ILEI, a Novel Key Regulator of Hepatocellular Carcinoma Progression

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ILEI requires oncogenic Ras for the epithelial to mesenchymal transition of hepatocytes and liver carcinoma progression

Abstract

Introduction

ILEI is expressed and differentially localized during TGF-β-dependent EMT of hepatocytes

ILEI collaborates with oncogenic Ras to induce and maintain EMT of hepatocytes

ILEI stimulates upregulation of PDGF-C/PDGF-Rβ and enhances proliferation and migration in oncogenic Ras-expressing MIM-R-GFP hepatocytes

The collaboration of ILEI with oncogenic Ras enhances tumor formation, nuclear accumulation of β-catenin and Stat3 tyrosine phosphorylation

ILEI enhances the growth rate of MIM-R-GFP-derived metastatic colonies

Cytoplasmic localization of ILEI correlates with tumor dedifferentiation in human HCCs

Discussion

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4. Assessing the role of Stat3 in the epithelial to mesenchymal transition of neoplastic hepatocytes

Abstract

Introduction

Results

Stat3 is activated during EMT

Expression of constitutive active (ca) and dominant negative (dn) Stat3 versions in Ras-transformed hepatocytes

Expression of constitutive active (ca) Stat3 in Ras-transformed hepatocytes reveals reduced tumor formation

Diminished metastatic colonization of hepatocytes co-expressing ca Stat3 and oncogenic Ras
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Summary:
The development of human hepatocellular carcinoma (HCC) is a complex process. Recently it has been shown that the synergy of transforming growth factor (TGF)-β with constitutive active Ras induces an epithelial-to-mesenchymal transition (EMT) of murine hepatocytes. This phenotypical change of tumor cells results in increased malignancy, a higher migratory potential and metastasis. The loss of E-cadherin, which represents an essential constitutive factor for the formation of adherens junctions, is a key event in EMT. In recent studies, a novel key regulator of malignant epithelial cells, referred to as interleukin like EMT inducer (ILEI), was identified by expression profiling of epithelial versus fibroblastoid breast cancer cells. To understand the role of ILEI in hepatocellular carcinoma we employed p19ARF deficient hepatocytes to study whether ILEI alone or in combination with oncogenic H-Ras is able to induce and maintain EMT. The role of ILEI on tumor formation was analyzed by injection of ILEI overexpressing cells subcutaneously in severe combined immunodeficient (SCID) mice. In further experiments an upregulation of components of the PDGF-signalling pathway was detectable. In addition an increase in activated Stat3 and nuclear β-catenin accumulation was observed in tumors derived from ILEI overexpressing cells. A human tissue array consisting of 69 patient samples was further used to analyze the distribution of ILEI in primary human HCC and revealed a strong cytoplasmic expression particularly in poorly differentiated tumors. In conclusion the obtained results indicate that ILEI requires oncogenic Ras to induce EMT via mechanisms involving PDGF-R/β-catenin and PDGF-R/Stat3 signaling. A further project focused on the role of activated Stat3 during EMT formation of neoplastic hepatocytes. In this study, the cooperative function of Stat3, oncogenic H-Ras and TGFβ1 was analyzed using the same cellular model of p19ARF null hepatocytic cells. Constitutive active (ca) and dominant negative (dn) mutants of Stat3 were introduced into hepatocytes and the ability to form tumors and metastasis was analyzed. These experiments revealed a tumor suppressive role of Stat3 together with H-Ras and p19ARF deletion in liver carcinogenesis. Expression profiling showed an upregulation of c-jun and MMP-3, which have been reported to play an important role in the establishment of EMT. Together, these results suggest a crucial function of Stat3 in combination with oncogenic Ras and TGFβ during hepatocellular tumor progression.
Zusammenfassung:


Stat3 an- oder abgeschalten werden. Dabei wurden zwei Gene, nämlich c-Jun und MMP-3 identifiziert, die eine wichtige Rolle bei der Induktion einer EMT spielen. Zusammengefasst führte diese Untersuchung zur Schlussfolgerung, dass Stat3 in Kombination mit onkogenem Ras und TGFβ eine wichtige Rolle bei der hepatozellulären Tumorentstehung einnimmt.
1. Introduction:

1.1 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is one of the most malignant diseases with poor survival due to delayed diagnosis which causes more than half of a million deaths per year (Parkin, 2001; Song et al., 2004). With regard to the survival rate, HCC is therefore the second most lethal cancer of the world (Llovet, 2006). Still today the mechanisms underlying this disease are poorly understood and biological markers such as α-fetoprotein are limited in their sensitivity and specificity. Further commonly used markers for HCC are des-γ-carboxy prothrombin and glypican-3 (Pang et al., 2008), ferritin, γ-glutamyltranspeptidase isoenzyme, alkaline phosphatase, α-1-antitrypsin and aldolase A (Lau, 2003). For quantification of the different stages of HCC, several frequently used markers are hepatoma-specific gamma-glutamyl transferase isoenzyme, an enzyme that is responsible for the degradation of glutathione and is increased during liver diseases and extrahepatic tumors (Yao et al., 2007). Another very important factor for the staging of HCC is the transforming growth factor (TGF)-β1, which is increased in more than 50% of all HCCs (Deryck, 1998). Due to the increased expression of this cytokine, TGF-β1 is therefore used as a marker for mid-stage HCC (Yao et al., 2007). For graduation of patients suffering from HCC, different staging forms are available, among them Child-Pugh’s Classification, Primack-Staging, and Okuda Staging for example (Okuda, 1985; Primack et al., 1975; Zhou et al., 2008). Worth mentioning is the tumor-node-metastasis (TNM) staging (Huang et al., 2005), emphasizing on different clinicophatological factors which are shown to influence the prognosis of HCC (Zhou et al., 2008). Beside all these pathological staging, the Edmonson-Steiner grading, a histological way of staging tumor patients, is always helpful for more accurate prognosis of patients with HCC (Zhou et al., 2008). The factors, which influence the stages of the disease and therefore the therapy are, according to criteria such as solitary or multiple tumors at stage I, no evidence of tumor thrombus in the trunk or portal vein at stage II, no extrahepatic metastasis at stage III and metastasis in distant organs or penetration of the visceral peritoneum at stage IV (Zhou et al., 2008). Because of these involved factors it is very difficult to treat patients with the right therapy. For example, the functionality of the liver is the main
cause for liver transplantation or partial hepatectomy (Edmonson, 1954). A lot of statistical analysis has to be done to find the right way of staging HCC patients and to treat them in the right way to increase the survival rate. 

The incidence of HCC reflects the aetiological factors and the ethnicity of regarding countries (Llovet et al., 2003). Infection with Hepatitis B (HBV) or C (HCV) virus, chronic alcohol abuse, haematochromatosis, steatohepatitis, and Aflatoxin B1 intoxication are among the well-defined major risk factors for liver cirrhosis and subsequent liver cancerogenesis (Bosch et al., 1999; Liaw et al., 1986). Gender can also be a factor, because males show a 3-fold higher incidence of hepatocellular diseases than females (Sherman, 2005; Parkin, 2001). Beside all these mentioned risk factors, the distribution of HCC also has an influence on the relevant people. For example, living in Asia or Africa can increase the possibility of suffering from a hepatitis B or hepatitis C infection. Also the consumption of contaminated food in this area can cause HCC. In western countries and Japan, the chance of suffering from hepatitis C is higher than in the rest of the world. The excessive consumption of alcohol, a main problem in western countries and hematochromatosis also lead to the development of HCC (Bruix, 2003). For a short overview about the risk factors causing HCC see Figure 1.

Still today the ability to detect slow growing tumors in the liver lacks of randomized controlled trials. Imaging techniques are very expensive and therefore can neither be deployed in African countries nor in Asia. Among these abilities to detect small nodules in the liver are precutaneous ultrasonography, multiphasic contrast-enhanced helical computed tomography, magnetic resonance imaging and positron emission tomography (Song et al., 2004). Once a tumour has been diagnosed, it has to be determined if the patient will be treated by surgery or by application of medicaments, including Sorafenib, an oral multikinase inhibitor blocking tumor cell proliferation by interfering with the Raf/mitogen-activated protein kinase/ extracellular signal regulated kinase and PDGF-R/VEGF-R (Zhu, 2007). Other molecular target agents for patients suffering on HCC suppress several signalling pathways, as for example Sunitinib and Bevacizumab, which block the vascularization of tumor tissue. Others, such as Gefitinib, Eplotinib or Lapatinib focus on the inhibition of the epidermal growth factor (EGF) pathway. Monoclonal antibodies against several involved signalling pathways were also discovered (Zhu et al., 2007), either blocking EGF-R of VEGF-R.
Many different treatment options are known to combat HCC. Among these therapies are liver resection, divided in local hepatectomy and orthotopic liver transplantation, local ablative therapy including injections of different cytotoxic agents, hepatic artery transcatheter treatments, systemic therapies including chemotherapy or immunotherapy and other treatments like gene therapy or supportive therapy (Lau, 2003). The applied treatment depends on the liver function of the patient. Good liver function goes along with partial hepatectomy. Liver transplantation can be done in patients with early HCC and decompensated cirrhosis. For late and severe HCCs, local ablative therapy is most commonly used. This includes injections of ethanol percutaneously (PEI) and radiofrequency ablation (RFA). All these therapies described so far give the patient a survival of 3 years with a rate of 46% to 77%. Another remarkable therapy is the transarterial chemoembolisation (TACE), where chemotherapeutics are injected using the transhepatic artery as the route of choice (Lau, 2002). Another way is the systemic therapy, where chemotherapeutics are used for HCC interventions. Among these chemotherapeutics are cis-platin, interferon-alpha, adriamycin and 5-fluorouracil, which showed promising results with a 3-year survival rate of 26% (Leung et al., 1999).

HCC is proposed to be a long-term result of a chronic liver inflammation effected by one of the major risk-factors. The disease itself can be divided in an early, intermediate or advanced and end-stage HCC (Llovet, 2003). To classify a patient in one of these stages several parameters are of high importance, like tumor size or colonization of distant organs. Due to the fact that more than 50% of HCC-patients show an activation of TGFβ1, this factor belongs to the group of the most important molecular factors involved in hepatocellular tumor progression (Bedossa, 1995; Factor, 1997; Zhao, 1998).
Figure 1: Mechanisms of hepatocarcinogenesis (Farazi & DePinho, 2006).

Previous investigations show that genetic and epigenetic alterations result in the dysregulation of key oncogenes and already known tumor-suppressor genes, as for example p53, p16 or E-Cadherin (Llovet, 2006). P53, which is frequently inactivated in HBV, HCV, and Aflatoxin B1 induces hepatocellular neoplasia, plays a major role in the progression of HCC, because it either contributes to initation or progression of tumor growth or both. Another important factor in HCC progression plays the Wnt-pathway target gene β-catenin, which translocates to the nucleus and activates several cancer-related genes like cyclin-D1, which leads to uncontrolled cell growth (Gregorieff & Clevers, 2005). Several genomic alterations can lead to the generation of liver-tumors including defects in chromosome segregation. Abnormal DNA-damage response pathways, as p53 or BRCA2 or genomic alterations, as hypermethylation of CpG-islands, hypomethylation of several genes result in an increased mutational rate and microsatellite instability (Herath et al., 2006). Also a defective DNA-mismatch repair belongs to the main modifications involved in the molecular main causes for HCC.
1.2 Epithelial-to-mesenchymal transition
Epithelial-to-mesenchymal transition (EMT) describes the process of cellular conversion of epithelial cells to a mesenchymal phenotype by losing the cell-to-cell contacts. This process is considered to play a crucial role in carcinogenesis. However EMT also plays a major role in embryonic development, especially in somitogenesis and muscle formation. Extracellular stimuli are the main source of signals which are necessary for cells to undergo such a conversion (Hugo et al., 2007). The upregulation of transcriptional repressors of E-Cadherin such as Snail and Zeb1 is involved in the transformation of epithelial cells to mesenchymal ones (Eger et al., 2005; Thiery, 2002). These newly formed fibroblastoid cells then show features of unlimited cell growth, persistent angiogenesis, evasion of apoptosis (Hanahan & Weinberg, 2000) and upregulation of typical mesenchymal marker genes. After this conversion, cells break through the basement membrane and invade the blood and lymphoid vessels to reach adjacent organs to set up colonies there (Eger and Mikulits, 2005). The main property of EMT does not only include the formation of primary tumors, but also gives cells the ability to leave solid tumors and build up cancerous colonies in distant organs. For this process cells have to undergo the reverse mechanism, also known as mesenchymal to epithelial transition (MET) after extravasation. This process and its involvement in tumor progression is depicted very clearly in Figure 2.

A major hallmark in this process plays the activation of several signaling pathways, upon them the receptor tyrosine kinases (RTK)s, mitogen-activated protein kinase (MAPK), integrin-linked kinase (Lackmann et al., 1998), phosphatidyl-inositol 3 kinase (PI3K) as well as the activation of small GTPases, as for example Rac and Rho (Schmitz et al., 2000), platelet derived growth factors (PDGF) and receptors, and transforming growth factor (TGF) β signalling (Eger et al., 2005; Heldin, 2004).

Among the major attributes of EMT, the loss of tight and adherens junctions and the loss of its major constituent such as E-Cadherin and ZO-1 respectively, is crucial. Along with this observation is the upregulation of the repressors of the marker genes as for example Snail, Twist, deltaEF1 or Slug. Also the accumulation of β-catenin in the nucleus after the release from the adherens-junction complex, consisting of E-cadherin, β-catenin and p120CTN, plays an important role in the EMT process (Zhai et al., 2008). Rearrangement of the cytoskeleton from cortical actin to actin stress fibres is an
indicator of migratory cells (Zavadil & Bottinger, 2005) and therefore a hallmark of ongoing EMT. Among the activated signaling pathways, the Ras/MAPK pathway plays a critical role in the establishment of mesenchymal cells.

![Diagram of EMT and MET](image)

**Figure 2:** Sites of EMT and MET in the progression of cancer (Thiery et al., 2002).

Several *in vitro* and *in vivo* models help to gain more information about the cellular processes involved in EMT. These models have been investigated the molecular events resulting in the identification of various important molecules for the ongoing EMT process. To study the process of EMT in different cancers, several cell lines were established overexpressing oncogenic Ras. These cells were then treated with TGFβ to undergo an EMT and can be used for further analysis. Not only *in vitro* models were generated, but also *in vivo* models were established employing knock-out experiments or transplantation studies or models for intravital imaging of all invasion (Eger et al., 2005). Under the most important mechanisms is the upregulation of transcriptional repressors for the expression of E-Cadherin, among them Twist, which plays a major role in metastasis formation (Karreth & Tuveson, 2004).
1.3 EMT and HCC progression

In recent studies several genes such as Laminin-5 or Stat5b were identified to cause EMT, alone or in combination with TGFβ1, in human HCC (Giannelli, 2005; Lee, 2006). As a new factor involved in human EMT, Laminin-5 was found during the formation of metastasis. Giannelli et al. found out that Laminin-5 was only expressed in tumor tissue compared to peritumoral sections. Also the E-cadherin repressors Slug, Snail and β-catenin showed an increased expression in HCC samples revealing a role for this factor in EMT. While E-cadherin expression was decreased in the samples, β-catenin accumulation in the nucleus was increased indicating an important role of these two factors during EMT. All investigated human invasive cells did not change their morphology when treated with TGFβ1, a main factor responsible for EMT induction in the liver, but showed a dramatic morphological change after treatment with Laminin-5 leading to scattered cells and long shaped cell bodies. Non-invasive cell lines induced with both TGFβ1 and Laminin-5 showed a phenotypical transition together with upregulation of Snail and downregulation of E-Cadherin. Furthermore, it has been shown that TGFβ1 can induce EMT with the help of Laminin-5 due to the induction of integrin α3β1 subunit which are the main receptors for Laminin-5.

Lee et al. found a second factor playing a role in HCC, especially in EMT. In particular, an induction of the isoform Stat5b and not Stat5a was shown to be activated in human HCC samples. Furthermore this process is influenced by HBV infection. During this infectious disease, HBX is transactivated and can induce the increased expression of Stat5b. An upregulation of this transcription factor leads to an increased invasion ability of investigated metastatic HCC cell lines. In these cell lines the expression of Stat5b upregulates E-cadherin repressors and therefore causes an EMT activation. This was the first hint for Stat5b and its correlation with the aggressive behaviour of cells causing HCC and the induction of EMT under activated HBX gene. Altogether these findings of Gianelli and Lee may help to generate new therapeutical targets for the treatment of HCC (Lee, 2006).
1.4 The role of TGFβ in hepatocellular cancer progression

The multifunctional cytokine TGFβ is responsible for the control of proliferation, differentiation and many other functions in a wide range of different cell types (Sporn et al., 1986; Dewilde et al., 2008). Studies on the function of TGFβ revealed a role in tumor suppression and tumor progression. At least three different TGFβ-ligands and two different receptors are known with a very high similarity (Abou-Shady et al., 1999). As a co-receptor and therefore necessary for the activation of this pathway, β-glycan was found (Feng, 2005; Shi, 2003). From these three ligands, the peptide TGFβ1 plays the most prominent role in tumor cells (Akhurst & Derynck, 2001). The role of this peptide in cancer influences the premalignant state, the loss of tumor suppression, the migratory potential of the cells and the modifications of the microenvironment after metastatic colonization (Massague, 2008).

The loss of the tumor suppressive function of TGFβ is frequently caused by disruptions of the receptors, which consist of characteristically short nucleotide repeats in their kinase region (Grady et al., 1998; Pardali & Moustakas, 2007; Massague, 2008). Also mutations of the target genes, the Smads, can occur and lead to an inhibition of the physiologic role of the TGFβ pathway. As recent findings, epigenetic alterations of several components of the pathway have been reported (Chen & Chang, 1997; Munoz-Antonia et al., 1996) which can lead to inhibition of the inhibitory effect of TGFβ on cell proliferation.

In the liver, the function of this growth factor can be classified in a cytostatic and an apoptotic one, depending on the physiological conditions of the surrounding liver tissue (Rossmanith & Schulte-Hermann, 2001). For activation of the TGFβ-signaling pathway TGFβ has to bind to its co-receptor, β-glycan. This binding induces a heterogenic complex formation of TGFβRII and TGFβRI. This process leads to the phosphorylation of the regulatory domain of TGFβRI by the kinase domain of TGFβRII and is therefore activated. After the activation of TGFβRI intracellular proteins, the so-called receptor activated Smads (R-SMADs), a subclass of Smad proteins, among them Smad2 and Smad3, are phosphorylated on their C-terminal serine residues. In normal cytoplasm Smad2 and Smad3 can bind to several proteins, including also the Smad anchor for receptor activation (SARA) and due to this binding, the R-Smads stay inactive in the
cytoplasm (Siegel et al., 2003). After the release of Smad2 and Smad3 from SARA, this complex forms a trimeric protein complex together with the Co-Smad Smad4 and is then shuttled in the nucleus of regarding cells (Pardali & Moustakas, 2007) where it activates several target genes. An overview of the TGFβ-pathway is shown by Figure 3. Due to the fact that in more than 50% of all human HCCs TGFβ1 is upregulated and influences the surrounding tissue by uncontrolled proliferation, more about the role of this factor has to be known. In last years, different mouse models have been generated to investigate the pro-tumorigenic activity in different tissues. In the skin, TGFβ1 suppresses the growth of tumors, but accelerates the rate of metastasis (Cui et al., 1996). For breast cancer models the direct induction of TGFβ1 did not affect primary breast tumor formation, but it increased the amount of lung metastasis. These results confirmed an autocrine action of TGFβ in promoting metastasis (Muraoka-Cook et al., 2004). For analyzing the overexpression of TGFβ ligands and their receptors further investigations were done emphasizing on the production and secretion of TGFβ. Again breast cancer cell lines expressing oncogenic H-Ras showed an induction of EMT and a higher migratory behaviour followed by a higher metastasis rate (Oft et al., 1996). The ability of TGFβ to induce the transition of epithelial cells to gain a mesenchymal phenotype is crucial for the formation of hepatic neoplasia (Moustakas et al., 2007). To explain the tumor promoting or pro-metastatic role of this polypeptide it is worth mentioning that cancer cells often overexpress TGFβ (Derynck, 1986). Due to this overexpression a phenotypical change of epithelial cells occurs, a process called EMT, which goes along with the upregulation of the expression of matrix-metalloproteinases (MMPs), which leads to a higher migratory potential of these cells. Recent observations claimed an induced neovascularization of tumorigenic colonies (Hasegawa et al., 2001; Ito et al., 1995) along with the formation of metastasis. A novel factor, found in breast cancer cell lines, playing a role in the formation of lung metastasis after TGFβ induction is angiopoietin-like protein-4 (Angptl4). Tumor cell-derived Angptl4 starts to disrupt the vascular endothelial cell-cell junctions, increases the blood pressure to passage more blood through the capillaries and facilitates the trans-endothelial passage of tumor cells (Padua, 2008).
Recent studies on therapeutic strategies against TGFβ focus mainly on the manipulation of the TGFβ-pathway (Yingling et al., 2004). Among these therapeutic approaches, low molecular weight inhibitors, enzymes modifying the small GTPases of the Ras family, neutralizing antibodies and antisense RNA-approaches against TGF-β signalling components have been established (Arteaga et al., 1993; Schlingensiepen et al., 2005; Witham et al., 2003). Despite all this knowledge about TGF-β signaling, major research has to be focused on the TGFβ-induced EMT in tumor progression.

1.5 The role of Platelet-derived growth factor signaling in cancerogenesis:
Platelet-derived growth factors (PDGF) are described as mitogens of myofibrolats and are involved in the conversion of epithelial cells to spindle-shape like mesenchymal cells (Heldin & Westermark, 1999). This highly conserved molecule family consists of five isoforms PDGF-A, -B, -C, –D and –E and two receeptors PDGFR-α and PDGFR-β,
which can form homodimers and heterodimers. They also have a very high similarity to vascular-endothelial cell growth factors (VEGF) and therefore play a role in blood vessel formation (Joukov et al., 1997). Beside these 5 PDGF-ligands, two different receptor tyrosine kinases, referred to as PDGF-R\(\alpha\) and PDGF-R\(\beta\), which are members of the cystein knot family of proteins and complete this signaling pathway. Both receptors consist of five immunoglobulin-like domains and intracellularly there is a common tyrosin kinase domain with an characteristically inserted sequence without kinase-homology (Claesson-Welsh et al., 1988; Matsui et al., 1989; Yarden et al., 1986). The binding affinity of the ligands to their receptors is shown in Figure 4.

**Figure 4:** Mammalian PDGF/PDGFR binding interactions (Soriano et al., 2003).

Upon receptor dimerization these two receptor isoforms can form homo- or heterodimers (Hammacher et al., 1989; Kanakaraj et al., 1991; Seifert et al., 1989). For activation of these receptor tyrosine kinases, autophosphorylation of tyrosine residues in *trans* at the receptor is necessary (Heldin & Ostman, 1996). After this process, phosphorylation of tyrosine residues in the activation loop induces the kinase domain and then the activated receptor provides SH2-docking sites for several signalling molecules. Among the SH2-binding molecules are members of the Src family, PI3K, phospholipase \(\gamma\), the tyrosine...
phophatase SHP-2, Grb2/Sos, STAT and the GTPase activating protein (GAP) for Ras (Heldin et al., 1998). Together with the classical MAPK pathway, PDGF signalling is claimed to play a role in tumor progression and metastasis formation (MacDonald et al., 2001). The activation of the PI3K- together with the PDGF-pathway is assigned to influence apoptosis protection and a higher migratory potential including the stimulation of small GTP binding proteins of the Rho-family (Barres et al., 1992; Hawkins et al., 1995; Hooshmand-Rad et al., 1997; Yao & Cooper, 1995). In recent investigations the physiological functions of PDGF are assumed to play a role in embryonic development, supported by the findings in PDGF-knock-out mice, which are dying during embryogenesis. Functions of this pathway are also the formation of the vascular system due to its relation to VEGF and tissue homeostasis, where it regulates the interstitial fluid pressure and stimulation of wound (Heldin & Westermark, 1999). On the other hand PDGF is described to induce diseases due to its dysregulation in many different tissues. Among these effects are developmental defects, inflammation, atheriosclerosis, fibrosis and cancer. Latest experiments showed a function of PDGF in HCC. Overexpression of PDGF-C is assumed to cause liver fibrosis, steatosis and HCC (Campbell et al., 2005). In recent findings it is shown that TGFβ1 causes PDGF activation and an autocrine PDGF secretion in cells expressing H-Ras (Gotzmann et al., 2006). Together with these findings, the accumulation of β-Catenin in the nuclei of tumorigenic cells was observed (Fischer et al., 2007). Similar results in breast-cancer cell lines revealed an important role of TGFβ1 and PDGF in formation of neoplastic tumors (Jechlinger, 2006). But still today the understanding of PDGF in tumour progression and metastasis formation and the cooperation with TGFβ signaling are not completely understood and therefore further investigations have to be done.

1.6 Wnt signaling and its role in cancer

The Wnt signaling is highly conserved having its role in embryonic development and cancer. This signaling pathway functions as a morphogen which is secreted beyond cell boundaries and influences the transcription of several factors in adjacent cells regulating processes such as proliferation, survival or differentiation. One of the major factors in
this pathway is β-catenin which can interact with E-cadherin at the cell-boundaries and can translocate into the nucleus and bind to the lymphoid enhancer factor (LEF) 1, which was discovered first in the early 90ties (Behrens et al., 1996; Huber et al., 1996). Further regulators of the Wnt-pathway were found by genetic analysis in Xenopus embryos and Drosophila (Behrens et al., 1998; Itoh et al., 1998; Zeng et al., 1997).

At the moment 19 Wnt genes in the human genome are known all playing a role in cell polarity, embryonic induction, cell fate determination and tissue hoemostatsis (Lee, 2006). There are three different major Wnt signalling pathways among them the canonical pathway (see Figure 5), which is characterized by the activation of Wnt target genes by β-catenin (Logan & Nusse, 2004). The second major pathway involves Rho A and Jun Kinase (Veeman et al., 2003). The third cascade contributing to the Wnt signalling pathways is calcium dependent and therefore activates the protein kinase C and calmodulin-dependent protein kinase II (Kuhl, 2000; Lustig & Behrens, 2003; Sheldahl, 1999; Slusarski, 1997). The classical canonical Wnt pathway starts with the binding of one of the 19 Wnt ligands to their cognate Frizzled (Fzd) receptors at the cell surface, from which 10 different are known so far. After binding of the ligand to its cognate receptor, proteins belonging to the dishevelled protein family are activated and therefore change the amount of β-catenin that can translocate to the nucleus (Birchmeier et al., 2008). These signalling steps thereafter lead to the release of β-catenin from the boundary complex consisting of α-catenin and E-cadherin and causes the translocation into the nucleus occurs. There β-catenin activates its partners Tcf/LEF1 and induces the transcription of Tcf/LEF1 bound target genes. In the absence of Wnt-ligands, β-Catenin gets phosphorylated by GSK3β and ubiquitinilated and afterwards degraded. Very low levels of β-Catenin in the cytoplasm lead to repression of Wnt target genes (Klaus & Birchmeier, 2008). Several inhibitors of this pathway are already known playing a role in different signalling steps. Dickkopf is a competitive antagonist for binding of Wnt/Fzd to LRP receptors (Roman-Gomez et al., 2004). In the absence of Wnt, TCF is able to repress target genes in the nucleus (Bienz, 1998; Brannon et al., 1997; Riese et al., 1997) by forming a complex together with the Wnt inhibitor Groucho, which causes the recruitment of histon deacetylases (Cavallo et al., 1998; Chen & Struhl, 1999)
Figure 5: The canonical Wnt-pathway (Eisenmann et al., 2005).

The role of this pathway in cancerogenesis is still poorly understood, but recent studies revealed several disorders caused by members of the Wnt pathway. Circumventing the inhibitory effect of Wnt signaling by downregulation of its repressor Dickkopf (Dkk) through hypermethylation or loss-of-function mutations were found in different tumors and play a role also in many other diseases (Caldwell et al., 2004; Terasaki et al., 2002; Vider et al., 1996), as for example in cancer, especially in HCC, non-small lung cancer and colon carcinoma (Fuerher, 2008). Wnt β-catenin signalling also plays a role in the cardiovascular system and the dysregulation of this pathway may lead to cardiovascular diseases as well as neurodegenerative diseases as for example Schizophrenia or Alzheimer’s disease (Klaus & Birchmeier, 2008).

In HCC patients the activated Wnt β-catenin pathway can be found in more than 50%. In these patients the accumulation of β-catenin in the nucleus results in a poor prognosis. On the other hand there are repots showing that the expression of β-catenin in the cytoplasm might result in a better curing (Austinat et al., 2008). Recent results identified a role of Wnt signalling in regeneration and ageing and therefore gave rise to the idea that this pathway may play a role in cancer stem cells. Several studies on this topic
showed an increase of nuclear accumulated β-catenin in regions responsible for the tumor progression not only in the liver but also in the colon (Jung et al., 2006). There accumulation of β-catenin causes an induction of EMT due to downregulation of E-cadherin and degradation of the adherens and tight junctions by matrix-metalloproteinase 14 (Brabletz et al., 2006) and therefore indicating an important role of β-catenin in tumor progression.

1.7 Signal transducers and activators of transcription
Another important pathway linked to tumor progression is the Signal transducer and activators of transcription (Stat) pathway, Stat3. This protein family consists of 7 Stat proteins which are highly similar in their protein structure (Darnell et al., 1994; Horvath, 2000; Lackmann et al., 1998; O'Shea, 1997). Each Stat protein shows a transactivation domain at the C-Terminus, followed by a SH2-domain and a DNA-binding domain in the center of the protein (Becker et al., 1998; Chen et al., 1998). Many receptors at the cell membrane such as cytokine receptors and receptor tyrosine kinases are able to activate these Stat proteins in the cytoplasm (Levy & Darnell, 2002).

Activation of Stat proteins occur after the binding of cytokines or growth factors to their cognate receptors. Receptor dimerization takes place followed by autophosphorylation of the Janus kinase (Roberts et al.), which is non-covalently bound to specific receptors (Levy & Darnell, 2002). As a next step the Janus kinases (Jak), which is now activated, phosphorylates the receptor and therefore provides binding sites for the SH2-domain of Stat molecules. These bound Stat proteins are then phosphorylated by the Jaks and translocate into the nucleus where they can activate several target genes (See Figure 6).
Several inhibitors of Stat activation can be found within a cell, among them SH2-containing phosphatases (SHP), proteins which dephosphorylate the receptor or suppressors of cytokine signalling (SOCS), which can block the entire signaling mechanism (Krebs & Hilton, 2001). Epigenetic inactivation of SOCS-3 apart from SOCS-1 may be involved in tumor progression and metastasis of HCC caused by HCV infection (Ogata et al., 2006). Downregulation of SOCS-3 is accompanied with constitutive expression of Stat3 and therefore leads to the upregulation of numerous tumor-promoting genes (Yoshikawa, 2001; Yoshimura, 2006).

The role of Stat proteins in cancer is still poorly understood but recent studies showed a function of Stat1, Stat3 and Stat5 in cancerogenesis. It has been shown that Stat1 has its role in the antiviral defense, inflammation and injury in the liver and is mainly activated by IFN-α/β and INF-γ. Stat3 itself acts in the acute phase response, hepatoprotection, liver regeneration, glucose homeostasis and hepatic lipid metabolism and is mainly activated by interleukin (IL)-6 and its related cytokines as well as IL-22. For the function of Stat5 in the liver, only few results are known among them the expression of a variety of hepatic genes such as cytochrome P-450, glutathione S-transferase, sulfotransferase enzyme, growth hormone receptor, serine protease inhibitor Sp12.1, insulin growth factor I and hepatocyte growth factor. These genes are involved in the metabolism, growth and differentiation in the liver. Stat5 is activated by a
wide variety of cytokines in the immune and hematopoietic system (Gao, 2005). Stat3 exists in two isoforms, Stat3\(\alpha\) and Stat3\(\beta\) which might have different fuctions. Stat3\(\beta\) on its own is able to induce and repress genes depending on different cellular environment (Dewilde et al., 2008). Further differences between these two alternatively spliced isoforms is the lack of 55 amino acid residues at the C-terminus which are replaced by 7 amino acids specifically for Stat3\(\beta\). At the level of expression, Stat3\(\beta\) shows a decreased expression but is able to be activated especially in hepatocytes at the same level as Stat3\(\alpha\) after inflammation (Biethahn et al., 1999; Hevehan et al., 2002). The function of the specific 7 amino acid tail is still poorly understood but may play a role in the DNA binding acticity (Yoo et al., 2002). Many tumors show an overexpression of activated Stat3 due to increased levels of IL-6. Introduction of dominant negative forms of Stat3 in mice enhanced the apoptotic potential of breast cancer cells showing the necessity of this protein for growth regulation (Garcia et al., 2001).

Among the oncogenic functions of Stat3 are its anti-apoptostic, mitogenesic, pro-survival and pro-proliferative, which can finally lead to malignant transformation. Also the dysregulation of the immune system has to be mentioned by inhibiting Th1 immunstimulatory molecules (Kortylewski et al., 2008). In recent studies of HCC it was shown that the activation of the Ras- and Jak/Stat pathway was enhanced in all HCCs which goes along with the suppression of at least 1 Ras inhibitor and 2 Jak/Stat inhibitors, namely the SH2-containing phosphatases (SHP) and the suppressors of cytokine signalling (SOCS). All these inhibitory factors cause a suppression of cytokines responsible for Stat3 activation (Calvisi et al., 2006).

1.8 The role of E-Cadherin in EMT

The adherenes-junction protein E-cadherin belongs to the cadherin superfamily of calcium dependent adhesion glycoproteins. The gene itself has 16 exons spanning 100 kD of genomic DNA. The structure is similar to that of cadherins and shows specific five extracellular cadherin repeats followed by a transmembrane region and a highly conserved intracellular tail domain. Knock-out experiments revealed a function of this gene in migration and tumor progression as well as invasion and metastasis (Larue et al., 1994). Therefore loss of E-Cadherin resulted in a transition of adenoma cells to
carcinoma cells and is the cause of the tumor progression and not a consequence (Chirstofori et al., 2003). The crosstalk of epithelial cells and cells of the surrounding tissue has great influence on the progression of neoplasia in the hepatocellular system. One of the most prominent factors playing a role in this process is the adherens junction protein E-cadherin (Giehl & Menke, 2008). Recent findings raise the question if epithelial cells dysregulate their shape or change their microenvironment to form tumors (Comoglio & Trusolino, 2005). In the liver, inflammation occurs and therefore is a trigger for the change of the microenvironment of the cells. Responsible for this process are cytokines and chemokines, which are secreted by several activated fibroblast and stellate cells (Bachem et al., 1998; Kleeff et al., 2007). In more than 50% of human HCCs TGFβ1 and members of the PDGF-family, which are secreted by immune cells are responsible for the progression of tumors in the inflammed liver (Esposito et al., 2004). Due to this process cells undergo EMT and are able to migrate to distant organs and to form colonies there. During EMT, one of the most important changes is the loss of the adherens junction protein E-cadherin (Gumbiner, 1996; Kemler, 1993). The loss of E-Cadherin is often transcriptionally silenced in human tissues caused by epigenetic changes of the promoter (Gilles et al., 1997; Rodrigo et al., 1999) either by hypermethylation or by acetylation.
Due to decreased p120^{ctn} content, E-cadherin is degraded (Ireton et al., 2002) and the cells therefore lose their tight adherens junction (for regular E-cadherin complex see Figure 8) and gain a more fibroblastoid phenotype. As a first factor involved in the downregulation of the adherens function of E-cadherin, Snail was found. Snail was also shown to cause EMT (Peinado et al., 2007; Batlle et al., 2000; Cano et al., 2000). Besides Snail, Slug, Zeb1, Zeb2 and Twist were shown to downregulate the transcription of E-cadherin and therefore lead to the phenotypical change important for EMT (Comijn et al., 2001; Eger et al., 2005). Also other repression mechanisms exist which alter the expression level of E-cadherin. For example, histon deactelyses are involved in the repression (Hemavaty et al., 2000), or even post-transcriptional regulation mechanisms play a prominent role in the regulation of EMT by downregulating the adherens junction protein.

TGF\(\beta\)1 plays a major role in the decreased expression of E-cadherin. Recent studies showed a collaboration of TGF\(\beta\)1 and \(\beta\)-catenin in the induction of EMT (Peinado et al., 2004). After increased expression of the profibrotic factor TGF\(\beta\)1, phosphorylation of \(\beta\)-
catenin takes place and the junctional complex is destabilized and dissolved (Janda et al., 2006).

1.9 The p19\textsuperscript{ARF} null hepatoctye cell model
To analyse molecular mechanisms in the liver, p19\textsuperscript{ARF} null hepatocytes were established to provide a cell model for in vitro studies. Due to mitogenic inactivity of hepatocytes, genetic alterations such as lack of p19\textsuperscript{ARF} were introduced. Of great interest was the ability to generate immortalized cells which are capable to repopulate a damaged liver and therefore show some important aspects of primary hepatocytes. Knock-out studies on the p19\textsuperscript{ARF}/MDM2/p53 pathway (see Figure 9), which plays a major role in the limited life-span of primary hepatocytes, revealed a unique opportunity to circumvent cell cycle arrest and senescence of hepatocytes (Lowe & Sherr, 2003; Mikula et al., 2004; Randle et al., 2001).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{ink4a_arf.png}
\caption{The Ink4A/Arf locus (Randle et al., 2001).}
\end{figure}

These immortalized cells derived from p19 null mice show similar properties as wild type hepatocytes, including genetic stability. These cells, which were called MIM1-4, were not able to form tumors after subcutaneous injection. All immortalized cells still expressed the liver specific markers such as albumin, alfa fetoprotein, HNF\textsubscript{\alpha} and HNF4\textsubscript{\alpha} and were
sensitive to Fas-induced apoptosis (Mikula et al., 2004). Tumor formation was only established after transformation of these hepatocytes with oncogenic H-Ras. These H-Ras transformed cells were used to establish a hepatocellular tumor model in vitro for further analysis of the role of ILEI in the liver tumorigenesis.

1.10 Interleukin-like EMT Inducer (ILEI) and its role in EMT induction and cancer progression

Expression profiling of mammary epithelial tumor cells involved in EMT and metastasis revealed a cluster of 30 specific genes involved in EMT (depicted in Figure 10). Upon this, a novel gene was identified, called interleukin-like EMT inducer (ILEI), which is necessary and sufficient to cause EMT, tumorigenesis and metastasis of normal epithelial cells (Mackenzie, 2006; Waerner et al., 2006).

ILEI on its own showed no sequence homology to already known genes (Zhu et al., 2002) and as a related protein to interleukins it shows the four helix-loop-helix bundles which are important for this family. Due to this, it belongs to a family of secretory proteins, among them cytokines, chemokines and other important regulators (Balkwill, 2004). Studies on ILEI found an elevation of translated and secreted ILEI in murine mammary epithelial cells that co-express oncogenic H-Ras and undergo EMT after treatment with TGFβ (Mackenzie, 2006). For studying the involved signaling pathways associated with ILEI-induced EMT, the authors focused on the Ras-pathway and TGFβ induced EMT (Janda et al., 2002). For this purpose cell lines overexpressing ILEI were generated, which showed an induced EMT-phenotype and reverted after treatment with siRNA against ILEI (Mackenzie, 2006). Inhibitor studies on the Erk/MAPK pathway revealed a necessary activation of the Ras-signaling pathway for ILEI-dependent EMT induction. In further experiments the biological function of ILEI was found to be of great impact for the formation of tumors and metastasis. This was studied by subcutaneous injections of overexpressing ILEI-cells in nude mice. These tumors showed the characteristic downregulation of E-cadherin and upregulation of Vimentin indicating an EMT (Mackenzie, 2006; Waerner et al., 2006). The formation of secondary tumors in the lung of these mice after injection of ILEI-overexpressing cells demonstrated the involvement of ILEI in metastasis formation (Waerner et al., 2006).
Waerner et al. could further show a relationship between ILEI and the formation of carcinomas in humans. Human colon carcinomas showed an increased expression pattern of cytoplasmic ILEI, which was prominent at the invasive front (Mackenzie, 2006). It was found that strong cytoplasmic ILEI expression in breast tumors correlated with increased metastasis and poor prognosis (Mackenzie, 2006; Waerner et al., 2006). Therefore ILEI could represent a novel diagnostic marker for the progression of carcinomas (Waerner et al., 2006).

![Figure 10: Identification of ILEI (picture kindly provided by T. Waerner).](image)

**1.11 Relevance of the study**
The major goal of the PhD thesis was to address the question whether ILEI is a crucial regulatory protein in hepatocellular EMT and HCC progression. Therefore the aim was
to characterise the role of ILEI in epithelial plasticity of hepatocytes and its role in cancer progression and metastasis. Further aims included the analysis of other key signalling pathways such as Erk/MAPK, Stat3 and Wnt/β-catenin. To bridge mouse and human hepatocarcinogenesis, a human tissue array was used to analyse ILEI expression and cellular localization. These studies revealed that ILEI is crucially involved in the EMT of malignant hepatocytes in cooperation with H-Ras through the activation of PDGF-R/β-catenin and PDGF-R/Stat3.

Due to the fact that Stat3 was activated in hepatocytes after treatment with TGFβ, a possible role of Stat3 in hepatocellular tumor progression was further investigated. This analysis revealed an upregulation of c-jun and MMP-3 in Stat3-overexpressing hepatocytes. Furthermore, studies on the tumorigenesis of Stat3 expressing hepatocytes in SCID mice led to the finding that Stat3 shows a tumor suppressive role rather than a tumor promoting one, which might depend on the deficiency of the p19ARF protein. Therefore, the crosstalk between Stat3 and p19ARF could play an important role in the EMT and hepatocellular carcinoma progression.
1.12 References


2. Epithelial Plasticity of Hepatocytes During Liver Tumor Progression

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Running title: Epithelial plasticity of hepatocellular carcinoma

Abbreviations: AFP, α-fetoprotein; CK, cytokeratin; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; HCC, hepatocellular carcinoma; HNF, hepatocyte nuclear factor, HSC, hepatic stellate cell; MFB, myofibroblast; M2-PK, M2-pyruvate kinase; PDGF, platelet-derived growth factor; TGF, transforming growth factor.
Abstract

Hepatocellular carcinoma (HCC) correlates with poor survival of patients due to delayed diagnosis and frequent recurrence after adjuvant treatment. The majority of malignant hepatic lesions shows a tremendous heterogeneity in differentiation patterns and molecular signatures which challenges efficient therapeutic intervention. Here we discuss aspects on which route hepatocytes progress towards epithelial lineage commitment during liver repopulation and further resume configurations of hepatocyte differentiation during liver tumorigenesis. We particularly focus on the epithelial to mesenchymal transition (EMT) of hepatocytes and its consequences upon the progression of HCC which depends on the synergy of transforming growth factor (TGF)-β signaling and the hyperactivation of Ras-subeffectors. Recent insights into the epithelial plasticity of malignant hepatocytes revealed that activation of platelet-derived growth factor (PDGF) links TGF-β signaling to nuclear β-catenin accumulation upon EMT. PDGF-dependent activation of β-catenin reduces cellular turnover and provides protection of malignant hepatocytes against anoikis, the latter known as a prerequisite for dissemination of carcinoma and a feature of metastatic cancer stem cells. Finally, we discuss the cancer cell fate determination by integrating the repertoire of hepatocellular differentiation in a novel concept of liver carcinoma progression.
Stem cell activity in the adult liver

The liver belongs to a typical low turnover tissue which shows an enormous regenerative potential in response to resection or acute chemical injury (Michalopoulos and DeFrances, 1997). The hepatocyte represents the most abundant and versatile cell type of the liver, and is the cellular origin of HCC. Although hepatocytes are highly differentiated and quiescent under healthy conditions, they possess an astonishing proliferative capacity and are the major source for hepatocyte replacement upon liver repopulation (Fausto, 2004). Besides the regenerative potential of hepatocytes, oval cells provide a liver stem cell repository with the ability to differentiate into both hepatocytes and bile duct epithelial cells (cholangiocytes), thus representing bipotent progenitor cells (Roskams, 2006; Shafritz and Dabeva, 2002; Thorgeirsson and Grisham, 2003). In addition, bone marrow-derived hematopoietic stem cells have been proven to undergo metaplasia into cells with hepatic epithelial cell lineage capability (Lagasse et al., 2000; Petersen et al., 1999) which is caused to a certain extent by the fusion with hepatocytes (Vassilopoulos et al., 2003; Wang et al., 2003). For all different liver stem cell compartments involved in tissue homeostasis and organ repair, corresponding ex vivo cultures have been essential for the study of underlying molecular mechanisms and the subsequent treatment of liver diseases (Forbes et al., 2002; Sell, 2001).

Immortalized hepatocytes yield liver progenitor cells capable to restore the damaged liver

The access to hepatocytes with liver reconstituting activity is hindered by multiple obstacles, among them the very limited potential to expand hepatocytes in culture. Innate surveillance mechanisms linked to tumor suppressor activities are responsible for blocking cell cycle and for rapidly evoking senescence of hepatocytes ex vivo (Sell, 2001). Inactivation of the p19ARF/MDM2/p53 pathway therefore offers an unique opportunity to obtain limitless lifespan by the ablation of both cell cycle arrest and senescence (Lowe and Sherr, 2003; Sherr, 2001). On the one hand, loss of p19ARF (p14ARF in human) decreases the growth-suppressive functions of p53, while maintaining the Arf-independent activities of this protein. On the other hand, disruption of p19ARF function is sufficient to circumvent senescence without losing genetic stability.
In a recent study, we showed that immortalized hepatocytes from p19ARF null mice display a high degree of epithelial organization as detected by the expression of the polarity marker ZO-1, and the adherens junctions and desmosomal marker proteins such as E-cadherin, β-catenin, p120-catenin and desmoplakin at cell-to-cell contacts (Gotzmann et al., 2006; Mikula et al., 2004). Importantly, these p19ARF null hepatocytes, referred to as MIM cells, exhibit functional differentiation by expressing hepatic markers such as e.g. albumin, α-fetoprotein (AFP), hepatocyte nuclear factor (HNF)-1α, HNF-4α, phenylalanine hydroxylase, ApoAI and ApoAII. Typical for hepatocytes, MIM cells are sensitive to Fas-mediated apoptosis induced by the Fas antibody Jo-2, and exogenous expression of either anti-apoptotic Bcl-2 or Bcl-XL efficiently reduces cell death events (Mikula et al., 2004).

To affirm that MIM cells represent functional hepatocytes, MIM cells expressing green fluorescent protein (GFP) were orthotopically transplanted into SCID mice after Fas-dependent apoptotic liver damage. MIM-GFP donor hepatocytes contribute to liver restoration, thus being able to reconstitute the liver in vivo (Mikula et al., 2004). Transplanted MIM-GFP cells are organized in ductular structures comparable to canals of Hering, whereas a lower portion of these hepatocytes localizes in small-sized clusters as well as in isolated cells scattered throughout the entire liver. The emergence of such small-sized clusters of transplanted MIM-GFP hepatocytes might be an effect caused by the Jo-2 mediated destruction of the liver architecture. Intriguingly, donor cells organized in canals display a hepatocyte-like morphology with a high cytoplasm to nucleus ratio as well as a cholangiocyte-like phenotype with poor cytoplasmic portions (Shafrritz and Dabeva, 2002).

Transplantation of apoptosis-protected MIM-GFP-Bcl-XL hepatocytes and successive Fas-mediated injury enhances donor-derived liver restoration, providing strong evidence that p19ARF null hepatocytes are able to generate liver progenitor cells. As observed with MIM-GFP hepatocytes, most of MIM-GFP-Bcl-XL donors localize in ductular structures, suggesting the potential of MIM hepatocytes to generate progenitor cells after successive liver regeneration. To determine whether MIM-Bcl-XL donors represent hepatic precursor cells, markers characteristic for the various liver cell types were used for co-localization with the transplantation marker GFP (Vessey and De La Hall, 2001). (i) AFP and albumin for oval cells and fetal hepatocytes, (ii) M2-pyruvate...
kinase (M2-PK) for oval cells, fetal hepatocytes and cholangiocytes, and (iii) cytokeratin (CK) 19 for cholangiocytes. This analysis showed that engrafted MIM-GFP-Bcl-X\textsubscript{L} donor cells stain positive for AFP, M2-PK, albumin and CK19, which led to the conclusion that immortalized hepatocytes are capable to form bile ducts and to express markers specific for both oval cells and embryonic hepatocytes after transplantation in vivo (Mikula et al., 2004).

In the context of the liver in vivo, the majority of MIM hepatocytes reside in ductular structures comparable with canals of Hering, and display characteristics of oval cells which differentiate via alternative routes into different epithelial lineages. This observation indicates that hepatocytes are not narrowed down to generate unipotent progenies, but are still equipped to shift into a bipotential precursor compartment. These data thus point to a broader plasticity of hepatocytic cell fate determination as expected. Studies on the reversibility of hepatoblast-derived differentiation into cholangiocytes and hepatocytes in vitro and in vivo support this hypothesis (Fougere-Deschatrette et al., 2006; Notenboom et al., 2003). Hence, the accessibility of the hepatic MIM model to genetic manipulation may provide novel insights into the differentiation repertoire of hepatocytes and specification of liver progenitor cells. Furthermore, knowledge about the molecular and cellular mechanisms of malignant MIM hepatocytes is both conceptually important and relevant for the understanding of hepatocellular tumorigenesis.

**Etiology and epidemiology of liver cancer**

Hepatocellular carcinoma (HCC) accounts for more than five percent of all cancer cases and is the fifth leading cause of cancer mortality worldwide (Kensler et al., 2003). Major risk factors well define the etiology of HCC and include viral infection with hepatitis B or C, dietary exposure to the fungal toxin aflatoxin or alcohol intoxication. While hepatitis B and aflatoxin interaction are the predominant reason for HCC in Asia and Africa, hepatitis C and alcohol abuses are the most frequent causative events in Western countries. Independent of the carcinogenic insult, chronic hepatitis and cirrhosis resulting from inflammation and fibrosis are present in almost eighty percent of HCC cases worldwide (Friedman, 2004). Thus, aetiological factors which generate fibrosis and cirrhosis with a lower frequency such non-alcoholic fatty liver disorders and non-
alcoholic steatohepatitits have also a potential role in the development of HCC (Farazi and DePinho, 2006). The cirrhotic lesions that commonly proceed dysplastic HCC-like foci and nodules already display genomic alterations which further accumulate during hepatocarcinogenesis (Lee and Thorgeirsson, 2005). Due to the lack of symptoms in the early phase of tumorigenesis and the rapid cancer progression, HCC are most frequently recognized at advanced stages.

**Molecular mechanisms of HCC**

In the healthy adult liver, quiescent hepatic stellate cells (HSC) represent the major site for vitamin A storage in cytoplasmic lipid droplets. During liver injury due to e.g. viral infection, HSC get activated to myofibroblasts (MFB) and show cellular remodeling required for tissue repair and fibrogenesis (Friedman et al., 2000). These changes in cell fate vastly contribute to the establishment of liver fibrosis and subsequent cirrhosis, which frequently lead to formation of HCC. Importantly, MFB surround the malignant parenchyma intra- and peritumorally upon progression of HCC, indicating their important role during cancer progression. The most frequent molecular alterations in human HCC include the overexpression and secretion of cytokines such as transforming growth factor (TGF)-β (Breuhahn et al., 2006; Rossmanith and Schulte-Hermann, 2001), the loss of tumor suppressors such as retinoblastoma, p53, p16\(^{INK4A}\) and p14\(^{ARF}\) (p19\(^{ARF}\) in mouse, (Levy et al., 2002; Tannapfel et al., 2001)), the loss of the cell adhesion component E-cadherin (Osada et al., 1996), the induction of the Wnt signaling pathway through stabilization of nuclear β-catenin (Lee et al., 2006a), and the constitutive activation of Stat3 (Signal Transducer and Activator of Transcription; (Levy and Darnell, 2002)).

We previously established a murine hepatocellular model of tumor progression based on MMH (Met murine, (Amicone et al., 1997)) and MIM hepatocytes which reflects changes leading to a metastatic phenotype through an epithelial to mesenchymal transition (EMT, (Eger and Mikulits, 2005; Fischer et al., 2005; Gotzmann et al., 2002; Gotzmann et al., 2004)). Such changes in epithelial plasticity during liver tumor progression have also been reported in human HCC cell lines and patients (Giannelli et al., 2005; Lee et al., 2006b; Lee et al., 2006c). In our cellular models employing MMH hepatocytes expressing the cytoplasmic domain of the Met receptor or
MIM hepatocytes lacking p19ARF, EMT is caused by the collaboration of hyperactive Ras and TGF-β. The gain in malignancy provided by EMT associates with loss of E-cadherin, nuclear localization of β-catenin as well as secretion of TGF-β1 which represent hallmarks of human HCC progression (Fig. 1). Fibroblastoid hepatocytes that have undergone EMT display upregulation of platelet-derived growth factor (PDGF)-A ligand and both PDGF receptor subunits along with autocrine PDGF secretion (Gotzmann et al., 2006). In accordance with these data, the analysis of human HCCs showed enhanced expression of PDGF receptors in cancerous liver tissue compared to the adjacent parenchyma indicating a functional implication of PDGF activation in human HCC (Chen et al., 2002; Murakami et al., 2005; Tsou et al., 1998; Xu et al., 2001). Loss-of-function of PDGF signaling upon EMT by the expression of the dominant negative (dn) PDGF receptor α causes a strong suppression of tumor formation (Gotzmann et al., 2006). Intriguingly, this TGF-β-induced PDGF signaling is essentially involved in the activation and nuclear localization of β-catenin in hepatocellular EMT since interference with PDGF receptor signaling abolishes nuclear β-catenin accumulation in vivo (Fischer et al., 2006). This finding is of particular relevance since about 50% of human HCC display nuclear accumulation of β-catenin (Buendia, 2000; de La Coste et al., 1998).

**Heterogeneity and stemness of hepatocellular carcinoma**

Unexpectedly, functional validation of active β-catenin by the constitutive expression of an indestructible version of β-catenin revealed suppression of tumor growth and inhibition of proliferation in vivo, whereas intervention with β-catenin by the expression of its negative regulator Axin causes larger tumor formation (Fischer et al., 2006). Although well known target genes of β-catenin such as c-myc and cyclin D1 are expressed, the presence of the cell cycle inhibitor p16INK4A is suggested to be responsible for growth arrest. Comparable to our findings, nuclear β-catenin accumulation and concomitant expression of cyclin D1 and p16INK4A has been observed at the invasive fronts of colorectal adenocarcinomas (Bae et al., 2001).

Furthermore, active β-catenin protects malignant hepatocytes from anoikis which confers the capability to survive in suspension (Fischer et al., 2006). This mechanism
represents an important prerequisite for the survival of spreading cells after intravasation into the vasculature. Activation of β-catenin in hepatocytes that have undergone TGF-β-mediated EMT therefore provides characteristics of cell cycle arrested and moreover anoikis-resistant cancer cells. The protection against detachment-induced apoptosis point to the presence of a cancer stem cell phenotype capable to disseminate which is stabilized by β-catenin. Thus, we propose that invasive cancer stem cells result from preceding EMT and acquired stemness, both regulated by developmental pathways such as TGF-β, PDGF and β-catenin signaling.

The route on which hepatocytes progress in malignancy is of particular importance for the understanding of HCC. Recent concepts on the tumor stem cell have been provided in breast cancer, glioblastoma and acute myeloid leukaemia, which suggest that the potential to disseminate are features of cancer stem cells rather than of differentiated tumor cells (Pardal et al., 2003). A conceptual extension is provided by recent studies which point to the presence of “migrating cancer stem cells” (Brabletz et al., 2005). These cells localizing at the invasive front of colorectal carcinoma have undergone EMT, a transient and reversible hallmark of disseminating tumor cells. They show growth arrest with a concomitant accumulation of the Wnt signaling component β-catenin, indicating reactivation of developmental programes. With respect to the liver, it is still a matter of debate whether hepatic cancer stem cells exist.

We hypothesize that different patterns of nuclear β-catenin occur, depending on the localization of neoplastic cells in the tumor and correlating with variations in the differentiation state of malignant hepatocyte subpopulations. At the inner area of the cancerous tissue, neoplastic hepatocytes might have low or are even devoid of nuclear β-catenin and exhibit proliferation. In contrast, malignant hepatocytes that have undergone EMT at the invasive front might harbor high amounts of nuclear β-catenin expression accompanied by growth arrest. We therefore propose a broad differentiation repertoire of neoplastic hepatocytes through changes in epithelial plasticity (Fig. 1). The fate of this liver tumor stem cell repository might be controlled by the tumor microenvironment which on the one hand directs dissemination of liver carcinoma cells at the tumor-host interface, and on the other hand induces differentiation of malignant cells at the inner area of the tumor. So far, genetic profiling of HCCs and respective
animal models are promising to molecularly unravel this enigma (Lee and Thorgeirsson, 2005; Thorgeirsson and Grisham, 2002). Within this context, a subpopulation of HCC cells has been identified to show upregulation of particular “stemness genes” in cancer cells (Chiba et al., 2006).

**Tumor microenvironment and HCC progression**

We recently investigated the interaction between malignant hepatocytes and activated HSC or MFB, the latter representing the main constituents of the tumor-associated connective tissue (Pinzani et al., 2005). In addition to MIM hepatocytes, we established p19ARF deficient immortalized HSC which show proper expression of characteristic marker proteins of activated HSC such as α-smooth muscle actin, glial fibrillary acidic protein, pro-collagen I and desmin (Proell et al., 2005). Most notably, these non-tumorigenic p19ARF null HSC undergo a further transition to MFB in vitro upon treatment with TGF-β, and thus provide a suitable cellular tool to analyze the molecular and cellular mechanisms involved in liver fibrogenesis.

By simultaneous xenografting of these well characterized hepatic cellular models in vivo, we showed that paracrine feedback mechanisms governed by activated HSC and MFB strongly affect the malignant progression of neoplastic hepatocytes through (i) induction of active TGF-β signaling, (ii) nuclear accumulation of β-catenin, and (iii) abrogation of E-cadherin mediated cell-to-cell contacts (Mikula et al., 2006). Genetic intervention with TGF-β signaling leading to loss of paracrine TGF-β regulation in neoplastic hepatocytes confirmed this finding. Hence, these data indicate that the tumor-progressive TGF-β signaling is induced by paracrine regulation in hepatocytes, and molecularly linked to activation of β-catenin. Extrapolation of these findings with recent data supports clarification of the scenario, how paracrine and autocrine TGF-β regulation of hepatocytes and non-parenchymal liver cells are executed (Fig. 2). Tumor-associated macrophages (TAM) comparable to activated Kupffer cells produce TGF-β which stimulates activated HSC to complete the transition to MFB. TAM, activated HSC and MFB each on its own secrete TGF-β, and positively regulate the progression of neoplastic hepatocytes in a paracrine fashion. Hepatocytes proceeding in tumorigenesis and displaying an EMT-like, invasive signature produce TGF-β themselves in an
autocrine fashion (Gotzmann et al., 2002). Since HCC develops from chronically injured liver involving stimulation of Kupffer cells and activation of HSC to MFB, these non-parenchymal cells are the major source of TGF-β, and thus being predominantly responsible for the progression of initiated tumor nodes. Future studies will focus on the determination of paracrine regulatory loops between cancerous cells and the host in order to unravel the molecular framework of the tumor-host crosstalk in the liver.

**Conclusion**

Activation of β-catenin in hepatocytes that have undergone TGF-β mediated EMT confers characteristics of cell cycle arrested and anoikis-resistant cancer cells. These features point to the presence of a dormant cancer stem cell phenotype in HCC which is stabilized by β-catenin and is involved in proximal and distal colonization. The quiescence of a subpopulation of liver tumor cells might render them more resistant to standard chemotherapy that targets proliferating cells and could therefore be responsible for post-operative disease recurrence, as often observed in HCC. These aspects implicate important consequences for therapeutic intervention of aggressive HCCs, suggesting TGF-β and PDGF as promising targets for anti-cancer drug development.
Acknowledgment

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References


Figure Legends

**Figure 1:** Linear view on the differentiation patterns of neoplastic hepatocytes and the heterogeneity of HCCs. Malignant hepatocytes at the tumor center show features of epithelial differentiation which is progressively lost towards the tumor-host border. Activated myofibroblasts (MFB) mainly presenting the tumor microenvironment provide TGF-β in a paracrine mode of regulation. A gradient in the amount of available TGF-β induces changes in the epithelial plasticity of neoplastic hepatocytes. Malignant hepatocytes close to the tumor-host interface are exposed to bulk TGF-β and undergo an EMT which is associated with an autocrine regulation and secretion of TGF-β and PDGF. The stroma compartment is boxed in light gray (upper panel), whereas tumor cells are trapezoidly boxed in darker gray (lower panel). The dashed line marks the tumor-host boundary.

**Figure 2:** The involvement of TGF-β in hepatocellular tumor-stroma interaction. The liver tumor microenvironment mainly consists of tumor-associated macrophages (TAM), activated hepatic stellate cells (HSC) and myofibroblasts (MFB). TAM secrete TGF-β which stimulates HSC to transdifferentiate in MFB. TGF-β produced by TAM and MFB induces the malignant progression of hepatocytes by changes in epithelial plasticity. Molecular and cellular events corresponding to the non-parenchymal tumor-stroma compartment are boxed in light gray (upper panel). Neoplastic hepatocytes undergo an EMT in response to TGF-β and are finally endowed with autocrine TGF-β signaling (trapezoidly boxed in darker gray, lower panel). Red arrows, paracrine TGF-β regulation; wended red arrows, autocrine TGF-β secretion; green to orange shaded arrows; activation of HSC to MFB; blue to purple shaded arrows, malignant progression of hepatocytes by EMT. The dashed line marks the tumor-host boundary.
Figure 1: Linear view on the differentiation patterns of neoplastic hepatocytes and the heterogeneity of HCCs
Figure 2: The involvement of TGF-β in hepatocellular tumor-stroma interaction
3. ILEI requires oncogenic Ras for the epithelial to mesenchymal transition of hepatocytes and liver carcinoma progression

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Abstract
In human hepatocellular carcinoma (HCC), epithelial to mesenchymal transition (EMT) correlates with aggressiveness of tumors and poor survival. We employed a model of EMT based on immortalized p19ARF null hepatocytes (MIM), which display tumor growth upon expression of oncogenic Ras and undergo EMT through the synergism of Ras and transforming growth factor (TGF)-β. Here we show that the interleukin-related protein ILEI, a novel EMT-, tumor- and metastasis-inducing protein, cooperates with oncogenic Ras to cause TGF-β-independent EMT. Ras-transformed MIM hepatocytes overexpressing ILEI showed cytoplasmic E-cadherin, loss of ZO-1 and induction of α-smooth muscle actin as well as platelet-derived growth factor (PDGF)/PDGF-R isoforms. As shown by dominant-negative PDGF-R expression in these cells, ILEI-induced PDGF signaling was required for enhanced cell migration, nuclear accumulation of β-catenin, nuclear pY-Stat3 and accelerated growth of lung metastases. In MIM hepatocytes expressing the Ras mutant V12-C40, ILEI collaborated with PI3K signaling resulting in tumor formation without EMT. Clinically, human HCC samples showed granular or cytoplasmic localization of ILEI correlating with well and poorly differentiated tumors, respectively. In conclusion, these data indicate that ILEI requires cooperation with oncogenic Ras to govern hepatocellular EMT via mechanisms involving PDGF-R/β-catenin and PDGF-R/Stat3 signaling.
Introduction

Hepatocellular carcinoma (HCC) accounts for 5.5% of all cancer cases worldwide (Kensler et al., 2003). Due to the aggressive behavior and the poor prognosis at advanced stages, HCC belongs globally to the third leading cause of cancer mortality (Bruix et al., 2004). HCC arise through abnormal proliferation and dedifferentiation of hepatocytes, caused by hepatitis B or C virus infections, dietary exposure to fungal hepatotoxins or alcohol intoxication (Thorgeirsson and Grisham, 2002). Most frequently occurring molecular alterations in HCC include the (i) loss of tumor suppressors involving p53, pRB, cyclins/cdk5 or the CDKN2A-encoded proteins p14ARF and p16INK4a (El-Serag and Rudolph, 2007; Tannapfel et al., 2001), (ii) loss of the cell-cell adhesion protein E-cadherin (Kondoh et al., 2001), (iii) activation of oncoproteins – e.g. receptor tyrosine kinases – causing strongly enhanced Erk/MAPK and PI3K signaling (Breuhahn et al., 2006), (iv) nuclear accumulation of Wnt/β-catenin (Lee et al., 2006a), and (v) aberrant regulation and secretion of cytokines such as transforming growth factor (TGF)-β (Rossmanith and Schulte-Hermann, 2001). While the knowledge of mechanisms involved in HCC is rapidly growing (Herath et al., 2006), the molecular pathogenesis of HCC is still poorly understood.

Epithelial to mesenchymal transition (EMT) is a developmental event increasingly recognized as a central process during cancer progression and metastasis (Grunert et al., 2003; Hugo et al., 2007; Thiery and Sleeman, 2006). Multiple molecular mechanisms have been identified to induce EMT (Huber et al., 2005), including TGF-β/TGF-βRI signaling which collaborates with other signaling effectors to disintegrate tight junctions and E-cadherin/β-catenin complexes at cell-cell contacts (Pardali and Moustakas, 2007; Thiery and Sleeman, 2006). In human HCC, Laminin-5 and TGF-β1 have been described to cooperatively induce EMT at the invasive front of metastatic tumors (Giannelli et al., 2005). In addition, signal transducer and activator of transcription (Stat)5b collaborates with the hepatitis B oncoprotein HBX to cause EMT and invasiveness of HCC (Lee et al., 2006b).

To study EMT in hepatocytes, we employed mouse MMH hepatocytes immortalized by transgenic expression of cyto-Met (Gotzmann et al., 2002) or a unique model of hepatocytes derived from p19ARF null mice. While wildtype mouse hepatocytes cannot be expanded due to mitotic inactivity and rapid dedifferentiation, p19ARF deficient MIM1-4
hepatocytes proliferate and show characteristics of normal hepatocytes such as expression of albumin and the ability to restore the liver after injury (Mikula et al., 2004). In both MMH and MIM1-4 hepatocytes, the synergistic action of oncogenic Ras and TGF-β signaling induces EMT resulting in a highly malignant, invasive phenotype (Gotzmann et al., 2006; Gotzmann et al., 2002). Studies of EMT in MIM hepatocytes by employing Ras subeffector mutants revealed that Erk/MAPK signaling is sufficient to induce and maintain hepatocellular EMT in cooperation with TGF-β. On the contrary, the selective activation of PI3K signaling by expression of V12-C40-Ras showed lack of EMT and tumor formation (Fischer et al., 2005). Furthermore, the molecular collaboration between oncogenic Ras and TGF-β signaling causes the upregulation of platelet-derived growth factor (PDGF)/PDGF-R components which are responsible for the nuclear accumulation of β-catenin, the latter representing a hallmark of human HCC (Fischer et al., 2007).

Recently, expression profiling of polysome-bound mRNA revealed ILEI as a novel regulator of EMT (interleukin-1-like EMT inducer; Waerner et al., 2006). In the mammary EpH4/EpRas model, overexpression of ILEI causes EMT, tumor growth and metastasis upon tail vein injection. Moreover, RNAi-mediated suppression of ILEI prevents EMT and metastasis in various murine and human cellular models (Waerner et al., 2006), suggesting that ILEI is both necessary and sufficient for these processes. Importantly, ILEI localization in cytoplasmic particles was identified as an independent parameter predictive for metastasis development and shortened survival of breast cancer patients. Cytoplasmic ILEI localization was also observed in nuclear β-catenin-positive tumor cells which have undergone EMT at the invasive front of colon carcinomas (Waerner et al., 2006).

In this study we analyzed the function of ILEI in MIM hepatocytes and determined, how ILEI affects EMT and liver carcinogenesis. We found that ILEI required the cooperation with oncogenic Ras to induce and maintain EMT of hepatocytes in a TGF-β-independent fashion. Ras/ILEI expressing MIM hepatocytes exhibited upregulation of PDGF/PDGF-R associated with Stat3 activation and nuclear accumulation of β-catenin. In human HCC samples, cytoplasmic localization of ILEI correlated with poor differentiation and prognosis as recently observed in breast cancer patients.
Results

**ILEI is expressed and differentially localized during TGF-β-dependent EMT of hepatocytes**

First, we analyzed the expression of endogenous ILEI by immunohistochemistry in experimental tumors derived from epithelial hepatocytes expressing oncogenic Ras (MIM-R-GFP) and the same cells after EMT upon long-term treatment with TGF-β1, resulting in fibroblastoid MIM-RT cells (Fischer et al., 2007). ILEI was hardly detectable in normal liver, whereas prominent expression of ILEI was observed in orthotopic tumors formed by MIM-R-GFP and MIM-RT cells (Figure 1a). Moderately differentiated tumors generated by epithelial MIM-R-GFP cells showed granular staining of ILEI (Figure 1a). In contrast, poorly differentiated tumors generated from fibroblastoid MIM-RT cells exhibited cytoplasmic staining of ILEI (Figure 1a). Furthermore, pulmonary metastatic colonies of fibroblastoid MIM-RT cells after tail vein injection revealed a mixed cytoplasmic and granular localization of ILEI, suggesting a partial reversal of EMT in metastatic nodules. From these data we concluded that ILEI is expressed in mouse hepatocellular carcinoma but not in normal liver, and is subject to differential localization during EMT and cancer progression.

**ILEI collaborates with oncogenic Ras to induce and maintain EMT of hepatocytes**

We assessed the role of ILEI in hepato cellular EMT by its stable overexpression in immortalized MIM1-4 hepatocytes, either alone (MIM1-4-ILEI), together with oncogenic Ras (MIM-R-ILEI) or in cooperation with the PI3K-hyperactivating Ras mutant V12-C40 (MIM-C40-ILEI). Overexpression of total ILEI was verified by Western analysis, showing a 2-4 fold increase in all cell types (Figure 1b).

ILEI expression on its own or together with V12-C40-Ras did not result in significant morphological changes in culture (Figure 2), neither in sparse nor dense monolayers (Supplementary Figure S1a). ILEI overexpression in oncogenic Ras-transformed MIM-R-GFP hepatocytes, however, induced a shift from an epithelial to a fibroblastoid morphology, regardless of cell density (Figure 2 and Supplementary Figure S1b), similar
to MIM-RT cells having undergone Ras/TGF-β-mediated EMT (Supplementary Figure S1b).

In line with their epithelial morphology, MIM1-4-ILEI hepatocytes showed plasma membrane localization of the epithelial markers ZO-1, E-cadherin and β-catenin similar to MIM1-4-GFP control cells, suggesting intact epithelial cell-cell contacts (Supplementary Figure S2a). Furthermore, ILEI did neither affect β-actin localization nor elevate the mesenchymal marker α-smooth muscle actin (α-SMA) expression (Supplementary Figure S2b). In addition, ILEI failed to induce TGF-β/Smad signaling since Smad2/Smad3 were entirely cytoplasmic in both MIM1-4-GFP and MIM1-4-ILEI cells. Yet, MIM1-4-ILEI cells showed a weak increase of active, nondestructible β-catenin in nuclei (Supplementary Figure S2a).

Coexpression of ILEI and V12-C40-Ras in MIM1-4 cells (MIM-C40-ILEI) resulted in a similar marker expression as in MIM1-4-ILEI hepatocytes, i.e. plasma membrane localization of ZO-1, E-cadherin, β-catenin and β-actin (Figure 2). MIM-C40-ILEI cells, however, displayed elevated nuclear accumulation of β-catenin as compared to MIM1-4-GFP cells as well as enhanced expression of α-SMA (Supplementary Figure S3). In contrast, ILEI plus oncogenic Ras in MIM-R-ILEI cells led to a stable EMT phenotype resembling the one observed in MIM-RT cells (Fischer et al., 2005). MIM-R-ILEI cells showed a fibroblastoid morphology, strong reduction of ZO-1, cytoplasmic redistribution of E-cadherin and relocalization of β-actin from the plasma membrane to stress fibers (Figure 2). Furthermore, MIM-R-ILEI cells showed strong nuclear accumulation of β-catenin (Figure 2) and enhanced expression of α-SMA (Supplementary Figure S3). In addition, ILEI induced a partial shift of nuclear to cytoplasmic Smad2/Smad3 localization in both MIM-C40-GFP and MIM-R-GFP cells (Supplementary Figure S3), suggesting that TGF-β/Smad signaling is inhibited rather than activated. This independence of ILEI action from TGF-βR signaling was confirmed by (i) the reduction of secreted TGF-β levels by ILEI in all cell types (Supplementary Figure S4), (ii) the inability of the TGFβ-RI/II kinase inhibitor LY02109761 [Lacher, 2006 #3213] to reverse MIM-R-ILEI cells to an epithelial phenotype (data not shown), and (iii) the absence of reporter activities in MIM-R-ILEI cells after transfection of Smad2- or Smad3-dependent Luciferase.
constructs (data not shown). These results demonstrate that ILEI requires oncogenic Ras but not TGF-β/Smad signaling to induce and maintain hepatocellular EMT in vitro.

**ILEI stimulates upregulation of PDGF-C/PDGF-Rβ and enhances proliferation and migration in oncogenic Ras-expressing MIM-R-GFP hepatocytes**

Next, we analyzed the expression of several EMT-specific genes in MIM-R-GFP, MIM-R-ILEI and MIM-RT cells. Proteins strongly reduced (E-cadherin) or elevated (MMP-9) in MIM-RT cells showed weaker down- or upregulation in MIM-R-ILEI cells, respectively (Figure 3a). With the exception of Slug, the EMT-inducing transcription factors Twist (Yang et al., 2004) and ZEB1/Δ-EF1 (Eger et al., 2005) were induced in MIM-R-ILEI cells, but surprisingly not in MIM-RT cells (Figure 3a). Since an autocrine loop of PDGF/PDGF-R is essential for hepatocellular EMT (Gotzmann et al., 2006) and the EMT in EpH4/EpRas cells (Jechlinger et al., 2006), we focused on the effect of exogenous ILEI on the expression of different PDGF and PDGF-R isoforms in these cells. Interestingly, ILEI induced a strong increase of PDGF-C in MIM1-4-ILEI, MIM-C40-ILEI or MIM-R-GFP-ILEI cells (Figure 3b). In contrast, the cooperation of ILEI and oncogenic Ras in MIM-R-ILEI cells was necessary to induce PDGF-Rβ expression, which was very low in MIM-R-GFP cells and undetectable in MIM1-4-GFP and MIM-C40-GFP hepatocytes expressing or lacking exogenous ILEI. These findings suggest an involvement of an autocrine PDGF-C/PDGF-Rβ loop in ILEI-induced EMT of MIM-R-GFP hepatocytes.

We further assessed the effect of exogenous ILEI and PDGF/ PDGF-R signaling on the proliferation of parental MIM1-4-GFP and MIM-R-GFP hepatocytes. MIM1-4-GFP and MIM-R-GFP cells proliferated with doubling times of 23 hours and 15 hours, respectively (Figure 4a and 4b), while exogenous ILEI expression hardly altered doubling times (MIM1-4-ILEI, 22 hours; MIM-R-ILEI, 15 hours). Expression of a dominant-negative (dn) PDGF-R mutant, however, strongly inhibited proliferation of MIM-R-ILEI cells (doubling time of MIM-R-ILEI-dnP cells, 31 hours; Figure 4a). Control MIM-R-GFP-dnP hepatocytes expressing the dn PDGF-R showed proliferation kinetics comparable to MIM-R-GFP hepatocytes as described recently (data not shown; (Fischer et al., 2007)).
In line with the TGF-β-independent function of ILEI in hepatocytes described above, MIM1-4-GFP and MIM1-4-ILEI cells underwent proliferation arrest and cell death with similar kinetics. TGF-β, however, inhibited early proliferation of MIM1-4-GFP cells (during the first 5 cell divisions) more strongly than of MIM1-4-ILEI cells (Figure 4b). Therefore, active TGF-β/Smad signaling causes growth arrest and cell death in MIM1-4 cells lacking or expressing ILEI, except that ILEI may exert early and transient protective effects on proliferation. In contrast, Ras-expressing MIM-R-ILEI and MIM-R-GFP-hepatocytes were both protected from TGF-β-dependent cell cycle arrest and cell death, showing exponential proliferation in the presence and absence of TGF-β (Figure 4c; data not shown).

Another parameter important in EMT is the migratory potential of cells as analyzed by Transwell filters. Interestingly, ILEI enhanced the migration not only of MIM-R-GFP hepatocytes (as expected from their fibroblastoid phenotype) but also of MIM-C40-GFP cells (Figure 4d). This correlates with the fact that MIM-C40-ILEI but not MIM-C40-GFP cells are tumorigenic (see below). This ILEI-dependent increase in migratory activity also required autocrine PDGF/PDGF-R signaling since its blockade by dn PDGF-R in MIM-R-ILEI-dnP cells reduced migration to control levels in MIM-R-GFP cells (Figure 4d). From these data we concluded that PDGF-R signaling enhanced both cell proliferation and migration of hepatocytes that have undergone Ras/ILEI-mediated EMT.

The collaboration of ILEI with oncogenic Ras enhances tumor formation, nuclear accumulation of β-catenin and Stat3 tyrosine phosphorylation

We next investigated the tumorigenicity of ILEI-overexpressing hepatocytes and found that exogenous ILEI expression in MIM-1-4-ILEI cells did not cause tumor growth (data not shown). Overexpression of ILEI in MIM-R-GFP (MIM-R-ILEI) cells, however, resulted in a 4.5-fold increase of tumor size irrespective of the absence or presence of dn PDGF-R (MIM-R-ILEI-dnP cells; Figure 5a). Tumor growth of control MIM-R-GFP-dnP hepatocytes expressing the dn PDGF-R was slightly lower as compared to MIM-R-GFP cells (data not shown; (Fischer et al., 2007; Gotzmann et al., 2006)). Interestingly, ILEI induced tumorigenicity of MIM-C40-GFP hepatocytes (Figure 5b), which are strictly nontumorigenic in the absence of ILEI (Figure 5b, (Fischer et al., 2005)). Remarkably,
tumors derived from MIM-C40-ILEI cells showed a delay in tumor formation (detectable after 14 days) and a slower growth rate as compared to MIM-R-ILEI-induced tumors but reached similar volumes and tumor weights as MIM-R-ILEI tumors (Figure 5c).

Immunohistochemical analysis of sections from epithelial MIM-R-GFP- and MIM-R-GFP-dnP-derived tumors showed that E-cadherin and β-catenin localized at cell boundaries of most tumor cells, suggesting the presence of functional cell adhesion complexes (Figure 6a). In contrast, tumors generated from MIM-R-ILEI cells showed aspects of EMT *in vivo* such as cytoplasmic E-cadherin, partial loss of plasma membrane-localized β-catenin and strong nuclear staining of β-catenin (28% of cells; Figure 6a and 6b). Although MIM-R-ILEI and MIM-R-ILEI-dnP-induced tumors resulted in comparable volumes (Figure 5a), intervention with PDGF-R signaling in MIM-R-ILEI-dnP tumors caused reexpression of plasma membrane-localized E-cadherin and reduced β-catenin nuclear translocation (Figure 6a). The restoration of E-cadherin expression at cell borders of MIM-R-ILEI-dnP cells was also prominent in cell culture when compared to MIM-R-ILEI cells (Supplementary Figure S5). In conclusion, ILEI in collaboration with Ras induced a similar PDGF-R-dependent EMT phenotype as observed in MIM-RT tumors, i.e weak, cytoplasmic E-cadherin staining and accumulation of nuclear β-catenin generated by oncogenic Ras plus prolonged TGF-β exposure (Supplementary Figure S6; (Fischer et al., 2007).

PDGF-R signaling causes tyrosine phosphorylation of Stat3 (pY-Stat3) via Src activation (Garcia et al., 2001). Thus, we analyzed the above tumor types for pY-Stat3 and total Stat3 as well as ILEI expression. Interestingly, MIM-R-ILEI-induced tumors (cytoplasmic ILEI staining) showed intense nuclear staining for pY-Stat3 in 27% of cells, while only 8% and 11% of cells in MIM-R-GFP and MIM-R-GFP-dnP tumors (both granular ILEI staining) showed nuclei positive for pY-Stat3, respectively (Figure 7a and 7b). As expected, blockade of PDGF-R signaling in MIM-R-ILEI-dnP cells reduced pY-Stat3 expression to intermediate levels and still showed cytoplasmic ILEI staining (Figure 7a), in line with their unaltered tumorigenicity (Figure 5a). In contrast, immunohistochemical staining for total Stat3 was similar in all three cell types (Figure 7a and 7b). These data show that the Ras/ILEI induced upregulation of PDGF/PDGF-R isoforms causes nuclear accumulation of both activated β-catenin and pY-Stat3.
ILEI enhances the growth rate of MIM-R-GFP-derived metastatic colonies

We further asked whether ILEI overexpression enhances metastasis of MIM-R-GFP hepatocytes and whether metastasis requires PDGF-R signaling. Thus, we analyzed the effect of ILEI overexpression on metastasis and its requirement for PDGF-R signaling in MIM-R-GFP cells already causing liver tumors which metastasize to the lung. MIM-R-GFP, MIM-R-GFP-dnP, MIM-R-ILEI or MIM-R-ILEI-dnP cells were injected into the tail vein of SCID mice and further analyzed for lung colonization. While the frequency of lung metastases was similar in the four groups of mice, the size of lung metastases was slightly enhanced in mice receiving MIM-R-ILEI cells but strongly reduced in those injected with MIM-R-ILEI-dnP cells (Supplementary Figure S7a and S7b). MIM-R-ILEI-dnP cells showed almost no metastases with more than 100 cells while about 40% of MIM-R-ILEI-derived metastases contained >100 cells (Supplementary Figure S7b). In addition, immunohistochemical staining of sections from these metastases revealed that ILEI was predominantly localized in the cytoplasm of MIM-R-GFP and MIM-R-ILEI cells, while MIM-R-ILEI-dnP-derived metastases showed also granular areas overlapping with nuclei (Supplementary Figure S7c). These data suggest that ILEI-induced PDGF signaling increases the growth rate of metastatic colonies from hepatocytes expressing oncogenic Ras.

Cytoplasmic localization of ILEI correlates with tumor dedifferentiation in human HCCs

Recently, it has been described that cytoplasmic expression of ILEI was strongly enhanced in metastatic breast carcinomas and tumor-host borders of invasive colon carcinomas (Waerner et al., 2006). In an attempt to bridge mouse and human hepatocarcinogenesis with respect to the importance of ILEI expression and localization for HCC patient prognosis, a tissue array covering 69 human HCC samples was immunohistochemically analyzed. ILEI staining intensities were distinguished between no, weak or strong staining (Figure 8a). In addition, we further discriminated between granular (G) and cytoplasmic staining (C) to evaluate the intracellular localization of ILEI (Figure 8a). The important parameter yielding a clear result was postoperative, histological grading (pG0 to pG3) which is described to predict good to bad prognosis (Edmondson and Steiner, 1954; Zhou et al., 2007). Granular staining was not predictive for differentiated HCC with good prognosis. Interestingly, moderately or strongly

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dedifferentiated tumors (pG2-pG3 grades) were positive for strong cytoplasmic staining at a higher frequency (66%) than pG0 (28%). In contrast, weak cytoplasmic ILEI staining predicted pG0 (72%) than dedifferentiated tumors (pG2-pG3; 34%). These data suggest that strong cytoplasmic ILEI staining can predict poor differentiation and prognosis of human HCC.
Discussion

We used p19ARF deficient hepatocytes as a physiologically relevant model to analyze the function of ILEI during hepatocarcinogenesis. Here, we demonstrate that ILEI strictly requires oncogenic Ras to cause TGF-β-independent hepatocellular EMT and tumor progression of hepatocytes. In cooperation with hyperactivated PI3K signaling, exogenous ILEI expression did not affect the epithelial phenotype of hepatocytes, but induced migratory and tumorigenic abilities. ILEI plus oncogenic Ras induced both PDGF-C/PDGF-Rβ, expression which associated with nuclear accumulation of β-catenin and pY-Stat3. Using a dominant-negative PDGF-R mutant, we showed that PDGF-R signaling is causally involved in Ras/ILEI mediated hepatocellular EMT. Analysis of a human HCC tissue array suggested a correlation between dedifferentiation / poor prognosis with overexpression of cytoplasmically localized ILEI.

Human hepatocarcinogenesis involves ubiquitous, hyperactive Ras signaling as a central event caused by epigenetic silencing of Ras inhibitory proteins (e.g. RASSF1A, NORE1A; (Calvisi et al., 2006; Macheiner et al., 2006). Accordingly, oncogenic Ras and exogenous TGF-β cooperate in normal MIM1-4 hepatocytes to induce and maintain EMT, resulting in an invasive, malignant phenotype which associated with autocrine TGF-β signaling (Fischer et al., 2005). This study demonstrates that exogenous ILEI requires cooperation with oncogenic Ras to cause EMT and hepatocellular carcinoma progression in normal MIM1-4 hepatocytes since overexpression of ILEI on its own failed to induce changes in morphology, loss of epithelial markers, migration or tumorigenicity in MIM1-4 cells (Figures S1a and S2a). In nontumorigenic mammary EpH4 cells, however, ILEI alone was sufficient to induce reversible, density-dependent EMT, tumor growth and lung metastasis after tail vein injection (Waerner et al., 2006). In contrast to the mammary EpH4 model, exogenous ILEI also failed to induce EMT and metastasis in the nontumorigenic MIM-C40 cells, but rendered them migratory and tumorigenic. These results indicate that oncogenic Ras, which causes constitutive hyperactivation of multiple signaling pathways, is strictly required by ILEI to induce EMT and tumor progression in hepatocytes. A possible reason why ILEI plus V12-C40-Ras caused EMT, tumor and metastasis formation in mammary EpC40-ILEI cells could be the fact that these cells show ILEI-enhanced phospho-Erk/MAPK signaling, perhaps
cooperating with PI3K hyperactivation to induce EMT. Thus, ILEI probably activates endogenous Ras, since the Ras inhibitor L739.749 abolished EMT in mammary EpC40-ILEI cells (Waerner et al., 2006).

A major difference concerning EMT induction by Ras/ILEI versus Ras/TGF-β in both hepatocytes and mammary cells was that ILEI-induced EMT neither involved nor required autocrine TGF-β secretion. In contrast to mammary cells, MIM1-4-GFP, MIM-C40-GFP and MIM-R-GFP cells secreted TGF-β at rather high levels which were 2-4-fold reduced by ILEI expression (Figure S4). In this line, MIM-C40-GFP and MIM-R-GFP cells showed nuclear localization of Smad2/3, which was partially shifted to a cytoplasmic localization in MIM-C40-ILEI and MIM-R-ILEI cells (Figure S3). These cells were also negative for Smad2- and Smad3-dependent activation of luciferase reporter constructs (data not shown). Importantly, the TGF-βRI/II kinase inhibitor LY02109761 failed to revert EMT in MIM-R-ILEI cells (data not shown), suggesting that ILEI-induced EMT of Ras expressing hepatocytes occurs independently of TGF-β activation, a finding also valid for ILEI-induced EMT in mammary epithelial cells (Waerner et al., 2006).

Recently, we showed that TGF-β/Ras-induced hepatocyte EMT led to activation of PDGF/PDGFR signaling which triggers β-catenin to nuclear translocation (Fischer et al., 2007). Here we demonstrate that Ras/ILEI-induced EMT involved upregulation of PDGF-C and PDGF-Rβ, while epithelial MIM-1-4-ILEI and MIM-C40-ILEI cells expressed PDGF-C, but not PDGF-Rβ (Figure 3b). This suggests that an autocrine PDGF-C/PDGFRβ loop is important in Ras/ILEI-induced tumor progression, which is supported by the finding that transgenic PDGF-C expression in hepatocytes caused fibrosis and subsequent HCC (Campbell et al., 2005; Campbell et al., 2007). Bioactive PDGF-CC ligand showed high-affinity-binding to PDGF-Rα/α and PDGF-Rα/β dimers, inducing similar phospho-Erk1/2 levels as PDGF-AB (Pietras et al., 2003). Our idea that ILEI plus Ras-induced autocrine PDGF-C/PDGFRβ signaling could activate β-catenin was supported by the finding that a dominant-negative PDGF-Rα mutant (dnP) – inactivating both PDGF-Rα/α and PDGF-Rα/β dimers – was sufficient to reduce nuclear β-catenin levels both in vitro (data not shown) and in vivo (Figure 6). Our observation, however, that dnP could not suppress nuclear β-catenin levels of MIM-R-ILEI tumors down to
those observed in MIM-R-GFP-derived tumors, suggests additional mechanisms
contributing to nuclear β-catenin activation.

Our finding that Stat3 activation caused by PDGF-R signaling contributes to
Ras/ILEI-induced hepatocellular EMT is supported by reports that silencing of the Stat3-inhibitory proteins SOCS1 or SOCS3 by methylation promotes HCC formation by
stimulating tumor cell proliferation, survival, migration and angiogenesis (Ogata et al.,
2006a; Ogata et al., 2006b; Yang et al., 2007; Yoshikawa et al., 2001). Furthermore,
avtivation of Ras and Stat3 by epigenetic inactivation of their endogenous protein
inhibitors was ubiquitously detected in human HCC (Calvisi et al., 2006). Thus, the
induction of pY-Stat3 by PDGF signaling during Ras/ILEI-induced EMT could be
particularly relevant for HCC progression. Whether PDGF-R signaling causes
phosphorylation of Stat3 via Jak kinases or Src, the latter shown to act as a Stat3 kinase
downstream of PDGF-R signaling (Silva, 2004), remains to be clarified.

In this study, the novel EMT-inducer ILEI is shown to cause EMT and tumor
progression in normal hepatocytes expressing oncogenic Ras, suggesting that ILEI
substitutes for tumor-progressive functions of TGF-β signaling in the liver. Since MIM1-4
hepatocytes are more comparable to normal cells than the murine EpH4 or human
HMEC mammary epithelial cell models, it was not entirely unexpected that ILEI required
oncogenic Ras to cause EMT and metastasis in MIM1-4 cells. Yet, ILEI was sufficient for
EMT induction in the EpH4 model through cooperation with chemokine-dependent
activation of endogenous Ras. The requirement of Ras/ILEI to cause EMT and tumor
progression in hepatocytes, together with a putative intracellular mechanism of ILEI
action, renders ILEI comparable to a rapidly growing number of epithelial polarity/vesicle
trafficking regulators in Drosophila, which in cooperation with Drosophila Ras cause
lethal, invasive larval tumors (Giebel and Wodarz, 2006).
Materials and Methods

Cell culture

MIM-R and MIM-C40 hepatocytes were generated by stable retroviral transmission of MIM1-4 cells with oncogenic v-Ha-Ras or C40-V12-Ras, respectively (Fischer et al., 2005). MIM1-4-GFP, MIM-R-GFP and MIM-C40-GFP cells were obtained by stable retroviral transmission of MIM1-4, MIM-R and MIM-C40 cells with pMSV-green fluorescent protein (GFP), respectively, and subsequent cell sorting. MIM1-4-ILEI, MIM-R-ILEI, and MIM-C40-ILEI cells were generated by stable transmission of MIM1-4, MIM-R and MIM-C40 cells with pMSV harboring bicistronic ILEI and GFP (Waerner et al., 2006), respectively, and subsequent cell sorting. To stably interfere with PDGF signaling, MIM-R-ILEI cells were transmitted with pMSV bicistronically expressing the dominant-negative (dn) PDGF-receptor α and red fluorescent protein (Fischer et al., 2007), resulting in MIM-R-ILEI-dnP cells. Details about culture conditions of MIM hepatocytes are available as Supplementary Information on the Oncogene website.

Western blot analysis
Details are available as Supplementary Information on the Oncogene website.

Confocal immunofluorescence microscopy
Cells were fixed and permeabilized as described earlier (Gotzmann et al., 2006). Primary antibodies were used at following dilutions: anti-β-catenin (BD Transduction Laboratories (TL), Lexington, UK), 1:100; anti-active-β-catenin (Upstate, Lake Placid, USA), 1:100; anti-E-cadherin (TL), 1:100; anti-α-smooth muscle actin (Dako, Carpinteria, USA), 1:100; anti-Smad2/3 (TL), 1:100; anti-ZO-1 (Zymed, San Francisco, USA), 1:100; phalloidin (Molecular Probes, Eugene, USA), 1:40. Nuclei were visualized using To-PRO3 (Invitrogen, Carlsbad, USA) or DAPI (Roche, Basel, Switzerland) at dilutions of 1:10.000 and 1:1.000, respectively.
**Tumor formation in vivo**

1 x 10^6 cells in 50 µl Ringer solution were either orthotopically transplanted into the liver by injection into the spleen or subcutaneously injected into immunodeficient CB-17 SCID recipient mice as described (Fischer *et al.*, 2007; Mikula *et al.*, 2004). Tumor volume and weight were determined after subcutaneous tumor formation as recently described (Fischer *et al.*, 2007). Tumor incidences were equal to 100%, i.e. all injected malignant hepatocytes gave rise to experimental tumors. All experiments were performed twice using three mice per cell type and carried out according to the Austrian guidelines for animal care and protection.

**Immunohistochemistry**

Paraffin-embedded sections of tumor tissues were stained with the following antibodies: anti-β-catenin (Transduction Laboratories (TL), Lexington, UK), 1:100; anti-active β-catenin (Upstate, Lake Placid, USA), 1:100; anti-Stat3 (Cell Signaling, Beverly, USA), 1:100; anti-phospho-Stat3 (Cell Signaling, Beverly, USA), 1:100; anti-E-cadherin (TL), 1:100; anti-ILEI (Waerner *et al.*, 2006), 1:100. More details are available as Supplementary Information on the Oncogene website.

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Extraction of poly(A)^+^mRNA and cDNA synthesis were performed as recently described (Gotzmann *et al.*, 2006). The PCR amplification products were analyzed by agarose gel electrophoresis. Details about primer sequences are available as Supplementary Information on the Oncogene website.

**Proliferation kinetics**

1 x 10^5 cells were seeded in triplicate and the number of cells in the corresponding cell populations was determined periodically in a multichannel cell analyzer (CASY; Schärfe Systems, Reutlingen, Germany). Cumulative cell numbers were generated from the absolute cell counts and their calculated dilution factors (Gotzmann *et al.*, 2002).
Transwell assay
Details are available as Supplementary Information on the Oncogene website.

Tissue array
Tissue array (ORIDIS Biomed, Graz, Austria) contained paraffin-embedded specimens of tumors and adjacent normal tissue collected from 69 female and male HCC patients which were surgically removed between 1995 and 2004. Protocols of the retrospective analyses were approved by the local institutional review board of the Medical University of Graz. All histological specimens were reviewed for histological type and grade. Core biopsies with diameter of 0.6 mm were taken from each donor paraffin block. 4 µm thick sections of each tumor and adjacent tissue were arrayed in triplicate. After antigen retrieval in sodium-citrate buffer (pH 6.0), the anti-ILEI antibody (Waerner et al., 2006) was used at a dilution of 1:300. More details are available as Supplementary Information on the Oncogene website.
Acknowledgement

The authors thank ORIDIS Biomed for providing HCC tissue arrays and immunohistochemical staining. This work was supported by grants from the Austrian Science Fund, FWF, grant number SFB F28 and P19598-B13.
References


Figure legends

Figure 1: ILEI expression in moderately or poorly differentiated experimental liver tumors and in ILEI-transduced hepatocytes. Epithelial MIM-R-GFP hepatocytes, or MIM-RT cells that have undergone EMT, were orthotopically transplanted into the spleen or injected by tail vein. Liver tumors were collected after 20 days and processed for histology and immunohistochemistry. (a) Sections from wild type BALB/c livers (control), MIM-R-GFP and MIM-RT generated liver tumors, and lung metastases induced after tail vein injection of MIM-RT cells were stained with Hematoxylin & Eosin (H&E) or immunohistochemically stained with anti-ILEI antibody. Scale bar represents 50 µm. Insets show staining of tumor sections at 4-fold higher magnification to reveal endogenous ILEI localization. (b) ILEI-overexpressing MIM1-4-ILEI, MIM-C40-ILEI and MIM-R-ILEI cells or empty vector expressing MIM1-4-GFP, MIM-C40-GFP and MIM-R-GFP cells were analyzed by immunoblotting using anti-ILEI and anti-β-actin antibodies. The expression of β-actin is shown as a loading control.

Figure 2: ILEI causes the disassembly of epithelial junctions, cytoskeletal reorganization and β-catenin activation in cooperation with oncogenic Ras. Phase contrast images of MIM-C40-GFP, MIM-C40-ILEI, MIM-R-GFP and MIM-R-ILEI cells are shown in the top panels (bar: 50 µm). The lower panels show confocal immunofluorescence images of these cell types after staining with specific antibodies against ZO-1, E-cadherin (E-cad), total β-catenin (total β-cat), activated, nondestructible β-catenin (nondestr. β-cat) and phalloidin to visualize β-actin. Blue-green staining: DNA. Bar in confocal images: 15 µm. Insets (green border): Cells marked by white dots are shown without DNA staining to better visualize cytoplasmic versus nuclear staining of activated β-catenin (green asteriks in main panel mark DNA staining).

Figure 3: Expression of EMT markers and PDGF / PDGF-R isoforms in hepatocytes before and after ILEI-overexpression. (a) Expression levels of transcripts for E-cadherin, the E-cadherin-repressing transcription factors Slug, Twist and ZEB-1, and the matrix metalloproteinase MMP-9 in MIM-R-GFP, MIM-R-ILEI and MIM-RT cells (positive EMT control), as determined by linear semi-quantitative RT-PCR. (b) Individual transcript
levels of three PDGF isoforms (PDGF-A, -B, -C) and two PDGF-receptor isoforms (PDGF-R and PDGF-Rβ) were analyzed by linear semi-quantitative RT-PCR in MIM1-4-GFP-, MIM1-4-ILEI-, MIM-C40-GFP, MIM-C40-ILEI-, MIM-R-GFP- and MIM-R-ILEI-cells. Constitutive levels of rhoA mRNA are shown as a loading control.

**Figure 4:** ILEI modulates migratory behavior of hepatocytes but has little effect on proliferation. (a-c) Proliferation kinetics were determined by cumulative cell numbers. (a) Proliferation kinetics of untreated MIM-R-GFP, MIM-R-ILEI and MIM-R-ILEI-dnP cells. (b) Proliferation kinetics of MIM1-4-GFP and MIM1-4-ILEI cells as well as (c) MIM-R-GFP and MIM-R-ILEI cells during treatment with TGF-β1 (1 ng/ml). (d) Control cells (MIM-C40-GFP and MIM-R-GFP), ILEI-overexpressing cells (MIM-C40-ILEI and MIM-R-ILEI) and ILEI-overexpressing MIM-R-GFP cells harboring a dominant-negative PDGF-R (MIM-R-ILEI-dnP) after transmigration through Transwell filters. Migrated cells were visualized by Hoechst staining under UV-light and counted (numbers represent transmigrated cell numbers per standard microscopic field). Error bars denote S.E.M.

**Figure 5:** ILEI enhances tumor formation in synergy with oncogenic Ras and induces tumorigenicity in nontumorigenic hepatocytes expressing the PI3K-hyperactivating Ras mutant V12-C40. Tumors were generated in SCID mice by subcutaneous injection of MIM-R-GFP (a, white bars), MIM-R-ILEI-dnP (a, dark gray bars), MIM-R-ILEI cells (a, black bars) and MIM-C40-ILEI (b, light gray bars). Control MIM-C40-GFP hepatocytes (b, hatched bars) failed to form tumors. (a, b) Kinetics of tumor formation as determined by calculation of tumor volumes. (c) Weights of tumor tissues collected after 20 days (MIM-R-GFP, white bar; MIM-R-ILEI, black bar; MIM-R-ILEI-dnP, dark gray bar) or 49 days (MIM-C40-ILEI, light gray bar). Statistically significant weight differences (p<0.0005) between MIM-R-GFP and MIM-R-ILEI tumors are indicated with ***. (a-c) One representative experiment out of three is shown. Error bars denote S.E.M.

**Figure 6:** Ras plus ILEI expression increases nuclear β-catenin levels *in vivo* which requires PDGF-R signaling. (a) Experimental tumors obtained after subcutaneous injection of MIM-R-GFP, MIM-R-GFP-dnP, MIM-R-ILEI or MIM-R-ILEI-dnP cells into
SCID mice were collected after 20 days and processed for histology (H&E) and immunohistochemistry using anti-E-cadherin (E-cad), anti-β-catenin (total β-cat) or anti-activated, nondestructible β-catenin (nondestr. β-cat) antibodies. Bar, 50 µm. Insets show staining of tumor sections at 5-fold higher magnification to reveal details of E-cadherin localization at cell membranes and nuclear β-catenin. (b) Quantitative evaluation of cells for nuclei expressing activated, nondestructible β-catenin. Error bars depict S.E.M. from three independent experiments.

Figure 7: The cooperation of ILEI and Ras activates Stat3 in a PDGF-dependent fashion. (a) Subcutaneous tumors were generated in SCID mice, collected after 20 days and processed for immunohistochemistry, employing anti-ILEI, anti-phospho-Stat3 (pY-Stat3) and anti-total Stat3 (total Stat3) antibodies for staining. Bar: 50 µm. Insets: 5-fold enlarged images to reveal details in granular versus cytoplasmic ILEI staining and nuclear pY-Stat3 staining. (b) Quantitative evaluation of cell nuclei positive for tyrosine-phosphorylated Stat3 (pY-Stat3; gray bars) and total Stat3 (black bars). Statistically significant differences (p<0.05) in expression of pY-Stat3 are indicated with *.

Figure 8: Intensity and pattern of ILEI expression predicts tumor dedifferentiation and prognosis of human HCCs. A tissue array containing 69 human HCC samples and respective control liver tissues was immunohistochemically stained with anti-ILEI antibody. (a) Shown are representative examples for no (negative), weak or strong granular and cytoplasmic ILEI staining of the HCC tissue array. Bar: 200 µm. (b) The intensity of granular (gran; left panel) and cytoplasmic (cyto; right panel) ILEI staining (weak including negative or strong) was correlated with postoperative histological grading (pG) of the HCC in the array ranging from well-differentiated (pG1) to moderately (pG2) and poorly dedifferentiated HCCs (pG3). pG2 and pG3 were grouped together because of low sample numbers for pG3. While strong granular staining slightly correlated with more differentiated HCC, strong cytoplasmic staining correlated with poorly differentiated HCC and thus bad prognosis. The comparison of the tumor grading distribution between the groups cytoplasmic weak and cytoplasmic strong was
significant (p<0.05) showing a higher percentage of pG1 and pG2-3 tumors in the group cytoplasmic strong than in group cytoplasmic weak.

**Supplementary Materials and Methods**

**Cell culture**

Immortalized p19ARF null MIM1-4 hepatocytes were grown in RPMI 1640, 10% fetal calf serum (FCS), 40 ng/ml recombinant human TGF-α (Sigma, St.Louis, USA), 30 ng/ml human IGF-II (Sigma, St. Louis, USA), 1.4 nM Insulin (Sigma, St. Louis, USA) and antibiotics as previously described (Mikula et al., 2004). MIM1-4-GFP, MIM1-4-ILEI and MIM-C40-GFP hepatocytes were propagated in RPMI 1640, 10% FCS and growth factors as described for MIM1-4 hepatocytes, whereas all other cell types were cultured in RPMI 1640 and 10% FCS without additional growth factors. MIM-RT cells were obtained by prolonged TGF-β1 treatment (1 ng/ml) of MIM-R(-GFP) hepatocytes as previously described (Fischer et al., 2005). Fibroblastoid MIM-RT and MIM-R-ILEI cells were grown on tissue culture plastic, whereas epithelial hepatocytes required rat tail collagen-coated culture dishes (Gotzmann et al., 2002). All cells were kept at 37°C and 5% CO₂, and routinely screened for the absence of mycoplasma. The TGF-βRI/II kinase inhibitor LY02109761 (a kind gift of Eli Lilly and Company, Indianapolis, USA; (Lacher et al., 2006)) was used at a concentration of 1 µM.

**Western blot analysis**

Immunoblotting was performed as previously described (Gotzmann et al., 2002). The primary antibodies were used at the dilutions: anti-ILEI (1:1.000; (Waerner et al., 2006)); anti-β-actin (Sigma, St.Louis, USA), 1:2.500. Horseradish peroxidase-conjugated secondary antibodies (Calbiochem, LaJolla, USA) were used at dilutions of 1:10.000.
**Immunohistochemistry**
Biotinylated secondary antibodies were employed and visualization was performed with the vectastain ABC kit using diaminobenzidine as substrate (Vector Laboratories, Burlingame, USA).

**Reverse transcriptase polymerase chain reaction (RT-PCR)**
The following forward and reverse primer were used for the specific amplification, respectively: Slug, 5’-CATTTCAACGCTCCAAGAAGC-3’ and 5’-GAAGCAGCCAGGTCTGGAG-3’; Twist, 5’-GCTCCTCTGCTCTACCCTCC-3’ and 5’-TGCTAGTGGACGCGGACAT-3’. All other primers used have been previously described (Gotzmann *et al.*, 2006; Gotzmann *et al.*, 2002).

**Enzyme-linked immunosorbent assay (ELISA)**
For determination of latent TGF-β1 secretion into the medium, cells were grown in serum-free RPMI containing 10 mM Hepes and antibiotics for 24 hours. Aliquots of cell culture supernatants were used directly for ELISAs according to the instructions of the manufacturer (Bender MedSystems, Vienna, Austria). All assays were performed in triplicate. Values were normalized to background measurements from respective growth media and calculated on the basis of a TGF-β1 standard curve. Values of secreted proteins are expressed per 1 x 10^6 cells and per ml supernatant.

**Colonization of tumor cells in vivo after tail vein injection**
5 x 10^4 cells in 25 µl Ringer solution were injected into the tail vein of immunodeficient CB-17 SCID recipient mice. Xenografted mice were sacrificed after 14 days. Subsequently, the lung was removed and subjected to histological and immunohistochemical analyses. All experiments were performed twice in triplicate and carried out according to the Austrian guidelines for animal care and protection.

**Transwell assay**
4 x 10^4 cells were seeded on Transwell filters with a pore size of 8 µm (Corning Costar, Cambridge) and incubated for 24 hours. Visualization of migrated cells through
Transwell filters using Hoechst 33258 has been recently outlined (Fischer et al., 2007). Migrated cells were quantified by counting per microscopic field at a ten-fold magnification. Assays for each condition were performed twice in triplicate.

**Tissue array**
Secondary antibody was employed and visualization was performed with the ChemMate Envision Kit (DAKO, Carpinteria, CA, USA). Two independent researcher (C.L. and M.M.) evaluated the immunostaining on triplicate tissues by using weak and strong staining intensities of ILEI-positive areas, which covered at least 50% of the total tumor area. Comparison of the tumor grading distribution between the groups cytoplasmic weak and cytoplasmic strong was done by the Jonckheere-Terpstra test in SAS (SAS 9.2, SAS Institute Inc., Cary, NC, USA).

**Supplementary Figure legends**

**Supplementary Figure S1** Morphology of ILEI-overexpressing hepatocytes. Shown are phase contrast images from hepatocyte populations, seeded either at low (top subpanels; sparse) or high density (bottom subpanels; dense) on tissue culture plates. (a) MIM1-4 hepatocytes expressing GFP (MIM1-4-GFP), ILEI (MIM1-4-ILEI), the Ras mutant V12-C40 plus GFP (MIM-C40-GFP) or V12-C40-Ras in combination with ILEI (MIM-C40-ILEI). (b) MIM1-4 hepatocytes expressing oncogenic Ras plus GFP (MIM-R-GFP), Ras plus ILEI (MIM-R-ILEI), or Ras plus ILEI and dn PDGF-Rα (MIM-R-ILEI-dnP). MIM-RT cells representing MIM-R-GFP hepatocytes after prolonged TGF-β1 treatment are shown as controls. Bar, 50 µm.

**Supplementary Figure S2** Overexpression of ILEI on its own in hepatocytes fails to induce changes in their epithelial phenotype. Confocal immunofluorescence images of MIM1-4 hepatocytes expressing either GFP (MIM1-4-GFP) or ILEI (MIM1-4-ILEI) are shown. (a) For expression and localization of epithelial cell markers, cells were stained with specific antibodies against ZO-1, E-cadherin (E-cad), total β-catenin (total β-cat)
and activated, nondestructible β-catenin (nondestr. β-cat). Insets: Cells are shown without DNA staining to visualize nuclear versus cytoplasmic staining of activated, nondestructible β-catenin. (b) To monitor β-actin localization and α-smooth muscle actin (α-SMA), cells were stained with phalloidin and anti-α-SMA antibody, respectively. Nuclear or cytoplasmic localization of Smad2 and Smad3 were detected using an antibody staining both Smads. Bar, 15 µm.

**Supplementary Figure S3** ILEI causes TGF-β-independent upregulation of α-SMA and reduces nuclear Smad2/Smad3 in hepatocytes coexpressing oncogenic Ras or V12-C40-Ras. Shown are confocal immunofluorescence images of MIM-C40-GFP, MIM-C40-ILEI, MIM-R-GFP and MIM-R-ILEI cells after staining with anti-α-SMA and anti-Smad2/3 antibodies. Insets in lower panels show Smad localization without nuclear staining to better visualize nuclear Smad in the GFP control cells and the partial shift of Smad2/3 into the cytoplasm upon ILEI expression. Bar, 15 µm.

**Supplementary Figure S4** ILEI does not stimulate but reduce TGF-β1 secretion of MIM hepatocytes coexpressing oncogenic Ras or the Ras mutant V12-C40. Cell culture supernatants from the cell types indicated (see legend to Figure S1) were harvested and analyzed by ELISA to determine the amount of latent TGF-β1 secretion after normalization to cell numbers.

**Supplementary Figure S5** Expression of dominant negative PDGF-R (dnP) restores E-cadherin localization at cell boundaries of MIM-R-ILEI cells. Confocal immunofluorescence images of MIM-R-GFP, MIM-R-GFP-dnP, MIM-R-ILEI and MIM-R-ILEI-dnP after staining with specific antibodies against E-cadherin (E-cad) or total β-catenin (total β-cat) are shown.

**Supplementary Figure S6** The cooperation of Ras and TGF-β in MIM-RT-induced tumors show EMT *in vivo*. Tumor tissues were collected 20 days after subcutaneous injection of epithelial MIM-R-GFP or fibroblastoid MIM-RT cells into SCID mice. (a)
Shown are images of tumor sections after H&E staining (top) and immunohistochemical staining employing anti-E-cadherin (E-cad), anti-total β-catenin (total β-cat) and anti-activated, nondestructible β-catenin (nondestr. β-cat). Bar: 50 µm. Insets show staining of tumor sections at 5-fold higher magnification to reveal E-cadherin localization (plasma membrane staining in MIM-R-GFP cells changing to weak cytoplasmic staining in MIM-RT cells) and increasing nuclear localization of total and activated β-catenin in fibroblastoid MIM-RT cells. (b) Quantitative evaluation of cells harboring nuclei stained for activated, nondestructible β-catenin.

Supplementary Figure S7 ILEI enhances metastasis formation in a PDGF-dependent fashion. 5 x 10^4 of MIM-R-GFP, MIM-R-ILEI, MIM-R-GFP-dnP or MIM-R-ILEI-dnP cells were each injected into the tail vein of SCID mice and lung colonization was evaluated 14 days after cell injection. (a) Micrographs of lungs bearing MIM-R-GFP- and larger MIM-R-ILEI-derived metastases. (b) H&E-stained lung sections from the four types of metastatic lungs were evaluated for cell numbers per metastasis (grouped into <50 cells, 50-100 cells and >100 cells) in all metastases from 3 lungs resected. MIM-R-ILEI cells generated slightly increased lung metastases whereas those derived from MIM-R-ILEI-dnP cells were strongly reduced. In accordance with recently published data (Jechlinger et al., 2006), control MIM-R-GFP-dnP cells showed a metastatic colonization into the lung with even smaller colonies as compared to those derived from MIM-R-GFP cells. (c) Shown are images of metastatic lung sections after H&E staining (top insets, outline of metastases indicated by dotted lines) and after ILEI-staining (main panel; bottom insets with black frames showing 4-fold magnification). While MIM-R-GFP- and MIM-R-ILEI-induced metastases show general cytoplasmic staining (including nuclei), MIM-R-ILEI-dnP-derived metastases show diffuse or granular (arrows) staining of the nuclear area only (outlines of nuclei indicated by dotted lines).
**Supplementary References**


Figure 1

A

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B

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</table>
Figure 2
Figure 4

A

- MIM-R-GFP
- MIM-R-ILEI
- MIM-R-ILEI-dnP

Cumulative cell number

Days in culture

B

- MIM1-4-GFP
- MIM1-4-ILEI

Cumulative cell number

Days in culture (+TGF-β1)

C

- MIM-R-GFP
- MIM-R-ILEI

Cumulative cell number

Days in culture (+TGF-β1)

D

Transmigrated cells/field

MIM-C40-GFP
MIM-C40-ILEI
MIM-R-GFP
MIM-R-ILEI-dnP

Bars represent mean ± SD.
Figure 6

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A

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H&E

E-cad

Total β-catenin

Nondestr. β-catenin

B

![Bar chart showing nondestr. β-catenin positive cells](chart.png)
Figure 7

(A) Immunohistochemical analysis of MIM-R-GFP, MIM-R-GFP-dnP, MIM-R-ILE1, and MIM-R-ILE1-dnP showing the expression of ILE1, pY-Stat3, and total Stat3.

(B) Quantitative analysis of Stat3 positive cells for MIM-R-GFP, MIM-R-GFP-dnP, MIM-R-ILE1, and MIM-R-ILE1-dnP, showing the proportion of pY-Stat3 and total Stat3 positive cells.
Figure 8

A

negative

granular

weak

strong

cytoplasmic

B

% cases in pG categories 0, 1, 2, 3

n = 41

gran-weak

gran-strong

n = 28

n = 34

cyto-weak

n = 35

cyto-strong

Intensity of granular and cytoplasmic ILEI staining

pG0

pG1

pG2-3
Supplementary Figure S4

![Bar graph showing latent TGF-β1 levels in different cell lines](image)

- MIM1.4-GFP
- MIM1.4-ILEI
- MIM-C40-GFP
- MIM-C40-ILEI
- MIM-R-GFP
- MIM-R-ILEI
- MIM-R-ILEI-dnP
- MIM-RT

Y-axis: latent TGF-β1 (ng/10^6 cells)
Supplementary Figure S6  C Lahsniag et al.

A

MIM-R-GFP  MIM-RT

H&E

E-cad

total β-catenin

nondestr. β-catenin

B

nondestr. β-catenin

β-catenin positive cells

MIM-R-GFP  MIM-RT
Supplementary Figure S7

A

Lung metastasis (14 days)

MIM-R-GFP

MIM-R-ILEI

B

Metastases of different sizes (percent of total)

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C

MIM-R-GFP

MIM-R-ILEI

MIM-R-ILEI-dnP
4. Assessing the role of Stat3 in the epithelial to mesenchymal transition of neoplastic hepatocytes

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Running Title: The role of STAT3 in neoplastic tumor progression

Key Words: Hepatocytes, HCC, STAT3, TGFβ, epithelial to mesenchymal transition, tumor progression
Abstract
Epithelial to mesenchymal transition (EMT) is a crucial process in development and plays an important role during tumor formation and metastasis. One of the most important events involved in EMT is the loss of the adherens junction protein E-cadherin, which is caused by the cooperation of transforming growth factor beta (TGFβ) and oncogenic Ras signaling. Here we show that the signal transducer and activator of transcription (Stat) 3 is activated by Tyr705 phosphorylation in collaboration with TGFβ and oncogenic Ras signaling during EMT formation. To study the role of StatT3 in hepatocellular EMT, we generated p19ARF deficient hepatocytes expressing oncogenic Ras in combination with either constitutive active or dominant negative versions of Stat3. Interestingly the gain-of-function of Stat3 revealed a decreased tumor formation together with a diminished metastatic colonization of the lungs. In contrast, loss–of-function of Stat3 resulted in the opposite effect revealing a tumor suppressive role of Stat3 in liver carcinogenesis. Expression profiling studies of these cell lines revealed a Stat3-dependent upregulation of c-jun, an important factor in the development of liver tumors, and the induction of matrix-metalloproteinase (MMP) 3, which has been reported to play a crucial role in the establishment of EMT. These results point to an important role of Stat3 together with oncogenic Ras in liver tumor progression.
Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancerous disease worldwide and the third common cause of mortality due to its aggressive progression (Kensler, Qian et al. 2003; Bruix et al. 2004; El-Serag, Gilger et al. 2006). The major risk factors for this disease are viral infections like hepatitis B and C, alcohol abuse and fungal toxins like Aflatoxin B1 (Thorgeirsson and Grisham 2002). Beside all these environmental factors, genetic and epigenetic alterations play an important role in the formation of HCC. Among them are the dysregulation of key signal transducers and known tumor-suppressor genes such as for example β-catenin, p16, p53 and E-cadherin (Toyosaka, Okamoto et al. 1996; el-Assal, Yamanoi et al. 1997; Lee, Park et al. 2006; Llovet 2006). A major event in hepatocellular tumor progression is the epithelial-to-mesenchymal transition (EMT), which leads to the dissociation of cell-cell contacts followed by a dramatic phenotypical change of the tumor cells (Yang, Mani et al. 2004; Hugo, Ackland et al. 2007). Involved in the transformation of epithelial cells to mesenchymal, fibroblastoid cells is the upregulation of transcriptional repressors of E-cadherin such as Snail or Zeb1 (Hanahan and Weinberg 2000). The loss of E-cadherin associates with a higher migratory potential of cells and metastasis formation (Thiery 2002). Studies on EMT in human liver cancer revealed novel factors involved in this process among them signal transducer and activator of transcription (Stat) 5b (Lee, Man et al. 2006) and Laminin-5 (Bergamini, Sgarra et al. 2007).

Especially in HCC the constitutive activation of Stat molecules might play a major role in neoplastic formation (Calo et al. 2003). Among the 7 different members of Stat molecules, Stat3 is known to serve as an oncoprotein (Bromberg et al., 2000). Currently 3 different isoforms of Stat3 termed, Stat3α, Stat3β and Stat3γ (Akira et al. 1994; Hevehan et al. 2002) are described and their functions are poorly understood. Full length Stat3, also known as Stat3α, consists of 6 different domains, among them the coiled-coil domain, necessary for interactions with other proteins, as for example CBP/p300, followed by the DNA-binding domain, the SH2-domain and the transactivation domain, which is truncated in Stat3β (Dewilde et al. 2008). After binding of cytokines to their cognate receptors, receptor dimerization occurs followed by an phosphorylation step of the non-covalently bound Janus kinases (Jak) (Wheadon et al. 1999; Darnell 2002).
Activated Jak phosphorylates the receptor and therefore expose SH2-binding sites for cytosolic Stat molecules (Darnell 2002). The now bound Stat molecules dimerize and translocate into the nucleus where they start to activate several target genes (Aaronson and Horvath 2002).

Another important factor involved in the formation of HCC due to its ability to cause EMT is transforming growth factor (TGF)β (Rossmanith 2002) which is a multifunctional peptide with many different functions such as control of proliferation and differentiation of normal tissue (Sporn et al. 1986; Rossmanith et al., 2001). After binding of one of the three TGFβ ligands to the TGFβRII, a heterodimeric formation of TGFβRI and TGFβRII takes place (Massague 2000), which causes the phosphorylation of the regulatory subunit of TGFβRI followed by the activation of the receptor associated SMAD proteins Smad2 and Smad3 (Massague 2000; Heldin, Eriksson et al. 2002; Pardali and Moustakas 2007). In more than 50% of human HCC, cancerous cells secrete TGFβ and therefore cause EMT and uncontrolled growth of the surrounding cells. The established autocrine loop of secreted TGFβ influences the migratory potential of neoplastic hepatocytes. In recent studies it has been shown that the collaboration of TGFβ1 and Laminin-5 causes hepatocellular EMT (Lee et al., 2006) and tumor progression in an α3-integrin-dependent fashion (Fuchs et al., 2008; Tang et al., 2008). Studies on breast cancer stem cells revealed an important role of TGFβ in cooperation with Stat3-signalling (Tang et al., 2008).

In this study we addressed the role of Stat3 during the Ras-mediated EMT and tumor progression of hepatocytes. In particular, constitutive Stat3 activates in collaboration with oncogenic Ras associated with a strong tumor suppression which might depend on p19ARF deficiency. The cooperation of Stat3 and Ras further showed an increase of c-jun and MMP-3, which might play a role in the acquisition of an EMT phenotype.
Results

Stat3 is activated during EMT

Stat3 is known to be involved in tumor progression, however the role of Stat3 in hepatocellular EMT is an unresolved issue. Therefore we first analyzed the expression of Stat3 by immunohistochemistry of subcutaneous tumors derived from epithelial hepatocytes expressing oncogenic H-Ras (MIM-R) and the same cells after treatment with TGFβ (MIM-RT) over 14 days. Activated Stat3 (pStat3) was hardly detectable in the control liver tissue and in the MIM-R tumors, whereas in the tumors derived from MIM-RT cells a strong expression of pStat3 was detectable (Figure 1A). The expression of total Stat3 was not altered in all three analyzed tumor samples. To verify this finding, a Western-Blot containing MIM-R and MIM-RT cell lysates was carried out which revealed phosphorylated Stat3 in MIM-RT but less in MIM-R (Figure 1B). These data show that Stat3 is activated by Tyr705 phosphorylation during hepatocellular EMT.

Expression of constitutive active (ca) and dominant negative (dn) Stat3α and β versions in Ras-transformed hepatocytes

For a better understanding of the role of Stat3 in tumor progression we retrovirally transmitted MIM-R hepatocytes, which were already expressing oncogenic Ras with several Stat3 constructs (Figure 2A). Further, as a control, a wt construct of Stat3 and a version lacking the transactivation domain and the tyrosine responsible for the phosphorylation and therefore for the activation of Stat3 were also transformed in MIM-R cells (Figure 2A). For both ca versions of Stat3, the Stat3α and β isoforms carried each two point mutations in their SH2-domain, A662N664C, leading to the formation of disulfide bridges causing a constitutive dimerization of the Stat3 monomers and therefore a permanent activity. The dn forms were constructed by exchanging the aminoacid tyrosine 705 into a phenylalanine resulting in a blockade of the phosphorylation of the amino acid and therefore in a permanent inactive form of both Stat3α and Stat3β isoforms (Besser et al. 1999). All Stat3-transfected hepatocytes showed a more than 85% GFP-purity and were stably diploid (Table 1).
For studies on the DNA binding activity of all Stat3 versions, we performed an electrophoretic mobility shift assay (EMSA) either in the presence interleukin (IL)-6, or in the presence of an inhibitor of Stat3 expression, the Jak inhibitor (Figure 2B). A difference in the strength of DNA binding was observed between the Stat3\(\alpha\) and the Stat3\(\beta\) isoform which was already shown in previous reports (Dewilde et al. 2008). Usually the expression-ratio of Stat3\(\beta\) in comparison to Stat3\(\alpha\) is much lower, but during inflammation the amount of both isoforms is almost equal. After overexpression of Stat3\(\beta\) this factor is constitutively tyrosine-phosphorylated and can then bind DNA and promote transcription due to an extended half-life of the phosphorylated dimer (Dewilde, et al., 2008). For a better understanding of the EMSA experiment, the signals were quantitative analyzed (see Figure 2C).

Regarding these results, all used constructs seem to work in the predicted way and cause either upregulation or downregulation of Stat3 in our hepatic MIM cell system. Worth mentioning is the stronger DNA binding activity of the Stat3\(\beta\) isoform compared to the other versions (Figure 2B).

Expression of constitutive active (ca) Stat3 in Ras-transformed hepatocytes reveals reduced tumor formation

We next examined the tumorigenic role of Stat3 in combination with oncogenic Ras. Ca Stat3\(\alpha\), and in particular, Stat3\(\beta\) overexpression caused smaller tumors than the control tumors generated by MIM-R cells alone (Figure 3A). The volume of Stat3\(\beta\) tumors were 4-fold reduced compared to MIM-R-control tumors. The greatest tumorigenecity showed the dnStat3\(\beta\) tumors compared with all others. The difference in size was 2-fold higher to the MIM-R and 6-fold higher to the caStat3\(\beta\) tumors leading to the conclusion that Stat3 in combination with oncogenic Ras rather has a tumor suppressive role. Also very interesting was the finding that the \(\Delta S683\)Stat3 version without transactivating domain also showed an increase in tumor size compared to MIM-R control tumors (Figure 3B).

In contrast control wtStat3 version showed only very small tumors. These results suggest a tumor suppressive role of Stat3 in combination with oncogenic Ras in hepatocytes lacking p19\(^{ARF}\).
**Diminished metastatic colonization of hepatocytes co-expressing ca Stat3 and oncogenic Ras**

For further analysis of Stat3, we investigated the metastatic role of caStat3 and dnStat3. For this reason we injected the hepatocytes transmitted with oncogenic H-Ras and either Stat3\(\alpha\) or Stat3\(\beta\) into the tail vein of SCID mice and analyzed the colonization of the lung with these cells. The results showed a tumor-suppressive role of caStat3 in metastasis formation and therefore a reduced metastatic behaviour due to the decreased amount of lung colonies. As depicted in Figure 4, metastasis produced by caStat3\(\alpha/\beta\) led to a 2- to 4-fold decrease of colony formation and also to small secondary tumors in the respiratory organ compared with the MIM-R cell as a control. Expression of dnStat3 versions in hepatocytes resulted in a 2-fold decrease in the amount of secondary colonies and large metastasis of dnStat3\(\alpha\). WtStat3 and \(\Delta S683\)Stat3 cells showed also a decreased amount of metastasis in the lung. Colonies derived from the overexpression of wtStat3 resulted in small metastasis and for the expression of \(\Delta S683\)Stat3, the secondary lung colonies were large. Therefore these data suggest a metastasis-suppressive role of Stat3 in the p19\(^{ARF}\) deficient model of hepatocellular carcinoma progression.

**Upregulation of c-jun and of matrix metalloprotease 3 (MMP3) in hepatocytes co-expressing ca Stat3 and oncogenic Ras**

For further investigation we performed by expression profiling to identify genes, which are upregulated in response to the different expression mutant Stat3 versions. For this purpose we used a commercial array covering 99 genes involved in Stat-signalling (see Figure 5). Among the influenced genes, c-jun, a gene, which in combination with c-fos constitutes the AP-1 complex (Rahmsdorf 1996), showed a 6- to 10-fold upregulation in MIM-RT and caStat3\(\alpha\) compared to the expression level in the control MIM-R-cells. Also the downregulation of c-jun in the dnStat3-cells is worth mentioning and therefore c-jun might play a role in tumor suppression of caStat3 expressing malignant hepatocytes (see Figure 5).

Another upregulated gene dependend on Stat3 was the matrix-metalloproteinase (MMP)-3, which is known to play a role in epithelial-to-mesenchymal transition (Nagase, Meng et al. 1999) due to its opportunity to degrade extracellular matrices (Lijnen,
Maquoi et al. 2002). The upregulation of MMP-3 was between 30-fold in MIM-RT and 90-fold in MIM-R-caStat3β-cells compared to the expression level in the MIM-R cells. Therefore caStat3 might play a role in tumor initiation and progression due to the upregulated c-jun and MMP-3. These data obtained by expression profiling further suggest the Stat3-dependent upregulation of c-jun and MMP-3 and might play a prominent role during hepatocellular carcinoma progression.
Discussion

In this paper we focused on the role of activated Stat3 and its ability to modulate EMT and hepatic tumor progression. Stat3 in combination with a p19ARF deficiency showed a tumor suppressive role in a cellular EMT model employing H-Ras transformation. These tumors showed a decreased tumor formation compared with the control tumors. We could also show an increased activation of at least two target genes of Stat3, namely c-Jun and MMP-3, during EMT progression. However further studies are required to identify the mechanisms underlying the tumor-suppressive role of Stat3.

Recent findings have shown that Stat3 is also activated in other tumors than in hepatocellular cancerogenesis. For example a role of Stat3 was detected in primary breast tumors, where inhibition of Stat signaling downstream of Src or Janus kinases showed an abrogated constitutive Stat3 DNA binding, inhibited cell proliferation and induced apoptosis (Bowman, et al., 2000; Garcia and Jove, 1998). Also an increased expression of Stat3 in multiple myeloma (Catlett-Falcone, et al., 1999), head and neck cancer (Grandis, et al., 1998), lung cancer (Fernandes, et al., 1999) and lymphoma (Lund, et al., 1997; Lund, et al., 1999; Nielsen, et al., 1997; Sun, et al., 1998; Weber-Nordt, et al., 1996; Zhang, et al., 1996) implied a tumor-promoting role of Stat3 (Bowman, et al., 2000).

In this study we used a cellular model of p19ARF null hepatocytes for analyzing the role of Stat3 in tumorigenesis. Expression of caStat3a or caStat3b nor their dominant negative versions on its own resulted in an undetectable phenotype with respect to EMT formation and tumorigenesis. Thus we focused on the cooperative role of Stat3 and oncogenic H-Ras. CaStat3 overexpressing cells in combination with oncogenic H-Ras were injected in mice causing a decreased tumor formation compared to control cells without activated Stat3. However, experiments done with the MMH-D3 hepatocytic cell line showed contrary effects resulting in an increase of tumor formation under the influence of activated Stat3 (caStat3, data not shown). Noteworthy, MMH-D3 hepatocytes have been immortalized by transgenic cyto-Met expression (Amicone et al., 1987), and express p19ARF at levels comparable to primary hepatocytes (data not shown). Therefore these results revealed a tumor progressive role of Stat3 in a p19ARF background, which was lost upon expression in a p19ARF-positive genetic background.
Next we studied the necessity and sufficiency of Stat3 for EMT in these cells. We could show that neither caStat3α/β nor dnStat3α/β modulated the TGFβ-dependent EMT in vitro (data not shown).

To have a more detailed insight into the ability of Stat3 to cause EMT in hepatocytes, also the role of other Stat family members should be analyzed in this process. A compensatory role of other Stat-family members such as Stat1 or Stat5, could explain the suppressive role of caStat3 in p19ARF deficient hepatocytes due to the fact that both Stat molecules are also described as growth inhibitors and activator of apoptosis (Bromberg, 2000).

The expression of caStat3 and dnStat3 showed an unexpected effect in our investigations and revealed a tumor suppressive role of Stat3 in a p19ARF mouse background. This tumor suppressive role of Stat3 depends on the combination with oncogenic H-Ras in a p19ARF null background. Similar results were found with c-Fos, a factor involved in the AP-1 complex and JunB (Eferl, 2003). Therefore, this study describes a novel tumor suppressive role of Stat3 in hepatocellular tumor progression. The upregulation of MMP3 and c-Jun in hepatocytes overexpressing ca Stat3 isoforms in collaboration with H-Ras is the matter for further experiments helping to mechanistically understand the tumor suppressive role of Stat3. Both factors are important in generating an EMT phenotype and therefore seem to play a role in tumor progression rather than in tumor suppression. Due to the fact that cells which undergo an EMT show a higher migratory potential than epithelial cells, this effect can be combined with increased tumor formation. The upregulation of c-jun and MMP-3 together with caStat3 in combination goes along with a decreased tumor formation and colonization of adjacent organs. All these results represent the basis for further questions which will be addressed in future investigations.
Materials and methods

Cell culture
MIM-R hepatocytes were generated by stable retroviral transmission of immortalized p19ARF null hepatocytes (MIM1-4) with a construct bicistronically expressing oncogenic v-Ha-Ras and green fluorescent protein (GFP) as outlined recently (Fischer et al. 2005). In order to study the role of Stat3 in hepatocellular EMT, we used MIM-R hepatocytes expressing either wild-type Stat3 (MIM-R-wtStat3), a C-terminally truncated version of Stat3 (MIM-R-Stat3ΔC683), the constitutive active mutants of Stat3alpha or beta (MIM-R-caStat3α or -caStat3β) or the dominant negative mutants of Stat3alpha or beta (MIM-R-dnStat3α or -dnStat3β) after retroviral transmission (Cockerham and Zeng 1996; Reya, Duncan et al. 2003). All cells were grown in RPMI 1640 plus 10% fetal calf serum and antibiotics at 37°C and 5% CO₂, and were routinely screened for the absence of mycoplasma. Treatment of epithelial cells, such as MIM-R with TGF-β1 over 14 days resulted in stable fibroblastoid cell lines, termed MIM-RT. For long-term treatment of cells, TGF-β1 was added at a concentration of 1 ng/ml. Fibroblastoid cells were grown on plastic, whereas epithelial cells required culture dishes coated with rat tail collagen, prepared as described (Gotzmann, Fischer et al. 2006). For stimulation of the cells, interleukin (IL)-6 (R&D Systems, McKinley Place NE, USA) was added at a concentration of 20ng/ml and for the investigation of theDNA-binding activity, a Jak inhibitor (Calbiochem, LaJolla, CA, USA) was added at a concentration of 1µM. GFP-expression and the ploidy of the cells was measured by FACS analysis and described in Table 1.

Western blot analysis
The preparation of cellular extracts, separation of proteins by sodiumdodecylsulfate–polyacrylamide gel electrophoresis and immunoblotting were carried out as described recently (Gotzmann, Huber et al. 2002). The protein extract from 1x10⁵ cells per sample was loaded onto gels, and immunological detection of proteins was performed with the SuperSignal detection system (Pierce Chemical Company, Rockford, IL, USA). The following primary antibodies were used: anti-phospho-Stat3 (Cell Signaling, Beverly, MA,
USA; 1:1000; anti-Stat3 (Cell Signaling, Beverly, MA, US). Secondary antibodies (Calbiochem, LaJolla, CA, USA) were used at dilutions of 1:10 000.

**Electrophoretic mobility shift assay (EMSA)**

Culture dishes of adherent cells were rinsed twice with ice-cold PBS. Cells were scraped off and pelleted by centrifugation (1 min, 1000g). Pellets were mixed with two times of the volume with whole cell extract buffer (20 mM Hepes, pH 7.9; 20% Glycerol; 50 mM KCl; 1 mM EDTA; 1 mM DTT (Sigma); 400 mM NaCl; 5µg/ml Leupeptin (Boehringer Mannheim); 0.2 units/ml Aprotinin (Bayer); 1 mM PMSF (Boehringer Mannheim); 5 mM Na3VO4; 10 mM NaF; 5 mM b-glycerophosphate) and subjected to freeze-thaw cycles which were repeated 4 times. Extracts were centrifuged at 4°C for 20 minutes, and the supernatant was subjected to EMSAs. Oligo (m67 5’-dGATTTCCCGTAAATCAT-3’) was annealed at high equimolar concentration in a 200 µl reaction with annealing buffer (10x = 0.625x PCR Buffer II (Roche); 9.4 mM (MgCl₂) by heating up to 95°C and a slow cool down to room temperature. Annealed oligos were then diluted to 2.5 µM. Extracts (20 µg protein) from cell extracts were incubated with 0.5 – 1 µl hot probe (8,000 cpm/fmol) in a 20 µl reaction volume containing DNA-binding buffer (10 mM Tris; 1 mM DTT; 0.2 mM PMSF; 0.1 mM EDTA; 5% Glycerol; 50 mM NaCl; 0.1% NP40), 2 µl BSA (10µg/µl in 20 mM KPO₄, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol) and 2 µl Poly dI-dC (1 µg/µl; Roche Diagnostics) for 5 - 15 minutes at room temperature before loading on the gel. Complexes were separated on a non-denaturating 4% acrylamide (BIO-RAD; acrylamide:bis-acrylamide = 29:1). TBE was used as running buffer at 200V for about 3 hours. The gel was transferred onto filter paper (Whatman Chromatography Paper), dried on a vacuum gel dryer at 80°C for two hours and x-ray films (Kodak Biomax MR) were exposed at –80°C.

**RNA isolation and expression profiling**

RNA was isolated from cells using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Quality of the RNA was analyzed using the Agilent Bioanalyzer (Agilent Technologies). Expression profiling, including the preparation of cRNA and hybridization to JAK / STAT Signaling Pathway Oligo GEArrays® (Super Array,
Bioscience Corporation) was done according to the manufacturer’s protocol. Data analysis was performed using ImageQuant™ TL (Molecular Dynamics).

_Tumor formation in vivo_
Cells of the respective cell type were detached from the tissue culture plate, washed with PBS, and resuspended in Ringer solution. Subsequently, 1x10^6 cells in 100 μl Ringer solution were subcutaneously injected into immunodeficient SCID/BALB/c recipient mice. Tumor formation was periodically measured by palpation, and the tumor size was determined using a vernier caliper. Tumor volume was calculated from tumor size using the formula diameter x diameter x length/2, and the tumor weight in milligram was determined as described recently (Gotzmann, Huber et al. 2002). Tumor incidences were equal to 100%, which means that all injected cell populations gave rise to experimental tumors. All experiments were performed in triplicate and carried out according to the Austrian guidelines for animal care and protection.

_Intrasplenic transplantation of hepatocytes_
1x10^6 of cultured cells resuspended in 20 μl Ringer solution were injected into the spleen of SCID mice. All experiments were performed in duplicate and carried out according to the Austrian guidelines for animal care and protection.

_Immunohistochemistry of experimental tumors_
SCID mice were sacrificed and the obtained tumors, livers or lungs were fixed in 4% phosphate-buffered formaldehyde overnight at 4°C (Mikula, Fuchs et al. 2004). Four μm thick sections were stained with hematoxylin and eosin for standard microscopy. For immunohistochemical analyses, paraffin-embedded sections (4 μm) were treated with citric acid buffer (0,01M, pH 6.0) before staining with the following antibodies: anti-phospho-Stat3 (Tyr705) (Cell Signaling, Beverly, MA, USA), 1:1000; anti-Stat3 (Cell Signaling, Beverly, MA, US), 1:100. Corresponding biotinylated secondary antibodies were employed and visualization was performed with the vectastain ABC kit using diaminobenzidine as substrate (Vector Laboratories, Burlingame, USA).

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Figure legends

**Figure 1:** *Stat3 is activated during EMT.* (A) Epithelial MIM-R hepatocytes and corresponding fibroblastoid MIM-RT cells that have undergone EMT were each transplanted into the spleen of severe combined immunodeficiency (SCID) mice. The resulting experimental liver tumors were subjected to immunohistochemical analysis by employing an anti-pStat3 antibody (Tyr705, Cell Signaling technology, USA) which revealed a prominent activation of Stat3 in MIM-RT derived liver tumors. Insets show staining of tumor sections at 4-fold magnification. Nuclear pStat3 is indicated by arrows. (B) Epithelial MIM-R hepatocytes and fibroblastoid MIM-RT cells were processed for Western blot analysis. MIM-RT hepatocytes showed Tyr705 phosphorylation of Stat3 after long-term stimulation with TGF-β.

**Figure 2:** *Expression of constitutive active (ca) and dominant negative (dn) Stat3 versions in Ras-transformed hepatocytes.* (A) Schematic representation of the Stat3 constructs stably co-expressed in Ras-transformed hepatocytes. (B) Overexpressing Stat3 cells were stimulated with 20 ng/ml IL/6 prior to harvesting. Whole cell extracts were analyzed by electromobility shift assays (EMSA) using the M67 site containing a Stat3 consensus sequence. (C) Quantitative evaluation of (B). DNA binding could be diminished after incubation with 1 µM pan-JAK inhibitor in the parental cell line (Tang, et al.) and in cells overexpressing dn Stat3 versions but not after expression of either wt or ca Stat3.

**Figure 3:** *Expression of constitutive active (ca) Stat3 in Ras-transformed hepatocytes reveals reduced tumor formation.* Tumor volumes after subcutaneous injection of hepatocytes overexpressing oncogenic H-Ras and mutant Stat3 versions into immunodeficient SCID mice. (A) Reduced tumor formation was observed in Ras-transformed cells expressing ca Stat3 mutants. In contrast, larger tumor formation was associated with expression of dn Stat3β. (B) Similar effects on tumor growth were observed with wtStat3 and Stat3ΔC683.
**Figure 4:** *Diminished metastatic colonization of hepatocytes co-expressing ca Stat3 and oncogenic Ras.* Malignant hepatocytes showed metastatic colonization after tail vein injection. Hematoxylin/eosin staining of lung tissue sections revealed differences in number and size of metastatic colonies after expression of various Stat3 mutants in Ras-expressing hepatocytes. (B) Metastatic colonies were counted according to their size. Malignant hepatocytes expressing ca Stat3 showed a diminished metastatic potential.

**Figure 5:** *Upregulation of c-jun and of matrix metalloprotease 3 (MMP3) in hepatocytes co-expressing ca Stat3 and oncogenic Ras.* (A) Expression profiling of malignant hepatocytes after introduction of various Stat3 mutant versions revealed differential gene expression. Red encircled is the c-jun expression, black circles correspond to MMP-3 expression. (B) Evaluation of c-jun as well as MMP3 compared to MIM-R hepatocytes. Both genes were upregulated in cells that have undergone EMT (RT) and in cells that express ca Stat3.
Table 1: GFP positivity and polyploidy of used cell lines

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Figure 1: Stat3 is activated during EMT
Figure 2: Expression of constitutive active (ca) and dominant negative (dn) Stat3 versions in Ras-transformed hepatocytes
Figure 3: Expression of constitutive active Stat3 in Ras-transfromed hepatocytes reveals reduced tumor formation.
Figure 4: Diminished metastatic colonization of hepatocytes co-expressing ca Stat3 and oncogenic H-Ras
**Figure 5: Stat3 Microarray**

A

B

![Image of Stat3 Microarray showing different conditions and fold expression graphs for Jun and MMP3 genes.](image-url)
Acknowledgment

I would like to thank all the members of the Mikulits lab for their help, stimulating discussions and suggestions during this project. I would also like to thank the members of the Beug lab at the IMP and the service departments there for sequencing and for their assistance with microscopes and image processing. Finally I want to thank my supervisor Wolfgang Mikulits whose support and enthusiasm kept me going and without whom this work would not have been possible.
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RNA in situ hybridisation
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September 2007 Poster presentation at the Conference “Epithelial – Mesenchymal Transitions”, Krakow, Poland

Publications

