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Intercellular Adhesion Molecule 1 (ICAM-1) ein neuer Co-Rezeptor für c-Met

ZUSAMMENFASSUNG

Das Transmembranprotein CD44v6 wird in verschiedenen transformierten und normalen Zellen zur Aktivierung und Signalweiterleitung durch den „hepatocyte growth factor (HGF)“-Rezeptor c-Met benötigt.

Kollaboriert der c-Met Rezeptor in Abwesenheit von CD44, z.B. in Hepatozyten oder in CD44 Knockout Mäusen, mit anderen Transmembranmolekülen um aktiviert zu werden und Signale weiterzuleiten? Die Idee, dass solche Moleküle existieren, die Funktionen von CD44 übernehmen können, stammt von CD44 Knockout Mäusen, die im Grunde einen normalen Phenotyp aufweisen.

Um einen alternativen Co-Rezeptor für c-Met zu finden, habe ich Merkmale von CD44v6 untersucht, die für die Aktivität von c-Met essentiell sind: (i) Modulation der c-Met Signaltransduktion durch die ERM Proteine (ezrin/radixin/moesin), die die Verbindung zum Aktinzytoskelett vermitteln, (ii) Repression der c-Met Aktivierung durch Hyaluronsäure (HA), einer Komponente der extrazellulären Matrix und (iii) Komplexbildung mit c-Met.

Da ich mit Kompetitionsexperimenten und RNA Interferenz zeigen konnte, dass ERM Proteine in der c-Met Signalkaskade benötigt werden und dass HA in HepG2 Zellen tatsächlich die Signalweiterleitung durch c-Met hemmt, habe ich die Expression von Kandidaten untersucht, die für solche Funktionen in Frage kommen. Ein Protein Screen hat das „intercellular adhesion molecule-1“ (ICAM-1) als solches identifiziert. ICAM-1 ist ein Zelladhäsionsmolekül, das an ERM Proteine und an HA bindet. Blockierungsexperimente mit Antikörpern und siRNA Experimente haben bestätigt, dass ICAM-1 für die Aktivierung von c-Met und Signalweiterleitung nach der Stimulierung durch den Liganden HGF notwendig ist.

Diese Ergebnisse zeigen einen neuen Co-Rezeptor für c-Met und implizieren eine mögliche Substitution für CD44 Funktionen in CD44 Knockout Mäusen.

Intercellular Adhesion Molecule 1 (ICAM-1), a Novel Co-receptor for c-Met

ABSTRACT

In several transformed and normal cells the transmembrane protein CD44v6 is required for activation and downstream signalling of the hepatocyte growth factor (HGF) receptor c-Met. However in situations where CD44 is not expressed i.e. in hepatocytes or in CD44 knockout mice does the c-Met receptor collaborate with other transmembrane partners for its activation and signal transduction? The idea that such molecules exist that can overtake CD44 functions came from CD44 knock out mice, which display an essentially normal phenotype.

In order to find an alternative co-receptor for c-Met I analysed c-Met activation in cells devoid of CD44v6, the human hepatocellular carcinoma cell line HepG2. In these cells I checked for features of CD44v6 required for c-Met activity (i) modulation of c-Met signal transduction via the actin cytoskeleton linker, the ezrin/radixin/moesin (ERM) proteins (ii) repression of c-Met activation through the extracellular matrix component, hyaluronic acid (HA) and (iii) complex formation with c-Met.

Since I confirmed in competition and siRNA experiments the requirement of ERM proteins in c-Met downstream signalling and furthermore that HA can indeed repress signalling from c-Met in the HepG2 cells, I looked for the expression of candidate molecules that could mediate these functions. Such a protein screen identified the intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is a cell adhesion molecule implicated in binding to ERM proteins and HA. Antibody blocking and siRNA experiments confirmed that ICAM-1 is required for the activation of c-Met and signal transduction upon ligand stimulation by HGF.

These results demonstrate a novel co-receptor for c-Met and suggests that it might substitute for CD44 functions in CD44 knockout mice.

ABBREVIATIONS

AER	Apical ectodermal ridge
Arp2/3	Actin related protein 2/3
BSA	Bovine serum albumin
CAM	Cellular adhesion molecule
CD44H	CD44 haematopoietic
CD44s	CD44 standard
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal related kinase 1/2
ERM	Ezrin/Radixn/Moesin
F-actin	Filamentous actin
FGFR	Fibroblast growth factor receptor
Gab1	Grb2-associated binding protein 1
GAG	Glycosaminoglycans
GEF	Guanine nucleotide exchange factor
GF	Growth factor
gp85	Glycoprotein of Mr 85,000
Grb2	Growth factor receptor bound protein 2
HA	Hyaluronic Acid
HBEGF	Heparin-binding epidermal growth factor precursor
HGF/SF	Hepatocyte growth factor/scatter factor
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycan
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL-2	Interleukin 2
K	Kringle domain
LARG	Leukemia-associated RhoGEF

MBD	c-Met binding domain
NF2	Neurofibromatosis 2
NGF	Nerve growth factor
N-WASP	neural Wiskott-Aldrich syndrome
Pgp-1	Phagocyte glycoprotein-1
PI3K	Phosphotidylinsitol 3-kinase
PKC	Protein kinase C
PLCg	Phospholipase Cg
PMA	Phorbol myristate acetate
PSI	Plexin Semaphorin Integrin
Ras/MAPK	Ras-mitogen activated protein kinase
RHAMM	Receptor for hyaluronan-mediated motility
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription-polymerase chain reaction
sCD44	Soluble CD44
Sema 4D	Semaphorin 4D
SH2	Src-homology 2
Shc	Src-homology 2 containing protein
SHP2	SH2 containing protein tyrosine phosphatase 2
siRNA	small interfering RNA
SOS	Son-of-sevenless
TGF b-1	Transforming growth factor-beta1
TPR	Translocated promoter region
v	variant
VEGFR	Vascular endothelial growth factor receptor

CONTENTS

INTRODUCTION

General introduction

Physiological and pathological behaviour of cells requires their ability to sense their environment and transmit or process what they sense, producing tightly controlled responses such as proliferation or migration. The complex environment outside the mammalian cell comprises the extracellular matrix (ECM), which is made up of various different proteins such as collagen, fibronectin and glycosaminoglycans (GAGs). In addition there are a multitude of other macromolecules such as growth factors, cytokines or chemoattractants, which co-exist in the ECM. Cells sense the components of the environment by expressing a number of transmembrane molecules that are able to bind directly to the ECM components, the other external factors or molecules on other cells. Upon binding stable or transient complexes are formed and may elicit a number of downstream mitogenic or morphogenic consequences. One group of such molecules are the cellular adhesion molecules (CAMs).

CAMs embrace a large group of structurally different membrane molecules primarily involved in cell-cell or cell-ECM interactions. These interactions are important in maintaining tissue integrity and in processes requiring migration such as lymphocyte homing or metastasis. The major groups include selectins, integrins, the immunoglobulin (Ig) superfamily e.g. intercellular adhesion molecules (ICAMs), and proteoglycans such as the syndecan family or the CD44 family.

A different class of membrane molecules involved in sensing the extracellular environment are the growth factor (GF) receptors. They are essential molecules that bind their cognate ligands in the extracellular milieu resulting in a varied collection of growth, proliferative or differentiation responses. A subset of growth factor receptors are the receptor tyrosine kinases (RTK), which have the ability to add a phosphate group to specific tyrosine residues on a protein or substrate. There are 58 RTKs in humans, which make up about 20 subfamilies and are

expressed in a cell or tissue specific manner primarily involved in cell growth (ExPASy web database) e.g. the Epidermal growth factor receptor family (EGFR), Vascular endothelial growth factor receptor family (VEGFR), Fibroblast growth factor receptor family (FGFR), the nerve growth factor (NGF) receptor family Trk, or the hepatocyte growth factor/scatter factor (HGF/SF) receptor family c-Met.

Interestingly a number of CAMs and GF receptors can co-operate in response to external stimuli thus expanding the cellular repertoire. Of interest in this study is the interplay between the CAMs, CD44 and the GF receptor c-Met.

CD44

CD44 is a key adhesion molecule as well as a molecular modulator of extracellular cues expressed in a vast array of tissues. It was discovered in 1980 on leukocytes and cells of the haematopoietic system (Dalchau et al. 1980), then as a hyaluronate receptor (Lacy and Underhill 1987), a glycoprotein of Mr 85,000 (gp85) (Underhill et al. 1987), a phagocyte glycoprotein-1 (Pgp-1) (Mackay et al. 1988) and a Hermes antigen (Picker et al. 1989). Molecular sequencing established that these proteins were all one designated CD44 (Naor et al. 1997).

Gene structure

CD44 actually comprises of a family of type I transmembrane glycoproteins. The diversity of protein products is due primarily to differential alternative splicing of pre-mRNA transcripts as well as post-translational modifications.

Figure 1. *Schematic representation of the CD44 gene*. Exons 6-15 are subject to alternative splicing and are also known as the variant (v) exons v1-v10.

A single gene found on chromosome 11 in humans (Forsberg et al. 1989) and chromosome 2 in mice (Colombatti et al. 1982) encodes the CD44 proteins. The

gene, which spans approximately 50kb is organised into 20 exons (Fig. 1) (Screaton et al. 1992). Exons 1-17 encode the extracellular portion of which 10 exons (exons 6-15) are subject to alternative splicing and thus generates the CD44 variant (v) isoforms designated exons v1-v10 (Screaton et al. 1992; Screaton et al. 1993). Exon 18 encodes the transmembrane domain and the first three amino acids of the cytoplasmic domain (Screaton et al. 1992). The cytoplasmic domain encoded by exons 19 and 20 is also subject to alternative splicing. Exclusion of exon 19 generates a long tail of around 70 amino acids. Exon 19 inclusion generates a short tail of 3 amino acids due to an alternative stop codon (Goldstein et al. 1989; Thorne et al. 2004).

Protein structure

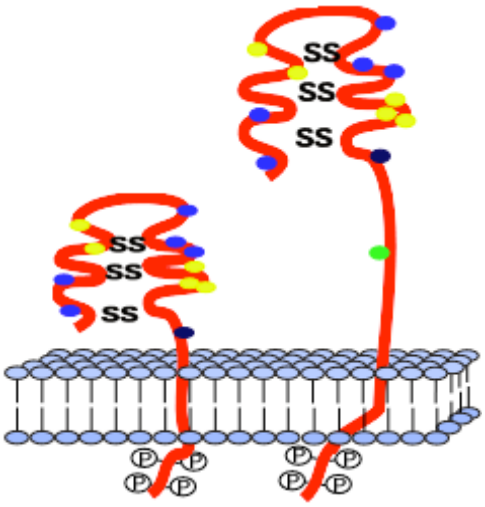

Figure 2. *A simplified representation of CD44 isoforms protein structure.* The smaller CD44s isoform (left) and a CD44 isoform containing all the variants are depicted (right). The N-terminal globular domain is subjected to extensive N-linked (light blue balls) and O-linked (yellow balls) glycosylation. The stem region is identified by the GAG chondroitin sulphate modification (dark blue balls). Inclusion of the variant exons increases the stem region and introduces the GAG heparan sulphate modification at exon v3 (green ball).

The smallest and most abundant isoform is the CD44 standard or haematopoietic (CD44s or CD44H respectively) form of 361 amino acids in human (or 363 amino acids in mouse) (Naor et al. 1997), where all the variant exons are excluded (Fig. 2 left). Inclusion of all the variant exons expands the protein by 381 amino acids in humans. The largest isoform in humans contains variant exons v2-v10 and is

found in keratinocytes (Bloor et al. 2001). In humans exon v1 contains a stop codon and is not expressed (Screaton et al. 1993).

Extracellular portion

The first 5 exons encode the “5’ constant” amino-terminal (N-terminal) domain (~180 amino acids), thus being common in all isoforms and are highly conserved amongst mammalian species (~85%). In humans, the N-terminal domain contains 6 cysteine residues involved in forming 3 disulphide bonds, which are thought to be determinants in forming a globular structure (Fig.2) (Goldstein et al. 1989). This domain is responsible for binding to the extracellular matrix component hyaluronic acid (HA), which has been mapped to exons 2 and 3 (Thorne et al. 2004). This region of ~100 amino acids is known as the Link-homology module due to its resemblance to the HA binding domains of cartilage link protein, aggrecan and tumour necrosis factor stimulated gene 6 (TSG-6) (Kohda et al. 1996; Bajorath et al. 1998). The N-terminal domain also contains sites for post-translational modifications. Five conserved sites of the consensus sequence Asn-X-Ser/Thr for N-glycosylation are found and are thought to modulate HA binding of CD44 in human and mouse cells (Fig 2) (Bartolazzi et al. 1996; English et al. 1998).

Membrane proximal region

Exons 4 and 5 together with exons 16 and 17 (3’ constant region) encode the membrane proximal stem region in the CD44s isoform. It is in this region that the insertion site for additional sequences of the variant isoforms is contained thus allowing for an enlarged membrane proximal portion (Tolg et al. 1993). In addition this region is subjected to extensive post-translational modifications. Indeed, the predicted size of the CD44s core protein has an apparent Mw of 37-38 kDa (Goldstein et al. 1989; Stamenkovic et al. 1989). Utilisation of the 6 N- and 7 O-linked glycosylation sites in humans (5 and 10 in mouse respectively) doubles the molecular weight to 80-95 kDa (Zhou et al. 1989; Lokeshwar and Bourguignon 1991). There are in addition four Ser-Gly motifs in human CD44

(three in mouse) that can be modified by GAGs (Goldstein et al. 1989; Stamenkovic et al. 1989). CD44s is modified with keratan sulphate and chondroitin sulphate (CS) (Fig.2) and is involved in modulating binding to ECM components in metastatic cells (Henke et al. 1996; Takahashi et al. 1996).

Variant isoforms

Inclusion of the variant exons v1-v10 expands the number of potential N- and O-linked glycosylation sites particularly at exons v2 and v8-v10 (Naor et al. 1997). Variant isoforms can also expand the GAG binding capabilities of CD44 by altering the specificity of ligand binding i.e. v6 and v7 containing isoforms bind directly to multiple GAGs like CS or heparin (Sleeman et al. 1997). The CD44v3 isoform has a consensus Ser-Gly-X-Gly motif for the modification of the GAG heparan sulphate (HS) (Bennett et al. 1995), which enables the binding of heparin-binding growth factors to CD44.

Soluble CD44

Soluble fragments of the extracellular portion of CD44 (sCD44) are detectable in culture supernatants and in normal sera from healthy and diseased patients (Ristamaki et al. 1994). sCD44 isoforms are shed from the membrane and may compete with cell surface CD44 for ligand binding. For example, sCD44 has been used to interfere with CD44-HA interactions inhibiting tumour cell proliferation (Yu et al. 1997). The major mechanism of CD44 release from cells involves proteolytic cleavage by metalloproteases and serine proteases (Bazil and Strominger 1994; Kajita et al. 2001).

Transmembrane and Cytoplasmic domains

The transmembrane domain contains Cys residues, which undergo fatty acid addition or palmitoylation. This modification may play a role in CD44-ankyrin binding (Bourguignon et al. 1991) and with signal transduction since it interferes with CD3 mediated signalling in lymphocytes (Guo et al. 1994). This domain may also be important in CD44 clustering as a small proportion of CD44s formed dimers after stimulation with phorbol myristate acetate (PMA) and the Cys

residues may help to form stable aggregates with implications for HA binding (Liu and Sy 1996).

The cytoplasmic tail of CD44 contains a number of serine residues that are subject to phosphorylation e.g. Ser325 phosphorylation may be important in HA mediated cell migration (Peck and Isacke 1998). The cytoplasmic tail also contains binding sites for molecules that associate with the actin cytoskeleton e.g. ezrin (Thorne et al. 2004). The best characterised cytoskeleton mediators are the ezrin/radixin/moesin (ERM) proteins. They are members of the band 4.1 superfamily, which have a filamentous (F)-actin binding site in their C-terminus. The association between CD44 and ezrin is modulated by phosphorylation of CD44 Ser291 by protein kinase C (PKC) *in viv*o (Legg et al. 2002). Importantly, the neurofibromatosis 2 (NF2) tumour suppressor protein merlin (moesin-ezrin-radixin-like) can also bind to the cytoplasmic tail of CD44 and is important in modulating growth with the ERM proteins (Morrison et al. 2001). Interestingly, a recent study demonstrated the neural Wiskott-Aldrich syndrome (N-WASP) protein can complex with CD44 linking it to the actin cytoskeleton via the actin related protein 2/3 Arp2/3 binding complex contributing to F-actin formation and tumour cell migration (Bourguignon et al. 2007).

In addition, numerous other proteins can form complexes at the cytoplasmic tail of CD44 such as the Rho-family of GTPases and members of the Src family of non-receptor tyrosine kinases potentially linking CD44 to downstream signalling pathways although whether their interaction is direct or functionally relevant is unclear (Ponta et al. 2003; Thorne et al. 2004).

Expression and Biological functions of CD44 proteins

The CD44 family of proteins are widely and dynamically expressed primarily on the epithelium of various tissues during embryogenesis, in the adult and in tumours. CD44s is the most ubiquitously expressed of the isoforms and can be found in most tissues of the adult organism with a more restricted pattern in the embryo (Wheatley et al. 1993; Terpe et al. 1994). The CD44 variant isoforms in contrast, have a highly limited and specific expression pattern in the embryo and

adult, mainly localised in regions of active cell growth such as the basal layers of stratified epithelium or the apical ectodermal ridge (AER) (Alho and Underhill 1989; Wheatley et al. 1993; Mackay et al. 1994; Terpe et al. 1994; Ruiz et al. 1995).

Physiological functions

Numerous antibody interference studies against all or specific CD44 isoforms have contributed to elucidating their functionally diverse physiological roles in activities such as adhesion, proliferation and migration. More specifically CD44 proteins are involved in processes including haematopoiesis (Miyake et al. 1990) and lymphocyte activation and homing (Huet et al. 1989; Shimizu et al. 1989; Jalkanen et al. 1990). In the haematopoietic system, strong expression of CD44s is observed and studies indicate that the expression of CD44 isoforms are dynamically regulated as is the case during T-lymphocyte activation (Naor et al. 1997; Ponta et al. 1998). CD44 isoforms are thus important in the maturation as well as the homing of haematopoietic progenitor cells (Khaldoyanidi et al. 1996; Khaldoyanidi et al. 1997). Unsurprisingly then, CD44 plays a role in inflammation and has been implicated in several inflammatory and autoimmune diseases (see pathological functions below) (Foster et al. 1998; Pure and Cuff 2001).

CD44 isoforms (particularly variant) are strongly expressed in the skin. RT-PCR analysis demonstrated that keratinocytes predominantly express CD44 molecules containing all the variant isoforms (CD44v2-v10 in humans) (Hudson et al. 1995; Bloor et al. 2001). In keratinocytes CD44-mediated proliferation and adhesion plays a role in wound healing, tissue integrity and maintaining skin elasticity (Hudson et al. 1995; Kaya et al. 1997).

Development

During embryogenesis, CD44 isoforms are highly expressed in some tissues, which also correlate with HA-mediated morphogenesis and organogenesis e.g. the heart or somites (Fenderson et al. 1993; Wheatley et al. 1993). Studies using antibody interference against CD44s and CD44v6 in pregnant rats demonstrated a role for CD44 isoforms during ontogeny (Zoller et al. 1997). For example anti-

CD44s caused delays in delivery and frequent intrauterine abortions. Anti-CD44v6 hampered development until day 16-18 of gestation (E16-18). In contrast, the large CD44v3-v10 isoform is expressed in the instructive epithelial layer of the developing limb, the AER. This isoform with its heparan-sulphate modification is necessary for growth factor presentation of fibroblast growth factor-8 (FGF-8) to the underlying mesenchymal cells of the developing limb, conveying a proliferative signal (Sherman et al. 1998). This CD44 variant isoform is required for correct outgrowth of limbs.

Pathological functions

CD44 isoforms are involved in a number of autoimmune diseases. Blocking antibodies against CD44 could reduce collagen induce rheumatoid arthritis in mouse models (Zeidler et al. 1995). Expression of CD44 on immune cells was involved in endothelial cell damage and interleukin 2 (IL-2) induced vascular leak syndrome (Rafi-Janajreh et al. 1999). CD44 variant containing isoforms have been implicated in inflammatory disease such as inflammatory bowel disease where their expression leads to increased survival of effector lymphocytes and a persistence of inflammation (Wittig et al. 2000).

A vast amount of data unequivocally illustrates the role of CD44 in tumour formation and metastasis. For instance, one of the first studies that investigated CD44 isoforms in cancer demonstrated that expression of specific variant isoforms was linked to lymphatic spread of tumour cells i.e. CD44v6 containing isoforms were able to confer metastatic potential to non-metastatic rat carcinoma cells (Gunthert et al. 1991; Rudy et al. 1993). Interestingly, mis-regulation of CD44 does not appear to be due to mutations in the gene itself but to factors that can influence the expression of the CD44 gene and which may also be implicated in carcinogenesis (Ponta et al. 2003). For example there is a correlation between growth factor regulated signalling pathways i.e. Ras-mitogen activated protein kinase (Ras/MAPK), and modulation of CD44 expression in tumour cell lines (Hofmann et al. 1993; Hamada et al. 1998). CD44 isoforms are expressed on a number of primary human tumours originating from cells of the

breast, brain, colon and skin (Hart et al. 1991; Dall et al. 1995; Naor et al. 1997; Reeder et al. 1998). Expression particularly of CD44 variant isoforms is often correlated with poor prognosis due to metastasis i.e. CD44v6 containing isoforms expression in colorectal carcinoma (Sleeman et al. 1995 Naor, 1997 #4148; Wielenga et al. 1998). In contrast, CD44 is downregulated in neuroblastomas and prostate carcinomas (Shtivelman and Bishop 1991; De Marzo et al. 1998), which also correlates with poor prognosis. The variant isoforms do not hold the monopoly in tumourigenesis e.g. CD44s is associated with growth of mouse melanomas (Bartolazzi et al. 1994). Certain isoforms have been reported to shed more easily in disease. Elevated levels of sCD44 and sCD44v6 are observed in patients with lymphoma (Ristamaki et al. 1994) and sCD44v6-10, 7-10 and 8-10 may play a role in determining rate of tumour formation (Bartolazzi et al. 1995).
Interestingly, the metastasis conferring CD44v6 containing isoforms are required for T and B lymphocyte activation (Arch et al. 1992). Clearly, the adhesive, migratory, proliferative and anti-apoptotic functions of CD44 offer an advantage to neoplastic development.

CD44 binding partners and molecular mechanisms

CD44 and Hyaluronic Acid

Hyaluronic Acid (HA) is a high molecular weight polysaccharide of the ECM consisting of repeats of the disaccharide unit D-glucuronic acid and N-acetyl-D-glucosamine component. HA acts as a space filling component of the ECM and is particularly enriched in pericellular matrices surrounding migrating and proliferating cells during embryogenesis, tissue repair as well as tumourigenesis (Pure and Cuff 2001). In addition HA is abundant in connective tissue such as the skin dermis or the lungs (Naor et al. 1997). Functionally HA is important in hydration of tissues, organisation of the ECM and through interactions with its principle cell surface receptor CD44, influences cell behaviour. Subsequently, CD44-HA interactions are important in many cell-ECM mediated processes including lymphocyte rolling, in the inflammatory response and tumourigenesis. Studies concerning the binding of CD44 isoforms to HA are conflicting however,

the cell type as well as the post-translational modifications of the CD44 isoforms needs to be considered in modulating CD44-HA interactions (Naor et al. 1997). Interestingly, CD44 is involved in mediating the internalisation of HA in several of cell types including macrophages (Thorne et al. 2004). A number of studies have demonstrated that this uptake is physiologically relevant. The absence of CD44 by knock out studies, lead to an abnormal accumulation of HA in tissues with consequences in wound healing and aggravated immune responses (Kaya et al. 1997; Teder et al. 2002; Nedvetzki et al. 2004).

CD44 as a membrane platform

CD44 interacts with a number of growth factors and matrix metalloproteinases (MMP) functioning as a specialised docking area in the control of cellular processes such as growth (Ponta et al. 2003). In mammary carcinoma and melanoma cells CD44 localises MMP-9 to cell surfaces, which is necessary for degradation of collagen IV, correlates with tumour cell invasiveness (Yu and Stamenkovic 1999), and it is important for tumour angiogenesis *in vivo* (Yu and Stamenkovic 2000). Furthermore, MMP-9 can cleave latent transforming growth factor β (TGF-β) leading to its activation and tissue remodelling (Yu and Stamenkovic 2000). In another study CD44HSPG (v3 containing isoform) recruits MMP-7 and heparin-binding epidermal growth factor precursor (pro-HBEGF) to the epithelial cell surfaces of tissues including the postpartum uterus and lactating mammary glands (Yu et al. 2002). This enables MMP-7, probably through proteolytic cleavage, to process the precursor to mature HBEGF, which can bind and activate its receptor ErbB4, one consequence of which is cell survival. The assembly of the surface complex is important in tissue remodelling of the female reproductive organs and disruption in localisation of the components i.e. MMP-7 has implications in the regulation of apoptosis. Recently, CD44 was shown to interact with a RhoA guanine nucleotide exchange factor (RhoGEF) the leukemia-associated RhoGEF (LARG) (Bourguignon et al. 2006). LARG acts as a linker between CD44 and the epidermal growth factor receptor

(EGFR) leading to HA dependent activation of EGFR and tumour growth in head and neck squamous cell carcinoma.

CD44 mediated growth factor presentation

The HBEGF-ErbB4 interaction also illustrates the ability of CD44 isoforms to mediate interactions between GFs and their receptors, in a GF presentation mechanism. The heparan-sulphate side chain of CD44v3 containing isoforms is able to bind a number of heparin-binding growth factors such as the HBEGF, FGF and HGF. During limb development, CD44HSPG presents FGF on one cell type to its high affinity receptor on another (Sherman et al. 1998). In Namalwa Burkitt's lymphoma cells transfection of the CD44HSPG sequesters HGF and this is necessary for activation of the c-Met receptor and downstream signalling (van der Voort et al. 1999).

CD44 as a co-receptor

Mechanistically, GF presentation is not the only way CD44 isoforms assist cell surface receptors, CD44 isoforms can also act as co-receptors. CD44 can interact with surface receptors like the ErbB family. The interaction between CD44 and ErbB2 is important for their activation in ovarian carcinoma cell growth and migration (Bourguignon et al. 1997; Bourguignon et al. 2007). CD44s may catalyse heterodimerisation between ErbB2 and ErbB3 in response to their ligand neuregulin in Schwann cell differentiation and growth of the peripheral nervous system (Ponta et al. 2003). In contrast to the GF presentation mechanism, a CD44v6 isoforms can act as a co-receptor for c-Met in different cell lines (discussed below) (Orian-Rousseau et al. 2002).

CD44 and ERM proteins

The ERM proteins are important modulators between membrane proteins and the actin cytoskeleton. CD44 can influence downstream signalling pathways in cells through binding to ERM proteins. In human kidney cells, the CD44-ERM interaction is required for signalling from the c-Met receptor to the extracellular

signal-related kinase1/2 (ERK1/2) (Orian-Rousseau et al. 2002). In a CD44v6 absent cell line introduction of constitutively active ezrin fused with tailless CD44v6 promoted HGF/SF dependent signalling (Orian-Rousseau et al. 2007). Conversely, down-regulation of ezrin by small interfering RNA (siRNA) abrogated HGF/SF downstream signalling. In addition, signalling from the c-Met receptor modulated by CD44v6-containing isoforms can be disrupted by overexpression of a CD44v6tailess-ezrin fusion protein mutated in the actin binding domain (Orian-Rousseau et al. 2007). Collectively these data demonstrate that ezrin and F-actin are necessary to propagate HGF dependent signalling.

CD44 knockout studies

CD44 total knockout mice

CD44 null mice have been generated by targeted deletion of exons 2 and 3, 4 and 5, and by an in-frame disruption of the leader peptide (Schmits et al. 1997; Protin et al. 1999; Yu et al. 2002). Surprisingly, in all reported cases homozygous mutant mice were born viable, fertile, and with the normal Mendelian ratio. Despite the expression pattern of CD44 proteins in the embryo and their role in limb outgrowth, CD44 null mice exhibited no developmental defects. CD44 null mice grew without abnormalities in size, cellularity or tissue architecture (Schmits et al. 1997). The homozygous mutant mice, although phenotypically normal, did however display some mild abnormalities. Altered tissue distribution of myeloid progenitor cells between the bone marrow and the spleen suggested that CD44 is involved in egress of these cells from the bone marrow (Schmits et al. 1997). CD44 is also implicated in lymphocyte recirculation *in vivo* as CD44 null lymphocytes exhibit impaired homing to the thymus and peripheral lymph nodes (Protin et al. 1999). Pregnant CD44 null mice displayed postpartum defects; uterine involution was accelerated and impairment in maintaining lactation, highlighting the importance of CD44 in female reproductive organ tissue remodelling (Yu et al. 2002).

Further, CD44 null mice displayed more pronounced phenotypes when challenged: 1) CD44 null mice display exaggerated granuloma response when challenged with heat-killed *Cryotsporidium parvum*, which reflects minor defects in the co-ordination of this immune response (Schmits et al. 1997). 2) Following induced lung injury, CD44 null mice die due to unresolved inflammatory response i.e. impaired clearance of apoptotic neutrophils, persistent accumulation of hyaluronan fragments at the site of tissue injury, and impaired activation of transforming growth factor-beta1 (TGFβ-1) (Teder et al. 2002). 3) When crossed into a Fas null background, CD44 null mice exhibited an increased autoimmune lymphoproliferative disease, which correlated with increased resistance of T-cells to activation-induced cell death (Do et al. 2003).

CD44 conditional knockout

An alternative approach to gain further insight into the biological role of CD44 was the creation of transgenic mice expressing an antisense CD44 cDNA (Kaya et al. 1997). In an antisense orientation, the entire mouse CD44s cDNA was cloned downstream of the bovine keratin-5 gene promoter. The result was a tissue specific loss of CD44 expression, which leads to a complete loss of CD44 in all layers of the epidermis and outer root sheath of hair follicles. Newborn mice did not display any skin abnormalities. However phenotypical deficits became more apparent with age. Loss of keratinocyte CD44 was accompanied by morphological alterations in the basal keratinocytes and loss of HA in interkeratinocyte spaces with accumulation of HA in the superficial dermis. This was reflected in the abnormal skin elasticity of the transgenic mice. In addition, defective keratinocyte proliferation in response to mitogen and growth factors was observed. This together with reduced skin elasticity contributed to delays in wound healing, which in comparison to control animals was delayed by 4-7 days. Delays in hair re-growth by 5 days could also be attributed to reduced keratinocyte proliferation in the hair bulb. That CD44 is important for skin proliferation and the correct hyaluronate homeostasis is clearly demonstrated by the severity of the skin phenotype.

Compensation of CD44

The defects in the conditional knock out mice coupled with the relatively normal nature of the CD44 total knockout mice indicates that a compensatory mechanism takes place whereby other genes may be differentially regulated to fulfil the functional role of CD44. That this compensation must take place during early embryogenesis is further substantiated by the apparent strong phenotypic discrepancies between the total and antisense knockout mice. Abrogation of CD44 expression by the antisense cDNA occurs at a later time point during embryogenesis (E11.5), i.e. a stage well after early limb outgrowth (Sherman et al. 1998).

In fact two independent studies have implicated different compensatory mechanisms for the loss of CD44. Studies of limb bud development in the CD44 null mice indicated that the proliferative mesenchymal cells increased their sensitivity to FGF (Wainwright 1998). Since in the wild type situation heparan-sulphate modified CD44 is required for FGF presentation, in the CD44 null mice these cells may express or utilise another HSPG.

In contrast, another group reported that the receptor for hyaluronan-mediated motility (RHAMM) compensates for CD44 in inflamed CD44 null mice (Nedvetzki et al. 2004). Using a collagen-induced arthritis challenge they demonstrated that joint inflammation in CD44 null mice was more severe than in the wild type situation. This was due to an enhanced ability of RHAMM to support the inflammatory cascade. The mechanism of compensation is not due to up-regulation of RHAMM, but rather that the loss of CD44 causes an accumulation of HA thus augmenting signalling through the ready available RHAMM.

These data demonstrate that the diversity of CD44's molecular actions appears to require different substitute mechanisms.

c-Met the receptor for hepatocyte growth factor (HGF)

c-Met is a receptor tyrosine kinase (RTK) that when bound by its ligand hepatocyte growth factor/scatter factor (HGF/SF) is involved in a number of cellular responses including proliferation, migration, invasion and differentiation.
c-Met was originally discovered as a proto-oncogene that mapped to chromosome 7 in humans (a region associated with disease formation) (Cooper et al. 1984a; Cooper et al. 1984b). In contrast HGF and SF were independently identified as a mitogenic factor (HGF) in primary rat hepatocytes and a factor involved in loss of cell contacts (SF) and migration in normal epithelial cells respectively (Nakamura et al. 1984; Stoker et al. 1987). Three independent studies demonstrated that both are the same molecule (Bottaro et al. 1991; Naldini et al. 1991b; Naldini et al. 1991c).

Structural features of HGF/SF and c-Met

HGF/SF is a heterodimeric protein that is homologous to the plasmin precursor plasminogen (Nakamura et al. 1989). A precursor pro-HGF/SF single chain protein of 83kDa is encoded in a single open reading frame. After proteolytic processing, the mature active form consists of two subunits linked by a disulphide bond, a 69kDa α subunit and a 39kDa β subunit (Nakamura et al. 1987; Nakamura et al. 1989). The α subunit contains an N-terminal domain and four kringle (K1-K4) domains, which are required for c-Met binding with the K1, K2 and K4 domains being sufficient for activation of the receptor (Hartmann et al. 1992; Holmes et al. 2007). In addition the β subunit contains a serine protease domain, which provides additional binding and is necessary for the full-scale activation of all signalling events including mitogenic activity (Lokker et al. 1992; Okigaki et al. 1992).
c-Met is the prototypic member of a family of RTKs, which include Ron and Sea (Huff et al. 1993; Ronsin et al. 1993). Like its ligand, c-Met is a heterodimer of 2

disulphide-linked subunits. Biosynthetic studies showed that c-Met exists as a glycosylated precursor protein of 190kDa, which undergoes proteolytic cleavage to form 50kDa α subunit and 145kDa β subunit. (Giordano et al. 1989a; Giordano et al. 1989b). The short α subunit is extracellular and contains a semaphorin-like sema domain. The β subunit comprises the rest of the ectodomain, the transmembrane domain and the cytoplasmic domain. The extracellular portion of the β subunit also contains a sema domain, as well as a cysteine rich plexin, semaphorin and integrin (PSI) domain and four Ig (Ig1-4) domains. Finally a juxtamembrane region and kinase domain makes up the cytoplasmic tail region.

Expression and biological functions of c-Met and HGF/SF

Expression analysis determined that c-Met is a broadly distributed protein, primarily expressed on epithelial cells (Chan et al. 1988; Di Renzo et al. 1991). It is found in a number of different organs of the developing and adult organism. For example strong expression of c-Met is found in such organs as the liver and the kidney (Andermarcher et al. 1996). In addition, c-Met is expressed in a number of tumour cells many of which are epithelial in origin (Di Renzo et al. 1991). The expression pattern of HGF/SF similarly follows that of its receptor however it is secreted by mesenchymal cells (Zarnegar and DeFrances 1993) normally in close vicinity to receptor expressing cells (Sonnenberg et al. 1993) signifying that HGF/SF stimulation is mainly paracrine.

Physiological functions

HGF/SF-c-Met signalling is important for proliferation of various cell types including hepatocytes (Machide et al. 2006), keratinocytes (Kan et al. 1991), endothelial cells (Rubin et al. 1991), melanocytes (Halaban et al. 1993) and renal cells (Ishibashi et al. 1992). It also stimulates the loss of cell junctions and the gain of motility (scattering) (Gherardi et al. 1989). Most strikingly it stimulates movements into artificial basement membrane (invasion) (Singh-Kaw et al. 1995). The HGF/SF-c-Met pathway induces differentiation responses in cells

known as branching morphogenesis, relevant for the development of the kidney, liver and mammary glands (Birchmeier et al. 1993; Bowes et al. 1999). Thus this process is involved in organogenesis and organ regeneration in adult tissues. Branching morphogenesis is a highly complex process, which also requires cells to proliferate, migrate, polarise, communicate with cells and the ECM, and invade the surrounding matrix, although it is not exactly understood how the HGF/SF-c-Met pathway modulates some of these events (Zhang and Vande Woude 2003). However organisation of cell proliferation and migration by the HGF/SF-c-Met pathway has been shown to be important in wound healing and the formation of new blood vessels (angiogenesis) in endothelial cells (Bussolino et al. 1992).

Development

The HGF/SF-c-Met signalling pathway is essential for proper development, which is highly dependent on the epithelial-mesenchymal expression between receptor and ligand. The generation of HGF/SF null or c-Met null mice resulted in embryonic lethality (E14.5) due to defects in the development of the placenta (Bladt et al. 1995; Uehara et al. 1995). Placental development requires stimulation of placental epithelial cells (trophoblasts) by HGF/SF producing mesenchymal cells (from the allantois). In addition abnormal cell morphology and number in the developing liver was observed (Schmidt et al. 1995) leading to reduced liver size. The loss of the HGF/SF-c-Met signalling pathway also severely affects the development of skeletal muscle as migration of the myogenic precursor cells is lost (Bladt et al. 1995). Interestingly, there is a correlation with the high expression of HGF/SF and c-Met during embryogenesis and their essential functions, in tissues like the placenta or the liver.

Cancer

The evidence for the involvement of the HGF/SF-c-Met pathway in cancer is compelling. Firstly, c-Met was originally identified as an oncogene where its transmembrane and C-terminal portion was fused with the N-terminus of translocated promoter region (TPR) called TPR-Met (Park et al. 1986), which is

ligand independent, constitutively active and highly transforming (Rodrigues and Park 1993). Secondly, targeted expression of HGF/SF to various tissues in transgenic mice lead to the formation of several types of tumours (Takayama et al. 1997) illustrating *in vivo* transforming capability. Thirdly, introduction of c-Met and HGF (or mutant c-Met) into cells conferred the properties of motility, invasiveness, and tumourgenicity to transformed cells (Jeffers et al. 1997; Jeffers et al. 1998; Christensen et al. 2005). Finally, alterations in c-Met or HGF/SF are implicated in human cancers i.e. mutations in c-Met, overexpression of c-Met and/or HGF/SF, and autocrine signalling from this pathway has contributed to transformation and metastasis of numerous tissue types including breast, colon, lung and pancreas (Maulik et al. 2002). Specifically, HGF/SF overexpression is correlated with breast cancer liver metastases (Eichbaum et al. 2006). In the instance of TPR-Met, although initially observed in chemically transformed cells, it has been detected in patients with gastric carcinomas (Yu et al. 2000). Since the HGF/SF-c-Met pathway is responsible for a multitude of cellular events it is not surprising to find them aberrantly organised in numerous cancers.

HGF/SF-c-Met signalling

HGF/SF binds c-Met at the α chain and the first 212 amino acids of the β chain (Bottaro et al. 1991; Gherardi et al. 2003). This combined region called the sema domain of c-Met is required for receptor dimerisation, which triggers autophosphorylation of tyrosine residues in the cytoplasmic domain (Naldini et al. 1991a; Gherardi et al. 2006). Phosphorylation of tyrosine residues (Tyr1234 and 1235) in the kinase domain are critical for receptor activation whilst phosphorylation of Tyr 1349 and 1356 in a cluster of amino acids C-terminal of the kinase domain is essential for activation of the multi-substrate docking site, which allows binding of proteins involved in downstream signalling form c-Met (Ferracini et al. 1991; Longati et al. 1994; Zhu et al. 1994; Pelicci et al. 1995).

c-Met effectors

A multitude of proteins are recruited to the activated c-Met C-terminus and include the multi-domain adaptor proteins growth factor receptor bound protein 2 (Grb2) (Ponzetto et al. 1994) Src-homology 2 (SH2) containing protein (Shc) (Pelicci et al. 1995) and Grb2-associated binding protein 1 (Gab1) (Weidner et al. 1996), and other signal transducers such as the p85 subunit of phosphotidylinsitol 3-kinase (PI3K) (Graziani et al. 1991), the Ras guanine nucleotide exchange factor son-of-sevenless (SOS) (Graziani et al. 1993) and Src kinase (Ponzetto et al. 1994). In many cases substrate interaction with c-Met is mediated through SH2 domains, whilst SH3 domains can bind to the other signal transducers (Birchmeier et al. 2003; Bolanos-Garcia 2005). c-Met activates a series of signalling pathways including the Ras-MAPK or PI3K pathway mainly through two major adaptor molecules Gab1 and Grb2 (Zhang and Vande Woude 2003).

Gab1 is a large multi-adaptor protein that is essential to c-Met signalling. The Gab1 knockout mice display almost identical phenotypes to the HGF/SF and c-Met null mice e.g. impairment in migration of muscle precursors, reduced liver size and placental defects (Sachs et al. 2000). Gab1 binds directly to c-Met through its unique c-Met binding domain (MBD) or indirectly via Grb2 (Weidner et al. 1996) and recruits a number of SH2 containing proteins i.e. PI3K, phospholipase Cγ (PLCγ), SH2 containing protein tyrosine phosphatase 2 (SHP2) and the Crk family of adaptors (Gao and Vande Woude 2005). Signalling mediated via Gab1 requires multifaceted co-ordination of these downstream effectors in physiological processes such as scattering and branching morphogenesis, and cell survival (Gual et al. 2000; Maroun et al. 2000; Fan et al. 2001; Zhang and Vande Woude 2003). A number of studies have also demonstrated the requirement of Gab1 and its effectors in cell transformation (Fixman et al. 1997; Mood et al. 2006; Watanabe et al. 2006).

The Grb2 adaptor protein contains an SH2 domain, which interacts with Tyr1356 of cytoplasmic c-Met (Ponzetto et al. 1994). Grb2 interacts with SOS through its

SH3 domains and links c-Met to the Ras-MAPK pathway since SOS activates Ras (Furge et al. 2000). c-Met signalling mediated by Grb2 is important in processes such as proliferation, migration, invasion and branching morphogenesis (Zhu et al. 1994; Maina et al. 1996; Maina et al. 2001). In addition Grb2 binding to c-Met is implicated in cellular transformation (Ponzetto et al. 1996).

In summary, the c-Met signalling pathways are complex. Differential contribution of the signalling pathways regulate signalling responses i.e. PI3K and Ras-MAPK are both required in scattering and tubulogenesis in MDCK cells (Khwaja et al. 1998). These data signify that the HGF/SF-c-Met signalling pathway are important for neoplastic as well as normal cellular processes including growth and invasion (Bardelli and Comoglio 1997).

c-Met signalling partners

c-Met functions may be influenced by input signals that complement its activity in a tissue-specific manner. For instance c-Met can bind to cell surface proteins, which aid in activation or signalling. These interactions may be required in regulating different threshold levels of signal output, which are thought to be important in modulating the different responses from c-Met.

c-Met and $\alpha 6\beta 4$ integrin

$\alpha 6\beta 4$ integrin is a cell surface adhesion protein usually involved in cell-ECM interactions and typically involved in adhesive structures. It also contributes to neoplastic invasion. $\alpha 6\beta 4$ integrin was found to interact with c-Met and act as a signal amplifier in invasive growth of carcinoma cells (Trusolino et al. 2001). HGF/SF stimulated activation of c-Met leads to phosphorylation of the $\beta 4$ cytoplasmic domain, which recruits SHC and PI3K leading to activation of Ras/MAPK pathway.

c-Met and Plexin B1

c-Met can form a complex with Plexin B1, a c-Met related protein (28% homology to c-Met) and the receptor for semaphorin 4D (Sema 4D) (Giordano et al. 2002). Semaphorins are important for control of axon guidance. Interestingly the association with c-Met allows Sema 4D induced (through binding to Plexin B1) tyrosine phosphorylation of c-Met and elicits invasion and branching morphogenesis. In addition Sema 4D and HGF can co-operatively induce invasive growth. Plexin B1 thus acts as a c-Met primer in normal and tumour invasion and expands the ligand repertoire.

CD44, a dedicated co-receptor in HGF/SF induced activation and downstream signalling of c-Met

The observations that CD44 and c-Met are both expressed in diverse tissues and up-regulated in number of cancers prompted investigations into a possibly co-operation between them. A CD44v6 containing isoform and c-Met were found to interact in a number of transformed and non-transformed cells (Orian-Rousseau et al. 2002). The consequences of the association are two-fold i) the CD44v6 extracellular domain is required for c-Met activation by organisation of a ternary complex between CD44v6, c-Met and HGF/SF. A short three amino acid region in CD44v6, RWH in humans, is essential for c-Met activation (Matzke et al. 2005) ii) the cytoplasmic tail of the CD44v6 isoform is required for HGF/SF dependent activation of the Ras/MAPK pathway. The transmission of signal depends on the interaction between CD44 and the ERM proteins and the recruitment of the actin cytoskeleton. The assembly of this complex at the membrane is important for HGF/SF dependent activation of Ras by its GEF, SOS (Orian-Rousseau et al. 2007).

Thus, the interaction between CD44v6 and c-Met at least in several tissues is a prerequisite for the receptor activation.

AIM

The principle aim of this thesis is to find proteins that can in the absence of CD44v6, act as an alternative co-receptor for the receptor tyrosine kinase c-Met. The idea that molecules exist that can take the place of CD44 functions came from the discrepancies in CD44 loss of function studies. By investigating cell systems where CD44 is not normally expressed or has been removed and exploiting the fact that c-Met can utilise co-receptors, I hope to find a molecule that may function in a similar fashion to CD44v6 as a co-receptor for c-Met.

MATERIALS AND METHODS

Materials

Chemicals: All general chemicals were, unless otherwise stated, supplied by Carl Roth GmbH & Co (Karlsruhe, Germany), Merck (Darmstadt, Germany), and Sigma Chemie GmbH (Deisenhofen, Germany) and were of the highest purity grade.

Oligonucleotides

RNA, small interfering RNA, (siRNA)

Human ICAM-1	20-25nt siRNA	(Santa Cruz)
Human Ezrin	Pool of 3 target-specific 20-25nt siRNAs	(Santa Cruz)
Mouse ICAM-1	20-25nt siRNA	(Santa Cruz)
Control	20-25nt non-targeting siRNA	(Santa Cruz)

Plasmids

pCI-neo ICAM-1	Gift from Dr. Oli Carpen (Sweden) (Heiska et al. 1998)
pCI-neo vector control	Promega
GST-CD44 cytoplasmic tail	Gift from Dr. C. Isacke (Legg and Isacke 1998)
GST-CD44 cytoplasmic tail mutated in ERM binding region	Gift from Dr. C. Isacke (Legg and Isacke 1998)
GST vector control	Gift from Dr. C. Isacke (Legg and Isacke 1998)
pGKCD44v6	Gift from Dr J Sleeman
pGK vector control	Gift from Dr. J Sleeman

Antibodies

Antibodies	Isotype	Specificity	Source
β4 Integrin (H-101)	Rabbit IgG	Human, mouse & rat	Santa Cruz
CD44v6 clone 1.1ASML	Mouse IgG1	Detection of CD44 exonv6 encoding epitope from rat	Purified from ascites
CD44/Pgp-1	Rat IgG1κ	Detection of all CD44 isoforms from mouse	SouthernBiotech
ERK 1/2 (K-23)	Rabbit IgG1	Human, rat, and mouse	Santa Cruz
Ezrin (3C12)	Mouse IgG1	Human, monkey, cow, rat and mouse	NeoMarkers
Hermes 3	Mouse IgG1	Detection of all CD44 isoforms from human	Gift from Sirpa Jalkanen, Turku, Finnland
ICAM-1 (CD54)	Mouse IgG1	Human	R&D systems
ICAM-1 (G-5)	Mouse IgG2a	Human	Santa Cruz
ICAM-1 (M-19)	Goat IgG	Mouse and rat	Santa Cruz
h-Met (C-12)	Rabbit IgG1	Human	Santa Cruz
m-Met	Mouse IgG2a	Mouse and rat	Santa Cruz
Moesin (E-10)	Mouse IgM	Human, rat and mouse	Santa Cruz
Phospho-p44/42 MAP Kinase	Rabbit IgG1	Human, rat and mouse	New England Biolabs
Phospho-tyrosine clone 4G10	Mouse IgG2bκ	Detection of tyrosine phosphorylated proteins	Upstate
Plakophilin 2a	Mouse IgG1	Human	Transduction Laboratories
Plexin-B1 (H-300)	Rabbit IgG	Mouse, rat & human	Santa Cruz

Radixin (C-15)	Goat IgG	Detection of Radixin (to a lesser extent Ezrin and Moesin) from rat, mouse and human	Santa Cruz
Syndecan-1 (C20)	Goat IgG	Mouse, rat and human	Santa Cruz
Syndecan-2 (M-140)	Rabbit IgG	mouse, rat and human	Santa Cruz
Syndecan-4 (H-140)	Rabbit IgG	human, rat and mouse	Santa Cruz

Secondary antibodies: HRP-Mouse IgG, HRP-rat IgG, HRP-rabbit IgG and HRP-Goat IgG were purchased from Dako company, Hamburg.
Isotype control antibodies: Rat IgG2a, Rat IgG2b, Mouse IgG2b were purchased from Southern Biotechnology Associates Inc., U.S.A. and Mouse IgG1 from R&D systems.

Peptides

Mouse CD44 exon v6 A: ETWFQ NGWQG
The peptide sequence corresponds to a part of mouse CD44 exon v6 sequence
Control peptide HNREQANLNSRTEETI

Enzymes and growth factors

Heparinase II (Sigma)
pro-HGF (R&D systems)

Cell lines and general cell culture media

Cell culture reagents

Trypsin, 0.25% Difco, Detroit
DMEM GibcoBRL Life Technologies, Karlsruhe

RPMI 1640	GibcoBRL Life Technologies, Karlsruhe
Foetal calf serum (FCS)	Bio-Whittaker, Belgium
Penicillin-Streptomycin	Invitrogen
L-Glutamine	GibcoBRL Life Technologies, Karlsruhe

Cell lines

Name	Description	Culture medium
HT29	Human colon adenocarcinoma cell line	DMEM, 10% FCS
HepG2	Human Hepatocellular carcinoma cell line	DMEM, 10% FCS
CD44WT or KO keratinocytes	Primary mouse keratinocytes isolated from the skin epidermis	4x Low Ca^{2+} MEM (see in keratinocyte cell culture below)
CD44WT or KO hepatocytes	Primary mouse hepatocytes isolated from the liver	DMEM, 10% FCS
BSp73AS10 (AS10)	Rat pancreatic carcinoma cell line	RPMI 1640, 10% FCS
AS14	AS10 cells transfected with CD44v4-v7, without 5G8 epitope (exon 15)	RPMI 1640, 10% FCS, 0.3g/l Geneticin (G418)

Methods

General Methods: A number of protocols and recipes for common buffers used in this project were taken from the laboratory manual of (Maniatis et al. 1989) unless otherwise stated. Aqueous solutions were prepared with water purified by the Milli-Q plus water purification system (Millipore, Molesheim, Germany).

General Cell culture

Unless otherwise stated, cells were maintained in a humidified (95%) atmosphere with 5% CO_2 at 37°C. The cell culture medium for each type of cell line has previously been described (see Materials, paragraph cell line).

Cells were frozen in freezing medium (10% DMSO in FCS). The cell suspension was transferred into pre-chilled cryo-vial, left on ice for 1 hour and then frozen at -80°C. For long-term storage, the frozen cells were transferred into liquid nitrogen.

Frozen cell were thawed fast in a 37°C water-bath, and transferred into a 15ml falcon tube containing pre-warmed fresh medium. The cells were spun and resuspended in fresh medium and plated in a flask or a Petri dish.

Murine Epidermal Keratinocytes (MEKs)

Medium

Media and media-salts were purchased from Biochrom AG (Berlin, Germany), fetal calf serum (FCS) from PAA (Cölbe, Germany) and penicillin-streptomycin and phosphate buffered saline without Ca^{2+} or Mg^{2+}(PBS) Gibco (Germany)

4xMEM "high Ca^{2+}"

(Minimal essential medium with 4 times the concentration of amino acids and vitamins)

Hanks solution (10x)	40ml
MEM Vitamin (100x)	16ml
MEM amino acids (50x)	32ml
MEM non-essential (100x)	16ml
L-Glutamine (200 mM)	5ml
Biocarbonate (7.5%)	16ml
Penicillin/Streptomycin (100x)	5ml
Sterile distilled water	to 500ml

The pH of the solution was adjusted with 1M NaOH to pH 7.2. After sterile filtration (Millipore Steritop, 0.22μm) the medium was supplemented with 10% FCS.

4xMEM "low Ca^{2+}"

Hanks solution (1x without Ca^{2+},Mg^{2+} with phenol red)	400ml
MEM Vitamin (100x)	16ml
MEM amino acids (50x)	32ml
MEM non-essential (100x)	16ml
L-Glutamine (200 mM)	5ml
Biocarbonate (7.5%)	16ml
Penicillin/Streptomycin (100x)	5ml
$MgCl_2$ (20 g/l)	5ml
Phenol red	1ml

The pH of the solution was adjusted with 1M NaOH to pH 7.2. After sterile filtration the medium was supplemented with 10% chelexed FCS (10ml normal FCS and 40ml chelexed FCS) and the calcium concentration adjusted to between 0.06-0.08mM using sterile $CaCl_2$.
Chelexed FCS was prepared by addition of 15g chelex-100 resin (Sigma) per 500ml FCS and stirred for 1.5 hours at 4°C followed by sterile filtration.
To starve cells, low Ca^{2+} MEM supplemented with 0.5% normal FCS and $CaCl_2$ (0.06mM) was used.

Coating culture dishes with collagen type IV

Collagen type IV (Santa Cruz Biotechnology) stock solution was thawed for several hours on ice then diluted to the appropriate concentration using cold

sterile 0.05M HCl. Cellstar™ (Greiner Bio One, Germany) tissue culture dishes were coated with between 1-10μg/ml of collagen IV for 1-2 hours at room temperature. Dishes were rinsed twice with PBS containing 1% penicillin-streptomycin, replaced in original packaging, sealed and stored at 4°C until required.

Murine Epidermal Keratinocyte culture

1-2 day old mice pups (B6,CD44-/- (Schmits et al. 1997)) or (C57Bl/6J wild type) were sacrificed by decapitation, washed with 70% ethanol, the limbs and tail amputated and the trunk skin removed. The skins were rinsed three times in PBS containing 1% penicillin-streptomycin and flattened with the dermal side down on tissue culture dishes. The skins were then floated on a 2.4U/ml solution of Dispase (Gibco) in PBS (ensuring the liquid did not flow onto the top epidermal side) overnight at 4°C.

The next morning the dispase solution was aspirated; the epidermal layer gently peeled away from the dermis, rinsed in PBS and floated outspread in dishes containing high Ca^{2+} MEM (approximately 1.5ml per skin layer). The pooled epidermal layers were then cut into smaller pieces and transferred with the medium into a sterile glass beaker. The epidermal pieces were stirred at low to intermediate speed for 30 min at room temperature.

The mixture containing disaggregated cells and tissue clumps was poured through a sterile cell strainer 70μm (BD Biosciences) (10 skins per cell strainer) into a 50ml falcon tube (Greiner Bio One, Germany). The epidermal cells were centrifuged at 1000 rpm for 10 min, seeded at 50% cell density in high Ca^{2+} MEM on collagen IV coated plates and placed in a 35°C, 5% CO_2 incubator. After 6 hours the medium was aspirated, cells gently washed once with PBS and low Ca^{2+} MEM gently added. The medium was changed after 24 hours and again 2-3 days later. Keratinocyte cell growth was monitored between culture days 2-5. Experiments were normally performed in this time.

Liver cell culture

Mice pups were sacrificed as described above in keratinocyte culture. Livers were dissected and rinsed in PBS (1x) and sequentially incubated for 5 min in solution A (EBSS without Ca^{2+}/Mg^{2+}, 0.5mM EGTA), 5 min in solution B (EBSS with Ca^{2+}/Mg^{2+}, 10mM HEPES pH 7.4) and 5 min in solution C (EBSS with Ca^{2+}/Mg^{2+}, 10mM HEPES pH 7.4, 0.3mg/ml Collagenase type I). Livers were washed with PBS (1x) and the cells dispersed by pipetting up and down several times with a glass pipette. The cells were then spun down by centrifugation seeded in DMEM containing 10% FCS in a plastic dish. The medium was changed with washing every day to remove haematopoietic cells. Once a pure hepatocyte culture was obtained, medium was changed every 2 days.

Amaxa Transfections

Cells were cultivated to 70-80% confluency before transfection according to the manufacturers instructions. On the day of transfection cells were trypsinised, centrifuged and all traces of medium removed. Cells were resuspended in the appropriate room temperature Nucloefector™ solution, $1x10^6$ cell/100μl for a 6-well plate and $3x10^6$ cells/100μl for a 10cm plate. 100μl of the cell suspension was then mixed separately with each oligonucleotide to be transfected in sterile eppendorf tubes and transferred to Amaxa cuvettes. Electroporation was carried out with the corresponding program for each cell type. Immediately after transfection, pre-warmed medium was added to cells (500μl) in the cuvettes then the cells were transferred to plates containing pre-warmed medium.

HA pre-treatment

High molecular weight HA (Helon Mw: $4\text{-}5x10^3$kDa, Pharmacia&Upjohn) (400μg/ml) was added to cells and incubated for 5 min at 37°C, then aspirated prior to HGF addition.

Peptide treatment

Peptides (100ng/ml) were added to cells and incubated for 5 min at 37°C, then aspirated prior to HGF addition.

Heparinase II treatment

Heparinase II (6U/ml) was added to cells and incubated for 3 hrs, then aspirated prior to HGF addition

Blocking antibody treatment

Human anti-ICAM-1 antibody or IgG_1 control (20μg/ml) were added to cells and incubated for 1 hr at 37°C, then aspirated prior to HGF addition

HGF induction, chemical cross-linking

$3x10^6$ cells were seeded in 10cm plates, serum starved for 24 hours, and finally induced with HGF (50ng/ml) at 37°C for 5 min. After washing the cells 2 times with PBS 1x, protein cross-linking in living cells was performed in 3ml PBS 1x containing 3mg DTSSP at room temperature for 30 min. The reaction was quenched using 40μl/ml of 20mM Tris-HCl pH 7.4.

The cells were then washed with ice-cold PBS 1x, lysed with either co-immunoprecipitation lysis buffer (25mM HEPES pH 7.4, 100mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1.25% CHAPS, containing protease inhibitors (10μg/ml Aprotinin and Leupeptin, 1mM PMSF, 10mM NaF and 2mM Na_3VO_4) or immunoprecipitation lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100).

Cell lysate preparation

In order to analyse protein expression by western blot, cells were washed with PBS 1x and lysed in 2x laemmli sample buffer (160mM Tris-HCl pH 6.8, 4% SDS, 16% glycerol, 0.1M DTT, 0.01% bromophenol blue). DNA was sheared through a 26-guage needle. The cell lysate was incubated 5 min at 95°C for

protein denaturation, and centrifuge for 1 min at 10 000 rpm before loading on SDS-PAGE gel.

Immunoprecipitation

Cells grown in 10cm plates were washed twice with ice-cold PBS 1x and lysed in 1ml of lysis buffer containing protease inhibitors as listed in the HGF induction section (above). After an incubation of 30 min on ice, lysed cells were scraped and pooled in a 1.5 ml eppendorf. The lysate was cleared by a centrifugation of 15 min at 13000g, at 4°C. The supernatant was transferred to a fresh tube. 5μg of antibody per ml of cell lysate was added and the mixture was rotated overnight at 4°C. 40μl of 50% beads slurry (previously equilibrated in lysis buffer) was added to the sample, and was rotated for 1 to 3 hours at 4°C. Immuno-complexes were recovered by centrifugation and washed 3 times for 1 min, with cold lysis buffer. Supernatant was removed, beads were then resuspended in 50μl of laemmli sample buffer and boiled at 95°C for 5 min. After spinning down the beads by centrifugation, the supernatant was loaded on a SDS-PAGE gel, or stored at -20°C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were electrophoretically separated on the basis of size using the method of Laemmli (1970). The resolving gel (8.5-10% acrylamide) and the stacking gel (5% acrylamide) were cast according to (Maniatis et al. 1989). Samples were run into the stacking gel at 100 V, and then run at 150 V during the day or 30 V overnight.

The running buffer contained 30.3 g/l Tris-base, 144g/l Glycine, and 1% SDS.

Western Blotting

After proteins were separated by SDS-PAGE, they were electrically transferred onto Immobilon membrane (Millipore, type PVDF, pre-soaked in methanol) at 35 V for at least 6 hrs in Transfer Buffer (25 mM Tris-base, 190 mM Glycine, 20% Methanol). Following the completion of the transfer, in order to reduce unspecific

binding of the antibodies to the PVDF membrane, the blot was incubated in TBS-0.2% Tween containing 5% bovine serum albumin (BSA) blocking solution, for 1 hour at room temperature with shaking. For detection of proteins of interest, the membrane was further incubated in TBS-0.2% Tween blocking solution containing the appropriate primary antibody (at the manufacturer-recommended dilution) for 1 hr at room temperature with shaking. After 3 washes (5 min each at room temperature with shaking), the membrane was incubated in blocking solution containing a 1:1500 dilution of HPR-conjugated secondary antibody. The membrane was then washed 5 times 5 min in TBS-Tween. The detection of specific proteins was achieved by enhanced chemiluminescence using ECL Western blotting detection reagents (Amersham) ECL hyperfilms (Amersham) according to the manufacturer's instructions.

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

Silver staining

After proteins were separated by SDS-PAGE, the gel slab was fixed in 50% methanol, 5% acetic acid for 1 hour. It was then washed first with 50% methanol for 10 min and second with water for another 10 min to remove remaining acid. The gel was sensitised by 1 min incubation in 0.02% sodium thiosulfate, and rinsed with 2 changes of distilled water for 1 min each. The gel was then submerged in ice-cold 0.1% silver nitrate solution and incubated for 20 min at 4°C. The gel slab was afterwards rinsed twice with water for 1 min and developed in 0.04% carbonate with intensive shaking. Once the developer solution turned yellow, it was replaced by fresh a one. It is essential that the development be carried out in an absolutely transparent solution. The development was achieved when the desired intensity of staining was obtained. The developer solution was then discarded and the gel was washed with 5% acetic acid.

Silver stained gels were stored in 1% acetic acid at 4°C.

RESULTS

Hepatocyte growth factor, HGF can activate c-Met and induce downstream signalling in a cell line that does not express CD44 molecules

It has been extensively shown in a number of transformed and non-transformed cell lines that CD44v6-containing isoforms are required for the HGF/SF induced activation of the c-Met receptor and signalling via the Ras/MAPK signalling pathway (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007). However, in several cases where c-Met can be activated by its ligand, CD44 isoforms are not expressed e.g. in hepatocytes and hepatanomas (Seelentag et al. 1995; Washington et al. 1997). To investigate the molecular interactions and activity of c-Met in the absence of CD44v6 I looked at the human hepatocellular carcinoma cell line HepG2. As expected, the HepG2 cells express the c-Met protein but no CD44 protein (Fig 3A). I used the HT-29 colon carcinoma cells, which have been shown to express both the CD44 isoforms including CD44v6 (Reeder et al. 1998) and c-Met (Orian-Rousseau et al. 2002) as a reference for their expression and c-Met activity (Fig 3).

Although the two cell lines do not derive from the same tissue, I needed a control system where I knew that c-Met utilised CD44 as a co-receptor. This was previously shown using antibodies against the v6-specific isoform of CD44, which in the HT-29 cells could block HGF induced activation of the c-Met receptor along with phosphorylation of ERK1/2 a downstream target of c-Met (Orian-Rousseau et al. 2002) and Fig. 3B.

Next I checked the activation status of c-Met upon stimulation with its ligand HGF in the HepG2 cells. Exponentially growing cells were serum starved for 48 hrs, stimulated with HGF and cell supernatants treated with an α-c-Met antibody (Ab). Immunoprecipitates and untreated lysates were examined for c-Met and ERK phosphorylation on western blots respectively. Fig 3B demonstrates that upon

ligand stimulation the c-Met receptor is phosphorylated in the HepG2 cells (upper left panel, right lane) and this leads to a subsequent phosphorylation of ERK (lower left panel, right lane). The corresponding activation status is depicted in the HT-29 cells (right panel). I concluded that the HepG2 cells were a suitable cell system to search for a new co-receptor for c-Met.

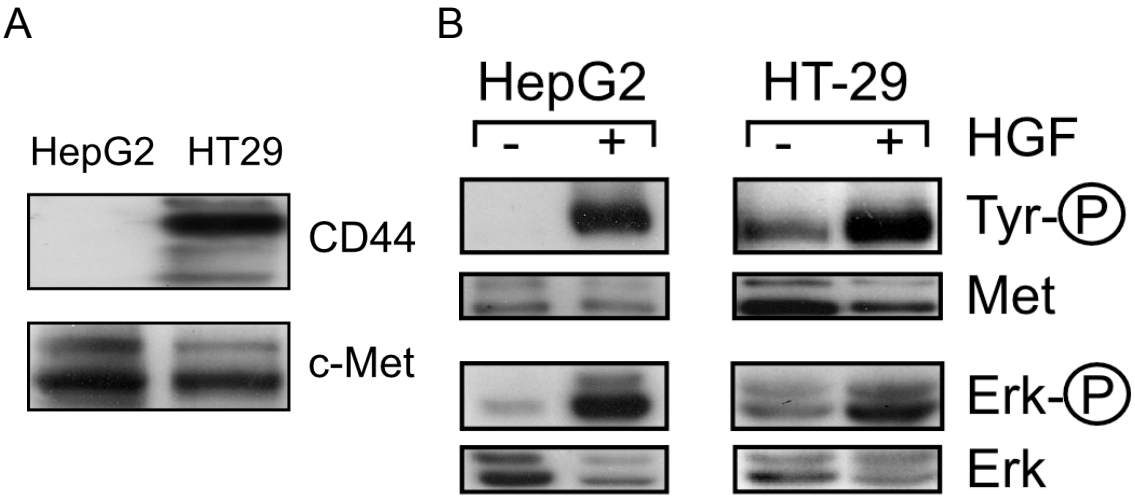

Figure 3. *c-Met is activated by it's ligand HGF and can signal downstream to ERK in the CD44 negative carcinoma cell line HepG2.* ***A***, Western blot analysis of total cell lysates from HepG2 and HT-29 cells using an α-pan-CD44 Ab to detect CD44 expression (upper panel) or a human α-c-Met Ab for c-Met (lower panel). ***B***, c-Met and ERK phosphorylation in HepG2 and HT-29 cells. Logarithmically growing HepG2 and HT-29 cells were serum starved for 48 hrs then either left untreated or induced for 5 min with HGF (50ng/ml). After cell lysis, immunoprecipitation was performed on cell supernatants with an α-c-Met Ab as described in "Materials and Methods". Immunoprecipitates were analysed on western blots, following SDS-PAGE separation (8.5%), using α-phospho-tyrosine Ab (α-pTyr) (first row) and α-c-Met Ab (second row). Untreated cell lysates were probed for ERK phosphorylation using a α-p42/44MAPK Ab (third row) and α-ERK (fourth row). c-Met and ERK are phosphorylated upon HGF stimulation in both cell lines.

Comparative Co-IP screen for c-Met interacting partners

Previous studies in the HT-29 cells demonstrated that only upon stimulation with HGF could CD44v6 interact with c-Met, forming a multimeric complex between the three proteins (Orian-Rousseau et al. 2002). Might a similar interaction occur between c-Met and a cell surface molecule in the HepG2 cells? I attempted an unbiased approach to find such molecules whereby I compared c-Met immunoprecipitates in the HepG2 cells versus the HT-29 cells and observed

potential differences in the protein expression or interaction pattern. Cells were induced with HGF, treated with the chemical cross-linker dithiobis-sulfosuccinimidylpropionate (DTSSP) to stabilise cell surface complexes, lysed and immunoprecipitated. c-Met precipitates were resolved on SDS-PAGE gels and proteins associated with c-Met were visualised by silver staining. Western blot analysis confirmed that c-Met was efficiently immunoprecipitated from cell lysates (Fig 4, inset). I detected a protein band of approximately 70kDa in the c-Met precipitate of the HepG2 cells that was not observable in the precipitate of the HT-29 cells (Fig 4). Protein sequencing of the band by Maldi-Tof revealed high homology to a cell junctional protein Plakophilin 2.

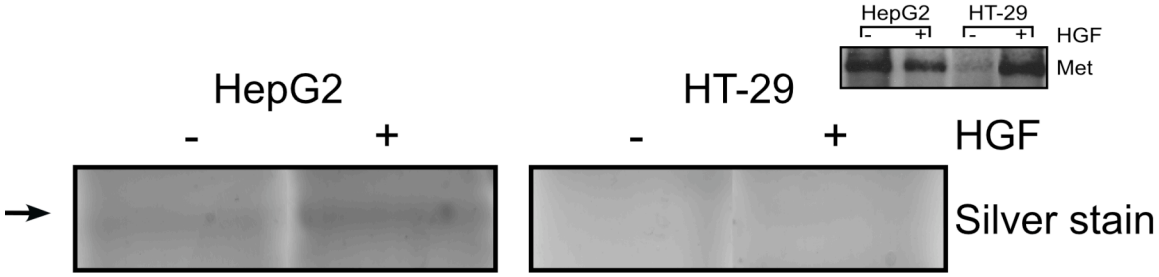

Figure 4. *A 70kDa protein is associated with c-Met upon HGF stimulation in the HepG2 cells but not the HT-29 cells.* Logarithmically growing cells were serum starved for 24-48 hrs. After HGF induction cells were crossed-linked with DTSSP for 30 min, lysed and immunoprecipitated with α-c-Met Ab. Following SDS-PAGE separation, immunoprecipitates were analysed on the silver stained SDS-PAGE gels (see Materials and Methods). Bands of interest were excised from the gel and sent analysed by Maldi-Tof protein sequencing. The arrow indicates the 70kDa protein band in the HGF stimulated HepG2 cells. Aliquots of the c-Met immunoprecipitates were loaded on a second gel and probed for c-Met pull down efficiency by western blot (inset).

Plakophilin 2 is a member of the armadillo protein family, which are characterised by a series of motifs known as the arm-repeats, and whose members include protein p120, plakoglobin, β-catenin and its sub-family members plakophilin 1 and 3 (Mertens et al. 1996; Bonne et al. 1998; Chen et al. 2002). The plakophilins are primarily localised to the juxtamembrane plaque of desmosomal junctions of simple and complex epithelia and the nucleus (Chen et al. 2002; Grossmann et al. 2004; Hatzfeld 2007).

An interaction between c-Met and plakophilin 2, had not previously been described and although plakophilin 2 is not a transmembrane protein, its dual-cellular localisation has led some groups to speculate that it may be involved in

the co-ordination of intercellular junctions and growth control (Chen et al. 2002; Hatzfeld 2007) My observation that it is differentially expressed between the two cell lines raised some intriguing possibilities towards a potential use in signalling from c-Met. To confirm the results I observed in the silver stain, I specifically looked for plakophilin 2 in co-immunoprecipitates of c-Met in both cell lines. Western blot analysis indicated that plakophilin 2 is expressed in both cell lines. Unexpectedly and contrary to the silver stain, plakophilin 2 was found to associate with c-Met in the HT-29 cells as well as the HepG2 cells (Fig 5).

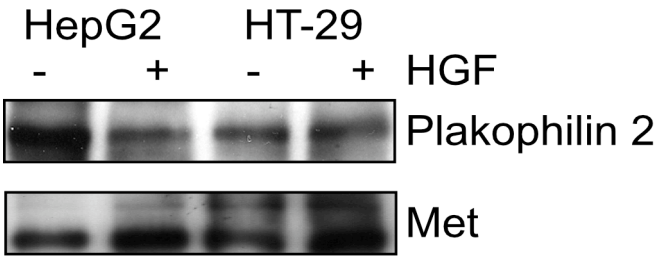

Figure 5. *Plakophilin 2 associates with c-Met in HepG2 and HT-29 cells.* Non-stimulated and HGF induced cells were treated with the chemical cross-linker DTSSP as previous. Following lysis, immunoprecipitation with c-Met Ab was performed. Cell samples were probed for the presence of c-Met and plakophilin 2 in the precipitates on western blots.

Plakophilin does not as first observed differentially interact with c-Met in the cell lines. It's interaction with c-Met seems to be independent of activation of the receptor (Fig 5). Ultimately it seems more plausible that a protein interaction between plakophilin 2 and c-Met may be involved in a crosstalk between receptors and junctional proteins and have nothing to do with the co-receptor function I was looking for.

Since c-Met in the absence of CD44v6 is activated and can signal in the Hep G2 cells, I asked if potential membrane proteins could function in a similar fashion to CD44v6 by examining some functions that may, in the HT-29 cells, be mediated by CD44 ((Orian-Rousseau et al. 2002) and Orian-Rousseau unpl.)

c-Met signalling is negatively affected by HA in HepG2 cells

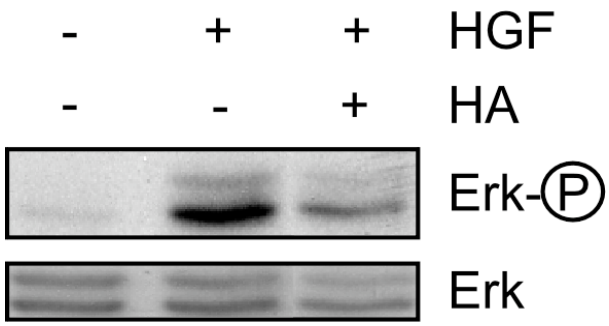

Figure 6. *Pre-treatment of HepG2 cells with hyaluronic acid (HA) inhibits c-Met signalling.* Serum-starved HepG2 cells were pre-treated with 400µg/ml of HA for 5 min, washed, and then induced for 5 min with HGF (50ng/ml). Cell lysates were resolved by SDS-PAGE (8.5%) and ERK phosphorylation was determined on a western blot using α-p44/42 MAPK Ab. ERK phosphorylation is reduced after cells are pre-treated with HA (lane 2 compared to lane 3).

CD44 is the principle receptor for the ECM protein, HA (Entwistle et al. 1996). HA-mediated processes like cell migration and cell adhesion require interactions with CD44 i.e. during inflammation infiltrating leukocytes adhere to the ECM. HA can also modulate growth through its interaction with CD44. It has been shown in several cell lines that pre-treatment of logarithmically growing cells with HA negatively influences their growth by induction of a molecular switch in proteins complexed at the cytoplasmic tail of CD44 (Morrison et al. 2001). In this “growth inhibitory” mode CD44 recruits the ERM-related protein merlin to its cytoplasmic tail, which results in its dephosphorylation and induction of its growth suppressive function (Morrison et al. 2001). In addition HA can affect GF dependent activation of ERK in the HT-29 cells (V. Orian-Rousseau in preparation). I investigated the influence of HA treatment on HGF dependent signalling in the HepG2 cells. Cells were pre-treated with HA and then stimulated with HGF to activate the c-Met

receptor. Compared to HA untreated cells, pre-treatment of cells with HA lead to a reduction in HGF dependent ERK phosphorylation (Fig 6, lane 3). This suggests that a HA binding protein on the HepG2 cells is involved in mediating c-Met signalling.

An ERM binding molecule and ERM proteins are involved in c-Met signalling in HepG2 cells

The ERM family of proteins are known to associate to the plasma membrane via membrane partners like CD44 and are important for modulating membrane traffic and in maintaining cell shape (Louvet-Vallee 2000). The ERM proteins also play a major role in c-Met signalling (Orian-Rousseau et al. 2007). In several cell lines, where CD44v6-containing isoforms were able to propagate HGF dependent signalling to ERK, transfection of the CD44v6-containing isoform minus the cytoplasmic tail abolished this signalling (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007). In addition, a CD44v6-constituitively active ezrin fusion protein was able to convey HGF induced downstream signalling to ERK in CD44v6 negative rat carcinoma cells Bsp73AS (Orian-Rousseau et al. 2007). Conversely, a GST-fused CD44 cytoplasmic tail but not one mutated in the ERM binding region could successfully abrogate HGF-dependent signalling to ERK in the human kidney carcinoma cell line 293 (Orian-Rousseau et al. 2002). These data suggest that c-Met downstream signalling to ERK requires the cytoplasmic tail of CD44v6 and ERM proteins in various cells (Orian-Rousseau et al. 2002; Orian-Rousseau et al. 2007).

In cells where CD44 is absent, another ERM binding molecule may take its place. To test this notion, I asked if in the HepG2 cells the soluble cytoplasmic tail of CD44 sequestering of ERM molecules could interfere with HGF dependent activation of ERK. HepG2 cells were transfected by electroporation with either the soluble tail of CD44 or the tail with a mutation in the ERM binding domain. Compared to the control vector (Fig 7, lane 2), HGF induced ERK phosphorylation in cells transfected with the CD44 tail was reduced (lane 4). However in cells transfected with an ERM binding mutant tail, ERK

phosphorylation was not affected (lane 6). This result implicates ERM-binding protein involvement in downstream transmission of c-Met signals in the HepG2 cells. Accordingly, the ERM proteins seem to be connected in these signalling events from the c-Met receptor.

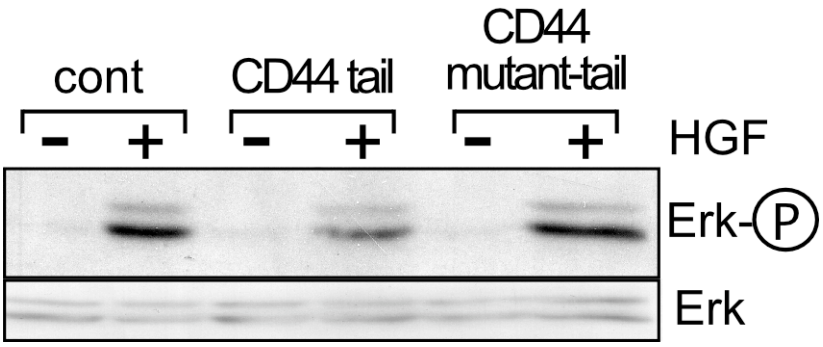

Figure 7. *A cytoplasmic portion of CD44 sequesters ERM proteins and affects c-Met signalling in HepG2 cells.* HepG2 cells were transiently transfected with a GST-CD44 cytoplasmic tail fusion construct, one mutated in the ERM binding region, or an empty vector (cont) by Amaxa electroporation. 24 hrs later cells were serum-starved for 24 hrs and then induced for 5 min with HGF (50ng/ml) at 37°C, subsequently ERK phosphorylation was determined by western blot analysis.

To further investigate the relevance of the ERM proteins in c-Met signalling I performed another independent experiment. I first determined which of the ERM proteins are expressed in the HepG2 cells. Western blot analysis of total cell lysates showed that ezrin was the most abundantly expressed of the ERM proteins in the HepG2 cells (Fig 8A). Recently, ezrin has been shown to be important for c-Met signal transduction in 293 cell and HGF induced scattering in the HT-29 cells (Orian-Rousseau et al. 2007). I looked at the effect of down-regulating ezrin protein expression on ERK phosphorylation in the HepG2 cells. siRNA oligonucleotides against ezrin were transfected by electroporation into cells and the c-Met signalling as determined by ERK-phosphorylation observed. Compared to a scrambled oligonucleotide, the siRNA against ezrin successfully reduced ezrin protein expression. In addition this reduction negatively affected HGF dependent ERK-phosphorylation. Taken together these data indicate that ERM proteins and specifically ezrin, through binding to plasma membrane molecules are necessary for c-Met downstream signalling.

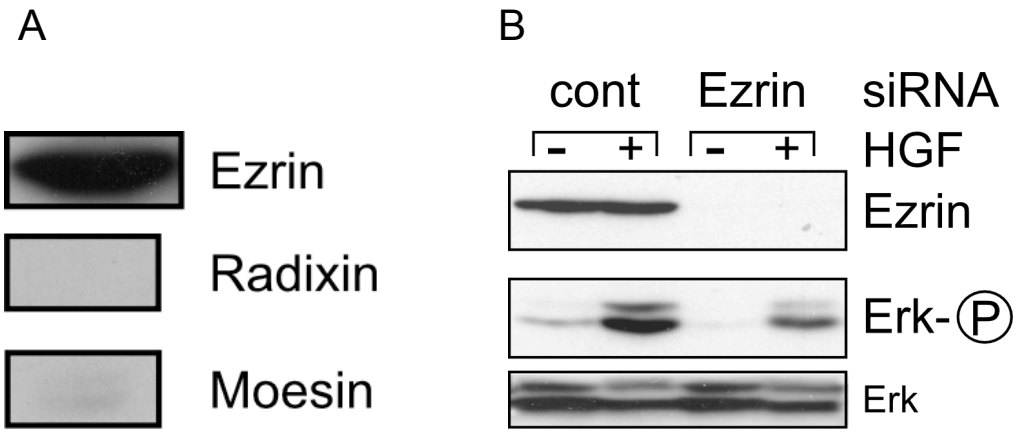

Figure 8. *c-Met signalling is inhibited by down-regulation of the ERM protein ezrin.* ***A*** Expression analysis of ERM proteins. Equal amounts of HepG2 total cell lysate was probed for ERM protein expression on western blots. Ezrin and not radixin or moesin is predominantly expressed. ***B*** Down-regulation of ezrin by siRNA. HepG2 cells were transfected by Amaxa electroporation with siRNA against ezrin (300pmol) or a scrambled oligonucleotide (cont). The following day cells were serum-starved for 24 hrs then induced with HGF as previous. Cell lysates were assayed for total ezrin expression and phosphorylation of ERK. Ezrin reduction leads to a decrease in ERK phosphorylation.

Since HA pre-treatment negatively affects c-Met signalling and ERM binding proteins and ezrin appear to be involved in c-Met signalling in the HepG2 cells. I wondered whether a membrane protein with such functions might act as a co-receptor, substituting for CD44v6.

Protein screen of cell lysates reveal a cellular adhesion molecule is distinctively expressed in the HepG2 cells compared to the HT-29 cells

I screened whole cell lysates of the HepG2 and HT-29 cells to evaluate the expression patterns of membrane proteins. It has previously been demonstrated that Plexin B1 and $\alpha 6\beta 4$ can independently act as co-receptors for c-Met (Trusolino et al. 2001; Giordano et al. 2002). c-Met was found to associate and could be activated by Semaphorin 4D, the ligand of Plexin B1 in liver progenitor cells (Giordano et al. 2002). In contrast, $\alpha 6\beta 4$ association with c-Met enhances the receptor's signalling potential via the Ras/MAPK pathway in mammary

carcinoma cells (Trusolino et al. 2001). I observed no difference in expression of Plexin B1 in the two cell lines and less expression of $\alpha 6\beta 4$ integrin (represented by $\beta 4$-integrin) in the HepG2 cells than compared to the HT-29 (Fig 9).

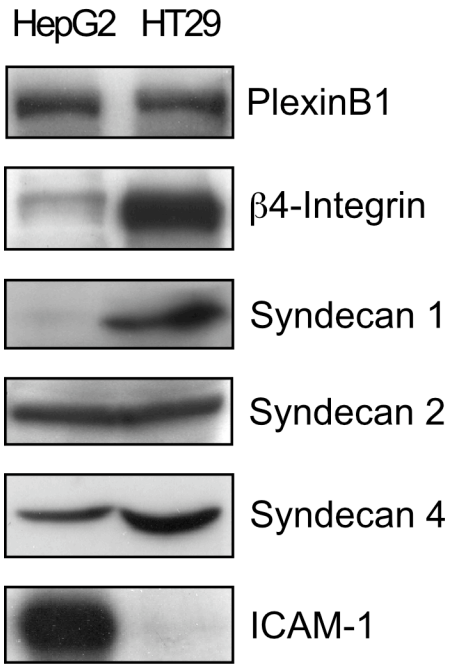

Figure 9. *Different membrane proteins expression pattern in the HepG2 and HT-29 cells.* Logarithmically growing cells were lysed and equal amounts of total cell lysates from the HepG2 and HT-29 cells were separated on SDS-PAGE. Lysates were screened for expression of the different membrane proteins by western blot analysis.

Since HGF is a heparin-binding GF and CD44HSPG isoforms have been implicated in presenting GF to their receptors including HGF and c-Met (van der Voort et al. 1999) I decided to look at other families of HSPGs. The Syndecans are a family of four proteins, Syndecan 1-4 whose similarities to CD44 also include binding to ERM proteins (Granes et al. 2000). Syndecan-1 was not expressed in the HepG2 cells whereas syndecan 2 and 4 were expressed in both cell lines albeit slightly less in the HepG2 cells in the case of the latter. Another important class of surface molecules that I tested were the intercellular adhesion molecules, ICAMs. The ICAMs are also a family of proteins consisting of ICAM 1-5, involved in leukocyte migration (Yang et al. 2004). Of the detectable proteins, only ICAM-1 showed a higher expression in the HepG2 cells versus the HT-29 (Fig 9, bottom row).

ICAM-1 is a plausible candidate because functionally it is similar to CD44. Both proteins are cellular adhesion molecules also expressed on leukocytes and it is therefore not surprising that they are involved in inflammatory cell trafficking (Jalkanen et al. 1990; Hallahan and Virudachalam 1997). Interestingly ICAM-1 is also a HA binding molecule (McCourt et al. 1994; Entwistle et al. 1996) and has been reported to associate with ERM proteins (Heiska et al. 1998), placing it in a feasible position as an alternative to CD44v6.

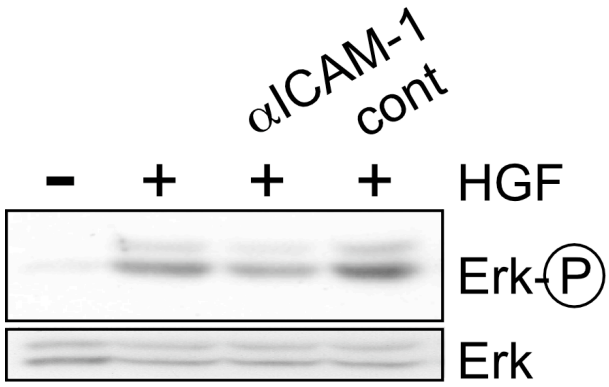

Figure 10. *An ICAM-1 Ab reduces HGF dependent ERK phosphorylation in HepG2 cells*. Logarithmically growing cells were serum starved for 48 hrs. Cells were then pre-treated with an α-ICAM-1 blocking antibody (20μg/ml) or an isotype-matched control (cont) for 1 hr, washed and induced with HGF. c-Met activity was determined by ERK phosphorylation on western blots.

α-ICAM-1 blocking Abs inhibits downstream signalling in a HGF dependent manner

To explore a relationship between ICAM-1 and c-Met in the HepG2 cells, I interfered with ICAM-1 on the cell surface by using an ICAM-1 blocking Ab and looked at its affect on c-Met activity. Serum starved cells were pre-treated with antibody then stimulated with HGF, downstream signalling to ERK was observed. Inducible ERK phosphorylation was reduced in cells pre-treated with ICAM-1 Ab but not with a control Ab (Fig 10). This indicated that ICAM-1 might have a role in c-Met activation or signalling in the HepG2 cells.

Abrogation of ICAM-1 expression leads to inhibition of c-Met activation and downstream signalling

In the HepG2 cells ICAM-1 is highly expressed. Would abolishing its expression have an affect on c-Met activation? To test this, I transfected cells with siRNA against ICAM-1 and evaluated the expression level of the protein on western blots. Compared to cells transfected with the control oligonucleotides, the ICAM-1 siRNA completely and successfully abolished the expression of endogenous ICAM-1 independent of HGF stimulation (Fig 11 first row).

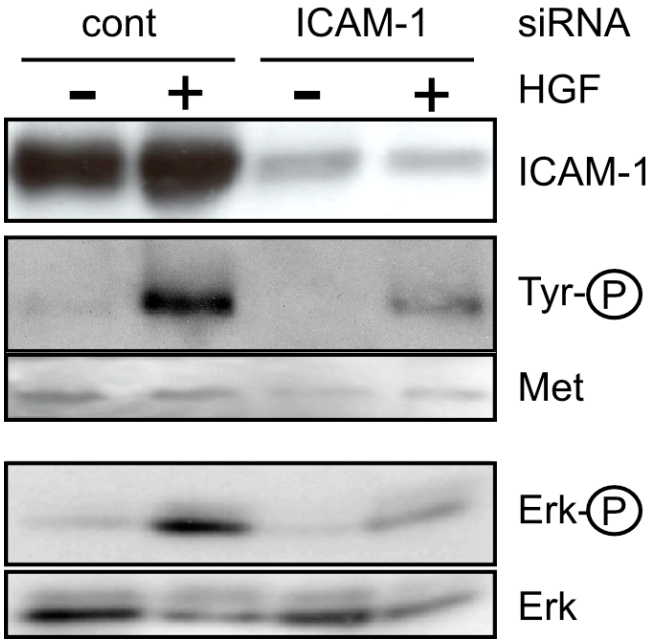

Figure 11. *ICAM-1 is important in c-Met activation and signal transduction to ERK upon stimulation with HGF.* HepG2 cells were transfected with siRNA against human ICAM-1 (400pmol) or a scrambled oligonuclotide (cont) by electroporation. Following stimulation with HGF, total cell lysates were analysed for ICAM-1 expression and ERK phosphorylation. c-Met was immunoprecipitated from cell lysates and analysed for tyrosine phosphorylation on western blots

In the same experiment I investigated the amount of c-Met activation and ERK phosphorylation under HGF stimulating conditions in the ICAM-1 silenced cells. HGF stimulation induces activation of c-Met and ERK phosphorylation in cells transfected with control oligonucleotides (Fig 11). However, HGF induced phosphorylation of c-Met and ERK are drastically reduced when ICAM-1 protein

levels are abolished (Fig 11). These results strongly implicate the requirement of ICAM-1 in the activation of the c-Met receptor and subsequent activation of ERK.

c-Met and ICAM-1 are associated in the HepG2 cells

The ability of ICAM-1 to assist c-Met is likely to depend on close interactions at the cell surface. I therefore investigated if c-Met and ICAM-1 associate by performing immunoprecipitation studies. Serum starved HepG2 cells were stimulated with HGF followed by chemical cross-linking with DTSSP to stabilise extracellular complexes. c-Met and ICAM-1 were immunoprecipitated from cell supernatants and the ability of each to co-immunoprecipitate with the other observed on western blots. c-Met and ICAM-1 were both successfully pulled out from cell supernatants (Fig 12). c-Met associates with ICAM-1 in the absence HGF and the association is slightly increased upon HGF stimulation (first row lanes 1 and 2).

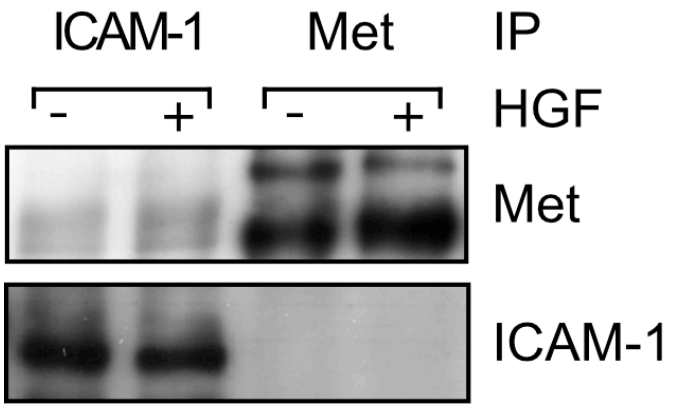

Figure 12. *ICAM-1 and c-Met associates in the HepG2 cells.* Non-stimulated and HGF stimulated cells were incubated with the chemical cross-linker DTSSP. After cell lysis, cell supernatants were divided and immunoprecipitation with a c-Met or ICAM-1 Ab was performed. Samples were probed for the presence of c-Met and ICAM-1 in both precipitates.

ICAM-1 is a co-receptor for c-Met

If ICAM-1 acts as a co-receptor for c-Met, then it's introduction into ICAM-1 negative cells may activate the receptor. The ASs cells are a rat pancreatic

carcinoma cell line stably transfected with the CD44 standard isoform. These cells express c-Met, however the receptor is not activated upon stimulation with its ligand, HGF. Transfection experiments with CD44v6 in these cells lead to activation of c-Met as well as signalling to ERK (Orian-Rousseau et al. 2002) and Fig 13. This demonstrated that CD44v6 containing isoforms were necessary and sufficient for c-Met activity in these cells. I examined if ICAM-1 could similarly enable activation of the receptor in these cells. To eliminate any potential interference from possible endogenous ICAM-1, I checked for the expression of ICAM-1 in these cells. Compared to control murine cells, the ASs cells do not express ICAM-1 (Fig 13A).

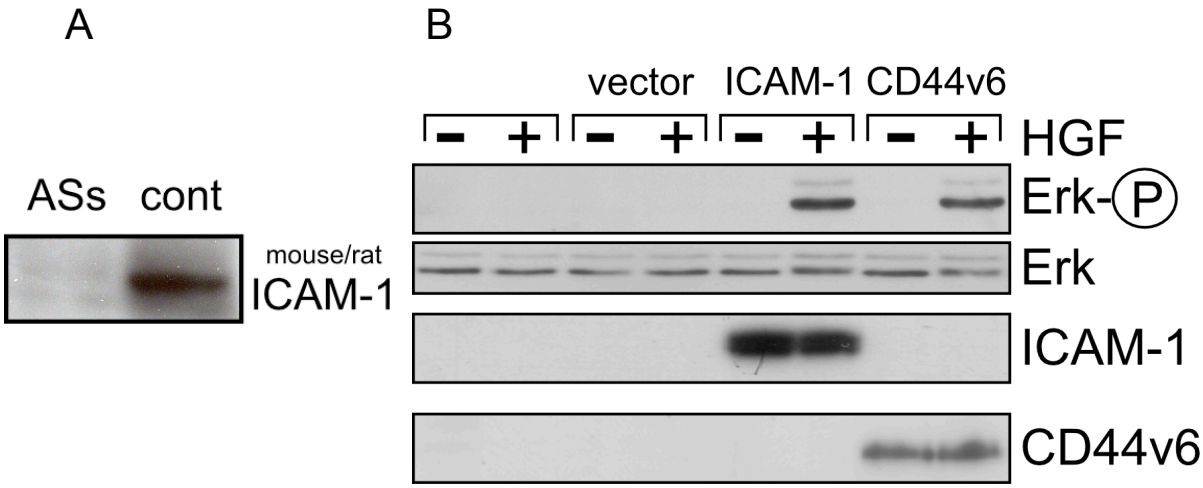

Figure 13. *ICAM-1 can activate c-Met activity and downstream signalling to ERK in ASs cells.* ***A*** The ASs cell do not express ICAM-1. Whole cell lysates from the ASs cells and primary mouse keratinocytes were analysed for expression of ICAM-1 by western blots. ***B*** Expression of ICAM-1 or CD44v6 induces HGF dependent ERK phosphorylation. ASs cells were transiently transfected with an empty vector or vectors containing ICAM-1 or CD44v6 by electroporation. 24 hrs after transfection cells were starved for 36 hrs induced with HGF (10ng/ml) and lysed. Whole cell lysates were analysed for expression of ICAM-1, CD44v6 and phosphorylation of ERK by western blot.

Next, I transiently transfected a vector expressing ICAM-1 (or CD44v6) by electroporation and observed c-Met activity as determined by ERK phosphorylation. As expected the ASs cells are not inducible upon HGF stimulation also in the presence of the vector control (Fig 13B). However, ERK was phosphorylated upon stimulation with HGF only when ICAM-1 (or CD44v6) was present (Fig 13B lanes 6 & 8). This activation was HGF dependent as the

presence of ICAM-1 (and CD44v6) alone was not able to induce ERK phosphorylation (Fig 13B lanes 5 & 7). In the ASs cells ICAM-1 expression is necessary to promote c-Met activity. This result clearly shows that ICAM-1 is able to function as a co-receptor for c-Met and further substantiates the findings for ICAM-1 as a co-receptor for c-Met in the HepG2 cells.

DISCUSSION

The cellular adhesion molecule family of CD44 and the growth factor receptor c-Met are well established as key mediators of cell growth and differentiation during development and in the aberrantly regulated process of tumour metastasis. Critically, CD44v6 containing isoforms are required for c-Met activation and downstream signalling in several transformed and normal cells including primary human keratinocytes. However in situations where CD44 is not expressed i.e. in hepatocytes or in CD44 knockout mice, does the c-Met receptor collaborate with other transmembrane partners for its activation and signal transduction? In this study I provide evidence that in the absence of CD44 the intercellular adhesion molecule-1 (ICAM-1) is required for the activation of c-Met and signal transduction upon ligand stimulation by HGF. In the same cells an ERM binding protein and ezrin are important for c-Met downstream signalling. Finally, in these cells a HA binding molecule is involved in modulating affects on c-Met activation.

The relevance of ICAM-1 expression in the HepG2 cells

ICAM-1 expression has been observed in various tumours including that of the liver (Sun et al. 1999; Rosette et al. 2005; Lin et al. 2006). The expression of ICAM-1 that I observed in the human hepatocellular carcinoma cell line HepG2 could be significant in their tumourigenesis. Interestingly and coincident with my observation is that ICAM-1 is up-regulated in human hepatocellular carcinomas as compared to normal human hepatocytes and this upregulation correlates with metastasis (Sun et al. 1999).

HGF/c-Met may also play a role in the carcinogenesis of liver cells (Luo et al. 1999; Son et al. 2006; Yang et al. 2007) and since ICAM-1 is important for c-Met in the HepG2 cells (Fig. 11) it is tempting to speculate that this interaction may play a role in potentiating hepatocellular carcinoma metastasis since both c-Met and ICAM-1 can contribute to metastasis.

How can ICAM-1 modulate activation of the c-Met receptor?

In the HepG2 cells c-Met and ICAM-1 can associate independently of HGF although this is slightly enhanced upon HGF addition (Fig. 12). A constitutive association is in contrast to CD44v6 where an association between c-Met and the former seems to be dependent only on the presence of HGF (Orian-Rousseau et al. 2002). However an inducible association between c-Met and ICAM-1 is not per se necessary for c-Met activation. This is substantiated by another co-receptor for c-Met plexin B1, which is also constitutively associated with the receptor (Giordano et al. 2002). In that case stimulation by the plexin B1 ligand Sema 4D leads to plexin B1 clustering, which drives oligomerisation and activation of c-Met. Clustering of ICAM-1 has been reported and is important for its surface localisation (Yang et al. 2006) but it is speculative as to whether this could impact c-Met dimerisation and activation.

Heparan sulphate (HS) modification of cell surface proteins like CD44 have previously been shown to play a role in binding growth factors like HGF in a GF-presentation mechanism in Namalwa lymphoma cells (van der Voort et al. 1999). Conversely, a HGF mutant that cannot bind to HS can still activate c-Met better (Hartmann et al. 1998). Thus heparan sulphation is not always necessary for growth factor presentation and this appears to be the case for CD44v6, which is not heparan sulphated. We have recently observed that HGF can bind to CD44v6 independent of the presence of c-Met. Yet in cells were c-Met is expressed but not CD44v6, HGF cannot bind to c-Met (Matzke, A Orian-Rousseau, V and Ponta H unpl). If this is so then it is plausible that a region in CD44v6 is necessary for binding and presentation of HGF to c-Met. Could this also be true of ICAM-1? Similarities in the extracellular domains of ICAM-1 and CD44 molecules apart from a common HA-binding motif are still to be determined. Yet introduction of ICAM-1 or CD44v6 into rat carcinoma cells promoted HGF induced activation of c-Met (Fig 13) suggesting that ICAM-1 and CD44v6 can modulate an interaction between HGF and its receptor. The question of whether ICAM-1 can bind to HGF has not yet been examined but

HGF binding studies to the HepG2 cells in the presence and absence of ICAM-1 will address this point and this will help to understand if ICAM-1, like CD44v6 can present HGF to c-Met thus stabilising an interaction between the ligand and receptor. Indeed the observation that upon HGF induction the association between ICAM-1 and c-Met is slightly enhanced might speak towards a more stable surface complex (Fig. 12).

The ECM may use ICAM-1 to modulate c-Met activity

It has previously been described that ICAM-1 can bind to HA on synovial fibroblasts (Hiramitsu et al. 2006) and contains several near-B(X7)B motifs described as the HA binding motif found in proteins like CD44 (Nelson et al. 1995). An interaction between ICAM-1 and HA could affect c-Met activation by inhibiting an interaction between HGF and c-Met, a potential one between HGF and ICAM-1 or c-Met and ICAM-1. Concurrent with this idea is the observation that ICAM-1 antibody blocking experiments (Fig 10) also negatively affects HGF dependent ERK phosphorylation. It is not clear if the antibody could mimic HA binding or if it too sterically hinders HGF binding ability. An interpretation of the activation status of the c-Met receptor itself would be required to eliminate an indirect effect on downstream signalling.

Mechanistically, HA has been proposed to be involved in a reorganisation of downstream partners that could affect proliferative responses (Morrison et al. 2001). Binding of HA to CD44 leads to the recruitment of the active form of the ERM related protein merlin, a negative growth regulator. In the CD44 negative HepG2 cells, ICAM-1 could potentially bind to merlin, as it has been described to interact with ERM proteins (Heiska et al. 1998). Recent data also suggest that active merlin may modulate signalling by inhibiting activation of Ras thereby affecting downstream targets such as ERK (Morrison et al. 2007).
A mechanistic modulation of signalling from HA by ICAM-1 like CD44 is plausible due to the general trend for ICAM-1 and CD44 to convey similar HA signals. ICAM-1 and CD44 have been described to modulate HA mediated inhibition of

MMP production in synovial fibroblasts during proinflammatory responses (Shimizu et al. 2003; Hiramitsu et al. 2006) and in chondrocytes both CD44 and ICAM-1 HA binding contributes to a reduction in anti-Fas induced apoptosis in osteoarthritis (Lisignoli et al. 2001). A growth inhibition signal propagated by HA in the absence of CD44 can potentially utilise ICAM-1.

Intracellular cues can be modulated by ICAM-1 in c-Met signal transduction

The loss of ICAM-1 expression in the HepG2 cells negatively effects c-Met activation and subsequently signalling to ERK. Since ICAM-1 can bind to ERM proteins (Heiska et al. 1998) like CD44, the potential mechanism for c-Met signalling in the HepG2 cells could be mediated via an ICAM-1-ERM interaction. This may be possible, as it has recently been demonstrated that a CD44v6-ERM interaction is required for HGF-dependent activation of Ras, which strictly requires formation of a complex comprising ERM proteins, Grb2, SOS, Ras and F-actin (Orian-Rousseau et al. 2007). Interestingly it had been speculated that ICAM-1 might transduce signals via adaptor proteins like Grb2 and SOS to ERK (Gardiner and D'Souza 1999).

Attempts to Co-IP endogenous ICAM-1 and ezrin, the predominantly expressed ERM protein in the HepG2 cells, were unsuccessful. However it is possible that an interaction between them may require mediation of phosphatidylinositol 4,5-bisphosphate (PIP_2). *In vitro* interaction studies between ICAM-1 and ezrin were much more enhanced in the presence of PIP_2 (Heiska et al. 1998). Indeed it appears that PIP_2 binding to ezrin is required for its phosphorylation at Thr567 (Fievet et al. 2004; Ivetic and Ridley 2004) and enhanced binding to CD44 (Hirao et al. 1996). Binding of ICAM-1 to merlin may also be mediated in this way since binding is between the N-terminal portion of ezrin and PIP_2, and merlin and ezrin have 61% homology in this region (Tsukita and Yonemura 1999).

CD44 candidate substitute molecules in primary mouse cells

The mild phenotype of CD44 total knock out (KO) mice suggests that the organism elicits a mechanism to manage CD44 functions. Such a mechanism of compensation for CD44 molecules must take into account molecules that

participate in similar functions of CD44, like c-Met activation. The finding that ICAM-1 could function as a co-receptor for c-Met in the HepG2 cells lead to the question as to whether it could function as a substitute molecule in the same capacity in CD44KO mice. Preliminary studies in primary mouse hepatocytes from mice using siRNA against mICAM-1 indicated that loss of ICAM-1 correlated with a reduction in HGF-dependent ERK phosphorylation in the CD44KO mice, which was not observed in the CD44 wild type (WT) mice (Fig.14B) although it is expressed in both situations (Fig 14A). What is still not clear is whether in the normal situation c-Met utilises CD44 isoforms in these cells (or another molecule). During mouse embryogenesis CD44 expression is differentially regulated in the liver until day E11.5 (Wheatley et al. 1993) but there are no studies on CD44 expression in newborn mice. It will be interesting to see if the hepatocytes from newborn mice make use of CD44 isoforms for c-Met activation

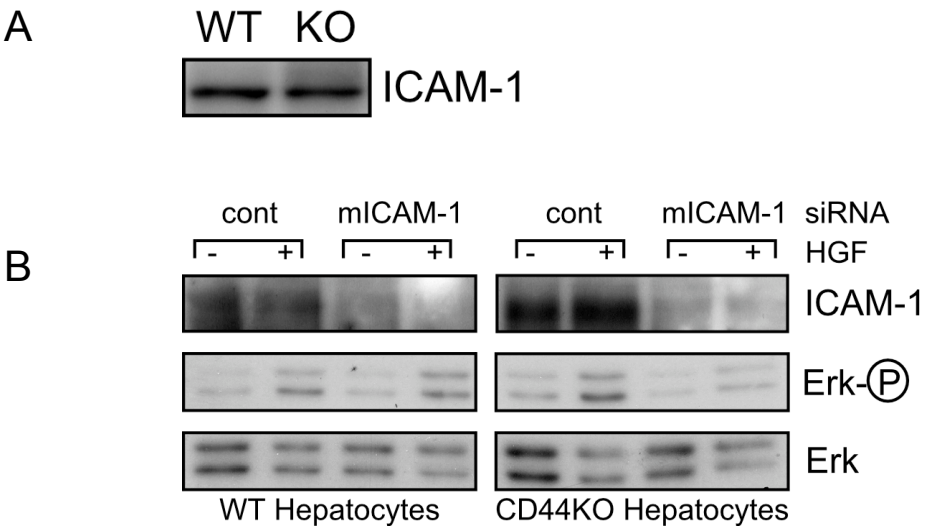

Figure 14. *Loss of ICAM-1 expression affects c-Met activity in CD44 KO primary mouse keratinocytes.* Hepatocytes were isolated from the livers of CD44WT and KO mice and placed into culture at 37°C, 5% CO_2. **A** ICAM-1 is expressed in both the WT and KO mouse hepatocytes **B** siRNA against ICAM-1 inhibits HGF-dependent ERK phosphorylation in the KO but not WT hepatocytes. One week after isolation, logarithmically growing cells were transfected with siRNA against mICAM-1 (300pmol) or a scrambled oligonucleotide. The cells were serum starved as previous and induced with HGF, c-Met activity was observed as determined by ERK phosphorylation on western blots.

In primary human keratinocytes, pre-treatment of cells with CD44v6 blocking antibodies inhibits HGF induced c-Met activation and downstream signalling to ERK (Orian-Rousseau et al. 2002). I isolated primary mouse keratinocytes from CD44WT and CD44KO mice and could observe that in the CD44WT keratinocytes, CD44 isoforms were expressed as compared to the KO (Fig.15A).

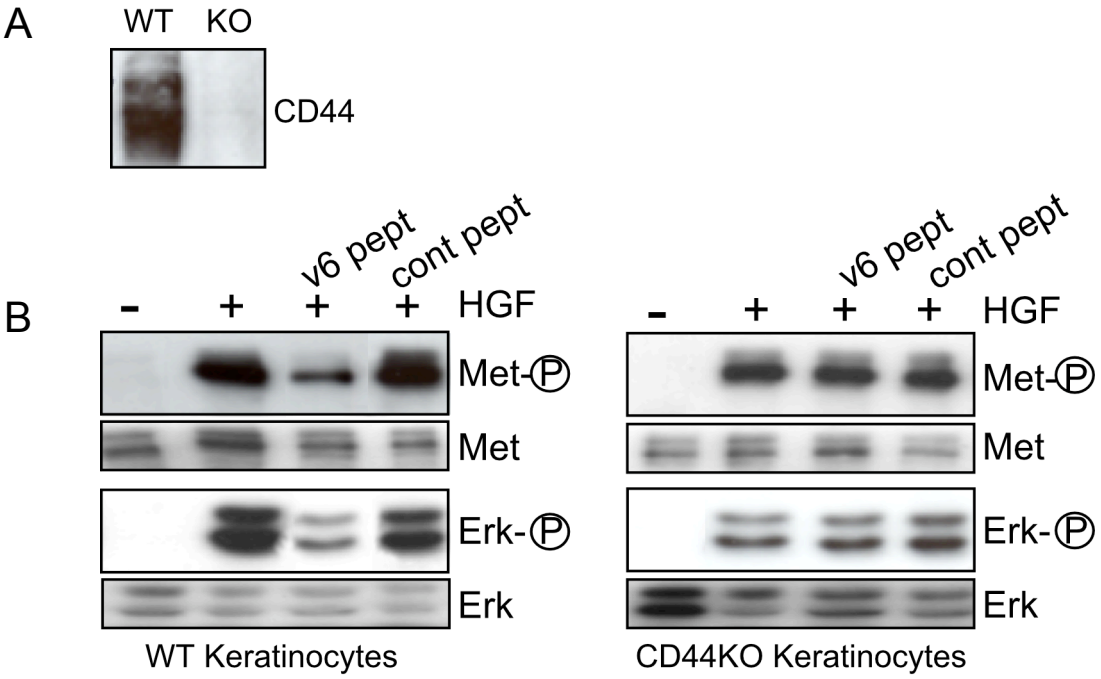

Figure 15. CD44 *Expression and requirement of CD44v6 for c-Met activity in primary mouse keratinocytes*. Primary cells were isolated as described in Materials and Methods. **A** CD44 isoforms are expressed in the cell lysates from WT but not KO keratinocytes. **B** A murine CD44v6 peptide blocks c-Met activation and subsequent downstream signalling in CD44WT but not CD44KO mouse keratinocytes. Logarithmically growing primary cells were serum starved for 24 hr. Cells were pre-treated with murine or control peptides (100ng/ml) 5 min 37°C, then induced with HGF (10ng/ml).

Since no inhibitory v6 anti-mouse antibodies are available, I designed short peptides against the corresponding region in mouse v6 that have been shown to be important for human v6 for c-Met activation (Matzke et al. 2005). Pre-treatment of keratinocytes with the murine peptide indeed blocked activation of c-Met in the CD44WT cells suggesting that CD44v6 acts as a co-receptor for c-Met in a manner similar to the human keratinocytes. The same peptide did not, of course, block c-Met activation in the CD44KO keratinocytes (Fig. 15B). The advantage of this system is that I can make comparisons based solely on the

presence or absence of CD44 in relation to c-Met. Importantly, in the CD44KO keratinocytes c-Met is still activated and the signal is transduced to ERK (Fig. 15B). This could mean that c-Met activation may be independent of co-receptor molecules, also in other CD44 negative cells or that c-Met utilises other accessory proteins. The evidence presented from mine and other studies suggest that cell surface receptors like c-Met, FGFR or ErbB form co-operative relationships with other surface molecules, which are required for their activation and signal transduction (Bourguignon et al. 1997; Wainwright 1998; Orian-Rousseau et al. 2002) and this may also be the case in the CD44KO

In the absence of CD44 an organism may cope with its loss by up-regulating or activating other molecules, with similar functional properties, ICAM-1 could be such a molecule. The keratinocytes from WT and KO mice express ICAM-1 (Fig. 16). Unfortunately, the primary mouse keratinocytes are highly sensitive and difficult to transfect therefore I was unable so far to clarify the relevance for ICAM-1 in these cells. Interestingly ICAM-1 is not particularly up-regulated in the CD44KO keratinocytes but syndecan-2 is in comparison to the WT keratinocytes (Fig 16). Thus syndecan-2, which provides similar functions to CD44 and ICAM-1 may well be another candidate for a substitute for CD44 in keratinocytes of CD44KO.

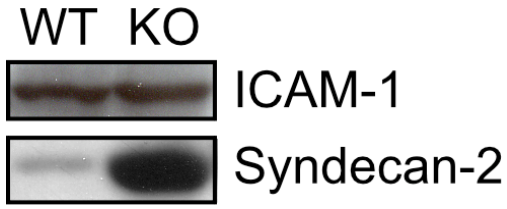

Figure 16. *Syndecan-2 and not ICAM-1 is differentially expressed in the CD44KO keratinocytes as compared to the CD44WT keratinocytes.* Logarithmically growing cells were lysed and analysed for protein expression by western blot.

Generally, syndecan-2 belongs to a family of HSPGs (Syndecans 1-4) that like CD44 are involved in cell-ECM communication, and have the ability to bind growth factors (Beauvais and Rapraeger 2004). Syndecan-1 was demonstrated

to bind HGF and promote c-Met signalling in multiple myelomas an apparent presentation mechanism that utilised the HS moieties (Derksen et al. 2002).
The syndecan proteins also exhibit a high degree of conservation in their cytoplasmic domain, which includes an ERM protein-binding domain (Beauvais and Rapraeger 2004). Studies have also shown that syndecan-2 can directly bind to ezrin thus linking syndecan-2 to the actin cytoskeleton and the ability to mediate signal transduction in CD44 negative cells (Granes et al. 2000; Granes et al. 2003).

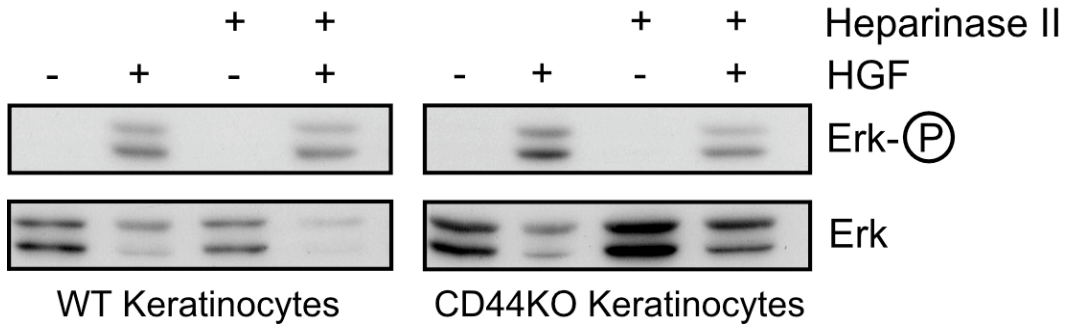

Figure 17. *A HSPG can modulate c-Met activation and subsequent signal transduction in KO but not WT keratinocytes.* Logarithmically growing cells were pre-treated with Heparinase II (6U/ml) at 37°C for 3 hrs. Cells were induced with HGF and assayed for ERK activation

Testing a potential role of syndecan-2 in c-Met activation in the CD44KO keratinocytes was hindered by their sensitivity to transfection and the lack of efficient antibodies against murine syndecan-2. I decided to take an indirect approach and examined the potential of a HSPG to affect c-Met activity. Keratinocytes from WT and KO mice were pre-treated with Heparinase II to remove all HS side chains from surface molecules. Preliminary data suggests that a HSPG via a HS moiety on the KO keratinocytes modulates c-Met activity (Fig 17). Whether this molecule is syndecan-2 still remains to be determined.

I have described a novel function for ICAM-1 as a co-receptor for c-Met, which is independent of any previously known ICAM-1 functions, largely described in terms of adhesion to leukocytes. ICAM-1 shares functional similarities like HA or

ERM binding capability to another co-receptor for c-Met, CD44v6. Although the question of ICAM-1 as a substitute molecule is still unclear the capacity for ICAM-1 to act as a co-receptor for c-Met supports the idea that different molecules can substitute for CD44 by different mechanisms.

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