bourguignon et al. 1997).

The best-characterised co-receptor function of CD44 isoforms for an RTK is the collaboration between CD44v6 and Met in epithelial cells. In several cancer cell lines and in primary cells the activation of Met by its ligand HGF depends on the presence of CD44 isoforms containing variant exon v6. Indeed, treatment with an antibody against CD44v6 (Orian-Rousseau et al.

2002) or a CD44v6 peptide (Matzke 2006) prevents Met activation and signal transduction. Furthermore, in a rat pancreatic cancer cell line that expresses CD44s but no variant isoforms, the RTK Met cannot be activated by its ligand HGF. HGF can induce Met activation only when CD44v6 is introduced into this system (Orian-Rousseau et al. 2002). There is evidence that CD44v6 directly interacts with HGF, as biotinylated HGF does not bind to cells expressing only Met (Bsp73AS), whereas it binds to the same cells stably transfected with CD44v6 (Bsp73ASs6) (Matzke 2006). In addition the presence of HGF induces the formation of a ternary complex between CD44v6, HGF and Met (Orian-Rousseau et al. 2002).

The CD44 isoform that collaborates with Met has to include the variant exon v6, as any other isoform plays no role in Met activation. Three amino acids in the variant v6 sequence are necessary for the co-receptor function of CD44v6. When these three amino acids are mutated, CD44v6 cannot act as a co-receptor for Met anymore (Matzke et al. 2005). Interestingly small peptides (at least 5 amino acids) containing the three important amino acids are able to block the co-receptor function of CD44v6. Besides their blocking effect on Met activation *in vitro* (Matzke et al. 2005), they also inhibit metastasis formation of rat pancreatic tumour cells expressing CD44v6 (Bsp73Ass6) in animals (Matzke, unpublished data). Interestingly these three important amino acids are not conserved in human (RWH), mice (GWQ) and rat (EWQ). This has to be taken into account when the co-receptor function of CD44v6 should be blocked. Antibodies against CD44v6 as well as the CD44v6 peptides act highly species specific and don't cross-react between human, mouse and rat CD44v6. Surprisingly the soluble ectodomain of rat CD44v6 is able to interfere with Met activation on human cells, and addition of the rat v6 peptide abrogates the blocking effect of the rat ectodomain. These data led to the hypothesis that the three amino acids in the v6 sequence are not directly involved in the interaction with the ligand or Met, but rather are required for the right conformation of CD44v6 that allows the function as a co-receptor.

Met requires the co-receptor CD44v6 also for being activated by a second ligand, namely InternalinB (InlB), a protein expressed by the bacteria *Listeria monocytogenes*. InlB is able to activate Met, and Met activation physiologically leads to receptor internalisation. This mechanism is used by the bacteria as they enter the cells by internalisation together with Met after InlB-induced Met activation. In conclusion CD44v6 acts as a co-receptor for Met activation induced by two different ligands, HGF and InlB.

Further investigation revealed that CD44v6 has an additional function as a co-receptor: it is also required for Met signalling. When the cytoplasmic domain of CD44v6 is overexpressed, induction with HGF leads to Met phosphorylation, but not to activation of downstream targets as

Ras or Erk. The excess of cytoplasmic domain seems to “neutralise” all cellular binding partners. In contrast, the overexpression of a cytoplasmic domain of CD44 mutated in the ERM binding site does not influence HGF-induced signalling, as it does not compete with the endogenous cytoplasmic domain. These results lead to the conclusion that the binding of ERM proteins to CD44v6 is necessary for signalling. ERM proteins can directly bind the actin cytoskeleton. This interaction is also required for Met signalling, as ezrin proteins with a defective actin binding site again block activation of downstream signalling components. Thus ERM proteins serve as linkers between the actin cytoskeleton and the cell membrane. CD44v6 recruits the cytoskeleton to the cell membrane via the ERM proteins and the assembly of this complex at the membrane is important for HGF-dependent activation of Ras by its GEF SOS (Orian-Rousseau et al. 2007).

RESULTS

1 CD44v6 acts as a co-receptor for Met in endothelial cells

The receptor tyrosine kinase (RTK) Met and its ligand hepatocyte growth factor/scatter factor (HGF/SF) are involved in the regulation of several cellular responses, including proliferation, survival and migration, the combined action of invasive growth (reviewed by (Bardelli and Comoglio 1997; Birchmeier and Gherardi 1998)). Misregulation of HGF and Met activity and upregulation of both proteins are found in numerous human tumours (reviewed in (Bardelli et al. 1997; Birchmeier et al. 2003)) and lead to poor prognosis in many cases. This suggests a role of both molecules in the metastatic process. However, Met and HGF are not only involved in tumour metastasis but also in angiogenesis. HGF is a potent proangiogenic factor that activates Met expressed on ECs and thereby contributes to the formation of new blood vessels (Bussolino et al. 1992; Grant et al. 1993; Rosen et al. 1993).

In several primary carcinoma cell lines Met needs a co-receptor in order to fulfil its function. This co-receptor is an exon v6 containing variant isoform of the transmembrane protein family CD44 (CD44v6). It is needed for receptor phosphorylation as well as for signal transduction (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007).

Since Met is expressed on vascular ECs and contributes to angiogenesis, the question arises if the co-receptor function of CD44v6 is also required in ECs.

1.1 CD44v6 is required for Met activation and signal transduction in endothelial cells

In order to analyse whether CD44v6 acts as a co-receptor for Met in angiogenesis, several primary ECs isolated from different kinds of human blood vessels were used. ECs can differ in shape, function and in the type of cell-cell junctions depending on the blood vessel where they are localised (reviewed by (Aird 2007)). ECs in arteries (HAOECs (**h**uman **ao**rtic **e**ndothelial **c**ells)) are subjected to high pressure compared to ECs in veins (HUVECs (**h**uman **u**mbilical **v**ein **e**ndothelial **c**ells)). In capillaries major exchange of gases and nutrients takes place. There the ECs (e.g. HCMECs (**h**uman **c**ardiac **m**icrovascular **e**ndothelial **c**ells)) are the only barrier to control this exchange.

When these ECs were treated with HGF, the receptor tyrosine kinase Met was phosphorylated and signalling to Erk – a downstream target of Met – was promoted (Figure 4).

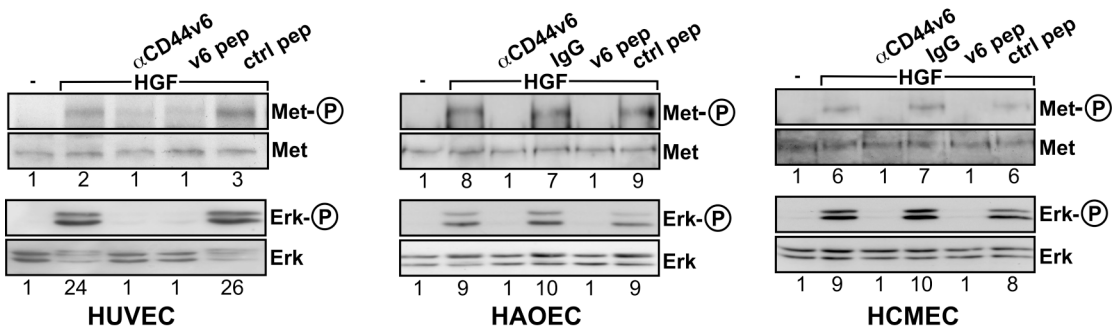

Figure 4: CD44v6 is required for activation of Met and downstream signalling in several ECs. Different ECs (HUVEC, HAOEC and HCMEC) were serum starved and incubated with several reagents (αCD44v6, v6 pep and the controls IgG and ctrl pep). The growth factor HGF was added and the cells were lysed. The proteins in the cell lysates were separated by SDS-PAGE and subjected to Western blot analysis. Activation of Met and of its downstream target Erk was detected by means of phospho-specific antibodies that detect phosphorylated forms of Met and Erk. In order to check equal loading of the different samples the membrane was incubated with stripping solution and reprobed with antibodies recognising all Met and Erk proteins. The numbers indicate the fold induction as calculated with the computer programme ImageJ.

The contribution of CD44v6 to Met signalling in these cells was analysed using different tools. Both, an antibody recognising the exon v6 encoded sequence (referred to as αCD44v6) and a peptide composed of 14 amino acids originating from the v6 region (referred to as v6 peptide) (Matzke et al. 2005), were able to prevent activation of Met and of its downstream target Erk when added to the cells before induction with HGF (see Figure 4). As incubation with a control antibody and a control peptide did not interfere with Met activation and signalling, these results show that CD44v6 is required for Met activation in all types of ECs that were used.

This result was confirmed by means of a third tool proving the involvement of CD44v6 in Met activation. A soluble form of CD44v6, which comprises only the extracellular domain (v6ECD) connected to a c-myc epitope and a polyhistidine tag, was able to block the HGF-induced activation of the signalling cascade to Erk (Figure 5). This blocking was highly specific as a mutated soluble CD44v6 (v6ECD mut) where just three amino acids in the v6 region are modified (mutation to alanins) (see introduction chapter 5.5) was not able to interfere with Met activation induced by HGF (Figure 5).

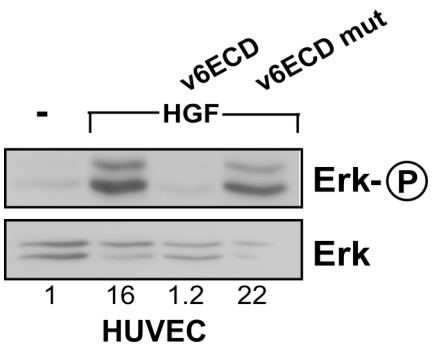

Figure 5: The soluble CD44v6 ectodomain interferes with HGF-induced signalling. HUVECS were incubated with the ectodomain of CD44v6 (v6ECD) or the ectodomain mutated in the v6 exon (v6ECD mut) after serum-starvation. Upon HGF treatment cells were lysed and the lysates were subjected to SDS-PAGE and Western blot analysis to detect activated Erk (see Figure 4).

Thus these experiments reveal that the receptor tyrosine kinase Met needs a co-receptor for activation not only in epithelial cells but also in ECs. In all cell types used this co-receptor is an isoform of CD44 containing the variant exon v6.

2 CD44v6 acts as a co-receptor for VEGFR-2 in endothelial cells

Besides HGF, numerous proangiogenic factors are described, e.g. VEGFs, angiopoietins, FGFs, PDGF and TGF-β. The most prominent one is the polypeptide VEGF-A that predominantly acts on endothelium (Plouet et al. 1989) where its two receptors VEGFR-1 and VEGFR-2 are expressed. VEGFR-2 collaborates with co-receptors like VE-cadherin (Carmeliet et al. 1999; Lampugnani et al. 2003; Lampugnani et al. 2006) or neuropilin-1 (NRP-1) (Soker et al. 1998) that are able to modify receptor activation and cellular responses. It is very likely that in addition other proteins can modulate VEGFR-2 signalling and act as co-receptors. Isoforms of the CD44 protein family might be promising candidates as they have already been shown to bind VEGF-A (Jones et al. 2000). CD44v3 can be modified by covalent binding of heparan sulphate (Bennett et al. 1995) and thereby interact with heparin binding growth factors like bFGF, HB-EGF or VEGF-A.

2.1 CD44v6 is required for activation of VEGFR-2 and signal transduction

In order investigate if an isoform of CD44 is necessary for VEGFR-2 activation, the three tools inhibiting CD44v6 activity (see results chapter 1.1) were used in an experiment where VEGFR-2 was activated by VEGF-A. As expected all ECs responded to VEGF-A165 (see introduction chapter 3.2) treatment with VEGFR-2 phosphorylation and signal transduction to Erk.

Surprisingly the same reagents that interfered with Met activation also inhibited VEGFR-2 phosphorylation. The antibody recognising CD44v6 as well as the CD44v6 peptide blocked VEGFR-2 activation and downstream signalling to Erk induced by VEGF-A165 (Figure 6). Thus CD44v6 is also necessary for a functional VEGF-A165/VEGFR-2 system in these cells.

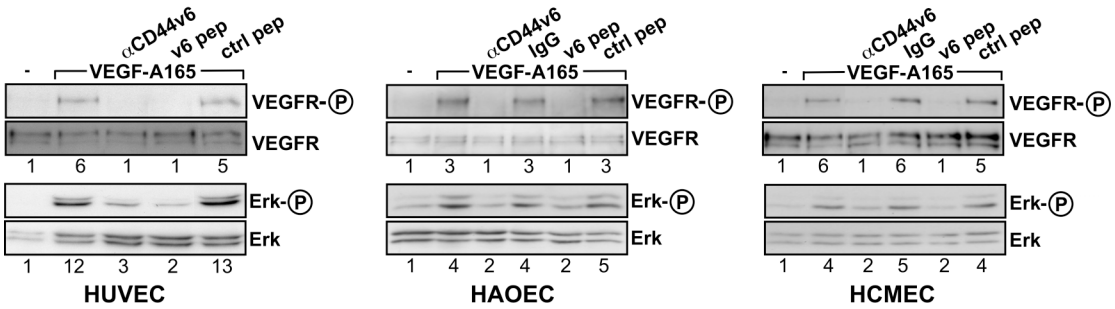

Figure 6: Activation of VEGFR-2 and signal transduction induced by VEGF-A165 depends on CD44v6. Different ECs (HUVEC, HAOEC and HCMEC) were serum starved and incubated with several reagents (αCD44v6, v6 pep and the controls IgG and ctrl pep). The cells were then induced with VEGF-A165 and lysed. The cell lysates were loaded on SDS-PAGE and subjected to Western blot analysis. Activation of the receptor tyrosine kinase VEGFR-2 and of its downstream target Erk was detected by means of antibodies that detect phosphorylated forms of VEGFR-2 and Erk. The loading control was performed with antibodies recognising total VEGFR-2 and Erk levels. The numbers indicate the fold induction as calculated with the computer programme ImageJ.

Supporting this result, the soluble CD44v6 ectodomain (v6ECD) impaired VEGF-A165 induced activation of Erk, whereas the mutated form (v6ECD mut) was not able to interfere (see Figure 7 left part).

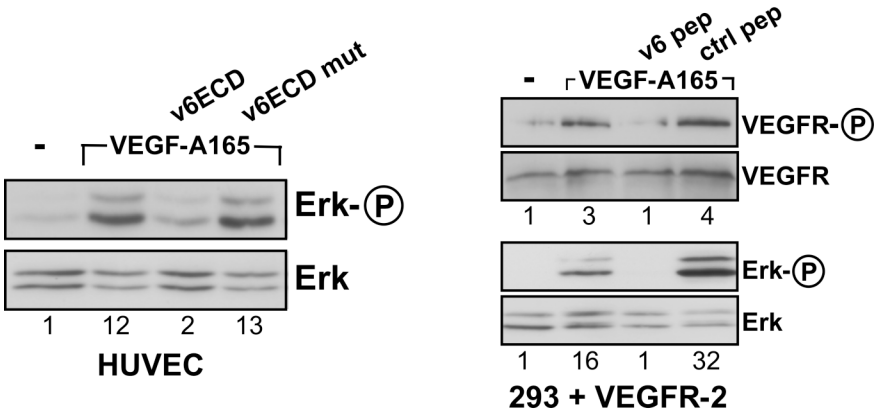

Figure 7: CD44v6 is important for VEGF-A-induced activation of VEGFR-2 and signalling to Erk. Left part: The experiment was performed as described in Figure 5 except that the cells were induced with VEGF-A165. Right part: 293 cells were transfected with an expression vector containing the human VEGFR-2 cDNA and serum starved. Pre-incubation with the CD44v6 peptide was performed prior to induction with VEGF-A165. Western blot analysis was performed with phospho-VEGFR-2 and phospho-Erk antibodies.

In addition a further cell system was used in order to test the role of CD44v6 for VEGFR-2 signalling. Recombinant human VEGFR-2 was introduced into the epithelial kidney cell line 293, which can be transiently transfected with high efficiency. As in ECs, VEGFR-2 and Erk were phosphorylated upon VEGF-A165 treatment and this activation was blocked in the presence of the CD44v6 peptide (Figure 7 right part).
In conclusion both receptor tyrosine kinases Met and VEGFR-2 need a CD44v6 isoform for activation by their ligands HGF and VEGF-A165 in ECs.

2.2 CD44 variant 6 alone is sufficient for activation of VEGFR-2 in endothelial cells

The three reagents CD44v6 antibody, CD44v6 peptide and CD44v6 ectodomain directly address the exon v6 sequence of CD44 and are able to block VEGFR-2 activation. But due to alternative splicing v6 can be combined with other variant exons to form other variant isoforms that might also be inhibited by the same reagents. Especially exon v3 of CD44 is interesting as it encodes the only sequence that can be heparan-sulphated (Bennett et al. 1995) and thereby can directly bind VEGF-A165 (Jones et al. 2000). Additionally VEGF-A165 requires heparin-like molecules on the cell surface in order to bind its receptor (Gitay-Goren et al. 1992). Thus a CD44v3 isoform containing also exon v6 might be the co-receptor for VEGFR-2.
An exon-specific RT-PCR analysis of HUVEC mRNA showed that several isoforms of CD44 are expressed in HUVECs (see Figure 8). The use of different forward primers that anneal to the variant exons of CD44 in combination with one common reverse primer that binds to an exon in the standard region give rise to DNA fragments of different sizes depending on the combination of the variant exons in the mRNA (see Figure 8). The ladder formation in the agarose gel indicates that the variant exons v6 to v10 are expressed in one protein. As the bands of variant 3 are not in a ladder together with the bands corresponding to variant 6, both variants are probably not expressed in one protein but rather in separate molecules. Thus it is very unlikely that CD44v3 is involved in the co-receptor function for VEGFR-2.

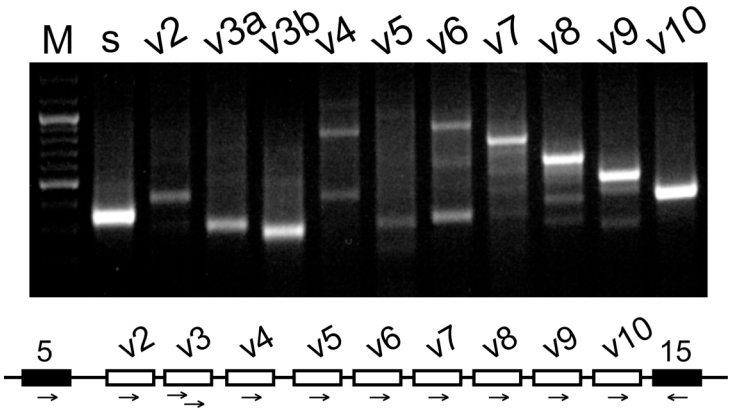

Figure 8: HUVECs express several variant isoforms of CD44, but variants 6 and 3 are expressed in separate molecules. cDNA was obtained by RT-PCR from total mRNA extractions. The cDNA was analysed by using exon-specific PCRs with different forward primers that anneal to sequences in the variant exons of CD44 together with a reverse primer that binds to the standard region of CD44. PCR products were separated via an agarose gel. Multiple signals per lane show that the specific exon is present in different CD44 isoforms. The bands that are organised in a ladder indicate that the corresponding exons are organised in one CD44 protein.

In order to see if the ligand itself needs to bind heparin to become bioactive, VEGF-A121, a shorter isoform of VEGF-A165 that does not contain the heparin binding domain encoded by exon 7, was used for EC stimulation. VEGF-A121 was able to activate VEGFR-2 similarly to the heparin binding isoform VEGF-A165 in ECs as well as in epithelial cells transfected with the VEGFR-2 expressing vector (Figure 9).

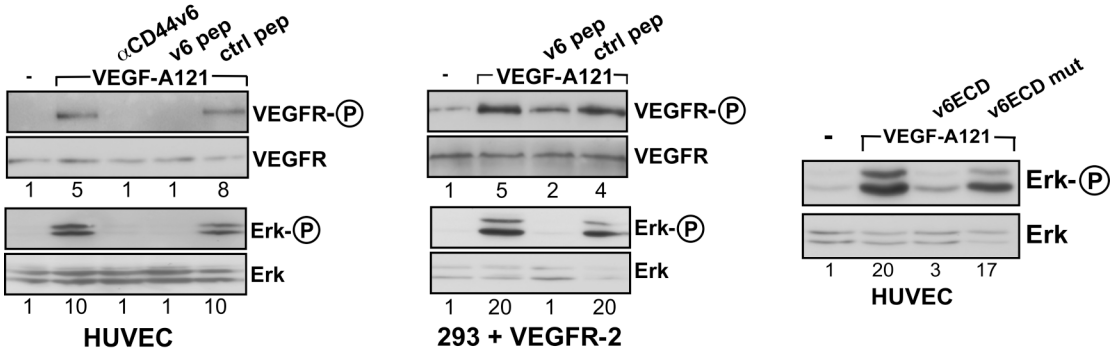

Figure 9: VEGF-A121 requires CD44v6 for activation of VEGFR-2 and signalling to Erk. Left and middle part: HUVECs or 293 cells transfected with an expression vector for VEGFR-2 were serum starved and incubated with CD44v6 antibody, CD44v6 peptide or a control peptide as indicated. After induction with VEGF-A121 the cells were treated as described in Figure 6. Right part: Incubation with the CD44v6 ectodomain (v6ECD) or the mutated CD44v6 ectodomain (v6ECD mut) before treatment with VEGF-A121 and further analysis was performed as described in Figure 5. The numbers refer to fold induction, calculated with the computer programme ImageJ.

This activation and signal transduction depended again on CD44v6 as it could be blocked with the CD44v6 antibody and the v6 peptide. Furthermore the soluble extracellular part of CD44v6 (v6ECD) was also able to interfere with VEGF-A121 induced signalling as it did after induction with VEGF-A165.

Finally, CD44v6 is sufficient for VEGFR-2 activation as demonstrated by transfection of non-inducible cells by a CD44v6 isoform. These cells became then inducible by VEGF-A165. For this experiment the pancreatic carcinoma cell line Bsp73AS (referred to as AS), that only expresses the smallest isoform CD44s, and its transfectant Bsp73ASs6 (referred to as ASs6), that in addition expresses a CD44 isoform containing exclusively variant exon v6, were used. After transient transfection with an expression vector encoding for VEGFR-2 both cell lines expressed VEGFR-2 (Figure 10). The AS cells showed a weak background tyrosine phosphorylation of the receptor that was not enhanced when treated with the ligand VEGF-A165. Although the VEGFR-2 in the CD44v6 expressing cells (ASs6) was phosphorylated stronger in absence of the ligand, there was a further increase of receptor activation after induction with VEGF-A165 (Figure 10).

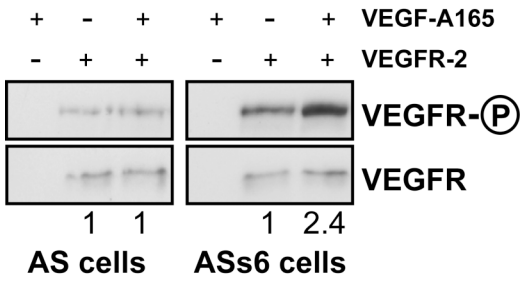

Figure 10: Exon v6 in the variant part of CD44 alone suffices for VEGFR-2 activation. Rat pancreas carcinoma cells expressing only CD44s (AS cells) or CD44v6 in addition to CD44s (ASs6) were transiently transfected with an expression vector coding for human VEGFR-2. Induction with VEGF-A165 and detection of activated VEGFR-2 was done as described in Figure 6. The numbers refer to fold induction, calculated with the computer programme ImageJ.

Thus a CD44v6 isoform containing exclusively exon v6 as a variant exon is able to fulfil the co-receptor function for activation and signal transduction of VEGFR-2 and no additional variant exons are needed.

As CD44v6 and VEGFR-2 cooperate in receptor activation and signalling, they should be in close proximity in the membrane. In order to test if they form a complex, a co-immunoprecipitation experiment was performed. Endogenous CD44 was pulled down from

HUVEC lysates either untreated or induced with VEGF-A165 and the precipitate was examined for the presence of VEGFR-2. CD44 and VEGFR-2 are indeed associated, even in absence as well as in presence of VEGF-A165. Both form a complex that appears to be constitutive and not inducible by ligand activation (see Figure 11). This is in contrast to the situation observed for CD44 and Met in epithelial cells were the complex formation of both proteins is triggered by the ligand HGF (Orian-Rousseau et al. 2002).

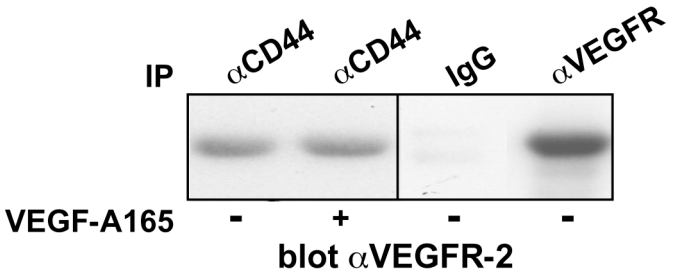

Figure 11: CD44 and VEGFR-2 form a complex. HUVEC cells were serum starved, induced with VEGF-A165 where indicated, and lysed. For protein pull down the lysates were incubated with antibodies recognising CD44, antibodies against VEGFR-2 as a positive control or an unrelated IgG antibody (negative control) and protein A/G agarose beads to pull down the antibody complexes. The antibody complexes were solubilised in sample buffer, separated by SDS-PAGE and blotted with an VEGFR-2 antibody.

2.3 The cytoplasmic domain of CD44 promotes signal transduction of activated VEGFR-2

So far the molecular mechanism of action of CD44v6 for VEGFR-2 is very similar to the situation observed for Met. However the contribution of CD44v6 to Met activation is not restricted to the extracellular domain. Also the intracellular part has a role and contributes to signal transduction (Orian-Rousseau et al. 2002). Indeed, CD44 has a binding site for ERM proteins (Legg and Isacke 1998) that are able to bind the actin cytoskeleton (Turunen et al. 1994). Thereby they serve as linkers that connect the cytoskeleton with the cell membrane. This connection is absolutely required for the HGF-dependent activation of Ras, which is a crucial step in the signalling cascade (Orian-Rousseau et al. 2007).

To investigate if VEGF-A signalling also needs the actin cytoskeleton linked to the membrane via CD44 and ERM proteins, two experiments were performed. Both approaches show that the linkage of the cytoskeleton to the cell membrane via ERM proteins and CD44 is necessary also for VEGFR-2 signalling.

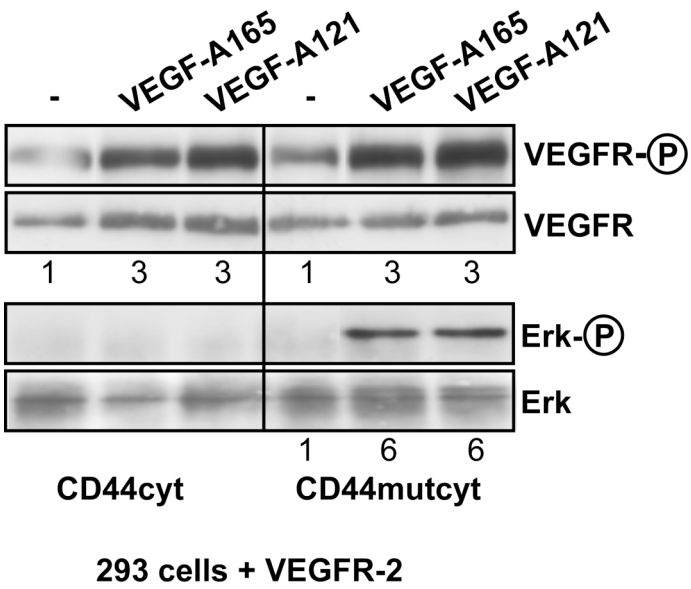

Figure 12: The cytoplasmic part of CD44 is involved in VEGF-A-induced signalling. 293 cells were transfected with an expression vector for VEGFR-2 together with a vector coding for a GST-fused cytoplasmic part of CD44 (CD44cyt) or a fusion protein in which the ERM binding region of CD44 is mutated (CD44mutcyt). The cells were serum starved and induced with either VEGF-A165 or VEGF-A121. The cell lysates were subjected to SDS-PAGE and Western blot analysis. VEGFR-2 and Erk activation were analysed as described in Figure 5. The numbers refer to fold induction, calculated with the computer programme ImageJ.

An expression vector coding for the cytoplasmic part of CD44 fused to GST (CD44cyt) was transiently introduced into 293 cells together with a vector coding for VEGFR-2. Overexpression of CD44cyt did not abrogate VEGFR-2 phosphorylation by its ligands VEGF-A165 or VEGF-A121 (Figure 12), but the signal transduction to Erk was strongly inhibited. The cytoplasmic domain seems to compete with the endogenous intracellular part of CD44 and sequesters proteins that are essential for signal transfer. Introduction of a cytoplasmic tail that is mutated in the binding site for ERM proteins (CD44mutcyt) was not able to interfere with signalling showing that the ERM proteins recruited to CD44 are important for cellular responses induced by VEGF-A.

What is the function of ERM proteins? This question was answered by utilising an expression vector coding for a dominant negative ezrin protein lacking the binding site for F-actin. Addressing just one of the ERM proteins is justified, as ezrin is the predominant ERM protein in 293 cells. Expression of this truncated ezrin resulted in a reduced phosphorylation of Erk upon induction with VEGF-A165 or VEGF-A121 (Figure 13).

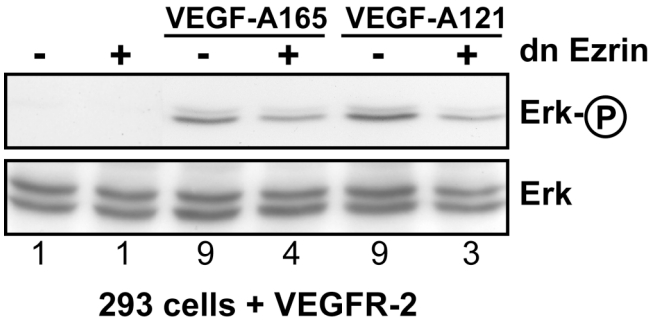

Figure 13: VEGF-A-induced signalling requires ezrin binding to the actin cytoskeleton. 293 cells were transfected with a vector encoding an ezrin protein that is not able to interact with the actin cytoskeleton (+) or an empty vector (-) in addition to VEGFR-2. After serum starvation, the cells were incubated with VEGF-A, lysed and proteins were analysed by SDS-PAGE (see Figure 4). The numbers indicate fold induction, calculated with the computer programme ImageJ.

These experiments show that signal transduction upon VEGFR-2 activation depends on the recruitment of the actin cytoskeleton to the cell membrane via CD44 and ERM proteins. Apparently both receptor tyrosine kinases, Met and VEGFR-2, share a common mechanism to transduce signals.

3 CD44v6 is required for physiological ligand-induced behaviour

The previous results reveal the important role of CD44v6 as a co-receptor for the two different receptor tyrosine kinases Met and VEGFR-2 on ECs. Both need CD44v6 for receptor activation induced by HGF or VEGF-A as well as for signal transduction. How relevant is the co-receptor function for ligand-induced cellular responses?

Several *in vitro* assays mimic angiogenesis and serve as models for the investigation of EC behaviour. Indeed, angiogenesis is characterised by different consecutive steps, and the contribution of CD44v6 to that process was analysed. At the beginning the basement membrane surrounding the existing vasculature is locally degraded in order to allow the ECs to dissociate from the united cell structure, sprout and form new branches. Proliferation and migration towards an angiogenic stimulus is followed by assembly and finally formation of a complete new capillary (Hanahan and Folkman 1996).

3.1 CD44v6 is involved in sprouting of endothelial cells

Early events in angiogenesis can be monitored in the spheroid sprouting assay. Clusters of HUVEC cells, so-called spheroids, were generated in hanging drops and embedded in a three-dimensional collagen matrix (Alajati et al. 2008). They were overlaid with medium containing the blocking substances and the growth factors. Activated by the growth factors VEGF-A165

and VEGF-A121 the ECs started to grow out of the spheroids (Figure 14). This ability to sprout in response to VEGF-A treatment was blocked completely when the cells were treated with the v6 peptide. The control peptide did not influence VEGF-A-induced sprouting. Sprouting of the ECs was quantified by analysing the total length of all sprouts originating from one spheroid.

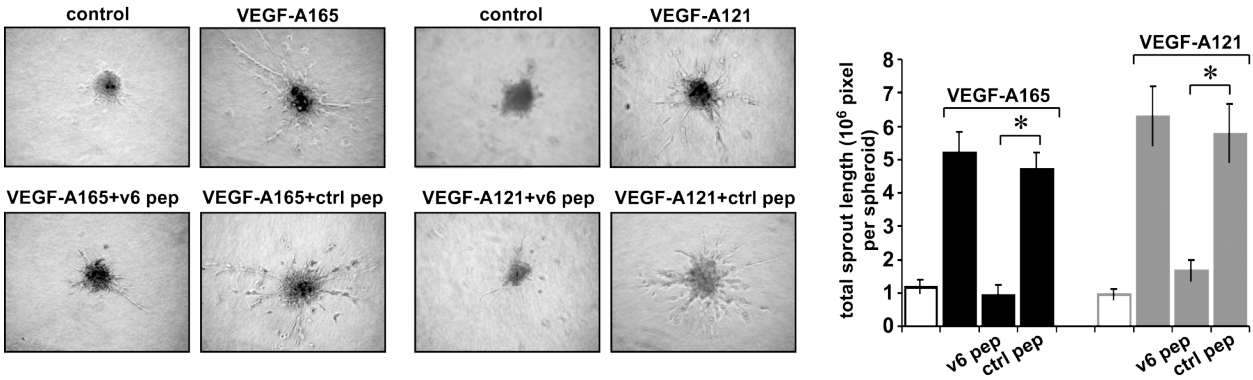

Figure 14: VEGF-A-induced spheroid sprouting depends on CD44v6. Spheroids of HUVECs were generated in hanging drops overnight, then collected by centrifugation and embedded in a collagen gel. Where indicated the spheroids were treated with CD44v6 peptide or control peptide before they were induced with VEGF-A165 or VEGF-A121. Pictures were taken after 48 hours and the total sprout length of the spheroids was quantified (right part). Standard deviation was calculated from three independent experiments and statistical analysis was performed by Student's t-test. $P < 0.05$ was considered as statistically significant and indicated by an asterisk.

Thus, sprouting from a cell aggregate induced by VEGF-A, an experiment that mimics the early steps of angiogenesis, requires CD44v6. The co-receptor function is therefore an important element of the physiological cellular behaviour.

3.2 Endothelial cell migration requires CD44v6

EC migration is another crucial step in the process of angiogenesis. The ECs leave the EC monolayer of existing blood vessels and migrate towards an angiogenic stimulus before they reassemble to a newly formed vessel. Angiogenic growth factors – amongst them HGF and VEGF-A – belong to these angiogenic attractants. HGF (reviewed by (Bardelli and Comoglio 1997; Birchmeier and Gherardi 1998)) as well as VEGF-A (Yoshida et al. 1996) contribute to cellular migration. Thus the requirement for CD44v6 for cellular migratory responses was tested.

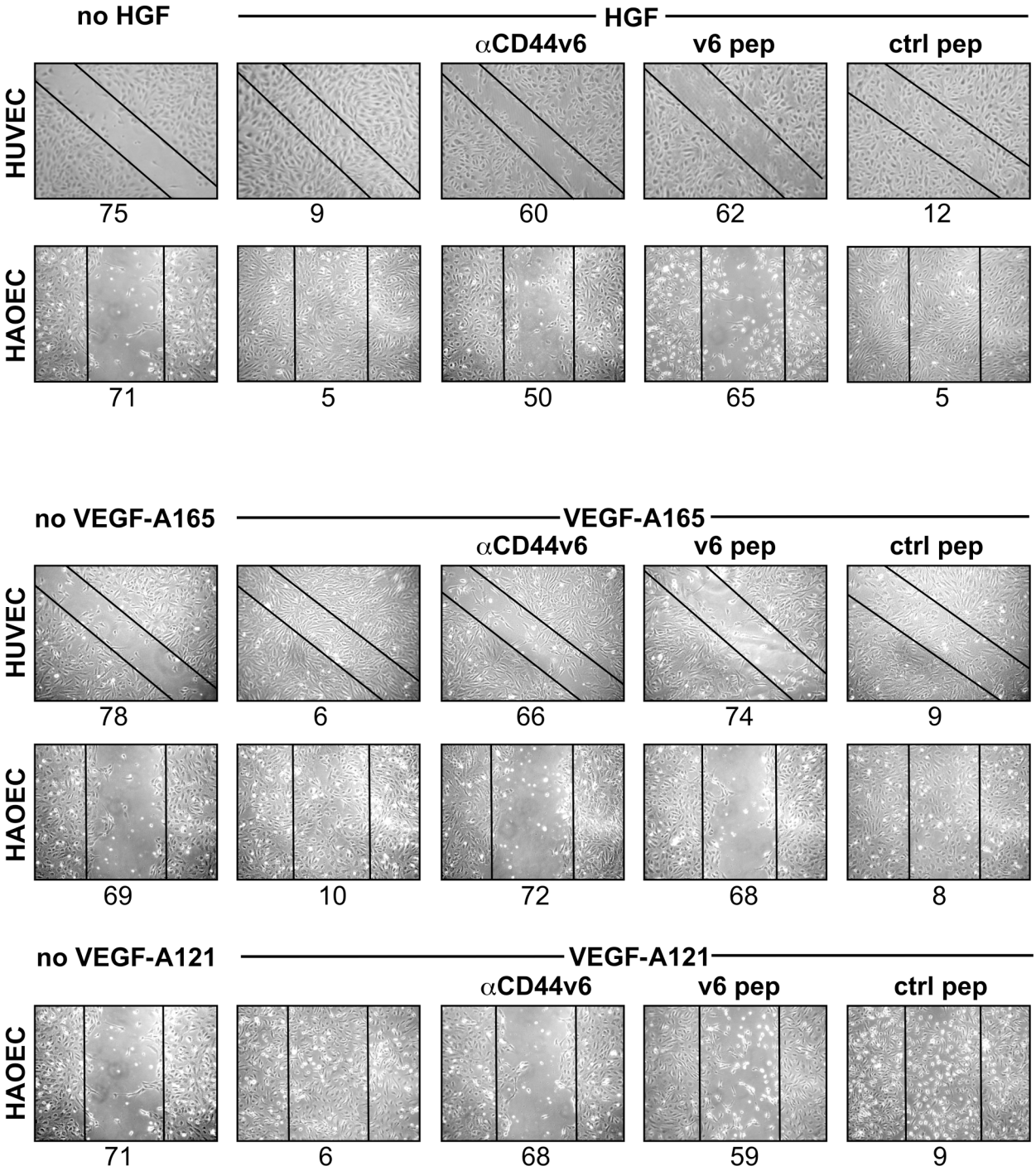

Figure 15: CD44v6 is required for EC migration. HUVECs and HAOECs were grown to confluence and a wound was made into the cell monolayer with a sterile pipette tip. After washing the cells were incubated with the antibody against CD44v6, the CD44v6 peptide or the control peptide as indicated. The ligands HGF, VEGF-A165 or VEGF-A121 were added and migration of the ECs was analysed after 24 hours. The numbers indicate the percentage of free area in the scratch area quantified by the computer program ImageJ.

In a scratch closure assay migration of either vein or artery ECs was tested. These cells were grown to confluence and a scratch was made into the confluent monolayer using a sterile pipette-

tip. The cells lining the scratch border started to migrate into this empty space in order to close the gap (Figure 15). In presence of either HGF, VEGF-A165, or VEGF-A121 the cells migrated faster than in absence of the ligands and the scratch was closed within one day. The EC migration was reduced to basal levels when the CD44v6 antibody or the CD44v6 peptide interfered with receptor activation (Figure 15).

To rule out that proliferation and not migration was involved in the scratch closure in this experiment, the growth factors and the blocking reagents were tested for their ability to influence proliferation of ECs. The substances alone or in combination were given to HUVEC cells and the incorporation of radioactive labelled thymidin was measured.

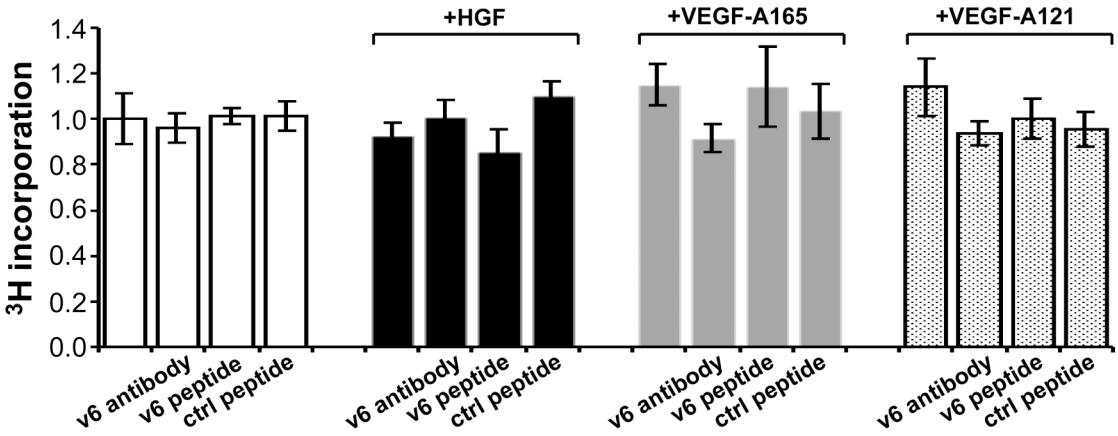

Figure 16: The ligands HGF, VEGF-A165 or VEGF-A121 do not induce EC proliferation. HUVECs were treated as described in Figure 15. 16 hours before the experiment was stopped tritium-labelled thymidin was added. The cells were washed, trypsinised and the incorporated radioactivity was measured with a liquid scintillation counter. Standard deviation was calculated from three independent experiments.

Neither the growth factors HGF, VEGF-A165 or VEGF-A121 nor the blocking reagents v6 peptide or CD44v6 antibody influenced EC proliferation in this time period of 24 hours (Figure 16). Thus the closure of the scratch was exclusively due to growth factor-induced EC migration. Both types of ECs used in this experiment required CD44v6 for this migration.

3.3 Tube formation of endothelial cells depends on CD44v6

Migration, reorganisation and assembly of ECs to new vessels is mimicked by the tube formation assay. There the ECs undergo morphological changes when they come in contact with components of the extracellular matrix in a three-dimensional gel. Within a few hours they

elongate and aggregate to form capillary like structures that contain a lumen (Kubota et al. 1988).

The extracellular matrix used in this experiment is produced by the Engelbreth-Holm-Swarm mouse sarcoma and resembles the basal membrane. It is called Matrigel and its main components are collagen IV, laminin, entactin and heparan sulphate proteoglycans, but the mixture also contains growth factors that are produced by this sarcoma.

In the experiments a Matrigel reduced in the growth factor content was used. However, when HUVECs were seeded on this Matrigel they already formed a tubular network showing that the Matrigel was not completely devoid of growth factors (see Figure 17). This tubular network was more branched and considerably more complex upon treatment with the growth factors HGF, VEGF-A165 or VEGF-A121. Again the co-receptor CD44v6 was needed for this particular cellular behaviour because the tube and network formation on top of Matrigel was completely inhibited in presence of the CD44v6 antibody or the CD44v6 peptide. CD44v6 contributed to the process of tube formation of ECs induced by HGF, VEGF-A165 and VEGF-A121.

For quantification of this assay the number of branching points as well as the total vessel length was determined. The quantification revealed that treatment with the CD44v6 antibody or CD44v6 peptide reduced the number of branching points at least by half compared to the systems treated with growth factor alone. Also the total vessel length was decreased to 45 to 60 % in presence of CD44v6 antibody or CD44v6 peptide.

All these *in vitro* assays mimicking various steps in the complex process of angiogenesis reveal the importance of CD44v6 for cellular responses following both HGF- and VEGF-A-induced signalling. The typical behaviour of ECs is impaired in presence of CD44v6 antibody or CD44v6 peptide. This indicates that CD44v6 promotes physiological features of ECs.

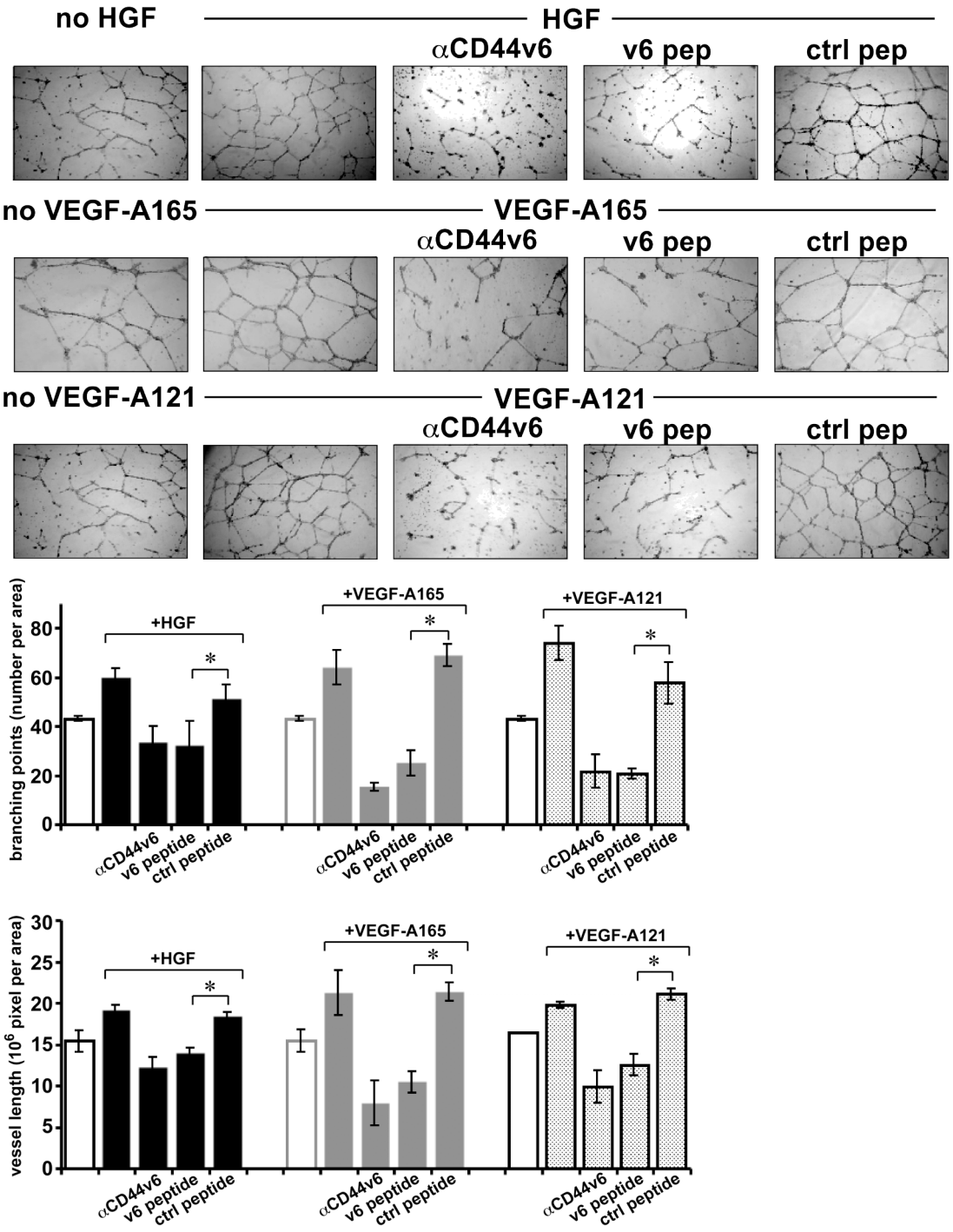

Figure 17: CD44v6 is required for EC tube formation on Matrigel. HUVEC cells were seeded on top of a Matrigel layer and incubated with CD44v6 antibody, CD44v6 peptide or control peptide as indicated. After 10 min HGF, VEGF-A165 or VEGF-A121 was added. Pictures were taken 24 hours later (upper part) and the total vessel length as well as the number of branching points was quantified with the computer program ImageJ (lower part). Standard deviation was calculated from three independent experiments and statistical analysis was performed by Student's t-test. $P < 0.05$ was considered as statistically significant and indicated with an asterisk.

4 CD44v6 containing isoforms play a role in angiogenesis *in vivo*

The previous data show that CD44v6 plays an important role in EC responses to the growth factors VEGF-A and HGF. The angiogenic process in a living organism however is more complex. There are several different pro- and antiangiogenic factors that tightly control angiogenesis (Folkman and Klagsbrun 1987). They act in concert to establish a functional blood vessel network.

In the following experiments it was tested whether CD44v6 blocking reagents have an effect *in vivo* when several angiogenic factors contribute to the new vessel formation. As CD44v6 is a co-receptor for HGF and VEGF-A, the influence will be observed only if the dominant signal is HGF or VEGF-A or both.

4.1 The response of endothelial cells to the stimulus of isolated angiogenic Langerhans islets depends on CD44v6

In order to get closer to an *in vivo* situation, the effects of the CD44v6 peptide and the CD44v6 antibody were tested in an *ex vivo* experiment using the RIP1Tag2 mouse model. These transgenic mice develop pancreatic β-cell tumours as they express an oncogene (simian virus 40 large T antigen: Tag) under the control of the rat insulin promoter 1 (RIP1) (Hanahan 1985; Alpert et al. 1988). At 4 to 5 weeks of age some of the Langerhans islets become hyperplastic and induce angiogenesis (Folkman et al. 1989) by production of angiogenic factors. Finally a few of them form carcinomas at around 12 weeks of age.

The angiogenic hyperplastic islets of Langerhans were isolated by collagenase perfusion. These isolated islets were able to stimulate an angiogenic response when co-cultured with HUVEC cells in a collagen matrix. Attracted by the mixture of angiogenic factors produced by the islets, the ECs started to migrate towards this stimulus and organised themselves into capillary like structures (Folkman et al. 1989). The angiogenic response of the ECs was reduced when the human CD44v6 of the ECs was blocked by means of the CD44v6 antibody or the v6 specific blocking peptide, whereas the control peptide did not interfere with the angiogenic response. In agreement with published data around 60 % of islets stimulated an angiogenic response in the control experiment (Gannon et al. 2002) (see Figure 18).

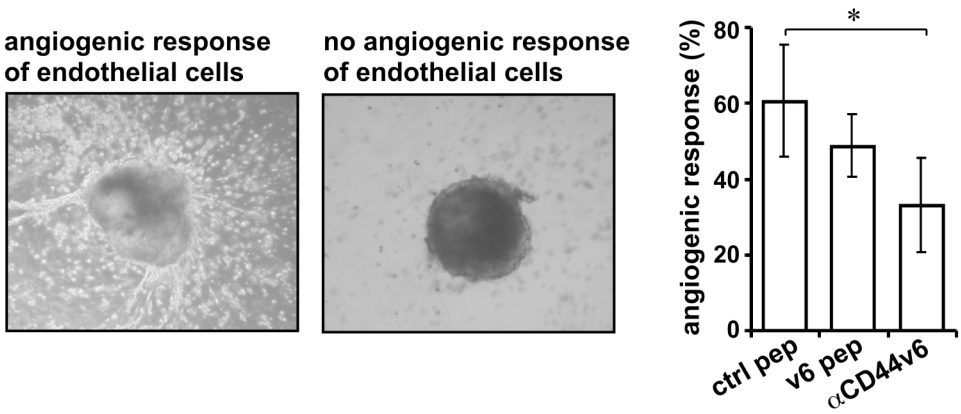

Figure 18: The response of HUVECs to a mixture of angiogenic factors produced by angiogenic Langerhans islets can be reduced by CD44v6 specific inhibitors. HUVEC cells were mixed with CD44v6 antibody, CD44v6 peptide or control peptide and then suspended in a collagen gel. Hyperplastic angiogenic Langerhans islets isolated from the pancreas of 8-9 week old RIP1Tag2 mice were placed on top of the HUVEC/collagen mixture and overlaid with medium. Every third day new antibody or peptide was added and pictures were taken after 5 to 7 days. The number of islets that induced an angiogenic response of the ECs is given as percentage of all analysed islets. For quantification 60 islets per condition were analysed. Both the CD44v6 antibody and the CD44v6 peptide reduced the angiogenic response, and the reduction obtained with the antibody is significant ($p < 0.05$).

This *ex vivo* experiment shows that blocking of CD44v6 affects the behaviour of ECs in situations where a combination of angiogenic growth factors stimulates ECs.

4.2 CD44v6 is required for the formation of a vascular network in a spheroid-based *in vivo* angiogenesis assay

Do ECs need CD44v6 for new vessel formation *in vivo*? This question was addressed at first in the spheroid-based *in vivo* angiogenesis assay. Spheroids of HUVEC cells embedded in a Matrigel/fibrin matrix together with the angiogenic growth factors FGF-2, VEGF-A165 or a combination of both can engineer a vasculature when grafted into the flank of SCID mice (Alajati et al. 2008). These growth factors induce the ECs in the spheroids to organise themselves into new blood vessels. VEGF-A165 induces the formation of a network with many sprouting ends that are not connected to the mouse vessels. FGF-2 is able to cause a network connected to mouse vessels that is covered with mural cells. When administered together both ligands show an additive effect (Alajati et al. 2008). Although the angiogenic stimulus used in this assay was injected in combination with the ECs, the natural environment of the plug with either activating or inhibiting substances influenced the vessel formation over a period of three weeks.

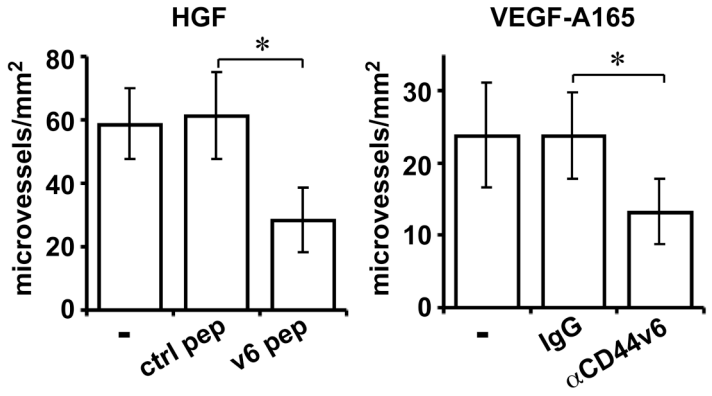

Figure 19: CD44v6 contributes to *in vivo* vessel formation of endothelial cell spheroids. Spheroids of HUVEC cells were generated as hanging drops overnight. The spheroids were collected by gentle centrifugation, mixed with CD44v6 antibody, CD44v6 peptide or the according controls and the growth factors HGF or VEGF-A165 and suspended in a matrix of Matrigel, EC growth medium and fibrinogen. Thrombin was added and the matrix was injected subcutaneously into the flank of SCID mice. Every other day the antibodies or peptides were injected in proximity to the Matrigel/fibrinogen plugs. The plugs were isolated after three weeks, fixed in 4 % formaldehyde and section were immunohistologically stained with an antibody against the EC marker CD34. For quantification five different plugs and at least three sections per plug were analysed. Significant reduction of the vessel number is indicated by an asterisk ($p < 0.05$).

HGF was used for the first time in this spheroid-based *in vivo* angiogenesis model to induce the formation of a new vasculature. Figure 19 shows that the ligand HGF was able to initiate the reorganisation of HUVEC cells within the Matrigel/fibrin plug. HGF even seemed to be more potent than VEGF-A165 in respect to the number of microvessels formed per plug. The reagents affecting CD44v6 on the ECs like the CD44v6 antibody or the CD44v6 peptide reduced the number of microvessels and impaired vascularisation mediated by HGF as well as by VEGF-A165.

4.3 CD44 is involved in tumour vascularisation

The previous experiments revealed the contribution of CD44v6 to angiogenesis in animals. However they were just partially done under physiologic conditions, e.g. the Langerhans islets that secrete a physiologic mixture of pro- and antiangiogenic factors were removed from the original tissue, isolated and cultured in cell culture dishes. The spheroid-based experiment was performed *in vivo*, but additional growth factors were added. In order to prove the importance of the co-receptor function of CD44v6 for angiogenesis under exclusive *in vivo* conditions, two orthotopic tumour models in mice were chosen that already had been used to study tumour-induced angiogenesis.

The oestrogen independent human breast cancer cell line MDA-MB231 (described in (Yano et al. 1992; Missbach-Guentner et al. 2008)) and the human pancreatic cell line L3.6pl (described in (Bruns et al. 1999)) were implanted orthotopically into the respective mouse tissue. After one week of tumour growth, the animals were split in groups and treated either with the CD44v6 antibody or the CD44v6 peptide or controls injected intraperitoneally three times per week. As the isoforms of CD44 differ in different species, the antibody and the peptide against CD44v6 act species specific. The reagents used in this experiment addressed mouse CD44v6, so they interfered with CD44v6 only in mouse tissue, but they did not directly affect the behaviour of the human tumour cells.

Interestingly the two cancer types respond differently to the treatment with anti-CD44v6 reagents.

The breast cancer cells (MDA-MB231) give rise to tumours with volumes varying from 125 mm^3 to 667 mm^3 after 4 weeks of treatment. The tumour sizes are not significantly changed in animals treated with CD44v6 blocking reagents in comparison to control-treated animals. However vascularisation of the tumours occurred faster in control-treated animals. Using the flat-panel detector volume computed tomography (fpVCT), all contrast agent containing blood vessels with a diameter of more than 150 µm can be visualised. Smaller vessels can be detected when they contain a high amount of contrast agent (Missbach-Guentner et al. 2008). The tumour vasculature in animals treated with control immunoglobulin was already well vascularised after a period of three weeks and the vessel network continuously grew until the end of the experiment (Figure 20). In contrast the tumours of the animals treated with CD44v6 antibody contained just a few blood vessels after a period of three weeks. The vessel number and size increased over time, however it was always clearly reduced in comparison to the IgG treated tumours (Figure 20).

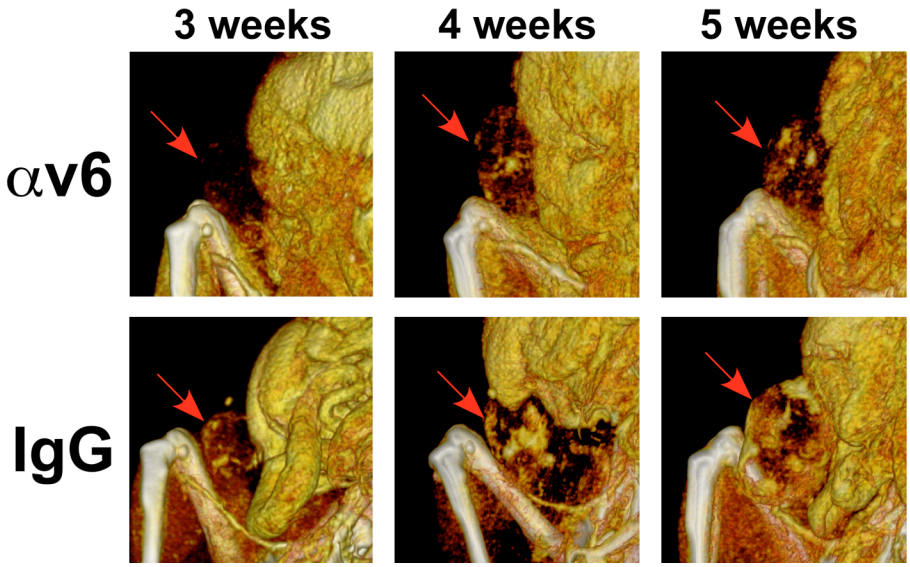

Figure 20: Treatment with the CD44v6 antibody reduces vascularisation of MDA-MB231 tumours. Breast cancer cells (MDA-MB231) were orthotopically implanted into the mammary fat pad of SCID mice. After one week of tumour growth, the animals were divided into two groups of six animals each and treated either with CD44v6 antibody or IgG control three times per week intraperitoneally. Tumour vascularisation was followed by pfVCT after injection of contrast agent into the tail vein.

These data obtained from the fpVCT cannot be used for quantification because the visualisation of the vessel depends on the distribution and the stability of the contrast agent. Therefore tumour sections were stained for the EC marker CD31 in order to quantify the vessel density.

	IgG	αCD44v6	ctrl peptide	v6 peptide
average vessel number (per mm^2)	**247.6**	**220.1**	**240.3**	**183.7**
stdev	22.5	20.1	24.4	31.7
significance		0.025		0.007
average vessel size (µm^2)	**192.9**	**203.9**	**207.8**	**211.4**
stdev	11.5	33.7	28.1	31.7

Figure 21: Blocking of CD44v6 reduces the microvessel density in MDA-MB231 tumours. Sections of tumours developed from MDA-MB321 breast cancer cells were stained for the EC marker CD31 and vessel number and vessel size were quantified with the computer program ImageJ. Five to six independent tumours were analysed and at least three sections per tumour were quantified. Statistical analysis was performed by Student's t-test. $P < 0.05$ was considered as statistically significant.

Analysis of vessel number confirmed that the microvessel density was significantly reduced in tumours of animals treated with CD44v6 antibody and peptide compared to control animals (Figure 21). Additional examination revealed that the vessel size was not influenced by anti-CD44v6 treatment.

Tumours of the pancreatic cancer cells (L3.6pl; the experiments were performed together with Alexandra Matzke) responded much more to treatment with CD44v6 peptide. After three weeks of treatment with CD44v6 peptide the tumour volume was drastically reduced compared to tumours treated with a control peptide or PBS (Figure 22, left part). Also the microvessel density as well as the vessel size was affected. Treatment with CD44v6 peptide reduced the number of blood vessels per mm^2 by more than 50 %, and the size of the single vessels is reduced to half in control treated animals (Figure 22, middle and right part).

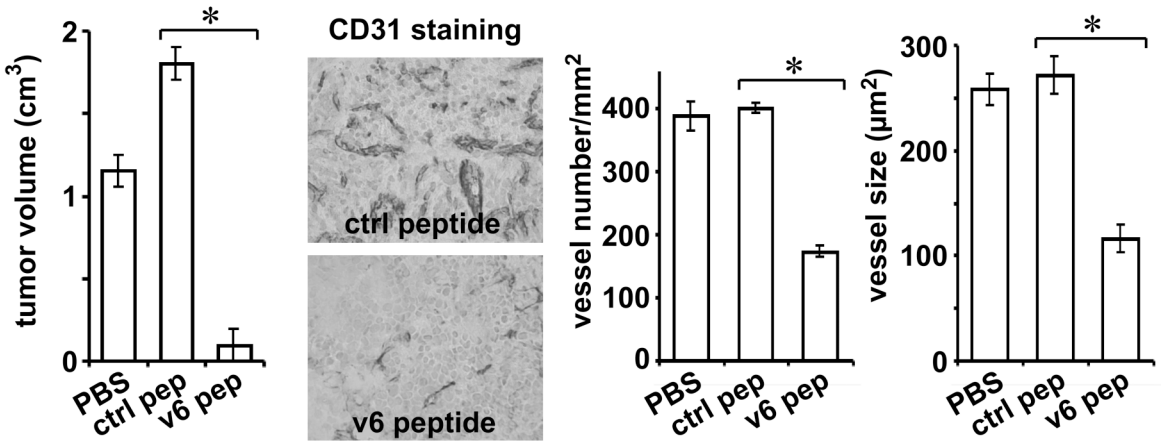

Figure 22: Treatment with the CD44v6 peptide blocks tumour growth and tumour vascularisation. Pancreatic cancer cells (L3.6pl) were orthotopically implanted into the tail of the pancreas of nude mice. After one week of tumour growth, the animals were divided into three groups of five animals each and treated either with CD44v6 peptide, control peptide or PBS three times per week intraperitoneally. After three weeks of treatment the tumours were extracted and tumour volume was measured with a calliper (left part). Additionally sections of the tumours were stained for the EC marker CD31 (middle part) and vessel number and vessel size were quantified with the computer program ImageJ (at least three sections per tumour) (right part). Statistical analysis was performed by Student's t-test. $P < 0.05$ was considered as statistically significant and indicated by an asterisk.

Both these orthotopic tumour models demonstrate that CD44v6 is involved in tumour angiogenesis *in vivo*. Depending on the tumour type, its contribution to tumour vascularisation and tumour growth differs.

In conclusion all experiments show that the receptor tyrosine kinases Met and VEGFR-2 need the co-receptor CD44v6 for their function in ECs. CD44v6 is required for receptor activation as well as signal transduction to Erk. When CD44v6 is blocked by means of the CD44v6 antibody or the CD44v6 peptide, ligand-induced EC migration, sprouting and tube formation is impaired and most importantly tumour angiogenesis *in vivo* is reduced.

DISCUSSION

This work shows that CD44 isoforms containing variant exon v6 are required for activation and signal transduction of the receptor tyrosine kinases Met and VEGFR-2 in several endothelial cells originating from different blood vessels (HUVECs, HAOECs and HCMECs). CD44v6 acts therefore as a co-receptor for both RTKs. An antibody directed against CD44v6, a CD44v6 peptide and a soluble ectodomain of CD44v6 are able to block receptor activation as well as downstream signalling, subsequently inhibiting ligand-induced migration, sprouting and tube formation. Most importantly these two reagents addressing CD44v6 also interfere with angiogenic processes *in vivo*. Indeed in two orthotopic tumour models performed in mice the microvessel density was significantly decreased in tumours that were treated with these two reagents.

For long it was believed that RTKs and their ligands work in an isolated manner: the ligands were thought to activate their respective receptor alone and induce all cellular responses. Now there is increasing evidence that additional molecules contribute to the receptor activation and even to signal transduction. Therefore the collaboration between RTKs and co-receptors is now accepted as a common principle (Orian-Rousseau and Ponta 2008). For example following RTK/co-receptor pairs are known to work together: EGFR and E-cadherin (Qian et al. 2004), FGFR and syndecans (Steinfeld et al. 1996), FGFR and NCAM (Francavilla et al. 2007), PDGFR and integrins (Woodard et al. 1998), TGF-b and syndecan-2 (Chen et al. 2004), VEGFR-2 and VE-cadherin (Carmeliet et al. 1999; Lampugnani et al. 2003; Lampugnani et al. 2006) and VEGFR-2 and neuropilin (Soker et al. 1998; Whitaker et al. 2001; Soker et al. 2002). Also for Met collaboration with several co-receptors has been shown, e.g. $\alpha 6\beta 4$ integrin (Rabinovitz and Mercurio 1996; Trusolino et al. 2001), plexinB1 (Giordano et al. 2002; Basile et al. 2005; Conrotto et al. 2005) and neuropilin (Sulpice et al. 2007). The best-characterised co-receptor for Met on epithelial cells is however CD44v6 (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007).

This work now shows that CD44v6 also acts as a co-receptor for Met on endothelial cells, and moreover, it is in addition a co-receptor for a second RTK, namely for VEGFR-2.

The collaboration between RTKs and co-receptors allows expanded and highly regulated receptor activation and cellular responses. For example Sema4D, a ligand for plexins, is able to induce Met-dependent signalling when Met and plexinB1 form a complex (Giordano et al.

2002). Therefore the collaboration of Met with the co-receptor plexinB1 broadens the spectrum of ligands that induce Met activation. In addition the intracellular part of a co-receptor can serve as a platform for signalling molecules. When Met is in a complex with the integrin α6β4, Met activation by its ligand HGF leads to phosphorylation of α6β4. Signalling molecules can bind both to the Met docking site and the phosphorylated co-receptor and strengthen and broaden signalling (Trusolino et al. 2001). The expression pattern of co-receptors in different cells can determine the tissue specificity of RTK activation and influence the cellular responses.

How can one single molecule, namely CD44v6, control activation and function of two different receptor tyrosine kinases involved in many important cellular processes like growth and motility? And even more strikingly, how can one small peptide (14 amino acids), addressing CD44v6, interfere with the function of two structurally different receptor tyrosine kinases and their two different ligands?

One explanation could be that the co-receptor directly binds the ligands in order to increase the ligand concentration in proximity of the respective receptor or present them to their receptor. This assumption is supported by the finding that rat pancreatic cancer cells that express Met but no CD44v6 (Bsp73AS) are not able to bind HGF, whereas the same cells stably transfected with CD44v6 (Bsp73ASs6) do bind HGF as measured in FACS analysis (Matzke 2006). Furthermore this binding of HGF to Bsp73AS cells can be blocked with the CD44v6 peptide (Matzke, unpublished data).

These data indicate that CD44v6 might bind HGF on cells. However, there is no doubt that *in vitro* purified Met directly binds its ligand HGF in the absence of CD44v6 (Park et al. 1994). How to explain this discrepancy? The situation on cells must differ from the conditions that exist in solution when purified proteins are used. One hypothesis is that the ligand/receptor complex *in vivo* needs to be stabilised by additional molecules, the co-receptors, allowing receptor dimerisation and autophosphorylation. This might explain why it has never been demonstrated that Met dimerises *in vitro* in the presence of HGF.

Another hypothesis is that the receptors are in a negative conformation in the cell membrane, possibly to prevent uncontrolled receptor activation. Such a mechanism has been suggested for the epidermal growth factor receptor (EGFR). EGFRs exist as pre-formed, but inactive dimers (Teramura et al. 2006). Association with the co-receptor might then enable ligand binding and receptor activation. In addition the co-receptors could also induce clustering of the receptors in order to promote RTK activation.

VEGF-A might also bind directly to the co-receptor CD44v6. This has to be tested in further experiments.

For the co-receptor function of CD44v6 the protein conformation seems to be important. This is concluded from experiments analysing the species specificity of CD44v6. The v6 peptides and v6 antibodies used in this work act in a species specific manner. This means that a mouse peptide/antibody only blocks mouse CD44v6, a human peptide/antibody blocks human CD44v6 and a rat peptide/antibody blocks rat CD44v6. In contrast, the CD44v6 ectodomain (v6ECD) homologue of the rat CD44v6 is able to interfere with mouse or human RTK activation, showing no species specificity. Therefore incubation of human cells with rat v6ECD inhibits HGF-induced activation of Met and downstream signalling (Orian-Rousseau et al. 2002). However, simultaneous treatment with a rat CD44v6 peptide released inhibition of the rat CD44v6 ectodomain. In addition a rat CD44v6 protein introduced into a human cell system is sufficient for Met activation by HGF (Matzke, unpublished). This suggests that the critical region in the v6 sequence, which is addressed by the v6 peptide and antibody, is not directly involved in interactions with either the ligands or the receptors, but rather that this sequence is required for the correct conformation of the total CD44v6 protein. Loss of the right conformation might prevent the protein to act as a co-receptor for Met and VEGFR-2. A change in the structural conformation might also explain how this peptide can interfere with two different ligand/receptor systems, if the binding of both ligands depend on the correct conformation.

The intracellular part of CD44 is necessary for signal transduction from both Met and VEGFR-2. After activation of Met in epithelial cells, the cytoplasmic domain of CD44 recruits ERM proteins that bind the actin cytoskeleton and thereby allow Ras activation by its GEF SOS (Orian-Rousseau et al. 2007). Similar events seem to regulate VEGFR-2 signalling, as ezrin, a member of the ERM proteins, also links the cytoskeleton to the membrane.

Also other RTKs profit from the cytoplasmic domains of their co-receptors (reviewed in (Orian-Rousseau and Ponta 2008)), e.g. the cytoplasmic domain of syndecan-4 is instrumental for fibroblast growth factor receptor 1 (FGFR-1) signalling induced by FGF-2 (Volk et al. 1999). The intracellular domain of syndecan-4 seems to recruit signalling molecules (Horowitz et al. 2002) and interact with the ERM proteins (Granes et al. 2000; Tkachenko et al. 2005). Interestingly many proteins that can act as co-receptors, such as syndecans and ICAMs, share similar features with CD44v6. Besides the fact that they are transmembrane molecules, their extracellular domains often interact with components of the ECM (McCourt et al. 1994; Entwistle et al. 1996), whereas the intracellular parts have been shown to associate with ERM proteins (Heiska et al. 1998; Granes et al. 2000). The collaboration of RTKs with co-receptors

broadens the number and variety of docking sites for adaptors and signalling molecules, so the fine-tuning of signalling is increased after receptor activation.

All blocking experiments in this work were performed with an antibody or a peptide directed against the exon v6 sequence of CD44. Therefore the co-receptor for Met and VEGFR-2 is most likely a CD44 isoform containing variant exon v6. But due to alternative splicing exon v6 can occur in various combinations with other variant exons on the cell. So the blocking reagents might influence sequences of other exons when expressed together with v6 in the same protein. Interesting are the CD44 isoforms expressing variant exon v3, as this is the only sequence that can be modified by heparan sulphate. And both ligands, HGF and VEGF-A165, are known to bind heparin (Ferrara and Henzel 1989; Lyon et al. 1994). Even more, CD44v3 isoforms can directly bind VEGF-A165 (Jones et al. 2000), and VEGF-A165 needs heparin-like molecules in order to potentiate the affinity to its receptor VEGFR-2 (Gitay-Goren et al. 1992). Is a CD44v3 sequence important for the co-receptor function? The sequences of the variant exons v3 and v6 seem not to be located on one CD44 protein and are rather expressed on independent molecules as it has been shown in an exon-specific analysis of EC mRNA. When CD44v6 is blocked, CD44v3 should not be affected. As inhibition of CD44v6 impairs RTK activation and signalling, a CD44v3 sequence seems not to be important for the co-receptor function. In addition experiments with a rat pancreatic carcinoma cell line expressing variant exon v6 as the only variant CD44 isoform (ASs6) showed that CD44v6 is sufficient for activation of VEGFR-2 by VEGF-A165 and CD44v3 and its heparan sulphate residues are not required for VEGFR-2 activation.

The induction of VEGFR-2 also occurs via VEGF-A121, a VEGF-A isoform that is not able to bind heparin, implying that VEGFR-2 activation itself is independent of heparin-like molecules. VEGF-A121 activates the VEGFR-2 and promotes signalling, and this activation can be blocked with the CD44v6 antibody and CD44v6 peptide. What is then the role of HS? It has been shown that the non-heparin binding VEGF-A121 is a less potent mitogen compared to VEGF-A165 (Keyt et al. 1996) and has a lower biological activity (Ogawa et al. 1998). Based on these data one can assume that the presence of heparin-like molecules is not necessary for activation of VEGFR-2 by its ligands per se, but they are required for the full VEGF-A-induced cellular responses.

Possible candidates that could provide the VEGF-A/VEGFR-2 system with heparin are neuropilins. It is known that they are able to bind heparin and VEGF-A165, and their presence enhances VEGF-A165 binding to VEGFR-2 (Soker et al. 1998; Mamluk et al. 2002). Huge

complexes involved in VEGFR-2 signalling containing several co-receptors and other proteins with different functions might exist. All of them contribute to the broad diversity and precise fine-tuning of the cellular responses induces by one single ligand.

An interesting case is the activation of Met by InternalinB (InlB), a ligand produced by *Listeria monocytogenes* (Niemann et al. 2007). The bacteria use InlB to activate Met on host cells and Met activation results in internalisation of the receptor. They make use of this internalisation process and enter the host cells together with Met. In this case, the presence of heparin is required for InlB-induced Met activation, as it induces Met clustering, and thereby allows internalisation of *L. monocytogenes* (Niemann et al. 2007). But here again a CD44v6 isoform as a co-receptor is sufficient for Met activation and internalisation, and the HS motif on CD44v3 is not involved in this process (Jung et al. 2009).

HGF as well is a growth factor that binds heparin and HSPGs, and it forms a trimeric complex together with heparin and Met (Lyon et al. 1994; Gherardi et al. 2003). Similar to the situation of VEGFR-2, HSPGs are not required for Met activation by HGF, as the removal of HA moieties on cell surface proteins by heparinase does not influence Met activation by HGF (Orian-Rousseau et al. 2002). In addition, an HGF mutant devoid of heparin binding sites is a better inducer of Met than wild type HGF (Gherardi et al. 2003).

This work and previous results (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007) highlight the important role of CD44v6 for activation and signal transduction of Met and VEGFR-2. These RTKs are both required for embryonic development, e.g. Met for placenta development, and VEGFR-2 for formation of a vasculature (Bladt et al. 1995; Shalaby et al. 1995; Uehara et al. 1995; Shalaby et al. 1997). This suggests that also CD44 is essential during embryogenesis. Surprisingly CD44 knockout mice develop normally. They are born at normal Mendelian ratio, they are fertile and they show no abnormalities in size or tissue architecture (Schmits et al. 1997). Only some minor defects like impaired homing of lymphocytes to lymph nodes or thymus and altered tissue distribution of myeloid progenitor cells can be observed (Schmits et al. 1997; Protin et al. 1999). Interestingly transgenic mice expressing a CD44 antisense cDNA under control of a keratin-5 gene promoter exhibit strong phenotypes (Kaya et al. 1997). From the time when this tissue specific promoter gets activated (E11.5), CD44 function is impaired in keratinocytes. This results in alterations of the skin structure, delayed wound healing and hair regrowth (see introduction chapter 5.4).

This contradiction between the mild phenotype observed in total knockout animals and the more severe phenotype in the transgenic animals suggests that the loss of CD44 can be compensated

during early stages of development, whereas the knock down at later time is not counterbalanced. Other genes might be differentially regulated in order to substitute for CD44 and overtake the functional role of CD44 isoforms. Such a substitution has been found in limb development. In wild type animals, CD44v3 is required for limb development as it binds the ligand FGF and presents it to its high-affinity receptor (Sherman et al. 1998). This function seems to be overtaken by other HSPGs in the CD44 knockout animals (Wainwright 1998).
Another study supports the existence of a substitute molecule. In genetic studies where $CD44^{-/-}$ and $Met^{+/-}$ mice were crossed, around 70 % of the progenies with a $CD44^{-/-}$ $Met^{+/-}$ genotype die shortly after birth due to a breathing defect. The synaptic transmission in the respiratory rhythm-generating network is impaired, and alterations in the phrenic nerve are found (Matzke et al. 2007). This haploinsufficiency of Met in the CD44 negative background leads to the conclusion that CD44 and Met collaborate *in vivo*. In addition, CD44 must be substituted in CD44 knockout mice, otherwise there would be a phenotype. This substitute protein is not as efficient as it is present in $CD44^{-/-}$ $Met^{+/-}$ mice and the animals yet die.
Finally, ICAM-1 acts as a substitute molecule for CD44 in CD44 null mice (Olaku et al, in preparation). This protein is a co-receptor for Met in liver regeneration in CD44 knockout mice, whereas CD44 fulfils this function in the wild type animals.

The relevance of CD44v6 for tumour angiogenesis *in vivo* is demonstrated by the effect of the CD44v6 antibody and CD44v6 peptide in two orthotopic tumour models. Both the human breast cancer cells MDA-MB231 and the human pancreatic cancer cells L3.6pl are known to give rise to well-vascularised tumours, and in both cases the microvessel density is significantly reduced when the animals are treated with CD44v6 antibody or CD44v6 peptide, the reduction in vessel number being more pronounced in the pancreatic tumours. Obviously the pancreatic tumours are more sensitive to the therapy. Whereas the size of the breast cancer tumours was not affected by CD44v6 blocking compared to control animals, the volume of the pancreatic tumours was drastically reduced when the CD44v6 peptide was injected. In addition the average size of the vessels in the tumours was decreased.
The different response of both tumour systems to the treatment regarding tumour growth might be based on intrinsic properties of different cell types. The tumour cells originate from different organs and might produce different proangiogenic factors. The growth factors PDGF and FGF for example activate their receptors independently of CD44v6 (Matzke et al. 2005 and own unpublised data). If tumour angiogenesis is mainly induced by such CD44v6-independent

factors, the blocking effect of CD44v6 antibody and CD44v6 peptide will be less drastic than in cases where tumour angiogenesis is primarily induced by HGF and VEGF-A.

Interestingly CD44v6 is not only involved in angiogenesis but also in tumour progression and metastasis formation. Metastasis formation of rat pancreatic cancer cells expressing CD44v4-7 can be inhibited by treatment with CD44v6 antibodies (Günthert et al. 1991), and introduction of CD44v6 into non-metastatic rat pancreatic cancer cells gives rise to a metastatic phenotype (Matzke, unpublished). Taken together these data and the data reported in this thesis imply that CD44v6 is required in ECs for new vessel formation as well as in tumour cells for invasive and metastatic properties. Consequently blocking of the co-receptor function of CD44v6 addresses both endothelial cells and tumour cells. On the one hand tumour vascularisation is impaired, resulting in a reduction of tumour size and a lower number of possible entry sites of metastatic cells into the blood circulation. On the other hand proliferation and migration of tumour cells is reduced and there is less invasion into surrounding tissues. Therefore reagents that inhibit the co-receptor function of CD44v6 might reduce tumour angiogenesis and metastasis.

Developing a therapy to cure cancer is the goal of numerous efforts. Targeting pathologic angiogenesis is a promising way, as most tumours have to recruit new blood vessels in order to get enough oxygen or to metastasise. An effective inhibition of tumour angiogenesis would prevent tumour growth of several different tumour types, and the malignancy of various tumours could be decreased by one treatment. In addition this antiangiogenic therapy could also be used for other diseases, such as age-related macular degeneration, diabetic retinopathy, psoriasis and arthritis, where hyper-vascularisation causes the symptoms.

So far various approaches were used to interfere with angiogenesis. The most prominent drug that is used in the clinic is bevazicumab (Avastin), a humanised monoclonal antibody that neutralises VEGF-A. Also the VEGFRs are targets of antiangiogenic drugs, e.g. sunitinib that inhibits the tyrosine phosphorylation of VEGFRs. Antiangiogenic drugs are used in combination with other anti-cancer therapies like chemotherapy or radiotherapy in order to increase the success of treatment. Unfortunately these treatments are not able to cure cancer, although they improve life quality and prolong the life span for a couple of month. Therefore further investigations are necessary and as many as possible targets should be addressed by the therapies.

Although antibodies have very high target specificity, their large size is a big disadvantage. Cell surface binding peptides are an interesting alternative to antibodies. They are small in size, their

synthesis is easy and cheap, and in addition they can be chemically modified in order to reduce proteolytical degradation (Ponta, unpublished). Depending on their size small one's might even bypass an immune response.

The CD44v6 peptide used in this work is a promising molecule for further studies in order to develop a new antiangiogenic therapy. Addressing one single protein, namely CD44v6, the peptide interferes with activation and function of at least two different strong activators of angiogenesis, Met and VEGFR-2. It reduces angiogenesis and tumour growth as well as the invasive properties of several cancer cells. This pronounced effect on tumour progression is the ideal prerequisite for a drug that might be used for therapy of many cancer types.

MATERIAL AND METHODS

1 Material

1.1 Chemicals and cell culture equipment

All chemicals used in this work were of highest purity grate. The water used for aqueous solutions was desalted.

2-Propanol	Merck, Darmstadt, Germany
Acetic acid	Merck, Darmstadt, Germany
Acrylamid/N,N'-Methylenbisacrylamid (37,5:1)	Roth, Karlsruhe, Germany
Agarose	Peqlab, Erlangen, Germany
Ampicillin sodium salt	Roth, Karlsruhe, Germany
Aprotinin	Sigma-Aldrich, Steinheim, Germany
APS (Ammonium peroxodisulphate)	Roth, Karlsruhe, Germany
Bromophenol blue	BioRad, München, Germany
BSA (bovine serum albumine)	PAA, Pasching, Austria
Calcium acetate	Roth, Karlsruhe, Germany
Collagenase	Roche, Mannheim, Germany
DMEM	Invitrogen, Karlsruhe, Germany
DMSO	Fluka, Neu-Ulm, Germany
DNAse	Sigma-Aldrich, Steinheim, Germany
dNTP	Peqlab, Erlangen, Germany
DTT (Dithiothreitol)	Roth, Karlsruhe, Germany
ECGM MV	Promocell, Heidelberg, Germany
ECGM	Promocell, Heidelberg, Germany
ECL western blotting reagents	Thermo Scientific, Rockford, IL
EDTA	Roth, Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidiumbromide	Roth, Karlsruhe, Germany
eXia 160	Binitio Biomedical, Ottawa, Canada
FCS (fetal calf serum)	PAA, Coelbe, Germany
Fluorescent mounting medium	Dako, Hamburg, Germany
Formalin	Roth, Karlsruhe, Germany
G418 (Neomycin)	Calbiochem, Bad Soden, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
Goat serum	Dako, Hamburg, Germany
Hepes	Roth, Karlsruhe, Germany
Hoechst dye 33258	Sigma-Aldrich, Hamburg, Germany
Hydrochloric acid fuming	Merck, Darmstadt, Germany
Hydrogen peroxide	Roth, Karlsruhe, Germany
Igepal	Sigma-Aldrich, Steinheim, Germany
Isovist 300 (contrast agent)	Bayer-Schering, Berlin, Germany
Leupeptin	Sigma-Aldrich, Steinheim, Germany
Kanamycin sulphate	Roth, Karlsruhe, Germany

Magnesium chloride hexahydrate	Roth, Karlsruhe, Germany
Methyl cellulose	Fluka, Buchs, Switzerland
Mounting medium (fluorescent)	Dako, Hamburg, Germany
Paraffin, Paraplast Bulk	McCormick Scientific, St. Louis, MO, USA
PBS	Invitrogen, Karlsruhe, Germany
Pen/Strep	Invitrogen, Karlsruhe, Germany
PMSF (Phenylmethylsulfonyl-fluoride)	Sigma-Aldrich, Steinheim, Germany
Proteinase K	Sigma-Aldrich, Taufkirchen, Germany
Protein G plus Agarose	Calbiochem, Bad Soden, Germany
Protein A Agarose	Calbiochem, Bad Soden, Germany
Rabbit serum	Dako, Hamburg, Germany
RMPI 1640	Invitrogen, Karlsruhe, Germany
SDS (Sodiumdodecylsulphate)	Roth, Karlsruhe, Germany
Sodium chloride	Roth, Karlsruhe, Germany
Sodium fluoride	Sigma-Aldrich, Steinheim, Germany
Sodium hydrogen carbonate	Roth, Karlsruhe, Germany
Sodium hydroxide	Roth, Karlsruhe, Germany
Sodium orthovanadate	Sigma-Aldrich, Steinheim, Germany
Taq polymerase	Fermentas, St. Leon-Rot, Germany
TEMED	Roth, Karlsruhe, Germany
Thermo Cycler MJ Research PTC-200 Peltier Thermal Cycler	Scientiffic Support, Hayward, CA, USA
^{3}H thymidine	Hartmann Analytic, Braunschweig, Germany
Tris base	Roth, Karlsruhe, Germany
Tris-HCl	Roth, Karlsruhe, Germany
Trypsin 0.25 %	Becton Dickinson, Heidelberg, Germany
Trypsin 0.04 %/EDTA 0.03 %	Promocell, Heidelberg, Germany
Trypton/Pepton	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany
Xylene	Merck, Darmstadt, Germany
Yeast extract	Roth, Karlsruhe, Germany
Zinc acetate	Roth, Karlsruhe, Germany
Zinc chloride	Roth, Karlsruhe, Germany

1.2 Hardware and consumables

Agarose gel electrophoresis chamber	Peqlab, Erlangen, Germany
Bacteria petri dishes	Greiner, Nürtingen, Germany
Cell harvester, 96 well	Tomtec, Hamden, CT, USA
Cell incubator	Thermo Scientific, Wilmington, DE, USA
Centrifuge Megafuge 1.0	Heraeus Instruments, Hanau, Germany
Volume computed tomography prototype flat-panel detector	GE Global Research, Niskayuna NY, USA
Cooling centrifugeJ2-HS	Beckmann, Stuttgart, Germany
Cryostatic vials	Corning Incorporated, Corning, NY, USA
Developer for X-Ray-films	Kodak, New Haven, CT, USA
Digital Camera Canon Power Shot	Canon, Krefeld, Germany
Eagle eye	Peqlab, Erlangen, Germany
ECL Hyperfilm	Amersham, Freiburg, Germany
Electrophoresis chambers	Hoefer, Holliston, MA, USA

Electroporation cuvettes	Peqlab, Erlangen, Germany
Electroporator Gene Pulser	BioRad, München, Germany
Freezer -80°C	New Brunswick, Edison, NJ, USA
Freezer -20°C	Liebherr, Ochsenhausen, Germany
Fridge	Siemens, Munich, Germany
Glass fibre filter	Wallac, Turku, Finland
Heatblock Thermomixer Comfort	Eppendorf, Hamburg, Germany
Reaction tubes 1,5 ml	Eppendorf, Hamburg, Germany
Immobilon-P (PVDF membrane)	Millipore, Bedford, USA
Liquid scintillation counter	Wallac, Turku, Finland
Microscope	Leica, Wetzlar, Germany
Microscope	Zeiss, Jena, Germany
Microscope, inverted	Olympus, Hamburg, Germany
Microtome	Leica, Wetzlar, Germany
NanoDrop Photometer	Thermo Scientific, Wilmington, DE, USA
Needles	Braun, Melsungen, Germany
Pipetboy Costar Stripettor	Corning, Tokyo, Japan
Pipettes	Gilson, Paris, France
Pipette tips	Stein Labortechnik, Remchingen, Germany
Power Supply	Hoefer, Holliston, MA, USA
Shaker	Fröbel Labortechnik, Lindau, Germany
Sterile filters	Roth, Karlsruhe, Germany
Syringes	Braun, Melsungen, Germany
Table-Centrifuge	Eppendorf, Hamburg, Germany
Tissue culture plastics	Greiner, Nürtingen, Germany
Tissue processor Hypercenter XP	Shandon, Frankfurt, Germany
Vicryl suture	Ethicon, Norderstedt, Germany
Vortex	Janke & Kunkel, Staufen, Germany
Westernblot chamber	BioRad, München, Germany
Whatman 3MM paper	Whatman, Part of GE Healthcare, Munich, Germany

1.3 Antibodies

1.3.1 Primary antibodies

Antibody	Isotype	Epitope	Company
CD31 (MEC 13.3)	rat monoclonal	mouse CD31	BD Biosciences, Heidelberg, Germany
CD34 (QBEND-10)	mouse monoclonal	human, cynomolgus monkey and rhesus monkey CD34	Novocastra, Newcastle upon Tyne, UK
CD44 pan (IM7)	rat monoclonal	mouse and human CD44	BD Biosciences, San Diego, CA
CD44v6 (Biwa)	mouse monoclonal	human CD44v6	Bender, Wien, Österreich
CD44v6 (9A4)	rat monoclonal	mouse CD44v6	Caltag Laboratories, Burlingame, CA

Erk-1 (K-23)	rabbit polyclonal	human, rat and mouse Erk1/2	Santa Cruz, Heidelberg, Germany
hMet (C-12)	mouse polyclonal	human Met	Santa Cruz, Heidelberg, Germany
Phospho-Met (Tyr1234/1235)	rabbit polyclonal	phosphorylated human, rat and mouse Met	Cell signalling, Beverly, England
phospho-p44/42 MAPK (Thr202/Tyr204)	rabbit polyclonal	phosphorylated human, rat and mouse Erk	Cell signalling, Beverly, England
Phospho-Tyrosine (4G10)	mouse monoclonal	phosphorylated tyrosine proteins	Upstate, Temekula, CA
Phospho-VEGFR-2 (Tyr1175)	rabbit monoclonal	phosphorylated human VEGFR-2	Cell signalling, Beverly, England
VEGFR-2 (A3)	mouse monoclonal	human, rat and mouse VEGFR-2	Santa Cruz, Heidelberg, Germany
Isotype control antibodies	mouse IgG rat IgG		NatuTec, Frankfurt, Germany

1.3.2 Secondary antibodies

goat-anti-rabbit-HRP — Dako, Hamburg, Germany
goat-anti-mouse-HPR — Dako, Hamburg, Germany
biotinylated rabbit-anti-rat IgG — Dako, Hamburg, Germany
goat-anti-mouse-Alexa 488 — Invitrogen, Karlsruhe, Germany

1.4 Animals and organisms

1.4.1 Animals

SCID (severe combined immunodeficiency) mice
strain C.B-17/IcrHanHsd-Prkdc-scid — Harlan, Horst, Netherlands

Nude mice
strain Hsd:Athymic Nude-Foxn1 nu/nu — Harlan, Horst, Netherlands

RIP1Tag2 mice
used in collaboration with Imke Albrecht and Gerhard Christofori, Department Biomedicine, University of Basel, Switzerland (Schomber et al. 2007)

1.4.2 Cell lines

Cell line	Description	Culture medium	Starving medium	Source
HUVEC	human umbilical vein	endothelial cell growth medium MV with supplements	endothelial cell growth medium	Promocell, Heidelberg, Germany; Provitro, Berlin, Germany
HAOEC	human aorta	endothelial cell growth medium MV with supplements	endothelial cell growth medium MV	Promocell, Heidelberg, Germany
HCMEC	human cardiac microvasculature	endothelial cell growth medium with supplements	endothelial cell growth medium MV	Promocell, Heidelberg, Germany
293	donkey kidney	DMEM + 10 % FCS	DMEM	American tissue culture collection, ATCC, Wesel, Germany
Bsp73AS (AS)	rat pancreatic carcinoma	RMPI 1640 + 10 % FCS	RMPI 1640	(Günthert et al. 1991)
Bsp73ASs6 (ASs6)	Bsp73AS cells transfected with CD44v6 (containing exon 15)	RMPI 1640 + 10 % FCS + G418	RMPI 1640	(Günthert et al. 1991)
MDA-MB231	human mammary carcinoma	DMEM + 10 % FCS		American tissue culture collection, ATCC, Wesel, Germany

1.4.3 Bacterial strain

E.coli DH5α:
Recombination defect suppressive strain for plasmid expression
Genotype: F- 80d*lac*Z M15 (*lac*ZYA*arg*F)U169*deo*R*rec*A1*end*A1*hsd*R17($r_K^- m_K^+$) phoA*sup*E44 thi-1*gyr*A96relA1

1.5 Kits

Qiagen Plasmid Maxi Kit	Qiagen, Hilden, Germany
Nucleospin RNA L purification kit	Macherey-Nagel, Düren, Germany
Biotin blocking system	Dako, Hamburg, Germany
StreptABComplex	Dako, Hamburg, Germany
DAB substrate kit	Biozol, Eching, Germany

1.6 Growth factors and special reagents

HGF
Gift from Vande Woude, Van Andel Institute, Grand Rapids MI, USA

VEGF-A165
Gift from Kurt Ballmer-Hofer, Paul Scherrer Institut, Villigen, Switzerland

VEGF-A121
Gift from Kurt Ballmer-Hofer, Paus Scherrer Institut, Villigen, Switzerland

ratCD44v6 ectodomain and rat CD44v6 ectodomain mutated in v6 region
Gift from Kurt Ballmer-Hofer, Paus Scherrer Institut, Villigen, Switzerland

Matrigel (growth factor reduced)	BD Biosciences, Heidelberg, Germany
Collagen	BD Biosciences, Heidelberg, Germany
Thrombin	Calbiochem, Beeston, UK
Fibrinogen	Calbiochem, Beeston, UK
PageRuler prestained protein ladder	Fermentas, St. Leon-Rot, Germany
GeneRuler DNA ladder mix	Fermentas, St. Leon-Rot, Germany

1.7 Peptides

human CD44v6 peptide: KEQWFGN**RWH**EGYR
NMI Peptides, Reutlingen, Germany

murine CD44v6 peptide: QETWFQN**GWQ**GKNP
NMI Peptides, Reutlingen, Germany

control peptide: HNREQANLNSRTEETI
Gift from Jonathan Sleeman, Forschungszentrum Karlsruhe, Germany

1.8 Plasmid constructs

human VEGFR-2 pBE
Cloned into pEGFP-C1 from Clontech, Mountain View, CA
Gift from Kurt Ballmer-Hofer, Paus Scherrer Institut Villigen, Switzerland

GST-CD44 wild type and mutant cytoplasmic tail
Cloned into pGEX-KG (Legg and Isacke 1998)
Gift from Clare Isacke, Breakthrough Breast Cancer Research Centre, London, UK

DN ezrin
The sequence of wt ezrin without the last 29 aa encoding for the actin binding site was cloned into pCB6 (Algrain et al. 1993).
Gift from Monique Arpin, Institut Pasteur Paris, France

2 Methods

2.1 Tissue culture methods

Cells were cultured under sterile conditions in a humidified (85 %) incubator with 5 % CO_2 at 37° C. All experiments were performed in a sterile clean bench. Adherent cells were passaged when they reached about 80 % confluency.

2.1.1 Passaging cells

Old growth medium was removed by aspiration and the cells were washed once with PBS. Trypsin containing solution (0.25 % trypsin) was added and the cells were incubated at 37° C until they started to detach from the plate. Trypsin-dependent digestion was stopped with serum containing medium, cells were collected by centrifugation, resuspended in fresh culturing medium and distributed in new tissue culture plates or flasks.

2.1.2 Freezing and thawing cells

Cells were trypsinised as described above and collected by centrifugation. They were resuspended in freezing medium (10 % DMSO in FCS) and transferred into cryostatic vials. The vials were slowly frozen in a isopropanol containing box at -80° C and transferred to liquid nitrogen.

Cell lines stored in liquid nitrogen were thawed rapidly in a waterbath (37° C) and immediately mixed with pre-heated growth medium. Cells were collected by centrifugation to remove freezing medium and transferred into tissue culture plates containing growth medium. ECs were directly transferred into tissue culture plates with pre-heated growth medium after thawing. The medium was replaced with fresh medium after 24 hours.

2.1.3 Seeding cells

Cells were trypsinised as described above, collected by centrifugation and resuspended in growth medium. 10 µl cell suspension were transferred into a Neubauer counting chamber and counted by using a bright field microscope to obtain the cell number per ml. The planned number of cells was mixed with culture medium and distributed in tissue culture pates for the experiments.

2.1.4 Transfecting 293 cells

293 cells were seeded at a concentration of 2×10^5 cells in 6-well-plates 24 hours before transfection. Cells were transfected with Lipofectamine 2000 according to the manufacturer's

protocol. To transfect one well, 10 µl Lipofectamine 2000 reagent were diluted in 250 µl DMEM and incubated for 5 min at room temperature. 4 µg of vector DNA were filled up to 250 µl with DMEM without serum and mixed with the Lipofectamine 2000 solution. The mixture was incubated for 20 min at room temperature (in all 500 µl per well). In the meantime old medium on the cells was removed and replaced by 1.5 ml fresh DMEM without serum. 500 µl of DNA-transfection reagent-mixture was applied to the cells before they were transferred to the incubator. After 6 hours incubation, medium was replaced again by pre-warmed growth medium containing serum. Cells were allowed to express the newly inserted protein for 48 hours before the experiments were started.

2.1.5 Transfecting Bsp73AS cells

The rat pancreatic carcinoma cells were transfected by electroporation. 3 x 10^6 cells were mixed with 5 µg of vector DNA on ice and transferred into a 4-mm electroporation cuvette. Electroporation was performed at 250 F, 0.28 kV. Pre-warmed medium containing serum was added to the cuvette and the cells were distributed into 6 wells of a 6-well plate. Following the transfection the cells were grown for 24 hours then starved for additional 24 hours before they were used for the experiments.

2.1.6 Activation of Receptor Tyrosine Kinases and downstream target Erk

Cells were seeded at a concentration of 1.5 x 10^5 in 6-well plates. After 24 hours the growth medium was replaced by basal medium without serum to remove all growth factors that are components of the serum. After a starvation period of 24 hours, the cells were activated with the growth factors HGF (20 ng/ml) at 37° C for 5 min and with VEGF-A165 and VEGF-A121 (40 ng/ml) at 37° C for 8 min. When indicated, the cells were pre-treated with blocking reagents directly before growth factor treatment. The CD44v6 specific antibody (100 µg/ml), the CD44v6 peptide (100 ng/ml) and the CD44v6 ectodomain (0.5 µg/ml) (or according controls) were added in serum free medium for 10 min at 37° C followed by induction with growth factors as described above. The cells were washed with cold PBS before cell lysis.

2.1.7 Co-immunoprecipitation

For co-immunoprecipitation 1.5 x 10^6 HUVECs were seeded in 10 cm plates, serum starved for 24 hours and induced with 50 ng/ml VEGF-A165 for 8 min at 37° C. After induction, the cells were washed with cold PBS and lysed in 1 ml lysis buffer for 30 min on ice. The cells were transferred into reaction tubes and cleared by centrifugation (10000 rpm, 20 min, 4° C). 5 µg of a

pan CD44 antibody were added to the supernatant (containing 1000 µg total protein) and mixed over night at 4° C. Immunoprecipitation was performed with agarose beads (mixture of protein A and protein G) for 2 hours at 4° C. The mixture was centrifuged at 10000 rpm for 2 min at 4° C and the precipitate was washed 3 times with 500 µl lysis buffer. The remaining pellet was dissolved in 25 µl SDS lysis buffer to separate the proteins from the beads and boiled for 5 min to denature the proteins and the proteins were subjected to Western blot analysis.

Lysis buffer: 25 mM Hepes pH 7.5; 100 mM NaCl; 10 mM $MgCl_2$; 1 mM EDTA; 10 % Glycerol; 1 % Igepal; 10 mM NaF; 1 mM PMSF; 1 mM Na-orthovanadate; 1 mM aprotinin; 1 mM leupeptin

SDS lysis buffer: 125 mM TrisHCl pH 6.8; 4 % SDS; 20 % Glycerol; 0.01 % bromophenol blue; 100 mM DTT

2.2 Protein methods

2.2.1 Protein extraction and cell lysis

In order to determine protein activation by Western blot, cells were washed with cold PBS and lysed in SDS lysis buffer. Cells were transferred to a reaction tube and DNA was sheared through a 24 gauge needle to reduce viscosity and allow better protein separation. Denaturation of proteins was obtained by the presence of DTT in the lysis buffer, which destroys the disulfide bonds and boiling the samples for 5 min. The samples were spun down for 1 min at 12000 rpm before loading on a SDS-Page gel.

SDS lysis buffer: 125 mM TrisHCl pH 6.8; 4 % SDS; 20 % Glycerol; 0.01 % bromophenol blue; 100 mM DTT

2.2.2 Separation of proteins via SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins can be separated electrophoretically on the basis of their size. The detergent SDS (sodium dodecyl sulphate) equalizes the charge of the protein by forming aggregates with the positive charged structures. All proteins migrate to the positive pole of an electric field. The gel is composed of two parts. In the stacking gel (5 % acrylamide) the proteins are focussed whereas they are separated by their size in the resolving gel (8-10 % acrylamide). Two vertical glass plates with a distinct distance according to the size of the spacers serve as a mold. The components of the resolving gel are thoroughly mixed, poured into that mold and overlaid with 70 % ethanol. After polymerization the ethanol is removed and the stacking gel is filled into the mold. A comb is inserted to get spaces for loading the samples. The gel is put into an

electrophoresis chamber, the reservoirs are filled with running buffer and the protein samples are loaded on the gel. The electrophoretic separation occurs at a voltage of 100-130 V.

Running buffer: 25 mM Tris; 192 mM Glycine, 0.1% SDS

Stacking gel: 5 % acrylamide; 0.1 % SDS; 0.1 % APS; 0.1 % TEMED; 0.125 M Tris pH 6.8

Resolving gel: 8-10 % acrylamide; 0.1 % SDS; 0.1 % APS; 0.06 % TEMED; 0.375 M Tris pH 8.8

2.2.3 Western blotting

The proteins separated by SDS-PAGE were electrophoretically transferred to a PVDF (Polyvinylidene difluoride) membrane in transfer buffer at 34 V for at least 6 hours at 4° C. The PVDF membrane was activated with Methanol for 1 min and equilibrated in transfer buffer. The acrylamide gel was placed onto the PVDF membrane and air bubbles were removed. Both were embedded in three layers of Whatman paper soaked in transfer buffer, placed into a cassette and put into the transfer chamber filled with transfer buffer. The proteins migrate to the membrane in direction of the positive electric pole.

Proteins were detected by immunoblotting. Unspecific binding of antibodies was blocked with 5 % BSA in TBS-T buffer for one hour at room temperature. The membrane was incubated with primary antibodies diluted in blocking buffer at concentrations recommended by the supplier for 3 hours at room temperature or over night at 4° C. After washing 3 times for 5 min each in TBS-T buffer the membrane was treated with secondary antibodies coupled to horseradish peroxidase (HRP) for 1 hour at room temperature. The membrane was washed again 3 times in TBS-T buffer. Detection of specific protein signals was achieved using enhanced chemiluminescence (ECL) western blotting detection reagents and ECL hyperfilms as described in the manufacturer's instructions. In order to reprobe the membrane with different antibodies, the membrane was incubated in stripping solution for 45 min at 50° C and treated again as described above.

Transfer buffer: 20 mM Tris; 192 mM Glycine; 10 % Methanol

TBS-T buffer: 20 mM Tris; 140 mM NaCl; 0.2 % Tween 20; pH 7.6

Stripping buffer: 62.5 mM TrisHCl pH 6.8; 2 % SDS; 5 mM DTT

2.3 DNA methods

2.3.1 RNA isolation and cDNA synthesis

5 x 10^6 HUVEC cells were lysed and RNA was isolated and purified with the Nucleospin RNA L kit according to the manufacturer's protocol. RNA concentration was measured with a NanoDrop photometer. For denaturation 2 µg RNA was diluted to 40 µl with RNase free water and incubated at 70° C for 3 min, then quickly cooled down in ice water. This 2 µg RNA was subjected to reverse transcription, which was performed with 20 U of AMV reverse transcriptase in 80 µl reactions containing 80 U RNasein, 400 ng of oligo $d(T)_{18}$ primer and nucleotides. Reactions were incubated for 45 min at 41° C followed by heat inactivation of the reverse transcriptase at 70° C for 15 min. The cDNA was stored at -80° C.

2.3.2 Exon-specific RT-PCR

The exon-specific RT-PCR was done as described before (König et al. 1996). Different sequence specific oligonucleotides recognizing sequences in the mRNA of exon 5 and all variant exons of CD44 (all together 11 forward primers) were used separately with one reverse primer complementary to the mRNA of exon 15. DNA fragments were amplified with the thermo-stable Taq-DNA polymerase in a Thermo-Cycler and afterwards loaded on a 1 % agarose gel containing ethidium bromide.

PCR mixture:

cDNA template	5 µl of reverse transcriptase reaction
5 x PCR buffer	6 µl
dNTPs (10 mM)	0.5 µl
forward primer	10 pmol per primer
reverse primer	10 pmol
Taq polymerase (1 U/µl)	0.25 µl
H_2O	to 30 µl

PCR program:

1 cycle:	1 min	95° C
36 cycles:	30 sec	95° C
	1 min	50° C
	2 min	72° C
1 cycle:	5 min	75° C
1 cycle:	hold at 4° C	

2.3.3 Separation of DNA fragments via agarose gel electrophoresis

Nucleotides carry a negative charge according to the phosphate residues. In an electric field they move towards the positive charged anode and are separated by size. In order to build a gel the

agarose was mixed with TAE buffer and dissolved by boiling. After cooling down ethidium bromide was added and the solution was poured into an electrophoresis chamber equipped with a comb where it solidified. The gel was overlaid with TAE buffer and the DNA samples mixed with 10 x DNA loading buffer were loaded into the pockets. The separation was performed at 80 to 100 V. DNA can be visualized by UV light because the ethidium bromide intercalates into the DNA double helix.

TAE buffer: 40 mM Tris pH 8.3; 2 mM EDTA; 40 mM sodium acetate

10 x DNA loading buffer: 15 % Ficoll 400; 0.5 mM EDTA pH 8; 0.25 % bromophenol blue; 0.25 % xylene cyanol

2.4 In-vitro experiments

2.4.1 Wound closure assay

This assay mimics the ability of cells to migrate into a wound after injury. HUVECs and HAOECs were seeded in 12-well plates at a concentration of 2.5 x 10^5 cells per well. After 24 hours the cells form a confluent monolayer in which a scratch is inserted using a sterile pipette tip. Medium was aspirated to remove scratched cells and replaced by new growth medium containing blocking reagents as indicated (αCD44v6 100 µg/ml, v6 peptide 100 ng/ml, ctrl peptide 100 ng/ml). After an incubation time of 10 min at 37° C growth factors were added to induce migration (HGF 20 ng/ml, VEGF-A165 40 ng/ml, VEGF-A121 40 ng/ml). Pictures of the cells were taken 24 hours after induction using a Canon Power Shot S620 digital camera. The computer program ImageJ was used for quantitative evaluation. An area in the wound was defined and the cell free area was measured. The efficiency of wound closure is represented as percentage of cell free area.

2.4.2 Thymidine incorporation

HUVEC cells were seeded in 96 well plates at a concentration of 10^4 cells per well. The next day the cells were treated with blocking reagents (100 µg ml CD44v6 antibody, 100 ng/ml v6 peptide and control peptide). After 10 min incubation at 30° C the growth factors were added (HGF 20 ng/ml, VEGF-A165 40 ng/ml, VEGF-A121 40 ng/ml) for 24 hours at 37° C. 16 hours before the experiment was stopped the tritium-labelled thymidine (^{3}H thymidine) was added to each well at a concentration of 1 µCi per well. At the end the cells were washed twice with PBS and detached from the plate with 50 µl trypsin (40 min at 37° C). In order to detect the incorporated

radioactivity the cells were transferred to a glass fibre filter with a cell harvester and analyzed with a liquid scintillation counter.

2.4.3 Spheroid sprouting

Spheroids were formed to investigate the behaviour and competences of EC aggregates in a three-dimensional environment. Spheroids were generated in hanging drops. HUVECs were suspended in growth medium containing 0.25 % (wt/vol) methylcellulose at a concentration of 3 x 10^4 cells per ml. Drops of 25 µl (containing 750 cells each) were put on cell culture plastics and incubated up side down in the incubator at 37° C over night that the cells form one spheroid per drop. Spheroids were rinsed off the plate with 10 % FCS in PBS and collected by gentle centrifugation (5 min. 800 rpm). 48-well plates were coated with 100 µl of growth medium containing 1 mg/ml rat tail collagen and 0.6 % (wt/vol) methylcellulose at 37° C for 20 min. Spheroids were resuspended in the same mixture (30 spheroids per 400 µl) on ice and distributed in the wells to get 30 spheroids per well. After solidification of the collagen at 37° C for 30 min, the matrix was overlaid with 250 µl starving medium containing blocking reagents as indicated (αCD44v6 100 µg/ml, v6 peptide 100 ng/ml, ctrl peptide 100 ng/ml calculated to complete well content) and induced by VEGFA-165 and VEGF-A121 (40 ng/ml). Pictures were taken after 48 hours and sprout length was determined using the computer program ImageJ.

Methylcellulose stock solution: 1.2 % methyl cellulose in EC growth medium

2.4.4 Tube formation on Matrigel

Matrigel is a mixture of extracellular matrix components and very viscous. It starts to solidify at room temperature, therefore it was kept on ice as long as possible and pipetted with chilled pipette tips. Wells of a 48-well plate were coated with 150 µl of growth factor reduced Matrigel mixed with ice cold HUVEC starving medium in equal parts and incubated at 37° C for 30 min. HUVECs were seeded on these Matrigel coated plates at a concentration of 2.5 x 10^4 cells per well in 500 µl starving medium. Where indicated blocking reagents were added (αCD44v6 100 µg/ml, v6 peptide 100 ng/ml, ctrl peptide 100 ng/ml calculated to complete well content) and after 10 min at 37° C the growth factors were added additionally (HGF 20 ng/ml, VEGF-A165 40 ng/ml, VEGF-A121 40 ng/ml). Pictures were taken after 24 hours. The quantification was performed by counting the branching points and the total vessel length per field using the computer program ImageJ.

2.5 Experiments with animals

2.5.1 Islet culturing assay

Isolation of hyperplastic Langerhans islets from Rip1Tag2 mice at the age of 8 to 9 weeks was done by pancreas perfusion with a collagenase/DNase solution (0.3 units/µl collagenase type IV and 10 µg/ml DNase I in HBSS Hank's buffered salt solution) as described previously (Folkman et al. 1989). Mice were sacrificed, the entry of bile duct into duodenum was closed with a fine clamp and perfusion of the pancreas via the bile duct with a bent 30 gauge needle was started. Approximately 2 to 3 ml of collagenase solution was applied with mild pressure. The pancreas was isolated, chopped with small scissors and incubated at 37° C for 45 min. Digested pancreas was resuspended in RMPI 1640 containing 10 % FCS and 20 mM Hepes. Langerhans islets were picked under a dark field illumination microscope.

HUVECs were suspended in RMPI 1640 containing 10 % FCS and 20 mM Hepes (2×10^5 cells per ml) and mixed on ice with a bovine collagen solution (2.4 mg/ml Vitrogen®, completed with 10 mM NaOH and 0.075 % $NaHCO_3$ to allow solidification and Glutamine in RMPI 1640) in equal parts. Blocking reagents (αCD44v6 100 µg/ml, v6 peptide 100 ng/ml, ctrl peptide 100 ng/ml) were added as indicated and the mixture was quickly transferred into 24-well plates (350 µl per well, 3.5×10^4 cells per well). The isolated Langerhans islets were put on top of this three-dimensional collagen matrix (4 to 5 per well) and co-cultured with HUVECs. Every third day new blocking reagents were added. After 5 to 7 days the response of ECs to the angiogenic islets was determined. Approximately 60 islets per condition were analyzed.

2.5.2 Spheroid-based *in vivo* angiogenesis assay

This assay was done as described previously (Alajati et al. 2008). HUVECs were suspended in growth medium containing 0.25 % (wt/vol) methylcellulose at a concentration of 4×10^3 cells per ml. Drops of 25 µl (containing 100 cells each) were put on cell culture plastics and incubated up side down in the incubator at 37° C over night that the cells form one spheroid per drop. Spheroids were rinsed off the plate with 10 % FCS in PBS, washed once with EC growth medium and collected by gentle centrifugation (5 min 800 rpm). The spheroids were resuspended in a matrix made of EC basal medium and Matrigel (growth factor reduced) in equal parts and fibrinogen at a final concentration of 2 mg/ml on ice. The growth factors HGF or VEGF-A165 were mixed with this matrix at a concentration of 500 ng/ml. Where stated, the mixture also contained the blocking reagents αCD44v6 or CD44v6 peptide (20 µg/ml each). Thrombin (0.4 U) was added and the matrix was injected subcutaneously on each side lateral to

the abdominal midline region into 4-6 weeks old SCID mice. All in all, one plug consists of 600 µl matrix containing 500 spheroids.

Every second day mice were treated with the blocking reagents αCD44v6 or CD44v6 peptide (20 µg per plug) by injection in proximity to the Matrigel/fibrinogen plugs. Mice were sacrificed 21 days after implantation by cervical dislocation and constructs were retrieved and fixed over night in 4 % formaldehyde for immunohistological analysis.

Methylcellulose stock solution: 1.2 % methyl cellulose in EC growth medium

2.5.3 Orthotopic mouse tumour model

In this experiment the human mammary carcinoma cells MDA-MB231 were implanted orthotopically into the mammary fat pad of female SCID mice. 1 x 10^6 cells were harvested from subconfluency, washed with sterile PBS and resuspended in 50 µl of PBS. Female SCID mice were anesthetized with 75 mg/kg ketamine hydrochloride and 15 mg/kg xylazine by peritoneal injection. The cells were implanted with a 29 gauge syringe very slowly into the mammary fat pad of the fourth mammary complex and the needle was removed carefully with a delay of 1 min. The incision was closed using an interrupted Vicryl suture for the skin. After tumour growth for one week, the animals were split in groups (6 to 7 animals per group) and treated with 20 µg of anti-mouse CD44v6 (clone 9A4), control IgG, mouse v6 peptide or control peptide three times per week by intra-peritoneal injection. Tumour growth was monitored once per week using a calliper or the flat-panel detector volume computed tomography (see below). The tumour volume measured by calliper was calculated as 0.5 x length x width x height. Five weeks after tumour cell implantation the animals were sacrificed by cervical dislocation, the tumours were excised, externally measured, divided in halves and fixed in 4 % formalin or zinc fixative for 24 hours for immunohistological analysis.

The human pancreatic cancer cells L3.6pl were injected orthotopically as described previously into male nude mice (Bruns et al. 1999). In brief, a small left abdominal flank incision was made and the spleen was exteriorised. 5 x 10^5 cells in 40 µl Hanks buffered salt solution were injected into the subcapsular region of the pancreas just beneath the spleen using a 30 gauge needle. An appearing fluid bleb was considered as a sign for successful subcapsular intra-pancreatic injection. One week after injection of the tumour cells, mice were randomly assigned to groups of 5 animals each and treated with PBS, control peptide (20 µg) or CD44v6 peptide (20 µg) three times per week (intraperitoneal injection). Three weeks after initiation of the treatment the tumours were isolated and tumour volume was calculated as 0.5 x length x width x height. The tissues were processed as described above.

Zink fixative: 0.5 g/l calcium acetate, 5 g/l zinc acetate, 5 g/l zinc chloride in 0.1 M Tris pH 7.4

2.6 Detection methods

2.6.1 Flat-panel detector volume computed tomography imaging

The tumour growth was monitored with a non clinical volume computed tomography prototype flat-panel detector as described previously (Missbach-Guentner et al. 2008). The mice were placed perpendicularly to the z-axis of the system after anaesthesia with 0.8-1 % vaporized isoflurane. 150 µl of the iodine-containing contrast agent Isovist 300 were applied intravenously into the tail vein of the mice 30 sec before scanning. For better demonstration of small blood vessels, Isovist 300 was replaced by the blood pool agent eXia 160 that was used 90 sec before the scan at the day of dissection. All data sets were acquired with the same protocol: 500 views per rotation, 4 sec rotation time, 360 used detector rows, tube voltage of 80 kVp and a current of 100 mA. A modified Feldkamp algorithm was used for image reconstruction resulting in isotropic high-resolution volume data sets (512 x 512 matrix, with an isotropic voxel size of approximately 100 µm). For tumour segmentation and volume estimation data sets were analyzed with voxtools 3.0.64 Advantage Workstation 4.2 (GE Healthcare, UK).

2.6.2 Immunohistological analysis

The Matrigel/fibrin plugs and the tumour tissues were processed in a tissue processor and embedded in paraffin. 7 µm sections of paraffin blocks were deparaffinised and dehydrated.

In order to block unspecific binding, the Matrigel/fibrin plug sections were incubated with 10 % goat serum in PBS for 60 min prior to the staining with the antibody against human CD34 (clone QBEND/10, 20 µg/ml in blocking solution, 2 hours). The sections were washed three times with TBS-T washing buffer and incubated with the goat-anti-mouse Alexa Fluor 388 secondary antibody (10 µg/ml) for 45 min. Nuclei were counterstained with Hoechst dye 33258 for 5 min at a concentration of 5 µg/ml after washing with TBS-T washing buffer for three times. The sections were mounted with fluorescent mounting medium, dried over night and analyzed with an Olympus IX50 inverted microscope. Fluorescent structures were manually counted, referred to the complete matrix area and calculated as vessel number per mm^2 with the computer program cell^F.

In the tumour sections the antigens were retrieved with 8 µg/ml Proteinase K in TE buffer for 10 min at 37° C, washed three times with PBS and peroxidases were inactivated with 3 % H_2O_2 for 12 min. Endogenous biotin was blocked with the biotin blocking system according to the

manufacturer's instructions (10 min avidin solution, washing, 10 min biotin solution, washing). Before the sections were incubated with the primary antibody against mouse CD31 (0.5 µg/ml in blocking solution) over night at 4° C, unspecific binding was blocked with 10 % rabbit serum in PBS for 1 hour. The next day the sections were washed three times with PBS and the biotinylated rabbit-anti-rat antibody was added at a concentration of 2 µg/ml for 45 min followed by three washing steps and treatment (30 min) with a streptavidin-peroxidase conjugate (StreptABComplex) as described in the manual. The sections were developed with DAB substrate system (3,3'-diaminobenzidine), dehydrated and mounted with a xylene soluble mounting medium. With the computer program ImageJ all stained structures were analyzed for vessel number per mm^2 and average vessel size.

TBS-T washing buffer: 10 mM Tris pH 7.5; 100 mM NaCl; 0.1 % Tween 20

TE buffer: 10 mM Tris; 1 mM EDTA; pH 8

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