Forschungszentrum Karlsruhe
in der Helmholtz-Gemeinschaft
Wissenschaftliche Berichte
FZKA 6884

CD44 function as a growth factor co-receptor

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Von der Fakultät für Chemie und Biowissenschaften
der Universität Karlsruhe (TH)
genehmigte Dissertation

Forschungszentrum Karlsruhe GmbH, Karlsruhe
2003

Impressum der Print-Ausgabe:

Als Manuskript gedruckt

Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe

Mitglied der Hermann von Helmholtz-Gemeinschaft
Deutscher Forschungszentren (HGF)

ISSN 0947-8620

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Zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

an der Fakultät für Chemie und Biowissenschaften

der Universität Karlsruhe (TH)

genehmigte

DISSERTATION

von

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aus Jiangxi, China

2003

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Tag der mündlichen Prüfung: 07.05.2003

Die Funktion von CD44 als Ko-Rezeptorfunktion von CD44 für Wachstumsfaktorrezeptoren

Zusammenfassung

CD44 bezeichnet eine Familie von Transmembranglykoproteinen, die in einer Vielzahl von physiologischen und pathophysiologischen Prozessen involviert sind.
Spleissvarianten von CD44, die von Exon v6 kodierte Sequenzen enthalten, spielen in der Tumormetastasierung eine wichtige Rolle. Die hier vorgestellte Arbeit zeigt, dass CD44 Isoformen, die von Exon v6 kodierte Sequenzen enthalten, als Ko-Rezeptor für den c-Met-Rezeptor agieren, einem Tyrosinkinase-Rezeptor, der in Zellwachstum und Invasierung involviert ist. Der cMet-Rezeptor und sein Ligand “Hepatocyte growth factor” (HGF) sind an der Tumormetastasierung beteiligt. Meine Ergebnisse zeigen, dass eine Kooperation zwischen CD44, HGF und c-Met stattfindet. Eine CD44-Isoform, die von Variantenexon v6 codierte Sequenzen enthält, wird für die Aktivierung von c-Met durch HGF/SF in von Ratte und Mensch stammenden Krebszellen benötigt.
In der nicht metastasierenden Zelllinie Bsp73 AS, die ausschließlich die CD44 Standardform exprimiert, führt HGF nicht zur Aktivierung von c-Met. Durch Transfektion mit unterschiedlichen CD44 v6 enthaltenden Isoformen, werden die Zellen HGF-induzierbar. Antikörper gegen zwei von CD44 Exon v6 kodierte Epitope verhindern die Autophosphorylierung von c-Met. Die CD44 Isoform wird zur Bildung eines Signalkomplexes benötigt, der zumindest HGF, c-Met und CD44v6 tragende Isoformen enthält.
Bei dieser Funktion als Ko-Rezeptor für einen Wachstumsfaktor könnte es sich um einen allgemeinen Mechanismus handeln. Hierzu habe ich die Involvierung von CD44 Isoformen in der Signaltransduktionskaskade der EGF-Rezeptor Familie untersucht. Meine Ergebnisse zeigen, daß HB-EGF, EGF und Amphiregulin ihre Rezeptoren in Abhängigkeit von CD44 aktivieren. CD44 v6 spezifische Antikörper können in die Signaltansduktion über HB-EGF, EGF und Amphiregulin sowohl auf der Ebene von Erk als auch auf der Ebene des Rezeptors eingreifen.

Die hier vorgestellte Arbeit zeigt, dass CD44 Isoformen als Ko-Rezeptor für mehrere Tyrosinkinase-Rezeptoren fungieren und das Zellwachstum unterstützen.

Abstract

CD44 is a family of broadly distributed trans-membrane glycoproteins that are involved in a variety of physiological and pathological processes.

CD44 splice variant proteins containing exon v6 encoded sequence have been implicated in tumour metastasis. The work presented in this thesis shows that CD44 isoforms containing exon v6 encoded sequences act as co-receptor for the c-Met receptor, a tyrosine kinase receptor that is involved in growth control and invasive growth. The c-Met receptor and its ligand hepatocyte growth factor (HGF) have also been implicated in tumour metastasis. My results show the cooperation between CD44, HGF and c-Met. A CD44 isoform containing variant exon v6 encoded sequences is strictly required for c-Met activation by HGF/SF in rat and human carcinoma cells.

In a non-metastatic cell line BSp73AS cells which only expressed CD44 standard form, HGF can not activate c-Met. Upon transfection with the CD44 bearing v6 encoded sequences, the cells become HGF inducible. Antibodies against two CD44 exon v6-encoded epitopes inhibit autophosphorylation of c-Met. The CD44 isoform is required for the assembly of signalling complex containing at least HGF, c-Met and CD44 v6 bearing isoform.

Furthermore, this growth factor co-receptor function could be a more general mechanism. I have investigated the involvement of CD44 isoforms in the signalling by the EGF receptor family. My results show that HB-EGF, EGF and Amphiregulin activate their receptors in a CD44 dependent manner. CD44 v6 specific antibodies can interfere with HB-EGF, EGF and Amphiregulin signalling both at Erk level and at receptor level.

In general, my work shows that CD44 isoforms function as co-receptors for several tyrosine kinase receptors and promote cell growth.

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Abbreviations:

°C	Degrees Celsius
AER	Apical ectodermal ridge
APS	Ammonium persulfate
AR	Amphiregulin
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD44E	Epithelial CD44
CD44H	Hemopoietic CD44
CD44s	CD44 standard
CD44v	CD44 variant
cm	Centimeter (10^{-2} meter)
CS	Chondroitin sulfate
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTSSP	Dithiobis (sulfosuccinimidyl propionate)
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylendiamine-N, N- tetracetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycol bis (2-aminoethylether) tetraacetic acid)
ERK	Extracellular signal-regulated kinase
ERM	Ezrin/radixin/moesin
Fab	Fragment of antigen binding
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluoresein isothiocyanate

g	gram
GAG	glycosaminoglycan
GEF	Guanine nucleotide exchange factor
GGF	Glial cell growth factors
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HA	Hyaluronate
HB-EGF	Heparin-binding Epidermal Growth Factor
HEPES	N-hydroxyethylpiperazine-N'-2-ethansulphoxide
HER	Human Epidermal growth factor receptor
HEV	High endothelial venules
HGF/SF	Hepatocyte growth factor/scatter factor
HPC	Hematopoietic progenitor cells
HPRC	Hereditary papillary renal carcinoma
hr	hour
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycan
ICAM	Intercellular adhesion molecule
IL	Interleukin
kDa	Kilo-dalton
m	Milli
M	molar
MHC	Major histocompatibility complex
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MDCK	Madin Darby canine kidney
merlin	Moesin, ezrin, radixin-like protein
mg	Milli-gram
min	minute
MIP-1□	Macrophage Inflammatory-1-beta
ml	Millilitre (10^{-3} litre)
MMPs	Matrix metalloproteinases
MW	Molecular weight
N-CAM	Neural cell adhesion molecule

NDFs	Neu differentiation factors
NF2	Neurofibromatosis type II
NP-40	Nonidet P-40
NRG	Neuregulin
O. D	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3k	Phosphatidylinositol 3-kinase
PLC	Phosphatidylinositide specific phospholipase C
PTP	Protein tyrosine phosphatase
RT	Reverse transcription
RTKs	Receptor tyrosine kinase receptors
SDS	Sodium dodecyl sulphate
sec	Second
SOS	Son-of-sevenless
SSH	Suppressive subtractive hybridization
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-□	Transforming growth factor -beta
Tiam-1	T lymphoma invasion and metastasis 1
tPA	Tissue type plasminogen activator
TRIS	Tris-(hydoxymethyl)-aminomethane
uPA	Urokinase-type plasminogen activator

Prologue

Cancer is one of the major causes of human death among all diseases in modern society. The development of cancer is a multi-step process depending on genetic alterations that drive the progressive transformation of normal human cells into malignant derivatives (Renan et al., 1993). Two classes of gene mutations have been identified in cancer development, including oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function. These genes code proteins such as growth factors, growth factor receptors, adhesion molecules, cytoskeleton and signaling molecules, transcription factors or cell cycle gate controlling proteins, which have been closely correlated with malignancy (Liotta and Stetler-Stevenson, 1991, Nestl et al., 2001, Ozanne et al., 2000, Saez and Martin, 1995, Webb and Vande Woude, 2000).

The most life-threatening event in cancer formation is metastasis, a process in which cancer cells detach from a primary tumor and migrate to distant sites to form secondary tumors. These distant settlements of tumor cell metastases are the cause of 90% of human cancer deaths (Sporn et al., 1996). One of the earliest successful screens for metastasis-associated genes identified variant isoforms of CD44 (Günthert et al., 1991). Later, numerous other metastasis-associated genes were identified by suppressive subtractive hybridization (SSH) or differential display. These genes were over-expressed in metastatic tumor cells of different origins as well as in primary metastases in comparison to non-metastatic sister cell lines or parental tumors. Interestingly, among these genes, c-Met and ezrin were found co-expressed with CD44 in metastatic cell lines (Nestl et al., 2001). My work shows that a CD44 isoform containing exon v6 encoded sequences is required for c-Met activation by HGF in various cells. A signaling complex is assembled that consists of CD44, HGF and c-Met.

Members of the ErbB family of receptor tyrosine kinases have also been implicated strongly in human cancer (Yarden et al., review 2001). My results show that HB-EGF, EGF and Amphiregulin activate their receptors in a CD44 dependent manner. CD44 v6 specific antibodies can interfere with HB-EGF,

EGF and Amphiregulin signaling both at the Erk level and at the receptor level.

1. Introduction:

1.1 CD44

CD44 is the designation of a family of type I transmembrane glycoproteins that are widely expressed on a variety of cells and tissues derived from epithelial, mesenchymal, hematopoietic and mesodermal origins. They originate from one gene but diverge by alternative splicing and post-transcriptional modification. CD44 proteins play important roles in various physiological and pathological processes such as hematopoiesis, immune response (lymphocyte activation and homing), development, wound healing, inflammation and tumor progression (Herrlich et al., 1998, Naor et al., 1997, Ponta et al. 2003).

CD44 was first described as a granulocyte-T lymphocyte antigen (Dalchau et al., 1980). Afterwards, CD44 was given various structural or functional names by different researchers, such as phagocytic glycoprotein (Pgp-1), LY-24, extracellular matrix receptor III (ECMR III), In(Lu) related-p80, HUTCH-1, GP90hermes, Homing cell adhesion molecule (H-CAM) (Hughes et al., 1981, Trowbridge et al., 1982, Gallatin et al., 1989, Haynes et al., 1983, Jalkanen et al., 1986). Later, a number of independent studies showed all these names refer to the same molecule (Mackay et al., 1987, Omary et al., 1988, Stefanova et al., 1989). CD44 cluster designation was assigned by the Third International Workshop on Leukocyte Differentiation Antigens (Cobbold et al., 1987).

1.1.1 Structure of CD44 gene

All CD44 proteins are encoded by a single gene that consists of 20 exons (Screaton et al., 1993, Tölg et al., 1993). Among seventeen exons that code for extracellular domain, the first five exons (exon1-5), exon 15 and 16 are present in all CD44 isoforms, whereas exon 6-14 are subjected to alternative splicing (designated as variant exons, v1-v10) which theoretically can generate 768 different isoforms (van Weering et al., 1993). So far at least twenty different CD44 transcripts have been identified (Günthert et al., 1991, Stamenkovic et al., 1991, Rudy et al., 1993). Exon 15 and 16 code the membrane proximal region of the extracellular domain. Exon 17 which codes the transmembrane domain is present in all CD44 isoforms. Exon 18 and 19 code for the intracellular portion of CD44 and can also be alternatively spliced. Differential utilization of exon 18 and 19 generates the short version (three amino acids) and the long version (70 amino acids) of cytoplasmic tail respectively. The short version of the cytoplasmic tail is only rarely expressed (Goldstein et al., 1990, Screaton et al., 1992).

1.1.2 Structure of CD44 Proteins

The smallest CD44 isoform is the CD44 standard form (CD44s) in which all variant exons are excised. CD44s is composed of 270 amino acids establishing the extracellular domain, 23 amino acids establishing the trans-membrane domain and 70 amino acids establishing the intracellular domain (Stamenkovic et al., 1989, Brown et al., 1991). Besides the CD44s, there are numerous other CD44 isoforms, for example, epithelial CD44 (CD44E) which contains CD44v8-10, a 230 kDa isoform which contains CD44v3-10 is expressed in keratinocytes (Hofmann et al., 1993).

Extracellular domain

The first five exons of CD44 encode a globular domain whose structure is partly determined by disulfide bridges formed between cysteine residues (Goldstein et al., 1989). There are six cysteine residues in the amino-terminus of extracellular domain of CD44 which are highly conserved throughout all

examined species with the exception that rat only has five (Günthert et al., 1991). Within this region a stretch of 90 amino acids shows 80-90% sequence similarity among mammalian species and has considerable homology to both the cartilage link protein and proteoglycan core protein (Deak et al., 1986, Neame et al., 1986, Stamenkovic et al., 1989, Doege et al., 1987). This domain, which is also named link domain, is responsible for HA-binding (see paragraph ligands of CD44). The amino-terminal globular domain of CD44s is separated from the transmembrane domain by a short stem structure of 46 amino acids. The stem structure can be enlarged by sequences encoded by the alternatively spliced variable exons.

The CD44 extracellular domain is heavily modified by glycosylation. Amino acid analysis showed that the extracellular domain of CD44 contains numerous clusters of serine and threonine residues together with prolines and acidic residues that are sites for glycosylation. In addition in this region there are five conserved consensus sites for N-glycosylation (Asn-X-Ser/Thr) and four serine-glycine dipeptides for modifications by glycosaminoglycans (GAGs). CD44 usually contains GAGs modifications, the amount of which is dependent on the cell type. Multiple sites for N- and O- linked glycosylation and GAGs attachment are present also in the sequence encoded by variant exons, for example CD44E (CD44v8-10) can be additionally modified by both N- and O-linked glycosylation (Brown et al., 1991). The v3 exon contains a Ser-Gly-Ser-Gly GAG attach-site which enables isoforms containing this exon to be modified by heparan sulfate (HS) or chondroitin sulphate (CS) (Bennett et al., 1995). Heparan sulfate modified CD44 can bind to several heparin-binding growth factors (see below paragraph ligands of CD44).

Except hyaluronan, other GAGs are covalently attached to protein cores to form proteoglycans (Ruoslahti et al., 1989). CD44 is able to bind such proteoglycans that are modified by GAGs, such as, versican (Kawashima et al., 2000), aggrecan (Fujimoto et al., 2001), serglycin (Toyama-Sorimachin et al., 1995), interferon-□ (Hurt-Camejo et al., 1999) and the MHC class II invariant chain (Naujokas et al., 1993). It is still not clear whether these interactions have functional relevance.

The extracellular domain of CD44 can be shed from cell surfaces (Bazil and

Strominger, 1994). Shedding of CD44 increases during tumor dissemination (Katoh et al., 1994, Ritamaki et al., 1997). The proteases responsible for cleavage of CD44 remain unknown although some studies show that matrix metalloproteinases and serine protease may be involved (Bazil and Strominger, 1994). Certain CD44 variant isoforms are reported to shed from the cell surface spontaneously more readily than CD44s (Bartolazzi et al., 1995). Soluble CD44 could act as potential antagonists to membrane-bound CD44: Murine mammary carcinoma cells tansfected with cDNAs encoding soluble isoforms of CD44 displayed a marked reduction in their ability to internalize and degrade hyaluronate (Yu et al., 1997).

The Transmembrane domain and the cytoplasmic tail of CD44

The transmembrane domain of CD44 spans 23 amino acids. It is encoded by exon 17 that is highly conserved among mammalian species. A cysteine at position 286 appears to participate in certain situations in the formation of CD44 clusters (Liu et al., 1997).

The cytoplasmic tail of CD44 consists of 70 amino acids. It shows 80-90 % sequence similarity among species (Isacke et al., 1994), which implies that this domain mediates important functions. Two classes of functions are predicted: 1) functions leading to changes in the avidity or affinity of CD44 for ligands (“inside-out” signaling); 2) functions activated as a consequence of ligand binding that mediate (or trigger) further downstream events (“outside-in” signaling).

The cytoplasmic tail of CD44 can be phosphorylated on serine residues. There are six serines in the cytoplasmic domain of CD44. The phosphorylation of CD44 cytoplasmic domain may influence protein-protein interactions. Mutational analysis indicates that Ser323 and Ser325 can be phosphorylated in vivo. Ser325 is the major residue that is phosphorylated, accounting for approximately 90% of the phosphorylation of CD44 (Peck et al., 1998). However, Ser323 is required for phosphorylation of Ser325 since mutation of Ser323 abolishes phosphorylation on Ser325 (Neame et al.,

1992). Residue Ser291 has the properties of a protein kinase C (PKC) consensus site (Kalomiris et al., 1989). One recent study shows that phosphorylation of Ser291 modulates the interaction between CD44 and ezrin in vivo, and that this phosphorylation is critical for CD44-dependent directional cell motility (Legg et al., 2002).

1.1.3 Ligands of CD44

It is now well established that CD44 is a major cell receptor for HA (Lesley et al., 1998). CD44 proteins have the ability to bind ECM components other than hyaluronan, for example, fibronectin (Ehnis et al., 1996) collagen (Wayner et al., 1987, Carter et al, 1988) and laminin (Jalkanen et al., 1992). The physiological function of these interactions in vivo remains unclear.

Hyaluronan (HA) is a glycosaminoglycan consisting of a linear polymer of repeating disaccharides, D-glucuronic acid/ N-acetyl-D-glucosamine, linked by □(1□ 3) and □(1□ 4) glucosidic linkages. HA is a major component of the extracellular matrix (ECM) and is principally involved in filling intercellular spaces and providing cellular support (Wight et al., 1992). HA polymers can have molecular weights of several million kDa and may aggregate with further HA polymers via hydrophobic bonds (Scott et al., 1992).

Although CD44 isoforms are expressed on the cell surface of a variety of cells, and any CD44 isoform contains link domain, many cells that expressed CD44 do not bind to HA constitutively. The binding of HA to CD44 can be regulated. One example is that in lymphocytes CD44 proteins appear to exist in three states with respect to HA-binding: 1. Active state: CD44 bind to HA constitutively. 2. Inducible state: CD44 bind to HA only in the presence of inducing antibodies or physiological stimuli such as cytokines or phorbol ester. 3. Inactive state: CD44 cannot bind to HA even in the presence of inducing mAb (Lesley et al., 1993).

Several evidences show that CD44 clustering is important for HA binding. CD44 v4-7 containing variant proteins oligomerize and bind to HA better than CD44s (Sleeman et al., 1997). In human Jurkat cell line, PMA-induced HA

binding depended on the presence of a cysteine in the CD44 transmembrane domain. This cysteine residue appeared to be important for CD44 clustering (Liu et al., 1997), however, other studies showed that this cysteine seemed to be irrelevant for CD44 aggregation in a murine T cell line (Li et al., 1998). HA binding was induced by an antibody against CD44 (IRAWB14) when applied in bivalent forms allowing oligomerisation, but not as monovalent Fab fragment (Lesley et al., 1993).

Besides hyaluronan, the extracellular domain of CD44 can also bind growth factors and matrix metalloproteinases (MMPs) which may mediate important functions. Several growth factors and cytokines such as members of the fibroblast growth factor (FGF) family, heparin binding growth factors or macrophage inflammatory-1□ (MIP-1□) can bind to heparan sulfate modified CD44. FGF2, and heparin binding epidermal growth factor (HB-EGF) have been shown to bind to CD44 v3-immunoglobulin fusion protein. Enzymatic removal of heparan sulphate modifications abrogated the interaction (Bennett et al., 1995). MMPs were shown to bind to CD44 that seems to be required for the effective function of MMPs (Yu et al., 1999, Yu et al., 2000) (see paragraph Mechanism of CD44 function in tumor growth and metastasis).

Ligands binding to the cytoplasmic domain of CD44

Several intracellular partner proteins of CD44 have been characterized, for example ankyrin, members of the ERM family (ezrin, radixin and moesin) and merlin. ERM family proteins and ankyrin may serve as linkers between CD44 and cytoskeleton.

It was demonstrated that CD44 binds to ankyrin-coated sepharose beads in vitro (Kalomiris et al., 1988). Using different plasmid constructs in which various portions of the CD44 cytoplamic domain had been deleted (Lokeshwar et al., 1994), the ankyrin binding domain was mapped to a region between Asn304 and Leu318. The CD44-ankyrin interaction has been implicated in hyaluronan-dependent cell motility and adhesion (Lokeshwar et al., 1994).

The association of ERM family proteins with CD44 is mediated by a basic

amino acid motif located at the cytoplasmic tail of CD44. The binding of CD44 to ERM proteins plays an important role in regulating cell migration and shaping the plasma membrane (Tsukita et al., 1994).

Merlin, a tumor suppressor protein related to ERM proteins, shares 46% sequence identity to ezrin and is most homologous in its amino-terminal. Merlin is activated at high cell density and blocks proliferation. Its growth inhibitory function is associated with dephosphorylation and depends on the interaction with the cytoplamic tail of CD44 (Morrison et al., 2001).

Signal transduction via CD44

Transmembrane receptors are able to transduce extracellular signals from the outside to the inside of the cells. There are some reports that signals are induced by ligands binding to CD44. However, it is not clear whether signals are transmitted directly through CD44 or indirectly through other receptors bound to CD44. The cytoplasmic tail of CD44 recruited several intracellular proteins involved in signaling processes, such as, Src, Lck, Fyn, PKC and Rho GTPase (Kalomiris et al., 1989, Bourguignon et al., 2000, Oliferenko et al., 2000, Bourguignon et al., 2001, Hirao et al., 1996). The associations of these proteins and CD44 might be indirect. One example is that $P56^{Lck}$ (a member of the non-receptor kinase Src family) is associated with CD44 in T lymphocytes (Taher et al., 1996). However, since the cytoplasmic domain of CD44 does not possess the CXCP sequence that is considered to be a $P56^{Lck}$ binding motif, it seems that $P56^{Lck}$ might associate with CD44 indirectly (Lesley et al., 1998).

1.1.4 CD44 Functions

CD44 proteins have many important functions such as functions in the immune system, cell homing, hematopoiesis, embryonic development and tumorigenesis. These functions are described below.

1.1.4.1 CD44 involvement in HA internalization and degradation

Several studies show that CD44 participates in the internalization and intracellular degradation of HA. Bovine chondrocytes, transformed mouse fibroblast (SV-3T3), and human and hamster alveolar macrophages have been shown to bind and internalize labeled HA in a CD44 dependent manner (Culty et al., 1992, Hua et al., 1993). CD44 itself does not posses HA degrading activity (Culty et al., 1992). Cleavage of HA by macrophages can be blocked by chloroquine and NH_4Cl which inhibited lysosomal acid hydrolases. Degradation of HA following CD44 mediated internalization may facilitate locomotion of mobile cells, such as macrophage and invasive tumor cells (Naor et al., 1997).

1.1.4.2 CD44 in embryonic development

Though many studies have shown the expression of CD44 proteins during rat, mouse, chicken and human embryonic development (Wirth et al., 1993, Wheatley et al., 1993, Terpe et al., 1994, Campbell et al., 1995, Yu and Tool, 1996), the requirement for CD44 in developmental processes have been defined only in a few cases.

One such case is the vertebrate optic chiasm, an X-shaped array of axons formed by the retinal neurons as they enter the brain. The first retinal ganglion cell axons arriving at the embryonic mouse ventral diencephalon meet an inverted v-shaped neuronal array defining the midline and posterior boundaries of the future optic chiasm. These neurons express CD44 and L1, a molecule known to promote retinal axon outgrowth. CD44 appears to block the crossing of axons over the midline, possibly by blocking continued axon outgrowth in the direction of the midline and antagonizing the function of the

L1 cell adhesion molecule (Sretavan et al., 1994, Sretavan et al., 1995). L1/CD44 neurons may serve as an anatomical template for retinal axon pathways at the embryonic mouse ventral diencephalons (Sretavan et al., 1994).

CD44 proteins are also required for the development of the thymus and the limb bud. During thymus development, only cells expressing CD44v from fetal liver and adult bone marrow could efficiently populate fetal thymic stroma and develop into mature T cells. In fetal thymic organ cultures anti-CD44v (v6, v7, v10) antibodies blocked thymocyte development. CD44 variant proteins were required for the initial interaction of hematopoietic progenitor cells with the thymic stroma. During limb development, CD44 v3 containing isoforms are crucial for the presentation of FGF-8 to limb mesenchymal cells and their subsequent proliferation. These CD44 splice variants are temporally and spatially co-localized with FGF-8 in the apical ectodermal ridge (AER) throughout early limb development and bind FGF proteins in a heparan sulfate dependent manner. Interference with this mechanism by treating the AER with CD44 specific antibodies completely blocks the stimulatory capacity of the AER by preventing CD44 mediated FGF-8 presentation (Sherman et al., 1998).

1.1.4.3 CD44 in Hematopoiesis

CD44 is highly expressed on bone marrow hematopoietic progenitor cells (Kansas et al., 1990). This expression appears to be relevant for the development of the hematopoietic system, since the mAb against CD44 can interfere with B cell lymphopoiesis and myelopoises in vitro in the long term bone marrow culture (Miyake et al., 1990, Rossbach et al., 1996).

Several studies show that CD44 variant proteins also play a role in hematopoiesis. CD44s and CD44 variant proteins might play different roles in myelopoiesis, which is suggested by the following observation: in human long term bone marrow culture, both antibodies recognizing amino-terminal epitope (mAb 25-32) and antibodies against CD44 v6 epitope (mAb VFF18) inhibited

myelopoiesis, however, only mab 25-32 affected myeloid colony formation. It was suggested that mAb 25-32 targeted an early precursor cell compartment and mAb VFF18 targeted later stages in myelopoiesis (Moll et al., 1998). It was reported that different C44 variant antibodies triggered differential stimulations of hematopoiesis. Both CD44 v4 and v6 antibodies acted on bone marrow macrophages can stimulate GM-CSF production, but only v6 antibodies can stimulate interleukin-6 (IL-6) production. This may explain why both CD44 v4 and v6 antibodies stimulated myelopoiesis and only CD44 v6 antibodies stimulated lymphopoiesis (Khaldoyanidi et al., 2002).

Another functional contribution of CD44 in hematopoiesis can be concluded from the finding that CD44 was identified as a major E-selectin ligand on human hematopoietic progenitor cells. E-selectin plays a critical role in mediating tissue-specific homing of T cells into skin, and of primitive hematopoietic progenitor cells (HPCs) into bone marrow (Dimitroff et al., 2001).

1.1.4.4 CD44 in inflammation

Both CD44 and hyaluronan play important roles in inflammatory disease (Pure et al., 2001). The administration of KM81 (an antibody that blocks the HA binding to CD44) reduces inflammatory symptoms, while the treatment with IRAWB14 (an antibody that enhance HA binding) aggravated the disease (Mikecz et al., 1999). HA expression on cultured endothelial cell lines and primary endothelial cultures was increased, maybe induced by the pro-inflammatory cytokines TNF-α and IL-1β, as well as bacterial lipopolysaccharide (Mohamadzadeh et al., 1998). The interaction of low molecular weight HA with CD44 may serve as a potent regulatory signal in inflammation. At inflammatory sites, low molecular weight HA is produced and accumulated, which can induce the expression of many genes involved in the inflammatory response, for instance, chemokines (macrophage inflammatory protein-1 β, cytokine responsive gene-2 and monocyte chemoattractant protein-1) (Mckee et al., 1996), Interleukin-12 (Hodge-Dufour et al., 1997), TGF-β1 (Okawara et al., 2000) and transcriptional factor nuclear factor

kappaB (NF□B) (Rockey et al., 1998).

1.1.4.5 CD44 in lymphocyte homing and leukocyte extravasation

Lymphocyte homing is a process in which blood lymphocytes return to lymphoid organs. A CD44 specific antibody, Hermes-1, can interfere with human lymphocyte binding to the endothelium of high endothelial venules (HEV), indicating that CD44 is involved in lymphocyte homing (Jalkanen et al., 1986). A murine lymphocyte homing specific antibody, MEL-14, cross-reacted with human CD44 (Jalkanen et al., 1987). Therefore it was believed that CD44 might be a lymphocyte homing receptor. However, the cloning of the gene encoding the MEL-14 epitope showed that the lymphocyte homing receptor is L-selectin (Lasky et al., 1989, Siegelman et al., 1989).

The leukocytes in the bloodstream can extravasate into various tissues. The extravasation of leukocytes includes several steps. The primary event during which leukocytes begin to interact with the vascular wall and are retarded in their movement is known as “rolling”. This interaction is followed by a secondary “firm” adhesion of the rolling cells to the vasculature wall and subsequent extravasation (Laurence et al., 1991). In the most majority of cases, the primary adhesion event is mediated by selectins and their carbohydrate ligands. Members of the integrin family are responsible for the secondary (firm) adhesion events (Carols et al., 1994). Under certain conditions, CD44 also plays a role in leukocyte extravasation. CD44s and its ligand HA have been implicated in mediating leukocyte rolling on endothelial surfaces. The rolling could be abrogated by the treatment with CD44 specific antibodies, KM201, KM81, or by competition with excessive HA (DeGrendele et al., 1996).

These data show that CD44 is not the homing receptor, but it is somehow involved in homing. Furthermore CD44 may play a role in the motility and extravasation of some lymphocytes by mediating lymphocyte rolling.

1.1.4.6 CD44 and lymphocyte activation

CD44 is believed to play a role in the activation of immune cells. Some CD44 antibodies act as co-stimulators to enhance CD2 or CD3 mediated induction of proliferation of human peripheral T-cells (Sommer et al., 1995). Other CD44 antibodies inhibit CD3 antibody-mediated T-cell proliferation (Rothman et al., 1991). Furthermore, certain CD44 antibodies stimulate human T-cell proliferation even in the absence of other stimuli (Pierres et al., 1992, Galandrini et al., 1993). Similar to CD44 antibodies, a ligand of CD44 hyaluronate could also act as a co-stimulatory for human T cell proliferation in conjunction with CD3/TCR-mediated stimuli (Galandrini et al., 1994).

Besides the CD44 standard form, CD44 variant proteins also seem to promote lymphocyte activation and proliferation. In CD44v4-7 transgenic mice, the response of T cells to activating stimuli was accelerated (Moll et al., 1996).

1.1.5 CD44 in Cancer Progression

Studies with experimental animals and observations with human cancers show that CD44 proteins play important roles in the metastatic spread of tumor cells. The first evidence for the involvement of CD44 variant protein in metastasis came from the experiment that a CD44 variant protein (CD44v4-7) conferred metastatic potential upon transfection into non-metastatic rat pancreatic cells (Günthert et al., 1991). Other CD44 variant proteins were also shown to be involved in tumor metastasis. For instance, the expression of CD44v6,7 in the non-metastatic pancreatic carcinoma cells was sufficient to establish metastases in the lung and lymph node upon subcutaneous injection in syngeneic animals (Rudy et al., 1993).

In many human cancers, such as colorectal cancer, Non-Hodgkin's lymphoma, breast cancer and melanoma, CD44 variant proteins can be used as diagnostic and prognostic marker (Wielenga, et al., 1993, Pals et al., 1997, Kaufmann et al., 1994).

In some cases, however it seems that not CD44 variant proteins but CD44s is involved in tumor growth. Human melanoma cells expressing CD44s impressively displayed accelerated local tumor growth in immuno-deficient mice in comparison to cells expressing CD44E (CD44v8-10) (Bartolazzi et al., 1994). Similarly, the human Burkitt lymphoma cell line (Namalwa cells) expressing CD44s showed more efficient local tumor growth and metastatic spread in immuno-deficient mice in comparison to its parental cells that did not express CD44 (Sy et al., 1991). It is interesting that introduction of CD44E (CD44v8-10) or other CD44 variants (e.g. CD44v6-10) into Namalwa cells further reduced their ability to develop tumor in the animals (Sy et al., 1991, Sy et al., 1992).

CD44 proteins can not only promote tumor growth but also suppress tumor growth under certain conditions. CD44 was found as a metastasis suppressor for prostatic cancer. The decreased expression of CD44s is involved in the progression of prostatic caner to a metastatic state (Gao et al., 1997). Over-expression of CD44s in AT3.1 (a prostatic cancer cells) suppressed the metastatic ability of the cells (Gao et al., 1998). Thus, CD44 may function as tumor suppressor gene in the case of prostatic cancer.

1.1.6 Mechanism of CD44 function in tumor growth and metastasis

1.1.6.1 The role of CD44 as an adhesion molecule in tumor progression

Adhesive properties of tumor cells must be altered in order to detach from the primary tumor (Schirrmacher et al., 1985). CD44 proteins anchor the cells to the extracellular matrix (ECM). The extracellular portion of CD44 was subjected to proteolytic cleavage by an MMP on several cancer cell lines (Okamoto et al., 1999) and in human tumors (Okamoto et al., 2002). Inhibition of CD44 cleavage blocked tumor cell migration. Motility of cells in the extracellular matrix requires the recognition of ECM proteins. Tumor cells attach to ECM components via a number of cell surface adhesion molecules (Blood et al., 1990, Liotta et al., 1991). Thus, adhesion molecules such as CD44, integrins, selectins and cadherins are required for metastasis

processes.

CD44 may facilitate the assembly and organization of the pericellular matrix (Knudson et al., 1993), which could protect tumor cells form immune surveillance (Naor et al., 1997). Pre-treatment with anti-CD44 antibody blocked the pericellular matrix assembly by chondrocytes (Knudson et al., 1996).

1.1.6.2 The role of CD44 as a hyaluronan receptor during tumor progression

There are conflicting data on the relevance of HA-CD44 interaction in tumor progression. HA-CD44 interaction played an important role in the invasion and migration of human glioma cells (Okada et al., 1996). A melanoma cell line established from an invaded lymph node expressed high levels of CD44s that binds HA. This cell line was able to form local tumors and metastases in lung and other organs, following s.c. injection into immuno-deficient mice. In contrast, a melanoma cell line established from the primary tumor of the same patient did not express CD44, and failed to form metastases (Guo et al., 1994). This finding indicates that HA-CD44 interaction is required for melanoma metastases.

In mammary carcinoma cells, soluble CD44 which competes with endogenous cell surface CD44 to bind HA reduced metastases impressively in vivo, suggesting that CD44-HA interaction facilitates the migration of tumor cells to distal organs (Yu et al., 1997, Peterson et al., 2000).

Migration of cells appears to be controlled via activation of Rho GTPase upon HA binding. The activation of RhoA, Rac1, cdc42 leads to formation of stress fiber, focal adhesion plaques, lamillipodia, microspikes and membrane ruffling in fibroblast. In a mouse mammary epithelial cell line (EpH4), HA binding to CD44 activated Rac1 and induced formation of lamellipodia, which were inhibited by anti-CD44 mAb or micro-injection of a dominant negative mutant of Rac (Oliferenko et al., 2000).

However, the HA-CD44 interaction seems to be not required for the tumor outgrowth and metastasis of a rat pancreatic carcinoma cell line (Sleeman et al., 1996). The CD44v4-7 transfectant demonstrated increased HA binding compared with its parental cell line. However, when HA binding was abolished by transfection of hyaluronidase cDNA, the cells maintained the ability to induce local tumor growth and lung metastasis (Sleeman et al., 1996). Thus, in this case, the tumor growth and metastasis is independent of HA-CD44 interaction.

1.1.6.3 CD44 as a surface receptor for MMPs and their substrate

Matrix metalloproteinases (MMPs) play an important role in promoting tumor metastasis by inducing proteolysis of several ECM components (Werb et al., 1997). Several studies showed that CD44 is required for the function of MMPs.

It was reported that CD44 associated with MMP-9 on the surface of mouse mammary carcinoma and human melanoma cells. CD44-associated cell surface MMP-9 promoted cell-mediated collagen IV degradation in vitro and mediated tumor cell invasion of G8 myoblast monolayers. Disruption of CD44-MMP-9 cluster formation by over-expression of soluble or truncated cell surface CD44 inhibited tumor invasiveness in vivo (Yu et al., 1999). In addition, CD44 provided a cell surface for proteolytically active MMP-9 which was required for promoting tumor invasion and angiogenesis (Yu et al., 2000). In this case, MMP-9 could also proteolytically cleave latent TGF-□ into active form (Yu et al., 2000).

These data indicate that CD44 proteins can serve as “platforms” for enzymes such as MMPs, which is required for their effective functions.

Thus, CD44 proteins may contribute to tumor growth and metastasis in different ways: Regulation of adhesion and migration over ECM, function as a platform for enzymes like MMPs and function as a hyaluronan receptor.

1.1.7 Loss of function experiments in mice

CD44 is expressed on numerous cell types and tissues during embryogenesis and in the adult organisms and CD44 proteins mediate many important functions as described above. Thus, it is reasonable to predict that CD44 proteins play essential roles during embryogenesis and in the adult organisms. To study the physiological roles of CD44 in vivo, researchers used different approaches to generate animals lacking functional CD44 in all or a few tissues. One approach is the generation of CD44 null mice by homologous recombination (Schmits et al., 1997, Protin et al., 1999, Yu, et al., 2002); another is the use of an antisense construct to specifically abolish cell surface CD44 expression in keratinocytes (Kaya et al., 1997).

1.1.7.1 CD44 gene Knock-out mice

CD44 deficient mice were produced by the deletion of 3' end of exon 2 and the entire exon 3 of CD44 gene and insertion of neomycin gene expression cassette (Schmits et al., 1997). Surprisingly, the CD44 knock out mice were born and developed normally. Mild abnormalities were observed in myeloid progenitor migration and bone marrow colonization. The mice also developed exaggerated granuloma responses to cryotosporidium parvum infection. Tumor studies showed that SV-40-transformed CD44 deficient fibroblasts were highly tumorigenic in nude mice, whereas reintroduction of CD44s into these fibroblasts resulted in a dramatic inhibition of tumor growth. This is consistent with the finding that CD44 may function as a tumor suppressor gene (Gao et al., 1997, Gao et al., 1998).

1.1.7.2 Transgenic mice with selective suppression of CD44 in keratinocytes

An alternative approach used to study CD44 function in vivo was the generation of transgenic mice that express an antisense CD44 cDNA under the control of the keratin-5 promoter. These mice lack detectable CD44

expression in skin keratinocytes and corneal epithelium. They displayed abnormal hyaluronate accumulation in the superficial dermis and corneal stroma. Furthermore, distinct morphological alterations of basal keratinocytes and cornea, and defective keratinocytes proliferation in response to mitogen and growth factors were observed. The transgenic mice showed a severe skin phenotype, including decrease in skin elasticity, impaired local inflammatory response and tissue repair, delayed hair re-growth, and failure of the epidermis to undergo hyperplasia in response to carcinogen treatment (Kaya et al., 1997).

This study indicated the existence of two major functions of CD44 in skin: regulation of keratinocyte proliferation in response to extracellular stimuli and maintenance of local hyaluronate homeostasis (Kaya et al., 1997, Kaya et al., 1999).

Different results have been generated by studies of CD44 null mice and CD44 transgenic mice. It seems that the loss of CD44 expression at late stage of development is not tolerated the way it is when absent from early development onward. Probably the majority of CD44 functions are compensated for during early embryogenesis before major differentiation events have taken place (Ponta et al., 2003).

As mentioned before, the signaling through growth factors and their tyrosine kinase receptors is very important for cell proliferation. When tumor cells migrated to new sites, they must settle and proliferate to form secondary tumors. Signaling through growth factors and their receptors, for example, HGF/c-Met and EGF/ErbB, is very important for cell proliferation as growth signal. Like CD44 variant proteins, HGF/c-Met and EGF/ErbB have also been strongly implicated in tumorigenesis.

1.2 HGF/c-Met

Hepatocyte growth factor (HGF), also known as “scatter factor” (SF) is a multifunctional cytokine. HGF induced signaling is mediated by c-Met receptor, the product of the c-Met proto-oncogene.

Structure of HGF

HGF/SF was independently identified by its abilities to induce the proliferation of primary hepatocytes and to induce the dissociation of epithelial cells (scattering) respectively (Nakamura et al., 1986, Gohda et al., 1988, Zarnegar et al., 1989). Subsequent structural and functional studies showed that HGF and SF is the same molecule (Miyazawa et al., 1989, Nakamura et al., 1989, Naldini et al., 1991).

HGF is secreted as a biologically inactive precursor (about 100 kDa) which is activated through proteolytic cleavage (Naka et al., 1992). Several proteases are able to activate HGF. These include urokinase type (uPA) and tissue type (tPA) plasminogen activator, HGF activator and HGF converting enzyme (Naldini et al., 1992, Mars et al., 1993, Mizuno et al., 1994, Shimomura et al., 1995). The cleavage gives rise to a 60 kDa □ subunit and a 30 kDa □ subunit, which are linked by a disulfide interchain bridge (Nakamura et al., 1989, Rubin et al., 1991). The □ subunit contains four kringle domains (K1-K4). The □ subunit is homologous to the protease domain of plasminogen, but has no

catalytic activity due to the substitution of a serine by a tyrosine at its catalytic domain.

Several splice variant or mutant forms of HGF were reported. For example NK2 is a variant in which the amino-terminal domain of HGF is linked to the first two kringle domains in the □ subunit. NK2 behaves as an HGF antagonist (Chan et al., 1991). Another variant has been described which only has one kringle domain and functions as a partial HGF agonist (Cioce et al., 1996, Jakubczak et al., 1998). In another mutant form, fifteen nucleotides in the first kringle domain are deleted. This variant changed potency in the stimulation of DNA synthesis with different cells comparing with normal HGF. Probably the alteration of the biological activity is due to the change of its tertiary structure (Shima et al., 1994).

Structure of c-Met

The c-Met tyrosine kinase receptor is the receptor binding HGF. c-Met was identified as the product of the c-Met oncogene (Cooper et al., 1984). The c-Met protein is synthesized as a single chain 170 kDa precursor. This precursor is converted into the mature protein by proteolytical cleavage. The mature protein contains one □ and one □ chain. 50 kDa □ chain and 145 kDa □ chain form a heterodimer of 190 kDa by disulfide linkage. The heterodimer is anchored at the membrane by the transmembrane domain of □ chain. The cytoplasmic part of c-Met contains a tyrosine kinase domain and a “docking site” for multiple signaling molecules.

Signaling through HGF/c-Met

Upon activation by HGF, c-Met undergoes autophosphorylation of tyrosine residues (Tyr^{1234} and Tyr^{1235}) within the kinase domain. Phosphorylation of these two tyrosines leads to the phosphorylation of other two tyrosine

residues (Tyr^{1349} and Tyr^{1356}) in a cluster of amino acids in the carboxy-terminus of c-Met. A short sequence (Y^{1349}VHVNATY^{1356}) is the docking site in c-Met. The docking site binds adaptor proteins or signal transducers with Src homology-2 (SH-2) domain, phosphotyrosine binding (PTB) domain, and c-Met binding domain (MBD) (Pelicci et al., 1995, Ponzetto et al., 1994, Weidner et al., 1996). A large signaling complex was recruited to the cytoplasmic tail of activated c-Met, composed of the adapter proteins Grb2 (Ponzetto et al., 1994), Shc (Pelicci et al., 1995), Gab1 (Weidner et al., 1996) and Crk/CRKL (Garcia-Guzman et al., 1999, Sakkab et al., 2000), the signal transducers phosphotidylinositol-3-OH kinase (PI3K) (Graziani et al., 1991) the signal transducer and activator of transcription-3 (Stat3) (Boccaccio et al., 1998), phospholipase C-□ (PLC-□) (Ponzetto et al., 1994), the Ras guanine nucleotide exchange factor son-of-sevenless (SOS) (Graziani et al., 1993), the Src kinase (Ponzetto et al., 1994), and the SHP2 phosphatase (Fixman et al., 1996). Interaction of c-Met with these adapter proteins and signal transducers can occur directly via the docking sites of c-Met, for instance Grb2 and PI3K. Some proteins such as SOS might associate with c-Met indirectly via other adapter proteins and signal transducers.

Normal biological function of HGF/c-Met signaling

HGF/c-Met signaling has been shown to trigger a variety of cellular responses dependent on the cellular context. For example in vitro studies have revealed that activation of c-Met by HGF leads to cell proliferation, stimulation of cell dissociation and motility i.e. scattering, stimulation of cell movement through the extracellular matrix, i.e. invasion, and branching morphogenesis (Furge et al., 2000, Jeffers et al., 1998). The cellular responses to c-Met stimulation by HGF are important in mediating a wide range of biological activities including embryonic development, wound healing, tissue regeneration, angiogenesis, growth, invasion, and morphogenic differentiation (Jeffers et al., 1997).

c-Met is predominantly expressed on a wide variety of epithelial cells, whereas HGF is expressed by stromal cells. This reciprocal expression pattern points to their important roles in epithelial-mesenchymal interactions underlying branching morphogenesis and tubulogenesis during development of organs such as lungs, kidney, and mammary glands (van der Voort et al., 2000). Knock-out of the HGF or c-Met genes in the mouse genome causes embryonic lethality due to defects in liver and placenta, which indicated that they play important roles in mammalian development (Schmidt et al., 1995).

HGF/c-Met signaling in tumorigenesis

Aberrant HGF/c-Met signaling has been described in a variety of human cancers. Mutations in c-Met, over-expression of c-Met and/or HGF, and expression of c-Met and HGF by the same cell can all contribute to tumorigenesis (Jeffers et al., 1997, Jeffers et al., 1998). Cell lines with aberrant c-Met activation via one of these mechanisms are both highly invasive and metastatic (Jeffers et al., 1997).

Recently, a genetic connection between c-Met and hereditary papillary renal carcinoma (HPRC) has established a direct role for this receptor in the development of this human cancer. By using comparative genomic hybridization and linkage analysis, the HPRC gene was mapped to a region of chromosome 7 where the c-Met gene is located. Sequencing of the c-Met coding region from affected members of HPRC families as well as from tumor samples of patients with sporadic papillary renal carcinoma identified nine mutations. All mutations were missense mutations and were located within the tyrosine kinase domain of the c-Met receptor (Schmidt et al., 1997).

ErbB receptors are a family of tyrosine kinase receptors that also have been strongly implicated in human cancers. In a variety of cancer cell types, the ErbB receptors become hyper-activated by a range of mechanisms, including over-expression of ligands, over-expression of receptors or constitutive activation of receptors (Yarden et al., 2001). Aberrant receptor signaling promotes deregulated growth control and the onset of malignancy.

1.3 EGF/ErbB family

The ErbB receptors belong to type I tyrosine kinase receptors. ErbB receptors are hetero- or homo-dimers of four ErbB proteins including ErbB-1 (ErbB gene product) and proteins encoded by the ErbB-2 (neu/HER-2), ErbB-3 (HER-3) and ErbB-4 (HER-4) genes. Most of ErbB proteins contain an extracellular ligand-binding domain, a hydrophobic membrane-spanning region, and a cytoplasmic tyrosine kinase domain. ErbB-3 differs from the others in that it contains a nonfunctional tyrosine kinase domain. ErbB receptors are bound and activated by a variety of growth factors of the EGF family (See Table-1). Although no ligand is yet known for ErbB-2, it has a central role in the response to several growth factors via hetero-dimerization with other members of the ErbB family.

All EGF growth factors except for Decorin (Lozzo et al., 1999) contain the EGF domain, which is sufficient and necessary for receptor activation. This domain consists of six cysteines characteristically spaced over about 50 amino acids and is folded into a three-loop secondary structure by disulphide linkage. There are also many non-EGF motifs in some EGF growth factors, for example, Neuregulins contain immunoglobulin, kringle and cysteine-rich domains. However, little is known about the physiological function of these domains. All mammalian EGF growth factors are produced as transmembrane precursors and give rise to the mature soluble proteins by specific proteolytic cleavage.

Table 1. Ligands of the ErbB receptors

Ligands binding only to ErbB-1	Ligands binding to ErbB-1 / 4	Ligands binding to ErbB-3/ 4
Epidermal growth factor (EGF) Amphiregulin (AR) Transforming growth factor-□ (TGF-□) Decorin	Bactecellulin (BTC) Epiregulin (EPR) Heparin binding EGF (HB-EGF)	Neuregulin-1 (NRG-1) Neuregulin-2 (NRG-2) Neuregulin-3 (NRG-3)

Signaling through ErbB receptor

Binding of EGF growth factors to ErbB receptors induces receptor dimerisation and trans-phosphorylation of tyrosines located in the cytoplasmic domain. This event is believed to be the principal mechanism of activating ErbB receptors (Ullrich et al., 1990). There is a docking site at the cytoplasmic tail of the ErbB receptors. After receptor activation, the docking site of the receptor can bind a variety of adaptor proteins and signal transducers that contain SH-2 or PTB (phospho-tyrosine binding) domains, such as Shc (Pelicci et al., 1992), Grb2 (Buday and Downward, 1993), Src (Anderson et al., 1990), Cbl (Levkowitz et al., 1996), PLC□ (Cohen et al., 1996) and PI3K (Soltoff et al., 1994). ErbB receptors can couple to different signal transduction pathways depending on the cellular context. The primary pathway that mediates ErbB control of gene expression is the Ras-Raf-MAPK pathway (Brunner et al., 1994)

In mammalian cells, binding of most EGF growth factors to ErbB-1 induces the clustering of ligand-receptor complexes in coated pits, followed by endocytosis of the complexes and ultimately lysosomal degradation or

recycling of both EGF and its receptor. Interestingly, TGF-□ stimulated ErbB-1 does not undergo endocytosis and degradation (Ebner and Derynck, 1991). Other ErbB proteins (ErbB-2,3,4) do not undergo the rapid endocytic response to ligand activation (Baulida et al., 1996).

Normal biological function of ErbB proteins

All ErbB proteins play important roles in normal development. It is now well established that ErbB-1 is expressed throughout development and in a variety of undifferentiated as well as differentiated cells (Gospodarowicz et al., 1981). Among the functions attributed to ErbB-1 activity are the proliferation and development of specific epithelial territories in the embryo, including branch point morphogenesis and maturation of early embryonic lung tissue, skin development, and the promotion of survival of early progenitor cells of the cleft palate (Warburton et al., 1992, Abbott et al., 1991). Absence of ErbB-1 expression is lethal as a result of major defects of epithelial development in many organs, including those involved in tooth growth and eye development (Miettinen et al., 1995, Threadgill et al., 1995).

ErbB-2, 3 and 4 are involved in the interaction between nerves and their target cells, for example, muscle, glia and Schwann cells; and they are essential for cardiac and neural development (Yarden et al., 2001). In the embryonic rat, ErbB-2 was detected in the nervous system, connective tissue, skin, intestine, lung, and kidney (Kokai et al., 1987). The phenotype of ErbB-2 knock out mice shares characteristics with both Neuregulins and ErbB-4 knockout animals (Lee et al., 1995) which implies these three genes are closely relevant in development and essential for the activation of an overlapping pathway. ErbB-3 knockout mice developed severe neuropathies and lack Schwann cells, underscoring the importance of this receptor in peripheral nerve development (Riethmacher et al., 1997).

ErbB family signaling in tumorigenesis

All members of the ErbB family of proteins are implicated in tumorigensis. Both over-expression and structural alterations of ErbB-1 are frequent in human malignancies. Over-expression of ErbB-1 is a very frequent genetic alteration in brain tumors; amplification of the gene occurs in 40% of glioblastomas. Over-expression of ErbB-1 is associated with higher grade, higher proliferation and reduced survival (Wikstrand et al., 1998).

In a significant proportion of tumors, gene amplification is accompanied by rearrangement. The most common mutation deletes part of the extracellular domain, yielding a constitutively active receptor (Wong et al., 1992). Recent studies identified an identical alteration in carcinomas of the lung, ovary and breast, suggesting broader implication of ErbB receptors for human cancer (Moscatello et al., 1995).

ErbB-2 is one of the most frequently altered genes in human cancers. ErbB-2 is found amplified in human adeno-carcinomas of breast, stomach, and ovary (Yokota et al., 1986, Kraus et al., 1987, Slamon et al., 1989). ErbB-3 is mainly associated with breast carcinomas (Kraus et al., 1989). ErbB-4 is often found expressed in childhood medullo-blastomas, co-expression of ErbB-4 and ErbB-2 has a prognostic value in childhood medullo-blastomas (Gilbertson et al., 1997). Several studies found that co-expression of ErbB-2 with ErbB-1 or ErbB-3 in oral squamous cell carcinoma was significant and it critically improved the predicting power (Xia et al., 1999). Thus, it appears that signaling through heterodimer receptor containing ErbB-2 plays important role in tumorigenesis.

As described above, CD44, HGF, c-Met, EGF growth factors and ErbB proteins play important roles in a number of physiological and pathological

processes during embryonic development or in the adult organism, especially in regulation of cell proliferation. These proteins also have very important functions in tumor growth and metastasis. The molecular mechanisms of how these proteins contribute to tumorigensis are still not clear.

1.4 Background and the aim of my project

In our group, Dr. Veronique Orian-Rousseau's work showed that CD44 is important for HGF/c-Met signaling. In human colon adenocarcinoma cells (HT-29) and rat pancreatic carcinoma cells (BSp73ASML), CD44 exon v6 specific antibodies can block the phosphorylation of the c-Met receptor upon induction with HGF. Phophorylation of Erk, a downstream target of the c-Met signaling pathway, can also be blocked by the same antibodies. Moreover, physiological events such as HGF induced cell scattering and chemotaxis could be abrogated by these antibodies (Orian-Rousseau et al., 2002).

How are CD44 variant proteins involved in HGF/c-Met signaling? Recent reports showed that CD44 isoforms played important roles in the activation of the proform of TGF-□1 and HB-EGF (Yu et al., 2002, Yu et al., 1999). Since HGF is also released as a proform that is activated by proteolytic cleavage (Naka et al., 1992), we ask whether CD44 is required for HGF proform activation. There was no significant CD44-dependent pro-HGF activation detected in HT-29 cells even after prolonged incubation in the absence of serum (Orian-Rousseau et al., 2002). Thus it seems that CD44 plays a role in HGF dependent signaling other than by activation of the HGF proform.

To unravel this mode of action of CD44 v6 was one goal of my work. Furthermore, I investigated whether a CD44 v6 involvement is restricted exclusively to c-Met signaling or is also required for other growth factor receptors signaling.

2. Materials & Methods

2.1 Materials

2.1.1 General chemicals

Agarose	Peqlab, Erlangen
APS (Ammonium persulfate)	Sigma, Deisenhofen
Aprotinin	Sigma, Deisenhofen
2-□ Mercaptoethanol	Roth, Karlsruhe
Bromphenol blue	Serva, Heidelberg
BSA (Bovine Serum Albumin)	Serva, Heidelberg
Chloroform	Merck, Darmstadt
DMSO (Dimethyl sulfoxide)	Fluka, Neu-Ulm
DTT (Dithiothreitol)	Sigma, Deisenhofen
DSP[Dithiobis(succinimidylpropionate)]	
DTSSP[(Dithiobis(sulfosuccinimidylpropionate)]	
	PIERCE, U.S.A.
EDTA	Merck, Darmstadt
Ethanol	Roth, Karlsruhe
Ethidium bromide	Sigma, Deisenhofen
Formamid	Merck, Darmstadt
G418	GIBCO, Eggenstein
Glycerol	Merck, Darmstadt
HEPES	Roth, Karlsruhe
Hyaluronic acid, (rooster Comb)	Sigma, Deisenhofen
Isopropanol	Merck, Darmstadt

Leupeptin	Sigma, Deisenhofen
Methanol	Roth, Karlsruhe
Nonidet P-40	Boehringer, Mannheim
NHS-LC-Biotin	Pierce, USA
NeutrAvidin™-HRP conjugated	Pierce, USA
PMSF	Sigma, Deisenhofen
Primer p(dT)15	Boehringe, Mannheim
Rotiphorese Gel30: Acrylamide/bis-acrylamide	
(30% / 0.8%)	Roth, Karlsruhe
SDS (Sodium lauryl sulfate)	Roth, Karlsruhe
TEMED	Sigma, Deisenhofen
Tris	Roth, Karlsruhe
Tris Hydrochlorid	Roth, Karlsruhe
Triton-X100	BioRad, Müchen
Tween-20	Serva, Heidelberg

All other general chemicals were purchased from Merck(Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) or Sigma(Deisenhofen).

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
Benchmark (prestained)	GIBCO
Unstained Precision Protein Standards	BIO-RAD
Protein A sepharose CL-4B	Amersham
Protein A-Agarose	Calbiochem
Protein G-Agarose	Calbiochem
ProteinG Plus/ProteinA-Agarose	Calbiochem
Complete (Protease inhibitor Cocktail tablets)	Roche
TRIzol® Reagent (Total RNA Isolation Reagent)	GIBCO

2.1.4 Growth factors:

HGF (Hepatocyte growth factor)	R&D system
HB-EGF (Heparin-binding EGF-like growth factor	R&D system
EGF (Epidermal growth factor)	R&D system
AR (Amphiregulin)	R&D system
HRG-β1 (Heregulin-beta1)	R&D system
TGF-α (Transforming growth factor alpha)	R&D system

2.1.5 Primers for mouse CD44:

CD44R-C2A	5'-GGCACTACACCCCAATCTTC-3'
CD44R-C13	5'-AAGACATCGATGCCTCCAAC-3'
CD44R-PV1	5'-GCCTCAACTGTGTACTCAAA-3'
CD44R-PV2	5'-GATGACTACCCCTGAAACAC-3'
CD44R-PV3	5'-ACGGAGTCAAATACCAACCC-3'
CD44R-PV4	5'-TGCAACTACTCCATGGGTTT-3'

CD44R-PV5	5'-TATAGACAGAAACAGCACCA-3'
CD44R-PV6	5'-TGGGCAGATCCTAATAGCAC-3'
CD44R-PV7	5'-CTGCCTCAGCCCACAACAAC-3'
CD44R-PV8	5'-CCAGTCATAGTACAACCCTT-3'
CD44R-PV9	5'-CAGAACTTCTCTACATTACC-3'
CD44R-PV10	5'-GGTCGAAGAAGAGGTGGAAG-3'

Primers for human CD44 (Synthesised by Brisner&Grob Biotech GmbH)

CD44-C2Ah	5'-CCAAGATGATCAGCCATTCTGG -3'
CD44-C12Ah	5'-ATGCAAACTGCAAGAATC-3'
CD44-C13h	5'-AAGACATCTACCCCAGCAAC-3'
CD44-pv2h	5'-GATGAGCACTAGTGCTACAG-3'
CD44v3-1h	5'-ACGTCTTCAAATACCATCTC-3'
CD44v3-2h	5'-TGGGAGCCAAATGAAGAAAA-3'
CD44-pv4h	5'-TCAACCACACCACGGGCTTT-3'
CD44-pv5h	5'-GTAGACAGAAATGGCACCAC-3'
CD44-pv6h	5'-CAGGCAACTCCTAGTAGTAC-3'
CD44-pv7h	5'-CAGCCTCAGCTCATACCAGC-3'
CD44-pv8h	5'-TCGAGTCATAGTACAACGCT-3'
CD44-pv9h	5'-CAGAGCTTCTCTACATCACA-3'
CD44-pv10h	5'-GGTGGAAGAAGAGACCCAAA-3'

2.1.6 Cell culture reagents:

Trypsin, 0.25%	Difco, Detroit
DMEM	GibcoBRL Life Technologies, Karlsruhe
RPMI 1640	GibcoBRL Life Technologies, Karlsruhe
New born calf serum	GibcoBRL Life Technologies, Karlsruhe
Foetal calf serum (FCS)	Bio-Whittaker, Belgium
Foetal calf serum Gold	PAA Laboratories GmbH, linz Austria
Penicillin-Streptomycin	Invitrogen
Glutamin	GibcoBRL Life Technologies

2.1.7 Cell lines:

Name	description	Culture Medium
HT29	human colon adenocarcinoma cell line	DMEM, 10%FCS, 0.2M glutamin,
hepG2		DMEM, 10%FCS
5G8 hybridoma		RPMI, 15%FCS, 0.2M glutamin
IM7 hybridoma		RPMI1640, 15%FCS, 0.2M glutamin
RT4-D6-P2T		DMEM, 10%FCS
RT4-D6-P2T-clone5.4 (RT5-4)	RT4-D6-P2T transfected with dox-inducible merlin	DMEM, 10%FCS, 0.5g/l G418, 1mg/l puzomycin
NIH3T3		DMEM, 10% new born calf serum

Immortalized fibroblast from CD44 +/- mice		DMEM, 10% new born calf serum
Immortalized fibroblast from CD44-/-		DMEM, 10% new born calf serum
BSp73ASML(ASML)		RPMI1640, 10%FCS
BSp73AS10 (AS10)		RPMI1640, 10%FCS
AS14	AS10 cells transfected with CD44v4-7, without 5G8 epitope(exon15)	RPMI1640, 10%FCS, 0.3g/l G418
DIabc-v	AS10 cells transfected with CD44v1-10	RPMI1640, 10%FCS, 0.3g/l G418
AS-s6.7	AS10 cells transfected with CD44v6-7, with 5G8 epitope(exon15)	RPMI1640, 10%FCS, 0.3g/l G418
AS-v6.7	AS10 cells transfected with CD44v6-7, without 5G8 epitope(exon15)	RPMI1640, 10%FCS, 0.3g/l G418
AS-s6	AS10 cells transfected with CD44v6, with 5G8 epitope(exon15)	RPMI1640, 10%FCS, 0.3g/l G418
AS-s7	AS10 cells transfected with CD44v7, with 5G8 epitope(exon15)	RPMI1640, 10%FCS, 0.3g/l G418
M4.2	AS10 cells transfected with CD44v4-7 lacking the cytoplasmic tail	RPMI1640, 10%FCS, 0.3g/l G418
HA-PCR#2	AS10 cells transfected with CD44v4-7, with R44 mutation in HA-	RPMI1640, 10%FCS, 0.3g/l G418

	binding domain	
E12.9	AS10 cells transfected with CD44v8-10	RPMI1640, 10%FCS, 0.3g/l G418
AS-CD44N	AS10 cells overexpressed CD44s	RPMI1640, 10%FCS, 0.3g/l G418
MDCK	Madin-Darby Canine Kidney	DMEM, 10%FCS

2.1.8 Antibodies:

Antibodies	Isotype	epitope	Source
Phospho-p44/42 MAP Kinase antibody	Rabbit IgG1	Detect phosphorylated Erk1/2 in human, rat, mouse species	New England Bio-labs
Erk (K-23)	Rabbit IgG1	Detect Erk1/2 in human, rat, mouse species	Santa Cruz
h-Met (C-12)	Rabbit IgG1	Detect c-met of human origin	Santa Cruz
m-Met	Mouse IgG2a	Detect c-met of mouse or rat origins	Santa Cruz
HGF-□	Rabbit IgG1	Detect HGF□ of human or mouse origins	Santa Cruz

anti-Phosphotyrosine clone 4G10	Mouse IgG2b	Detect tyrosine phosphorylated proteins	Upstate
anti-Phosphotyrosine clone PY20	Mouse IgG2b	Detect tyrosine phosphorylated proteins	BD company
EGF Receptor	Mouse IgG1	Detect EGF receptor of human or mouse origins	BD company
NF2 (C18)	Rabbit IgG1	Detect carboxyl terminus of NF2	Santa Cruz
NF2 (A19)	Rabbit IgG1	Detect amino terminus of NF2	Santa Cruz
human anti-CD44var(v6) clone VFF18	Mouse IgG1	Detect an epitope encoded by exon v6 on the variant portion of human CD44	Bender MedSystems
pan-CD44 antibody clone IM7	Rat IgG2b	Detect all isoforms of CD44 of mouse origins. Cross-reacts with human CD44	prepared from hybridomas
CD44 clone 5G8	Mouse IgG1	Detect all isoforms of CD44 of rat origins	prepared from hybridomas
anit-CD44v6 clone 1.1ASML	Mouse IgG1	Detect an epitope encoded by exon v6 on the variant portion of rat CD44	purified from ascites

Hermes3	Mouse IgG1	Detect all isoforms of CD44 of human origins	gift of Sirpa Jalkanen, Turku, Finnland

Second antibodies: HRP-Mouse IgG, HRP-rat IgG, HRP-rabbitIgG were purchased from Dako company, Hamburg.

Isotype control antibodies: Rat IgG2a, Rat IgG2b, mouse IgG2b´, mouse IgG1 were purchased from Southern Biotechnology Associates Inc., U.S.A.

2.2 Methods:

2.2.1 Cell Culture

All cells were maintained in a humidified (95%) atmosphere with 5% CO_2 at 37 °C. The culture medium for each cell line is described before (see Materials, paragraph cell line). The storage of cells was performed by harvesting cells and resuspending cell pellet with freezing medium (10%DMSO in FCS). The cell suspension was transferred into pre-chilled cryo-vial, left on ice for 1 hour and then froze at -80°C. For long-term storage, the frozen cells were transferred to liquid nitrogen. To thaw cells, the cryo-vial was taken out of liquid nitrogen and was thawed fast in 37 °C water bath. Then cells were transferred to 15ml falcon, washed with 10ml pre-warmed fresh medium, centrifuged, the cell pellet was resuspended with fresh medium and plated in a flask or petri dish.

2.2.2 Isolation of cell total RNA

10^6 cultured cells was washed with chilled PBS and lysed in 1 ml TRIzol Reagent (Gibco-BRL). The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per ml of TRIzol Reagent was added. The sample tubes were capped securely and shaken vigorously by hand for 15 seconds. After 3 minutes incubation at room temperature, the samples were centrifuged at 10,000 g for 15 minutes at 4 °C. Following centrifugation, the aqueous phase was transferred to a fresh tube, and the RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol per ml TRIzol Reagent used for the initial homogenization. Samples were kept at room temperature for 10 minutes and centrifuged at 12,000 g for 10 minutes at 4 °C. 1 ml of 75% ethanol was added to the RNA pellet and the pellet was mixed by vortex and precipitated by centrifugation at 7,500 g for 5 minutes at 4 °C. The RNA pellet was briefly dried at room temperature for 5

minutes and dissolved in 200 µl Rnase-free H_2O. 4µl of the RNA was diluted to 400µl and the concentration was measured. The RNA was stored at -80°C.

2.2.3 Reverse transcription PCR

3µl (5µg) of total RNA was mixed with 4µl of oligodT_{15} (100µg/ml) and 5µl of distilled water in a PCR tube. It was heated at 70°C for 10 minutes (Perkin Elmar 9600/2400) and quick chilled on ice. Following brief centrifugation, 4µl of 5µ first strand reaction buffer, 2µl of 0.1M DTT, 1µl of 10mM dNTPmix were added. The tube was incubated at 42°C for 2minutes. Reverse transcription was started by the addition of 1µl of superscript II reverse transcriptase. The reaction last 50 minutes at 42°C and was inactivated by heating at 70°C for 15minutes. The cDNA is used as a template for amplification in PCR.

The following components were added in a 0.5 ml micro-centrifuge tube which sitting on ice:

10µPCR buffer Minus Mg	10µl
10mM dNTPmix	2µl
50mM $MgCl_2$	3µl
Template DNA	4µl
Taq DNA polymerase (5U/µl)	0.5µl
Primer 5‘	2.5µl
Primer 3‘	2.5µl
Distilled H_2O	75.5µl

The components were mixed briefly. After denaturing at 94°C for 5 minutes, the PCR amplification was performed as follows for 35 cycles:

Denature	94°C	45 seconds
Anneal	50°C	1minutes
Extend	72°C	2minutes

The PCR was finished by incubation 10 minutes at 72°C. The samples were stored at –20°C.

2.2.4 Purification of antibodies

500 ml tissue culture medium was cleared first by centrifugation at 5,000 g for 10 minutes. The supernatant was transferred to a clean container. Solid ammonium sulphate was added slowly with stirring to a final concentration of 50% saturated. It was incubated at 4 °C overnight with gentle stirring.

The next day, the precipitation was obtained by centrifugation at 5,000 g for 10 minutes. The supernatant was discarded, while the pellet was resuspended in 10-20 ml 150 mM NaCl, 20 mM phosphate pH7.0. It was transferred to a dilysis bag and dialysed for 4 hours against 150 mM NaCl, 20mM phosphate pH7.0.

Ammonium sulphate precipitated protein solution was transferred to a falcon. The supernatant was collected after centrifugation at 10,000 g for 10 minutes. 2ml of 50% slurry of protein G plus agarose was added. The mixture was incubated with rotation for 2 hours (or overnight) at 4 °C. The beads was collected by centrifugation at 3,000 g for 5 minutes and washed 4 times with 20 mM phosphate pH7.0, 150mM NaCl. After washing, the beads were packed into Econopak column. The column was eluted with 100 mM glycine ph 2.5 after the excess fluid was drained off. 10□ 0.5 ml eluting fraction was collected. Each aliquot was neutralized by 50 □l 1M Tris pH 9.6. The column was eluted with 100 mM sodium bicarbonate pH 10.8. 10 fractions of elution were collected and each aliquot was neutralized with 87 □l 1M sodium citrate pH 5.0.

The concentration of protein was measured by reading the O.D. 280. The fractions containing significant amount of antibody was pooled and dialysed against PBS at 4 °C overnight. The final concentration of antibody was measured again by reading of O.D 280.

Absorbance at 280 /1.4 = Concentration (mg/ml)

The antibody was stored in –20 °C.

2.2.5 Preparation of Cell lysate:

To analyze some protein directly by western blot, cells grown in 24 well plate (or 12 well plate) were washed with PBS and lysed in 2× Laemmli sample buffer (160mM Tris HCl pH 6.8, 4%SDS, 16% glycerol, 0.1M DTT, 0.01% bromphenol blue). DNA was sheared through a 26-guage needle. The cell lysate was denatured at 95°C for 5 minutes before loading on SDS-PAGE. To do western-blot with anti-CD44 antibodies, such as IM7 or KM81, cells were lysed in non-reducing sample buffer without DTT.

2.2.6 Immunoprecipitation:

Cells were grown in 10 cm (or 15cm) plates, washed once with ice-cold PBS and lysed with 1ml of lysis buffer containing protease inhibitors (1×Complete^{TM} protease inhibitor cocktail, Boehringer Mannheim). After 15 minutes incubation on ice, lysed cells were scraped and pooled in a 1.5 ml eppendorf. The lysate was cleared by centrifugation for 15 minutes 13,000 g at 4°C. Supernatant was transferred to fresh tube. 5μg/ml of antibody was added and the tube was rotated overnight at 4 °C. 50 μl of 50 % beads slurry (balanced in lysis buffer) was added the sample was rotated for another 4 hours. Immuno-complexes were recovered by centrifugation and washed 4 × with cold lysis buffer. After the last wash, the supernatant was removed as much as possible and 50 μl of sample buffer was added. Samples were boiled at 95°C for 5 minutes before loaded on SDS-PAGE. Or the samples can be kept at –20°C.

2.2.7 Biotiny label HGF, activation of pro-HGF

25 μg HGF (R&D system) was dissolved in 50μl PBS, 2 μl sulfo-NHS-LC-biotin (5mg/ml) was added and mixed well. After 2 hours incubation on ice, the biotinylated HGF was transferred to mini dialyser (MWCO 10,000),

dialysed against PBS for 2 hours at room temperature. (Change fresh PBS after 2 hours). The dialysis was continued at 4 °C overnight after third change of PBS. The yield of biotinylation reaction was estimated by western blot analysis with HGF-□ antibody and comparison to known amounts of non-biotinylated HGF.

HGF was activated with 5 % serum at 37 °C overnight.

2.2.8 HGF induction, Chemical cross-linking

Cells were seeded at a concentration of 3□ 10^6 cells per 10 cm plates, serum starved for 24 hours, induced with HGF (50ng/ml) at 37 °C for 5 minutes. After 3□ wash with PBS, cross-linking of proteins on cells was performed in 2 ml PBS plus 3 mg DTSSP at room temperature for 1 hour. The reaction was stopped using 40□l of 20 mM Tris-HCl pH 7.4. The cells were then washed with chilled PBS, lysed with HEPES lysis buffer (25mM HEPES pH7.4, 100mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1.25 % CHAPS, 5ul/ml proteinase-inhibitor-mix (complete, Roche).

2.2.9 Separation of proteins by polymerylamide gel electrophoresis (PAGE)

The separating gel mixture is poured between two glass plates with spacers between and allowed to polymerize. The stacking gel mixture was poured on the top of separating gel and the comb was inserted in the gel to form the wells. The gel was rum in 1□ laemmli-running buffer (25mM Tris-HCl pH 8.3, 0.2 M glycine and 0.1% SDS) until the desired distance had been reached.

10% separating gel mix (25ml)	Gel-30	8.3ml
	1.5M Tris pH8.8	6.25ml
	10% SDS	0.25ml
	H_2O	10ml

	APS (10%)	250µl
	TEMED	15µl
Stacking gel mix (20ml)	Gel-30	3.4ml
	0.5M Tris pH6.8	5ml
	10% SDS	0.2ml
	H_2O	11.3ml
	APS (10%)	250µl
	TEMED	15µl

2.2.10 Immunoblot

After gel electrophoresis, proteins were transferred to Immunobilon-PVDF membrane (Millipore) using Bio-Rad transfer chambers containing transfer buffer (24 mM Tris base, 193 mM glycine, 10% methanol). Transfer was performed at 4 °C overnight.

The blots were incubated 1 hour in blocking buffer (5% BSA, 0.1% tween in 10 mM Tris pH 7.6, 100mM NaCl) at room temperature. Incubation with primary antibodies (normally 1µg/ml) was in blocking buffer for 2 hours at room temperature (for phospho- specific antibodies, overnight 4 °C). After 3× 10 minutes wash with TBS, 0.1% tween, the blots were incubated with HRP labeled secondary antibodies at room temperature for 1 hour. After again 3× 10 minutes wash with TBS, 0.1% Tween, the blots were developed by enhanced chemiluminescence (Amersham) and visualized with Hyperfilm-ECL (Amersham).

To reprobe with another antibody, the blots were stripped with washing buffer (0.063 M Tris-HCl pH 6.8, 2% SDS, 0.8g % DTT) at 50 °C for 40 minutes, and then probed as described before.

2.2.11 Silver staining

After electrophoresis, the gel slab was fixed in 50% methanol, 5% acetic acid in water for 20 min. It was then washed for 10 min with 50% methanol in water and additionally for 10 min with water to remove the remaining acid. The gel was sensitized by 1 min incubation in 0.02% sodium thiosulfate, and it was then rinsed with two changes of distilled water for 1 min each. After rinsing, the gel was submerged in chilled 0.1% silver nitrate solution and incubated for 20 min at 4 °C. After incubation, the silver nitrate was discarded, and the gel slab was rinsed twice with water for 1 min and then developed in 0.04% formalin [35% formaldehyde in water (Merck, Darmstadt)] in 2% sodium carbonate with intensive shaking. After the developer turned yellow, it was discarded and replaced with a fresh portion. It is essential that the developing is carried out in an absolutely transparent solution. After the desired intensity of staining was achieved, the development was terminated by discarding the reagent, followed by washing of the gel slab with 5% acetic acid. Silver-stained gels were stored in a solution of 1% acetic acid at 4 °C until analyzed.

2.2.12 FACS analysis

Cells were harvested with 5mM EDTA/PBS and resuspended in PBS/ 0.3%FCS, then the cells were transferred to a 96 well plate (0.5×10^6 cells/well) and stained with primary antibodies (10 µg/ml). After 30 min incubation at 4 °C, the cells were washed 2× with PBS/0.3% FCS and incubated with fluorescently labeled secondary antibodies. After 30 min incubation, the cells were washed again 2× with PBS/0.3% FCS, and analyzed by a FACS®star Plus Flow Cytometer (Becton Dickinson).

Results:

A CD44 variant isoform is required for c-Met activation.

Both c-Met and CD44 variant proteins were independently implicated in tumor metastasis (Di Renzo et al., 1995, Di Renzo et al., 2000, Günthert et al., 1991). CD44 proteins could bind some growth factors such as HB-EGF and FGFs, acting as a supportive molecule in the signaling induced by these growth factors (Bennett et al., 1995, Yu et al., 2002, Sherman et al., 1998). To explore whether CD44 can also bind HGF and support c-Met signaling, I have chosen two metastatic cell lines, BSp73ASML (ASML) and HT-29. Both these cell lines express several CD44 isoforms and c-Met. The relevance of CD44 variant proteins in ASML cells for the metastatic process was demonstrated by an experiment in which antibodies against an exon v6 encoded epitope interfered with tumor growth and metastasis (Seiter et al., 1993). The involvement of CD44 v6 containing isoforms in HT-29 cells for tumor metastasis was tested by expression of antisense CD44 v6. Down-regulation of CD44 v6 containing isoforms inhibited the metastatic spreading of these cells (Reeder et al., 1998).

If CD44 is involved in the signaling of c-Met in these cells, antibodies against CD44 might interfere with c-Met signaling. To test this assumption, HT-29 cells were starved, and then treated with HGF with or without CD44 v6 specific antibody pretreatment. c-Met was precipitated and subjected to SDS-PAGE. Phosphorylation of c-Met was detected by Western-blotting with the phosphotyrosine specific antibody 4G10. HGF induced an increase of c-Met tyrosine phosphorylation (Fig. 1A). This activation could be completely blocked by antibodies against CD44 exon v6. Thus, in fact a CD44 variant

isoform containing exon v6 encoded sequences appears to be required for c-Met activation.

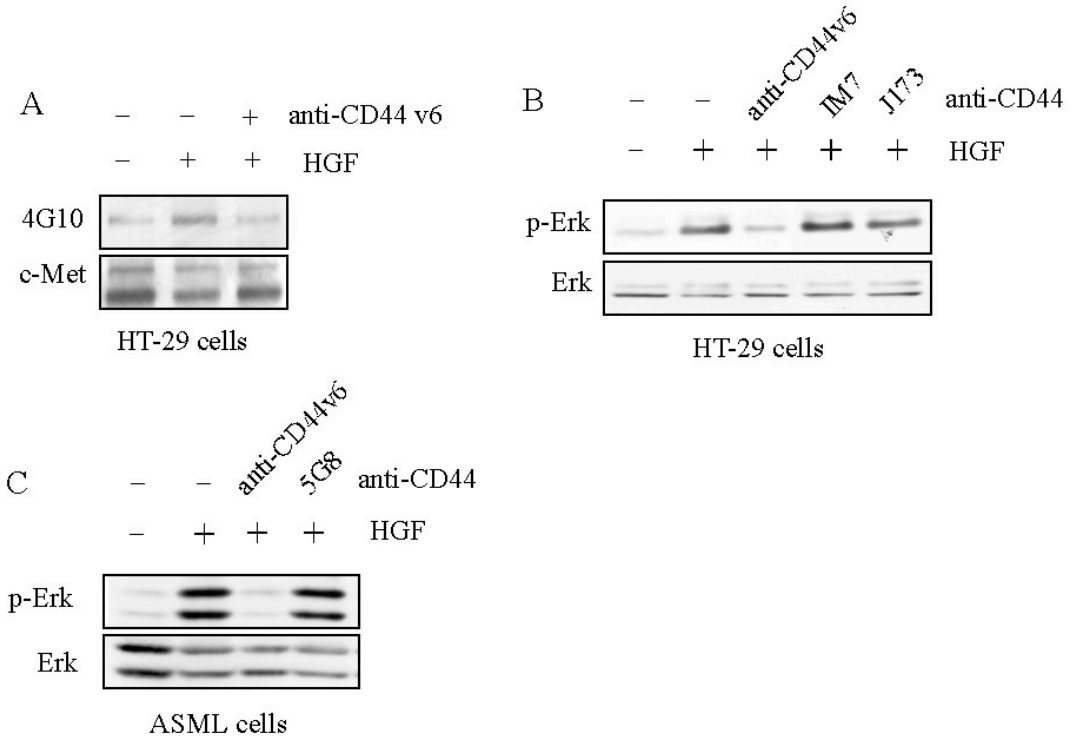

Fig. 1: A CD44 variant is required for c-Met activation.
A: HT-29 cells were seeded at a density of 3□10^6 cells /10cm plate, starved for 24 hours, and then induced with HGF (50ng/ml) for 5 min in the presence or the absence of anti-CD44 antibodies (50□g/ml). Subsequently, cells were lysed and c-Met was immunoprecipitated. Immune precipitates were resolved by SDS-PAGE (7%). c-Met phosphorylation was detected using the phosphotyrosine antibody 4G10 (upper panel). The membrane was stripped and re-probed with a c-Met antibody (lower panel).
B. C: HT-29 or ASML cells were plated in 24 well plate (10^5 cells/well), starved for 24 hours, and then induced with HGF (50ng/ml) for 5 minutes in the presence or absence of antibodies (50□g/ml). Afterwards cells were dissolved in 2□ sample buffer. The lysates were subjected to SDS-PAGE (10%) and Western blotting for phosphorylated Erk (p-Erk panels) and total Erk (Erk panels).

The original blots of Fig. 1 A, C were shown in appendix. Parts of the blots containing the bands that expected were scanned and shown in the figure.

The absence of any phosphorylation of c-Met upon treatment with antibodies against a CD44 v6 epitope suggests that all signaling components downstream of c-Met are also affected by antibody treatment. This was indeed the case, as shown for signal transduction to Erk (Fig. 1B, C). Both in HT-29 cells and in ASML cells, Erk phosphorylation was completely blocked by CD44 v6 specific antibodies.

The inhibition of Erk activation was specific for v6, since control antibodies IM7 and J173 (for HT-29) and 5G8 (for ASML) had no effects. IM7 and J173 recognize an amino-terminal epitope of CD44. This epitope is present in all CD44 proteins. 5G8 recognizes an epitope on exon 15 encoded sequences. This epitope is present only in the smallest CD44 isoform that is expressed in ASML cells (CD44s), but not in the variant isoforms (Günthert et al., 1991, Sleeman et al., 1996).

CD44 isoforms bearing exon v6 encoded sequence are sufficient for the cooperation with c-Met.

ASML and HT-29 cells express several CD44 isoforms as a result of alternative splicing of variant exons (Günthert et al., 1991, Reeder et al., 1998). To explore which variant sequences are required for cooperation with c-Met, I first analyzed which CD44 molecules were expressed in these two carcinoma cell lines and thus could be potential candidates for cooperation. Exon-specific RT-PCR (van Weering et al., 1993) permits to determine both the structure and the approximate abundance of the isoforms (Fig. 2). The “ladder” obtained with the variant exon specific primers (König et al., 1996,

van Weering et al., 1993) in combination with the size of each product demonstrated the structure of the CD44 isoforms. The approximate abundance of isoform transcripts can be estimated from the intensity and size of bands obtained with the constant region primers (Fig. 2, “c” lane).

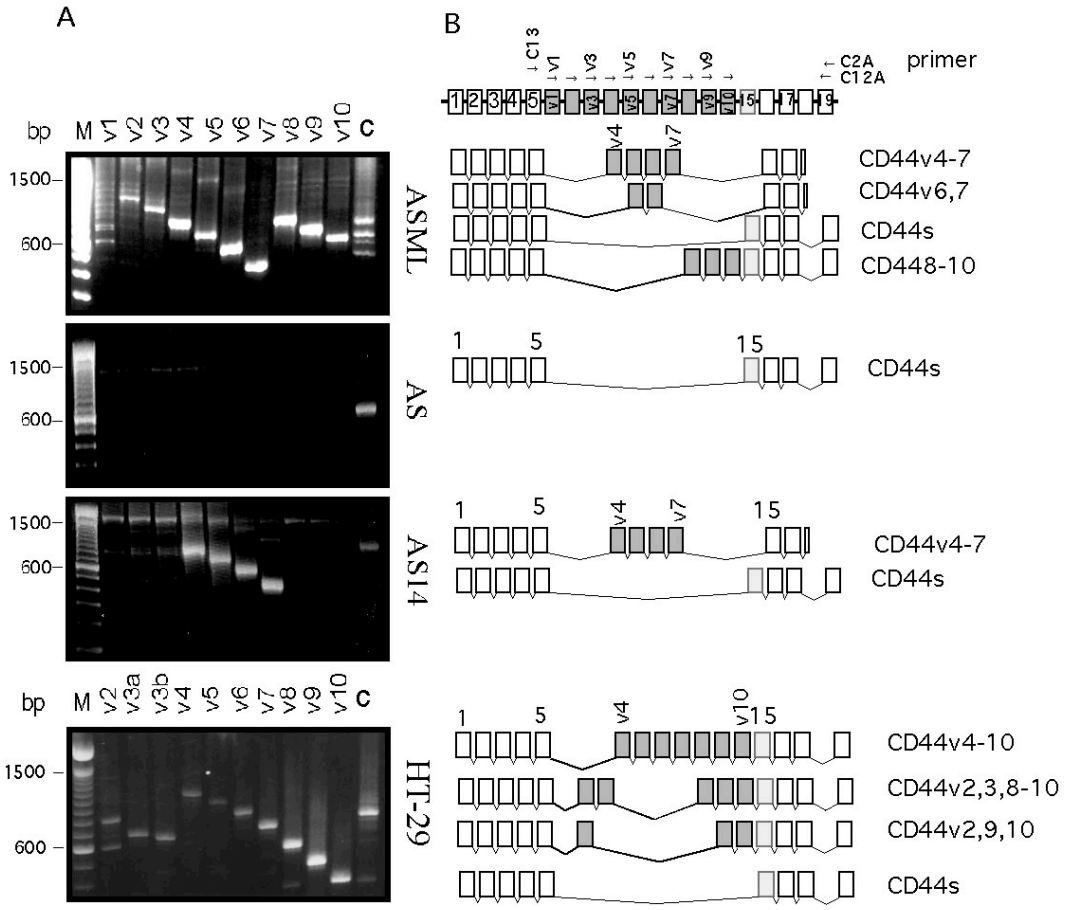

Fig. 2: Exon-specific RT-PCR analysis of CD44 isoforms in ASML, AS, AS14 and HT-29 cells.
A: RT-PCR analysis with mRNA derived from the cells as indicated were performed with exon-specific primers as described (see Materials and Methods). The marker lane (M) shows a 100 bp DNA ladder (Life Technologies, Karlsruhe).
B: Schematic representation of CD44 isoforms expressed in ASML, AS, AS14 and HT-29 cells. Constant region exons are numbered 1-5 and 15-19, variant exons v1-v10.

The predominant CD44 isoforms in ASML cells are CD44v4-7, CD44v6,7 and CD44s. Minor species are CD44v8-10 and possible CD44v2-7 (Fig. 2). HT-29 cells mainly express four isoforms CD44v4-10, CD44v2,3,8-10, CD44v2,9,10 and CD44s (Fig. 2). In both cell lines several isoforms thus carry the epitope encoded by exon v6 to which the inhibitory antibodies binds.

Table 2: AS transfectants:

Cell line	CD44 isoforms transfected
AS14	CD44v4-7Δ15
DIabc-v	CD44v1-10
AS-s6	CD44v6
AS-s7	CD44v7
AS-s6,7	CD44v6,7
AS-v6,7	CD44v6,7Δ15
E12.9	CD44v8-10
AS-CD44N	Overexpressed CD44s
HA-PCR#2	CD44v4-7, with R44 mutation in HA-binding domain
M4.2	CD44v4-7, lacking the cytoplasmic tail

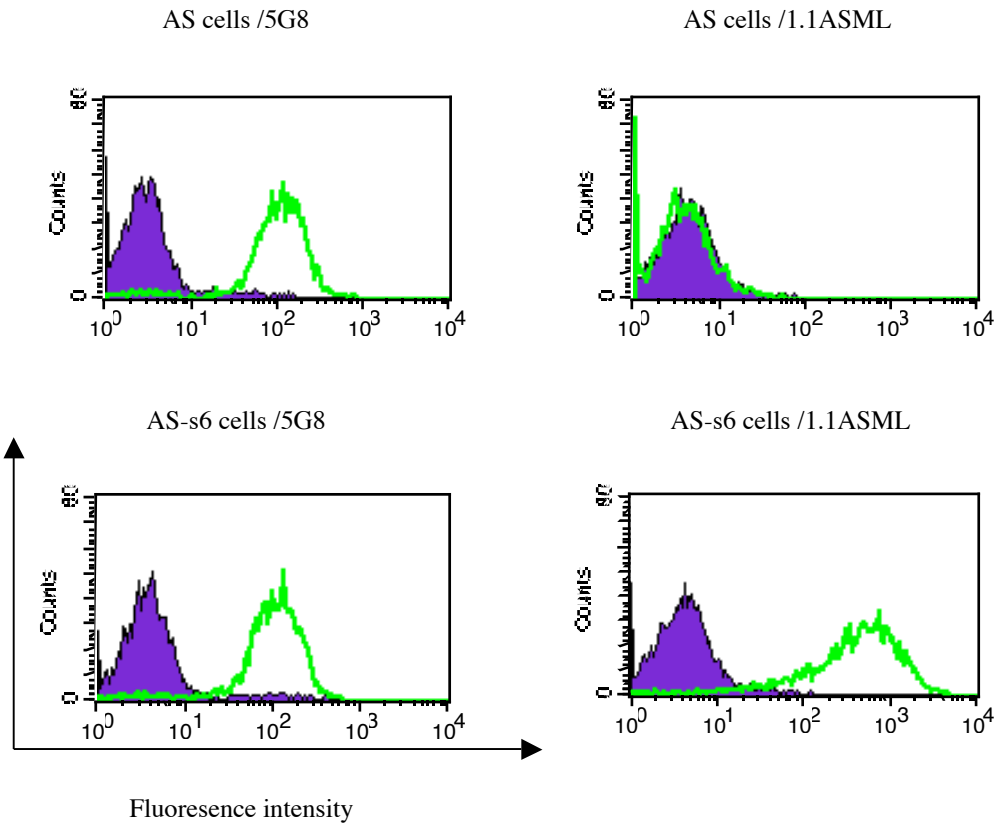

Fig. 3 FACS analysis with CD44 antibodies.

FACS analysis was performed as described in Materials and Methods on cells stained with 5G8 or 1.1ASML antibodies. Plots of fluoresence intensity against cell number are shown. In each case, the blue trace represents background staining, while the green trace represents the staining with the indicated antibodies.

In order to specifically define which variant exon sequences are required for cooperation with c-Met, I used rat pancreatic AS cells transfected with different isoforms of CD44. The parental AS cells only express CD44s (Fig. 2). Although it expresses levels of c-Met comparable to those in ASML cells (Fig. 4), it is hardly induced by HGF as determined by phosphorylation of Erk (Fig. 5B, panel 1). The names of stable transfectants of AS cells and which CD44 isoforms they were transfected with are listed in Table 2. The parental cell line AS cells and all transfectants express endogenous CD44s, thus they could be stained by pan-CD44 antibody 5G8. Only when the cells were transfected with CD44 exon v6 bearing isoforms, they could be stained by CD44 v6 specific antibody (1.1ASML).

Additional over-expression of CD44s in AS cells did not induce a response to HGF treatment (Fig. 5B, panel 2). However, the AS cells became HGF inducible upon transfection with CD44 isoforms bearing v6 encoded sequences. Even in AS-s6 cells that were transfected with the CD44v6 sequence, the cells became highly inducible by HGF (Fig. 4B, panel 10). In contrast CD44 isoforms without v6 such as CD44v8-10 and CD44v7 did not support Erk activation (Fig. 5B, panel 3, 4).

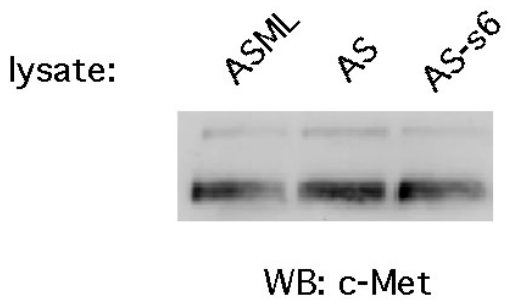

Fig. 4 Western blot with c-Met specific antibody.
The lysates of ASML, AS and AS-s6 were resolved on SDS-PAGE, Western blotting was performed with c-Met specific antibody. Equal amout of loading was checked by DC Protein Assay (Bio-Rad).

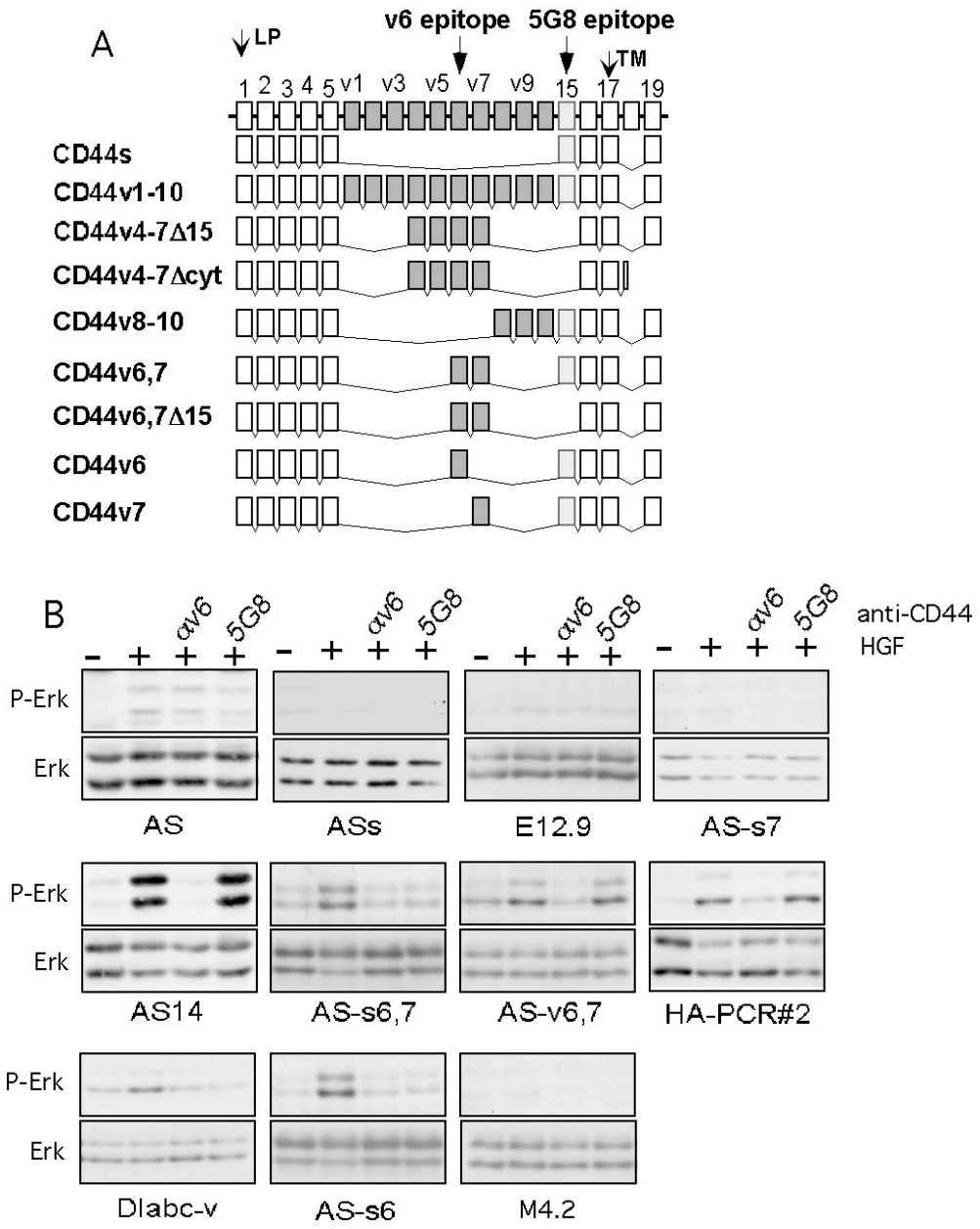

Fig. 5: CD44 isoforms containing the exon v6 sequence are required for c-Met activation.

A: Schematic representation of CD44 isoform structures. Constant region exons are numbered 1 to 5 and 15 to 19, variant exons v1 to v10. LP: Leader peptide; TM: Transmembrane region.

B: Cells were plated in 24 well plates at 10^5 cells/well, starved overnight and induced with HGF (50ng/ml) for 5 min in the presence or absence of CD44 antibodies. Activation of c-Met in AS cells stably transfected with various CD44 isoforms was measured using phosphorylation of Erk as read-out. The loading controls were performed by stripping the blots and reprobing with an anti-Erk antibody. CD44 isoforms that these cells were transfected with are listed in Table 2.

The cell lines analyzed also included transfectants expressing v6-containing CD44 with or without the exon 15 sequence (which encodes the epitope for 5G8). For example AS-s6,7 cells are AS cells transfected with CD44v6,7 plus exon 15, while AS-v6,7 cells are AS cells transfected with CD44v6,7 without exon 15 (Table 2). Lack or presence of exon 15 had no influence on the cooperation of v6-containing CD44 proteins with c-Met. However, HGF-dependent Erk phosphorylation could be inhibited by 5G8 antibodies in cases where the exon 15 sequence was present (Fig. 5B). Both v6 and exon 15 sequences are part of the membrane-proximal stem structure of these variant isoforms, and they are close to each other. Antibodies to the membrane-distal amino-terminal end of CD44 did not affect the HGF induced signaling (Fig. 1B). Consistent with this result, mutation of the major hyaluronate binding site in the amino-terminal end of the protein did not disturb the cooperation with c-Met (Fig. 5B, panel 8). We conclude from the analysis of the different transfectants that the presence of the v6 sequence is sufficient for c-Met signaling.

CD44v6 isoforms, c-Met and HGF associate into multimeric complex.

How do CD44 v6 containing isoforms support c-Met signaling? It was reported that heparan sulfate modified CD44 proteins bound HS-binding growth factors such as FGFs (Bennet et al., 1995). We therefore asked whether heparan sulfate modification was also required for CD44 to bind HGF. Any CD44 variant protein containing exon v3 can be modified by heparan sulfate (Bennett et al., 1995). From the result of the RT-PCR analysis of CD44 isoforms, we knew that in HT-29 cells isoforms containing exon v3 were expressed (Fig. 2). FACS analysis with heparan-sulfate specific antibodies showed that there were also heparan-sulfate modified proteins in HT-29 cells (Fig. 6). Western blotting revealed that also CD44 proteins were heparan sulfate modified in HT-29 cells (Fig. 7).

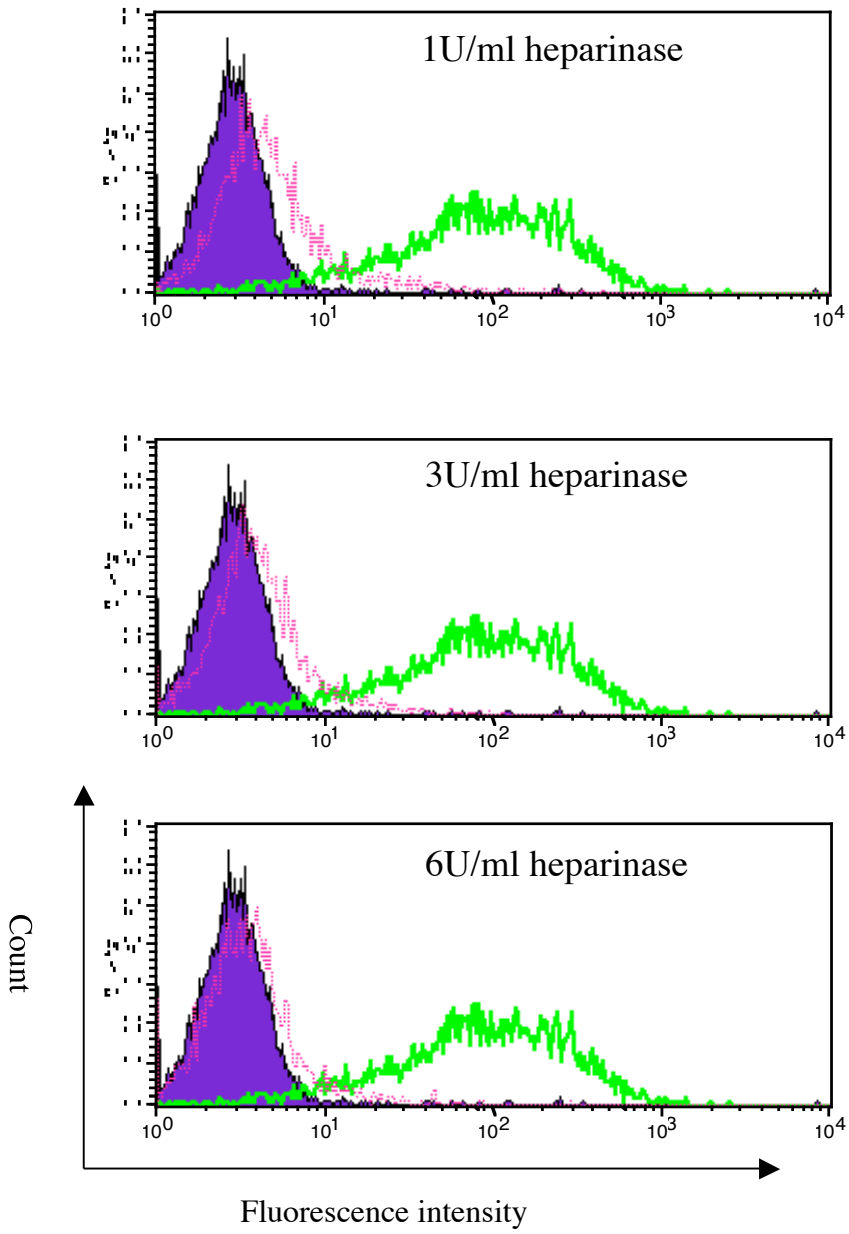

Fig. 6: Heparinase treatment abrogated heparan sulfate modifications of proteins.
10^6 HT-29 cells were harvested with 5mM EDTA/PBS, resuspended in PBS and incubated with heparinase II for 3 hours (concentration is indicated). The cells were incubated with anti-heparan sulfate antibody for 30 min, then washed with PBS and incubated with fluorescent labeled secondary antibodies. The cells were washed with PBS, and analyzed by a FACS®tar Plus Flow Cytometer. In each case, the blue trace represents negative control where cells were stained without primary antibodies. The green trace represents the staining with heparan sulfate antibody without heparinase digestion, while the red trace represents the staining with heparan sulfate antibody after heparinase digestion (concentration is indicated).

To examine whether heparan sulfate modified CD44 proteins are required for HGF/c-Met signaling, HT-29 cells were incubated with heparinase II prior to HGF treatment. FACS analysis showed that at a concentration of 6U/ml heparinase II almost all heparan sulfate residues could be removed (Fig. 6). However, HT-29 cells remained HGF inducible after removal of heparan sulfate residues (Fig. 8). So, at least in our case, heparan sulfate modification of CD44 is not required for c-Met activation.

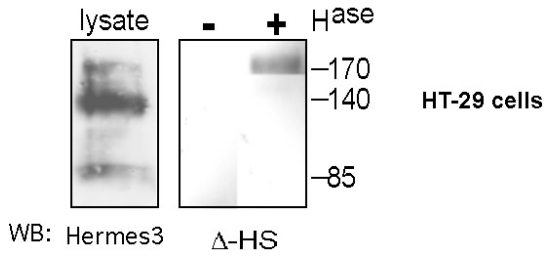

Fig. 7: Western-blot to detect heparan sulfate modified CD44 protein in HT-29 cells.
HT-29 cells were incubated with heparinase II (6U/ml) at 37 °C for 3 hours. Cells were lysed in 2□ sample buffer and subjected to SDS-PAGE. Western blotting was performed with □-heparan sulfate antibody (right panel). This antibody reacts with a heparan sulfate neo-epitope that is generated by digestion of heparan sulfate with heparinase (anti-heparan sulfate antibody does not work in Western blotting). The lysate was also probed using Hermes3 antibody (left panel).

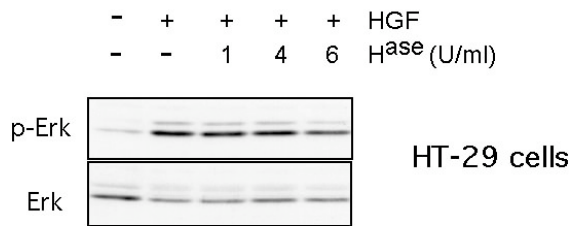

Fig. 8 Heparan sulfate modification of CD44 is not required for HGF induced signaling.
HT-29 cells were plated in 24 well plate (10^5 cells /well), starved overnight and then induced with HGF (50ng/ml). Prior to HGF induction, the cells were treated with Heparinase II at concentrations as indicated. The cells were lysed in 2□ sample buffer and subjected to SDS-PAGE. Western-blotting was performed with p-Erk (upper panel) and Erk antibodies (lower panel).

HGF is secreted as a biologically inactive proform (Naka et al., 1992). Thus, one possibility how CD44 could support HGF/c-Met signaling would be that it is required for pro-HGF activation. However, CD44 can support the activation of c-Met only when the cells was stimulated with mature HGF. CD44 is not involved in the cleavage of proform HGF in both HT-29 and AS transfectant cells (Orian-Rousseau et al., 2002), suggesting that CD44 might play a role in HGF/c-Met signaling other than proform HGF activation, which may require the close contact among these proteins. To demonstrate an association of c-Met, HGF and CD44 proteins, co-immunoprecipitations were performed using HT-29 and AS-s6 cells. Cells were incubated with biotinylated HGF (I used biotinylated HGF since we can detect HGF or HGF containing complexes by Western-blotting with avidin). Cell membrane proteins were cross-linked with dithiobissulfo-succinimidylpropionate (DTSSP) (I used DTSSP to stabilize the protein complexes. The cross-linker DTSSP is widely used for conjugating cell surface proteins. DTSSP molecules contain a disulphide bond which can be cleaved in the presence of DTT). Then the cells were lysed and immunoprecipitations were performed using different antibodies. The precipitated proteins were resolved by SDS-PAGE under reducing conditions and Western blotting was performed with CD44 specific antibodies (Hermes3 for HT-29 and 5G8 for AS-s6). With HT-29 cells, the major CD44 isoform (140 kDa) co-precipitated with anti-HGF and anti-c-Met antibodies (Fig. 9A). With AS-s6 cells, a CD44 isoform (120 kDa) co-precipitated with a c-Met antibody (Fig. 9B), suggesting that this isoform is CD44v6 (it is not contained in untransfected AS cells). Interestingly CD44s was not precipitated by c-Met antibody (Fig. 9B), demonstrating the specificity of the CD44 v6 isoform in the complex formation.

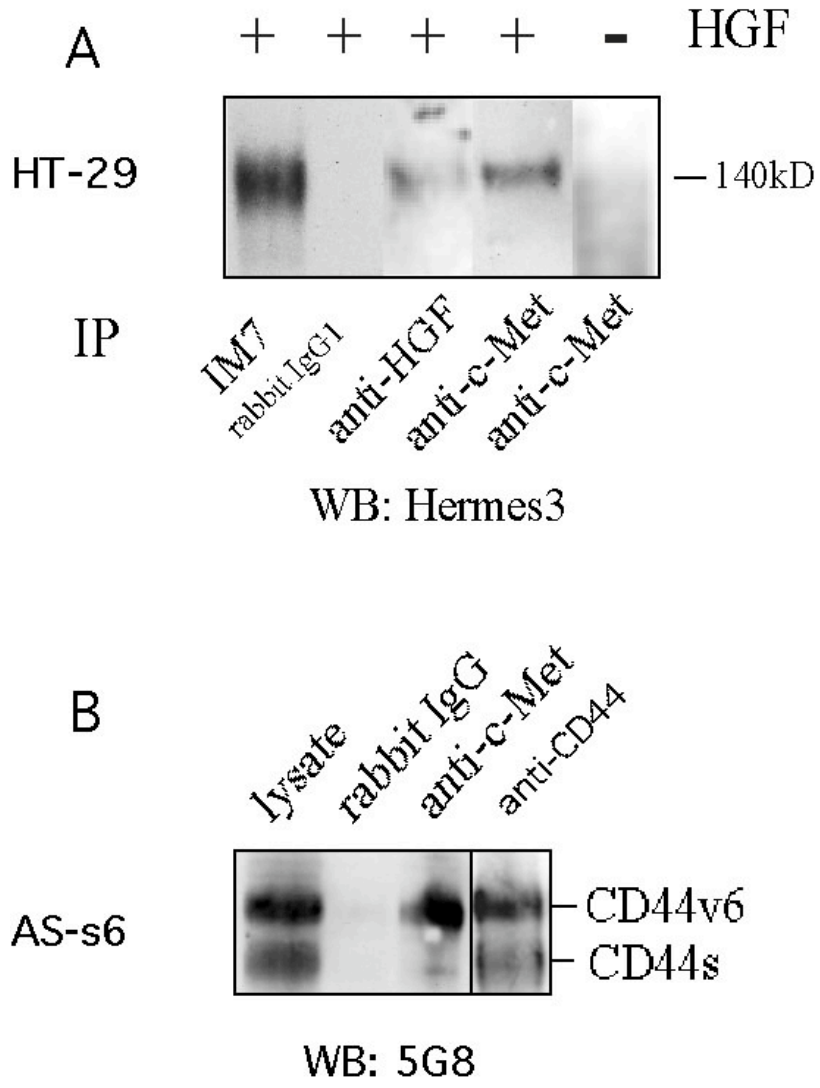

Fig. 9: CD44 was co-immunoprecipitated with HGF and c-Met.
HT-29 cells (Fig. 9A) or AS-s6 cells (Fig. 9B) were plated in 10 cm plates ($3 \square 10^6$ cells /10cm plate), starved overnight, induced with HGF (50ng/ml) as indicated and cross-linked with DTSSP (see Materials and Methods). Cells were lysed and immunoprecipitations were performed with different antibodies as indicated. Immunoprecipitated proteins were separated by 7% SDS-PAGE and Western blotting was performed using anti-CD44 antibodies (Hermes3 for HT-29 cells, 5G8 for AS-s6 cells).

HGF was also co-precipitated with anti-c-Met and anti-CD44 antibodies (Fig. 10A, lane 2, 6). AS-s6 cells were incubated with biotinylated HGF. Cell membrane proteins were cross-linked with DTSSP. Then the cells were lysed and immunoprecipitations were performed with different antibodies. The precipitated proteins were subjected to SDS-PAGE and Western blotting was performed with avidin to detect biotin-labeled HGF. HGF was co-precipitated with anti-c-Met and anti-CD44 antibodies (Fig. 10, lane2 and lane6). In the lane that is indicated as “anti-HGF(preclear)”, the cell lysate was first precleared by precipitation with anti-HGF antibody and then the immunoprecipitation was performed with anti-HGF antibody. The band was not detected in this lane (Fig. 10). Thus, the disappearance of this band after the depletion of HGF from cell lysate demonstrated that this band is HGF.

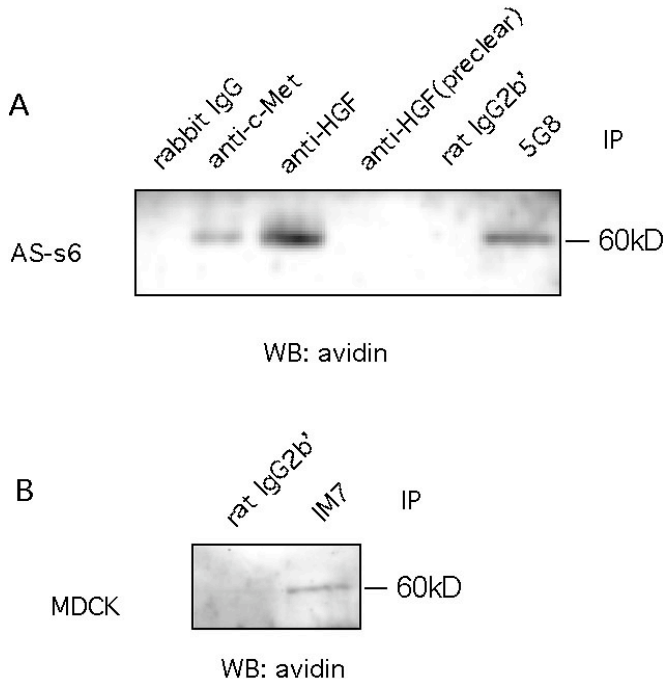

Fig. 10: HGF was co-immunoprecipitated with CD44 and c-Met.
AS-s6 cells (Fig. 10A) or MDCK cells (Fig. 10B) were plated in 10 cm plates ($3\square 10^6$ cells /10cm plate), starved overnight, induced with biotinylated HGF (50ng/ml). Cell membrane proteins were cross-linked with DTSSP (see Materials and Methods). Then the cells were lysed, immunoprecipitation was performed with different antibodies or isotope control. Immunoprecipitated proteins were separated by 7% SDS-PAGE. HGF was detected by Western blotting with avidin. For preclearing (anti-HGF(preclear)) lysates were precleared by immunoprecipitation with anti-HGF antibodies and then immunoprecipitation was performed with anti-HGF antibody.

MDCK cells are polarized epithelial cells in which the function of HGF/c-Met was intensively studied. I wanted to test whether CD44 is required for HGF/c-Met signaling in this cell line. Since MDCK is a canine cell line, the antibodies that I used for immunoprecipitation in HT-29 and AS cells, such as c-Met antibody, cannot be used. Furthermore, anti CD44 v6 antibody is not available for canine cell line, therefore I cannot examine the inhibition of HGF signaling in MDCK cells. Fortunately CD44 antibodies IM7 cross-react with canine CD44 (Trowbridge et al., 1982). MDCK cells were induced with biotin-labeled HGF. Cell membrane proteins were cross-linked with DTSSP. Then the cells were lysed and immunoprecipitation was performed with IM7 or isotype control. The precipitated proteins were subjected to SDS-PAGE. Western blotting was performed with avidin. HGF was co-precipitated by IM7 from MDCK cells (Fig. 10B, lane 2). Thus, CD44 was also involved in HGF/c-Met signaling in MDCK cells.

The associations of CD44/c-Met and CD44/HGF are demonstrated by the co-immunoprecipitation experiments using several different cells: HT-29, AS-s6 and MDCK.

HGF, c-Met and CD44 v6 containing isoforms form a multimeric complex

CD44 could be co-precipitated by c-Met and HGF antibodies (Fig. 9), and HGF could be co-precipitated by c-Met and CD44 antibodies from cells in which the activation of c-Met by HGF was dependent on CD44 v6 (Fig. 10). This gave us a hint that CD44, HGF and c-Met were assembled in one complex. To test this assumption, cells were induced with biotinylated HGF. Cell membrane proteins were cross-linked with DTSSP. The cells were lysed and an immunoprecipitation was performed with c-Met specific antibody. The precipitated proteins were separated into two halves and run on a non-reducing gel. Half of the gel was transferred to PVDF membrane (another half of the gel containing the same samples was kept in 4°C). Western blotting was performed with avidin to detect HGF or complexes (or a complex) containing HGF. A large protein complex with an estimated mass of more than 600 kDa was resolved by SDS-PAGE under non-reducing conditions from AS-s6 cells (Fig. 11A).

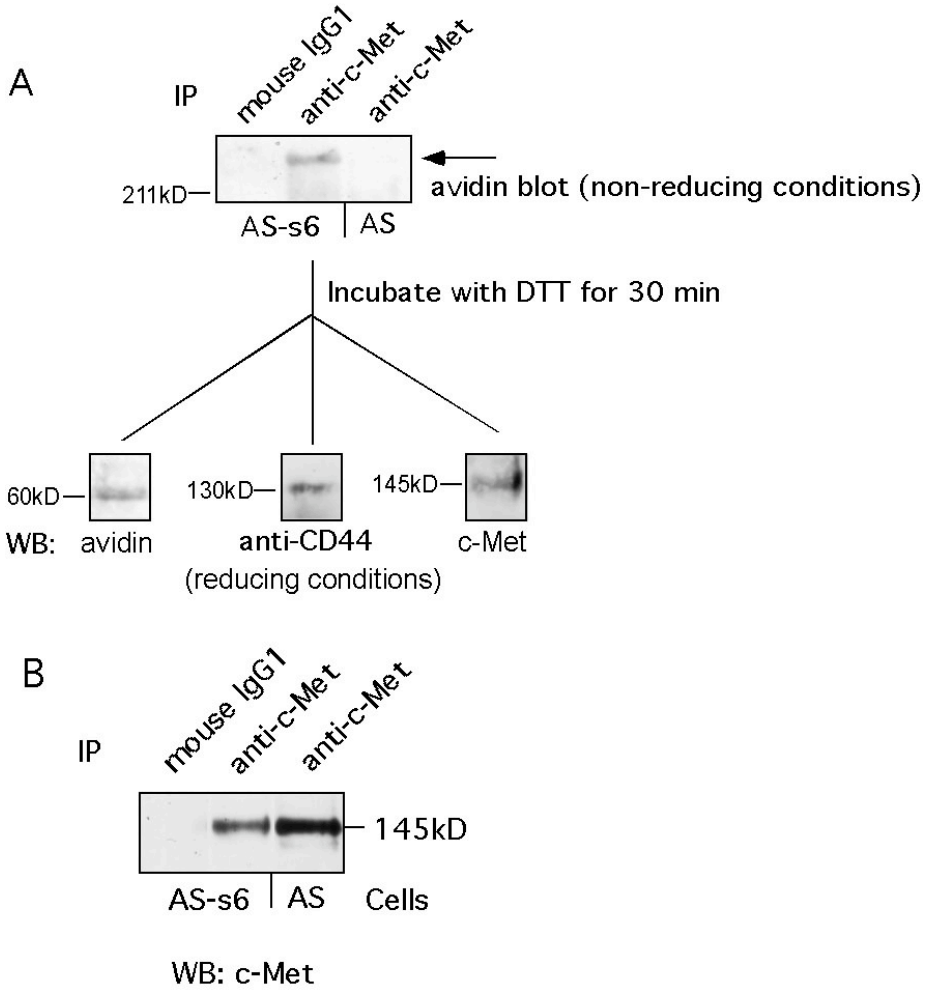

Fig. 11: CD44, c-Met and HGF form a multimeric complex.
A: AS-s6 or AS cells were plated in 10 cm plates (3×10^6 cells /10cm plate), starved overnight and induced with biotinylated HGF (50ng/ml). Cell membrane proteins were cross-linked with DTSSP. Then the cells were lysed and immunoprecipitations were performed with c-Met specific antibody or mouse IgG (isotope control). Immunoprecipitated proteins were resolved under non-reducing conditions on SDS-PAGE (7%). A single band was identified by Western blotting with avidin. The gel slice corresponding to the band shown in Fig. 11A was excised from the gel which was kept in 4 ^{O}C, incubated with DTT and re-ran on a SDS-PAGE. Western blots with avidin, CD44 antibody (5G8), and c-Met are shown.
B: Western blot for c-Met expression in AS-s6 or AS cells.
AS-s6 or AS cells were plated in 10 cm plates (3×10^6 cells /10cm plate), starved overnight and induced with biotinylated HGF (50ng/ml). Cell membrane proteins were cross-linked with DTSSP. Then the cells were lysed and immunoprecipitations were performed with c-Met specific antibody or mouse IgG (isotope control). The immunoprecipitated proteins were resolved under reducing condition by SDS-PAGE. Western blotting was performed with c-Met specific antibody.

To examine whether HGF, CD44 and c-Met were in this complex, the gel slice corresponding to the band shown in Fig. 11A was cut out from the half-gel which was kept in 4°C. The gel slice was incubated with DTT to cleave the disulphide bridges of the cross-linker. Subsequently portions of the gel were applied to a second SDS-PAGE. c-Met was detected by Western-blotting with c-Met specific antibody, in addition a CD44 isoform and biotinylated HGF were detected using CD44 specific antibody (5G8) or avidin (Fig. 11A). The result shows that in AS-s6 cells there exists an HGF-inducible multimeric complex containing at least CD44, HGF and c-Met.

This multimeric complex was not found in AS cells which express only CD44s (Fig. 11A), even though they contain more c-Met (Fig. 11B).

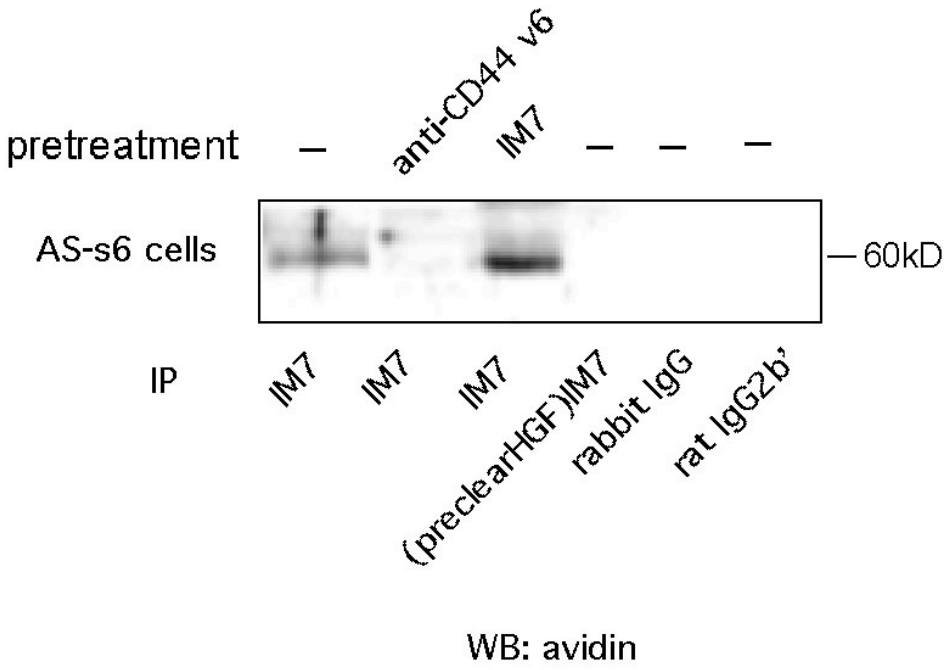

Fig. 12: Co-immunoprecipitation of HGF with CD44 can be abrogated by pretreatment with CD44 v6 specific antibody.
AS-s6 cells were plated in 10 cm plates (3×10^6 cells /10cm plate), starved overnight, induced with biotinylated HGF (50ng/ml) with or without antibody pretreatment. Membrane proteins were cross-linked by DTSSP. Cells were lysed and immunoprecipitations were performed with the CD44 specific antibody IM7 or with isotope control antibodies. HGF was detected by Western blotting with avidin. In the lane that was indicated as "(preclearHGF)IM7", the cell lysate was first precleared by immunoprecipitation with an anti-HGF antibody and then immunoprecipitation was performed with IM7.

CD44 v6 specific antibodies prevent the formation of the signaling complex.

How do CD44 v6 specific antibodies block c-Met activation? One possibility is that they may block the formation of the signaling complex containing CD44, HGF and c-Met. To test this assumption, AS-s6 cells were induced with biotin-labeled HGF with or without CD44 antibody pretreatment. Cell membrane proteins were cross-linked with DTSSP. The cells were lysed and an immunoprecipitation was performed with IM7 antibody. Western blotting was performed with avidin to detect HGF. Without any antibody pretreatment, HGF co-precipitated with IM7 (Fig. 12, lane1). The induced formation of the immunoprecipitable complex (here precipitated by IM7 recognizing the amino-terminus of CD44) was prevented by pretreatment of CD44 v6 specific antibodies (Fig. 12, lane2), but not by IM7 that recognize the amino-terminal domain of CD44 (Fig. 12, lane3). The depletion of HGF from the cell lysate using HGF antibodies abrogated the co-immunoprecipitation of HGF by IM7 (Fig. 12, lane4).

Thus, several independent experimental data indicate CD44 v6 specificity in c-Met activation: inhibition of c-Met activation by anti-v6 antibodies but not by antibodies that recognize the amino-terminal domain of CD44. Lack of c-Met activation in CD44 v6 negative cells and restoration by transfection with CD44 v6 containing isoforms. Identification of a multimeric complex composed of CD44v6 containing isoform, HGF and c-Met. Thus, a CD44 isoform containing exon v6 encoded sequences acts as a co-receptor for c-Met by establishing a multimeric signaling complex, which is strictly required for c-Met activation in several carcinoma cell lines.

CD44 isoforms are required for signaling induced by some EGF growth factors.

To test whether the growth factor co-receptor function of CD44 v6 as determined for HGF/c-Met is a general mechanism of CD44, I studied whether CD44 v6 is also involved in the signaling induced by several EGF growth factors (HB-EGF, EGF, Amphiregulin (AR), Heregulin and TGF-□). Signaling induced by EGF growth factors is mediated by the ErbB family receptors that belong also, like c-Met, to the class I tyrosine kinase receptors. ErbB family receptors are hetero- or homodimers that are composed of the ErbB-1, ErbB-2, ErbB-3 and ErbB-4 proteins. Several studies already suggested that CD44 proteins were involved in signaling of ErbB receptors. For example, ErbB-1 and ErbB-2 were co-precipitated with CD44 (Wobus et al., 2001, Bourguignon et al., 1997); CD44 facilitated Neuregulin-induced ErbB-2/ErbB-3 activation that is important for maintaining neuron-Schwann cell interactions (Sherman et al., 2000); CD44 isoforms were required for HB-EGF precursor processing as well as ErbB-4 activation (Yu et al., 2002). I studied the involvement of CD44 in the signaling of EGF growth factors. My results show that several EGF growth factors are CD44 v6 dependent, for example AR, HB-EGF and EGF.

As a first read out of signaling induced by EGF growth factors (HB-EGF, EGF, AR, TGF-□ and Heregulin), the phophorylation of Erk induced by these EGF growth factors was measured. All these EGF growth factors induced Erk phosphorylation in HT-29 cells (Fig. 13).

To study whether CD44 v6 is involved in the signaling induced by these EGF growth factors, HT-29 cells were treated with CD44 v6 antibody prior to growth factor induction. HB-EGF, EGF and AR induced Erk phosphorylations were blocked by CD44 v6 specific antibody, thus the signaling induced by these EGF growth factors is CD44 v6 dependent. In contrast, Heregulin and TGF-□ induced Erk phosphorylations were not affected by CD44 v6 specific

antibody (Fig. 13), thus the signaling induced by Heregulin and TGF-α is CD44 v6 independent.

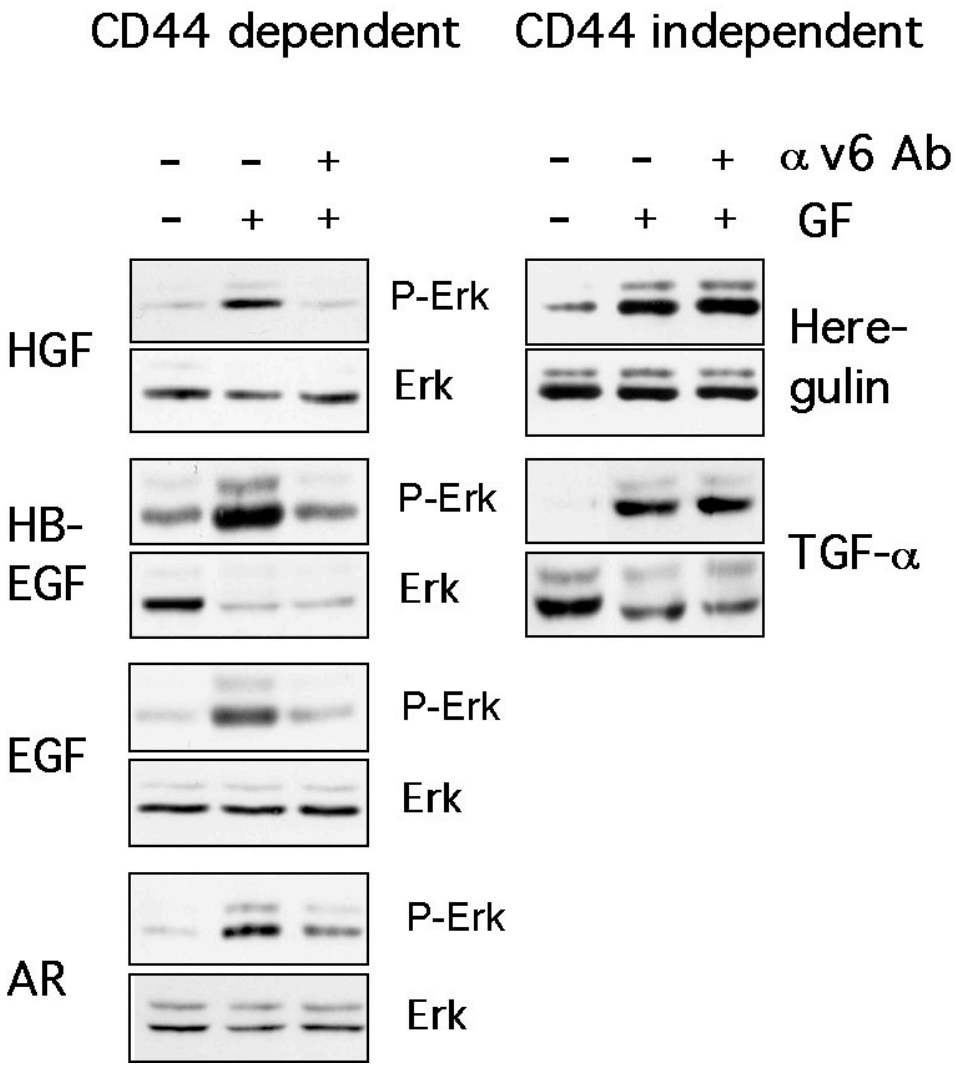

Fig. 13: Signaling induced by some EGF growth factors is CD44 v6 dependent.
HT-29 cells were seeded in 24 well plates (10^5 cells /well), starved overnight and induced with different growth factors as indicated with or without anti-CD44 v6 antibody pretreatment. Cells were lysed in 2× sample buffer. The lysates were subjected to SDS-PAGE (10%). Western blotting was performed with phosphorylated Erk antibody (P-Erk panels). The blots were stripped and reprobed with Erk antibody (Erk panels).

Interestingly, Heregulin induced Erk phosphorylation was inhibited to some extant by Hermes3 which is a pan-CD44 antibody (Fig. 14), suggesting that other CD44 isoforms than exon v6 containing ones might be involved in the signaling induced by Heregulin.

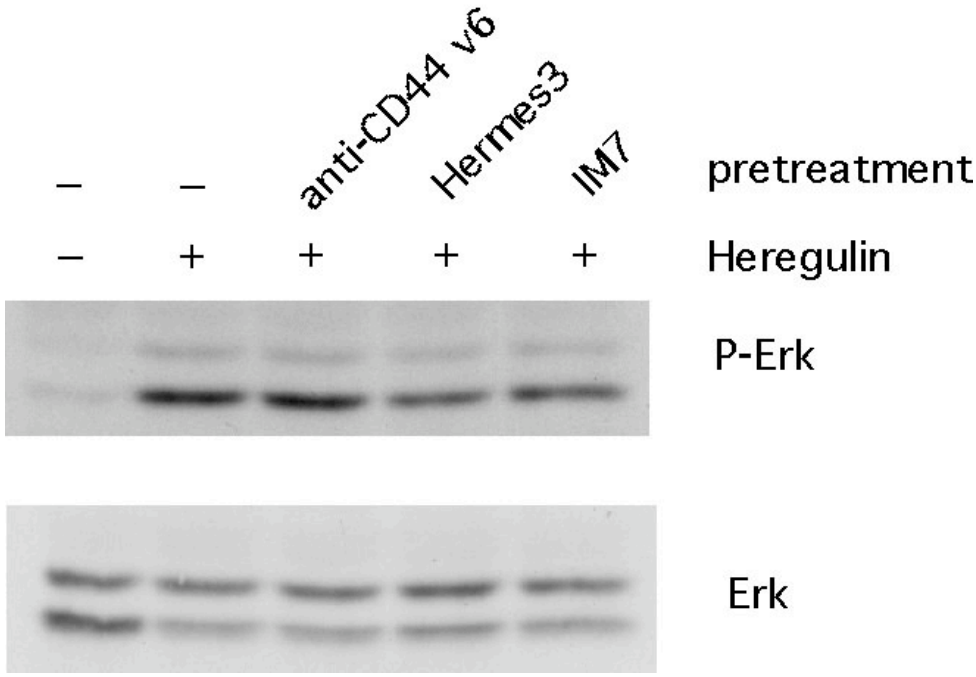

Fig. 14: Heregulin induced Erk phosphorylation was inhibited by pan-CD44 antibodies.
HT-29 cells were seeded in 24 well plates (10^5 cells /well), starved overnight and induced with Heregulin with or without CD44 antibodies pretreatment. Cells were lysed in 2 sample buffer. The lysates were subjected to SDS-PAGE (10%). Western blotting was performed with p-Erk antibody (upper panel). The blots were stripped and re-probed with Erk antibody (lower panel).

To determine whether the CD44 v6 protein is directly required for activation of the ErbB proteins in the case where the phosphorylation of Erk can be blocked with CD44 v6 specific antibodies, I measured the phosphorylation of EGFR (Fig. 15). For HB-EGF, EGF and AR, the phosphorylation of EGFR can

be completely blocked by CD44 v6 specific antibodies. EGFR phosphorylation induced by TGF-□ was not affected (Fig. 15) consistent with the results when the phosphorylation of Erk was measured.

Although ErbB-2 does not bind to EGF growth factors directly, it can associate with other members of the ErbB family proteins. In HT-29 cells ErbB-2 co-precipitated with EGFR antibody (Fig. 16A). HB-EGF and Heregulin induced also phosphorylation of ErbB-2 (Fig. 16B,C). Therefore the interference of a CD44 v6 antibody with ErbB-2 phosphorylation was also examined. Consistent with the result of EGFR phosphorylation, HB-EGF induced ErbB-2 phosphorylation was completely blocked by CD44 v6 specific antibody (Fig. 16B), however, Heregulin induced phosphorylation of ErbB-2 was not affected (Fig. 16C).

Thus, both HB-EGF and Heregulin can induce ErbB-2 phosphorylation, but only the activation of ErbB-2 by HB-EGF is dependent on CD44 v6 containing isoforms, whereas CD44 v6 containing isoforms are not required for the activation of ErbB-2 by Heregulin.

AS a summary, my work shows that a CD44 isoform containing exon v6 encoded sequence is required for c-Met activation in various cells. A signaling complex is assembled consisting of HGF, c-Met and CD44. The signaling induced by HB-EGF, EGF and AR is also CD44 v6 dependent. CD44 v6 antibody can interfere with signaling induced by these EGF growth factors both at Erk level and at receptor level.

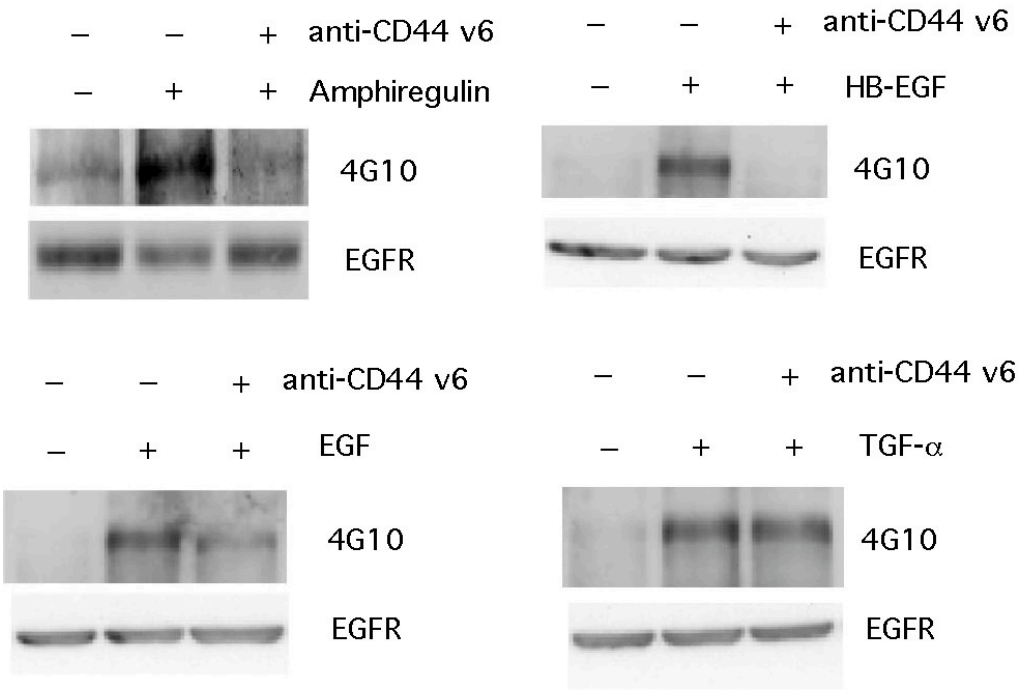

Fig. 15. EGFR phosphorylation induced by HB-EGF, EGF and AR but not by TGF-□ was blocked by CD44 v6 specific antibody.
HT-29 cells were seeded in 10 cm plates (3□10^6 cells /10cm plate) and starved overnight, induced with growth factors as indicated with or without CD44 v6 antibodies pretreatment. Cells were lysed with lysis buffer plus 10mM Na_3VO_4 (to inactive tyrosine phosphatases) and proteinase inhibitors. Immunoprecipitation was performed with EGFR specific antibodies. Phosphorylation of EGFR was detected using phosphotyrosine-specific antibody 4G10 (4G10 panels). The blots were stripped and reprobed with EGFR antibody (EGFR panels).

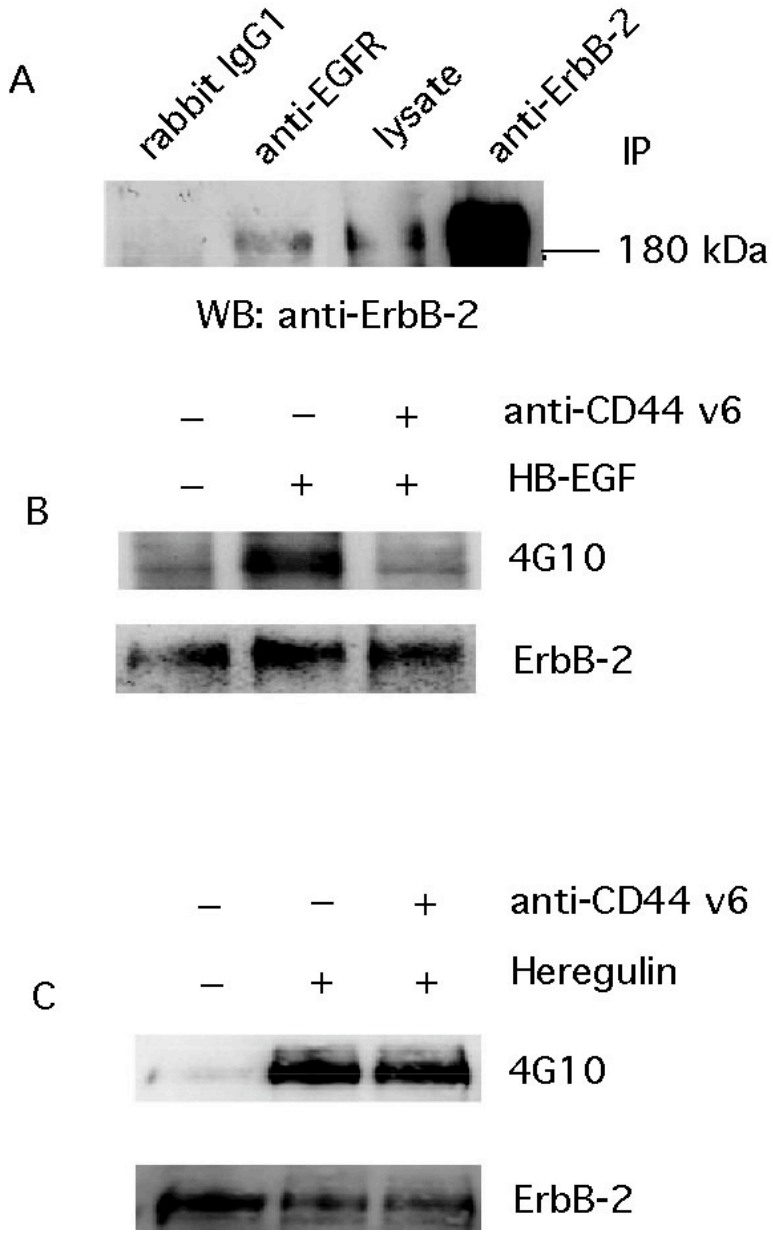

Fig. 16: ErbB-2 phosphorylation induced by HB-EGF but not by Heregulin was blocked by anti-CD44 v6 antibody.
A: HT-29 cells were plated in 10 cm plates. Cell membrane proteins were cross-linked with DTSSP. Then the cells were lysed and immunoprecipitation was performed with EGFR, ErbB-2 antibodies or isotope control. Immunoprecipitates were subjected to SDS-PAGE (7%) and Western blotted with ErbB-2 specific antibody was shown.
B. C: HT-29 cells were plated in 10 cm plates (3×10^6 cells /10cm plate), starved overnight and induced with growth factors as indicated with or without CD44 v6 antibody pretreatment. Cells were lysed with lysis buffer plus 10mM Na_3VO_4 and proteinase inhibitors. Immunoprecipitations were performed with ErbB-2 specific antibodies. Phosphorylation of ErbB-2 was detected using the phosphotyrosine-specific antibody 4G10 (4G10 panels). The blots were stripped and re-probed with ErbB-2 (ErbB-2 panels).

Discussion:

Both HGF/c-Met and CD44 were shown to be involved in tumor metastasis formation independently (Bardelli et al., 1997a, Bardelli et al., 1997b, Günthert et al., 1991, Naor et al., 1997). c-Met was found over-expressed and amplified in a variety of aggressive tumors such as thyroid and colorectal carcinomas. In the case of colorectal carcinomas, c-Met over-expression and amplification conferred a selective advantage for the capability of metastasizing to the liver (Di Renzo et al., 1995). The work presented in this thesis shows that in several types of cells the activation of c-Met by its ligand HGF depends on the function of CD44 proteins containing the exon v6-encoded protein sequence. It could be that CD44 and HGF/c-Met cooperate in tumor metastasis.

It was reported that heparan sulfate modification was required for CD44 to support c-Met activation. CD44 variant proteins containing sequences encoded by exon v3 carry the consensus Ser-Gly-Ser-Gly sequence for GAGs addition and are modified with heparan sulfate in a variety of cell types (Jackson et al., 1995, Bennett et al., 1995). Heparan sulfate modified CD44 proteins can function as low affinity receptors for some growth factors such as HB-EGF and FGFs (Bennett et al., 1995, Sherman et al., 1998). In Burkitt lymphoma cells (Namalwa cells), heparan sulfate modified CD44 (CD44-HS) could support c-Met activation (van der Voort et al., 1999). The majority of AS transfectants used in this thesis do not contain CD44 exon v3 encoded sequence, but still support c-Met activation. Although in HT-29 cells there were CD44 variant proteins that are modified by heparan sulfate (Fig. 6, Fig. 7), the CD44 protein that was co-immunoprecipitated by a c-Met antibody (about 140 kDa) does not contain heparan sulfate residues (Fig. 5, Fig. 7). Furthermore, HT-29 cells

can still be induced by HGF after the removal of heparan sulfate modifications by heparinase-II treatment (Fig. 8), indicating that heparan sulfate modifications are not required for CD44-dependent c-Met activation. This finding is consistent with the observation that though HGF can bind HSPG (Zioncheck et al., 1995) the function of HGF does not depend on HSPG. An HGF mutant that cannot bind to heparan sulfate retained its ability to activate c-Met (Hartmann et al., 1998). So it seems that only in Namalwa cells, heparan sulfate modification might be required for CD44-dependent c-Met activation.

Can CD44 isoforms containing other exons than v6 also support HGF/c-Met signaling? From the analysis of HGF induced Erk phosphorylation with AS transfectants that were transfected with different CD44 isoforms, I concluded that CD44v7 and CD44v8-10 could not support HGF/c-Met signaling (Fig. 5B, panel 3, 4). However, it is not ruled out that CD44 isoforms containing exons such as v4 or v5 could support HGF/c-Met signaling. A CD44 isoform containing all variant exons except v6 did not support c-Met activation (Orian-Rousseau et al., 2002). All these data indicate that only CD44 isoforms containing exon v6 encoded can support HGF/c-Met signaling.

Why are CD44 isoforms containing exon v6 encoded sequences required for HGF/c-Met signaling? An explanation is that CD44 v6 containing variant proteins may stabilize the HGF/c-Met signaling complex. A big signaling complex was found in AS-s6 cells that contain CD44v6 isoforms. There was no such complex or other HGF-containing complex detected in AS cells which only express CD44s (Fig. 11A), indicating that HGF could not bind c-Met in AS cells that do not express CD44 v6 containing isoforms. The binding of HGF to c-Met was measured using various cells (Naldini et al., 1991), it could be that CD44 v6 containing isoforms were

also expressed in these cells and cooperated with c-Met.

CD44 v6 containing isoforms may sequester negatively-regulating elements from c-Met which is required for c-Met activation. There seems to exist such negatively-regulating elements. In constitutively active oncogenic Tpr-met (the extracellular domain of c-Met is replaced by Tpr sequences which provide two strong dimerization motifs), the juxtamembrane domain is lost which contains such a negative control region (Vigna et al., 1999). When this region was included into the fusion protein, Tpr-met dependent cell transformation was inhibited (Vigna et al., 1999). The negative control region could be a binding site for a phosphatase. It was reported that several protein tyrosine phosphatases (DEP-1, PTP-S and PTPase LAR) interacted with c-Met as a negative regulator (Palka et al., 2002, Villa-Moruzzi et al., 1998, Kulas et al., 1996). Interestingly, the receptor-tyrosine phosphatase DEP-1 dephoshorylated particular tyrosine residues (Tyr1349 and Tyr1365), indicating that it may function in controlling the specificity of signals induced by c-Met (Palka et al., 2002). There exist other negative regulators of c-Met, such as c-Cbl (Peschard et al., 2001, Taher et al., 2002) and ganglioside GD1a (Hyuga et al., 2001). c-Cbl binds also to the juxtamembrane domain of c-Met (Peschard et al., 2001).

The cytoplasmic tail of CD44 also plays an important role in HGF/c-Met signaling pathway. Although c-Met could still be phosphorylated upon HGF induction in AS cells transfected with tail-less mutant of CD44v4-7 (Orian-Rousseau et al., 2002), downstream signaling such as phosphorylation of MEK and Erk were blocked (Orian-Rousseau et al., 2002 and Fig. 5B, panel 13). Thus, the cytoplasmic tail of CD44 is not required for c-Met phosphorylation but it is required for downstream signaling of HGF/c-Met pathway. At which step it is required is still

unclear.

The cytoplasmic tail of CD44 can associate with ERM proteins which function as linkers between cytoskeleton and plasma membrane proteins (Tsukita et al., 1999). The association of CD44-ERM-cytoskeleton proteins seems to be important in signal transduction. Sequestering of ERM proteins from CD44 by excessive soluble CD44 tails in the cytoplasm caused block of HGF-induced signal transfer, while the cytoplasmic tail with a mutation in ERM binding site could not block the signal transfer (Orian-Rousseau et al., 2002). Interestingly, ezrin, one member of ERM proteins, was found to be a substrate for c-Met both in vivo and in vitro (Jiang et al., 1995, Crepaldi et al., 1997). When ERM proteins were replaced by merlin in the association with CD44, the signal transduction was blocked (Morrison et al., 2001), indicating that signal transduction requires the association of CD44-ERM-cytoskeleton proteins.

Although our data show that CD44 v6 containing isoforms are required for HGF-induced c-Met signaling in several cell lines, in some cases however CD44 variant proteins appear to be not required for c-Met signaling. For example, in a CD44 negative cell line HepG2 and in immortalized fibroblast derived from CD44 null mice, c-Met can still be activated by HGF although these cells do not express CD44 (Fig. 17). How does c-Met signal in these cells? One possibility is that c-Met is able to signal independently of accessory proteins. Another possibility is that other proteins than CD44 function as co-receptors in these cells. It seems that such a co-receptor is required for c-Met signaling. This is supported by the observation that although AS cells express high level of c-Met, c-Met cannot be activated in these cells unless they were transfected with CD44 v6 containing isoforms (Fig. 5). The protein that substitutes for CD44 co-receptor in CD44 negative cells should have a close association with c-

Met. This should allow us to co-precipitate such protein with c-Met specific antibody. My preliminary data showed that a protein could be co-immunoprecipitated with c-Met from CD44 negative cells but not from CD44 positive cells. This protein might substitute for CD44 co-receptor function in CD44 null mice.

CD44 proteins are also required for the signaling induced by EGF growth factors. Phosphorylation of Erk induced by HB-EGF, EGF and AR could be blocked by CD44 v6 specific antibodies, similar to the observation for HGF induced signaling (Fig. 13). However, the role that CD44 plays in EGF growth factors signaling might be different from that in HGF signaling. Firstly, heparan sulfate modification is not required for CD44 to support c-Met signaling (Fig. 6, Fig. 8). HB-EGF could bind to heparan sulfate modified CD44 (CD44-HS) and the binding was eliminated by pretreating the protein with heparintinase or by blocking with free heparin (Bennett et al., 1995). The interaction of HSPG-HB-EGF modulated its bioactivity. Heparitinase or chlorate treatment of bovine aortic smooth muscle cells (BASMC cells) diminished the ability of HB-EGF to stimulate BASMC cells migration by 60-80% (Higashiyama et al., 1993). Secondly, CD44 proteins did not participate in the activation of the proform of HGF (Orian-Rousseau et al., 2002). The proform of HB-EGF is processed in a CD44 dependent way (Yu et al., 2002). In my experiments, I used active form of HB-EGF and the signaling is still depends on CD44 v6 containing isoform. Thus, besides pro-HB-EGF activation, CD44 might play another role in HB-EGF signaling.

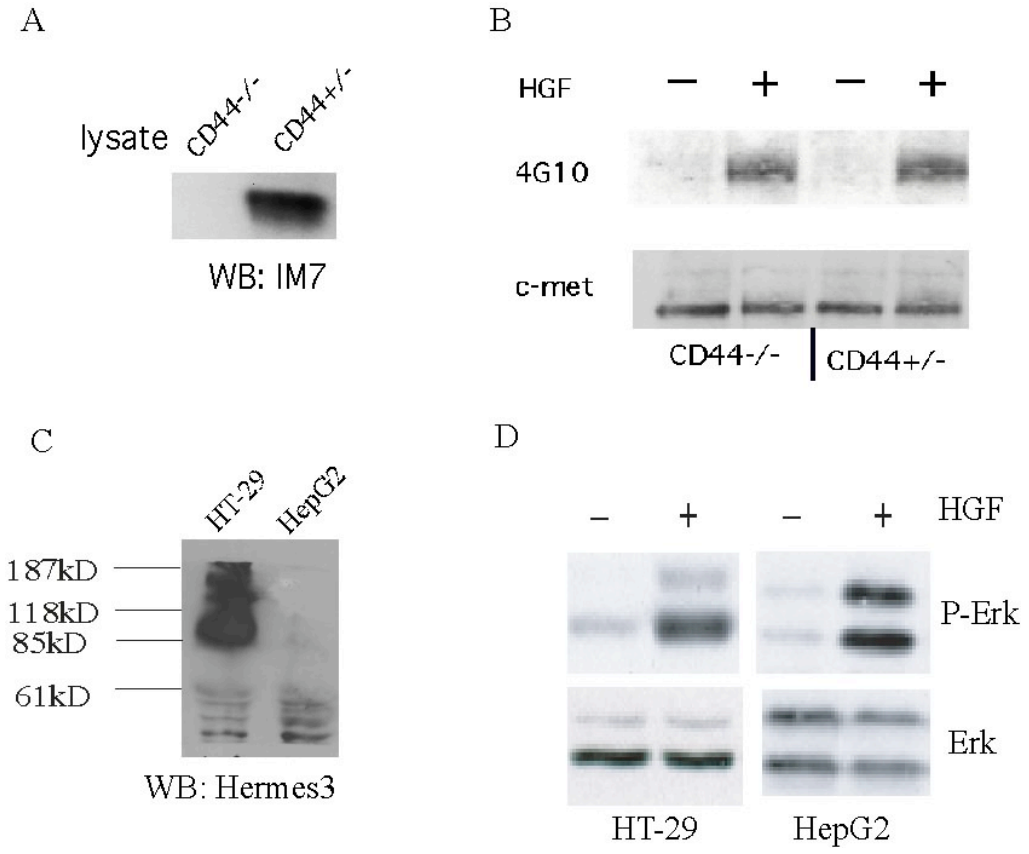

Fig. 17 c-Met can be activated in immortalized fibroblasts derived from CD44 null mice and HepG2 cells

A. The lysates of immortalized fibroblast from CD44+/- or CD44 -/- mice were loaded on non-reducing SDS-PAGE (equal amount of loading was checked by DC Protein Assay (Bio-Rad)). Western blotting was performed with CD44 specific antibody IM7.
B. 3×10^6 CD44+/- or CD44 -/- fibroblast were plated in 10 cm plates, starved overnight, induced with HGF, cells were lysed with lysis buffer plus 10 mM Na_3VO_4 and proteinase inhibitors. In the lysate an immuno-precipitation was performed with a c-Met specific antibody. The precipitated proteins were resolved on SDS-PAGE and blotted onto a membrane. Phosphorylation of c-Met was detected using phosphotyrosine antibody 4G10 (upper panel). The blot was stripped and reprobed with c-Met antibody (lower panel).
C. The lysates of HT-29 and HepG2 cells were subjected to SDS-PAGE (equal amount of loading was checked by DC Protein Assay (Bio-Rad)). Western blotting was performed with CD44 specific antibody Hermes3.
D. HT-29 or HepG2 cells were seeded in 24 well plate (10^5 cells/well), starved overnight and induced with HGF (50ng/ml). The cells were lysed in 2× sample buffer. The lysates were subjected to SDS-PAGE. Western blotting was performed with P-Erk (P-Erk panel) and Erk (Erk panel) antibodies.

How can heparan sulfate modified CD44 (CD44-HS) modulate the bioactivity of AR and HB-EGF? There are several possible mechanisms: HSPG may concentrate these growth factors on the cell surface (Klagsbrun et al., 1991), HSPG may stabilize heparin-binding growth factor dimerization and facilitate receptor dimerization (Yayon et al., 1991).

In the case of AR, it was reported that heparan sulfate is essential for AR induced mitogenic signaling. Both soluble heparin and heparan sulfate inhibited AR induced mitogenesis in various cells (Johnson et al., 1994). However, CD44-HS could not bind AR (Bennett et al., 1995). Therefore, it seems that other HSPG than CD44-HS might be involved in AR signaling. EGF could not bind HSPG (Higashiyama et al., 1993). Thus, in the cases of EGF and AR, heparan sulfate modification is not required for CD44 to support growth factors signaling. CD44 might play a same role as that in HGF signaling.

Surprisingly, CD44 v6 specific antibody blocked EGF induced EGFR and Erk phosphorylation, whereas it did not have an effect on TGF-□ induced EGFR and Erk phosphorylation (Fig. 13, Fig. 15). These two growth factors share the same receptor. Different EGF growth factors binding to ErbB receptors may induce differential receptors phosphorylation and recruitments of different intracellular signaling molecules (Olayioye et al., 1998, Sweeney and Carraway, 2000). It is possible that EGF and TGF-□ induce differential phosphorylation of ErbB receptors and signal through different downstream networks. TGF-□ induced signaling is independent of CD44 and EGF induced signaling is dependent on CD44.

Besides HGF and EGF growth factors studied in this thesis, several other

growth factors also require CD44 for their functions. Heparan sulfate modified CD44 could bind FGFs and vascular endothelial growth factor (VEGF) (Bennett et al., 1995, Jones et al., 2000). During limb development, Heparan sulfate modified CD44 was required for the presentation of FGF-8 to its high affinity receptor on mesenchymal cells. CD44 v3 and v6 specific antibodies blocked FGF presentation and inhibited limb outgrowth (Sherman et al., 1998). In the case of TGF-□, CD44 isoforms provided a cell surface to process pro-TGF-□ into mature form (Yu et al., 1999).

Can CD44s also function as a supportive molecule for growth factor signaling? Heregulin induced Erk phosphorylation was inhibited by a pan-CD44 antibody Hermes3 but not by CD44 v6 specific antibody (Fig. 14), suggesting that isoforms other than CD44 v6 containing variants might be involved in Heregulin signaling. CD44s facilitated Neuregulin-induced ErbB-2/ErbB-3 activation that is important for maintaining neuron-Schwann cell interactions (Sherman et al., 2000). In this case, CD44s was required for supporting Neuregulin signaling.

Why does the activation of a tyrosine kinase receptor depend on the cooperation with transmembrane proteins? It is possible that CD44 monitored the cellular environment through binding to hyaluronic acid. High-molecular-weight hyaluronic acid bound to amino-terminal domain of CD44 that changed the intracellular partners of CD44 and blocked the signaling of cell proliferation (Morrison et al., 2002). When cells were induced by growth factors at low cell density, CD44 associated with ERM proteins and support growth factor signaling. CD44 assembled different complexes depending on extracellular conditions. Thus, CD44 may function as an interpreter of the extracellular environment and a molecule switch between cell growth-permissive and growth-arrest states.

Interestingly it seems that different size hyaluronic acid has different effect on cells proliferation. CD44 stimulation by low-molecular-weight hyaluronic acid induced upregulation and tyrosine phosphorylation of c-Met in human chondrosarcoma cells (Suzuki et al., 2002). On the contrary, hyaluronan oligosaccharides inhibited growth of several types of tumor in vivo. In vitro the oligomers inhibit anchorage-independent growth of several tumor cells (Ghatak et al., 2002). All these data support the idea that CD44-HA interaction plays an important role in the regulation of cell proliferation.

Proteins other than CD44 can also function as co-receptors. For instance, N-CAM was crucial for FGFR-4 signaling by assembling a big signaling complex containing N-cadherin, FGFR-4, PLC□ and other molecules (Cavallaro et al., 2001). Syndecan-2 acted as a co-receptor for GM-CSF in osteoblasts. Down-regulation of syndecan-2 expression by antisense oligonucleotides inhibited the mitogenic activity of GM-CSF (Modrowski et al., 2000). Neuropilin-1 functioned as a co-receptor for VEGF, enhancing its binding to VEGFR-2 and its biological activity (Robinson et al., 2001).

Interestingly, in one of these cases, like CD44, the cytoplasmic tail of adhesion molecule was also important for regulating signal transduction. □-catenin bound to E-cadherin and exerted a negative control on signal transduction downstream of insulin receptor, but upstream of Ras/MAPK. □-catenin knock out keratinocytes exhibited sustained activation of Ras/MAPK cascade (Vasioukhin et al., 2001).

Thus, CD44 isoforms containing exon v6 encoded sequence function as a co-receptor in c-Met signaling pathway. CD44 may also support other growth factors signaling by different mechanisms: CD44 isoforms participate in the proform of growth factors activation, CD44-HS functions

as a low affinity receptor for growth factors. CD44 co-receptor function seems to be a general mechanism. Other molecules such as N-CAM, syndecan-2 and Neuropilin-1 can also function as co-receptors in growth factors signaling.

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appendix: original blot for Fig. 1 A, C