ABSTRACT CD44 isoforms act as coreceptors for the receptor tyrosine kinases c-Met and VEGFR-2. However, Cd44 knockout mice do not show overt phenotypes, in contrast to Met and Vegfr-2 knockout mice. We hypothesized that CD44 is being compensated for by another factor in Cd44 null mice. Using RNAi technology and blocking experiments with antibodies, peptides, and purified ectodomains, as well as overexpression studies, we identified intercellular adhesion molecule-1 (ICAM-1) as a new coreceptor for c-Met in Cd44-negative tumor cells and in primary hepatocytes obtained from Cd44 null mice. Most strikingly, after partial hepatectomy, Cd44v6-specific antibodies inhibited liver cell proliferation and c-Met activation in wild-type mice, whereas ICAM-1–specific antibodies interfered with liver cell proliferation and c-Met activation in Cd44 knockout mice. These data show that ICAM-1 compensates for Cd44v6 as a coreceptor for c-Met in Cd44 null mice. Compensation of proteins by members of the same family has been widely proposed to explain the lack of phenotype of several knockout mice. Our experiments demonstrate the functional substitution of a protein by a heterologous one in a knockout mouse.

INTRODUCTION

In response to its ligand hepatocyte growth factor (HGF), the receptor tyrosine kinase (RTK) c-Met elicits many different signaling pathways mediating cell proliferation, migration, differentiation, and survival. Under physiological conditions these pathways converge to promote tubulogenesis. In cancer cells, deregulation of these processes promotes invasive growth and leads to tumor progression and metastasis (reviewed in Birchmeier et al., 2003).

Recently, several studies have shown that c-Met recruits helper molecules to either amplify signaling (e.g., β4-integrin) or to expand its ligand-binding repertoire (e.g., plexin B1), or even to allow the activation of the receptor by its ligand (e.g., CD44v6) (reviewed in Orian-Rousseau and Ponta, 2008).

We have extensively studied the coreceptor function of CD44 isoforms containing the exon v6 (abbreviated CD44v6) for c-Met. The CD44 family of transmembrane proteins comprises proteins that differ in their extracellular domain by insertion of combinations of 10 variant exons (v1–v10). Isoforms containing exon v6 are particularly interesting, as they have been shown to play decisive roles in the metastatic process. This function is probably due to their coreceptor function for c-Met. This coreceptor function is twofold. On one hand, the extracellular part of CD44v6 is necessary for c-Met activation. On the other hand, the cytoplasmic domain of CD44 promotes downstream signaling (reviewed in Orian-Rousseau, 2010). It binds ezrin, radixin, and moesin (ERM) proteins that link transmembrane proteins to the cytoskeleton and play an important role in the dynamics of the plasma membrane and migration (reviewed by Manget et al., 1999). In the case of the c-Met receptor, activation of the ERM proteins and recruitment of the actin cytoskeleton lead to activation of Ras (Orian-Rousseau et al., 2007).

The coreceptor function of CD44v6 for c-Met relies on three amino acids in exon v6—EWQ in rat, GWQ in mouse, and RWH in human—which are strictly required for c-Met activation. Peptides
comprising these amino acids, the smallest being 5-mers, completely inhibit the activation of the c-Met receptor (Matzke et al., 2005) and the metastatic spread of several cancer cell lines (Ponta and Orian-Rousseau, 2009).

Despite the strict dependence of c-Met activation on CD44v6 in a variety of primary and transformed cells, data obtained from knockout mice seemed to rule out that such collaboration occurs in vivo. The Met (and Hgf) knockout mice are embryonic lethal (reviewed in Birchmeier et al., 2003) and die from a placental defect between E12.5 and E16.5. This is in striking contrast to the Cd44 knockout mice, which show no overt phenotype during development and only have mild abnormalities in the adult. This is even more surprising given that the activation of c-Met in primary human keratinocytes is strictly dependent on CD44v6, and limb outgrowth relies on CD44v3 heparan sulfate isoforms (reviewed in Ponta et al., 2003).

An explanation for this striking difference between the Cd44 knockout mice and the Met and Hgf knockout mice could be that the function(s) of CD44 is replaced by another protein in the Cd44 null mice. This hypothesis is strongly supported by the data obtained for another type of “knockout” mouse in which CD44 was down-regulated by means of CD44 antisense sequences expressed under the control of the keratinocyte K5 promoter (Kaya et al., 1997). Accordingly, these mice do not express CD44 in the keratinocytes. Surprisingly, and in clear contrast to the total Cd44 knockout mice, the newborn mice have severe skin alterations, such as delay in wound healing, local inflammatory responses, and hair regrowth. These data strongly suggest that CD44 functions can be substituted during early embryogenesis (in the knockout mice), whereas at later times (when the K5 promoter becomes active) CD44 can no longer be replaced. Knocking down Cd44 late in embryogenesis is then detrimental for the animals.

Recently we have provided genetic evidence for cooperation between CD44 and c-Met in vivo. Mice with a Cd44 null background show haploinsufficiency for c-Met, in contrast to mice with a Cd44 homozygote or heterozygote background (Matzke et al., 2007). They die from a breathing defect due to impairment of synaptogenesis (in the pre-Bötzing complex) and alterations in the phrenic nerves. These data can be explained only if CD44 and c-Met cooperate in normal mice in establishing proper nerve functions and if the Cd44 functions are substituted by those of another protein or proteins in the knockout mice. This substitute protein has, however, to be functionally less efficient in order to explain the haploinsufficiency.

Here we demonstrate the identification of a substituting protein in Cd44 null mice. In human hepatoma cells, in which c-Met can be activated in the absence of CD44, we analyzed the expression of adhesion molecules that have been described to bind ERM proteins and therefore might be potential coreceptors for c-Met. Among these molecules, intercellular adhesion molecule-1 (ICAM-1) was identified as a new coreceptor for c-Met. In the CD44-negative human hepatoma cell line HepG2, ICAM-1 mediates signal transduction from the activated c-Met receptor. Furthermore, ICAM-1 substitutes for CD44v6 in murine hepatocytes. Whereas in wild-type mouse hepatocytes the activation of the c-Met receptor was strictly dependent on CD44v6, ICAM-1 took over this function in CD44 null murine hepatocytes. This substitution also occurred during liver regeneration, in which c-Met plays a decisive role (Borowiak et al., 2004). c-Met activation and liver regeneration were inhibited by the CD44v6 antibody in wild-type mice, whereas in the Cd44 null mice they were blocked with an ICAM-1 antibody.

RESULTS

The putative coreceptor for c-Met in HepG2 hepatoma cells uses ERM proteins to promote signaling

To test whether the coreceptor function of CD44v6 for c-Met can be substituted by another protein, we first examined a cell line that lacks CD44 expression but allows activation of c-Met. Such cells are, for example, the human hepatoma HepG2 cells. They do not express any CD44 isoform, including CD44v6 (Figure 1A), but the c-Met receptor is expressed and can be activated by its ligand HGF (Figure 1A). In these cells c-Met activation and signaling could not be blocked by a CD44v6 peptide, in contrast to what is observed in human colon carcinoma cells HT29 (Figure 1A; see also Matzke et al., 2005).

We assumed that c-Met also requires a coreceptor in HepG2 cells and that this potential coreceptor should provide functions similar to CD44v6, for example, binding of ERM proteins to mediate downstream signaling from the activated c-Met receptor (Orian-Rousseau et al., 2007). If that was the case, the expression of the cytoplasmic CD44 domain should compete with the ERM binding of the putative coreceptor and block downstream signaling in the HepG2 cells. We transfected the HepG2 cells either with plasmids expressing the cytoplasmic domain of CD44 or a mutated version of the CD44 cytoplasmic domain where the ERM-binding site was not functional (Legg and Isacke, 1998). Indeed, expression of the CD44 cytoplasmic domain abrogated HGF-induced Erk phosphorylation, whereas expression of the mutated version or transfection with the empty vector had no effect (Figure 1B). Neither the cytoplasmic domain nor the mutated form had an effect on c-Met activation (Figure 1B). This indicated that in HepG2 cells c-Met signaling was dependent on a protein with binding features for ERM proteins, similarly to what occurs in cells expressing CD44v6 (Orian-Rousseau et al., 2007).

This result was confirmed by experiments in which ezrin expression was down-regulated by small interfering RNA (siRNA; Figure 1B). Among the ERM proteins, ezrin is by far the most abundantly expressed protein in HepG2 cells and was therefore chosen. The repression of ezrin expression severely impaired c-Met signaling to Erk (Figure 1B).

ICAM-1 is a new coreceptor for c-Met

According to the results presented in Figure 1, the potential coreceptor substituting for CD44v6 should contain a binding site for ERM proteins. Therefore we investigated whether such a protein might be present in the HepG2 cells. Proteins of the ICAM and syndecan families have been shown to bind ERM proteins. ICAM-1, -2, -3, and -5 interact with the ERM proteins, whereas no significant binding has been demonstrated for ICAM-4 (Hamada et al., 2003). Among the syndecan family members, syndecan-2 can connect to the cytoskeleton via ezrin (Granes et al., 2000). The expression of these potential coreceptors for c-Met in HepG2 cells was compared with their expression in HT29 cells, in which the coreceptor function of CD44v6 has been well defined (Orian-Rousseau et al., 2002; Figure 2A). ICAM-1 was the only protein that was strikingly overexpressed in HepG2 cells as compared with HT29 cells (Figure 2A). In a first instance, we tested whether ICAM-1 might act as a coreceptor for c-Met.

To unravel a potential role of ICAM-1 in the activation and signaling of c-Met, we repressed its expression in HepG2 cells by siRNA or used antibodies to interfere with its function. siRNA against ICAM-1 drastically decreased the amount of ICAM-1 (Figure 2B). In these settings, both HGF-induced activation of c-Met and HGF-induced signaling to Erk were severely repressed as compared with the control siRNA-treated cells. Along the same lines, a blocking
In HT29 cells, the coreceptor function of CD44v6 for c-Met could be inhibited by incubation with the CD44v6 ectodomain. No effect was observed upon treatment with the ICAM-1 ectodomain (Figure 2D). In contrast, in HepG2 cells, the ICAM-1 ectodomain abrogated HGF-induced Erk phosphorylation. Surprisingly, in these cells the CD44v6 ectodomain also repressed signaling (Figure 2D).

The CD44v6 ectodomain, as well as the ICAM-1 ectodomain, might be able to bind HGF. Indeed, the binding of HGF to HT29 cells as monitored by fluorescence-activated cell sorting (FACS) analysis is abrogated by CD44v6-specific antibodies but not by ICAM-1–specific antibodies. In contrast, the opposite is observed in HepG2 cells: CD44v6 antibodies have no effect on HGF binding to the cells, whereas ICAM-1 antibodies interfere with this binding (Figure 2E).

To demonstrate that ICAM-1 can indeed function as a coreceptor for c-Met, we expressed ICAM-1 in cells in which the activation of c-Met was dependent on the transfection of a coreceptor. Such cells are the rat pancreatic carcinoma cells BSp73AS. These cells express neither CD44v6 (Günthert et al., 1991) nor ICAM-1 (Figure 3A). We have already shown that the transfection of CD44v6 into these cells rendered them responsive to HGF and allowed the activation of c-Met (Orian-Rousseau et al., 2002; Figure 3B), whereas transfection of CD44 isoforms lacking the exon v6 had no effect, demonstrating the specificity of the CD44v6 coreceptor function.

When these cells were transfected with a plasmid expressing ICAM-1 instead of CD44v6, they also responded to HGF by activation of Erk (Figure 3B). These results demonstrate that ICAM-1 can act as a coreceptor for c-Met, similar to CD44v6.

The coreceptor ICAM-1 and c-Met should thus be in close proximity and should coimmunoprecipitate. Indeed, in HepG2 cells, c-Met could be brought down using the ICAM-1 antibody. Moreover, ICAM-1 can be brought down by immunoprecipitation of c-Met. The complex between c-Met and ICAM-1 seems to be increased in the presence of HGF, similar to what we observed in the case of CD44v6 and c-Met (Orian-Rousseau et al., 2002; Figure 3C).

In conclusion, our experiments demonstrate that the activation of the c-Met receptor in the CD44-negative HepG2 cells was dependent on ICAM-1 and on ERM proteins. Indeed ICAM-1 has features similar to CD44v6 in that it enables binding of HGF to cells and it can recruit ERM proteins (Heiska et al., 1998), which is a prerequisite for signal transduction (Figure 1B).

**ICAM-1 substitutes for CD44v6 as a coreceptor in Cd44 knockout hepatocytes**

To test whether ICAM-1 could substitute for CD44v6 in Cd44 null mice, we first established specific tools to prove the CD44v6 coreceptor function in murine cells. We used mouse-specific CD44v6 antibodies [developed in rats (Khaldoyanidi et al., 2002)] and murine-specific peptides that should interfere with its coreceptor function for c-Met (Matzke et al., 2005) and tested them in mouse 3LL.
cells. In these cells CD44v6 is expressed, and the c-Met receptor can be activated by HGF (Figure 4, A and B). Indeed, the CD44v6-specific antibodies and several v6-specific peptides that cover the CD44v6 sequence shown to be critical for c-Met activation were able to abrogate c-Met activation and signaling. As expected, incubation with a rat-specific v6 antibody recognizing a different epitope (Khaldoyni et al., 2002) and a rat-specific peptide had no effect on c-Met (Figure 4B). Furthermore, the CD44v6 ectodomain also inhibited HGF-induced Erk phosphorylation (Figure 4C). Of interest, although 3LL cells express ICAM-1 (Figure 4A), the ICAM-1 ectodomain did not interfere with HGF-dependent Erk phosphorylation.

To test the function of c-Met in vivo, we focused on the liver, since it has been previously shown that c-Met plays a critical role during the regeneration of this organ (Borowiak et al., 2004). In contrast to human hepatocytes, which do not express any CD44 isoform (Heider et al., 1993), mouse hepatocytes express CD44 (Kennel et al., 1993). Most important, they express larger isoforms containing the exon v6 (Figure 4A). In these mouse primary hepatocytes, the c-Met receptor could be activated by HGF, and the v6-specific antibodies, peptides, and the CD44v6 ectodomain repressed this activation (Figure 4C). Of interest, also in these wild-type hepatocytes the ectodomain of ICAM-1 did not inhibit HGF-dependent Erk phosphorylation, although ICAM-1 is expressed (Figure 4, A and C).

Hepatocytes isolated from Cd44 knockout mice do not express CD44, as expected, but do express ICAM-1 in similar amounts to wild-type hepatocytes (Figure 4A). In addition, in Cd44 null hepatocytes the c-Met receptor could be activated by HGF treatment. To test whether ICAM-1 functions as a coreceptor in these cells, we abrogated ICAM-1 expression by siRNA (Figure 5A) or inhibited ICAM-1 activity with an ICAM-1–specific antibody (Figure 5B). Both treatments repressed c-Met activation, although they had no effect on wild-type hepatocytes (Figure 5B). Furthermore, the expression of the ICAM-1 ectodomain competed with c-Met–dependent Erk activation only in Cd44 knockout hepatocytes (Figure 5C).

In conclusion, ICAM-1 not only functions as a coreceptor for c-Met in human hepatoma cells, but it also appears to replace the CD44v6 function in mouse hepatocytes obtained from Cd44 knockout mice.

ICAM-1 replaces CD44v6 in liver regeneration of Cd44 null mice

The relevance of c-Met in adult animals is illustrated by its physiological role in liver regeneration (Borowiak et al., 2004). Partial hepatectomy led to an increase of HGF production, followed by activation of the c-Met receptor, which then triggered proliferation of hepatocytes. Targeted inactivation of c-Met interfered with this proliferation (Borowiak et al., 2004). We therefore used partial hepatectomy to study the relevance of the CD44v6 and c-Met interaction in vivo and to test whether this function of CD44v6 is substituted by ICAM-1 in the Cd44 knockout mice.
In accord with the protocol for induction of liver cell proliferation after partial hepatectomy (Mitchell and Willenbring, 2008), we removed the left lateral and medial lobes and determined proliferation in the remaining lobes by staining with Ki67-specific antibodies 48 h postsurgery, the time at which proliferation reaches its maximum. The number of Ki67-positive cells was increased to 40% in wild-type animals and animals treated with control antibodies (Figure 6). In contrast, if the animals received an injection of CD44v6-specific antibodies, the number of Ki67-positive cells only reached ∼11%. Injection of ICAM-1-specific antibodies had only a minor effect on proliferation.

FIGURE 3: ICAM-1 is a novel coreceptor for c-Met. (A) Expression of ICAM-1 in primary mouse hepatocytes and rat BSp73AS cells (AS cells) measured by Western blot. (B) HGF-induced Erk phosphorylation in BSp73AS cells transiently transfected (Amaxa) with expression vectors for ICAM-1 or CD44v6 (Materials and Methods) or an empty vector as indicated. Bottom, the expression of ICAM-1 and CD44v6 (1.1ASML antibody) monitored in Western blots. (C) Coimmunoprecipitation of ICAM-1 and c-Met in HepG2 cells (see Materials and Methods). Proteins in cell lysates were immunoprecipitated with the antibodies as indicated, and the Western blots were done using a c-Met or an ICAM-1 antibody.

FIGURE 4: CD44v6 is a coreceptor for c-Met in WT mouse hepatocytes and in the mouse 3LL cells. (A) Expression of CD44v6 (9A4 antibody) and ICAM-1 (11C81 antibody) in 3LL cells or hepatocytes derived from Cd44+/+ (WT) or Cd44−/− (KO) mice measured by Western blot analysis. (B) HGF-induced c-Met or Erk phosphorylation in 3LL cells or hepatocytes obtained from WT mice treated or not with blocking reagents such as CD44v6 peptides (mpept, murine-specific CD44v6 peptides; rpept, rat-specific CD44v6 14-mer peptide; see Materials and Methods) or antibodies (mαv6, 9A4; rαv6, 1.1ASML) as indicated. (C) HGF-induced c-Met and Erk activation in 3LL cells and in mouse hepatocytes (WT) treated or not with the ICAM-1 ectodomain (ICAM-1 ECD) or the CD44v6 ectodomain (see Materials and Methods) as indicated.
influence on the number of Ki67-positive cells. When the experiment was performed with Cd44 knockout mice, proliferation occurred to a similar extent to that in wild-type mice and was not influenced by intravenous application of control antibodies or CD44v6-specific antibodies (Figure 6A). The injection of ICAM-1 antibodies, however, reduced the amount of Ki67-positive cells to ∼12%. By Western blot analysis we demonstrated that the effect on proliferation is accompanied by an effect on c-Met activation. CD44v6 antibody inhibited c-Met phosphorylation in livers obtained from wild-type mice after partial hepatectomy, and ICAM-1 antibody interfered with phosphorylation of c-Met in livers from Cd44 knockout mice obtained after partial hepatectomy (Figure 6B). We verified that the inhibition of proliferation was not due to induction of apoptosis by the injected antibodies by staining liver sections with anti–cleaved lamin A. In contrast to mice treated with anti-Fas antibody for control, we could not detect apoptotic cells in livers injected with either CD44v6 or ICAM-1 antibodies (Figure 6C).

These results clearly show that also during liver regeneration ICAM-1 takes over the coreceptor function of CD44v6 for c-Met in Cd44 knockout mice.

**DISCUSSION**

In a variety of cells the c-Met receptor recruits the coreceptor CD44v6 in order to induce its activation. We have shown that ICAM-1 can take on the coreceptor function for c-Met in cells that do not express CD44v6. In such cells, namely human hepatoma cells HepG2, ICAM-1 can be communoprecipitated with c-Met, and siRNAs against ICAM-1 abrogate HGF-induced c-Met and Erk activation. Most strikingly, ICAM-1 is also the coreceptor for c-Met in hepatocytes isolated from Cd44 knockout mice. In these mice, ICAM-1 substitutes for CD44v6 as a coreceptor for c-Met–induced proliferation of hepatocytes in liver regeneration.

ICAM-1 and CD44v6 provide similar coreceptor functions to c-Met. These functions are required for the activation of c-Met and for subsequent signaling. Both ICAM-1 and CD44v6 can bind ERM proteins. As in the case of CD44, the binding of ERM proteins to ICAM-1 is direct, as shown by means of binding assays using cytoplasmic ICAM-1 peptides and purified ezrin (Heiska et al., 1998). Similar to CD44, this binding is dramatically increased in the presence of phosphatidylinositol phosphates such as P(4,5)P2 (Heiska et al., 1998).

The binding of ERM proteins to CD44v6 is essential for c-Met signaling. Indeed, these proteins recruit the actin cytoskeleton to mediate signaling, promoting activation of Ras (Orian-Rousseau et al., 2007). The requirement of the actin cytoskeleton for signaling suggests that a scaffold is needed to bring together signaling molecules at the docking site of c-Met. In cells that lack CD44v6, this activation of the mitogen-activated protein kinase (MAPK) pathway by c-Met is mediated by ICAM-1 and also depends on ERM proteins. Of interest, other receptor tyrosine kinases, such as the vascular endothelial growth factor receptor-2, which also requires CD44v6 for its activation, also use ERM proteins to induce signaling (Tremmel et al., 2009). Even when RTKs are independent of CD44v6, as in the case of platelet-derived growth factor receptor, ERM proteins are necessary for signaling (Morrison et al., 2001). Thus the activation of ERM proteins and their binding to the cytoskeleton might be a common pathway needed to mediate signal transduction.

The functional substitution of the c-Met coreceptor CD44v6 by ICAM-1 in Cd44 knockout mice can explain the differences between the phenotypes observed in the Cd44 null mice as compared with the Cd44 null mice. It might also explain the striking difference between the lack of developmental phenotype observed in the Cd44 null mice as compared with the antisense knock down of CD44 isoforms in the keratinocytes (starting at E 9.5). Indeed, in the latter mice a skin phenotype was observed, in contrast to the Cd44 null mice. This result suggests a time window for the substitution of CD44. Finally, it might also explain why the phenotypes observed in the Cd44 null mice are restricted to the adult stage (reviewed in Ponta et al., 2003).

A lack of phenotype in several knockout mice has been claimed to be due to compensation in particular by other proteins of the same family, but only a few examples of such compensation have been demonstrated. For example, members of the MAPK family, including Erk, Jnk, and p38, can compensate for each other to a certain extent
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RHAMM). In inflammatory diseases such as collagen-induced arthritis (CIA) inhibition of CD44 reduces the pathology (Mikecz et al., 1995). Surprisingly, in Cd44 knockout animals CIA is even more severe than in wild-type animals (Nedvetzki et al., 2004). It is suggested that RHAMM binds to hemagglutinin in the absence of CD44, inducing the activation of inflammatory genes and increasing the severity of the disease.

Genetic data revealed haploinsufficiency of c-Met in a Cd44 null background (Matzke et al., 2007) indicating that the potential substitute for CD44v6 has to be less efficient than CD44v6 (see discussion in Matzke et al., 2007). Here we show that ICAM-1 is indeed less efficient than CD44v6 as a coreceptor for c-Met. In wild-type murine hepatocytes and 3LL cells, c-Met activation is only dependent on CD44v6, although both CD44v6 and ICAM-1 are expressed (Figure 4). Thus, when in competition with ICAM-1, CD44v6 is the functional coreceptor and ICAM-1 only takes over this function in the absence of CD44v6, as observed in HepG2 cells or Cd44 null hepatocytes. This observation is further corroborated by competition experiments using the ectodomain of CD44v6 or ICAM-1. The ectodomain of ICAM-1 inhibits the coreceptor function for c-Met only in cells in which ICAM-1 acts as the coreceptor, whereas the ectodomain of CD44v6 inhibits c-Met activation in cells that are dependent on either CD44v6 or ICAM-1 (Figures 2D, 4C, and 5C). A likely explanation for this competition is that the ectodomains compete for the binding of HGF and this binding is stronger in the case of CD44v6 than of ICAM-1. Such a binding is suggested by the FACS analysis shown in Figure 2E.

In addition to providing a new insight into gene compensation, this article has also described functions of CD44v6 and of ICAM-1 in the liver that are different from functions described previously. CD44 appears to play a role in migration of cytotoxic lymphocytes into an injured liver (Kimura et al., 2009), and ICAM-1 was shown to be responsible for the production of interleukin-6 after partial hepatectomy (Selzner et al., 2003). Here CD44v6 and ICAM-1 appear to play a role in the activation of c-Met and in proliferation of hepatocytes that occurs during liver regeneration.

Although there is no doubt that functional substitution of proteins occurs in several knockout mice, until now no direct demonstration of such compensation had been given. Here, we not only give evidence for the compensatory mechanisms occurring in the Cd44 null mice, but also demonstrate the functional replacement of the coreceptor CD44v6 for c-Met by ICAM-1.
MATERIALS AND METHODS

Animals and cell lines
CS7Bl/6J mice from Charles River Laboratory (Sulzfeld, Germany) (WT) and Cd44 knockout mice (KO; Schmits et al., 1997) were used for the isolation of hepatocytes and partial hepatectomies. The HT29 cells (Fogh et al., 1977) and HepG2 cells (ATCC no. HB-8065) were routinely grown in DMEM medium (Invitrogen, Karlsruhe, Germany) plus 10% fetal calf serum (FCS; PAA Laboratories, Coelbe, Germany). The BSp73AS cells (Matzku et al., 1983) and the Lewis lung carcinoma cell line (3LL; Eisenbach et al., 1983) were grown in RPMI 1640 medium (PAA Laboratories) plus 10% FCS.

Isolation and culture of mouse hepatocytes
Primary mouse hepatocytes were isolated from WT and Cd44 KO mice as follows: 1- to 2-d-old pups were killed by decapitation and briefly washed with 70% ethanol. Livers were resected and rinsed in phosphate-buffered saline (PBS) and then sequentially incubated for 5 min in Earle’s balanced salt solution (EBSS; Invitrogen) without Ca²⁺/Mg²⁺, 0.5 mM ethylene glycol tetraacetic acid; EBSS with Ca²⁺/ Mg²⁺, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4; and EBSS with Ca²⁺/Mg²⁺, 10 mM HEPES, pH 7.4, 0.3 mg/ml collagenase I (Invitrogen). Livers were washed with PBS and the cells dispersed by pipetting up and down several times. The cells were plated and cultured at 37°C in DMEM containing 10% FCS. Positive staining using the cytokeratin-18 (Acris Antibodies, Herford, Germany) and E-cadherin (BD Transduction Laboratories, Heidelberg, Germany) antibodies revealed 95% purity of the hepatocyte population (unpublished data).

Partial hepatectomy and antibody injections
All animals were handled according to German regulations for animal experimentation. The animal experiments were approved by the Regierungspräsidium Karlsruhe (35-9185.81/G-83/04).

Partial hepatectomy was performed on 8- to 12-wk-old male mice (n > 3) under anesthesia with inhaled isofluorane. Two-thirds hepatectomy was performed using a standard protocol (Mitchell and Willenbring, 2008). Briefly, the abdominal cavity was opened with longitudinal incision, and the left-lateral and median lobes were ligated and removed. The abdominal wall was closed by suture, and animals were recovered on a 37°C heating block. When required, mice received intravenous injections into the tail vein of 2 μg/kg of CD44v6 or ICAM-1 antibodies or immunoglobulin G (IgG) at the time of surgery. Mice were killed at 48 h after partial hepatectomy, and the remaining liver tissue was subsequently divided, with one part processed for histology and the other placed on dry ice and stored at −80°C.

anti-Fas/CD95 injections
Induction of apoptosis in the mouse liver was performed as previously described (Jakob et al., 2008). Briefly, nonhepatectomized animals received intravenous injections in the tail vein with anti-Fas antibody or control IgG (0.25 μg/g; BD Biosciences, Heidelberg, Germany) and were killed 4 h later. Apoptosis was detected with the nuclear protein anti–cleaved lamin A (Cell Signaling, Frankfurt am Main, Germany) as described (Jakob et al., 2008). Lamin A is cleaved in a caspase-dependent way during apoptosis.

Histology and immunohistochemistry
Liver tissues were fixed in 4% neutral buffered formalin (Merck, Darmstadt, Germany) overnight, dehydrated, embedded in paraffin, and sectioned (5 μm). Proliferation was analyzed on deparaffinized and rehydrated sections using the Ki67 mouse-specific antibody (TEC-3, DakoCytomation, Hamburg, Germany). Expression of Ki67 was detected using the DAB substrate system 3,3′-diaminobenzidine (Vector Labs, Eching, Germany), followed by counterstaining with Mayer’s hematoxylin (Labonord, Mönchengladbach, Germany).

Protein extraction from liver tissue
The frozen liver tissue was homogenized in 1 ml of lysis buffer (5 mM HEPES, pH 7.4; 2 mM MgCl₂) containing protease inhibitors. The lysates were centrifuged at 6300 × g for 5 min at 4°C, and the supernatant was transferred to another tube and centrifuged at 13,000 × g for 5 min at 4°C. Equal amounts of protein after Bradford analysis were used for immunoprecipitation.

Antibodies, ectodomains, and peptides
The human pan-CD44 antibody Hermes-3 was a gift from S. Jalkanen, (Turku, Finland); the rat CD44v6 antibody 1.1ASML and the mouse CD44v6 antibody 9A4 have been described (Matzku et al., 1989; Khaldoonyadi et al., 2002). The pan-CD44 antibody IM7 was from BD Biosciences (San Diego, CA). The antibodies Erk 1 (K-23), ICAM-1 (G-5), ICAM-1 (M-19), ICAM-2 (H-159), ICAM-3 (N-19), ICAM-5 (N-16), h-Met (C-12), m-Met (B-2), syndecan-1 (C20), synde can-2 (M-140), syndecan-4 (H-140), and tubulin (TU-02) were all from Santa Cruz Biotechnology (Heidelberg, Germany). The ezrin antibody 3C12 was purchased from NeoMarkers (Wedel, Germany). The ICAM-1 (16-0542) antibody and the IgG used for injections were from eBioscience (Frankfurt, Germany), and the ICAM-1 antibody (11C81) was used in immunoprecipitations and blocking experiments and the human HGF antibody (AF-294-NA9) used for FACS analysis were from R&D Systems (Wiesbaden, Germany). The Fas antibody (lot 25078) and its corresponding IgG were from BD Biosciences. Antibodies directed against phospho-Erk (phospho-p44/42), phospho-Met (D26), and Met (25H2) were purchased from Cell Signaling Technology. Secondary antibodies labeled with horseradish peroxidase were purchased from DakoCytomation, and the fluorescein isothiocyanate (FITC) anti–mouse IgG (115-096-071) was from Jackson ImmunoResearch Europe (Newmarket, United Kingdom). The recombinant mouse soluble ectodomain of ICAM-1/Fc chimera was from R&D Systems. The expression vector for producing the CD44v6 soluble ectodomain (CD44v6 ECD) in Pichia pastoris has been described (Tremmel et al., 2009). CD44v6 human and rat peptides (14-mer) and the control peptide have been described (Matzku et al., 2005). The murine CD44v6 peptides used in blocking experiments were as follows: A, ETWFQGNGWQG; B, NGWQGKPNPI; and C, QETWFQGNGWQKNP; the critical sequence for the CD44v6 coreceptor function is underlined (Matzku et al., 2005). The sequence of the control peptide is HNREQANLNI SRTTEETI.

Constructs and siRNA oligonucleotides
The ICAM-1 expression vector, pCI-neo ICAM-1, was a kind gift from Olli Carpén (Uppsala, Sweden; Heiska et al., 2005). The constructs encoding the CD44 cytoplasmic domain (CD44cytD) and the mutant cytoplasmic domain (CD44mut-cytD) have been described (Legg and Isacke, 1998) and were obtained from C. Isacke (Breakthrough Breast Cancer Research Center, London, United Kingdom).

Three human siRNAs for ICAM-1 were used as a pool: GCCUCAG-CACGUACCUCUA was from Santa Cruz Biotechnology, and GGAA-CAACCGGAAGGUGAUUU and GCUCAAAGUGUCUAAAGGAUUU
were from Thermo Scientific (Lafayette, CO) using the DHarmacon siRNA design tool. Human siRNA for ezrin (pool of three: GGAAA-CAUCUCUUUCAUGA, CCAGCGUCAGAAUCAACA, GACUCU-GUUUGCUUGUGU) and mouse siRNA for ICAM-1 (pool of three: CCAACUGGAGACUGUUGAGt, CCAUCAGCUUGAUAUUCGUtt, and CAGUACUGCUUGCUAGUUt) were from Santa Cruz Biotechnology. Control siRNA (SC-37007) was from Santa Cruz Biotechnology.

**HGF induction**

Cells were serum starved for 24 h and induced with recombinant HGF (10 ng/ml) and a generous gift from George Vande Woude, Van Andel Institute, Grand Rapids, MI) at 37°C for 5 min. Where indicated, cells were treated with peptides (100 ng/ml), antibodies (100 μg/ml), or IgG control (100 μg/ml) for 5 min at 37°C prior to HGF treatment or with soluble ectodomain domain (0.5 μg/ml) for 10 min at 37°C. The cells were washed with ice-cold PBS, followed by lysis in SDS sample buffer containing 100 mM dithiothreitol (DTT). The boiled lysates were subjected to SDS-PAGE gel electrophoresis, followed by Western blotting. To detect activated Erk, the membrane was probed with an antibody against phosphorylated Erk. For the loading control the membrane was stripped (62.5 mM Tris, pH 6.8, 2% SDS, 0.8% DTT) and probed with the Erk antibody or tubulin antibody. To detect activated c-Met, a phospho-Met-specific antibody was used.

**Com immunoprecipitation experiments**

The HepG2 cells (3 x 10⁶ cells in 10-cm plates) were induced with HGF as described. The cells were then incubated in lysis buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1% Igepal [Sigma-Aldrich, St. Louis, MO], 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na orthovanadate, 1 mM aprotinin, and leupeptin) for 30 min on ice, followed by centrifugation (10 min, 12,000 rpm). The cleared lysates were incubated with 5 μg of ICAM-1 antibody, 5 μg of Met antibody or IgG controls, respectively (as indicated in Figure 3) at 4°C for 2 h overnight and then precipitated with protein A/G agarose beads (Merck). The precipitates were washed three times in lysis buffer, resuspended in sample buffer (+DTT), and subjected to SDS-PAGE, followed by Western blot analysis.

**Transfections**

Cells were cultivated to 70–80% confluence before transfection using an Amaxa nucleofector Kit V (Amaxa Biosystems, Lonza, Köln, Germany) or Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For the Amaxa, briefly, trypsinized cells were resuspended at room temperature in transfection solution (1 x 10⁶ cells/100 μl for a six-well plate and 3 x 10⁶ cells/100 μl for a 10-cm plate). The cell suspension (100 μl) was then mixed with the respective oligonucleotides and plasmids in sterile Eppendorf tubes and transferred to Amaxa cuvettes. Electroporation was carried out with the corresponding program for each cell type. Immediately after transfection, prewarmed medium was added to cells (500 μl) in the cuvettes and then the cells were transferred to plates containing prewarmed medium. For the Lipofectamine 2000, HepG2 cells or primary hepatocytes were cultivated to 70% in a six-well plate. Lipofectamine was added to serum-free DMEM according to the manufacturer's instructions, incubated for 30 min with the respective oligonucleotides, and then added to the cells and incubated at 37°C. Four hours later the transfection solution was exchanged with prewarmed medium. Experiments were performed 24 h later. Transfection efficiency of hepatocytes as estimated using a rhodamine-labeled siRNA was 79.5%. The survival rate after transfection was measured using a trypsin blue staining and corresponded to 86%.

**FACS analysis**

A total of 1 x 10⁶ cells was incubated with human CD44v6 or ICAM-1 antibody (100 μg/ml) for 1 h at 37°C prior to induction with HGF. The cells were detached using 5 mM EDTA in PBS, centrifuged at 1200 rpm for 3 min, and washed three times with PBS. Cells were incubated with HGF antibody (5 μg/ml) in PBS for 1 h on ice, followed by three washes and incubation with FITC anti-mouse IgG (1 μg/ml) for 30 min on ice in the dark. After three washes the cells were resuspended in PBS and measured with a FACSscan (Becton Dickinson, Franklin Lakes, NJ).

**Quantification and statistical analysis**

The number of Ki67-positive cells was determined by counting 10 fields of 200× magnification per liver section. Data are expressed as percentage of positively labeled hepatocytes. All quantifications are given as mean ± SD. Differences between the various conditions were analyzed by paired Student's t-test, and p < 0.05 was considered as statistically significant. The quantification of the Western blots was performed using the ImageJ computer program (National Institutes of Health, Bethesda, MD). All experiments were repeated at least three times, with similar results.

**ACKNOWLEDGMENTS**

We thank Susanne Brema, Selma Huber, and the animal facility of the Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, for their help with the animal experiments. This work was supported by the Mildred Scheel Stiftung (H.P. and V.O.-R.).

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