The ligand-specific co-receptor function of CD44 for receptor tyrosine kinases

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Zusammenfassung

Listeria monocytogenes, ein gram-positives Bakterium, verursacht die Krankheit Listeriose. Eine Möglichkeit, wie *L.monocytogenes* Wirbeltierzellen infizieren kann, ist das Binden des Bakteriums an die Rezeportyrosinkinase (RTK) Met auf der Wirtszelle durch das bakterielle Protein InlB. Dieses Binden führt zur Aktivierung von Met und schließlich zur Aufnahme in die Zelle. Der erste Teil meine Doktorarbeit zeigt, dass die Infektion von nicht-phagozytotischen Zellen mittels InlB zusätzlich vom Ko-Rezeptor CD44v6 abhängig ist. Desweiteren kann diese bakterielle Infektion mit einem CD44v6-Peptid blockiert werden.

Zusätzlich zu der Ko-Rezeptorfunktion von CD44v6 für InlB und Met, die ich gezeigt habe, wurde CD44v6 bereits als Ko-Rezeptor für die Induktion von Met und VEGFR-2 durch ihre authentischen Liganden HGF and VEGF-A identifiziert. Im zweiten und Hauptteil meiner Doktorarbeit habe ich untersucht, ob diese Ko-Rezeptorfunktion von CD44v6 spezifisch von den Liganden, den Rezeptoren oder beiden bestimmt wird. Dazu habe ich den ErbB1-Rezeptor verwendet, der von sieben strukturell völlig unterschiedlichen Wachstumsfaktoren angesprochen werden kann.

Ich zeige, dass die Ko-Rezeptorfunktion von individuellen CD44-Isoformen Liganden-spezifisch ist. Durch Peptidblockierung, siRNA- und Überexpressionsexperimente konnte ich demonstrieren, dass die Aktivierung von ErbB1 durch TGF-α völlig unabhängig von CD44 abläuft. EGF und ER benötigen CD44v6, wohingegen HB-EGF von CD44v3 für eine ErbB1-Aktivierung abhängt. Die cytoplasmatische Domäne von CD44 bindet an ERM Proteine wie Ezrin, Radixin und Moesin. Ezrin-Hemmung oder Überexpression zeigte, dass die ErbB1-Liganden auf das ERM-Protein Ezrin angewiesen sind, um die Signaltransduktion vom aktivierten Rezeptor in das Zellinnere zu ermöglichen.

Interessanterweise ist CD44v6 auch essentiell für die Aktivierung der ErbB-Rezeptoren in Brustkrebszellen, in denen die Aktivierung der ErbB Rezeptoren den Transformationsprozess vermittelt. In diesen Zellen zeigte ein Ko-Immunopräzipitationsexperiment, dass ErbB1 und CD44v6 nach Induktion durch EGF einen Komplex bilden.

Zusammenfassend zeigen meine Ergebnisse, dass verschiedene CD44-Isoformen mit RTKs in liganden-spezifischer Art und Weise zusammenarbeiten. CD44-

Isoformen müssen spezifische Funktionen zur Verfügung stellen, die nützlich für die Aktivierung von RTKs sind. Dies könnte die Vielfalt der CD44-Proteinfamilie erklären.

Abstract

Listeria monocytogenes, a gram positive bacterium, is the causative agent for the disease listeriosis. One way that *L.monocytogenes* uses to infect cells is the binding of the bacterium to the receptor tyrosine kinase (RTK) Met present on host cells through the bacterial protein InlB. This binding leads to activation of Met and finally to the uptake into the cell. The first part of my PhD work shows that the bacterial infection of non-phagocytic cells via InlB is in addition dependent on the co-receptor CD44v6. Furthermore, bacterial infection can be blocked by a CD44v6 peptide.

In addition to the co-receptor function of CD44v6 for InlB and Met that I demonstrated, CD44v6 has been previously identified as the co-receptor for the induction of Met and VEGFR-2 by their authentic ligands HGF and VEGF-A. In the second and main part of my PhD I analysed whether this co-receptor function of CD44v6 is specifically determined by the ligands, the receptors or both. To this end, I have used the ErbB1 receptor that can be addressed by seven structurally different ligands. I showed that the co-receptor function of distinct CD44 isoforms for ErbB1 is ligand-specific. By means of blocking experiments using the CD44v6 peptide or siRNA and overexpression experiments I could demonstrate that the activation of ErbB1 by TGF-α is completely independent of CD44. EGF and ER require CD44v6, whereas HB-EGF relies on CD44v3 for the activation of ErbB1. The CD44 cytoplasmic domain can bind to ERM proteins (the main representatives are Ezrin, Radixin and Moiesin). Ezrin downregulation and overexpression experiments revealed that all the ErbB1 ligands, including TGF-a which is independent of CD44 isoforms, depend on the ERM-protein ezrin and its binding to the cytoskeleton for signal transduction.

Interestingly, the co-receptor function of CD44v6 is also instrumental for the activation of the ErbB receptors in breast cancer cells, in which these receptors mediate the transformation process. In these cells, a co-immunoprecipitation revealed that upon EGF-induction, ErbB1 and CD44v6 are found in a complex together.

Altogether, my results indicate that several CD44 isoforms collaborate with RTKs in a ligands specific fashion. They must be able to provide specific functions that are

useful for the activation of RTKs. This might explain the diversity the CD44 family of proteins.

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Abbreviations

AR	Amphiregulin
BC	Betacellulin
CAM	cellular adhesion molecule
CD44	Cluster Of Differentiation 44
CD44s	CD44 standard
ECM	extracellular matrix
EGF	Epidermal Growth Factor
ER	Epiregulin
FGF	Fibroblast Growth Factor
FGF-R	Fibroblast Growth Factor Receptor
HA	Hyaluronan
HB-EGF	Heparin-binding EGF-like Growth Factor
HGF	Hepatocyte Growth Factor
InlB	Internalin B
Met	Mesenchymal-epithelial Transition Factor
NRG-1	Neuregulin-1
PDGF	Platelet-derived Growth Factor
RTK	receptor tyrosine kinase
TGF-α	Transforming Growth Factor Alpha
v	variant
VEGF	Vascular Endothelial Growth Factor
VEGF-R	Vascular Endothelial Growth Factor Receptor

1. Introduction

1.1 Prologue

In order to communicate with their environment and to transfer signals to the intracellular machinery, cells are dependent on proteins such as receptor tyrosine kinases (RTKs), present on their cell surface (reviewed in Schlessinger, 2000). These proteins recognize signals that regulate differentiation, migration, proliferation or apoptosis, functions that are essential in almost all physiological processes a cell takes part in. The signal received by cell surface receptors is in most instances given by a ligand that binds to these receptors and activates them. For RTKs, ligand binding usually induces dimerization and trans-phosphorylation of the cytoplasmic part of the receptor. The tyrosine kinase of the RTK phosphorylates tyrosine residues in its cytoplasmic tail that can then serve as docking sites for numerous signaling molecules (reviewed in Schlessinger, 2000).

It has long been believed that for the activation of RTKs, only binding of their ligands is necessary. Yet this idea turned out to be too simplistic in most cases. In the activation process of a specific RTK, a co-receptor is often needed as an auxiliary molecule. Cell adhesion molecules (CAMs) provide diverse functions to the RTKs that span from ligand presentation to the induction or amplification of signaling.

The collaboration of RTKs with CAMs increases the diversity of responses that can be achieved by the receptors under physiological conditions. However, CAMs also have pathological functions as they are involved in a variety of cancers (reviewed in Cavallaro & Christofori, 2004). CAMs play a role in a wide variety of cellular functions such as signal transduction, intercellular adhesion and apoptosis (reviewed in Juliano, 2002) that are oftentimes deregulated in cancer. They are in particular involved in the metastatic process, the step responsible for the death of the cancer patients. One of the earliest successful screens for metastasis-associated genes has identified members of the CD44 family of proteins (Gunthert et al, 1991).

1.2 The CD44 family of proteins

1.2.1 Introduction

Cell-cell as well as cell-matrix interactions play important roles in physiological processes like differentiation, migration and growth but also in pathological processes such as inflammation or metastasis. A group of proteins that play a key role in these instances is the CD44 family of cell adhesion molecules. CD44 was first discovered in 1980 as an antigen recognized by a monoclonal antibody that was raised against human leukocytes (Dalchau et al, 1980). It was then described as granulocyte-T lymphocyte antigen. Other members of the CD44 family with molecular weigths ranging from 80-200 kDa were independently described by different groups using methods such as cloning of CD44 cDNA and subsequent Northern hybridization or genomic Southern blot analyses. The different groups gave CD44 several names such as class III extracellular matrix receptor (ECM III) (Carter & Wayner, 1988), Hermes (Goldstein et al, 1989), HUTCH-1 (Gallatin et al, 1989), HCAM (Goldstein & Butcher, 1990) and phagocyte glycoprotein 1 (Pgp-1) (Hughes et al, 1983).

1.2.2. The CD44 gene

All CD44 proteins are encoded by one single gene that consists of 20 exons (Screaton et al, 1993; Tolg et al, 1993). This gene codes for 3 regions corresponding to the extracellular part (exon 1-17), the hyprophobic transmembrane domain (exon 18) and the intracellular domain (exon 19 and 20) of CD44. The exons 1-5 and 16-20 are present in all isoforms of CD44 and are therefore called the constant exons.

In addition to exon 1 - 5, the DNA that encodes the extracellular domain of CD44 comprises 10 alternatively spliced exons (exons 6-15) that have been designated as variant exons v1 (exon 6) – v10 (exon 15) (**Illustration 1**). Due to a stop codon in the human v1 exon only the exons v2-10 are expressed in human cells. Additionally a second splice acceptor site is present in exon v3, that also allows the synthesis of two different v3 splice variants (Screaton et al, 1993; Screaton et al, 1992; Tolg et al, 1993).

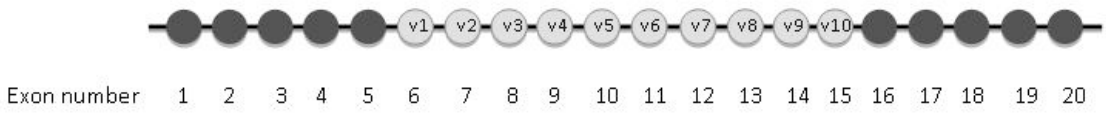

Illustration 1: Schematic representation of the CD44 gene. Exons 5-15 are subjected to alternative splicing and are also known as variant (v) exons v1-10

The differential splicing of the 10 variant exons in theory makes the expression of 768 different CD44 isoforms possible (van Weering et al, 1993). So far at least 20 different CD44 transcripts have been identified (reviewed in Naor et al, 1997a).

1.2.3 The expression of CD44

CD44 standard (CD44s) is the smallest isoform of CD44. It does not contain any variant exon and is ubiquitously expressed in vertebrates in developing and adult organisms (Brown et al, 1991; Stamenkovic et al, 1989). In contrast, the expression of CD44 variants is restricted to few cell types such as activated immune cells, monocytes, dendritic cells, proliferating epithelial cells and keratinocytes (Arch et al, 1992; Lesley et al, 1993; Stamenkovic et al, 1991). An isoform of CD44 that includes the variant exons v8-10 (also designated as CD44E or epithelial CD44) for example is preferentially expressed on epithelial cells (Stamenkovic et al, 1991) whereas the largest isoform of CD44 containing the exons v2-10 is expressed on human keratinocytes (Bloor et al, 2001; Hofmann et al, 1991) (**Illustration 2**).

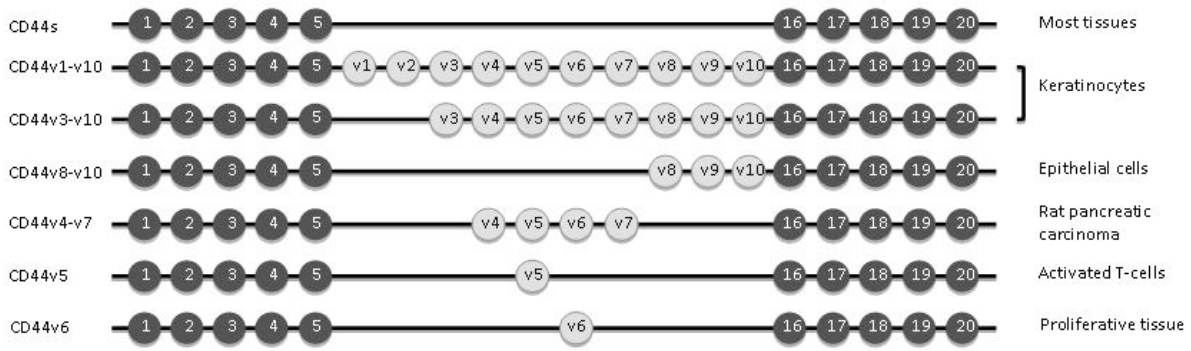

Illustration 2: CD44 transcripts expressed in different cells. CD44s is ubiquitously expressed in vertebrates in developing and adult organisms. The expression of CD44 variants is restricted to a few cell types (Based on Ponta et al., 2003).

Variant isoforms of CD44 are also expressed in a huge variety of cancers. The expression of the CD44 proteins in cancer is extensively described in paragraph 1.2.7

1.2.4 Alternative splicing

The differential expression of the CD44 isoforms is achieved by means of alternative splicing. This process is regulated by tissue specific factors. While mesenchymal cells predominantly splice out variant exons by a process called exon skipping, differentiated epithelial cells like keratinocytes and cells of the apical ectodermal ridge express a CD44 protein that contains all variant exons of CD44 that are maintained by a mechanism called exon inclusion (Hudson et al, 1995; Sherman et al, 1998). Inactive lymphocytes on the other hand express the standard isoform of CD44 but upon activation start to transiently express isoforms of CD44 that contain the variant exon v6 (Arch et al, 1992; Koopman et al, 1993).

Alternative splicing is regulated by mitogenic signals. Indeed, proliferating cells tend to express variant isoforms of CD44. For example the activation of the Ras-Erk pathway triggers inclusion of variant exons into the mature RNA (Weg-Remers et al, 2001).

Two types of *cis*-acting elements that control alternative splicing can be found in the variant exons. So-called splice enhancers are necessary to recognize a specific variant exon and are utilized in exon inclusions. Splice silencers on the other hand are used to prevent constitutive exon inclusion, a mechanism that is crucial for exon skipping (König et al, 1998). These elements differ at least to some extent between the variant exons (König et al, 1998; Matter et al, 2000). One *trans*-acting factor that regulates alternative splicing is the protein Sam68. It recognizes the silencer sequences in variant CD44 exons and can be directly phosphorylated by the Erk kinase. The phosphorylation of Sam68 presumably determines the assembly of spliceosomal complexes and auxilliary factors that recognize the so-called pyrimidine-rich stretch of introns (König et al, 1998; Matter et al, 2000).

1.2.5 The structure of CD44

The extracellular domain

The extracellular part of CD44 is divided into an aminoterminal globular domain and a variable stem region. The first five constant exons of the CD44 gene code for the aminoterminal globular protein domain that contains 90 amino acids. This domain is conserved between different species (homology of 80-90%) (Goldstein et al, 1989). It contains four highly conserved cysteins that can form intramolecular disulfid bonds. This domain, that is a homologue of the cartilage link protein and the proteoglycan core protein is called the link domain (Deak et al, 1986; Doege et al, 1986; Neame et al, 1986; Stamenkovic et al, 1989). The link domain is flanked by two more cysteins on either side that are required for its proper folding and for binding of the glycosaminoclycan (GAG) hyaluronan (HA). HA is the main binding partner of CD44 (Banerji et al, 1998). Additionally to the HA binding, the link domain facilitates binding to chondroitin and keratin sulphate. Binding to other gylcosaminoglycanes (GAGs) is not achieved through the link domain but by other not yet defined regions of the extracellular part. Affinity to GAGs is primarily dependent on posttranslational modifications (reviewed in Day & Sheehan, 2001).

The aminoterminal domain of CD44 can be modified by glycosylations that are cell type specific (reviewed in Day & Sheehan, 2001). The location of most of the N-glycosylated residues can be found here (reviewed in Day & Sheehan, 2001). One region of the aminoterminal domain that is adjacent to the membrane-proximal domain can also be modified by O-glycosylations.

The extracellular domain comprises binding sites for a variety of extracellular matrix (ECM) components such as HA, collagen, laminin and fibronectin. In the extracellular domain, four Ser-Gly motives can be modified by GAGs, one out of which can be used for binding to chondroitin sulphate (Goodfellow et al, 1982; Greenfield et al, 1999). These binding capabilities make CD44 a decisive factor for the matrix-dependent cell migration (Borland et al, 1998; Naor et al, 1997b).

The stem region

The stem region is a membrane-proximal part of the extracellular region of CD44 that has a minimal size of 46 amino acids (CD44s). Yet, by alternative splicing, the variable exons (v1-10) can be inserted between the amino acids 202 and 203 (in humans), expanding the stem region by up to 381 amino acids.

The sequences encoded by variant exons include several O- and N-glycosylation sites. For example CD44E (CD44v8-10) can be additionally modified by N- and O- linked glycosylation (Brown et al, 1991). The region encoded by the variable exon three includes a Ser-Gly-Ser-Gly GAG attachement site that enables modification by heparin sulphate or chondroitin sulphate (Bennett et al, 1995). Heparin-sulphated CD44 can bind to several heparin-binding growth factors, e.g. HB-EGF (Bennett et al, 1995).

The stem region separates the amino-terminal globular domain from the transmembrane domain. It includes a proteolytic cleavage site through which it can be shed from cell surfaces. Soluble CD44 blocks the binding of HA to CD44 molecules present on the cell surface (reviewed in Bazil & Strominger, 1994; Katoh et al, 1994). The shedding of CD44 can increase during tumor dissemination (Katoh et al, 1994). The proteases responsible for the cleavage of CD44 are serine proteases and metalloproteinases, such as the metalloproteinase Adam 10 (reviewed in Bazil & Strominger, 1994; Murai et al, 2006).

The transmembrane- and cytoplasmic region

The transmembrane region of CD44 encompasses 23 hydrophobic amino acids and one cystein residue. The cystein residue might take part in the aggregation of CD44 molecules by creating disulfid bonds between different CD44 proteins (Liu & Sy, 1996).

The cytoplasmic domain of CD44 is 70 amino acids long. In some rare cases, the exons coding for the cytoplasmic domain, can be alternatively spliced resulting in a short version (three amino acids in human) (Goldstein & Butcher, 1990; Screaton et al, 1992). The cytoplasmic domain shows 80-90% sequence homology among species (Isacke, 1994). This implies that this domain mediates important functions. It contains four highly conserved serine residues (Ser 291, Ser 316, Ser 323 and Ser

325 in human). The phosphorylation of these residues influences their role in different cellular processes. For example, Ser 325 is phosphorylated in all resting cells where it is required for HA mediated cell migration (Peck & Isacke, 1998). Upon HA-binding this serine residue is dephosphorylated and Ser 291 gets phosphorylated by protein kinase C (PKC). These changes in phosphorylation impair binding of ERM (ezrin, radixin, moesin) proteins, cytoplasmic linkers of the cell membrane and the cytoskeleton, to CD44 (Legg et al, 2002). Inhibition of ERM-binding leads to a block in migration. Indeed, Ser 291 is part of a membrane-proximal basic stretch of amino acids that these ERM proteins can bind to (Legg & Isacke, 1998).

The cytoplasmic domain also serves as a docking site for the cytoplasmic protein ankyrin that mediates the contact of CD44 with the cytoskeletal component spectrin (Bourguignon et al, 1992). This binding is required for HA-dependent cell adhesion and motility. The interaction between CD44 and ankyrin can be enhanced by palmitoylation of the transmembrane domain (Bourguignon et al, 1991; Lokeshwar et al, 1994).

1.2.6 CD44 and the ERM proteins

ERM proteins are members of the band 4.1 superfamily named after the erythrocyte membrane protein band 4.1 (reviewed in Niggli & Rossy, 2008). ERM proteins are involved in many cellular functions, including the control of cell shape, adhesion, motility, and the modulation of signaling pathways. Their main role is the connection of cell membrane proteins to the actin cytoskeleton. This connection is achieved either through their direct binding to single-pass proteins (proteins that pass the cell membrane one time) or indirectly by binding to adaptor proteins such as EBP50 and E3KARP that then bind multi-pass proteins (reviewed in Niggli & Rossy, 2008).

ERM proteins that connect cell membrane proteins to the cytoskeleton can mediate signal transduction from RTKs to downstream targets. The ERM protein ezrin for example is involved in the signal transduction of the Met signaling pathway (Orian-Rousseau et al, 2007). It links the co-receptor CD44v6 for the RTK Met to the actin cytoskeleton and through this enables downstream signaling to Ras and the signal-related kinases 1,2 (Erk 1,2).

Interestingly, a relative of the ERM proteins, merlin, can counteract the activation of signaling through these proteins. Merlin binds to CD44 but not to the cytoskeleton and thereby interferes with downstream signaling (Morrison et al, 2001).

1.2.7 The role of CD44 in physiological and pathological processes

The different isoforms of CD44 trigger cellular processes such as adhesion, proliferation, migration and survival that are important in physiological processes such as development, haematopoiesis and lymphopoiesis.

1.2.7.1 Physiological functions

Haematopoiesis

CD44 proteins are involved in the differentiation of haematopoietc progenitor cells and in the formation of lymphoid and myeloid lineages. In long-term bone marrow cultures (LTBMCs), a CD44 pan antibody interfered with the development of progenitor cells as well as with their differentiation into lymphoid and myeloid lineages (Khaldoyanidi et al, 1996; Miyake et al, 1990).

Also, specific variant isoforms of CD44 are important for hematopoiesis. Antibody treatment of LTBMCs with monoclonal CD44v4 or CD44v6 antibodies stimulates myelopoiesis and induces the production of granulocyte-macrophage colony stimulating factor (GM-CSF). CD44v6 antibody treatment alone on the other hand stimulates lymphopoiesis and the production of interleukin 6 (IL-6) (Khaldoyanidi et al, 2002; Rossbach et al, 1996) demonstrating that different CD44 variants play different roles in myelopoiesis and lymphopoiesis.

Lymphocyte homing and activation

The binding of CD44 to HA plays a decisive role in lymphocyte homing to specific lymphoid organs such as the lymph nodes (Shimizu & Shaw, 1991). The homing of bone marrow cells to the thymus is also mediated by CD44. Indeed, bone marrow cells that were treated with CD44 antibodies were not able to reach the

thymus (O'Neill, 1989) whereas their homing to the lymph nodes was not influenced by such a treatment (Camp et al, 1993).

CD44 appears to be involved in the rolling of lymphocytes. In this process, lymphocytes that traverse the blood stream are slowed down by rolling along the blood vessel border prior to their extravasation into the surrounding tissue. This lymphocyte rolling can be blocked by antibodies directed against CD44 or by HA-treatment of the lymphocytes (DeGrendele et al, 1996).

CD44 co-receptor function

CD44 proteins are able to bind different enzymes or growth factors thereby modulating their action. The heparin-decorated isoform CD44v3 for example binds to the heparin binding growth factors b-FGF (FGF-R ligand) and HB-EGF (ErbB ligand) (Bennett et al, 1995; Sherman et al, 1998). Interestingly, another heparin-binding ErbB ligand, Amphiregulin, was shown not to interact with CD44v3 (Bennett et al, 1995), showing that modifications other than heparin influences the binding.

Through its interaction with growth factors, CD44 can act as a co-receptor for different RTKs. This co-receptor can be mediated in different ways. CD44 can for example act as a docking platform for metalloproteinases that account for the activation of ligand proforms into their active form. CD44 can associate with the matrix metalloproteinase 7 (MMP-7) in the activation process of the ErbB4 receptor in the mammary gland. By recruiting the pro-form of HB-EGF and MMP-7, CD44 enables the maturation of pro-HB-EGF into HB-EGF (see also chapter 1.3.8) (Yu et al, 2002).

Another molecular mechanisms through which CD44 can act as a co-receptor is ligand presentation. In the apical ectodermal ridge of the limb bud CD44v3 can bind FGF-ligands and present them to their authentic receptor FGF-R that is expressed in the cells of the underlying mesenchyme, a type of undifferentiated loose connective tissue. Activated FGF-R promotes proliferation and limb outgrowth (Sherman et al, 1998).

Another well characterized co-receptor function of CD44 variant isoforms for an RTK is the collaboration between Met and CD44v6 (**Illustration 3**). In presence of HGF a complex containing HGF, Met and CD44v6 has been identified (Orian-Rousseau et al, 2002). CD44v6 is instrumental in the activation of Met, a process that

has been found to be important for proliferation and migration in primary as well as invasion and metastasis formation in several cancer cell lines. CD44v6 not only presents the ligand HGF to Met but features yet another molecular function, namely the mediation of downstream signaling by recruiting the ERM-protein ezrin. Since the co-receptor function of CD44v6 is specifically dependent on exon v6, the minimal v6 amino acid sequence required for Met activation was identified using linker scan mutations of the v6 sequence, replacing three amino acids at a time by alanines or glycine (in place of alanine) (Matzke et al, 2005). This linker scan analysis identified a sequence of three amino acids that is essential for the co-receptor function of CD44v6. Peptides containing the amino acid sequences EWQ in rat, GWQ in mouse and RWH in human, that are at least 5 amino acids in length can block the co-receptor function of CD44v6. This leads to an inhibition of Met-phosphorylation and signal transduction to its downstream targets Ras and Erk (Matzke et al, 2005). The CD44v6 peptide therefore inhibits the activation of Met with the same specificity as a CD44v6-antibody or a soluble ectodomain of CD44v6 (Matzke et al, 2005; Orian-Rousseau et al, 2002).

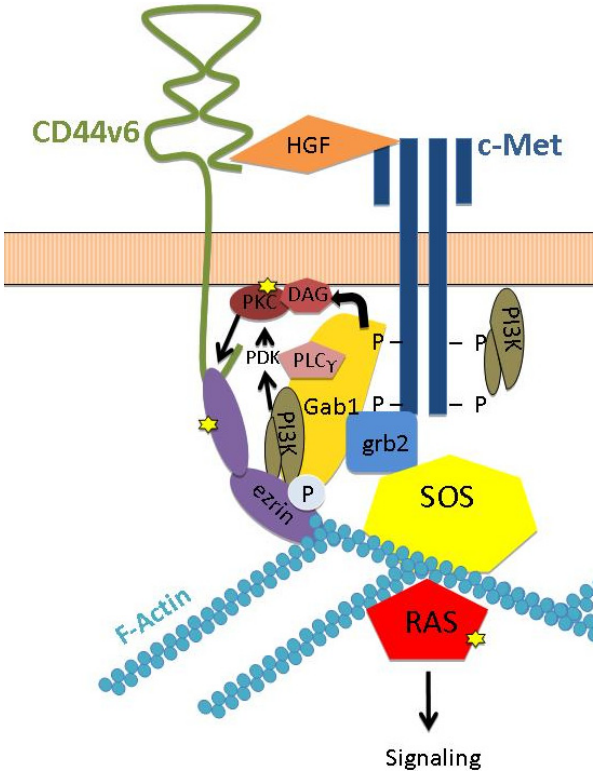

Illustration 3: CD44v6 is a co-receptor of Met. CD44v6 enables the activation of the Met RTK. It presents HGF to the receptor and mediates downstream signaling through the ERM protein ezrin (Based on Orian-Rousseau et al., 2002)

While for the activation of Met the extracellular domain of CD44 including the exon v6 and its transmembrane domain are sufficient the downstream signal transduction to Ras and the Erk kinase is dependent on the binding of ERM proteins to the cytoplasmic domain of CD44. The transfection of a CD44v6 isoform lacking its cytplasmic tail still allowed HGF-induced Met-activation but inhibited downstream signaling to Ras and Erk. In addition, an intracellular competition experiment by overexpression of the cytoplasmic domain of CD44 allowed Met receptor activation but blocked signal transduction to Ras and to the Erk kinase (Orian-Rousseau et al, 2002). If the cytoplasmic part of CD44 was mutated in its ezrin binding site no inhibition of signal transduction was observed (Orian-Rousseau et al, 2002). These data show that the interaction between ERM-proteins and CD44v6 is necessary for Met signaling. The binding of ezrin to the cytoskeleton is an essential step in downstream signal facilitation as shown by means of an ezrin-construct deleted in the F-actin binding domain, drugs that disturbed the integrity of the actin cytoskeleton and by the association of components of the HGF-induced signaling pathway with F-actin in Triton X-100 insolubility assays (Orian-Rousseau et al, 2007).

CD44v6 acts also as a co-receptor for the VEGFR-2 receptor, an RTK that promotes proliferation, angiogenesis and survival. A CD44v6 peptide, a CD44v6 antibody and the soluble ectodomain of CD44v6 were able to interfere with VEGFR-2 activation through its ligand VEGF-A and inhibit VEGF-A-induced migration, sprouting, tube formation and angiogenesis of endothelial cells (Tremmel et al, 2009).

CD44 has also been suggested to function as a co-receptor for the ErbB-family of proteins. This is discussed in section 1.3.8.

CD44 knockout

Mice with a gene knockout preventing expression of all CD44 isoforms that were generated by the deletion of the 3’ end of exon 2 and the entire exon 3 of the CD44 gene (Schmits et al, 1997) or by and insertion of a neomycin gene expression cassette into the reading frame of the leader peptide of the CD44 gene (Protin et al, 1999) are viable. They only show a mild phenotype, namely a deficite in haematopoiesis, homing and migration of the lymphocytes to the lymph nodes and in settlement in the thymus.

This is especially striking considering the role CD44 proteins seem to have in development. In addition to their involvement in limb outgrowth (see co-receptor section in this paragraph) (Sherman et al, 1998), CD44 also plays an important role in the migration of foetal liver cells and in their maturation in the thymus. During their maturation only CD44 expressing foetal liver cells and adult cells of the bone marrow are able to settle in the thymus and develop into mature T-cells (Kawakami et al, 1999). Additionally, CD44s is expressed in epithelial cells of the thymus and in thymocytes eight weeks after fertilization. Two weeks later the expression pattern of these cells changes and they start expressing the CD44 variants v4, 6 and 9. Antibodies against these isoforms of CD44 are able to inhibit the development of thymocytes in early stages of foetal organ cultures (Mackay et al, 1994; Terpe et al, 1994b).

The co-receptor function of CD44v6 for Met and VEGFR-2 should be physiologically indispensable. Met for example is an essential protein for development and mice bearing disruptions of the Met gene, the gene encoding its ligand HGF or of Met docking proteins such as Gab-1 are not viable (Bladt et al, 1995; Sachs et al, 2000; Uehara et al, 1995). In absence of CD44, Met and VEGFR-2 should not be able to function (Orian-Rousseau et al, 2002; Tremmel et al, 2009), which should also lead to lethality of the CD44 knockout mice. This however is not the case.

Selective suppression of CD44 in mouse keratinocytes

If CD44 is switched off during late embryonic development, in contrast to the germline knockout, a drastic phenotype is observed. CD44 antisense sequences under the control of the keratin-5-promoter (K5) were used in this case. This promoter gets only activated in the skin of the mice at day 9,5 of the embryonic development. The inactivation of CD44 by these antisense sequences in the skin results in severe shortcomings in wound healing and hair development. Also, the skin of the animals shows a thickened and brittle consistency. Additionally, a change in the hyaluronic hayluronan metabolism was observed (Kaya et al, 1997).

These differences between the inactivation of CD44 in the classical knockout and the transgenic animals expressing antisense sequences in keratinocytes imply that the loss of function of CD44 at various times in development has different

consequences. An explanation might be that a substituting molecule is taking over the co-receptor function of CD44 at early stages of development before reaching critical steps of differentiation. This compensation does not occur at later developmental stages, e.g when the K5 promoter is inactive.

Further data indicate that CD44 plays a role in vivo and indicate that the potential substitute molecule in the cd44 germline knockout is not as efficient as CD44. Indeed, a crossing between $cd44^{-/-}$ and $met^{+/-}$ gave a striking result: 70% of $cd44^{-/-}$ $met^{+/-}$ mice died at birth due to breathing defects caused by impaired synaptic transmission in the respiratory rhythm-generating network and alterations in the phrenic nerve (Matzke et al, 2007). This haploinsufficiency of Met is only observed in a CD44 null background and therefore suggests that CD44 indeed cooperates with Met *in vivo*. Furthermore, in CD44 null mice the function of CD44 as a co-receptor for Met must be substituted by another protein that is not as effective as CD44.

In order to identify a co-receptor for Met activation in absence of CD44v6, the CD44-negative human hepatocelllular carcinoma cell line HepG2 was used. In this cell system, that does not express CD44v6 yet still allows Met-activation, a different transmembrane-molecule, ICAM-1, is able to enable Met-activation. Met-activation and signaling can be blocked by specifically addressing the ICAM-1 receptor and inhibiting its function (Olaku et al, 2011).

In order to evaluate whether ICAM-1 could substitute for CD44v6 in CD44-knockout mice, a partial hepatectomy was performed in wild-type as well as in CD44-knockout mice. The relevance of Met in adult animals is illustrated by its physiological role in liver regeneration (Borowiak *et al.*, 2004). And indeed, in wild type mice, CD44v6-specific antibodies inhibited liver cell proliferation and Met activation, whereas ICAM-1-specific antibodies interfered with liver cell proliferation and Met activation in CD44 knockout mice (Olaku et al, 2011). These data show that CD44 can be functionally substituted by a heterologous protein, ICAM-1.

1.2.7.2 Pathological functions

Inflammation

CD44 and its ligand HA have been implicated to play a role in several inflammatory diseases (reviewed in Pure & Cuff, 2001). In a rheumatoid arthritis mouse model, inflammation in the murine joints can be reduced by specifically blocking the interaction between CD44 and HA by means of a CD44 antibody. In contrast, the enhancement of this binding by means of a different CD44-specific antibody increases the inflammatory symptoms (Mikecz et al, 1999). Furthermore, under inflammatory conditions, CD44 expression and HA-production on endothelial cells is increased due to transcriptional activation by pro-inflammatory cytokines like TNFalpha and IL-1beta (Mohamadzadeh et al, 1998). On these cells, CD44 then interacts with HA which might lead to an extravasation of the cells (Mohamadzadeh et al, 1998). Additionally, at the site of an inflammation or injury, low molecular weight HA (sHA) is produced and its binding to CD44 can induce the expression of genes involved in inflammatory responses such as TGF-β1 (McKee et al, 1996; Ohkawara et al, 2000).

Tumourigenesis

Animal experiments and observations in human cancers suggest that CD44 proteins play important roles in the metastatic spreading of tumor cells. The most important evidence for the involvement of CD44 variants in metastasis was found by transfection of a CD44 variant protein (CD44 v4-7) into non-metastatic rat pancreatic cells. This CD44 variant conferred metastatic potential to the cells (Gunthert et al, 1991). The metastatic abilities mediated by CD44 could be abolished by inhibiting its function. Treatment of colorectal tumor cells with different CD44-specific antibodies for example leads to a block in tumor progression as well as metastasis (Naor et al, 1997b; Sleeman et al, 1995; Wielenga et al, 1998).

It seems that in some instances, the expression of CD44 variants is controlled by oncogenes like c-Ha-ras. In cloned rat embryonic fibroblasts (CREF) the activated ras oncogene induces CD44 promoter activity leading to the expression of CD44s as well as its metastatic variants (Hofmann et al, 1993).

In many human cancers the expression of variant CD44 proteins can be used as diagnostic and prognostic markers (Kaufmann et al, 1995b; Pals et al, 1997; Wielenga et al, 1993). The expression of CD44v7 and v8 in cervical cancer (Kainz et al, 1995) or that of CD44v6 in colorectal carcinomas (Naor et al, 1997a; Sleeman et al, 1995; Wielenga et al, 1998) has been connected with the involvement of CD44 in metastasis formation.

In some instance however, it seems that not CD44 variants but CD44s is involved in tumor growth as for example the transfection with CD44s of a CD44-negative Burkitt lymphoma line renders it metastatic (Sy et al, 1991). Additionally, CD44s enhances tumor growth of CD44-negative melanoma cells whereas in contrast transfection with the CD44 variant v8-10 inhibited tumor growth in these cells (Sy et al, 1991; Sy et al, 1992).

Suprisingly, in some cases, CD44s can also function as a tumor suppressor. The first evidence for this was found in neuroblastomas, where CD44s is downregulated (Shtivelman & Bishop, 1991). The absence of CD44s correlates with histological dedifferentiation, N-myc amplification and reduced survival probability (Terpe et al, 1994a). In prostate cancer, CD44s is not expressed and its overexpression represses tumor metastasis (Gao et al, 1997).

Bacterial infection

CD44 facilitates direct interactions between bacteria and host cells and is also involved in signaling events that make these cells more susceptible to infection. *Streptococcus pyogenes* for example can attach to cells through its HA-rich cell wall. CD44 molecules on the target cell surface bind to this HA and trigger the tyrosine phosphorylation of several cytoplasmic proteins within the target cell. They also induce cytoskeletal rearrangements that cause ruffles and extension of lamellipodia. Through this rearrangement process, the connection of the affected cell to its neighbouring cells gets loosened due to reduced tight junction connections. The bacterium is then able to invade the subepithelial tissue (Cywes et al, 2000). Consequently, mice deficient in CD44 are resistant to oropharynx colonization by S. pyogenes (Cywes et al, 2000).

In order to be taken up and internalized by their host cells, bacteria like *Shigella flexneri* or *Listeria monocytogenes* also recruit CD44. *S.flexneri*, a bacterium

that causes bacillary dysentry in humans is the prototype of bacteria that enter cells via a so-called trigger mechanism (reviewed in Veiga & Cossart, 2006). *S.flexneri* secretes several proteins that are necessary for the infection process and one of them, IpaB, binds to CD44s (Skoudy et al, 2000). This binding is instrumental for its entry into host cells as blocking its function by means of CD44 specific antibodies inhibts bacterial entry. The entry process of *S.flexneri* is dependent on CD44 binding to the ERM protein ezrin (Skoudy et al, 1999).

The bacterium *Listeria monocytogenes,* is another pathogen that makes use of CD44 for its entry process. *L. monocytogenes* is a gram-positive, rod-shaped and food-borne bacterium that is the causative agent for the disease listeriosis(Newell et al, 2010). It can infect at least 37 different species of mammals and 17 different species of birds. The pathogen can also be found in the ground soil and on most plants (reviewed in Ivanek et al, 2006). Due to this fact, contact with the bacterium is almost unavoidable for humans. *L. monocytogenes* can thrive in the intestinal tract of animals or humans for extended periods of time without causing an infection and it is believed that at least 5% of the human population is a carrier of the bacterium without showing any symptoms (reviewed in Barbuddhe & Chakraborty, 2009).

The entry into the host organism of the bacterium is achieved primarily through the intestinal tract where *L. monocytogenes* is able to cross the intestinal barrier by active epithelial cell invasion or passively by phagocytosis through macrophages (reviewed in Barbuddhe & Chakraborty, 2009). Within the epithelium cells the bacterium is able to move freely from one cell to another due to a cytoskeleton-based translocation mechanism (Pizarro-Cerdá et al., 2004) and eventually enters the blood flow and the lymphatic system. After intestinal translocation the liver is the first organ that is infected by the pathogen (Scholing et al, 2007). Here it multiplies until the immune system of the host eliminates it. If such a response does not kill the bacterium, it can spread to other organs like for example the uterus and the brain.

L. monocytogenes very rarely infects healthy organisms, although pregnant women, unborn and new-born babies sometimes contract Listeriosis. Individuals with a deficit in the immune system like for example cancer, HIV or organ transplantation patients on the other hand are at a serious risk to be infected by *L.monocytogenes* (Patil et al, 2007; Rivero et al, 2003; Wiesmayr et al, 2005). The symptoms of an actual outbreak of Listeriosis last from 7 to 10 days, the most common of which are fever, muscle ache, vomiting or diarrhea. If the infection spreads to the nervous

system, it can also cause meningitis leading to headache, stiff neck, loss of balance or convulsions. Infection with *L. monocytogenes* can lead to death in one third of the patients, sometimes even after a treatment with antibiotics (Rivero et al., 2003).

The invasion and intracellular reproduction of *L. monocytogenes* is mediated by 2 gene clusters. The cluster that was first identified is called listeria pathogenicity island 1 (LIPI-1) and codes for a collection of proteins that mediate the escape of the pathogen from the phagocytotic vacuole, its cytosolic reproduction, actin-based motility and cell-cell spreading (Vazquez-Boland et al, 2001). The second gene cluster only incorporates two genes, that form a common operon: Internalin A (InlA) and internalin B (InlB). InlA and InlB code for invasion factors that are necessary for the attachement and invasion of non-phagocytic target cells (Cabanes et al, 2002; Glaser et al, 2001).

InlA mediates the recognition and invasion of epithelial cells through specific interaction with E-cadherin, a transmembrane adhesion protein that is involved in cell-cell adhesion complexes. The InlA-E-Cadherin interaction induces the interaction of E-cadherin with α-catenin that together with β-catenin achors E-cadherin to the actin cytoskeleton. The formation of this adhesion complex causes local cytoskeletal changes that mediate the uptake of the bacterium into its host cells (Hamon et al, 2006).

InlB on the other hand mediates the recognition and invasion of epithelial and endothelial cells through binding to the RTK Met (Shen et al, 2000). Like its physiological ligand, HGF, InlB induction of Met leads to the activation of the RTK and the activation of the Erk-Ras and PI3K signaling pathway. HGF and InlB do not share any sequential or structural homologies (Machner et al., 2003). They bind to different regions in the extracellular domain of the Met RTK (Niemann et al., 2007).

The activation of Met through InlB leads to its clathrin-dependent endocytosis together with the InlB-bound bacterium. This process is triggered by a mono-ubiquitination of Met through the ubiquitin ligase cbl (reviewed in Veiga & Cossart, 2005). By local polymerisation of actin filaments that is mediated by the Arp2/3 complex, pseudopodes are extended around the bacterium that is eventually completely covered by the cell membrane and transported into the cytoplasm where it is contained within a phagosome (reviewed in Ireton, 2007).

For the activation of Met, InlB is dependent on the variant CD44 isoform v6 as the inhibition of CD44v6 by a specific blocking peptide, a CD44v6 antibody or v6-

specific siRNA blocks the activation of Met through InlB (Jung et al., 2009). Consequently, signaling, scattering and the entry of InlB-coated beads into host cells are also impaired. For this process, the ERM protein ezrin is required (Jung et al., 2009).

CAMs like CD44 are often recruited as co-receptors by RTKs. They usually mediate the activation of these receptors or have additional functions like the recruitment of cytoplasmic proteins. The RTK Met for example can be activated by InlB and HGF. This activation is dependent on its cooperation with CD44v6 (Orian-Rousseau et al., 2002; Jung et al., 2009). Thus two structurally different ligands can activate the Met receptor by cooperating with the same co-receptor. This suggests that for its activation, Met requires the co-receptor CD44v6 independently of the ligand it is addressed by. However, CD44v6 not only acts as a co-receptor for Met. It also cooperates with the VEGFR-2 receptor where it mediates receptor activation through the VEGFR-2 ligand VEGF-A (Tremmel et al., 2009). These data suggest that CD44v6 might not be recuited by the RTK it works together with but by the ligands that address these RTKs. Indeed, this seems also to be the case for the heparin sulphated isoform CD44v3. CD44v3 cooperates with the FGF-receptor in limb outgrowth. It presents FGF-4 and FGF-8 to underlying mesenchymal cells that express the FGF-receptor (Sherman et al., 1998). However during the maturation of the female breast CD44v3 is also recruited by the ErbB4 RTK (Yu et al., 1999).

In order to test whether the ligands that address specific RTKs are decisive factors that dictate the need for specific isoforms of CD44 as co-receptors or whether a specific RTK requires a specific CD44 isoform, the ErbB family of receptors is an optimal candidate. The ErbB family contains four different receptor proteins (ErbB1-4) that form different homo- and heterodimers in order to bind their different ligands. The ErbB4 receptor recruits CD44v3 as a co-receptor. Therefore, other ErbB receptor proteins might also be dependent on CD44 isoforms. The ErbB1 receptor alone can be addressed by seven different ligands, whereas some of these ligands can also bind to additional receptor proteins. Thus, it can be evaluated whether for its activation by these seven ligands, ErbB1 is always dependent on the same CD44 isoform or whether the ligands determine which isoform is required.

1.3 The ErbB family of receptors

1.3.1 Introduction

The ErbB-family is also designated the subclass I of the receptor tyrosine kinase superfamily. It contains four different receptor proteins: EGFR or ErB1, ErbB2, ErbB3 and ErbB4. In rodents the ErbB2 receptor is referred to as „Neu“. The human forms of the ErbBs are named Her 1-4 (reviewed Bublil & Yarden, 2007).
In order to bind to their ligands, the ErbB receptor proteins form homo- and heterodimers. Due to the various homo- and heterodimers of the four ErbB receptors, a high degree of signal diversity is achieved. The ErbBs are expressed in various tissues throughout the development and in the adult organism where they control proliferation, differentiation and cell survival. The ErbBs also play a paramount role in human cancers (reviewed in Hynes & Stern, 1994; Salomon et al, 1995).

All ErbB receptors share an extracellular ligand-binding region, a single membrane spanning domain and a cytoplasmic domain that includes a tyrosine kinase domain (reviewed in Bublil & Yarden, 2007). Under physiological conditions, the activation of the receptors is controlled by several ligands that are expressed in a developmental stage- and tissue-specific manner (reviewed in Riese & Stern, 1998). The ligands of the ErbB receptors belong to the EGF-related family of growth factors. A ligand induction of the ErbB RTKs leads to activation of their intracellular kinase domain and subsequently to phosphorylation of specific tyrosine residues in their cytoplasmic domain. These modified residues can then serve as docking sites for a variety of signaling molecules whose recruitment leads to the activation of intracellular signaling pathways such as the Ras-Erk and the PI3K (Olayioye et al, 1998; Yarden, 2001).

The ErbB signaling family has been highly conserved during evolution. In Nematodes like for example *Caenorhabditis elegans* one single receptor (LET-23) and one ligand (LIN-3) exists (reviewed in Chang & Sternberg, 1999). In insects, e.g. the fruitfly *Drosophila melanogaster,* one receptor (DER) exists that can be activated by four different ligands (reviewed in Reich & Shilo, 2002; Schweitzer & Shilo, 1997). Interestingly an additional ligand (Argos) can block receptor activation by competing

with the other ligands (reviewed in Reich & Shilo, 2002). Such a regulation by competition is not found in higher organisms.

In higher vertebrates four different ErbB receptors bind to a multitude of ligands.

1.3.2 The EGF-related ligand family

In vertebrates, the „EGF-related ligand family“ constitutes the ligands of the ErbB receptors. All these growth factors are produced as transmembrane precursors. Their ectodomains are processed by proteolysis, a step that leads to the shedding of the mature soluble protein. Various studies have identified the ADAM metalloproteinases as being responsible for the cleavage of the ErbB pro-ligands.

The ErbB ligands differ in their ability to bind to the ErbB receptors. Based on this binding specificity they can be divided into three groups (reviewed in Olayioye et al., 2000) (**Illustration 4**):

(1) Amphiregulin (AR), Epigen (EPG), Epidermal Growth Factor (EGF) and Transforming Growth Factor Alpha (TGF-α) that exclusively bind to ErbB1

(2) Betacellulin (BC), Epiregulin (ER) and Heparin-binding EGF-like growth factor (HB-EGF) that show dual specificity. These growth factors can bind to either ErbB1 or ErbB4.

(3) The third group is formed by the Neuregulins (NRGs) that can be seperated into two subgroups based on their receptor specificity:

(3.1) NRG-1 and NRG-2 bind to ErbB3 and ErbB4 while

(3.2) NRG-3 and NRG-4 can only bind to ErbB4

The group of the Neuregulins is further diversified by alternative splicing.

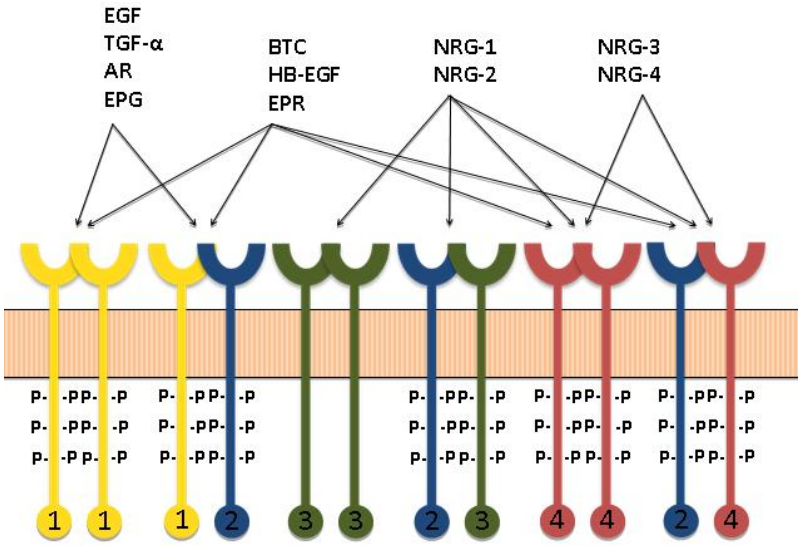

Illustration 4: The binding specificity of the ErbB ligands. The ErbB ligands can bind to 11 different ligands by forming homo- and heterodimers (Based on Olayioye et al., 2000)

No direct ligand has so far been found for the ErbB2 receptor. It is only adressed by heterodimerization with the other ErbB receptors. The ErbB2 receptor is often used as a signal enhancer for the other ErbBs (Beerli et al, 1995; Graus-Porta et al, 1995).

1.3.3 Expression of the ErbB ligands

The ErbB ligands usually act over short distances as autocrine or paracrine factors. Some ligands like e.g. EGF that is found in all body fluids or Nrg-1 are widely expressed (Meyer & Birchmeier, 1995). Also Epiregulin, TGF-α and HB-EGF are expressed in many different cell types. ER is expressed in macrophages and in the placenta (Toyoda et al, 1997). TGF- α is produced for example in brain cells and keratinocytes (Partanen, 1990) whereas HB-EGF is for example produced by macophages and keratinocytes (Hashimoto et al, 1994; Higashiyama et al, 1991). Other ErbB ligands show a more restricted pattern. Nrg-4 expression for example is found in the pancreas (Harari et al, 1999) while Nrg-3 expression is restricted to the developing and adult nervous system (Zhang et al, 1997).

In order to investigate the role of ErbB-ligand function during development, different knockout mice for ErbB ligands were created. Suprisingly, HB-EGF was the only ErbB ligand whose absence during development resulted in the postnatal death

due to malformed heart valves, hypertrophic cardiomyocytes and hypoplastic lungs (Iwamoto et al, 2003; Jackson et al, 2003). Mice with disruptions in the genes encoding AR (Luetteke et al, 1999), BC (Jackson et al, 2003), EGF (Luetteke et al, 1999), ER (Lee et al, 2004; Shirasawa et al, 2004), TGF-α (Luetteke et al, 1999; Mann et al, 1993) are viable. This is even the case in the triple-null mice (AR, EGF, TGF-α) (Luetteke et al, 1999). These experiments point to a high degree of functional redundancy within the ErbB ligand family since it is highly unlikely that they are physiologically dispensable.

Despite being viable, many of the knockout mice show phenotypes. If the mice were stressed they showed further homeostasis defects in previously apparently normal tissues. For AR-deficient mice for example only a defect in mammary gland development was reported initially (Luetteke et al, 1999). Further studies however revealed impaired liver regeneration (Berasain et al, 2005a; Berasain et al, 2005b) and significantly less growth of tibial trabecular bone than wild-type mice (Qin et al, 2005). Notably, several of the phenotypes observed in mice lacking individual ErbB1 ligands such as for example hair follicle and eye lid closure defects in TGF-α deficient mice (Luetteke et al, 1999; Mann et al, 1993) or heart-valve defects and lung immaturity in HB-EGF deficient mice (Iwamoto et al, 2003; Jackson et al, 2003) are found in mice with an ErbB1-receptor knockout (Chen et al, 2000; Miettinen et al, 1995; Sibilia & Wagner, 1995; Threadgill et al, 1995) indicating that the ErbB1 receptor primarily mediates the signaling of these ErbB ligands.

1.3.4 The structure of the ErbB receptors, their ligand binding and dimerization

The crystal structure of ligand bound ErbB1 has been resolved. The extracellular domain of the ErbB receptors consists of 4 different regions. For the binding of EGF (Ogiso et al, 2002) and TGF-α (Garrett et al, 2002) the domains I and III play a decisive role as they contact the ligand from two opposite sites and form a kind of a binding pocket the ligand fits into. The domain II contains a loop-like motif that helps in the dimerization process of two ErbB receptors. The protruding arm on one ErbB1 molecule directly contacts domain II in a second ErbB1 receptor and vice versa.

The ErbB ligands themselves do not interact. In an ErbB-dimer they are kept apart and bind in monomeric form. Consequently they cannot mediate dimerization.

This finding is in clear contrast to the dimerization process of other RTKs (reviewed in Schlessinger, 2000). PDGF or VEGF for example form homodimers and by binding one receptor per each molecule help in the receptor dimer assembly. FGF on the other hand stays in monomeric form while binding but two FGF molecules are brought together by binding simultaneously to a heparinsulphate proteoglycan (HSPG).

Inactive ErbBs are kept in a so-called tethered conformation. In the ErbB1 receptor, the domains II and IV interact intra-molecularly keeping the domains I and III that are involved in binding, apart (Ferguson et al, 2003). The ErbB3 and ErbB4 receptors have a conformation similar to the one of ErbB1 (Bouyain et al, 2005; Cho & Leahy, 2002). Upon ligand binding the conformation of the receptors changes, they enter a so-called open conformation in which domain I and III are bound to the ligand and the dimerization arm is exposed (i.e. bound to another ErbB receptor) (**Illustration 5**).

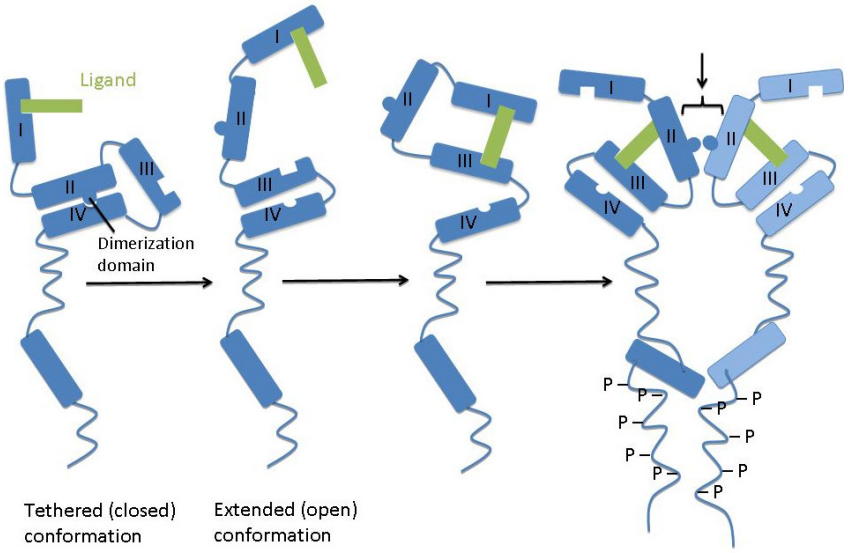

Illustration 5: The ligand binding process of the ErbB receptors. An ErbB ligand first interacts with region I of the ErbB receptor it interacts with. This interaction brings region I and III closer together until they finally both bind the ligand. Upon entering this binding pocket, the extracellular domain of the ErbB receptor undergoes a conformational change that extends it dimerization arm and allows dimerization (Based on Baselga & Swain, 2009).

Inactive ErbB2 receptors show a major structural difference in comparison to the other ErbBs (Cho et al, 2003; Garrett et al, 2003). They are found in a conformation, similar to that of ligand bound ErB1 and ErbB3. Their domains II and IV interact and the dimerization arm is extended. In this conformation the domains I and III that are normally used for ligand binding are kept too close together and no ligand can „fit“ in between them (Garrett et al, 2003).

Despite their extended dimerization arm, ErbB2 receptors do not form homodimers under physiological conditions because of an electrostatic repulsion between the two ErbB2 dimerization loops (Garrett et al, 2003). In contrast, when ErbB2 is overexpressed, which is the case in many human cancers, ErbB2 homodimers can be formed and are constitutively activated (reviewed in Holbro et al., 2003). Under physiological conditions however, the ErbB2 receptor needs to heterodimerise in order to mediate signaling (Klapper et al, 1999).

The ErbB3 receptor is also dependent on heterodimerization in order to signal. In contrast to ErbB2, ErbB3 can bind ligands but has substitutions of critical residues in the kinase domain (Guy et al, 1994). This domain is therefore called the „kinase dead domain“. Despite the substitutions in its kinase domain ErbB3 retains sufficients kinase activity to effectively trans-autophosphorylate its intracellular region (Shi et al, 2010).

1.3.5 The signaling pathways activated by the ErbBs

The binding of the ErbB ligands to the ErbB receptor proteins mediates their activation. The intrinsic tyrosine kinase activity leads to an autophosphorylation of C-terminal tyrosine residues (reviewed in Heldin, 1995; Weiss & Schlessinger, 1998) that can then act as docking sites for proteins that contain Src homology 2 (SH2)- and phosphotyrosine binding (PTB) domains (reviewed in Shoelson, 1997; Sudol, 1998). These domains mediate the docking of several downstream proteins such as the adaptor proteins Shc, Crk, Grb2, Grb7 and Gab1, the kinases Src, Chk, the PI3K via its regulatory subunit p85 and the tyrosine phosphatases SHP 1 and 2 (reviewed in Shoelson, 1997). For each ErbB protein a distinct C-terminal autophosphorylation pattern has been described that is recognized by specific effector proteins (reviewed in Shoelson, 1997).

The ErbB proteins show a large overlap in the signaling pathways they can activate. All of the ErbB receptors mediate the activation of the mitogen-activcated protein (MAP) kinase via Shc or Grb2 but each member of the ErbB family has preferences for specific downstream proteins. In heterodimers, the ErbB3 receptor for example is the most efficient activator of the PI3K because of its multiple binding sites for these proteins (Prigent & Gullick, 1994). ErbB1 is the only ErbB protein that can bind to Cbl and Eps15, both proteins that are involved in the downregulation of

the ErbB1 RTK (Fazioli et al, 1993; Levkowitz et al, 1996). Possibly due to its ubiquitination through Cbl, the ErbB1 receptor is the most efficiently internalized ErbB receptor and undergoes degradation more readily than the other ErbBs (Sorkin & Goh, 2009).

The heterodimerization of the ErbB receptors follows a hierarchical order, in which the ErbB2 receptor is the preferred dimerization partner of all other ErbB receptors (Graus-Porta et al, 1997; Tzahar et al, 1996). Heterodimers containing the ErbB2 receptor have an increased ligand affinity due to a slow ligand-disassociation rate (Karunagaran et al, 1996). This leads to an increased activation of the corresponding signaling pathways (Beerli et al, 1995; Graus-Porta et al, 1995). Additionally, biological responses that are mediated by all of the ErbB receptors, such as proliferation, morphological differentiation and migration/invasion are enhanced in cells expressing ErbB2 (Beerli et al, 1995; Spencer et al, 2000).

The heterodimerization of the ErbB receptors is not only used to amplify a signal, it also gives rise to signal diversification. The SH2- and PTB-binding proteins that are recruited to an activated ErbB receptor are defined by the specific pattern of phosphorylated tyrosine residues in the C-terminus of the protein. The phosphorylation pattern responsible for this specific binding capacity is modulated by heterodimerization of the ErbB receptors (reviewed in Olayioye et al, 1998). An ErbB1 homodimer for example can recruit Grb2 while an ErbB1/ErbB4 heterodimer is not able to do so (Olayioye et al, 1998). If ErbB2 homodimerises due to a mutation in the transmembrane domain on the other hand it is considerably more potent in binding Shc than an ErbB1/2 heterodimer (Olayioye et al, 1998). These data point to the fact that the signal given by a specific heterodimer of ErbBs is not simply the sum of their individual signaling properties but instead acts as a completely independent signaling unit with unique signaling properties.

1.3.6 ErbB receptors in development

In development the involvement of the ErbB receptors has been shown by using genetically modified mice (Olayioye et al, 2000). Null mutations in individual ErbB loci are lethal. ErbB2 knockouts die during midgestation (day 10.5) due to trabeculae malformation in the heart (Lee et al, 1995). The same phenotype is observed in ErbB4 knockout mice (Gassmann et al, 1995). A further role for ErbB2

has been demonstrated in the development of the peripheral nervous system (Morris et al, 1999). ErbB3 knockout mice die by day 13.5 due to defective heart valve formation. Additionally, these animals have defects in neural crest cells and lack Schwann cell precursors (Erickson et al, 1997; Riethmacher et al, 1997).

The only exceptions are ErbB1 knockout mice that (depending on the genetic background of the host) reveal an embryonic or perinatal lethality. The causes of death in all ErbB1 knockout mice are abnormalities in multiple organs amongst which the brain, skin and gastointestinal tract have been found (Miettinen et al, 1995; Sibilia et al, 1998; Sibilia & Wagner, 1995; Threadgill et al, 1995).

These data indicate that the ErbB receptors have essential roles in modulating certain aspects of vertebrate embryogenesis and postnatal development. They seem to play a particular important role in the development of the heart.

1.3.6.1 Heart development

The heart is the first organ that forms during development and is needed to support the growth of the developing embryo. ErbB signaling has been shown to play an essential role in its development and function. In midgestation, the ErbB2/4 and ErbB2/3 heterodimers regulate the formation of the cardiac trabeculae and of the cardiac cushion. ErbB1 signaling on the other hand is involved in the regulation of the formation of the cardiac valves. On top of that, during and after the perinatal stages, ErbB2/4 signaling drives the maintenance of cardiomyocytes (reviewed in Olayioye et al., 2000). In addition to these signaling events crosstalks between the ErbB receptors and other signaling cascades have been implicated that further modulate heart development (reviewed in Iwamoto & Mekada, 2006).

The ErbB receptors also play essential roles in mammary gland development.

1.3.6.2 Mammary gland development

At birth, the mammary gland consists of a rudimentary system of ducts that undergoes extensive remodelling and extension at puberty. During pregnancy, the lobuloalveolar cells undergo proliferation and at parturition the mammary fat pad is completely filled with milk-producing lobules (reviewed in Weiss & Schlessinger, 1998). The ErbB1 receptor works together with ErbB2 in the maturation of the breast. It has a modulatory effect on side branching and promotes alveolar development and differentiation. The ErbB1 receptor mediates ductal growth whereas the ErbB2/4 heterodimer promotes lobuloalveolar differentiation and is involved in maintenance of lactation (Olayioye et al., 2000). In ErbB2 knockout mice, an impairment in duct formation has been found potentially due to structural defects in the terminal end buds of the growing ducts (Jackson-Fisher et al, 2004). The ErbB3/4 heterodimer is required for proliferation in pregancy and lactation. (Olayioye et al., 2000).

In summary, these data suggest that the main role of ErbB1 in mammary gland development is the promotion of ductal growth, while ErbB2-4 have essential roles in lobuloalveolar differentiation and lactation.

In addition to their physiological functions the ErbB receptors are also involved in several of human cancers.

1.3.7 The ErbB receptors in cancer

The activation of the ErbB receptors, particularily ErbB1 and 2 is deregulated in many human cancers. This deregulation, caused by either overexpression or mutations of the ErbB encoded proteins or by autocrine ligand production, and is characterized by uncontrolled proliferation and migration of cancer cells.
The most common type of ErbB1 mutations is the so-called type III mutation (Voldborg et al, 1997). In this case, a deletion of the extracellular domain of the protein leads to constitutive activation of the receptor. ErbB1 proteins carrying a type III mutation are involved in glioma, ovarian, as well as breast cancer (Ekstrand et al, 1992; Moscatello et al, 1995).

Overexpression of ErbB1 on the other hand has been found in squamous-cell carcinomas of head and neck, non-small cell lung cancer, ovarian, lung and breast

cancer (Bartlett et al, 1996; Grandis & Tweardy, 1993; Klijn et al, 1994; Rusch et al, 1997) where it is as a strong prognostic marker for reduced recurrence-free and overall survival (Nicholson et al, 2001). While overexpressed, the ErbB1 receptor is still dependent on induction through a ligand (Di Fiore et al, 1987) in order to signal. The ErbB1 receptor is co-expressed with several of its ligands. TGF-α for example is often found to form an autocrine loop that leads to the deregulated activation of ErbB1, for example in lung, colon and breast cancer (Salomon et al, 1995; Umekita et al, 2000).

In contrast to the ErbB1 receptor, no activating mutation has been found for ErbB2 so far. Its activation is mainly due to overexpression, often by means of gene amplification. The increased abundance of ErbB2 molecules in target cells leads to spontaneous dimerization of the ErbB2 proteins and constitutive activation. This kind of receptor activation is found for example in lung, ovarian and stomach cancer (Lemoine et al, 1991; Salomon et al, 1989; Tateishi et al, 1991) but is especially important in breast cancer where it has been linked to a poor clinical prognosis and resistance to therapy (Ross & Fletcher, 1998a).

The ErbB3 receptor has been connected with a resistance against therapies targeting the ErbB2 receptor. Upon blocking of the ErbB2 receptor for example by tyrosine kinase inhibitors (TKIs), the ErbB3 phosphorylation level is increased. This leads to an activation of ErbB3 signaling that promotes cell survival in absence of ErbB2 signaling (Sergina et al, 2007). In breast cancer, the ErbB3 receptor has been shown to work together with ErbB2, mediating tumor cell division (Holbro et al, 2003). Their co-expressed has been found in many human breast cancers (Sithanandam & Anderson, 2008).

The ErbB4 receptor seems to play no role in most human cancers. No activating mutations of ErbB 4 have been identified and no statistically relevant correlation with clinical prognosis was found. In breast cancer, however, the expression of the ErbB4 receptor seems to correlate with endocrine resistance (Sutherland, 2011).

1.3.8 The ErbB receptors and CD44

Interestingly, several crosspoints between CD44 proteins and ErbB receptors have been unraveled. One main role of the CD44 protein family is due to its binding to HA. This function of CD44s as a HA receptor has been shown to promote the activation of an oncogenic form of ErbB2 in ovarian carcinoma cell lines (Bourguignon et al, 1997). CD44s interacts with the neuronal Wiskott-Aldrich syndrome protein (N-WASP) upon HA-binding. By this interaction it stimulates the Arp2/3 complex that facilitates filamentous actin formation, ovarian cancer cell migration and the activation of the ErbB2 kinase, leading to the transcription of TCF/LEF regulated genes (Bourguignon et al, 2007c).

Another CD44 isoform, CD44v3, is involved in several ErbB-related processes. CD44v3 can bind to the guanine nucleotide exchange factor Vav2. These two proteins then interact with ErbB2-bound Grb2. Upon HA-binding of CD44v3, the complex of these 4 proteins facilitates the activation of Rac1 and Ras signaling that is required for human ovarian tumor progression (Bourguignon et al, 2001).
The cooperation of CD44v3 with ErbBs is also involved in physiological processes like female reproductive organ remodeling. CD44v3 binds to the ErbB ligand HB-EGF (Bennett et al., 1995). CD44v3 recruits the matrix metalloproteinase 7 (MMP-7) and pro-HB-EGF to the cell surface of postpartum uterine and lactating mammary gland epithelium (Yu et al, 2002). After this recruitment, MMP-7 mediates the maturation of pro-HB-EGF into HB-EGF. CD44v3 might then bind to activated HB-EGF and present it to the ErbB4 RTK. This leads to increased phosphorylation of ErbB4. The interaction of MMP-7 with pro-HB-EGF has been shown to be instrumental in the tumorigenesis of cutaneous squamous cell carcinoma cell lines (Kivisaari et al, 2010) as well as in the tumorigenesis of mammary epithelial cell lines (Lynch et al, 2007).
CD44 has also been found to colocalize and coimmunoprecipate together with ErbB1 as well as with ErbB2 in several breast cancer cell lines (Wobus et al, 2001).

Altogether, the ErbB receptors are a family of RTKs that is expressed in many tissues throughout the body. Its 4 different receptor proteins can form homo- and heterodimers to bind to 11 different ligands. These ligand-dimer interactions account for its high degree of signal diversity. This signal diversity is one of the reasons that the ErbB family is involved in many different physiological and pathological

processes. ErbB receptors are for example involved in heart as well as in breast development, where their different receptor pairs and single ligands seem to have specific functions. ErbB signaling is also involved in pathologial situations. In many human cancers like lung, breast or stomach cancer, the activation of the ErbB family drives tumorigenesis and metastasis and its expression is connected with a bad clinical prognosis.

Aim of the PhD thesis

The first part of my PhD thesis is a continuation of my diploma work where I showed that the activation of the RTK Met, is dependent on CD44v6 for its activation by Inlb, a bacterial protein used for invasion of the bacteria *Listeria monocytogenes*.

Since these data suggested that the invasion of *L.monocytogenes* could also depend on CD44v6, the first aim of my PhD thesis was to test whether the entry of these bacteria into host cells requires CD44v6 (Jung et al., 2009).

CD44v6 had been previously identified as the co-receptor for the HGF-based induction of the RTK Met (Orian-Rousseau et al., 2002). Thus two structurally different ligands can activate the Met receptor by cooperating with the same co-receptor. This suggests that for its activation, Met requires the co-receptor CD44v6 independently of the ligand it is addressed by. However, CD44v6 not only acts as a co-receptor for Met. It also cooperates with the VEGFR-2 receptor where it mediates receptor activation through the VEGFR-2 ligand VEGF-A (Tremmel et al., 2009). The identification of CD44v6 as the co-receptor of three structurally unrelated growth factors and two structurally unrelated RTKs gave rise to the question whether CD44v6 was recruited by the RTKs themselves or the ligands that address these receptors.

The heparan-sulphated CD44v3 serves as a co-receptor for the growth factors FGF-2 in limb development (Sherman et al., 1998) and HB-EGF in mammary development (Yu et al., 1999) and therefore also cooperates with two structurally diverse ligands.

The aim of the main part of my PhD thesis was to test whether growth factor ligands dictate the need for specific CD44 isoforms in order to activate their respective RTKs or whether a specific RTK requires a specific CD44 isoform. Therefore I studied the EGFR family of RTKs where the ErbB1 receptor is addressed by seven different ligands. By means of downregulation and overexpression studies as well as blocking experiments, I set out to test whether the ligands that address the ErbB1 receptor protein are dependent on CD44v6, CD44v3 or other CD44 isoforms. If so I wanted to find out whether the ErbB ligands dictate the need for these variant

isoforms. In this case, the mechanism of CD44 co-receptor function was to be further evaluated in a physiological and pathological context.

2. Material & Methods

2.1 Materials

2.1.1 General chemicals

Agarose	Peqlab, Erlangen
APS (Ammonium persulfate)	Sigma, Deisenhofen
Aprotinin	Sigma, Deisenhofen
Bromphenol blue	Serva, Heidelberg
BSA (Bovine Serum Albumine)	Serva, Heidelberg
DMSO (Dimethyl sulfoxide)	Fluka, Neu-Ulm
DTT (Dithiothreitol)	Sigma, Deisenhofen
EDTA	Merck, Darmstadt
Ethanol	Roth, Karlsruhe
Ethidium bromide	Sigma, Deisenhofen
G418	GIBCO, Eggenstein
Glycerol	Merck, Darmstadt
HEPES	Roth, Karlsruhe

Isopropanol	Merck, Darmstadt
Leupeptin	Sigma, Deisenhofen
Methanol	Roth, Karlsruhe
Nonidet P-40	Boehringer, Mannheim
PMSF	Sigma, Deisenhofen
Rotiphorese Gel 30: Acrylamide / bis-acrylamide (30% / 0,8 %)	Roth Karlsruhe
SDS (Sodium lauryl sulfate)	Roth, Karlsruhe
TEMED	Sigma, Deisenhofen
Tris Hydrochlorid	Roth, Karlsruhe
Triton-X100	BioRad, München
Tween-20	Serva, Heidelberg

2.1.2 Enzymes

Heparinase II	Sigma
Taq DNA Polymerase	GIBCO
Superscript II Rnase H^- Reverse Transcriptase	GIBCO

2.1.3 Other materials

3MM Whatman paper	Whatman, Maidstone
Immobilon-P membrane	Millipore
ECL western blotting detection reagents	Amersham Life Science
Protein-A Agarose	Calbiochem
Protein-G Agarose	Calbiochem

2.1.4 Growth factors

Amphiregulin	R&D Systems
Betacellulin	R&D Systems
Epidermal Growth Factor	R&D Systems
Epiregulin	R&D Systems
Hepatocyte Growth Factor	R&D Systems
Heparin-binding EGF-like growth factor	R&D Systems
Heregulin	R&D Systems
Transforming Growth Factor Alpha	R&D Systems

2.1.5 Cell culture reagents

Trypsin, 0.25% EDTA	GibcoBRL Life Technologies, Karlsruhe
DMEM	GibcoBRL Life Technologies, Karlsruhe
RPMI 1640	GibcoBRL Life Technologies, Karlsruhe
Foetal calf serum gold	PAA Laboratories GmbH, linz Austria
Penicillin-Streptomycin	Invitrogen
Glutamin	GibcoBRL Life Technologies

2.1.6 Cell lines

Name	**Description**	**Culture Medium**
HT 29	human colon adenocarcinoma line	DMEM, 10% FCS
BSp73AS (AS10)	Human pancreas carcinoma line	RPMI1640, 10% FCS
BSp73ASv6 (ASs6)	AS10 cells transfected with CD44v6	RPMI1640, 10% FCS, 0.3 g/l G418
HeLa	Human caervix carcinoma line	DMEM, 10% FCS
MCF 7	Human mammary pleural effusion cell line	DMEM, 10% FCS
MCF 10A	Human pre-neoplastic mammary cell line	DMEM/F12, 5% horse serum, 20 ng/ml EGF, 0,5 µg/ml Hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, 100 IU/ml Penicillin, 100 µg/ml streptomycin

Ecopack 2-293	Human embryonic kidney cell line used for production of high titer Moloney-based retroviral stocks by transient transfection	DMEM, 10% FCS, 4mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1mM sodium pyruvate

2.1.7 Antibodies

Antibodies	Isotype	Epitope	Source
α-ErbB1	Rabbit IgG1	Detects ErbB1 in H, M	Millipore
α-ErbB2	Rabbit IgG1	Detects ErbB2 in H, M, Mk, R	Millipore
α-Erk (K-23)	Rabbit IgG1	Detects Erk1/2 in H,R,M	Santa Cruz
α-CD44var(v6) clone VFF18	Mouse IgG1	Detects an epitope encoded by exon v6 on the variant portion of human CD44	Bender MedSystems
α-Phospho-ErbB1	Mouse IgG1	Detects phosphorylated ErbB1 in H, R, M, Ca	Millipore
α-Phospho-p44/42 MAP kinase	Rabbit IgG1	Detects phosphorylated Erk1/2 in H,R,M	New England Biolabs
α-CD44v3	Mouse IgG1	Detects an epitope encoded by exon v3 on the variant portion of human CD44	R&D Systems

Hermes 3	Mouse IgG1	Detects all isoforms of CD44 of human origin	Gift of Sirpa Jalkanen, Turku, Finnland
α-VSV-G	Mouse IgG1	Detects the VSV-G tag	Santa Cruz
α-Ezrin	Rabbit IgG1	Detects ezrin in H, M, R, Mk	Cell Signalling

2.1.8 Oligonucleotides

α-CD44v6 siRNA-1: 5' – AGU AGU ACA ACG GAA GAA ATT – 3'
α-CD44v6 siRNA-2: 5' – GGA UAU CGC CAA ACA CCC ATT – 3'
α-CD44v3 siRNA-1: 5' – AGG CAU UGA UGA UGA UGA AUU – 3'
α-CD44v3 siRNA-1: 5' – UGA AGA UGA AAG AGA CAG AUU – 3'

2.1.9 Plasmid constructs

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

Human ErbB1 pBabe puro
The sequence of human ErbB1 was cloned into pBabe puro (Morgenstern & Land, 1990)
Gift from Nancy Hynes, Friedrich Miescher Institute For Biomedical Research, Switzerland

Human ErbB3 pBabe puro
The sequence of human ErbB3 was cloned into pBabe puro (Morgenstern & Land, 1990)
Gift from Nancy Hynes, Friedrich Miescher Institute For Biomedical Research, Switzerland

2.2 Methods

2.2.1 Adherance and invasion assay (gentamicin protection assay)

(The assay was performed as described in Jung et al., 2009)

The adherence of *L. monocytogenes* (EGD or Δ*InlA2*, Δ*InlB2* and Δ*inlAB2*) to and the invasion into HeLa cells were measured with a gentamicin protection assay. For adherence assays, the HeLa cells were infected with log phase bacteria and a multiplicity of infection (moi) of 5 (~5 x 10^6 bacteria /1 x 10^6 cells per well) in DMEM tissue culture medium supplemented with 1% heat-inactivated FCS for 1 h at 37°C in 5% CO_2. For the inhibition studies HeLa cells were pre-incubated with the CD44v6 peptide and control peptide at a concentration of 100 ng/ml, Biwa (anti-CD44v6 antibody) and control mouse IgG at a concentration of 100 µg/ml for 30 min. After 1 h of infection the HeLa cells were washed three times with DMEM and were subsequently lysed by adding 500 ml of 1% Triton X-100. The number of cell-adherent bacteria was determined by plating appropriate dilutions of the lysate onto agar plates. Due to the lysis of the eukaryotic cells in this process, the calculation of cell-adherent bacteria also included bacteria that had invaded into HeLa cells. Therefore, the number of invaded bacteria was subtracted from the numbers of celladherent bacteria to calculate the actual number of adherent bacteria. For invasion assays, the HeLa cells were also infected with log phase *L. monocytogenes* and an moi of 5. Bacteria were incubated for 1 h at 37°C in 5% CO_2, and
washed three times with DMEM. Subsequently, the infected cells were incubated for 2 h in tissue culture medium supplemented with gentamicin (50 µg/ml) to kill extracellular bacteria. After three washes with DMEM, the HeLa cells again were lysed by adding 500 ml of 1% Triton X-100. The number of invasive bacteria was quantified by plating serial dilutions of the lysate onto agar plates.

2.2.2 General cell culture

Cells were cultivated in a humid (95%) atmosphere with 5% CO_2 at 37°C. All handling of the cells was performed under sterile conditions. The cultivated cells were passaged as soon as the reached a level of 80% confluency.

2.2.3 Passaging of cells

After the removal of the growth medium the cells were treated with 0,25% Trypsin/EDTA and incubated for 5 mins at 37°C. The disassociated cells were then taken up in growth medium and spun down at 1200rpm for 3 mins. The supernatant was removed, the cell pellet resuspended in growth medium and plated in new culture plates in the desired dilution.

2.2.4 Freezing down and thawing of cells

Cells were frozen down in special freezing medium (10% DMSO in FCS). The cell suspension was transferred into a pre-chilled cryo tube, kept on ice for a short while and then frozen down at -80 °C.
Frozen cells were thawed rapidly at 37°C in the waterbath and transferred into a new 50 ml falcon with pre-warmed growth medium. The cells were spun down, resuspended in new growth medium and plated on a petri dish.

2.2.5 Blocking with the CD44v6-specific peptide

3 x 10^5 were seeded per well of a 6-well plate and serum-starved for 24 hours. Afterwards the medium inside of each well was reduced to 1 ml and the respective peptide (v6 or ctl peptide) was added at a concentration of 100 ng/ml. The cells then were incubated together with the peptide at 37°C for 5 mins. Ligand concentration for all peptide blocking experiments was 20 ng/ml.

2.2.6 Induction of cells with growth factors

The cells were incubated with the growth factors specified in the particular experiment. Except for the peptide blocking and migration assays, ligand concentration was 5 ng/ml at all times. Prior to induction the cells were treated as indicated in the results section.

2.2.7 Cell lysate preparation

To assay protein expression via Western blot cells were washed 1x with PBS and afterwards lysed in 2x Laemmli sample buffer (160mM Tris-HCl pH 6.8, 4% SDS,

16% glycerol, 0.1M DTT, 0.01% bromphenol blue). The sample was sheared through a 26 gauge needle. In succession the lysates were boiled for 5 mins at 99°C in order to denature the proteins in the sample and then spun down at 10 000 rmp for 1 min. Eventually the samples were loaded onto an SDS-PAGE gel.

2.2.8 SDS-polyacrylamide gel electrophoresis (PAGE)

Proteins were separated electrophoretically based on their size through the Laemmli method (1970). The stacking- (5% acrylamide) and the separating gel (10% and 7.5& respectively acrylamide) were cast after (Maniatis et al, 1989). Samples were run at a voltage of 100V in the stacking and a voltage of 140V in the separating gel. The gel running buffer contained 30.3 g/l tris-base, 144 g/l glycin and 1% SDS.

2.2.9 Western blotting and antibody incubation

After separation by SDS-PAGE the proteins were transferred electronically onto an immobilon membrane (Millipore, type PVDF, pre-incubated in methanol). The transfer took place at a voltage of 20V for 1,5 hours and was buffered with semi-dry transfer buffer (20mM Tris, 192mM glycin, 10% methanol). After successful transfer the membrane was incubated with TBS-0.2 % Tween + 5 % BSA for one hour at room temperature under constant shaking in order to block any unspecific binding of the antibodies.
To detect the proteins of interest the membrane was incubated with the specific antibody in the dilution and conditions specified by the manufacturer (Most of the time this was over night, in TBS-T-0.2% tween + 5 % BSA at a dilution of 1:1000). In succession the membrane was washed 3 times with TBS-T and the membrane was incubated with the secondary HRP-conjugated antibody (at a dilution of 1:2000 in the appropriate blocking solution, see manufacturers specifications).
Detection of the proteins was done by enhanced chemiluminescence.

2.2.10 Co-Immunoprecipitation of ErbB1 and CD44v6

5 x 10^6 cells were seeded in 15 cm plates, serum starved for 24 hours and induced with EGF (5 ng/ml) or left untreated respectively in succession. Afterwards the cells were washed with ice-cold PBS and incubated with low salt RIPA buffer (10mM Trips

pH 7.4, 10mM NaCl, 3mM EDTA, 1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS) on ice for 30 mins. The cells were then scraped off of the plates and transferred into 1,5 ml eppendorf tubes. The samples were spun down for 20 mins at 4°C and 12 000 rpm and the supernatant was transferred to a new tube afterwards. 5 µg of antibody for each ml of cell lysate were added and incubated with the lysates for 1,5 hours at 4°C under constant shaking. 25 µl of each protein-A and protein-G agarose beads were then added to the samples and incubated for 2 more hours at 4°C under constant shaking.
The immuno-complexes were then isolated by centrifugation at 12 000 rpm and 4°C and washed 3x with ice-cold lysis buffer.
The supernatant was then removed and the immuno-complexes were resuspended in 25 µl 2x Laemmli buffer + 1M DTT and boiled at 99°C for 5 mins. Afterwards the supernatant was ready to be loaded onto an SDS-PAGE gel.

2.2.11 Transfection of target cells with siRNA

The particular cells were transfected transiently with Lipofectamin 2000 (Invitrogen) according to the manufacturers instructions.:
3 x 10^5 cells were seeded per well of a 6-well plate, cultivated for 24 hours and then transfected.
For each transfection seperate mixes were prepared:

1. 200 pmol siRNA in 250 µl of serum-free medium
2. 5 µl Lipofectamin 2000 in 250 µl serum free medium

The mixes were incubated for 5 mins at room temperature and afterwards transferred into one common eppendorf tube. After a further incubation period of 20 mins at room temperature the target cells were transfected with the complete mix.
The medium of the transfected cells was changed 6 hours after transfection and substituted with fresh medium. After 24 to 48 hours the cells were serum-starved and afterwards used for the successive experiments.

2.2.12 Transfection of HT29 cells with dominant-negative Ezrin

HT29 cells were transfected with Promofectin (Promocell) according to the manufacturers instructions:
3 x 10^5 cells were seeded per well of a 6-well plate, cultivated for 24 hours and then transfected.
For each transfection seperate mixes were prepared:

1. 4 µg DNA in 250 µl of serum-free medium
2. 10 µl Promofectin in 250 µl serum free medium

The mixes were incubated for 5 mins at room temperature and afterwards transferred into one common eppendorf tube. After a further incubation period of 20 mins at room temperature the target cells were transfected with the full 500 µl of the complete mix.
The medium of the transfected cells was changed 6 hours after transfection and substituted with new one. After 24 to 48 hours the cells were serum-starved and afterwards used for the successive experiments.

2.2.13 Heparinase II treatment of HT29 cells

HT29 cells were seeded at a concentration of 3 x 10^5 per well of a 6-well plate and serum starved 24 hours later. After starvation the medium in the treated 6-wells was reduced to 1 ml and heparinase II at a concentration of 6 U/ml was added. The treated cells were incubated together with the enzyme for 3 hours and afterwards treated directly with the indicated growth factors. Cells were then washed with cold PBS and lysed in 2x Laemmli buffer in order to examine them further.

2.2.14 Migration assay with HeLa cells

2 x 10^5 HeLa cells were seeded in the upper part (insert) of a Boyden chamber (6-well) and serum-starved after 24 hours. 20 ng/ml growth factor were then added to the lower part of the boyden chamber. Afterwards the cells were allowed to migrate over night.
On the following day the cells on the inside of the Boyden chamber insert were removed with a cotton swap (while the cells on the outside of the membrane of the

Boyden chamber insert were left alone). The cells on the membrane of the insert were then washed 3 times with PBS and fixed in 10% Formalin for 10 mins at room temperature. After 3 more washing steps the cells were stained for 15 mins at room temperature with 0.5% crystal violet. After having washed them for 3 more times, the cells were then incubated with 10% acetic acid for aproximately 2 minutes at room temperature and the optical density of the obtained solution was measured at a photometer at OD 600.

2.2.15 Transfection of BSp73AS cells with ErbB1

24 hours prior to transfection the packaging cell line Ecopack-2-293 was seeded in a 6-well plate at a concentration of 2 x 10^5 cells per well.
1,5 hours before transfection, the growth medium was replaced with growth medium containing 25 µM chloroquine.
Each 6-well was then transfected with 4 µg of the ErbB1 or the ErbB3 plasmid DNA by using the CalPhos Mammalian Transfection kit (Clontech). The transfection was done according to the manufacturer's instructions.
After 48 hours, the medium containing the virus was collected and filtered through a 0.45-µm cellulose acetate filter. The virus containing medium was then added to BSp73AS cells that had been seeded at a concentration of 0.5 x 10^5 cells per well 24 hours earlier at a concentration of 1:2 to normal growth medium. Polybrene to a final concentration of 8 µg/ml was added and the cells were incubated for 6-8 hours together with the virus. The virus containing medium was then replaced by normal growth medium and the cells were further incubated over night. Afterwards the cells were serum-starved and treated with the indicated growth factors.

2.2.16 CD44-variant-exon-specific RT-PCR analysis in MCF7 and MCF10A cells

The exon specific RT-PCR was performed as described previously (Orian-Rousseau et al. 2002, König et al. 1996). All primers, but the v8 forward primer (5'-CGC TTC AGC CTA CTG CAA AT-3') were identical to those used by König et al. (1996).

2.2.17 In vivo metastasis assay

All animals were handled according to German regulations for animal experimentation. The animal experiments were approved by the Regierungspräsidium Karlsruhe (35-9185.81/G-83/04). All mice were obtained from Harlan.

1 x 10^6 murine 4T1 cells (diluted in either 20 µg of control peptide or 20 µg of CD44v6 peptide in PBS) were orthotopically injected into the mammary fat pad of female BALB/c mice. In succession, 5 of the mice were injected intraperitoneally with a CD44v6-peptide (20 µg per mouse) and 5 of the mice were injected with an unspecific control peptide (20 µg per mouse) three times a week. After four weeks, the mice were sacrificed and the tumors as well as the lung and lymph nodes of the animals were extracted. These organs were then fixed in 4% Formalin, processed and embedded in paraffin.

3. Results

In my diploma work I showed that the activation of the receptor tyrosine kinase (RTK) Met is dependent on CD44v6 for its activation by Inlb, a bacterial protein used in the invasion process of the bacteria *Listeria monocytogenes*. CD44v6 and its link to the actin cytoskeleton through the ERM protein ezrin are also instrumental for the internalisation of InlB-coated beads into mammalian cells, a system that mimics the host cell invasion by *L.monocytogenes*.

The data collected during my diploma thesis suggested that CD44v6 might also be required for the invasion of host cells through *L. monocytogenes*.

3.1. The invasion of L. monocytogenes into host cells is dependent on CD44v6

To test whether this was the case, I measured the adherence of *L.monocytogenes* to and the invasion into HeLa cells in the presence of CD44v6 blocking reagents. This experiment, like all other experiments in this PhD thesis were repeated at least three times and gave similar results.

HeLa cells were pre-incubated with either a CD44v6 peptide or a CD44v6 antibody, an unspecific control peptide or an unspecific mouse control IgG. The cells were then incubated with bacteria in their log phase of growth for one hour. After infection, the HeLa cells were washed and either incubated with gentamicin containing culture medium to kill any extracellular bacterium or directly lysed. To determine the number of bacteria that had invaded, the gentamicin treated cells were lysed and serial dilutions of the lysates were plated on agar plates. The bacterial colonies on the plates were counted. The number of bacteria adherent to the cells was determined by substracting the bacteria that had invaded from the number of bacteria in the cell lysates that were not treated with gentamicin.

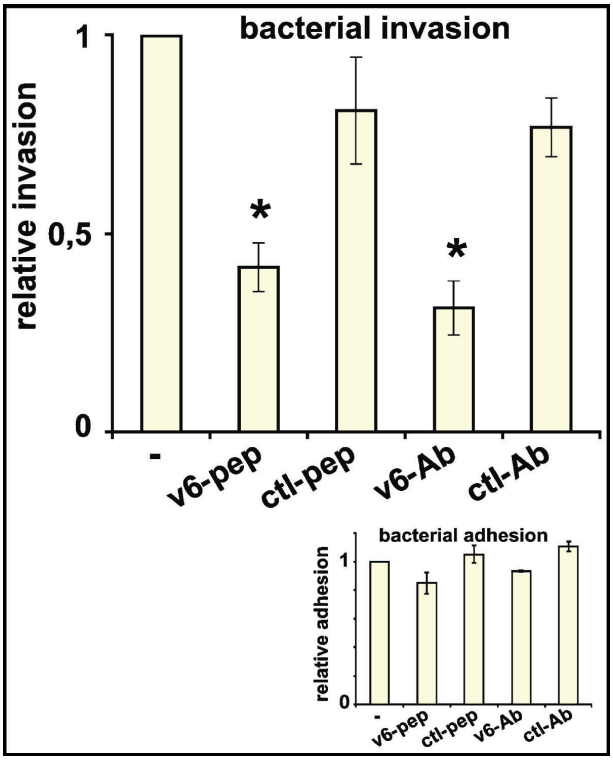

Figure.1: The invasion of HeLa cells through *L. monocytogenes* is dependent on CD44v6

The adherence to and the invasion into HeLa cells of *L. monocytogenes* were evaluated by means of a gentamicin protection assay. The HeLa cells were pre-incubated with the indicated CD44v6 blocking reagents (100 µg/ml for antibodies and 100 ng/ml for peptides). The cells were subsequently infected with log phase *L.monocytogenes* ΔinlA2 at a moi of 5. After 1 h of infection the cells were lysed and dilutions of the internalised or adhered bacteria were plated onto agar plates. The bacterial colonies were counted to determine the number of invasive and adherent bacteria. Where indicated by an asterix, the inhibition was significant.

Under physiological conditions, *L.monocytogenes* invades its host cells using both, the InlA as well as the InlB surface protein. To make sure that the infection of the bacteria was exclusively mediated by the Met receptor, a mutant strain with a chromosomal in-frame deletion in InlA (ΔinlA2) (Lingnau et al, 1995) was used. The invasion of HeLa cells using this strain was still about 60% as compared to wild type bacteria (data not shown). The invasion of a bacterial strain with a chromosomal in-frame deletion in InlB (ΔinlB2) (Lingnau et al., 1995) on the other hand was reduced to 20% of the wild type invasion rate (data not shown). This shows that InlB primarily mediates HeLa cell invasion whereas InlA only plays a marginal role. A double mutant (InlAB2) (Parida et al, 1998) almost did not invaded at all (data not shown).

A 60% reduction of invasion of the ΔinlA bacteria strain into HeLa cells was observed whereas a control peptide showed only a marginal effect (**Figure. 1**). Also in the case of the CD44v6 antibody an inhibition of approximately 70% of the

bacterial invasion could be measured in contrast to the control IgG antibody (**Figure. 1**). An invasion into HeLa cells of the ΔinlB2 mutant strain that lacks InlB was not affected by the v6 blocking reagents (data not shown) indicating that these reagents indeed specifically block the InlB-dependent *L. monocytogenes* invasion of HeLa cells.

In contrast to the drastic inhibition of invasion, the adherence of the ΔinlA bacteria was not significantly influenced by the CD44v6 peptide or the CD44v6 antibody (**Figure. 1 inset**). This indicates that the InlB-dependent invasion process of *L. monocytogenes* itself is dependent on CD44v6 and not the adherence of the bacteria to the cells.

In conclusion, the co-receptor function of CD44v6 is not only required for the activation of the Met RTK by InlB and its downstream signaling but is essential for the InlB-dependent entry process of *L. monocytogenes* into host cells.

These data and other results by our group indicate that two different isoforms of CD44, namely CD44v6 and CD44v3, collaborate with several RTKs activated by distinct ligands, such as HGF, InlB, VEGF-A, FGF and HB-EGF. The recruitment of CD44 isoforms for such a variety of RTKs and ligands might be due to two reasons. The recruitment of CD44 isoforms might be RTK-specific. Another possibility is that CD44 isoforms might act in a ligand-specific manner. In order to discriminate between these two possibilities I studied the EGFR family of RTKs where one receptor, namely ErbB1, is addressed by several different ligands.

3.2. EGF-induced activation of the ErbB receptors is dependent on CD44v6

In order to test whether CD44v6 plays a role in the activation of the ErbB family of receptors a peptide blocking experiment was performed in HT29 cells. The fact that this cell line expresses all ErbB receptors (Wu et al, 2009) and several CD44 variants including also exon v3 and v6 (Orian-Rousseau et al., 2002) makes it a perfect tool to evaluate the co-receptor function of CD44 proteins for the ErbB family.

The first ErbB1-ligand that I evaluated was EGF. To test whether the activation of the ErbB receptors by EGF was dependent on CD44v6, HT29 cells were pre-incubated with a v6-peptide that specifically inhibits the co-receptor function of CD44v6 (see chapter 1.2.7.1) or an unspecific control peptide. The cells were serum starved for 24 hours and induced with EGF. Phosphorylation of the Erk-Kinase, a

downstream target of the ErbB receptor family, was used as a read-out for ErbB-receptor activation. HGF was used as a control to test the blocking efficiency of the CD44v6 peptide since the induction of the Erk kinase through Met is dependent on CD44v6 (Orian-Rousseau et al., 2002).

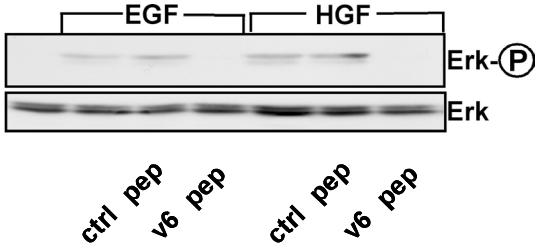

Figure.2: EGF-dependent induction of the ErbB receptors can be blocked by a CD44v6 specific peptide.
Serum-starved HT29 cells were pre-incubated for 5 min with 100 ng/ml of a CD44v6-specific peptide or a control peptide. Afterwards the cells were induced with 20 ng/ml of EGF and lysed. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF- and HGF-induced Erk-kinase phopshorylation.

EGF or HGF-induction of the HT29 cells lead to the phosphorylation of the Erk-kinase. The EGF- as well as HGF-dependent Erk- phosphorylation could be inhibited by pre-incubation with the CD44v6 peptide whereas a control peptide showed no effect. This suggests that EGF, is dependent on CD44v6 in order to activate the ErbB receptors (**Figure. 2**). This is similar to the situation observed in the case of Met and its ligand HGF.

3.3 The requirement for CD44 is receptor-independent

Besides EGF, five other ligands, namely AR, BC, ER, HB-EGF, and TGF-α, can activate the ErbB1 receptor. EGF, AR and TGF-α bind to either an ErbB1-homo- or an ErbB1/2-heterodimer. In addition to these dimers, BC, ER and HB-EGF can also bind to an Erb4/4 homo- or an ErbB2/4 heterodimer. In this section I investigated whether the activation of the ErbB receptors by the indicated ligands is also dependent on CD44v6.

To this end, the same blocking reagent and similar conditions as in section 2.2 were used. HT29 cells were serum-starved for 24 hours and pre-treated with the v6-specific or a control peptide. They were then induced with the indicated ErbB ligands. Erk-kinase phosphorylation was used as a read-out for receptor activation.

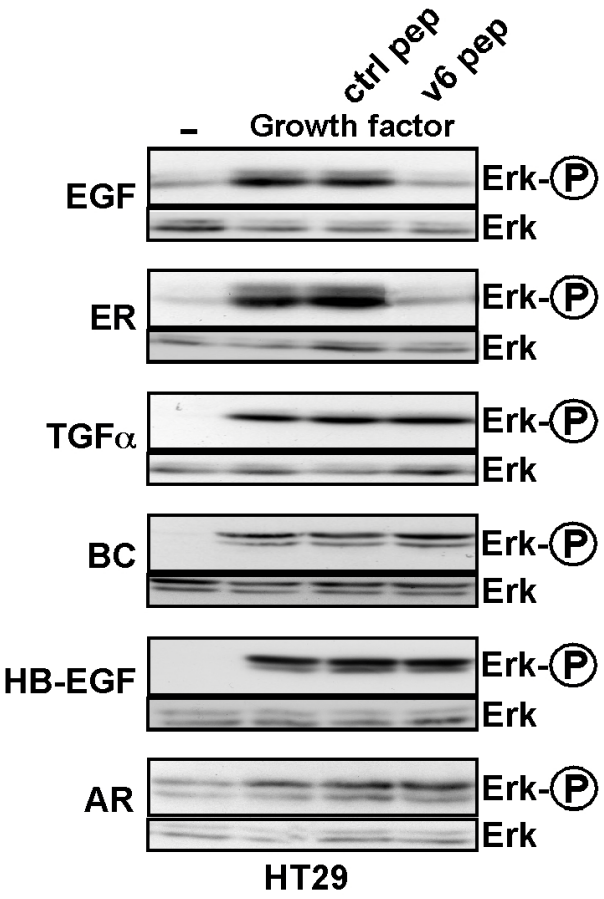

Figure.3: In contrast to EGF and ER, induction of the ErbB-receptors via TGF-α, BC, Her, HB-EGF or AR is completely independent of CD44v6
Serum-starved HT29 cells were pre-incubated for 5 min with 100ng/ml of a CD44v6-specific peptide or a control peptide. Afterwards the cells were induced with 20 ng/ml of various ErbB ligands as indicated. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect ErbB-ligands-induced Erk-kinase phopshorylation.

All ligands could activate the Erk-kinase. EGF- as well as ER-dependent Erk-phosphorylation was inhibited by pre-incubation of HT29 cells with the CD44v6 peptide whereas a control peptide showed no effect (**Figure. 3**). This shows that, besides EGF, ER is another ErbB1 ligand that is dependent on CD44v6 as a co-receptor. In contrast, AR, BC, HB-EGF and TGF-α-induced activation of the ErbB receptors could not be inhibited by blocking CD44v6 (**Figure. 3**). These ligands are independent of CD44v6 for their induction of the ErbB receptors. This is especially striking, since EGF and TGF-α address the same receptor pairs (ErbB1/1 or ErbB1/2). These data suggest that the specific CD44 isoform used as a co-receptor for ErbB-activation is determined by the ligand that activates the ErbB receptors and not by the receptor proteins themselves.

Using the CD44v6 peptide as a blocking reagent, EGF- as well as ER-dependent activation of the ErbB family was shown to be inhibited. To further confirm that EGF and ER require CD44v6 as a co-receptor the effect of the downregulation of CD44v6 expression was evaluated. HT29 cells were transfected with either CD44v6-specific siRNA or an unspecific control siRNA. HeLa cells were used in addition and treated in a similar way to rule out that our findings are cell type specific. Then the cells were serum-starved for 24 hours followed by induction with either EGF or ER.

In addition to Erk- phosphorylation, ErbB1 receptor phosphorylation was used as a read-out for the receptor activation. By probing for the ErbB1-phosphorylation status it was possible to determine whether a block in ErbB signaling was only affecting downstream targets like the Erk-kinase or also the ErbB1 receptor itself.

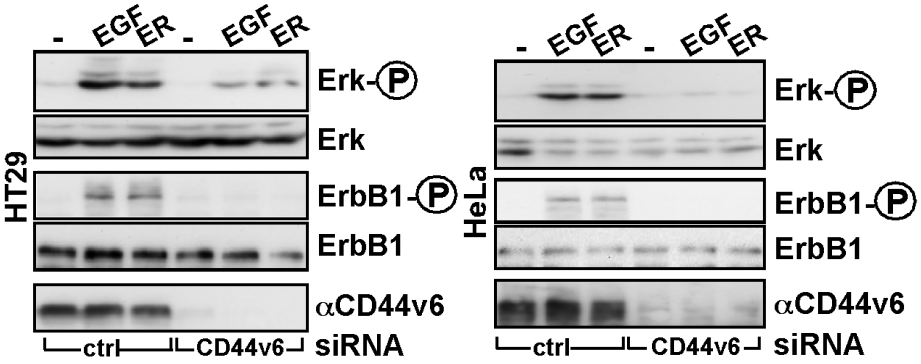

Figure.4: EGF- and ER-dependent induction of the ErbB receptors can be abolished by abrogating the expression of CD44v6 by means of siRNA
HT29 cells were transfected with CD44v6-specific siRNA or control siRNA as indicated. After transfection, the cells were serum starved for 24h. They were then induced with 5 ng/ml of EGF or ER as indicated. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF- and ER and -induced ErbB1 and Erk phopshorylation.

EGF and ER induction of the cells lead to ErbB1 and Erk phosphorylation. Upon transfection of CD44v6 specific siRNA into HT29 cells, the expression of CD44v6 was reduced as indicated by the western blot probed with a CD44v6 specific antibody (αCD44v6). This lead to an inhibition of EGF- as well as ER-induced signaling on the ErbB1- as well as on the Erk-level. A control-siRNA showed no effect on CD44 expression or ErbB signaling (**Figure. 4**). The downregulation of CD44v6 in HeLa cells also lead to an inhibition of EGF- as well as ER-induced ErbB signaling showing that the requirement for CD44v6 is not cell type specific (**Figure. 4**).

Using the CD44v6 peptide as a blocking reagent TGF-α induced phosphorylation of the Erk kinase could not be inhibited. To confirm that TGF-α does

not require CD44v6 as a co-receptor for its induction of the ErbB receptor family and to test whether TGF-α might possibly be completely independent of CD44, the effect of the downregulation of CD44v6 and the downregulation of all CD44 isoforms in HT29 cells was tested in the same experiment. Different HT29 cells were transfected either with CD44v6 specific siRNA, siRNA against all CD44 proteins (CD44pan), or an unspecific control siRNA. The cells were then serum-starved and induced with TGF-α or EGF. EGF was used as a control for the inhibiting effect of the CD44 downregulation. After growth factor induction, the activation of the ErbB family was measured on the level of ErbB1 and Erk.

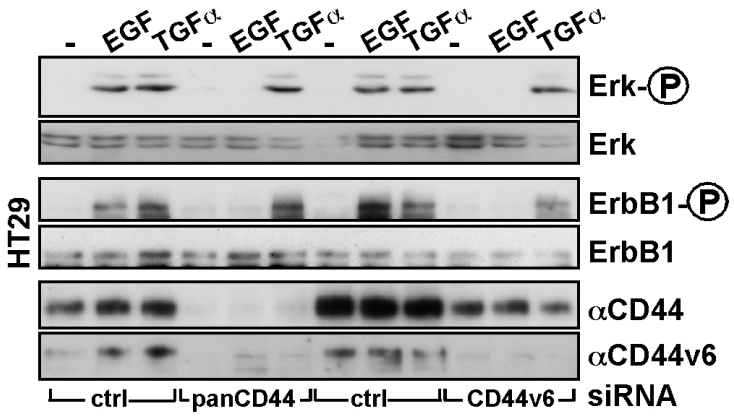

Figure.5: Induction of the ErbB-receptors via TGF-α is independent of CD44
HT29 cells were transfected with CD44v6-specific siRNA or control siRNA as indicated. After transfection, the cells were starved for 24h. They were then induced with 5 ng/ml of EGF or TGF-α as indicated. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF- and ER and -induced ErbB1 and Erk phopshorylation.

EGF as well as TGF-α induction of the HT29 cells lead to ErbB1- as well as Erk-phosphorylation. Upon down-regulation of CD44v6 a block in EGF signaling on the ErbB1 as well as on the Erk-level could be observed (**Figure. 5**). TGF-α-dependent signaling was not inhibited by a down-regulation of CD44v6. Even the transfection of CD44 pan siRNA did not lead to a block of TGF-α-dependent signaling although the expression of all CD44 isoforms was inhibited. As expected, EGF-dependent signaling could be abolished by down-regulation of all CD44 isoforms. An unspecific siRNA did not show any effect on either CD44 expression or ErbB receptor activation (**Figure. 5**).

Consistent with my previous data, induction of the ErbB receptors through TGF-α is not dependent on CD44v6. Moreover, activation of the ErbB receptors through TGF-α is independent of CD44, as even a complete down-regulation of all CD44 proteins does not abolish its ErbB activation.

3.4 HB-EGF-induced activation of the ErbB receptors is dependent on CD44v3

TGF-α is not the only ErbB1 ligand that is independent of CD44v6 for its induction of the ErbB receptors. Using the CD44v6 peptide I was not able to block BC, AR or HB-EGF-dependent activation of the Erk-kinase. HB-EGF and AR are heparan-binding growth factors (Cook et al, 1991; Higashiyama et al, 1992). Their binding to heparan might be instrumental for their activation of the ErbB receptors.

In order to test whether heparan sulphation is involved in the process of AR- and HB-EGF induced ErbB receptor activation, serum-starved HT29 cells were treated with heparinase, an enzyme that cleaves off heparin-sulphate of the surface of target cells. The cells were then induced with HB-EGF or AR. HGF was used as a control since for its activation of Met it is dependent on CD44v6, an isoform that is not heparin-sulphated (Orian-Rousseau et al., 2002). Therefore, removal of heparan sulphate should have no effect. The Erk-kinase was used as a read-out for receptor-activation since it is activated by Met- as well as by the ErbB receptors.

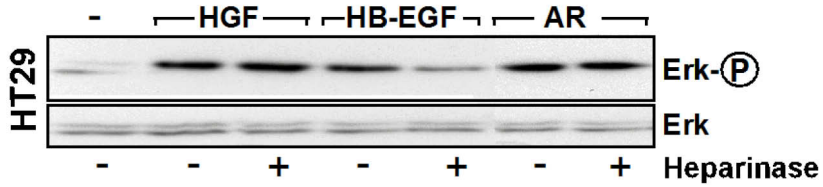

Figure.6: Induction of the ErbB-receptors via HB-EGF but not AR is dependent on heparin-sulphation

Serum-starved HT29 cells were pre-treated with heparinase at a concentration of 6U/ml for 3h and then induced with 5 ng/ml of HB-EGF or AR (HGF was used as a control). The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect the ErbB-ligands-induced Erk phopshorylation.

Induction of HT29 cells with HGF, HB-EGF or AR lead to Erk-phosphorylation. Upon Heparinase treatment, the activation of the ErbB-receptors through HB-EGF but not AR was abrogated (**Figure. 6**). This shows that for its induction of the ErbB receptors, HB-EGF is dependent on heparan-sulphation whereas AR is not.

In the CD44 protein family, CD44v3 is the only variant of CD44 that carries heparan-sulphate side chains. HB-EGF might bind to these heparan-sulphate side chains in order to activate the ErbB-receptors. And indeed, such a binding has already been shown (Bennett et al., 1995). In order to test whether CD44v3 mediates HB-EGF-dependent activation of the ErbB receptors, CD44v3 was down-regulated by RNAi technology.

HT29 cells were transfected with CD44v3-specific siRNA or an unspecific control siRNA. Then the cells were serum-starved for 24 hours followed by their induction with HB-EGF. EGF was used as a control since it is dependent on CD44v6 and its activation of the ErbB receptors should not be affected by the down-regulation of CD44v3. ErbB1- and Erk-phosphorylation was used as read-out for receptor activation.

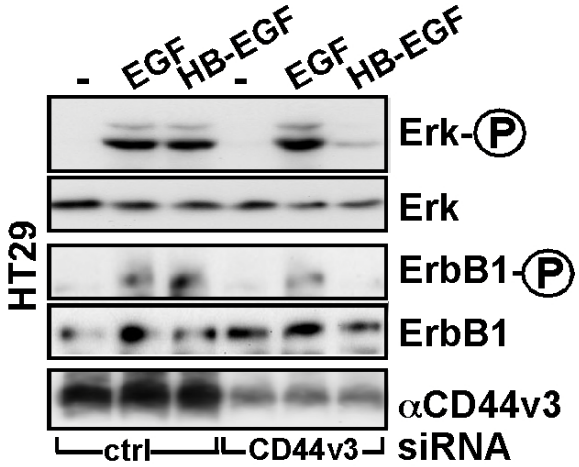

Figure.7: Induction of the ErbB-receptors via HB-EGF is dependent on CD44v3
HT29 cells were transfected with CD44v3-specific siRNA or control siRNA as indicated. After transfection, the cells were starved for 24h. They were then induced with 5 ng/ml of EGF or HB-EGF as indicated. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF- and HB-EGF and -induced ErbB1 and Erk phopshorylation.

Induction of the HT29 cells with EGF or HB-EGF lead to phosphorylation of the ErbB1 receptor and of the Erk-kinase. Upon downregulation of CD44v3 by means of siRNA the induction of the ErbB receptors through HB-EGF was blocked, whereas a control siRNA had no effect (**Figure. 7**). This suggests that HB-EGF is dependent on CD44v3 for its ErbB1-stimulating activity. In contrast, EGF is independent of CD44v3 (**Figure. 7**). In conclusion, depending on the ligand, specific CD44 variant isoforms can act as co-receptors for the same ErbB-receptor.

3.5 Evaluation of ErbB1-homodimer signaling

In order to activate the ErbB1 receptor, EGF, ER, HB-EGF and TGF-α bind to ErbB1 homodimers or ErbB1/2 heterodimers. ER and HB-EGF can additionally bind to an ErbB 4/4 homo- or an ErbB2/4 heterodimer. The cell lines that were used for my experiments (HeLa and HT29 cells) express all ErbB receptors. Under these

conditions the activation of the ErbB1 receptors through the indicated ligands might be mediated by ErbB homodimers as well as heterodimers. To undoubtedly study the effect of ligand induction on an ErbB1 homodimer, I changed to the BSp73AS pancreatic cell system. In these cells none of the four ErbB family members and only the standard isoform of CD44 (which includes no variant exon) are expressed. I introduced either the ErbB1 or the ErbB3 receptor into several transfected BSp73AS cell lines expressing specific CD44 variants (Rudy et al, 1993; Seiter et al, 1993; Sleeman et al, 1997; Sleeman et al, 1996b). ErbB3 is not able to bind to any of the ligands tested here and therefore was used as a control for infection.

Due to the very low transfection efficiency of the BSp73AS, I used a retroviral infection system. The ErbB1 and ErbB3 gene sequences were inserted into a lentiviral expression vector that was then introduced into the packaging cell line Ecopack 2-293. Ecopack 2-293 cells stably expresses the viral gag, pol, and env genes that are necessary for virus particle formation and replication. The retroviral expression vector provides the packaging signal, transcription and processing elements, and the target gene (ErbB1 or ErbB3). After the transfection of the retroviral vector into the ecopack 2 cells, they produce high-titer, replication-incompetent virus containing the gene of interest. This virus is then collected and can infect target cells and transmit target genes; however, it cannot replicate within the infected cells because the viral structural genes are missing.

After 48 hours, the cell medium containing the virus was collected and added to either BSp73AS cells that only express CD44s (AS cells) or to cells that express CD44s together with CD44v6 (ASs6 cells). These cells were serum-starved followed by their induction with either EGF or TGF-α. The expression of the ErbB1 receptor was detected by means of an ErbB1-specific antibody. ErbB1- and Erk-phosphorylation was then analyzed in ErbB1- and control-transfected cells in the presence or absence of EGF or TGF- α.

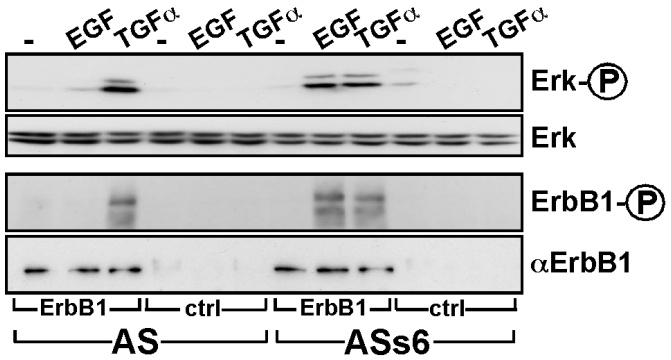

Figure.8: EGF can induce ErbB1 homodimers only in cells expressing CD44v6 whereas TGF-α can do so independently of CD44v6 expression
AS cells and the ASs6 cells were transfected with the ErbB1 expression vector receptor or a control vector by means of the retroviral ecopack 2-293 system as described in Material and Methods. Afterwards the transfected cells were starved and induced with 5 ng/ml of EGF or TGF-α. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF- and TGFa and -induced ErbB1 and Erk phopshorylation.

In AS- as well as in ASs6 cells expression of ErbB1 was detected. In both ErbB1-expressing cell lines TGF-α induced the activation of the ErbB1 receptor and of the Erk kinase. EGF on the other hand was only able to activate ErbB1 in ASs6 cells where CD44v6 is present. EGF is dependent on CD44v6 for the induction of ErbB1 homodimers. In the control cells no receptor activation could be measured (**Figure. 8**).

To test the requirement of CD44v6 for ER-based ErbB1-homodimer-activation, similar procedures as described above were performed. Cells were also induced with TGF-α as a control for an ErbB1 activation that is independent of CD44.

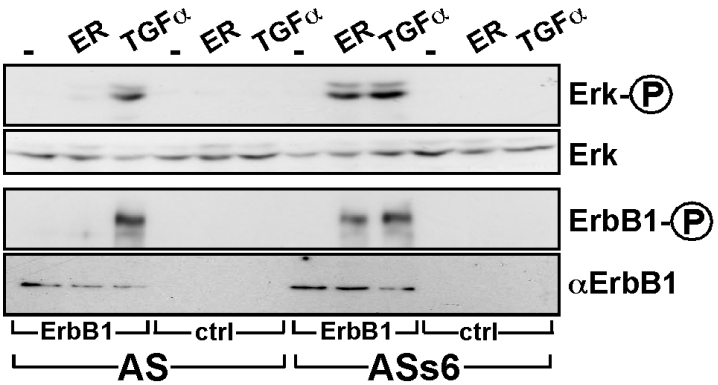

Figure.9: ER can only induce ErbB1 homodimers in cells expressing CD44v6
AS and ASs6 cells were transfected with the ErbB1 expression vector receptor or a control vector by means of the retroviral ecopack 2-293 system as described in Material and Methods. Afterwards the transfected cells were starved and induced with 5 ng/ml of ER or TGF-α. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect ER- and TGFa and -induced ErbB1 and Erk phopshorylation.

Similar to EGF, ER could only induce ErbB1 activation in cells that express CD44v6, whereas TGF-α was able to activate the ErbB1 receptor independently of CD44v6 expression (**Figure. 9**). ER is dependent on CD44v6 for the induction of ErbB1 homodimers. Control cells showed no receptor-activation.

To test whether HB-EGF can activate ErbB1-homodimers in the presence of CD44v3, ASs6 cells that express CD44s together with CD44v6 and cells that express

CD44s together with one isoform of CD44 that includes the sequences of all of its variant exons (ASv1-10) were infected with the ErbB1 receptor protein. The cells were then serum-starved and induced with either HB-EGF or EGF (as a control for CD44v6-dependent ErbB1 activation). The expression of the ErbB1-receptor was then detected and its activation evaluated by measuring ErbB1- or Erk-phosphorylation.

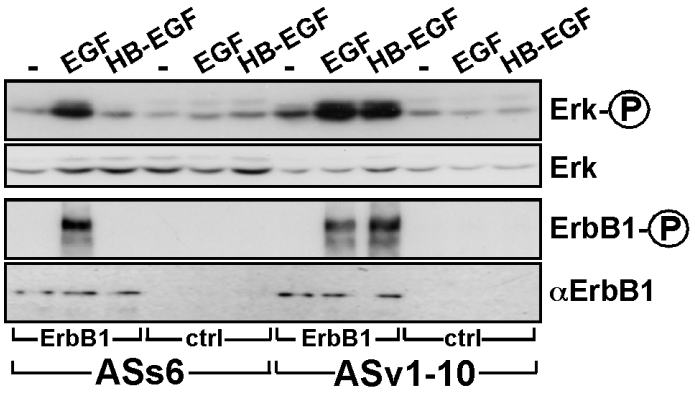

Figure.10: HB-EGF can induce ErbB1 homodimers only in cells expressing CD44v3

Ass6 cells and ASv1-v10 cells were transfected with the ErbB1 expression vector receptor or a control vector by means of the retroviral ecopack 2-293 system as described in Material and Methods. Afterwards the transfected cells were starved and induced with 5 ng/ml of EGF or HB-EGF. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF- and HB-EGF and -induced ErbB1 and Erk phopshorylation.

In cells that express CD44v1-10, HB-EGF could induce the ErbB1 receptor while it could not do so in the ASs6 cells where CD44v3 is absent. EGF was able to induce ErbB1 in ASs6 cells as well as in ASv1-10 cells since the v6 sequence is present in both transfectants. The control cells showed no receptor activation (**Figure. 10**). To directly address the CD44v3 isoforms, a CD44v3 expression vector has been cloned and will further be tested in similar experiments.

In summary, my data so far clearly indicate that ligands of the ErbB receptor family make use of specific variant isoforms of CD44 in order to induce their cognate receptors. While TGF-α appears to be independent of the CD44 family, EGF and ER depend on CD44v6 for their induction of the ErbB receptors. HB-EGF is dependent on CD44v3. The function of CD44 as a co-receptor is therefore strictly dependent on the ligand that induce the RTK and not on the RTK itself.

3.6 Blocking the activation of the ErbB family by inhibiting Ezrin

For the activation of the RTKs Met and VEGFR-2, CD44v6 has a two-fold function. First the extracellular domain of CD44v6 is involved in the presentation process of the authentic ligands of these RTKs (Orian-Rousseau et al., 2002; Tremmel et al., 2009). Secondly, the intracellular part of CD44v6 is used to enable downstream signaling. CD44v6 binds to the ERM protein ezrin that in addition binds to the actin cytoskeleton (Orian-Rousseau et al., 2002; Tremmel et al., 2009).

Does the intracellular domain of CD44 also mediate downstream signaling from the ErbB receptor family? If so, does the downstream signaling depend on ERM proteins and the cytoskeleton? In order to test this assumption, an RNAi experiment was performed in HT29 cells. Amongst the ERM proteins, ezrin is the most abundantly expressed one in these cells (Orian-Rousseau et al., 2007). Specific siRNA against ezrin was transfected into the HT29 cells in order to repress the expression of this ERM-protein whereas an unspecific siRNA was used as a control. The cells were then starved for 24 hours and induced with EGF, ER, TGF-α or HB-EGF in order to test whether an activation of the ErbB-downstream target Erk was still possible. HGF induction was used as a control.

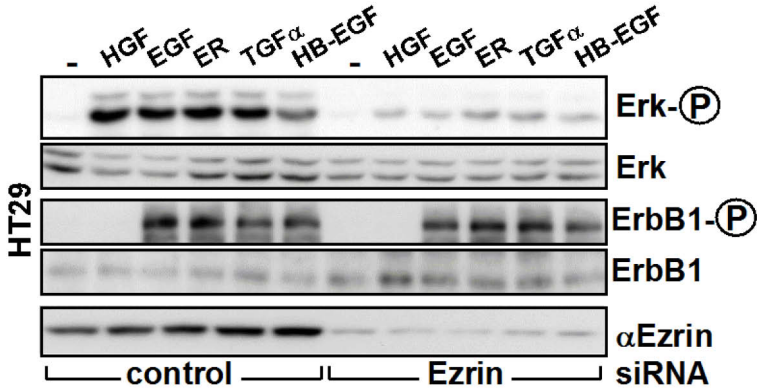

Figure.11: The downstream signaling of the ErbB receptors is dependent on Ezrin

HT29 cells were transfected with Ezrin-specific siRNA or control siRNA as indicated. After transfection, the cells were starved for 24h. They were then induced with 5 ng/ml of ErbB ligands as indicated. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect ErbB ligands-induced ErbB1 and Erk phopshorylation.

Upon downregulation of ezrin, EGF, ER, and HB-EGF-induced downstream signaling to the Erk kinase was inhibited, while there was no effect on the activation of the ErbB1 receptor itself (**Figure. 11**). In the cells transfected with a control siRNA, no inhibition of Erk was observed. Strikingly, even though CD44-independent TGF-α-induced signaling was blocked after downregulation of ezrin whereas ErbB1 activation was not inhibited (**Figure. 11**). This suggests that although TGF-α can induce the ErbB-receptors independently of CD44 its signal transduction to downstream targets like the Erk kinase depends on ezrin.

Altogether, the downstream signaling of all tested ErbB ligands is dependent on this ERM-protein.

For the Met and VEGFR-2 receptor (Orian-Rousseau et al., 2007; Tremmel et al., 2009), the link between ezrin and actin is required for downstream signaling. To evaluate whether this was also the case for the ErbB receptors, an ezrin-construct truncated in the last 29 aminoacids, was used. The protein encoded by this construct is unable to bind to the actin cytoskeleton and its dominant-negative function would suggest that a link between ezrin and actin is indeed required for ErbB signaling.

HT29 cells were transfected with the truncated ezrin construct or with the empty vector as an unspecific control. These cells were serum-starved and then induced with the ErbB1-ligands EGF, ER, TGF-α and HB-EGF. HGF was used as a control. The phosphorylation of the ErbB1 receptor and the Erk kinase was measured.

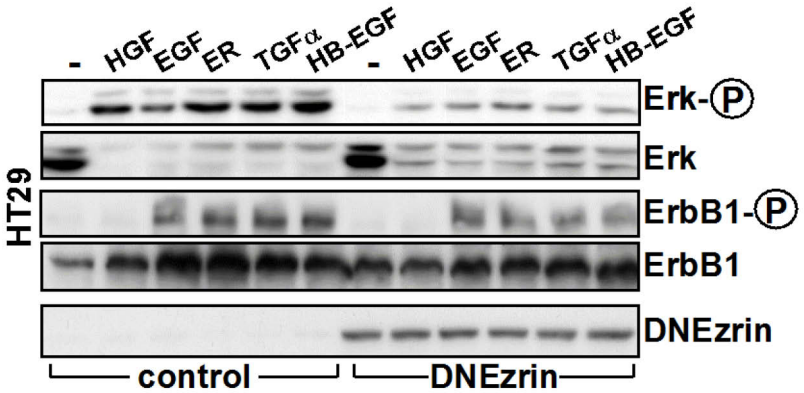

Figure.12: The downstream signaling of the ErbB receptors is dependent on Ezrin binding to the actin cytoskeleton

HT29 cells were transfected with an Ezrin-dominant negative construct or a control empty vector as indicated. After transfection, the cells were starved for 24h. They were then induced with 5 ng/ml of ErbB ligands as indicated. The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect ErbB ligands-induced ErbB1 and Erk phopshorylation.

Upon transfection of a truncated ezrin construct the downstream signaling induced by EGF, ER, HB-EGF and TGF-α was blocked whereas the ErbB1 activation itself was not abolished. In contrast, transfection with the empty vector did not have any effect (**Figure. 12**). This suggests that, EGF, ER, HB-EGF and TGF-α require the binding of ezrin to the actin cytoskeleton for signal transduction.

3.7 The role of CD44v6 in migration

The ErbB receptors have been postulated to play an important role in proliferation and migration (Normanno et al, 2005). By means of the CD44v6 peptide, I tested whether migration of HeLa cells induced by the ErbB ligands EGF and ER was dependent on CD44v6. TGF-α was used as a control as this ligand is not dependent on CD44 isoforms and TGF-α -induced migration should not be blocked. The experiment was performed in a Boyden chamber containing two chambers (an upper and a lower chamber) that are separated by a membrane. This membrane contains 8μm pores. Target cells were seeded into the upper chamber, whereas the lower chamber contained the chemoattractants, i.e. the ErbB ligands. In order to reach the chemoattractants in the lower chamber, the cells had to cross the membrane. Cells that were still present on the top of the membrane were wiped off using a cotton swab. Target cells that adhered to the lower side of the membrane were stained by crystal violet. They were then lysed and the staining was measured with a photometer.

HeLa cells were seeded in the upper compartment of the Boyden chamber, serum-starved, treated with the CD44v6 peptide and were then allowed to migrate through the pores of the membrane into the lower compartment, in which the ErbB ligands were present. The cells on the lower side of the membrane between the two compartments were fixed after their migration and stained with crystal violet. The number of cells that had migrated to the lower side of the membrane was determined after lysis of the cells using in a photometer. Statistical analysis was performed and presented as a diagram.

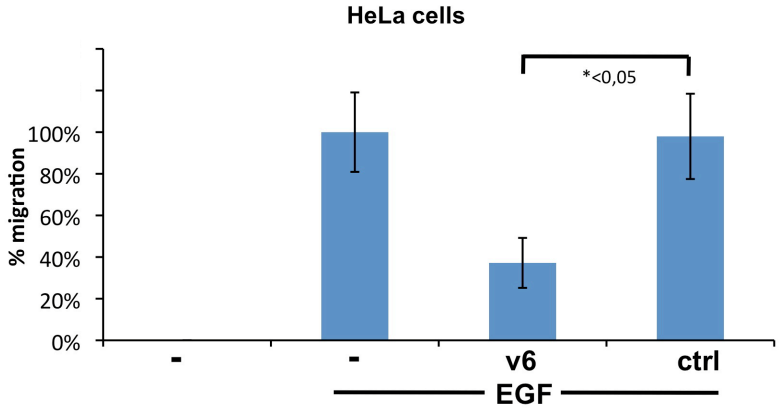

Figure.13: Migration induced through EGF is blocked by application of the v6-peptide
Hela cells were seeded in a Boyden chamber. The cells were serum-starved, pre-incubated with 100ng/ml of a CD44v6-specific peptide or a control peptide and afterwards treated with 20 ng/ml of EGF. After 24h , the migration of the treated cells was measured by means of a crystal violet staining. The cells were fixed, stained and lysed. The absorbance was measured at 600nm. Each determination represents the average of three individual wells. Error bars represent standard deviation (s.d.).
Data presented are representative of at least three separate experiments.

The HeLa cells responded to EGF and migration was observed. Control-peptide treated cells behaved like untreated cells. The v6-peptide treated cells showed a 60% reduction in migration . Thus, EGF-induced migration of HeLa cells can be significantly blocked by inhibiting CD44v6 (**Figure. 13**).

In the case of ER, a similar experiment was performed. The results were comparable to that obtained in the case of EGF (**Figure. 14**).

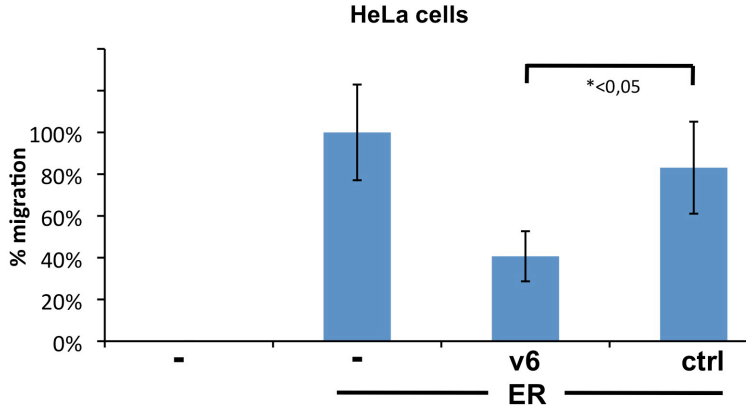

Figure.14: Migration induced through ER is blocked by application of the v6-peptide
Hela cells were seeded in a Boyden chamber. The cells were serum-starved, pre-incubated with 100ng/ml of a CD44v6-specific peptide or a control peptide and afterwards treated with 20 ng/ml of EGF. After 24h , the migration of the treated cells was measured by means of a crystal violet staining. The cells were fixed, stained and lysed. The absorbance was measured at 600nm. Each determination

represents the average of three individual wells. Error bars represent standard deviation (s.d.).
Data presented are representative of at least three separate experiments.

Then the experiment was repeated using TGF-α as a chemoattractant. No inhibition of migration was observed upon treatment with the CD44v6 peptide (**Figure. 15**) as expected.

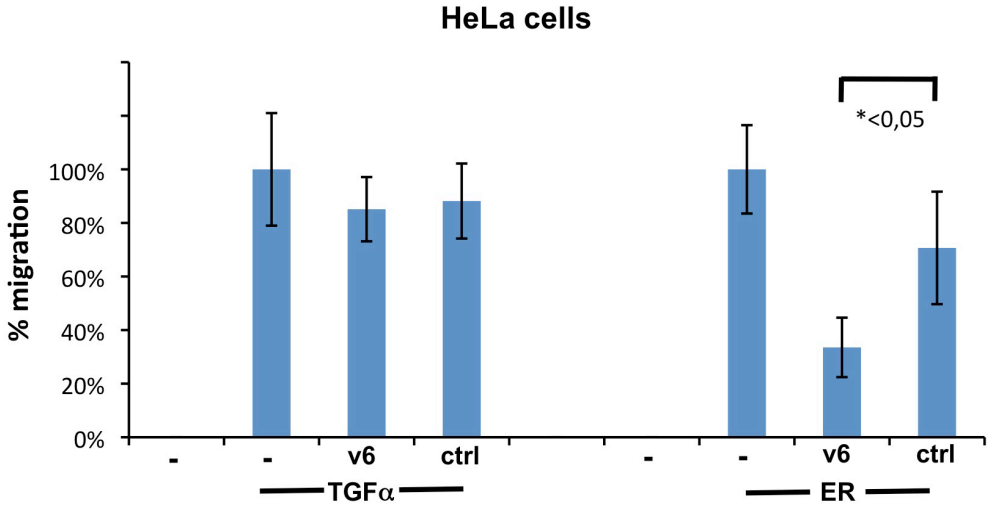

Figure.15: Migration induced through TGF-α is independent of CD44v6
Hela cells were seeded in a boyden chamber. The cells were serum-starved, pre-incubated with 100ng/ml of a CD44v6-specific peptide or a control peptide and afterwards treated with 20 ng/ml of EGF. After 24h , the migration of the treated cells was measured by means of a crystal violet staining. The cells were fixed, stained and lysed. The absorbance was measured at 600nm. Each determination represents the average of three individual wells and error bars represent standard deviation (s.d.).
Data presented are representative of at least three separate experiments.

Altogether, these results show that the migration induced by EGF and ER but not TGF-α is dependent on CD44v6.

3.8 The role of CD44v6 in breast cancer

The ErbB receptors play a paramount role in human breast cancer where the activation of the ErbB receptors, particularly ErbB1 and 2 have been shown to be deregulated (Hynes & MacDonald, 2009). The cells used in my experiments so far were colon- or cervix-carcinoma lines. In order to address the role of CD44v6 in breast cancer, MCF7 and MCF10A cells were used. MCF7 cells are pleural effusion cells from an invasive breast ductal carcinoma whereas MCF10A cells are pre-neoplastic mammary epithelial cells.

The expression of CD44 in these cell lines was examined by a run-off PCR analysis. MCF7 and MCF10A cells were lysed, the total RNA was isolated from the lysates and converted into cDNA via reverse transcription. Then PCR reactions were performed with specific forward primers for each variable exon of CD44 and one common backward primer in the constant region of CD44 (see illustration 6).

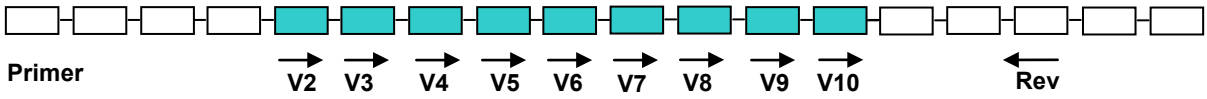

Illustration 6: Scheme of the primer pairs used for the detection of of CD44 mRNA by PCR

A run-off PCR permits the determination of both structure and approximate abundance of CD44 isoforms in the cells. The size of a specific band corresponds to the exons that are expressed between the two respective primers. Consequently, if the bands corresponding to adjacent exons form a ladder it indicates that they are expressed together in a larger isoform. A ladder formed for example by v2 to v10 suggests that the CD44 variants v2-10 are expressed as one transcript.

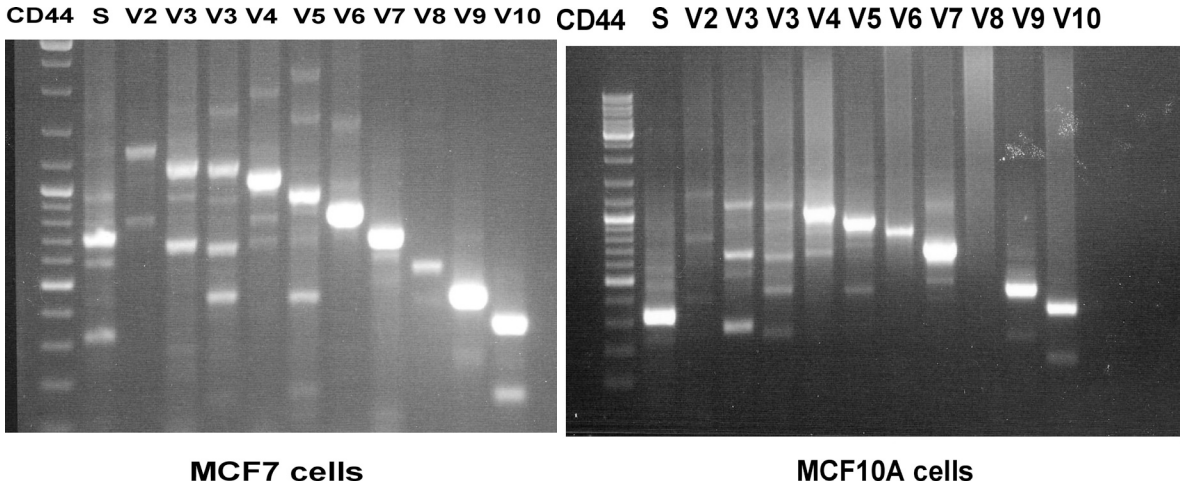

Figure.16: Detection of CD44 by PCR. MCF7 as well as MCF10A cells predominantly express CD44v2-v10 in one long isoform
The expression of CD44 transcripts was tested by run-off PCR. The total RNA of the target cells was isolated, transcribed into cDNA and the expression of CD44 variant transcripts was detected by PCR via different variant-exon specific forward primers and one common backward primer specific for a region in the constant part of CD44.

MCF7 as well as MCF10A cells express CD44v2-10 predominantly in one large transcript as well as several other smaller transcripts (**Figure. 16**). Thus, these

cells not only express the ErbB1 receptor (Gramlich et al, 1993; Rodriguez-Fragoso et al, 2009) but also CD44 variants that could act as co-receptors for the ErbB receptors.

To test whether CD44v6 acts as a co-receptor in MCF7 or MCF10A cells, I performed a peptide blocking experiment similar to those described in section 2.2. The cells were serum-starved for 24h and then pre-incubated with a v6 peptide or an unspecific control peptide. Then, the cells were induced with EGF or HGF as a control. As a read-out for ErbB-activation, the phosphorylation of the Erk-kinase was used.

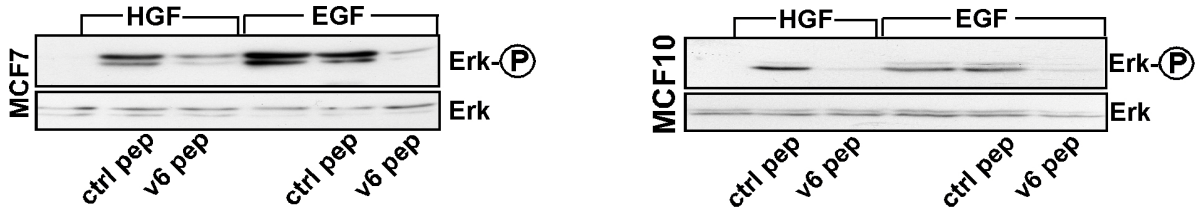

Figure. 17: CD44v6 acts as a co-receptor for EGF-dependent ErbB induction in breast cancer cells.
Serum-starved MCF7 and MCF10 cells were pre-incubated for 5 min with 100ng/ml of a CD44v6-specific peptide or a control peptide. Afterwards the cells were induced with 20 ng/ml of EGF.
The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF- induced Erk-kinase phopshorylation.

In MCF7 as well as in MCF10 cells, the activation of the ErbB receptors by EGF could be blocked by a CD44v6-specific peptide, whereas an unspecific control peptide showed no effect. CD44v6 is required as a co-receptor for EGF-induced ErbB-activation in these breast cancer cells (**Figure. 17**).

To further confirm this result, the CD44v6 expression was downregulated in MCF7 cells by CD44v6-specific siRNA.

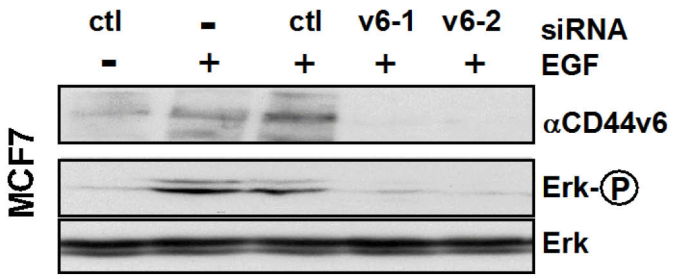

Figure. 18: CD44v6 acts as a co-receptor for EGF-dependent ErbB induction in breast cancer cells.
Serum-starved MCF7 cells were treated with CD44v6-specific siRNA or control siRNA respectively. After transfection, the cells were starved for 24h. They were then induced with 5 ng/ml of EGF.
The cell lysates were resolved by SDS-PAGE. Western-blotting was used to detect EGF -induced Erk phopshorylation.

SiRNA based downregulation of CD44v6 in MCF7 cells significantly reduced the activation of the ErbB receptors as deduced by the inhibition of the Erk signal. This confirms that the action of CD44v6 as a co-receptor for EGF-based ErbB activation is indeed required in breast cancer cells (**Figure. 18**).

3.9. Complex formation between CD44v6 and the ErbB1 receptor protein

The collaboration between the ErbB1-receptor and CD44v6 for EGF-dependent receptor activation in breast cancer cells suggests that these two proteins are located in close vicinity. In this final paragraph I tested whether ErbB1 and CD44v6 form a complex. To this end, a co-immunoprecipitation experiment was performed. Serum-starved MCF10 cells were treated with EGF (or left uninduced) and ErbB1 was subsequently immunoprecipitated with an ErbB1-specific rabbit antibody. The precipitates were then probed for the presence of CD44v6. As a control, a CD44v6 precipitate was loaded on the gel. The specificity of the IP was controlled using an unspecific rabbit IgG.

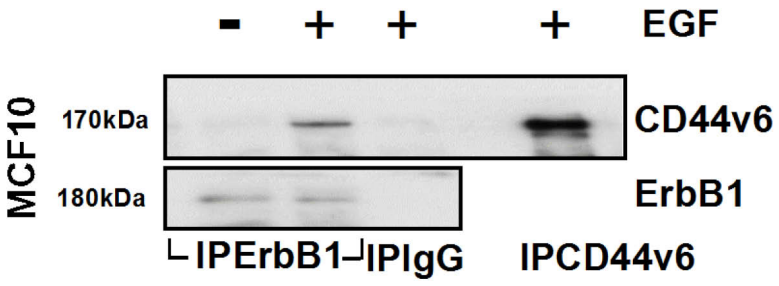

Figure.19: ErbB1 and CD44v6 are found in an inducible complex
Serum-starved MCF10 cells were either induced with 5 ng/ml of EGF or left untreated. Afterwards an ErbB1-Immunoprecipitation (or CD44v6-Immunoprecipitation as a control) was performed. The cell lysates were resolved by SDS-PAGE. A Western-blot was performed to detect ErbB1 and CD44v6.

In MCF10 cells, CD44v6 could be immunoprecipitated together with ErbB1. Interestingly, this complex formation was inducible and could only be found in cells that were treated with EGF. CD44v6 is in a complex with ErbB1 when it acts as a co-receptor for its EGF-based induction (**Figure. 19**).

In conclusion, my data show that in order to induce the ErbB receptors, several ErbB1 ligands are dependent on specific variant isoforms of CD44 (**Illustration 7**). While activation by TGF-α is independent of CD44, EGF and ER require CD44v6, whereas HB-EGF relies on CD44v3. This suggests that the requirement for CD44-isoforms as a co-receptor is ligand dependent.

In addition, all of the ligands that I have tested are dependent on the ERM-protein ezrin and its binding to the actin cytoskeleton for their downstream signaling to the Erk-kinase. Even TGF-α that does not depend on CD44, shares this requirement.

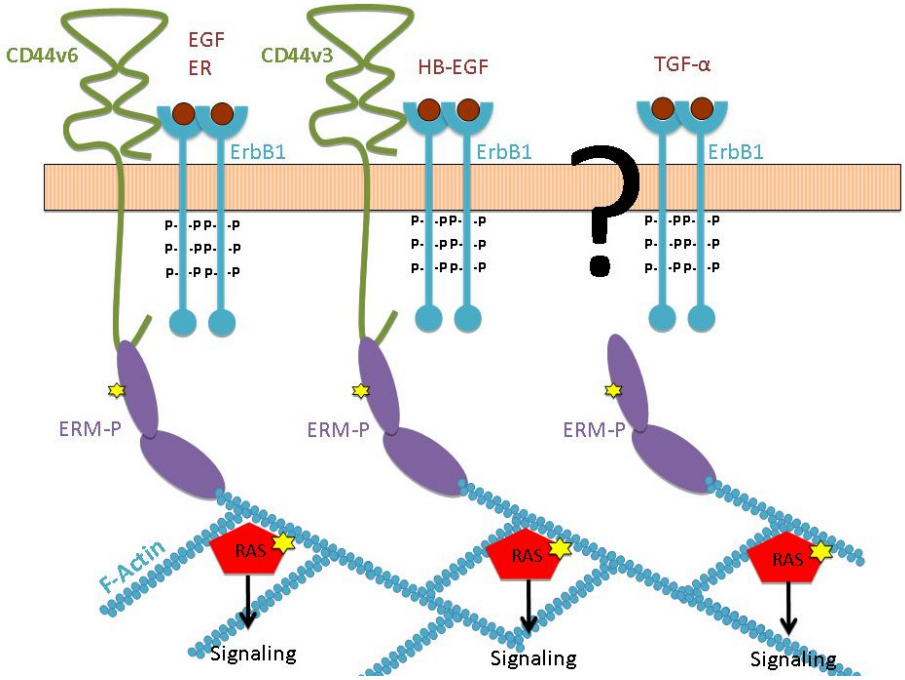

Illustration 7: Model of ErbB signaling: In order to induce the ErbB receptors, different ErbB ligands require specific CD44 isoforms. EGF and ER are dependent on CD44v6, HB-EGF requires CD44v3 and TGF-α is independent of CD44. For their signaling all of the ErbB ligands are dependent on ezrin binding to the actin cytoskeleton.

Furthermore, the EGF- and ER-dependent migration of HeLa cells was also dependent on the co-receptor function of CD44v6 whereas the TGF-α dependent migration of these cells was not.

Lastly, in breast cancer cells, CD44v6 also acts as a co-receptor for EGF and can be co-immunprecipitated together with the ErbB1 receptor upon EGF-induction.

4. Discussion

4.1 CD44 in bacterial invasion

In my PhD thesis I have shown that the co-receptor function of CD44v6 for the activation of the RTK Met by InlB is required for the invasion of *Listeria monocytogenes* into host cells. The uptake of the bacteria into HeLa cells was significantly blocked by a CD44v6 peptide or a CD44v6-specific antibody. The adhesion of the bacteria to the HeLa cells however was not significantly reduced by the CD44v6 peptide. This is in agreement with the fact that InlB does not mediate the adhesion of *L.monocytogenes* to their target cells. Normally other proteins play a role in this process. The autolysin ami for example promotes attachement of the bacteria to mammalian cells by means of its GW domains (Milohanic et al, 2001). D-alanin including lipoteichoic acids (LTAs) also facilitate binding of the bacteria to its target cells (Abachin et al, 2002). The bacterial proteins dltA, dltB, dltC and dltD catalyze the incorporation of D-alanine residues into bacterial cell wall-associated LTAs.

One of the first organs that is infected by *L.monocytogenes* is the liver where the bacterium usually multiplies before infecting other organs (Vazquez-Boland et al, 2001). Interestingly, the invasion of liver cells primarily depends on InlB (Vazquez-Boland et al., 2001). The reason why infection of liver is mainly dependent on InlB could be due to the importance of Met and CD44 in the liver. The Met RTK has an essential role in liver development and liver regeneration (Borowiak et al, 2004; Schmidt et al, 1995). In wildtype mice, an inhibition of CD44v6 by means of blocking antibodies leads to a block of liver regeneration and a lack of Met phosphorylation indicating that Met and CD44v6 collaborate in this process (Olaku et al, 2011).

The finding that CD44v6 is involved in the invasion process of *L.monocytogenes* is only one of many evidence that links the CD44 family to bacterial infection. In fact, although they depend on different entry mechanisms in order to infect their host cells, several pathogens share a need for CD44 to invade:

Neisseria meningitidis, for example, a bacterium that can cause meningitis. Following its initial adhesion to the cell surface mediated by so called bacterial type IV pilus like structures, *N.meningitidis* recruits CD44 in order to induce structural

changes within the cytoskeleton of its host cells to mediate its internalisation (Eugène et al, 2002).

Shigella flexneri, the causative agent of bacillary dysentry in humans, secretes several proteins that are necessary for its infection process and one of them, IpaB, binds to CD44s (Skoudy et al, 2000). This binding triggers cytoskeletal rearrangements and its entry into host cells.

Streptococcus pyogenes, a bacterium that can cause group A streptococcal infections, has an HA-rich cell wall. CD44 molecules on host cells can bind to the HA present on the bacterial surface and trigger the tyrosine phosphorylation of several of the host cell cytoplasmic proteins. This leads to cytoskeletal rearrangements and eventually to the uptake of the bacterium (Cywes et al, 2000). All CD44 proteins are able to bind to HA through their extracellular domain. HA might be a common feature of bacterial cell walls mediating their attachement to host cells. Indeed, HA is also an integral molecule of the capsule of the pathogen group *A streptococcus* where it enables the attachement of the bacteria to human keratinocytes (Schrager et al, 1998).

All CD44 isoforms share the ability to bind to ERM proteins through their cytoplasmic tail. This binding might be a general requirement for the bacterial entry into target cells. And indeed, the entry process of *S. flexneri* (Skoudy et al, 1999) as well as *L.monocytogenes* (Jung et al., 2009) into host cells is dependent on CD44 binding to an ERM protein. *N. meningitidis* also interacts with micro-villi-like structures that are rich in CD44 and ezrin prior to infection (Eugène et al, 2002; Merz et al, 1999).

The infection process of *S. pyogenes* (Cywes et al., 2000) and *S. flexneri (*Skoudy et al., 1999) as well as the invasion processes of *L. monocytogenes* (Jung et al., 2009) and *N. meningitidis (Eugène et al., 2002)* is mediated by cytoskeletal rearrengements. Ezrin binding to CD44 and the actin cytoskeleton might be required for signaling events that trigger these changes in the cytoskeleton. And indeed, the invasion process of *L.monocytogenes* depends on the activation of the Met RTK that is mediated by CD44v6 (Jung et al., 2009). Signal transduction via Met mediates actin remodeling (reviewed in Ivetic & Ridley, 2004) and ezrin binding to CD44v6 and Met is a requirement for signal transduction (Orian-Rousseau et al, 2007).

L.monocytogenes can move within infected cells and can even infect new cells by transfer directly from one cell to another. It seems that also this movement and the direct transfer is dependent on CD44 and its interaction with ezrin (Pust et al, 2005).

Altogether, these data suggest that ezrin is a common intracellular binding partner of CD44 for bacterial infection.

One year after the release of my paper on the co-receptor function of CD44v6 for Met induced by InB, a paper was published that challenged the need of CD44v6 for Listeria invasion (Dortet et al, 2010). In this paper they used different HeLa cells as their cells do not express CD44v6 in contrast to the cells that we use. Also in the HT29 cells that they used they could not show a dependency of Met on CD44v6. At this point I cannot explain this contradiction.

4.2 CD44 is a co-receptor for Receptor Tyrosine Kinases

Interestingly, CD44v6 not only mediates the activation of Met through InlB but is also involved in the activation of Met through its authentic ligand HGF. These two ligands share no structural homologies and bind to different regions of Met (Machner et al, 2003; Niemann et al, 2007). This suggests that for its activation, Met requires the co-receptor CD44v6 independently of the ligand it is addressed by. However, CD44v6 not only acts as a co-receptor for Met. It also cooperates with the VEGFR-2 receptor where it mediates receptor activation through the VEGFR-2 ligand VEGF-A (Tremmel et al., 2009). These data suggest that CD44v6 might not be recuited by the RTK it works together with but by the ligands that address these RTKs.

In order to test whether this is the case, I studied the EGFR family of RTKs where one receptor pair is addressed by several ligands. In my PhD thesis, I have shown that there is indeed a ligand specificity in the recruitment of CD44 isoforms by means of downregulation and overexpression studies as well as peptide blocking experiments. Although, EGF, ER, HB-EGF and TGF-α can activate the same receptor pairs (either ErbB1-homo- or ErB1/2 heterodimers), they show a specific recruitment of CD44 isoforms. While EGF and ER are dependent on CD44v6 in order

to induce the ErbB receptors, HB-EGF is dependent on CD44v3. TGF-α on the other hand is completely independent of the CD44 family.

CD44v6 is not the only CAM that is recruited by several structurally different growth factor ligands. The $\alpha_5\beta_3$ integrin for example is recruited by the growth factors FGF-1 (Mori et al, 2008), IGF-1 (Saegusa et al, 2009) and NRG-1 (Ieguchi et al, 2010). It can bind to these growth factors, presents them to their RTKs and promotes signaling through their RTKs. The $\alpha_9\beta_1$ integrin is also recruited by several ligands. It binds to Nerve GF (NGF), brainderived neurotrophic factor (BNF), and NT3 and presents them to the NGF receptor TrkA/NTRK1 (reviewed in Staniszewska et al, 2008).

4.3 CD44 as a mediator of ligand binding

Similar to the integrins, the contribution of CD44 isoforms in RTK activation could be direct binding of the ligands and presentation. Evidence suggests that CD44 can directly bind to growth factors. Upon transfection with CD44v3, a heparin-sulphated isoform of CD44, COS (CV-1 simian in origin carrying SV40 sequences) cells were able to bind to bFGF or HB-EGF whereas this was not the case in control cells (Bennett et al, 1995). The binding of these ligands was abolished by heparinase treatment. In Elisa studies with CD44v3-IgG constructs, a direct binding of bFGF and HB-EGF to CD44v3 was confirmed (Bennett et al., 1995).

Also, in the collaboration between CD44v6 and Met we have strong indications that CD44v6 is able to bind to HGF. In FACS analysis, rat pancreatic cancer cells that express Met but not CD44v6 are not able to bind to HGF unless transfected with a CD44v6 isoform. In addition, T47D cells that do not express Met but express CD44v6 are able to bind HGF. By means of a CD44v6 peptide, the binding of HGF to these cells can be blocked (Y.Volz and V.Orian-Rouseau, unpublished data). Elisa experiments performed with purified proteins as well as Biacore experiments show a binding of the CD44v6 ectodomain to HGF in the micromolar range (Y.Volz & V.Orian-Rousseau, unpublished data). FACS analysis and binding assays revealed that CD44v6 is also able to bind VEGF-A. Similar observations were made in my diploma thesis in the case of InlB. Indeed, in a FACS analysis, InlB was shown to

bind exclusively to cells expressing CD44v6 and CD44s as compared to cells that express CD44s alone (C.Jung & V.Orian-Rousseau, unpublished data).

Ligand presentation might be a common role of CAMs. Syndecan -1, -2 or -4 for example, are required for the binding of FGF-2 to FGFR-1 and for its subsequent activation (Steinfeld et al, 1996). Similarly, binding of the CAM Neuropilin-2 to VEGF-C and VEGF-D is required for the activation of the RTK VEGFR-3 (Staton et al, 2007).

CD44 could have the same function for the ErbB receptors – binding of their ligands and presentation. This presentation could be a requirement for the subsequent binding of the ligand to the ErbB receptor. Several ErbB ligands like for example EGF and TGF-α can bind directly to the ErbB1 receptor *in vitro* (Burgess et al, 2003). This seems to contradict a role for CD44 proteins in the ligand binding process. However, in *in vitro* studies the concentration of the ErbB ligands is higher than under physiological conditions. While the affinity for their ligands might be high enough for the ErbB receptors to allow ligand binding *in vitro*, *in vivo* the ligand availability might be too low and below the requirements for a direct binding of the ErbB receptors.

A possible binding of CD44 isoforms to ErbB ligands might help to concentrate them in a close vicinity to the receptor. In fact, CD44 variant proteins are able to form dimers or oligomers. In rat pancreatic cancer cells, splice variants of CD44 but not CD44s form molecular aggregates in the plasma membrane (Sleeman et al, 1996a). In addition to dimers, larger aggregates of CD44 variants could also be stabilized by chemical crosslinking (Sleeman et al, 1996a). Large aggregates of CD44 could bind to ErbB ligands, e.g. HB-EGF, thereby creating an area of growth factor enrichment around the RTK. With the ligand concentrated in this manner, the ErbB receptor can then bind to it.

To find out whether the ErbB ligands directly bind to CD44 variant isoforms, *in vitro* analyses have to be performed with purified proteins. In Elisa and Biacore assays, a possible binding of CD44 proteins to the ErbB ligands could be investigated. In addition to this, it has to be determined whether other variant isoforms are able to act as co-receptors for ErbB ligands other than EGF, ER and HB-EGF and can possibly also bind to them. The assays described in my phD thesis like for example RNAi and peptide blocking could be used to this end in addition to binding assays like Elisa and Biacore.

In addition to the ErbB ligands used in my phD thesis, several data suggest that CD44 isoforms are also involved in the activation of the ErbB receptors through additional growth factors. A blocking antibody addressing all isoforms of CD44 was able to inhibit NRG-1-induced activation of the ErbB receptors and NRG-1 was not able to activate the Erk-kinase in cells that express ErbB2, ErbB3 and CD44s whereas it could activate cells additionally expressing CD44v1-10 (L.Chen & V.Orian-Rousseau, unpublished data). Furthermore, CD44 appears also to be required for NRG-1-induced ErbB-activation in Schwann cells (Sherman et al, 2000).

It is possible that in addition to the binding of their ligands, CD44 proteins might have additional functions for the activation of the ErbB receptors.

4.4 Other possible functions of CD44 as a co-receptor

CD44 can form dimers and oligomers. This might correlate with their ability to support ErbB-signaling. In response to EGF and NRG-1, the heterodimerization of purified extracellular ErbB receptor domains could not be shown. This suggests that an auxiliary molecule might be required for heterodimerization *in vivo* (Ferguson et al, 2000). In addition, the heterodimerization of ErbB2 and ErbB3 could not be triggered *in vitro* in response to NRG-1 (Horan et al, 1995). And in fact, CD44 catalyzes the heterodimerization of ErbB2 and ErbB3 in response to NRG-1 in Schwan cell differentiation (Sherman et al., 2000).

All CD44 isoforms can bind to HA and this binding might also play a role in ErbB activation. Indeed, the binding of CD44s to HA stimulates the activation of the ErbB2 receptor in ovarian cancer, leading to increased tumor cell proliferation (Bourguignon et al, 2007b; Bourguignon et al, 2007c; Bourguignon et al, 1997). Also, CD44v3 stimulates ErbB2-induced cell proliferation upon HA-binding in ovarian cancer cells (Bourguignon et al, 2003). In fibroma cells of the jaw bone, the binding of CD44 to HA leads to a phosphorylation of ErbB1 and increased cell proliferation (Hatano et al, 2011). In turn, the synthesis of HA can be influenced by ErbB signaling, as EGF or NRG-1 induces the upregulation of the HA synthases Has2 and Has3 in organotypic keratinocyte cultures (Bourguignon et al, 2007a; Pasonen-Seppänen et al, 2003).

A binding of CD44 to HA seems to increase the phosphorylation of the ErbB receptors. The activation of ErbB receptors can lead to cell survival. This is especially

relevant in human cancers. Malignant cells are able to survive in conditions that would cause a growth arrest or apoptosis in normal cells (Hanahan & Weinberg, 2000). By cooperating with CD44, the ErbB receptors might mediate survival in cooperation with CD44 proteins. And indeed, the binding of HA to CD44 stimulates cell survival in colon carcinoma cells (Misra et al, 2008). Upon binding of HA to CD44, ErbB2 activation triggers the induction of cycooxygenase-2 (COX-2) (Misra et al, 2008). Two of the COX-2 metabolites, prostaglandin A2 and A1 can bind to p53 in the cytosol and inhibit its ability to cross into the nucleus, thereby blocking the apoptosis inducing function of this protein (Johnsen et al, 2004).

Our findings regarding Met activation revealed a different effect of HA. CD44 binding to HA produced by confluently growing cells leads to a switch of the intracellular binding partners of CD44. Instead of binding to ERM proteins, CD44 recruits merlin, a protein that can bind to CD44 but not to the actin cytoskeleton (Morrison et al, 2001). Merlin disrupts the link between CD44 and the cytoskeleton and thereby inhibits activation of Ras by growth factor receptors resulting in growth arrest (Morrison et al, 2007). The binding of HA to CD44 proteins cooperating with Met also inhibits the activation of the RTK itself (D.Koschut & V.Orian-Rousseau, unpublished data). According to these findings, binding of CD44 to HA could acts as a sensor of cell density and block RTK signaling.

Different sizes of HA seem to have different effects on cell proliferation. While in most situations, HA is found in high molecular form, at sites of inflammation low-molecular-weigh HA is poduced (sHA) (reviewed in Stern et al, 2006). In contrast to high molecular HA, the binding of CD44 to sHA, induces upregulation and tyrosine phosphorylation of Met in human chondrosarcoma cells (Suzuki et al, 2002).

In summary, positive effects have been described in the case of CD44 binding to HA for the ErbB receptors whereas inhibiting effects of such a binding are observed in the case of Met. The role of HA seems to differ depending on the cell type and the microenvironmental context. The mechanisms involved in these findings have to be evaluated by further studies.

While CD44 can bind to HA with its extracellular domain, its cytoplasmic tail is able to bind to ERM-proteins. These ERM proteins link CD44 to the actin cytoskeleton.

4.5 Ezrin as a mediator of downstream signaling

In my PhD I showed that CD44 isoforms need ezrin to enable signaling of the ErbB receptors. In order to mediate this signaling, ezrin needs to bind to the actin cytoskeleton. However, I have not shown that the direct link between ezrin and CD44 is required. By introduction of a CD44 construct mutated in the ezrin binding region into HT29 cells, it would be possible to test if this link is necessary for signaling.
In addition to the ErbB receptors, Met and VEGFR-2 signaling is dependent on ERM proteins. Interestingly in cells that don't express CD44v6, c-Met recruits a different co-receptor, ICAM-1, that also dependent on ezrin for signal transduction.
Even in situations where a co-receptor has not been identified so far, for example in the case of the PDGF receptor, ezrin is required for signaling (Morrison et al., 2001).

The CD44 isoforms that I tested do not function as a co-receptor for TGF-α, yet for the TGF-α induced signal transduction, the ErbB receptors are still dependent on ezrin. A different co-receptor most likely mediates ezrin recruitment in this case. The CAM integrin $\alpha_v\beta_3$ is a likely candidate for such a function since integrins, can bind to ERM proteins with their cytoplasmic tail (reviewed in Plow et al, 2009) and this particular integrin was suggested to be involved in the activation of the ErbB family by TGF-α (Ellis et al, 2007).

These data suggest that ERM proteins, and especially ezrin, are required to enable signaling. Further evidence for such a function comes from the downregulation of ezrin by siRNA in osteosarcoma cells that led to a reduction of MAPK- and Akt-activation (Khanna et al, 2004). Also, as discussed in the first paragraph, ezrin might be required for signal transduction in bacterial invasion. Furthermore, in some cancers that are characterized by unregulated signaling such as gastric cancer, ezrin is overexpressed (Zhao et al, 2011). The role of ERM proteins in signal transduction could be the recruitment of the actin cytoskeleton. Once recruited by these proteins, actin might provide a network where the components of a signaling event are brought together and synchronized. Actin could function both as a mechanical scaffold, holding signaling molecules in place and as a signal transducer that can directly modify the activation state of signaling proteins.

One direct involvement of the actin cytoskeleton in signaling is for example the activation of the transcription factor SRF. SRF can address a subset of target genes

in complex with its co-activator MAL (Treisman et al, 1998). Free G-actin can inhibit SRF activity by desposing of this coactivator (Miralles et al, 2003).

However, other evidence showing a role of the actin cytoskeleton in signaling rather suggests that actin is recruited as a consequence of signaling and not as a prerequisit for it. Rho GTPases for example are well known to regulate actin dynamics (Ridley, 2006). They are involved in processes such as the formation of membrane protrusions and vesicle trafficking and can activate two types of actin nucleators, WASP/WAVE proteins and Diaphanous-related formins (DRFs), which both mediate actin polymerization (reviewed in Ridley, 2006). Also, processes like cell migration are dependent on the signal induced reorganization of actin filaments (reviewed in Ridley et al, 2003).

4.6 The significance of the CD44 co-receptor function in breast cancer

Different members of the ErbB family of RTKs are either overexpressed or mutated in a wide variety of cancers (reviewed in Hynes & MacDonald, 2009). For example, high ErbB1 expression was found in a majority of carcinomas including mammary carcinomas and amplification of the ErbB2 gene can be found in 20-30% of metastatic breast cancer lesions. The tumorigenicity of these breast cancer types depends on the constitutive activation of the ErbB receptors.

In my PhD, I showed that CD44v6 enables the activation of the ErbB receptors through EGF in several breast cancer cell lines. CD44v6 should therefore play a role in breast cancer types that are dependent on EGF-based ErbB activation for their tumorigenicity. And indeed, the evaluation of 100 primary invasive breast tumors and 18 lymph node metastases connected the expression of CD44v6 with tumorigenity. The presence of these CD44 variants correlates with poor survival (Kaufmann et al, 1995a). Two more recent studies came to similar results (Brown et al, 2011; Yu et al, 2010).

However, there are conflicting data concerning the role of CD44v6 in breast cancer. A study, evaluating 109 patients with stage two breast neoplasia for example did not associate CD44v6 expression with disease free survival or overall survival at all (Morris et al., 2001). An explanation for the conflicting studies could be that CD44v6 does not act as a co-receptor for all breast cancer types. In cancer types where the activation of the ErbB-receptors is mediated by TGF-α for example,

CD44v6 would not be needed as a co-receptor since TGF-α is independent of CD44. Interestingly, a common type of breast cancer involves the expression of TGF-α in an autocrine loop in order to stimulate breast cancer cell growth (Scala et al, 1995; Umekita et al, 2000). In the case of constitutive ErbB2 activation, a common type of unregulated ErbB signaling in breast cancer, a ligand is not needed in order to activate the ErbB2 receptor (reviewed in Ross & Fletcher, 1998b). Since as discussed above, a likely role for CD44 action is ligand binding and presentation, it is questionable whether CD44 is needed at all to activate signaling in case of ErbB2 ligand-independent activation.

The importance of CD44v6 in breast cancer will have to be tested *in vivo*. Accordingly, at the end of my PhD I started such an experiment in a syngeneic breast cancer model. Murine 4T1 cells that are highly metastatic were used. In these cells, similar to MCF10A cells, the ErbB receptors can be activated by EGF and this activation is dependent on CD44v6 (A. Sahadevan & V.Orian-Rousseau, unpublished data). 4T1 cells were originally derived from a spontaneously arising BALB/c mammary tumor (Aslakson & Miller, 1992). When introduced orthotopically, 4T1 cells grow rapidly at the primary site and form metastases in the lung and the lymphnodes over a period of 3-6 weeks. The tumor growth and metastatic spread of 4T1 cells in BALB/c mice very closely mimics human breast cancer and is an animal model for stage IV human breast cancer (Tao et al, 2008). I orthotopically injected 4T1 cells into the mammary fat pad of female BALB/c mice. The mice were injected i.p. with a CD44v6 peptide or a control peptide 3 times a week. This experiment was finished before the end of my phD and the tumor sizes between CD44v6 peptide treated and control animals did not differ significantly at the end of the experiments. After the mice were sacrificed, the tumors as well as the lungs and lymph nodes of the mice were retrieved, fixed and embedded in paraffin. In order to test whether there was a difference in metastatic behaviour of the 4T1 cells between CD44v6 and control peptide treated animals, the paraffin blocks corresponding to lungs and lymph nodes are currently being analyzed by a pathologist.

Presently, breast cancer patients are treated with antibodies directed against the ErbB2 receptor like for example Trastuzumab or Pertuzumab to block aberrant ErbB2 signaling (reviewed in Baselga, 2010). Treatment of breast cancer patients with these antibodies oftentimes is not succesful however because the patients develop a resistance to the therapy. Interestingly, sometimes the Met RTK is

overexpressed and activated in cells that are blocked in ErbB2 signaling to compensate for the loss of ErbB2-activation (Mukohara, 2011; Shattuck et al, 2008). In these patients, a peptide blocking CD44v6 would be a useful tool since CD44v6 does not only act as a co-receptor for EGF- and ER-based ErbB2-signaling but is also instrumental in Met activation.

In some cases, HB-EGF-based ErbB signaling promotes breast cancer cell survival. Its blocking is thought to be a promising target for breast cancer therapy (Yotsumoto et al, 2009). Peptides that inhibit the function of CD44v3 could specifically address those cases of breast cancer that rely on HB-EGF expression for their tumorigenesis.

Further experiments could also be done in Wap-T or MMTV-Neu mice. These mice (reviewed in Hutchinson & Muller, 2000) could be used to analyze the possibility of CD44v6 peptide blocking in a further *in vivo* mammary carcinoma system. In these systems specific promoters induce the expression of proteins that are involved in breast cancer formation (like for example Neu) in the mammary gland. In these mice the occurence of breast cancer goes through specific stages that can be isolated.

4.7 Possible role of CD44 in mammary gland development

The mammary gland matures postnatally. Females are born with a rudimentary ductal tree. These breast ducts start to elongate at puberty and begin to grow into the mammary fat pad (reviewed in Zahnow, 2006). The ErbB receptors play a paramount role in the development and remodelling of the female breast (Zahnow, 2006). During breast development ErbB signaling regulates cell growth, differentiation and in particular lobuloalveolar growth. Therefore the role of the CD44 co-receptor function in breast development should be analyzed. The first evidence that such a role exists is the observation that pregnant CD44 knockout mice are impaired in the maintenance of post-partum lactation (Yu et al, 2002).

The mammary gland comprises two main sections. The epithelium that is in a state of constant proliferation, differentiation or apoptosis and the underlying stroma that regulates these processes by signaling to the epithelium (Su et al, 2011). The signals from the stroma have a significant impact on the morphology of the breast. In 3D culture systems, the contribution of the stroma that accounts for more than 80% of the female breast could be analyzed. When normal mammary epithelial cells are

cultured *ex vivo* in matrigel, a substance that mimicks the characteristics of the stroma, they form alveolar structures (so called mammospheres), that resemble their *in vivo* morphology. They become polarized, form spherical acini or branched tubules. In contrast, breast tumor cells form large, nonpolarized colonies without a lumen when grown in matrigel (Petersen et al, 1992). Blocking antibodies against ErbB1 reverse this change in morphology (Wang et al, 1998). In order to check if the co-receptor function of CD44 is needed to mediate the formation of defined morphological structures like mammospheres, mammary epithelial cells should be grown in matrigel in the presence of CD44 blocking reagents or after transfection with CD44-specific siRNA. The consequence of the CD44 inhibition on mammosphere morphology should then be analyzed.

A conditional inactivation of CD44 at specific stages in development and in specific tissues of the mammary gland will allow us to asses the co-receptor function of CD44 for the ErbB receptors in breast development. Our lab has produced a CD44 floxed mouse that would allow to perfom such studies. If this mouse was crossed with MMTV-Cre mice (Wagner et al, 2001; Wagner et al, 1997) or WAP-Cre mice (Wagner et al, 1997), the Cre-recombinase would be specifically expressed in the mammary gland during different stages of development and would lead to a block of CD44 expression. Eventually this could lead to phenotypic changes in mammary gland development.

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