THE ROLE OF HEPATOCYTE GROWTH FACTOR/ SCATTER FACTOR IN HEPATOBLASTOMA AND HEPATOCELLULAR CARCINOMA PROGRESSION

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"Research is the art of seeing what everyone else has seen, and doing what no-one else has done."
Anonymous

Zusammenfassung

Der Hepatozyten-Wachstumsfaktor (HGF/SF) ist ein im gesamten Organismus vorkommendes Molekül und besitzt mitose-, motilitäts-, angiogenese-, und transformationsfördernde Funktionen. Der Rezeptor für HGF/SF ist das Protein c-Met, welches durch das Proto-Oncogen *c-met* codiert wird. c-Met und/oder HGF/SF werden in vielen Tumoren übermäßig produziert, wodurch die Tumore hochsensitiv für HGF/SF-vermittelte Signale werden. Eine erhöhe Inzidenz bösartiger Neubildungen und postoperatives Wachstum von einzelnen Residualtumorzellen kann im Hepatoblastom (HB) und hepatozellulärem Karzinom (HCC) beobachtet werden, wenn der Serumspiegel von HGF/SF signifikant erhöht ist. Obwohl diese beiden Tumore zwei unterschiedliche Subtypen von Leberepithelialtumoren darstellen und sich deshalb hinsichtlich ihrer histologischen und klinischen Charakteristika deutlich voneinander unterscheiden, kann eine vergleichende Analyse der Auswirkungen von HGF/SF möglicherweise Aufschlüsse über die onkologischen Signalwege geben, die schließlich zur Tumorprogression führen. Die Ergebnisse dieser Studie zeigen, dass HGF/SF die Überlebensrate von Tumorzellen nach Induktion der Apoptose durch Cisplatin und Camptothecin, sowie Serumdepletion in einer Phosphoinositol 3-Kinase (PI3K)-abhängigen Weise positiv begünstigt. Weiterhin konnten Unterschiede in der HGF/SF-Sensitivität zwischen HB- und HCC-Zellen festgestellt werden, die auf unterschiedliche c-Met Expression zurückzuführen ist. Dadurch wird deutlich, dass HGF/SF zum Überleben von Tumorzellen beiträgt und damit die Tumorprogression fördert. Ausserdem konnte in dieser Studie HGF/SF als wesentlicher Faktor für eine erhöhte HB- und HCC-Zellmobilität identifiziert werden. HGF/SF induziert die Tumorzellwanderung, die durch eine vermehrte ‚Snail'-Produktion erreicht wird. Snail ist ein Transkriptionsrepressor, der eine entscheidende Rolle in der Epithelial-Mesenchymalen Transition (EMT) spielt. Snail reprimiert u.a. die Expression der E-cadherin und Claudin-3 Gene und ist für die HGF/SF-induzierte Zelldispersion essentiell. Entsprechend verhindert die shRNA-verursachte Verminderung der Snail-Produktion die HGF/SF-induzierte Zellmobilität. Die Erhöhung der Snail-Konzentration wird durch die Aktivierung der Mitogen-aktivierten Proteinkinase (MAPK) erreicht und durch den Transkriptionsfaktor „early growth response factor" (Egr)-1 vermittelt. Zusammenfassend konnte gezeigt werden, dass HGF/SF eine entscheidende Rolle in der Tumorzelldisaggregation, -migration und schliesslich Invasion spielt. HGF/SF sollte daher als ein Kandidat für eine therapeutische Intervention betrachtet werden.

Summary

Hepatocyte growth factor/scatter factor (HGF/SF) is a ubiquitously expressed molecule that elicits pleiotropic functions on epithelial cells, including mitogenic, motogenic, differentiating, angiogenic, and morphogenic effects. The receptor for HGF/SF is c-Met, a product of the proto-oncogene *c-met*, which is abundantly expressed in many tumors, rendering them highly receptive for HGF/SF signals. In hepatoblastoma (HB) and hepatocellular carcinoma (HCC), a high relapse incidence and post-operative residual tumor growth can be detected, when the serum levels of HGF/SF are markedly elevated, suggesting a link between this molecule and tumor malignancy. Although HB and HCC are two distinct subtypes of primary tumors arising from liver parenchymal cells and thus differ by many histo-clinical characteristics, comparative analysis of the impact of HGF/SF on these tumors may provide some clues on the oncogenic pathways leading to liver tumor progression. The results of this study demonstrate that HGF/SF mediates cytoprotective functions against the apoptotic inducers cisplatin, camptothecin, and starvation in a phosphoinositide 3-kinase (PI3K)-dependent manner, thereby contributing to chemotherapy resistance. Differences between HB and HCC cells regarding the sensitivity towards HGF/SF and HGF/SF-stimulated cellular responses were observed and are associated with c-Met expression. Furthermore, our experiments demonstrate that HGF/SF is a potent inducer of cell scattering and migration. HGF/SF triggers scattering of epithelial cells by upregulating the expression of Snail, a transcriptional repressor involved in epithelial-mesenchymal transition (EMT). Snail, which represses for example the expression of E-cadherin and claudin-3, is required for HGF/SF-induced cell scattering, since shRNA-mediated ablation of Snail expression prevents this process. HGF/SF-induced upregulation of Snail transcription involves activation of the mitogen-activated protein kinase (MAPK) pathway and requires the activity of early growth response factor (Egr)-1. Thus, HGF/SF plays a critical role in cell scattering, migration, and invasion. Together, these findings highlight the importance of HGF/SF in tumor cell survival and tumor progression and suggest that it should be considered as a candidate for therapeutic strategies.

1 Introduction

1.1 Hepatoblastoma (HB)

HB is the most frequent malignant primary hepatic tumor of early childhood, mainly affecting young children between six months and three years of age (Weinberg and Finegold, 1983). After neuroblastoma and nephroblastoma, primary epithelial tumors of the liver are the third most common intra-abdominal neoplasms in children (von Schweinitz *et al.*, 2000; Clatworthy *et al.*, 1974). HB has a fairly constant annual incidence of 0.5 – 1.5 diagnoses per 1 million children.

Although the etiology of this tumor is still largely unknown, there is increasing evidence that it arises and differentiates from early hepatic progenitor cells, which in animals are known as oval cells. These cells still present morphologic features recapitulating some of the developmental aspects of the liver, yet escape from normal cell proliferation control. A disturbance of normal cell proliferation and differentiation during hepatogenesis is suspected, since HBs exhibit a wide spectrum of epithelial and mesenchymal lines of differentiation (differentiated-fetal, less differentiated-embryonal, small cell-undifferentiated). Neoplasms, which occur in these differentiation states, are believed to derive from a pluripotent stem cell (Marceau, 1990; Ruck *et al.*, 1997).

At diagnosis, a striking observation is that HB patients often show highly elevated serum levels of α-fetoprotein and hepatocyte growth factor/scatter factor (HGF/SF). In addition, an increase of up to fourfold HGF/SF was detected in 10 out of 12 children 24 - 72 hours after liver resection (Lack *et al.*, 1982; von Schweinitz *et al.*, 1998; Weinberg and Finegold, 1983). This pre- and post-operative increase of HGF/SF serum levels is suspected to promote tumor cell proliferation and tumor progression after incomplete resection (von Schweinitz *et al.*, 2000). Distant metastases occur mostly in the lungs (Perilongo *et al.*, 2000), but metastasis of the central nervous system and even eye metastases have been described (Endo *et al.*, 1996; Miyagi *et al.*, 1984). Recently it was found, that a number of genetic conditions are related with an increased risk for developing HB, including Beckwith-Wiedemann syndrome, hemihypertrophy, and familial adenomatous polyposis (FAP) (Giardiello *et al.*, 1996; Hartley *et al.*, 1990).

The incidence of HB is highly increased in FAP individuals (Hughes and Michels, 1992; Polakis, 2000). FAP is an autosomal dominant disorder characterized by the development of colorectal adenomas during adolescence and young adulthood. If not

treated with prophylactic colectomy, colorectal cancer will develop in virtually all affected individuals before the fifth decade of life, accompanied with poor prognosis. The genetic disorder is caused by inherited germline mutations in the tumor suppressive adenomatous polyposis coli gene (APC) (Miyoshi *et al.*, 1992). Loss of APC function promotes β–catenin translocation to the nucleus and activation of target genes, which are important in cell proliferation including c-myc, c-jun, and cyclin D1 (Bienz and Clevers, 2000). It was previously reported that a significant subset (up to 48%) of sporadic HB harbors aberrations in various components of the Wnt/β-catenin pathway (see 1.7.7) (Koch *et al.*, 1999; Polakis, 2000; Udatsu *et al.*, 2001). This is one of the highest β-catenin mutation frequencies among solid tumors. Accordingly, nuclear accumulation of β-catenin and overexpression of its target genes is frequently found in HB (Koch *et al.*, 2005).

Cytogenetic studies have shown that frequent loss of heterozygosity (LOH) at chromosomal locations 1p36 and 11p15.5 often occurs in HB. Loss of the maternal allele in the latter case indicates that this region is imprinted (Hartmann *et al.*, 2000; Kraus *et al.*, 1996). Two important genes are located on 11p15.5, namely insulin-like growth factor (IGF)-2 and H19, which are reciprocally imprinted. IGF-2 is expressed monoallelically from the paternal allele whereas H19 is expressed exclusively from the maternal allele (Giannoukakis *et al.*, 1993; Zhang and Tycko, 1992). IGF-2 is a major fetal mitogen and growth factor whereas H19 codes for an untranslated RNA whose function is still under dispute (Barsyte-Lovejoy *et al.*, 2006; Jones *et al.*, 1998). Loss of imprinting (LOI) is an epigenetic alteration of the DNA, that leads to a biallelic expression predisposing cells to carcinogenesis and tumor growth. Accordingly, IGF-2 mRNA expression was increased in HB, whereas the suppressor gene H19 mRNA is markedly reduced, suggesting a disruption of the reciprocal regulation of the imprinted genes in these tumors (Hartmann *et al.*, 2000).

1.2 Hepatocellular carcinoma (HCC)

HCC is a malignant tumor, which arises from hepatocytes and accounts for a leading cause of worldwide cancer death with a 5-year survival rate of less than 5% without treatment (Parkin *et al.*, 1999). Even when treated by resection, the recurrence rates are the highest of any solid tumor, approaching 75 – 100% (Tung-Ping Poon *et al.*, 2000). HCC occurs mostly in people 50 to 60 years old and is more frequent in men than

women (2 - 4:1). It commonly develops in settings of liver cell injury, which leads to inflammation, hepatocyte regeneration, liver matrix remodeling, fibrosis, and ultimately, cirrhosis (Figure 1). Thereby, the vast majority of HCC worldwide (80%) is attributed to cirrhosis caused by alcohol abuse or other risk factors, including hepatitis B virus (HBV) and HCV infection, hemochromatosis, fatty liver disease, androgenic steroid use, and other metabolic disorders (Akriviadis *et al.*, 1998). Another high risk factor is the uptake of aflatoxin B1. This mycotoxin, produced by the fungus *Aspergillus*, contaminates food stored in humid conditions and is the most potent oncogenic agent for the liver (Sinha *et al.*, 1988).

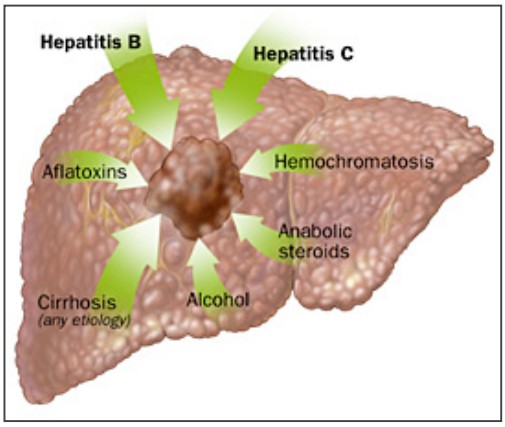

Figure 1: **Causes of hepatocellular carcinoma**. Various risk factors can cause HCC (© 2006 The Johns Hopkins University, http://hopkins-gi.nts.jhu.edu/).

The mechanisms by which these varied etiologies lead to cirrhosis and HCC are not well understood. Changed expression of growth factors and their receptors, along with overexpression of extracellular matrix (ECM) remodeling enzymes (matrix metalloproteinases, MMPs) are often seen in hepatic inflammation and chronic hepatitis. In general, this imbalance becomes more extensive as liver injury progresses through fibrosis, cirrhosis, or after surgical resection with subsequent cytokine and growth factor (*e.g.* HGF/SF) release and ischemia perfusion injury.

To date, liver transplantation is the most promising curative treatment option for non-virus-induced HCC, since systemic chemotherapy has low response rates and has never been shown to prolong patients' survival. Overexpression of the multidrug resistance gene (*mdr-1*) makes HCC an inherently chemotherapy-resistant tumor (Llovet *et al.*, 2003). Even repeated palliative treatment, such as chemoembolization cannot prevent the neoplasm progression, although it induces remarkable tumor necrosis. Finally, HCC has rarely been reported in patients younger than three years. In those patients the prognosis is much worse compared to the prognosis for patients with HB.

1.3 Hepatocyte growth factor/scatter factor (HGF/SF)

HGF/SF is a pleiotropic molecule that stimulates a wide variety of cellular responses including angiogenesis (Grant *et al.*, 1993), cellular motility (Stoker *et al.*, 1987), growth (Stoker *et al.*, 1987), invasion (Jeffers *et al.*, 1996), morphological differentiation (Maina *et al.*, 1997), embryological development (Schmidt *et al.*, 1995; Uehara *et al.*, 1995), tissue regeneration (Matsumoto and Nakamura, 1993), and wound healing (Nusrat *et al.*, 1994). It is mainly expressed by mesenchymal cells (*e.g.* by surrounding fibroblasts) and acts primarily on epithelial cells (Figure 2).

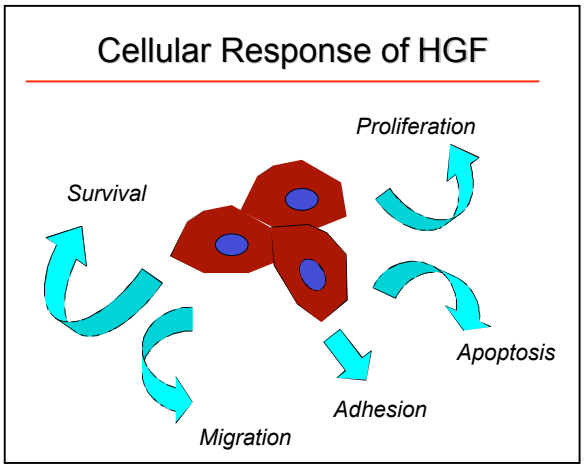

Figure 2: **HGF/SF has multiple effects on cells**. It controls a complex genetic program leading to cell-dissociation, migration in the extracellular matrix, growth, acquisition of polarity, tubule formation, survival, and apoptosis, depending on the concentration and cell type investigated.

HGF/SF contains four kringle domains, each an 80-amino acid double-looped structure that is defined by three conserved disulphide bonds, and a serine proteinase homology domain, which lacks enzymatic activity. A similar structure is found in plasminogen, a circulating pro-enzyme which after activation is responsible for the lysis of blood clots. Like plasminogen, HGF/SF is secreted as a single-chain, biologically inert glycoprotein precursor and is proteolytically cleaved into an active heterodimer consisting of a 60 kDa heavy chain and a 30 kDa light chain linked together by disulfide bonds (Figure 3) (Naldini *et al.*, 1991). Conversion of pro-HGF/SF is mediated by several serine proteases including the plasminogen activators uPA (urokinase plasminogen activator) and tPA (tissue-type plasminogen activator), HGF activator, coagulation factors X, XI, and XII, as well as a homologue of factor XII (Schwall *et al.*, 1996).

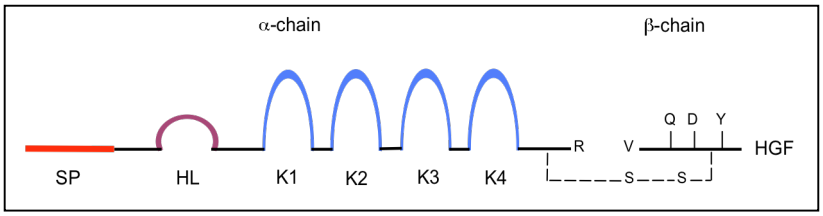

Figure 3: **Structure of HGF/SF.** HGF/SF is composed of α- and β-chains that are present after proteolytic cleavage. The α-chain contains four domains known as kringle (K1, K2, K3, K4), the signal peptide (SP) and the hairpin loop (HL). The β-chain contains the serine protease-like structure. Modified after (Trusolino and Comoglio, 2002)

1.4 c-Met

The c-Met receptor has originally been identified as the product of a human oncogene, *tpr-met*, resulting from chromosomal rearrangement. Here, the extracellular domain is replaced by a translocated promoter region (Tpr) sequence, which provides strong leucine-zipper dimerization motives leading to a constitutively active c-Met kinase activity (Cooper *et al.*, 1984; Park *et al.*, 1987). HGF/SF is the natural ligand for this receptor tyrosine kinase (RTK) c-Met, which is consistently expressed on most epithelial cells (Michalopoulos and Zarnegav, 1992). Since its initial characterization as a proto-oncogene, c-Met has been shown as an important mediator of both normal and neoplastic invasive growth (Trusolino and Comoglio, 2002). In fact, inactivation of the HGF/SF or *c-met* genes in mouse causes embryonal lethality between E12.5 and E15.5 (Maina *et al.*, 2001; Schmidt *et al.*, 1995).

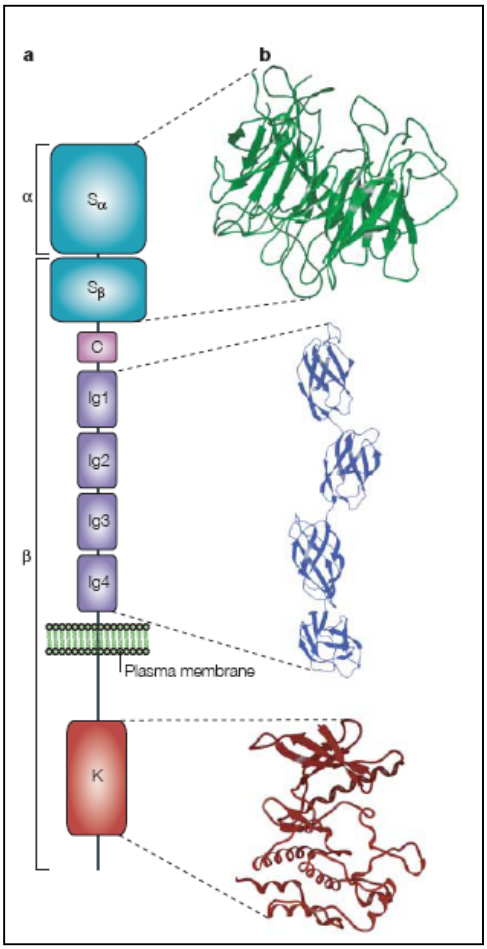

Figure 4: **The c-Met receptor tyrosine kinase.** (A) The domain structure of c-Met. S, sema domain; C, cysteine-rich domain; Ig, immunoglobulin domain; K, kinase domain; α and β refer to the subunits of the receptor that are present after proteolytic cleavage. (B) Three-dimensional models of the Sema, immunoglobulin and kinase domains of c-Met (Birchmeier *et al.*, 2003).

Like its ligand, the c-Met receptor is a disulphide-bridged heterodimer resulting from cleavage of a precursor molecule. The mature form of c-Met consists of an extracellular 50 kDa α-chain and a 185 kDa membrane-spanning β-chain. Recent mutagenesis experiments have shown that the α-chain and the first 212 amino acid residues of the β-chain are sufficient for HGF/SF binding (Gherardi *et al.*, 2003). This region is homologous to the Sema-domain of the semaphorin axon-guidance proteins and might fold into a β-propeller structure. The intracellular domain also comprises the juxtamembrane (JM) and kinase domains as well as a carboxy-terminal domain that is essential for downstream signaling (Ponzetto *et al.*, 1994). The JM region contains a docking site, which can act as a negative regulator of c-Met function. c-Cbl, an E3 ubiquitin ligase, can bind to phosphorylated Y^{1003} within this region via its tyrosine kinase binding (TKB) domain followed by c-Met ubiquitination,

endocytosis, transport to the endosomal compartment, and finally degradation (Hammond *et al.*, 2001; Jeffers *et al.*, 1997; Peschard *et al.*, 2001).

Upon ligand binding c-Met is autophosphorylated on the two tyrosine residues $Y^{1234/35}$, which enhance the intrinsic kinase activity, and phosphorylation of the tyrosine residues $Y^{1349,1359}$ results in formation of a unique multi-substrate docking site that serves as signaling scaffold for various downstream effector molecules (see 1.6 for detailed description). Disturbance of the functional integrity of this activation process, *e.g.* by overactivation mutations, are a major cause for malignant tumors (Birchmeier *et al.*, 2003).

The receptor activation is catalyzed by the kinase domain, which transfers the γ-phosphate group of ATP to tyrosine residues on protein substrates. Substrates of c-Met are multidomain proteins characterized by the presence of specific domains including the Src homology 2 (SH2), the phosphotyrosine binding (PTB) and Src homology 3 (SH3) domain. Some of the substrates that directly interact with c-Met include the growth factor receptor-binding protein (Grb) 2 (Ponzetto *et al.*, 1994), STAT3 (Boccaccio *et al.*, 1998), the p85 subunit of the phosphatidylinositol 3-kinase (PI3K) (Graziani *et al.*, 1991), Shc (Pelicci *et al.*, 1995), phospholipase C-γ (PLC-γ) (Weidner *et al.*, 1990), c-Src (Ponzetto *et al.*, 1994), and Grb2-associated binder (Gab)-1 (Weidner *et al.*, 1990). All these molecules play an important role in the signal transduction and regulation of cellular functions as described below.

1.5 c-Met signal transduction

Stimulation of the c-Met receptor has been linked to cellular responses associated with invasive behavior, such as cell dissociation, migration, scattering and reduced cellular adhesion, but also stimulation of cell proliferation and survival. Epithelial cells, in particular the Madin-Darby canine kidney (MDCK) cell line, respond to HGF/SF with scattering, *i.e.* colony dispersal, increased motility, and invasion into collagen or matrigel matrices (see (Thiery, 2002) for review). Moreover, MDCK cells, when cultured within a collagen matrix, form tubular structures upon HGF/SF stimulation (Boccaccio *et al.*, 1998). Tubular branching is a complex morphogenic coordination of cell growth, polarity, and movement and not yet completely understood.

Many attempts have been undertaken to define the role of each individual downstream effector pathway in specific cellular responses to c-Met activation. Early experiments showed that specific inhibitors of the ERK/MAPK or PI3K pathways could inhibit scattering of epithelial cells, indicating that both are important in disassembly of the adherens junctions, cell spreading and motility (Potempa and Ridley, 1998).

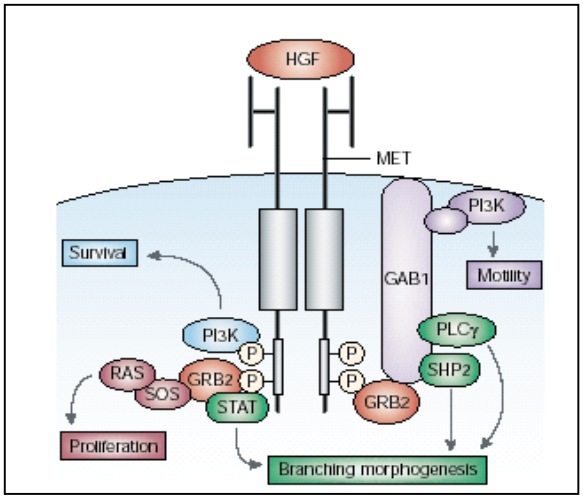

Figure 5: **Signaling pathways of Met.**
c-Met's activity is controlled by various downstream signaling molecules. Phosphatidylinositol 3-kinase (PI3K)-dependent signaling results in cell mobility and survival. Activation of the Grb2-SOS-Ras cascade leads to cell proliferation, whereas Jak/STAT (Janus kinase, Signal Transducers and Activators of Transcription), PLC-γ (phospholipase Cγ), and SHP2 (protein tyrosine phosphatase) are substantial for transformation processes (Trusolino and Comoglio, 2002).

An unique facet of c-Met relative to other RTKs is its reported interaction with focal adhesion complexes and non-kinase binding partners such as β4-integrins, CD44, and semaphorins, which may further add to the complexity of regulation of cell function by this receptor (Giordano *et al.*, 2002; Trusolino *et al.*, 2001; van der Voort *et al.*, 1999). However, these binding partners do not seem to be essential for short-term HGF/SF signaling, but might contribute to sustained c-Met activation (DiGabriele *et al.*, 1998; Hartmann *et al.*, 1998; Schwall *et al.*, 1996).

1.6 Adaptor proteins

Upon phosphorylation of the unique $Y^{1349,1359}$ docking site, several substrates can bind to the c-Met receptor. Most contain the Src-homology domain SH2, which predominately mediates these interactions. For steric reasons two substrates cannot simultaneously bind to the bidenate site of one c-Met molecule (Stefan *et al.*, 2001).

1.6.1 Grb2

Growth factor receptor binding protein (Grb) 2 is a small adaptor protein comprised of an SH2 domain flanked by two SH3 domains and thus is also able to bind to proline-rich ligands (Goudreau *et al.*, 1994; Terasawa *et al.*, 1994). Grb2 binding to the guanine nucleotide exchange factor (GEF) son-of-sevenless (SOS) establishes a link

between the RTK and Ras signaling with subsequent activation of the Raf-MEK1/2-ERK1/2 pathway (Li *et al.*, 1993; Maina *et al.*, 2001).

The c-Met receptor can only recruit one Grb2 through the SH2-domain-binding site at the tyrosine residue Y^{1356} (Fixman *et al.*, 1997). When the adaptor protein Shc is recruited and phosphorylated by the c-Met receptor, it can bind two Grb2 molecules through its SH2 domains (Rozakis-Adcock *et al.*, 1993). This leads to an amplification of Grb2 downstream pathways and can also strengthen the binding of Gab1 (see below) to the c-Met-Shc-$(Grb2)_2$ complex. Notably, Grb2 binding to c-Met is not essential for c-Met signaling in embryogenesis, indicating only a minor or enhancing role in the transmission of c-Met signals at this stage of development (Ieraci *et al.*, 2002).

1.6.2 Gab1

Grb2-associated binder (Gab)-1 is a versatile scaffolding adaptor protein, which binds to the phosphorylated tyrosine residue Y^{1349} of the c-Met receptor, but can also be indirectly recruited through Grb2. Gab1 is the most crucial substrate for c-Met signaling, since it binds signal-relay molecules, such as SH2-domain-containing protein tyrosine phosphatase2 (Shp2), PI3K, phospholipase C and Crk (Gu and Neel, 2003; Maroun *et al.*, 1999; Sachs *et al.*, 2000). Both Crk and Crk-like kinases bind to specific motifs in Gab1 and recruit C3G, a guanine-nucleotide exchange factor that regulates adherent junction positioning and cell adhesion by activating Rap1 (Knox and Brown, 2002; Knudsen *et al.*, 1994).

Knock-out studies of Gab1 revealed the same phenotypes as observed in *c-met* $^{-/-}$ mice, indicating an essential role of Gab1 in c-Met-mediated signaling (Sachs *et al.*, 2000). Indeed, recent experiments demonstrate that Gab1 is necessary for the c-Met-mediated induction of cell-cycle progression, proliferation, and oncogenic morphological transformation (Mood *et al.*, 2006). Notably, Gab1 also participates in the transmission of signals from other receptors, which is reflected by the presence of additional phenotypes in Gab1-mutant mice that are absent in *c-met*-mutant mice (Itoh *et al.*, 2000). However, a prolonged Gab1 phosphorylation, as it was observed in c-Met coupling, cannot be evoked by other growth factors (*e.g.* epidermal growth factor, EGF) (Maroun *et al.*, 1999).

1.6.3 Shc

Another important mediator of cell transformation downstream of c-Met is the adaptor protein Shc, which associates with c-Met and other RTKs as well. Upon

recruitment, $Y^{239/240}$ and Y^{317}-phosphorylated Shc binds to the SH2 domain of Grb2 to facilitate downstream responses, including activation of Ras-Raf-ERK-1/2 and PI3K/Akt pathways. In addition, although Grb2 can directly bind to RTKs, activation of some independent downstream signaling pathways relies on Shc's indirect recruitment of Grb2. Hence, Shc can activate and regulate distinct biological functions, *e.g.* c-Met-stimulated vascular endothelial growth factor (VEGF) production depends on the recruitment of Shc and not Grb2 (Saucier *et al.*, 2004).

Shc was initially identified as an SH2 containing proto-oncogene involved in growth factor signaling and thus hyper-phosphorylation of Shc has been seen in many different types of tumors, suggesting a crucial role in signal transduction (Ravichandran, 2001).

1.7 Activated downstream signaling cascades

1.7.1 Shp2

The SH2 domain containing tyrosine phosphatase (Shp2) is an important mediator of the ERK/MAPK pathway (Gu and Neel, 2003; Schaeper *et al.*, 2000). It normally has a low basal tyrosine phosphatase activity due to allosteric inhibition of its amino-terminal SH2 domain. Only when phosphorylated tyrosine residues of Gab1 bind to the SH2 domains of Shp2, is the inhibition relieved (Cunnick *et al.*, 2002). Experimental data show that a membrane-targeted (but not a cytoplasmic) mutant of Shp2 is sufficient to activate Ras and the ERK/MAPK pathway, whereas a mutant form of Gab1 that lacks the Shp2 binding sites blocks sustained but not transient activation of ERK/MAPK (Cunnick *et al.*, 2002; Maroun *et al.*, 2000). Shp2 possibly also has an adaptor function, given that Grb2 associates with phosphorylated tyrosine residues upon ligand stimulation (Vogel and Ullrich, 1996).

1.7.2 Ras-Raf-ERK/MAPK pathway

The mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes connecting cell surface receptors to critical regulatory targets within the cell. They phosphorylate specific serine and threonine residues of target proteins thereby controlling cell proliferation, differentiation and transformation (Chang and Karin, 2001; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). MAPKs also respond to chemical and physical stress, thus mediating cell survival and adaptation. At least four

distinctly regulated groups of MAPK are expressed in mammals, extracellular-regulated kinase-1/2 (ERK-1/2), Jun amino-terminal kinase (JNK 1/2/3), p38 proteins (p38α,β,γ,δ) and ERK5 (Chang and Karin, 2001).

In c-Met-induced signal transduction, MAPKs are activated through the Gab1/Grb2/SOS/Ras pathway: c-Met phosphorylation leads to the recruitment of Gab1 and Grb2 and association and activation of the Ras-GEF SOS, which in turn activates membrane-associated Ras. Ras then induces serine/threonine kinase activity of the MAPK kinase kinase (MAPKKK) Raf-1, which phosphorylates and activates MAPK kinases 1/2 (MEK-1/2). MEK-1/2 finally activate ERK-1/2 by phosphorylation of threonine and tyrosine residues in the regulatory Thr-Glu-Tyr (TEY) motif (Robinson and Cobb, 1997). ERK-1/2 then translocate to the nucleus to phosphorylate and activate transcription factors.

The ERK/MAPK pathway is a critical mediator of HGF/c-Met signaling and required for HGF/SF-elicited cell scattering, adherens junctions disassembly, proliferation and tubulogenesis (Khwaja *et al.*, 1998; Potempa and Ridley, 1998; Schaeper *et al.*, 2000; Tanimura *et al.*, 1998).

1.7.3 PI3K-Akt/PKB

Phosphatidylinositol-3 kinases (PI3Ks) have a central function in the control of metabolism, cell growth, proliferation and survival. The so-called class IA isoforms of PI3Ks are tightly associated with the p85 regulatory subunit that contains two SH2 domains with a high affinity for phosphorylated tyrosine residues of c-Met and other RTKs, as well as adaptor proteins (*e.g.* Gab1). Once activated, the 110 kDa-subunit of PI3K is brought in close proximity of the plasma membrane. There, it catalyzes the phosphorylation of membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP_2) at the 3' position of the inositol ring, producing the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). The generation of PIP_3 subsequently causes translocation of PIP_3-binding proteins to the plasma membrane (reviewed in Wymann *et al.*, 2003). Many of these molecules bind to PIP_3 through a pleckstrin homology (PH) domain, including the proto-oncogenic protein kinase B (PKB), also known as Akt (Brazil and Hemmings, 2001). The activation of class I PI3Ks is counteracted by phosphoinoside phosphatases, such as PTEN, which dephosphorylate PIP_3 to PIP_2 (Brazil and Hemmings, 2001; Wymann *et al.*, 2003).

Akt/PKB, besides its effects on glucose transport and activation of glycogen synthesis and glycolysis, promotes growth factor-mediated cell survival and anchorage-independent growth, both directly and indirectly. Akt/PKB-mediated phosphorylation of Bad, the pro-apoptotic Bcl-2 family member, leads to Bad dissociation from the Bcl-2/Bcl-x_L complex, thereby loosing its pro-apoptotic function (Dudek *et al.*, 1997; Fan *et al.*, 2000; Franke *et al.*, 1997b; Frisch and Francis, 1994; Igney and Krammer, 2002; Porter and Vaillancourt, 1998; Xiao *et al.*, 2001).

HGF/SF-induced PI3K/Akt signaling is required for scattering and branching morphogenesis in many cell types (Ponzetto *et al.*, 1994; Royal and Park, 1995). Overexpression of a constitutively active form of PI3K is sufficient to induce *in vitro* scattering and branching morphogenesis of MDCK cells (Khwaja *et al.*, 1998).

Another target of Akt/PKB is the glycogen synthase kinase-3β (GSK-3β). Phosphorylation of GSK-3β leads to its inactivation, thus preventing the association of GSK-3β, APC and free β-catenin and the successive degradation of β-catenin (see 1.7.7) (Bienz and Clevers, 2000; Polakis, 2000).

1.7.4 Jak/STAT pathway

The Janus kinase (Jak) family of cytokine receptor-associated protein tyrosine kinases and the signal transducer and activator of transcription (STAT) proteins have diverse biological functions, including roles in cell differentiation, proliferation, development, apoptosis, and inflammation (Figure 6)(Ishihara and Hirano, 2002). Thus, STAT proteins are induced by a wide variety of growth factors and cytokines (Levy *et al.*, 1988). Ligand binding to the cognate receptor results in activation of members of the Jak family (Shuai *et al.*, 1993) and tyrosine phosphorylation of STAT proteins. The latter then form homo- or heterodimers through their SH2-domains, and translocate to the nucleus, where they preferentially bind to response elements in the promoters of target genes and activate transcription (Sadowski *et al.*, 1993; Shuai *et al.*, 1993).

Aberrations in STAT signaling are found in numerous cancers and therefore this pathway is a potential target for anticancer therapy (Haura *et al.*, 2005). Constitutive activation and increased levels of STAT3 has been linked to increased survival of tumor cells and thus have been found in many human malignancies (Turkson and Jove, 2000). Constitutive activation of both STAT1 and STAT5 accompanies transformation of many cell types (Bowman *et al.*, 2000).

The role of the Jak/STAT pathway in c-Met signaling is controversial. Direct binding of STAT3 to the c-Met receptor and a requirement for STAT3 in the c-Met-dependent formation of branched tubules have been reported (Boccaccio *et al.*, 1998). However, other studies support STAT3 activation independent of c-Met binding, *e.g.* in anchorage-independent growth (Schaper *et al.*, 1997; Zhang *et al.*, 2002).

1.7.5 Src

The non-receptor tyrosine kinase c-Src is required for the loss of intra-cellular junctions and gain of cell-matrix adhesion during HGF/SF-dependent scattering and migration responses (Ponzetto *et al.*, 1994). Following HGF/SF stimulation, c-Src associates with c-Met and mediates cell motility and anchorage-independent growth by phosphorylating the tyrosine residues in the short cytoplasmic domain of E-cadherin, leading to its internalization (Pece and Gutkind, 2002). Other substrates of c-Src include focal adhesion kinase (FAK) and the adapter protein p130Crk-associated substrate ($p130^{Cas}$), both involved in cell adhesion, migration and transformation (Cary *et al.*, 1998). Analogously, in non-metastatic murine mammary carcinoma SP1 cells, where

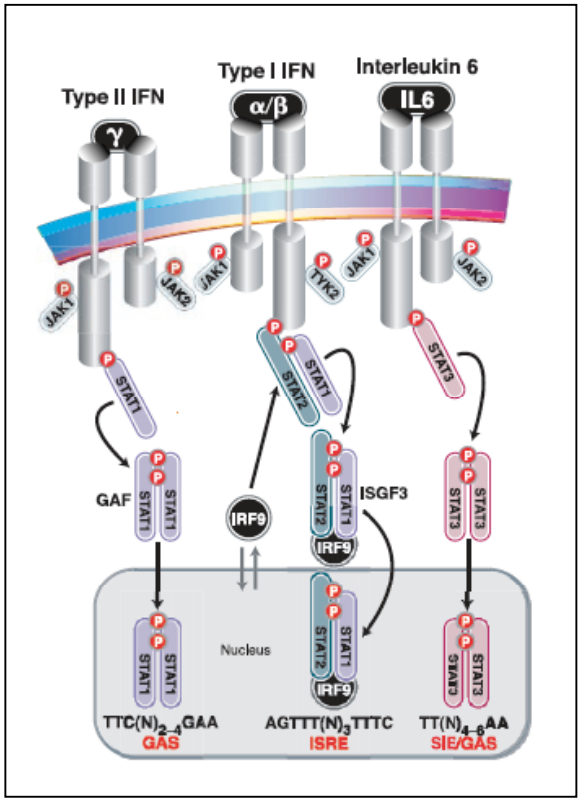

Figure 6: **Three examples of signaling in the JAK-STAT pathway**.

Specific ligand-receptor interactions generate active transcription complexes composed of distinct STAT proteins. *Left*: Type II IFN (IFN-γ) binding induces receptor tyrosine phosphorylation (P) by JAK1/2 proteins, producing a recruitment site for STAT1. STAT1 dimers translocate to the nucleus and activate transcription from IFN-γ target gene promoters containing IFN-γ activation sites (GAS). *Center*: Type I IFNs (IFN-α or IFN-β) stimulate the activity of JAK1 and TYK2 proteins, leading to STAT2 tyrosine phosphorylation. The STAT2 phosphotyrosine is a docking site for latent STAT1. The activated factor ISGF3 is a heterotrimer of STAT1 and STAT2 in association with IFN regulatory factor (IRF)-9, which alone can enter the nucleus, but is retained in the cytoplasm by interactions with STAT2. *Right*: IL-6 activates JAK1 and JAK2, producing a phosphotyrosine docking site for STAT3. STAT3 dimers translocate to the nucleus and activate transcription from target gene promoters containing a GAS-like element, sometimes referred to as the serum-inducible element (SIE). IL-6 also activates STAT1, leading to homo- and heterodimers of STAT1 and STAT3 (not illustrated; Aaronson and Horvath, 2002). ISRE, interferon-stimulated response element.

c-Src kinase activity is significantly reduced, HGF/SF-induced motility is blocked (Rahimi *et al.*, 1998).

1.7.6 Phospholipase C-γ (PLCγ)

PLCγ is another adaptor protein that directly binds to the phosphorylated tyrosine residue Y^{1356} of the c-Met receptor, but can also bind to Grb2. Although it is not essential for HGF/SF-induced scattering or proliferation of MDCK cells (Royal *et al.*, 1997), it was concluded that sustained recruitment of PLCγ to Gab1 plays an important role in branching tubulogenesis (Gual *et al.*, 2000). Furthermore, PLCγ has been shown to be involved in growth factor-induced mitogenesis, differentiation, development, metabolism, secretion, contraction and sensory perception (Noh *et al.*, 1995; Thackeray *et al.*, 1998; Wang *et al.*, 1998).

PLCγ forms stable complexes with RTKs through its SH2 domains (Anderson *et al.*, 1990; Margolis *et al.*, 1990). After activation by tyrosine phosphorylation, PLCγ hydrolyzes PIP_2 to form inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ stimulates the release of calcium ions from internal stores (Berridge *et al.*, 1998; Lipp and Niggli, 1996) and DAG activates protein kinase C (PKC) (Wahl and Carpenter, 1991). The correct balance of calcium between the endoplasmic reticulum and the mitochondria is necessary for cell survival (Berridge *et al.*, 1998; Dolmetsch *et al.*, 1998). Loss of this equilibrium, *e.g.* by a decrease in $InsP_3$ production, could be lethal for cells.

1.7.7 Wnt/β-catenin pathway

The highly conserved canonical Wnt/β-catenin pathway plays important roles in development and is frequently found upregulated in tumors (Bienz and Clevers, 2000; Polakis, 2000). In the absence of Wnt signals, β-catenin, in a complex with APC and Axin, is phosphorylated on serine/threonine residues by the GSK-3β and targeted for ubiquitin/proteasome-mediated degradation by the β-transducin-repeat-containing protein (β-TrCP). Upon Wnt binding to Frizzled receptors, Disheveled is activated, which subsequently blocks β-catenin degradation (Figure 7). Excess β-catenin translocates to the nucleus where it interacts with the HMG-box transcription factor TCF to modulate transcription of several target genes, such as cyclin D1, c-myc, c-jun, TCF-1, MMP-7,

CD44, β-TrCP, and Slug (Bienz and Clevers, 2000; Conacci-Sorrell *et al.*, 2003; Nelson and Nusse, 2004; Polakis, 2000).

The other central role of β-catenin is its function in the adhesion complex. As a member of adherens junctions it is, like γ-catenin, sequestered by cadherins and linked to the cytoskeleton, thereby mediating tight intercellular adhesion. Three conserved tyrosine residues in β-catenin regulate the integrity of the cadherin core complex: Y^{142}, Y^{489}, and Y^{654} (Aberle *et al.*, 1996). Phosphorylation of these residues results in dissociation of α-catenin from β-catenin with concomitant loss of cadherin adhesion. Liberated tyrosine-phosphorylated β-catenin does not interact with Axin (Danilkovitch-Miagkova *et al.*, 2001), thereby possibly inhibiting its ubiquitination and degradation through the proteasomal pathway, which finally leads to an accumulation of unbound phospho-β-catenin.

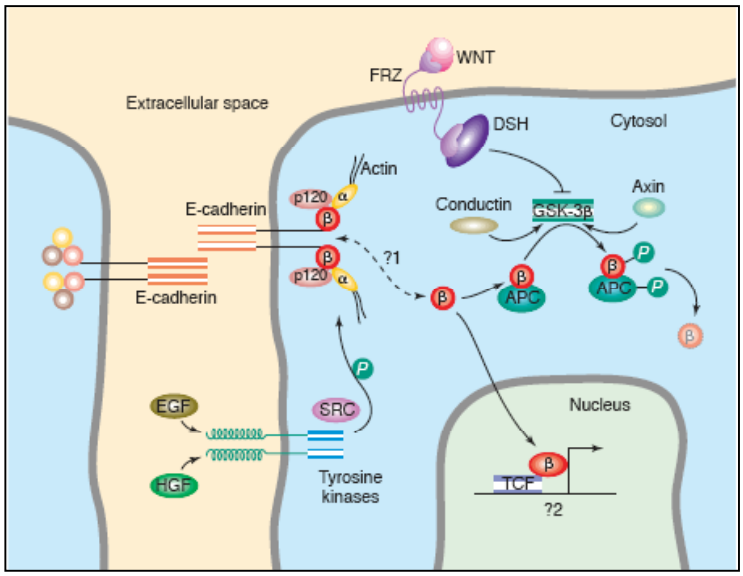

Figure 7: **The link between E-cadherin-mediated cell–cell adhesion and the WNT signalling pathway.**
In the absence of Wnt stimulation, the adenomatous polyposis coli (APC)/Axin/glycogen synthase kinase 3β (GSK-3β)/ complex earmarks β-catenin (β, red) for degradation by the proteasome. The levels of cytoplasmic β-catenin are low, and T cell factor (TCF) is repressed. After Wnt stimulation of the Frizzled (FRZ) receptor, Dishevelled (Dsh) is recruited to the membrane where it binds to Axin to inhibit the APC/Axin/GSK-3β complex. β-catenin accumulates and, after translocation into the nucleus, binds to TCF transcription factor to co-activate Wnt target genes. β-catenin is also involved in the formation of adherens junctions. These junctions are formed by the transmembrane protein E-cadherin, which is linked to the actin cytoskeleton by β-catenin and α-catenin (α, orange). EGF, epidermal growth factor; HGF, hepatocyte growth factor; P, phosphate; p120, $p120^{CAS}$; SRC, SRC tyrosine kinase (Christofori and Semb, 1999).

When E-cadherin function is lost, β-catenin is liberated from the adhesion core complex and serine-phosphorylated in the APC/Axin/GSK-3β-complex, resulting in its proteasomal degradation (Danilkovitch-Miagkova *et al.*, 2001).

Notably, there is a pool of β-catenin that is especially targeted by the HGF/SF receptor c-Met (Brembeck *et al.*, 2004). Binding of HGF/SF induces phosphorylation of β-catenin at the tyrosine residue Y^{142}, releasing the protein from a membrane-associated pool to associate with Bcl9-2, a *Drosophila* Legless homologue, which is responsible for

escorting β-catenin into the nucleus. This occurs concomitantly with the conversion of MDCK cells expressing Bcl9-2 to a mesenchymal phenotype (Brembeck *et al.*, 2004). These data are consistent with prior findings, where oncogenic c-Met mutations resulted in the accumulation of β-catenin and increased transcription of β-catenin/TCF target genes. Accordingly, in many cancer cells, loss of function of the tumor suppressor APC, mutations in β-catenin or inhibition of GSK-3β has been found associated with over-expression of proto-oncogenes.

Also for HB and HCC, frequent mutations of β-catenin and Axin resulting in the prevention of degradation and nuclear accumulation of β-catenin and activation of oncogenic target genes have been reported (Clevers, 2000; Koch *et al.*, 1999; Polakis, 2000; Satoh *et al.*, 2000).

The HGF/c-Met-mediated increase of oncogenic β-catenin/TCF target gene expression may play a pivotal role during EMT and tumorigenesis (Danilkovitch-Miagkova *et al.*, 2001; Muller *et al.*, 2002). However, to date it is not clear whether the translocated β-catenin originates from released E-cadherin/β-catenin junctions or whether c-Met is surrounded by its own pool of β-catenin as suggested by Monga and colleagues (Monga *et al.*, 2002).

1.8 Transcription factors

A frequent downstream event of signaling cascades is the activation of transcription factors, which bind to promoters of genes and thus mediate transcription of target genes. In HGF/c-Met-triggered cellular responses several transcription factors are involved, including c-Fos, Egr-1, JunB, JunD, and c-Myc (Johnson *et al.*, 1995; Maffe and Comoglio, 1998; Recio and Merlino, 2003; Weir *et al.*, 1994). Dysregulation of signaling cascades often leads to overactivation of transcription, which is considered as another hallmark of cancer (Hanahan and Weinberg, 2000).

1.8.1 AP-1

Transcription factors like c-Fos and c-Jun, which belong to the activated protein-1 (AP-1) transcription factor family, are responsible for a variety of cellular responses upon growth factor stimulation. It was previously shown that HGF/SF-induced AP-1 binding results from post-translational modification of c-Jun protein or expression of other members of the AP-1 family or both. In particular the activation of the Ras/MAPK

pathway by HGF/SF results in activation and stimulation of mRNA expression of AP-1 family members (*e.g.* c-jun and c-fos) (Maffe and Comoglio, 1998; Weir *et al.*, 1994). Moreover, by electrophoretic mobility shift assays (EMSA), an AP-1 binding site (TGAGTCA) was identified as the responsive element within the *c-met* core promoter region at position -158 to -152 (Seol *et al.*, 2000), indicating that c-Met induces a positive feedback loop.

1.8.2 Egr-1

The early growth response factor (Egr)-1 [also known as NGFI-A, Krox-24, and Zif268] encodes a nuclear, 82 kDa zinc finger protein capable of binding to specific GC-rich DNA sequences containing the consensus binding code GCG(G/T)GGGC, and it is known to regulate various genes, especially those important in injury stimuli (Sukhatme *et al.*, 1988; Sukhatme *et al.*, 1987). Egr-1 recognition elements were predominantly identified in promoters of genes encoding growth factors, cytokines and their receptors, and cell cycle control genes (for review see (Silverman and Collins, 1999)). Recently it was demonstrated that HGF/SF upregulates pro-angiogenic factors, such as PDGF and VEGF (Worden *et al.*, 2005), CD44v6 (Recio and Merlino, 2003), and angiotensin converting enzyme (ACE) (Day *et al.*, 2004), as well as fibronectin (Gaggioli *et al.*, 2005) through Egr-1 in a MAPK-dependent manner. Other target genes of Egr-1 play a vital role in cellular growth, development, and differentiation and include HGF/SF, TGFβ-1, FGF, IGF-II, TNF-α, IL-1β, and plasminogen activator inhibitor (PAI)-1 (Houston *et al.*, 2001).

NGFIA-binding proteins (Nab)-1 and -2 tightly regulate the expression and function of Egr-1. While Nab-1 is constitutively expressed, Nab-2 is activated by the same signals as Egr-1 and by Egr-1 itself (Kumbrink *et al.*, 2005). Nab-2 not only inhibits Egr-1 binding to DNA, it also directly interacts with the Egr-1 promoter (Cao *et al.*, 1993), thereby providing a strong negative feedback loop.

In summary, HGF/SF is capable of activating several pathways and inducing transcription of various target genes. The final biological outcome, however, depends on which pathway is activated and which co-signals derive from the cell microenvironment.

1.9 Mechanisms of apoptosis

In multicellular organisms, apoptosis is a naturally occurring process that is crucially involved in organogenesis and removal of altered or infected cells. Thus, it prevents the pathogenesis of various diseases such as cancer.

In this process, aspartate-specific proteases, the so-called caspases are the main effectors (Thornberry and Lazebnik, 1998). One mechanism to trigger these caspases is by ligand binding to the tumor necrosis factor (TNF) receptor family. This stimulation results in activation of the initiator caspase 8, which in turn activates the effector caspase 3.

Other apoptotic triggers, such as UV-radiation, γ-radiation, disruption of cell attachment, a process termed *anoikis*, or exposure to chemicals, lead to drastic changes of the mitochondria. Mediators include proteins of the Bcl-2 family, which consists of pro- and anti-apoptotic members. Among them are the pro-apoptotic proteins Bax and Bid as well as the anti-apoptotic protein Bcl-x_L, and Bcl-2 itself. When Bax is activated, its conformational change leads to an oligomerization at the outer membrane of the mitochondria and to pore formation. This promotes the efflux of cytochrome c and a second mitochondria-derived activator of caspases (Smac) into the cytoplasm. Cytochrom c, together with the apoptotic peptidase activating factor (APAF)-1 and caspase 9 form the so-called *apoptosome*. Subsequently, caspase 9 is activated, which then in turn activates caspase 3. Activated caspase 3 finally leads to destruction of cellular structures and causes cell death. Since activation of caspase 3 is a late event in apoptosis and is activated by both signaling pathways, it is a unique protein to study apoptosis (Frisch and Francis, 1994; Hengartner, 2000; Igney and Krammer, 2002).

1.10 EMT/ scattering/ invasion

Invasion of surrounding tissues and metastasis to distant organs have long been recognized as hallmark features of malignant cancers (Hanahan and Weinberg, 2000). Metastasis is a multi-step process characterized by dissociation of tumor cells from the epithelial layer, penetration through the basement membrane into the adjacent connective tissue, intravasation, survival in the blood stream, extravasation at a distant site and colonization of new terrain, with stimulation of neo-angiogenesis (Chambers *et al.*, 2002). In this process, the downregulation of adherens and tight junction proteins is a prerequisite. The central member of such adhesion junctions is the calcium-dependent

homophilic transmembrane protein E-cadherin. Intracellularly it is directly bound to β-catenin through the cytoplasmic domain. α-catenin then links the N-terminal region of β-catenin to α-actinin, and to several actin-binding proteins, *e.g.* zonula occludens (ZO)-1 and vinculin, suggesting that it plays a crucial role in organizing actin filaments at adherens junctions. However, it seems that the exact role of α-catenin has been misjudged so far (Burridge, 2006).

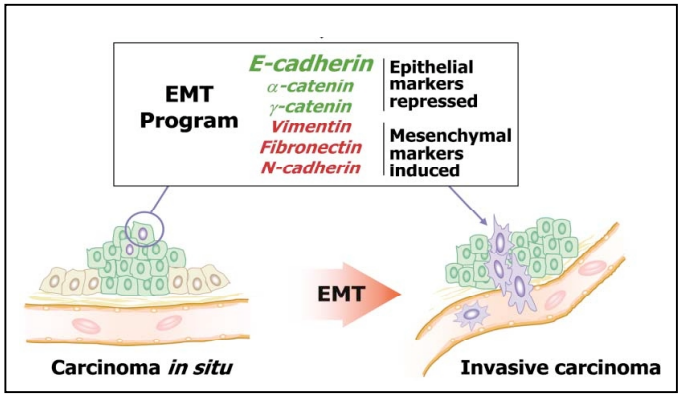

Figure 8: **EMT in tumor progression**.
During the multi-step process of epithelial-mesenchymal transition (EMT) early stage tumor cells (green) become more motile by accidental overexpression of EMT regulators (purple nuclei) resulting in downregulation of adhesion proteins like cadherins and catenins and concomitantly upregulation of mesenchymal markers (Kang and Massague, 2004).

Loss of cell-cell-adhesion together with morphological changes, loss of apico-basolateral cell polarization, induction of cell motility, decrease in cell-matrix-adhesion, and changes in the production or distribution of specific proteins, *e.g.* desmoplakin and vimentin, are typical characteristics of the epithelial-mesenchymal transition (EMT) (Grunert *et al.*, 2003; Thiery, 2002).

Table 1: Phenotypic modules of EMT and metastasis

category	***transitioning event***	***phenotypic module***
EMT	Epithelial release	Disintegration of cell-cell contacts Loss of cell polarity Repression of epithelial markers
	Migration	Cytoskeletal reorganization Locomotion Chemoattraction
	Invasiveness	Basement membrane degradation Interstitial matrix degradation Interstitial matrix synthesis
Metastasis	Intravasation/ extravasation	Anoikis resistance Apoptosis resistance Endothelial cell adhesiveness
	Metastatic growth	Autonomus growth potential Angiogenesis factors

(adapted from: Zavadil and Bottinger, 2005)

EMT plays a pivotal role during development by which epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility. Accumulating evidence points to a critical role of EMT-like events during tumor progression and malignant transformation, endowing the incipient cancer cell with invasive and metastatic properties (Boyer *et al.*, 2000). During development, tissue repair, and tumor metastasis, both cell-cell dissociation and cell migration occur and appear to be intimately linked (see Table 1).

In cancer progression, oncogenic EMT refers to clusters of malignant cells with altered epithelial characteristics and acquired, self-sustained migratory and highly matrix invasive phenotype. Oncogenic EMT is well documented *in vivo* and typically believed as complete and irreversible, in contrast to the reversible EMT observed during embryogenesis (Zavadil and Bottinger, 2005).

Loss of E-cadherin function is a key element in EMT, and thus E-cadherin is considered a tumor suppressor for two reasons: transcription of its gene is silenced in various carcinomas, and repression of a native form of E-cadherin in carcinomas is sufficient to reduce the aggressiveness of tumor cells (Cavallaro and Christofori, 2004; Perl *et al.*, 1998). Furthermore, there is a good correlation between the process of invasion and metastasis and loss of E-cadherin at the cell surface of cancerous cells (Birchmeier and Behrens, 1994).

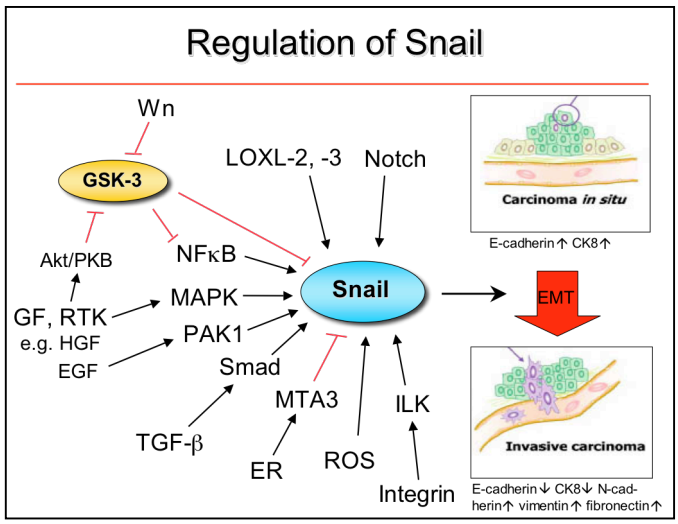

Figure 9: **Regulation of Snail**.

The function of Snail is tightly regulated by its stability, subcellular localization and different phosphorylation events. The subcellular localization is governed by the GSK-3β. GSK-3β phosphorylates serine residues, which is a signal for nuclear export and for ubiquitination and degradation, respectively.

ILK, integrin-linked kinase; PAK1, p21-activated kinase; GSK-3β, glycogen synthase kinase; TGF, transforming growth factor; EGF, epidermal growth factor; MTA3, metastasis-associated protein 3, ROS, reactive oxygen species; ER, estrogen receptor; LOXL, lysyl oxidase-like enzyme; CK8, cytokeratin 8.

Cadherins generally are regulated at both the mRNA and protein levels, by means of changes in subcellular distribution, translational or transcriptional events, and degradation. Normally, E-cadherin is repressed by transcriptional repressors that bind to the consensus sequence

CANNTG (termed E-box) in the E-cadherin promoter. Transcriptional repressors include the zinc-finger transcription factors Snail and Slug (Bolos *et al.*, 2003; Cano *et al.*, 2000; Nieto, 2002), δEF-1/ZEB-1 (Eger *et al.*, 2005), and SIP-1/ZEB-2 (Comijn *et al.*, 2001; Peinado *et al.*, 2004). In addition, the highly conserved basic helix-loop-helix (bHLH) transcription factors Twist (Yang *et al.*, 2004) and E12/47 (Cano *et al.*, 2000; Perez-Moreno *et al.*, 2001) were also found to control E-cadherin expression. Sugimachi *et al.* found an inverse correlation between the expression of Snail and E-cadherin in HCCs. Moreover, Snail overexpression and E-cadherin downregulation were associated with higher cancer invasiveness, suggesting a central role for Snail in tumor progression and metastasis (Figure 9) (Sugimachi *et al.*, 2003).

Besides mediating adhesion junctions, Snail has also been linked to loss of tight junction proteins such as claudins, occludin, and ZO-1 (Ikenouchi *et al.*, 2003; Ohkubo and Ozawa, 2004). In addition, Snail positively controls genes significantly involved in tissue remodeling, *e.g.* fibronectin, vimentin, RhoGTPases, and MMPs (Nieto, 2002).

1.11 c-Met, HGF/SF, and cancerogenesis

HGF/SF-triggered cell scattering and migration recapitulates many of the events that occur during cancer invasion, as tightly clustered epithelial cells break their cell-cell junctions and become single, migrating, invasive cells. Thus, dysregulation of c-Met and HGF/SF by mutation, gene rearrangement, gene amplification, constitutive kinase activation, and overexpression have been implicated as causative factors in the development and progression of numerous human cancers, including lung, breast, colorectal, prostate, pancreatic, gastric, hepatocellular, ovarian, renal, glioma, melanoma, and some sarcomas (see Table 2 for a comprehensive list). Activation of c-Met in cancer occurs most often through ligand-dependent autocrine or paracrine mechanisms and unfortunately, this over-activation often correlates with poor prognosis (Birchmeier *et al.*, 2003).

Table 2: HGF/SF, c-Met and cancer references

Category	Cancer type	HGF/SF expression	Met expression	Poor prognosis	Mutation of Met	*In vitro* studies	Animal model
Carcinomas	Bladder	Y	Y	Y	N	Y	Y
	Breast	Y (A)	Y	Y	N	Y	Y
	Cervical	N	Y (60%)	Y	N	N	N
	Cholangiocarcinoma	N	N	N	N	N	Y
	Colorectal	Y	Y	N	N	Y	Y
	Oesophageal	N	Y	N	N	N	N
	Gastric	Y	Y (46%)	Y	Y	Y	Y
	Head and neck	Y	Y	Y	Y	Y	N
	Kidney	Y	Y	N	Y	N	N
	Liver	Y	Y	Y	Y	Y	Y
	Lung	Y (A)	Y	Y	N	Y	Y
	Nasopharyngeal	Y	Y	Y	N	Y	N
	Ovarian	N	Y	N	Y	Y	Y
	Pancreas/Gall bladder	Y	Y	N	N	Y	Y
	Prostate	Y	Y	N	N	Y	Y
	Thyroid	Y (A)	Y	Y	N	Y	N
Musculoskeletal sarcomas		Y	Y	N	N	Y	N
	Osteosarcoma	Y	Y	N	N	Y	Y
	Synovial sarcoma	Y	Y	N	N	N	N
	Rhabdomyosarcoma	N	Y	N	N	Y	Y
Soft tissue sarcomas	MFH/Fibrosarcoma	Y	Y	N	N	N	Y
	Leiomyosarcoma	Y	Y	N	N	Y	Y
	Kaposi's sarcoma	Y	Y	N	N	N	Y
Haematopoietic malignancies	Multiple myeloma	Y (~85%)	Y	Y	N	Y	Y
	Lymphomas	Y	Y	N	N	Y	Y
	Adult T-cell leukaemia	N	Y (62%)	N	N	Y	N
	Acute myelogenous leukaemia	Y	N	N	N	Y	N
	Chronic myeloid leukaemia	Y	N	N	N	N	N
Other neoplasms	Glioblastomas/ Astrocytomas	Y (~80%)	Y (100%)	Y	Y	Y	Y
	Melanoma	Y	Y	N	N	Y	Y
	Mesothelioma	Y (~35–80%)	Y (~75–100%)	N	N	Y	N
	Wilms' tumor	Y	Y	N	N	N	N

The table shows significant references related to the roles of HGF/SF or c-Met in the types of cancer indicated. For each cancer type, headings correspond to studies showing: HGF/SF expression in tumor biopsies, c-Met expression in tumor biopsies, expression of HGF/SF or c-Met correlating with poor prognosis, sporadic or germline-activating mutations in c-Met, tumor cells *in vitro* expressing c-Met or HGF/SF, some with correlations to *in vitro* neoplastic-like activities, and animal models supporting the role of c-Met and HGF/SF in cancer, including human tumor xenografts in immunocompromised mice, mice with HGF/SF or c-Met transgenes, or other animal models displaying dependence on HGF/SF or c-Met in cancer development. A, autocrine; MFH, malignant fibrous histiocytoma; %, percentage of tumors examined that are positive; Y, citations available online (http://www.vai.org/vari/metandcancer); N, no report (Ref.: Birchmeier *et al.*, 2003).

Furthermore, ligand-independent activation of c-Met is also possible, *e.g.* by activating mutations (Park *et al.*, 1987). c-Met overexpression also has been attributed to a number of epigenetic mechanisms including tumor-secreted growth factors, tumor hypoxia, and activation of other oncogenes, *e.g.* activated Ras (Furge *et al.*, 2001; Ivan *et al.*, 1997). Further proof of the involvement of HGF/c-Met signaling in tumor progression comes from several *in vitro* and *in vivo* studies. Mouse and human cell lines that ectopically overexpress HGF/SF or c-Met, or both become tumorigenic and metastatic in athymic nude mice (Rong *et al.*, 1994).

In addition, c-Met mutations in the JM domain have been reported in some cancer subtypes (Table 2)(Lee *et al.*, 2000; Ma *et al.*, 2003). Mutations in this domain prevent downregulation of c-Met and leads to extended c-Met signaling (Peschard *et al.*, 2001). Therefore, a large number of studies show that HGF/SF, c-Met or both are frequently expressed in carcinomas, in other types of solid tumors, and in their metastases.

The switch from a begnin to a malignant tumor includes a step where the tumor cells become more motile. Therefore, cell-cell junctions have to be disassembled. Fujita and colleagues recently described a possible way in which HGF/SF may contribute to this process. c-Met activation by its ligand mediates disruption of cell-cell contacts through the tyrosine phosphorylation-dependent binding of the SH2-containing E3-ubiquitin-ligase Hakai to E-cadherin. Hakai then catalyzes the ubiquitination of the E-cadherin complex and enhances the subsequent E-cadherin endocytosis, resulting in a decrease of adherens junctions (Fujita *et al.*, 2002). Accordingly, an inverse correlation between E-cadherin and c-Met has been reported in colon epithelial cells (Boon *et al.*, 2005), further indicating the importance of this signaling cascade in tumorigenesis.

The diverse set of cellular functions regulated by c-Met influence critical steps during the metastatic process including: (i) migration, secretion of proteolytic enzymes and invasive growth of tumor cell during extravasation, (ii) anoikis/survival of tumor cells in the vasculature, (iii) arrest in capillary beds and invasive infiltration of tumor cells to form micrometastases, and (iv) cell growth and survival at diverse remote microenvironments supporting the growth of micrometastases. In addition, the increased production of HGF/SF by tumor and stromal cells in neoplams at both primary and metastatic sites may affect the recruitment of endothelial cells and formation of microcapillaries during tumor neovascularization (see Figure 10).

In conclusion, c-Met activation is not only a marker for cancer, but is also a marker of biological significance for malignancy. Especially in HCC, c-Met expression is correlated with increased incidence of intrahepatic metastasis and low patients' 5-years survival rates (Ueki *et al.*, 1997). Not surprisingly, HGF/SF and c-Met were therefore recently reported to be potential targets for chemotherapeutic intervention, highlighting their importance in tumor progression (Christensen *et al.*, 2005; Corso *et al.*, 2005; Jiang *et al.*, 2005).

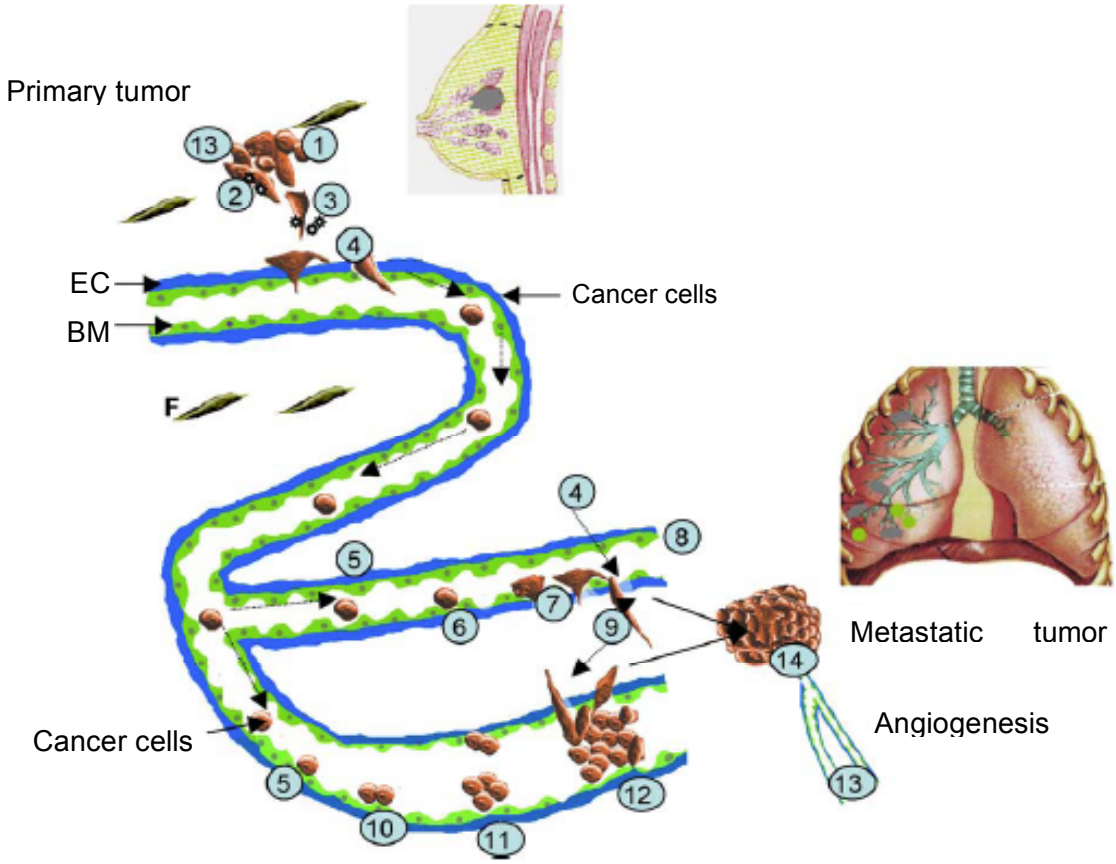

Figure 10: **HGF/SF participates widely in the metastatic cascade**.

A primary breast tumor and its metastatic spread to the lung are depicted. HGF/SF has been reported to affect the following events: (1) disruption of cell adhesion mechanisms, including E-cadherin, claudins, and possibly desmosomes; (2) stimulation of secretion and/or activation of proteolytic enzymes that are involved in matrix degradation, including MMPs, uPA, etc.; (3) the stimulation of motility by HGF/SF can be seen throughout the metastatic process; (4) breach of the basement membrane (BM), as a result of increased proteolytic enzymes and HGF/SF-induced motility of cancer cells; (5) 'the docking process' that allows tumor cells to loosely connect to endothelial cells (EC); (6) 'homing' of cancer cells; (7) HGF/SF increases tumor adhesion to the endothelium, mediated by cell adhesion molecules, including CD44; (8) HGF/SF is known to affect and disrupt tight junctions, thus allowing increased paracellular permeability; (9) 'extravasation process'; (10) division of adherent tumor cells followed by (11) formation of micro-emboli of tumor cells; (12) tumor emboli and embolism in microvessels which result in further disruption of the integrity of the endothelial monolayer and facilitate invasion of the endothelium by cancer cells; (13) HGF/SF acts as an angiogenic factor; and (14) promotes formation of metastatic tumors.

F: fibroblasts; BM: basement membrane; EC: endothelial cells; (Jiang *et al.*, 2005).

2 Aims of this study

The hepatocyte growth factor/scatter factor (HGF/SF) has been implicated in numerous effects on tumor progression. Strikingly, increased levels of this growth factor were found in serum of patients with hepatoblastoma (HB) and hepatocellular carcinoma (HCC) after surgery, accompanied by increased growth of residual tumors, higher relapse incidence, and poor prognosis (von Schweinitz *et al.*, 2000). A link between the high HGF/SF serum levels and the observed phenotype has been proposed. Comparative analysis of cell lines derived from different liver tumor subtypes could therefore help identifying general mechanisms by which HGF/SF promotes tumor progression. The general goal of this study was to unravel the molecular mechanisms underlying HGF/SF's function in the oncogenic process.

My studies at the Kantonsspital Basel and the Institute of Biochemistry and Genetics were aimed at characterizing in detail the effects of HGF/SF on HB and HCC growth, migration, survival, chemoresistance, and adhesion. Special consideration was given to the HGF/SF-induced signaling pathways underlying these cellular responses.

The main objectives of this study were to:

1.) investigate the role of HGF/SF on HB and HCC cell growth and progression,
2.) characterize the anti-apoptotic effect of HGF/SF on HB and HCC cells,
3.) unravel the molecular mechanisms which lead to HB and HCC cell scattering, migration, and invasion.

Therefore, the investigations of the molecular pathways in HB and HCC cells should help to define more targets for therapeutic strategies and provide further insights into normal and aberrant liver development.

3 Materials and Methods

3.1 Laboratory chemicals and biochemicals

General chemicals used were all purchased from Sigma/Fluka (Buchs, Switzerland) unless otherwise stated.

3.2 General maintenance of all cell lines

All cell lines were cultured at 37°C, 95% humidity, and 5% CO_2 in medium supplemented with 10% fetal calf serum (FCS, Sigma) and 2 mM glutamine (Invitrogen Basel, Switzerland). Prior to trypsinization, cells were washed once with 1x PBS (Sigma) to remove FCS. Cells were then detached with 1x trypsin/EDTA (Sigma). Hepatoma cells were maintained in RPMI1640 medium (Invitrogen), MDCK cells in Eagle's minimum essential medium (MEM, Sigma), and HEK293 cells in Dulbecco's modified Eagle's medium (DMEM, Sigma). For long-term storage, cells were deep frozen (liquid N_2) in 90% FCS, 10% DMSO.

3.3 Cell lines

3.3.1 HepT1

This cell line was established from a multifocal, poorly differentiated hepatoblastoma of a 34-month-old girl. It has been characterized by immunohistochemistry, electron microscopy, and molecular genetic analysis in the laboratory of Prof. Torsten Pietsch (Pediatric Oncology, Institute for Neuropathology, University of Bonn) (Pietsch *et al.*, 1996). The HepT1 cell line has been injected into nude mice and the resulting tumors show hematopoietic foci similar to those observed in the original tumor (Pietsch *et al.*, 1996; von Schweinitz *et al.*, 2000).

3.3.2 HuH6

This cell line was obtained through a biopsy from a hepatoblastoma of the right liver lobule of a one-year-old boy. These cells are strongly granulated and are positive for α-fetoprotein, albumin and CK18 and CK19, and resemble the original tumors in this respect (Doi, 1976). This cell line is commercially available from the Japanese Collection of Research Bioresources (JCRB).

3.3.3 HepG2

The liver epithelial HepG2 cell line is widely distributed and commonly used in research. It was originated from a liver biopsy of a 15-year-old Argentinean boy. This tumor is highly differentiated with trabecular cell order and positive for CKs, α-fetoprotein, albumin, and plasminogen. Desmin and vimentin could not be detected in this cell line (Knowles *et al.*, 1980). HepG2 is commercially available from the American Type Culture Collection (ATCC).

3.3.4 MDCK

Derived from a kidney of an apparently normal adult female cocker spaniel, the MDCK cell line was established by S. H. Madin and N. B. Darby (Gaush *et al.*, 1966), and has been widely used for research. It is highly receptive for HGF/SF signals and therefore has often been used for investigations of HGF/SF-induced cellular responses. This cell line is commercially available from ATCC.

3.3.5 HEK293

The epithelial cell line HEK293 was generated by transformation of human embryonic kidney cell cultures (hence HEK) with sheared adenovirus 5'-DNA, and was first described in 1977 (Graham *et al.*, 1977). Since then it has been commonly used and characterized in detail. This cell line is also commercially available from ATCC.

3.4 DNA plasmid constructs

For Snail promoter activation assays, a plasmid encoding for the mouse Snail promoter cloned upstream of a luciferase reporter was used (Peinado *et al.*, 2003). For normalization, the luciferase reporter construct p*Renilla* (Promega) was co-transfected.

To generate a construct expressing shRNA against Snail, Snail-shRNA 5'-GATCCAGGCCTTCAACTGCAAATAGTGTGCTGTCCTATTTGCAGTTGAAGGCCTTTTTTTGGAA-3' (forward) 5'-AGCTTTTCCAAAAAAGGCCTTCAACTGCAAATAGGACA-GCACACTATTTGCAGTTGAAGGCCTG-3' (reverse), and control-shRNA (mNCAM) 5'-GATCCCCGTACAAGGCTGAGTGGAAGTTCAAGAGACTTCCACTCAGCCTTGTACT-TTTTGGAAA-3' (forward) and 5'-AGCTTTTCCAAAAAGTACAAGGCTGAGTGGA-AGTCTCTTGAACTTCC-ACTCAGCCTTGTACGGG-3' (reverse) oligonucleotides were annealed and inserted into the pSUPER-retro-puro vector according to the manufacture's directions (OligoEngine, Seattle, WA, USA).

To create an inducible Snail expression system, a PCR fragment from the human constitutive active Snail-8SA cDNA (Figure 11, a gift from Dr. Mien-Chie Hung, University of Texas, Houston, USA) was generated using the following primers 5'-GCGCGGATCCA-CCATGCCGCGCTCTTTCCT-CG-3' (forward) and 5'-CGCC-GCTCGAGTCAGCGGGCA-CATGGTGAG-3' (reverse) and subcloned into the pcDNA5/FRT/TO vector at the BamH1 and Xho sites (performed by F. Lehembre).

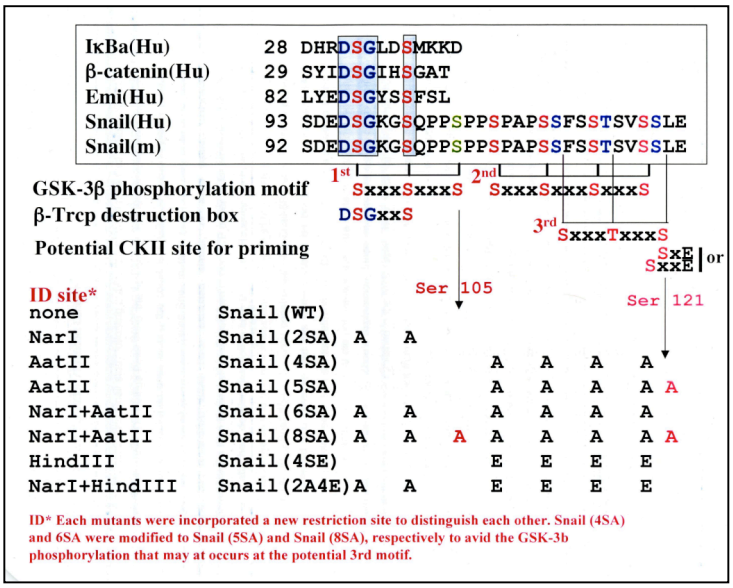

Figure 11: **Snail sequence and mutation sites**.
A stabilized version of Snail was generated by site-specific mutations of GSK-3β binding sites, resulting in nuclear accumulation of Snail, which is constitutive-active (Zhou *et al.*, 2004).

For studying Egr-1-mediated transactivation, plasmids encoding for a dominant negative (dn) Egr-1-GST fusion protein (pEBGN-Egr-1), empty vector (pEBGN; (Al-Sarraj *et al.*, 2005)) or a Nab-resistant mutant of Egr-1 (pCMV-FLAG-C2/Egr-1; (Al-Sarraj *et al.*, 2005)) were used (all Egr-1 plasmids kindly provided by Dr. Gerald Thiel,

Table 3: Plasmids used in this project.

Plasmid name	**Insert**	**Application**
pSUPER-RETRO-puro	sh-hSnail	ST
pSUPER-RETRO-puro	sh-mNCAM	ST
pMACS 4.1	CD4	TT, magnetic sorting
pcDNA5/FRT/TO	Snail-8SA	TT, ST
pCMV-FLAG-C2/Egr-1	FLAG-C2/Egr-1	TT
pxp1	murine Snail promoter	TT, luciferase assay
pEBGN-Egr-1	dnEgr-1	TT, luciferase assay
pEBGN	empty vector	TT, luciferase assay
pRL-CMV	*Renilla* luciferase	TT, luciferase assay

TT, transient transfection; ST, stable transfection.

University of Saarland Medical Center, Homburg, Germany). An overview over all plasmids used in this study is provided in Table 3.

3.5 Transfections and selection of transfected cells

HepG2 cells were transiently transfected using Fugene (Roche Diagnostics, Basel, Switzerland) or the Amaxa Nucleofactor II device (program P-27, Amaxa, Cologne, Germany) according to the manufacturer's instructions.

pSUPER-Snail-shRNA and pSUPER-mNCAM-shRNA were transfected into HepG2 cells using Fugene (Roche). Stable clones were obtained by selection with 150 μg/ml puromycin (Sigma) and were maintained under selection. Six out of 12 resistant clones were further analyzed and three showed reduced Snail mRNA levels.

The inducible Snail-8SA construct was transfected into HEK293-FlpInTRex cells using Lipofectamine reagent (both Invitrogen). Stable clones were obtained after selection with hygromycin B and blasticidin (both Invitrogen), according to the manufacturer's protocol. Snail-8SA-transfected HEK293-FlpInTRex cells were kept in FlpInTRex Medium (DMEM medium supplemented with 10% FCS, 2 mM glutamine, 15 μg/ml blasticidin, 100 μg/ml hygromycin B). Snail expression was induced by adding 1 μg/ml doxycycline (Sigma) to the culture medium.

For transient transfection of HEK293 cells, Metafectene was used according to the manufacturer's protocol (Biontex, Martinsried, Germany).

3.6 Magnetic sorting of CD4-positive HepG2 cells

HepG2 cells were transiently co-transfected with the eukaryotic vector pMACS 4.1 (Miltenyi Biotec, Bergisch Gladbach, Germany) encoding for a truncated, human CD4 cell surface receptor, incapable of inducing CD4-mediated signal transduction. After electroporation using the P-27 program of the Amaxa Nucleofector II device, cells were allowed to recover for three days. Cells were harvested with 2 mM EDTA in PBS and the cell suspension was mixed with MACS CD4 MicroBeads (Miltenyi Biotec) for magnetic labeling. Magnetic separation was performed by applying the labeled cells onto a MS column attached to an OctoMACS separator according to the instructions of the manufacturer (both Miltenyi Biotec). Positively selected cells were recultured for 48 h and then stimulated with 10 ng/ml HGF/SF for 8 h. Total RNA was isolated with the Trizol method according to the manufacturer's protocol (Invitrogen).

For Snail promoter activation assays (Peinado *et al.*, 2003), HEK293 cells in 24-well plates were transiently transfected at about 70% confluency with a total of 2 to 4 µg plasmid DNA by using Metafectene (Biontex, Martinsried, Germany) in a ratio to DNA of 2:1. Cells were incubated with DNA/Metafectene complexes for 24 h in normal medium at 37°C before lysis.

3.7 Immunoblot analysis

Cells were seeded in cell culture dishes of appropriate size and incubated with growth factors or inhibitors as indicated. After removal of the medium and two washing steps with ice-cold PBS, cells were lysed in RIPA$^+$ buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP40, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), 2 mM $CaCl_2$, 2 mM $MgCl_2$, and freshly added 1 mM dithiotreitol (DTT), 1 mM sodium fluoride (NaF), 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), 2 mM sodium orthovanadate (Na_3VO_4), and 1x protease inhibitor cocktail (Sigma)) and kept for 30 min on ice. After centrifuging at 12,000 x g for 10 min at 4°C, supernatant was frozen until subjected to analysis. Protein concentration was determined on a BioPhotometer (Vaudaux-Eppendorf, Schönenbuch, Switzerland) by utilizing the BioRad Protein Assay (BioRad, Reinach, Switzerland). 50 µg of cell lysates were boiled in Laemmli buffer (Laemmli, 1970), resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to an Immobilon P PVDF membrane (Millipore, Volketswil, Switzerland). Membranes were blocked with 5% non-fat milk in TBS (35 mM Tris-HCl (pH 7.4), 140 mM NaCl) containing 0.05% Tween-20. After primary and HRP-conjugated secondary antibody incubation (see Table 4 for antibodies used), ECL+ (Amersham Biosciences, Freiburg, Germany) or UptiLight (Interchim, Montluçon, France) were used for chemiluminescence detection of protein bands on superRX films (Fuji, Dielsdorf, Switzerland).

Table 4: Antibodies used for immunoblotting

Name	Source	Dilution	Company
HGF/SF	mouse	1:1000	R&D Systems
c-Met	mouse	1:1000	Cell Signaling Technology
p-Met (Tyr^{1349})	rabbit	1:1000	Cell Signaling Technology
p-p-Met ($Tyr^{1234/1235}$)	rabbit	1:1000	Cell Signaling Technology

p-Akt	rabbit	1:1000	Cell Signaling Technology
Akt	rabbit	1:1000	Cell Signaling Technology
p-GSK-3β	rabbit	1:1000	Cell Signaling Technology
GSK	rabbit	1:1000	Cell Signaling Technology
E-cadherin	mouse	1:2000	BD Transduction Lab
β-catenin	rabbit	1:2000	Sigma
hSnail, N-term	rabbit	1:400	Abgent
p-ERK-1/2	mouse	1:1000	Cell Signaling Technology
ERK-1/2	rabbit	1:1000	Cell Signaling Technology
β-actin	mouse	1:5000	Abcam
Vinculin	goat	1:1000	Santa Cruz Biotechnology
Egr-1	rabbit	1:500	Santa Cruz Biotechnology
p-STAT3	rabbit	1:1000	Cell Signaling Technology
STAT3	rabbit	1:1000	Cell Signaling Technology
p-STAT1	rabbit	1:1000	Cell Signaling Technology
STAT1	rabbit	1:1000	Cell Signaling Technology
anti-mouse-IgG	goat	1:5000	SouthernBiotech
anti-rabbit-IgG	goat	1:5000 – 1:10000	Transduction Labs
anti-goat-IgG	donkey	1:1000	Jackson ImmunoResearch

Abbreviation: p-, phospho-specific

3.8 MTT Assay

To assess cell viability, 100 µl pre-warmed MTT solution (5 mg/ml in PBS) was added to 1 ml of culture medium. After 1 h incubation, the medium was aspirated and 500 µl of solubilisation buffer (95% isopropanol, 5% formic acid) were added per 24-well. Then, the plate was transferred to a horizontal shaker for homogenization of the cell lysate. 100 µl of each sample were transferred to a 96-well microtiter plate (Becton Dickinson) and measured in a SpectraMax 340PC (Molecular Devices) at 560 nm.

3.9 ^{3}[H]-Tymidine incorporation

Stimulated cells were kept in growth and stimulation medium before 1 µCi/ml [^{3}H]-thymidine (Amersham) was added to each well. After 3 h incubation, incorporation was terminated by careful washing of the cells twice with 1 ml ice cold PBS and 1 ml of ice cold 5% TCA. The cells were then incubated for 30 min at 4°C before being washed once with ice cold PBS. At room temperature (RT), cells were then solubilized in 0.5 ml 0.5 N NaOH/0.5% SDS. Lysates were mixed by pipetting up and down and added to scintillation vials. After the scintillation cocktail (Ready Safe) was added, the samples were measured with a LS 6500 Liquid Scintillation Counter (both Beckman Coulter, Nyon, Switzerland).

3.10 Cell proliferation/ Cytometry

Cells were seeded in duplicate at 5 × 10^{5} cells/ well in 24-well plates. The next day, the cells were stimulated with various HGF/SF concentrations and incubated for the indicated time. Cells were then trypsinized in 1 ml trypsin/EDTA and 100 µl of the cell suspension was diluted with 10 ml Isoton II (Beckman Coulter). They were then counted with a Coulter Counter with particle size set between 8 and 12 µm.

3.11 Preparation of nuclear extracts

Stimulated cells were washed twice with ice-cold PBS and lysed in Low Salt buffer (20 mM Hepes (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.2% NP40, 10% glycerol, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 2 µg/ml aprotinin, 1 µg/ml leupeptin). After 10 min on ice, cell lysates were centrifuged at 3,000 x g for 5 min at 4°C). Supernatants were transferred to new Eppendorf tubes and used as cytosolic extracts. The remaining pellets were lysed in High Salt buffer (420 mM NaCl, 20 mM Hepes (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 2 µg/ml aprotinin, 1 µg/ml leupeptin) and incubated for 30 min on ice with intermittent vortexing. Samples were cleared by centrifugation for 5 min at 12,000 x g at 4°C, and supernatants were aliquoted, shock frozen on dry ice and stored at -80°C.

3.12 Electrophoretic Mobility Shift Assay (EMSA)

For the binding reaction, nuclear extracts (2 µl, 5 – 10 ng protein) were incubated at room temperature in 5x shift buffer (20 mM Hepes (pH 7.9)), 4% Ficoll, 1 mM $MgCl_2$, 40 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, 160 µg/ml poly [dI-dC] (Roche)) with 1 ng ^{32}P-ATP-labeled oligonucleotides (>10^5 cpm) for 20 – 30 min at room temperature.

Nucleoprotein-oligonucleotide complexes were resolved by a 5% non-denaturing PAGE at 400 V for 4 h at 4°C. Gels were dried for 1 h at 80°C, and exposed overnight at RT to Storage Phosphor Screens (Amersham Biosciences, Freiburg, Germany). Results were then digitized using a phosphor imaging system (PhosphorImager) and quantified by ImageQuant software (both Amersham Biosciences). The following oligonucleotides, corresponding to STAT-response-element sequences were used: mutated serum-inducible element (SIE-m67) = 5' CATTTCCCGTAAATCAT-3'; βCas = 5'-GATTTCTAGGAATTCAATCC-3' (Fasler-Kan *et al.*, 1998). The specificity of the DNA-protein complex was confirmed by adding 1 µl 1:10-diluted specific STAT antibodies (STAT1, sc346; STAT5, sc1656; STAT6, sc621; Santa Cruz Biotechnology) to the incubation mix, resulting in a stronger retention of the complex ('supershift').

3.13 Caspase 3-like protease activity

After incubation with the apoptotic inducers in the presence or absence of HGF/SF for the indicated time points, cells were washed three times with PBS and lysed with hypotonic extraction buffer (25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM Pefabloc, pepstatin, leupeptin and aprotinin (1 mg/ml each), and 0.1% Triton X-100). All lysates were cleared by centrifugation (15 min, 13,000 g, 4°C), and supernatants were frozen immediately and stored at -80°C until measurement.

The cleavage assay was carried out on opaque microtiter plates (DynaStar, Dynatech, Chantilly, USA) according to the method originally described by Thornberry (Thornberry and Lazebnik, 1998); generation of free 7-amino-4-trifluoromethylcoumarin (afc) was measured in assay buffer (50 mM HEPES (pH 7.4), 1% sucrose, 0.1% CHAPS, 10 mM – 25 mM DTT, and 50 µM fluorogenic substrate N-acetyl-asp-glu-val-asp-afc) after 120 min at 37°C using a microplate fluorometer SpectraMax Gemini EM (Molecular Devices) set to an excitation wavelength of 395 nm and an emission wavelength of 505 nm. Protein concentrations of corresponding samples were quantified with the BioRad protein assay (BioRad), and the specific caspase-3-like activity was calculated after 120 min as relative fluorescence units (FLU) per mg protein.

3.14 Cell scattering

Cells were seeded in 12-well tissue culture dishes at a density of $1x10^5$ per well. 24 h later, cells were incubated with HGF/SF (5 to 20 ng/ml) or PBS. When inhibitors were used, cells were pre-incubated with inhibitors for 1 h before addition of HGF/SF. Control cells were treated with 1 µl/ml DMSO. After 72 h, phase-contrast pictures were taken with a Nikon COOLPIX 995 (Egg/ZH, Switzerland) camera attached to a Leitz Labovert FS microscope (Leica, Heerbrugg, Switzerland).

3.15 Isolation of total RNA

Total RNA was isolated using Trizol reagent according to the manufacturer's instruction (Invitrogen). Shortly, subconfluent cell layers on 60-mm cell culture dishes were washed in 1x PBS and subsequently 1 ml of Trizol was added. After an incubation and centrifugation step, chloroform was added and after vigorous mixing shortly incubated. After phase separation, the upper RNA-containing aqueous phase then was transferred to a fresh Eppendorf tube and 0.5 ml isopropanol added. Precipitated RNA was washed with 1 ml of 75% ethanol and finally centrifuged at 7,500 x g at 4°C for 5 min. Pelleted RNA was air-dried completely and dissolved in diethylpyrocarbonate (DEPC)-treated deionized water (DEPC-H_2O). Before freezing, dissolved RNA was incubated for 10 min at 60°C.

3.16 Quantitative SYBR green real time RT-PCR (qPCR)

Total RNA (3 µg) was reverse transcribed with random hexamer primers using M-MLV reverse transcriptase RNAse (H-) (Promega, Wallisellen, Switzerland). To verify that the primers did not amplify genomic DNA, RNA samples were also incubated in the absence of the reverse transcriptase (-RT samples). Transcripts of various genes were detected by quantitative SYBR green RT-PCR (qRT-PCR) on an ABI Prism 7000 TaqMan using the SYBR green PCR MasterMix (both Applied Biosystems, Rotkreuz, Switzerland) with the primers listed in Table 5. Each PCR mixture contained cDNA, SYBR green MasterMix, and 300 nM primer pair in a final volume of 25 µl. The PCR conditions were an initial denaturation step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles consisting of 15 sec at 95°C, and 1 min at 60°C. PCR assays were performed in triplicates and relative mRNA expression was calculated of the mean value with the comparative C_t method ($\Delta\Delta C_t$) (Yuan *et al.*, 2006).

Table 5: Oligonucleotides used for qRT-PCR and qPCR

Name	forward primer	reverse primer	Application
Slug	5-GGACACATTAGAACTCACACGGG-3'	5'-GCAGTGAGGGCAAGAAAAAGG-3'	qRT-PCR
Twist	5-GCAGGGCCGGAGACCTAG-3'	5'-TGTCCATTTTCTCCTTCTCTGGA-3'	qRT-PCR
hSnail	5'-CACTATGCCGCGCTCTTTC-3'	5'-CACTATGCCGCGCTCTTTC-3'	qRT-PCR
h3'UTR-Snail	5'-TCCCGGGCAATTTAACAATG-3'	5'-TGGGAGACACATCGGTCAGA-3'	qRT-PCR
canine Snail	5'-TTGCCGACCGCTCCAA-3'	5'-CAGGG-ACATGCGGGAGAA-3'	qRT-PCR
canine β-actin	5'-ACGTTGACATCCGCAAGGAC-3'	5'-CAGGGCAGTGATCTCCTTCTG-3'	qRT-PCR
hRPL19	5'-GATGCCGGAAAAACACCTTG-3'	5'-CAGGGCAGTGATCTCCTTCTG-3'	qRT-PCR
Egr-1	5'-ACCGCAGAGTCTTTTCCTGACA-3'	5'-TCGAGTGGTTTGGCTGGG-3	qRT-PCR
Claudin-3	5'- CAGCAACATCATCACGTCGC-3'	5'- CAGTTCATCCACAGGCCCTC-3'	qRT-PCR
Snail	5'-TCGCTTCCTCCCCAGTGAT-3'	5'-TGGAGAGCGTGGCATTGAC-3'	qPCR
NCAM	5'-GTTCCATCAAAACGAACGAACCCG-3'	5'-CACTCGCAACTCGGAGATCC-3'	qPCR

3.17 Reporter gene assays

By using the Amaxa Nucleofactor II device, cells were transiently transfected with a mouse-Snail promoter cloned upstream of a firefly luciferase reporter (Peinado *et al.*, 2003). For normalization, the luciferase reporter construct p*Renilla* (Promega) was co-transfected. After transfection, cells were allowed to recover for 24 h and then incubated with 10 ng/ml HGF/SF or 2 mM LiCl.

Dual-Luciferase reporter (DLR) assays were performed in duplicate according to the directions of the manufacturer (Promega). Cells were lysed in passive lysis buffer and transferred to an opaque 96-well assay plate. Light units generated by both luciferase activities were recorded with a Berthold Mithras LB 940 luminometer (Berthold Technologies, Regensdorf, Switzerland). Luciferase activities were expressed as relative light units (RLU). Firefly luciferase activities (LAR) were always normalized to the control *Renilla* luciferase activity (RAR).

3.18 Chromatin immunoprecipitation (ChIP) analysis

The ChIP assay was performed using the ChIP assay kit (Upstate Biotechnology, Lucerne, Switzerland) following the instructions provided by the manufacturer. Shortly,

after stimulation with HGF/SF, histones were cross-linked to DNA for 10 min by adding formaldehyde to a final concentration of 1% to the medium. After two washing steps with ice-cold PBS containing protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml pepstatin A), cells were scraped off the plate and lysed in SDS lysis buffer for 10 min on ice. Cell lysates were sonicated five times with 10 sec pulses at 30% of maximum power with samples kept on ice during the whole procedure to shear DNA into fragments of 200 – 1000 bp length.

Sonicated cell suspension were then diluted in 10x ChIP dilution buffer (Upstate) containing protease inhibitors mentioned afore. For reduction of the background, 75 µl protein A agarose/salmon sperm DNA were added for 30 min at 4°C and kept under agitation. After removal of the protein A agarose/salmon sperm DNA, rabbit anti-Egr-1 (sc-189), rabbit anti-Snail (Abgent) in a concentration of 10 µg/ml each or unrelated rabbit IgG were used to immunoprecipitate DNA-containing complexes overnight. To collect the antibody/histone complex, 60 µl protein A agarose/salmon sperm DNA were added for 1 h and kept under agitation for 1 h at 4°C. Afterwards serial washing with Low Salt buffer, High Salt buffer, and LiCl immune complex wash buffer (all Upstate) were performed to remove unbound non-specific DNA. For the subsequent PCR, histone complexes were eluted from the antibody by addition of 250 µl elution buffer to the pelleted protein A agarose/antibody/histone complex. After incubation for 15 min at RT under agitation, the agarose was spun down, and the supernatant-containing DNA was mixed with 5 M NaCl and incubated for 4 h at 65°C to reverse cross-link histones to the DNA. Next, 10 µl 0.5 M EDTA, 20 µl 1 M Tris-HCl and 2 µl of 10 mg/ml proteinase K were added to the eluates and incubated for 1 h at 45°C. DNA was then recovered by phenol/chloroform extraction and ethanol precipitation. Pellets were washed with 70% ethanol and air-dried.

PCR was performed with primers 5'-GTTTCCCTCGTCAATGCCACGCTC-3' (forward) and 5'-CGTGCCGCGGCGCAATGAATGCAGC-3' (reverse) complementary to the Snail promoter region containing Egr-1 binding sites and primers 5’-TTTCGG-ATTCCCGCAGTGTGG-3’ (forward) and 5’-TAGGAAGTCACGGCCACTTGG-3’ (reverse) complementary to the E-box containing region of the Egr-1 promoter, respectively. DNA samples were analyzed by 2% agarose gel with ethidium bromide staining. This analysis was performed by F. Lehembre.

3.19 Immunofluorescence

Cells were grown on uncoated glass cover slips and washed twice with HEPES-buffered saline (HBS; 1 HEPES, 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 2 mM $CaCl_2$, 2 mM $MgCl_2$) before fixation in 4% paraformaldehyde in HBS for 20 min at RT. Cells were washed three times in HBS, permeabilized with 0.3% Triton X-100 in HBS for 10 min at RT, before they were washed three times in HBS. After blocking cells for 30 min with 3% bovine serum albumin (BSA) in HBS, primary antibodies in 3% BSA, in HBS were added (1 h at RT). Cells were washed three times in HBS, then secondary antibodies conjugated with different fluorochromes (Alexa Fluor 546, 568, 488 from various species (Molecular Probes)) were added (1 h at RT) in the dark. After washing the cells three times with 0.05% Tween-20, 0.1% BSA in HBS, 4',6-Diamidino-2-phenylindole (DAPI) (1 mg/ml) was added for 1 to 3 min in the dark, followed by two washing steps with HBS and one final washing step with deionized H_2O before the cover slips were mounted on glass slides with the fluorescent mounting medium Mowiol (12% glycerine, 4.8% Mowiol, 24% 0.2 M Tris-HCl (pH 8,5), 0,1% DABCO).

Table 6: Antibodies used for immunofluorescence stainings in this study

Name	Source	Dilution	Company
p-STAT1	rabbit	1:100	Cell Signaling Technology
p-STAT3	rabbit	1:100	Cell Signaling Technology
Anti-mouse-IgG-488	rabbit	1:600	Molecular Probes
Anti-mouse-IgG-546	rabbit	1:600	Molecular Probes
Anti-rabbit-IgG-488	goat	1:600	Molecular Probes
Anti-rabbit-IgG-546	goat	1:600	Molecular Probes
Cytokeratin 8	rabbit	1:250	Abcam
Cytokeratin 18	rabbit	1:250	Abcam
Cy3-conjugated anti-rabbit	goat	1:200	Jackson ImmunoResearch
Cy8-conjugated anti-rabbit	goat	1:200	Jackson ImmunoResearch

Abbreviation: p-, phospho-specific

3.20 Statistical analysis

Student's *t*-test was used to compare data between two groups. Values are expressed as mean ± standard deviation (SD) of at least triplicate measurements. Statistical analysis was performed using GraphPad Prism 4.0c (GraphPad Software, San Diego, USA). *P*-values < 0.05 were considered statistically significant.

4 Results

In HB and HCC patients, markedly elevated serum levels of HGF/SF are frequently found. HGF/SF, the ligand for the receptor tyrosine kinase c-Met, is a multipotent factor that regulates cell growth, motility, invasion, angiogenesis, and morphogenesis of various cells types. Especially during organogenesis and regeneration, it mediates crucial facets and seems to be a key modulator of epithelium-mesenchyme interactions. The aim of this study was to analyze the importance of this factor in HB and HCC progression.

4.1 HGF/SF as a mitogen for HB and HCC cells

HGF/SF induces cell proliferation in many cell types, thereby acting as a mitogen (Weidner *et al.*, 1993). This study aimed to investigate whether HGF/SF might promote HB and HCC cell proliferation. For this purpose HGF/SF's potential mitogenic activity was assessed by [^{3}H]-thymidine incorporation, cell counts, and MTT viability assays.

4.1.1 [^{3}H]-Thymidine incorporation

To quantitate the DNA synthesis rate after HGF/SF stimulation in comparison to untreated cells, HB (HuH6, HepT1) and HCC (HepG2) cells were incubated with various amounts of HGF/SF in normal growth medium for 48 h. Afterwards cells were harvested and [^{3}H]-thymidine incorporation was determined by liquid scintillation. HGF/SF incubation did not result in an increase of total [^{3}H]-thymidine incorporation in any of the cell lines tested (Figure 12, blue bars). The total cell number (Figure 12, red bars) was not significantly different in comparison to those of untreated cells. The ratio of incorporated [^{3}H]-thymidine per cell as a marker for DNA synthesis rate neither was changed (Figure 12, yellow bars), suggesting that HGF/SF does not have a mitogenic effect on human HB and HCC cell lines.

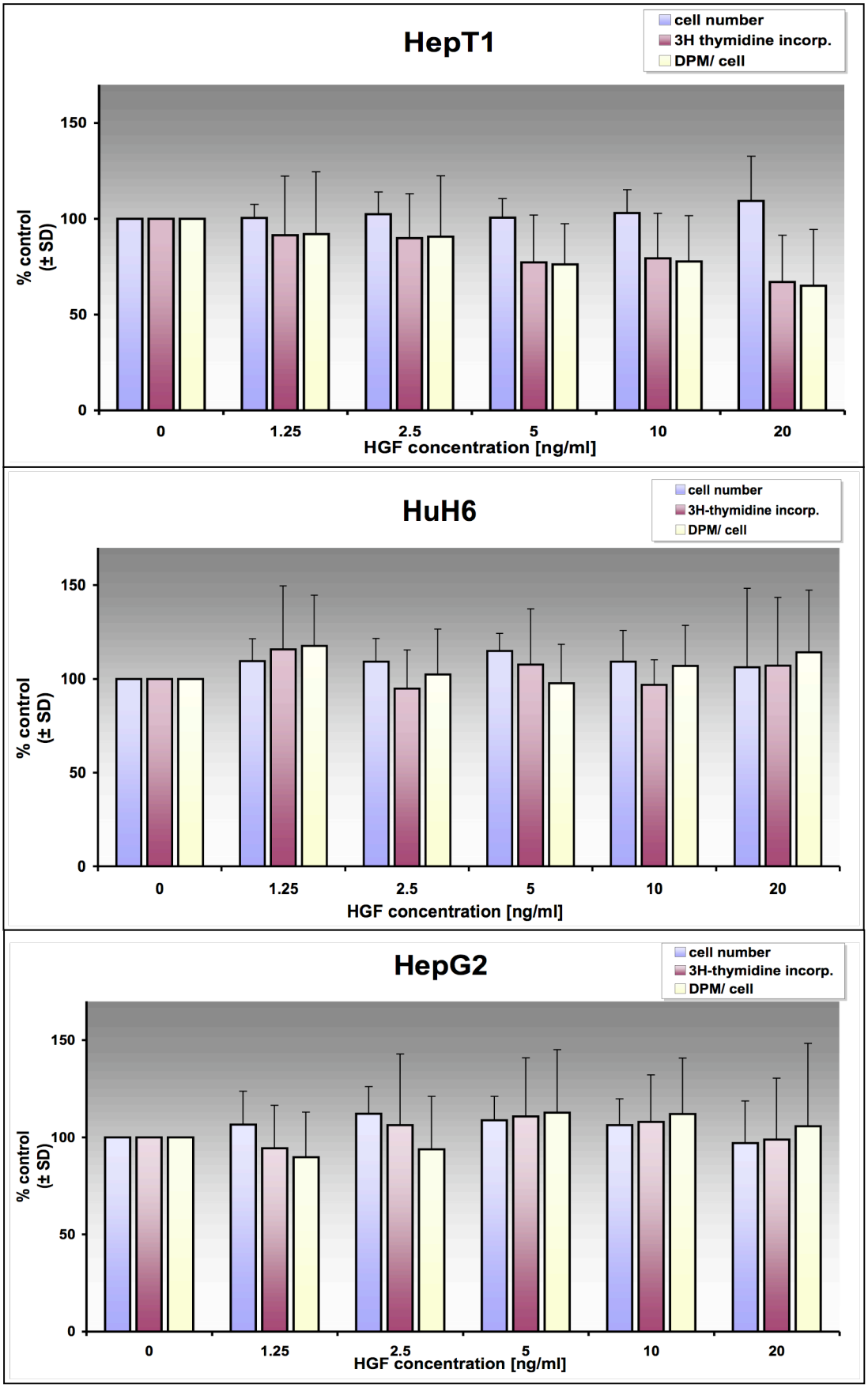

Figure 12: **DNA synthesis rates in HB and HCC cells**. [^{3}H]-thymidine incorporation and total cell numbers were assessed after incubation with various HGF/SF concentrations for 48 h. Values represent percent of control. Blue bars, cell number relative to control; red bars, total decays per minute (DPM) relative to control, yellow bars, DPM per cell relative to control.

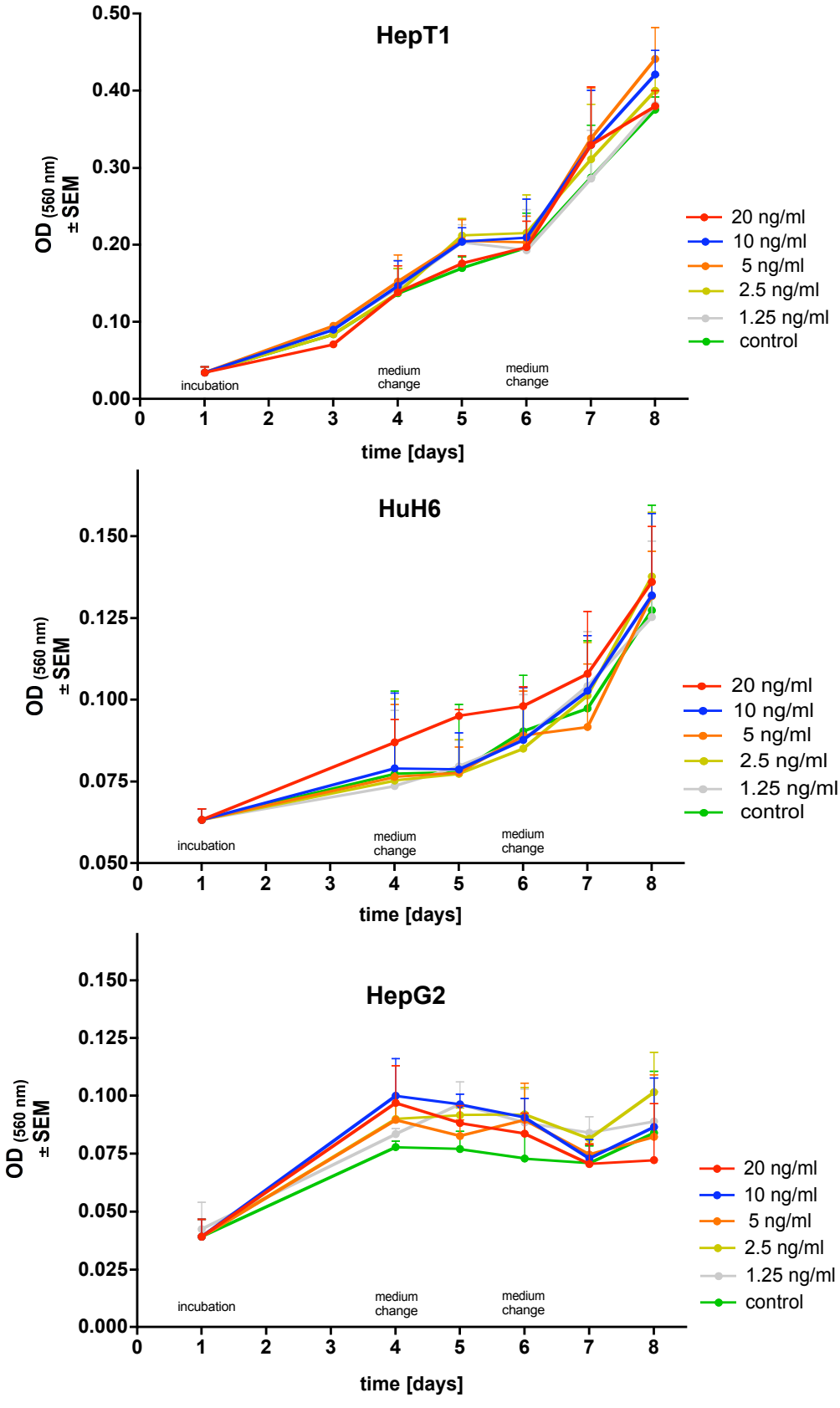

Figure 13: **Growth curves of HB and HCC cell lines**. Cells were cultured in the absence or presence of different HGF/SF concentrations and measured by the MTT assay at the time points indicated.

4.1.2 Cell viability

To check whether HGF/SF could affect long-term growth behavior, growth curves of the HB and HCC cell lines were generated for a time period of 8 days. Cells were grown in normal growth medium and incubated in the presence or absence of various HGF/SF concentrations. Cell viability was analyzed daily by the MTT method (Figure 13). Again, the presence of HGF/SF did not alter the growth rate of HB and HCC cells in comparison to untreated control cells.

As a positive control, MDCK cells, which are known to be receptive to HGF/SF signals (Weidner *et al.*, 1993), were treated with the same batch of human recombinant HGF/SF. Here, increased cell proliferation and the scattering phenotype were observed, indicating that the HGF/SF used was properly functional (data not shown). In conclusion, these experiments show that HGF/SF does not act as a proliferative factor for HB and HCC cells.

4.2 Integrity of the signal transduction pathways

Since the HGF/SF used showed full biological activity, it was speculated that disturbances of the signal transduction pathways downstream of the c-Met receptor in HB and HCC cells might explain the observed insensitivity towards growth stimulation. Thus, the activated pathways were investigated in detail by using specific antibodies against phosphorylated forms of downstream kinases and by performing electrophoretic mobility shift assays (EMSAs) on STAT protein targets.

4.2.1 Phosphorylation of the c-Met receptor

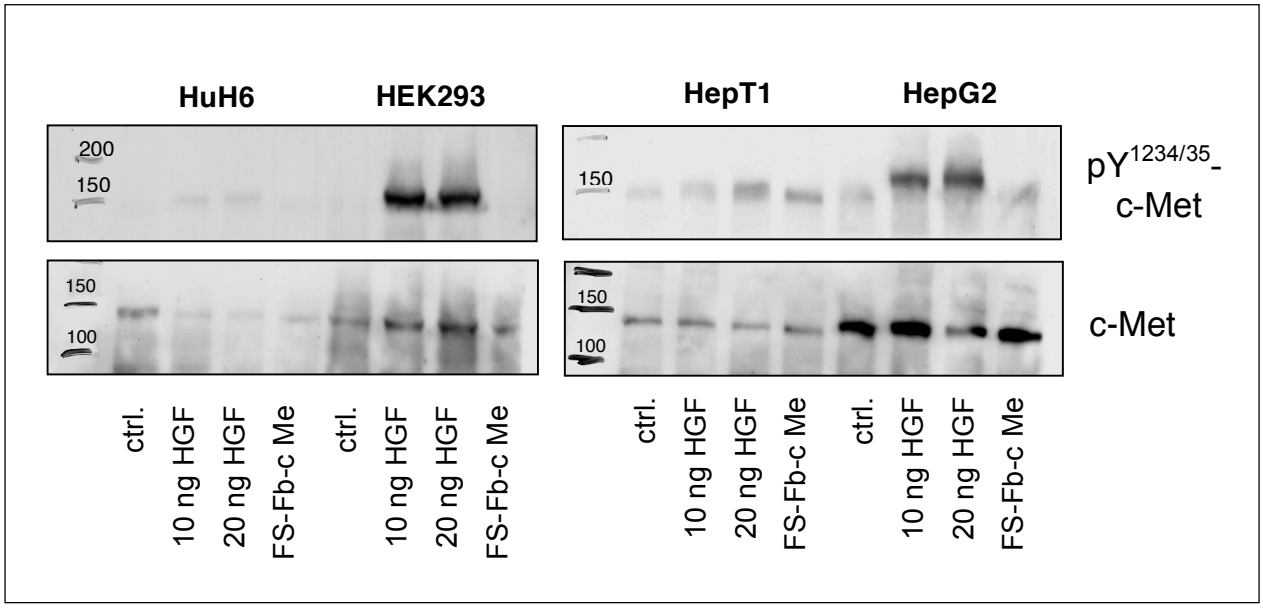

Figure 14: **Phosphorylation status of the c-Met receptor**. HB and HCC cells and for comparison, HEK293 cell were treated with various concentrations of HGF/SF and foreskin fibroblasts-conditioned medium (FS-Fb-c Me) for 30 min. After lysis, cells were subjected to immunoblot analysis with antibodies specific for tyrosine phosphorylated c-Met and total c-Met. Marker sizes are given in kDa.

Binding of HGF/SF leads to the phosphorylation of its receptor c-Met (Weidner *et al.*, 1993). To check whether this phosphorylation takes place in HB and HCC cells as well, cells were incubated with different amounts of HGF/SF in normal growth medium for 30 min. Afterwards, cells were lysed and subjected to immunoblot analysis with antibodies specific for tyrosine residues $Y^{1234/35}$-phosphorylated c-Met and total Met (Figure 14). Immunoblot analysis revealed that HB and HCC cell stimulation with HGF/SF triggered autophosphorylation of the c-Met receptor. Further, it was detected that the total amount of c-Met protein present in the HB cell lines HepT1 and HuH6 is lower than in HepG2 and HEK293 cells. Accordingly, those cell lines showed weaker c-Met phosphorylation upon HGF/SF stimulation.

Also, conditioned medium from foreskin fibroblasts (FS-Fb-c Me) as a possible natural source of HGF/SF was included in this study. However, this medium was not sufficient to induce c-Met phosphorylation in all cell line tested, suggesting none or low amounts of HGF/SF present.

4.2.2 Sustained phosphorylation of Akt/PKB

As mentioned above, PI3K is activated by HGF/SF signaling, which in turn phosphorylates Akt/PKB. To check whether HGF/SF stimulation is sufficient to activate Akt/PKB in HB and HCC cells, immunoblot analysis with antibodies specific for the phosphorylated form of this kinase was performed on lysates from cells treated with HGF/SF for different time points (Figure 15). Interestingly, HuH6 showed an increasing phosphorylation of Akt/PKB with time and peaked after 60 min. Similarly, Akt/PKB phosphorylation in HepT1 cells was the strongest after 60 min and declined afterwards. In contrast, the HCC cell line HepG2 showed increasing Akt/PKB phosphorylation levels, which were not yet reduced after 120 min. These immunoblots demonstrate that Akt/PKB is rapidly (within 15 min) and sustainably (for at least up to 2 h) phosphorylated upon HGF/SF treatment in all cell lines tested.

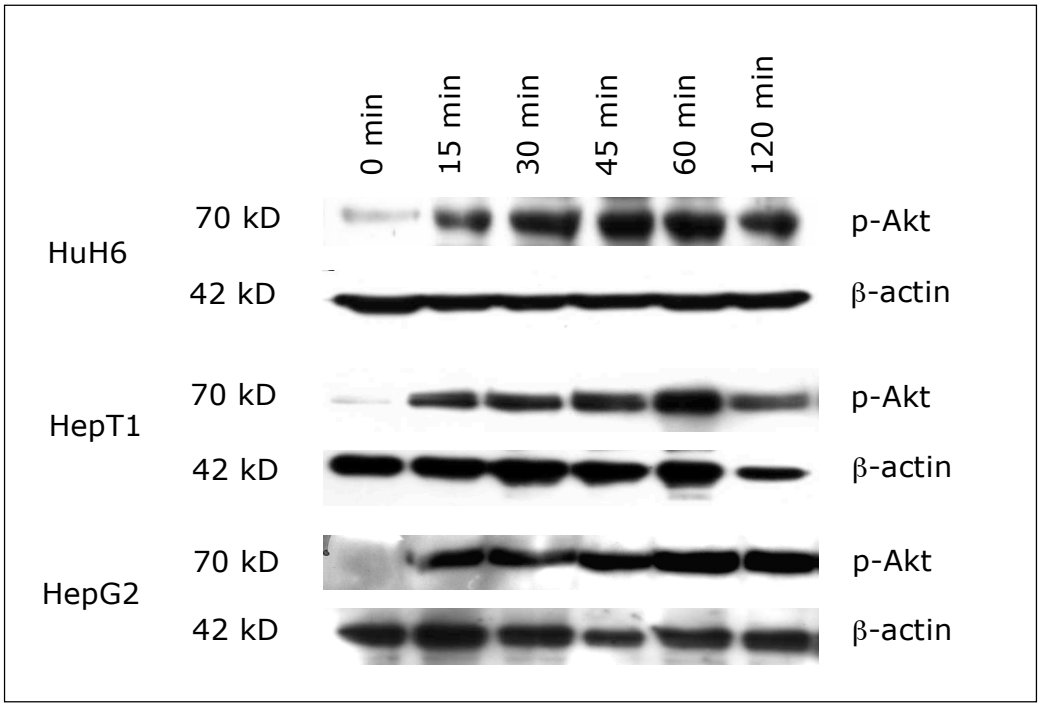

Figure 15: **HGF/SF-induced phosphorylation of Akt/PKB in HB and HCC cells**. Cells were incubated with 10 ng/ml HGF/SF for the time points indicated. After lysis, cells were subjected to immunoblotting with specific antibodies against phospho-Akt and β-actin.

4.2.3 Activation of MAPK/ERK-1/2 pathway

Numerous previous studies have shown that many HGF/SF-induced cellular responses are mediated through the activation of the p42/p44 MAPK (also called ERK-1/2) and hereby also plays a pivotal role in tumorigenesis (Birchmeier *et al.*, 2003). Therefore, the MAPK activation induced by HGF/SF stimulation was investigated in HB and HCC cells by immunoblotting (Figure 16).

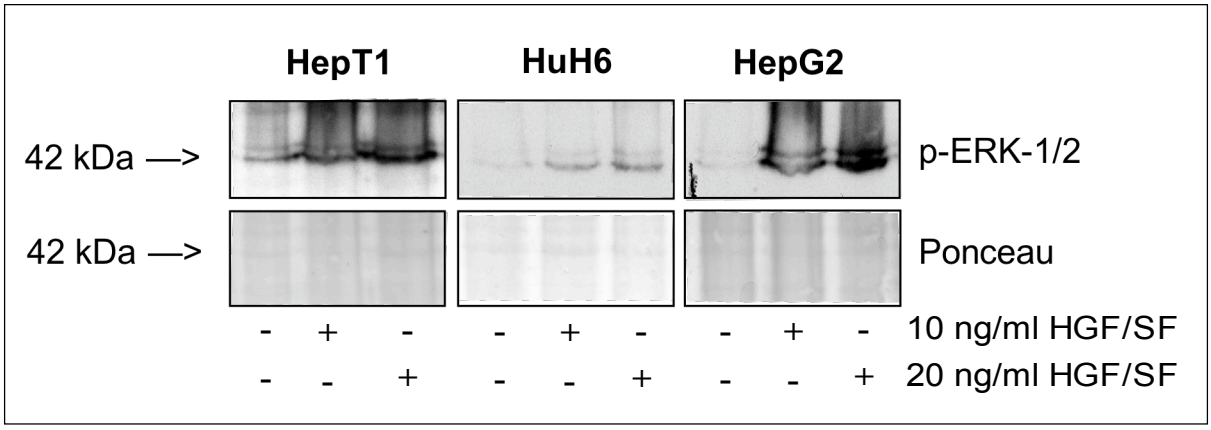

Figure 16: **HGF/SF-induced phosphorylation of Akt/PKB in HB and HCC cells.** Cells were treated with different concentrations of HGF/SF for 30 min. After cell lysis, immunoblot analyses with an antibody specific against phosphorylated ERK-1/2 were performed.

Indeed, when these cell lines were treated with different amounts of HGF/SF, phosphorylation of ERK-1/2 was clearly detectable. 10 ng/ml HGF/SF is already sufficient to induce ERK-1/2 phosphorylation within 30 min, which is not gradable with higher HGF/SF concentrations. Notably, the phosphorylation of MAPK/ERK-1/2 in HuH6 cells is modest in comparison to the HepT1 and HepG2 cells, which could be due to lesser c-Met phosphorylation as discussed above.

4.2.4 Activation of the Jak/STAT pathway

Many stimuli lead to STAT activation other than cytokine stimuli, including growth factors that use tyrosine kinase receptors (Aaronson and Horvath, 2002). For example, STAT3 is directly activated by many cytokines that use signaling receptor subunits similar to cytokine receptor gp130 (Ishihara and Hirano, 2002). Activation of STAT3 occurs in many solid and hematologic tumors and is correlated with growth stimulation and anti-apoptotic effects in malignancies (Bowman *et al.*, 2000). In addition, STAT3 is activated by the cellular non-receptor tyrosine kinase, c-Src. This is a striking example

of cross-talk and interconnection between functionally and conceptually distinct signaling pathways through a cellular proto-oncogene (Aaronson and Horvath, 2002).

To date little is known about a STAT involvement in transducing HGF/SF signals in HB cells. Therefore, the activation of this pathway in HB and HCC cells upon growth factor and cytokine stimulation, including IL-4, IL-6, IFN-α, IFN-γ, EGF, stem cell factor (SCF), and HGF/SF was analyzed. As a positive control, the Wilms' tumor cell line SKNEP and the B cell lymphoma cell line Daudi were included in this study (Figure 17 and Figure 18).

Cells treated with various cytokines and growth factors for different time points were lysed and fractionated. The nuclear fractions containing the STAT proteins bound to DNA were mixed with ^{32}P-radiolabeled oligonucleotides, corresponding to STAT-response element sequences: mutated serum-inducible element (SIE-m67) and β-casein (βCas). By non-denaturating PAGE, the DNA/protein complexes were separated according to their molecular weight. For supershift experiments, specific antibodies against the corresponding STAT protein were added, resulting in the formation of a larger complex that migrated slower through the gel. For visualization, gels were dried and exposed to a PhosphorImager screen.

When the SIE-m67 probe was used, STAT1 homodimers in Daudi cells as well as the STAT1 and STAT3 homo- and heterodimers in SKNEP cells could be detected within 20 min of IL-6 stimulation (Figure 17). Also HepT1, HepG2 and to a lesser extend HuH6 cells did respond to IL-6 treatment by activating STAT1. In HepG2 STAT1:3 heterodimers could additionally be detected. In contrast, HGF/SF treatment did not result in STAT1 or STAT3 phosphorylation (data not shown) and subsequent binding to DNA (Figure 17).

In the experiments where the βCas probe was used, the Daudi cell line clearly showed STAT1, STAT5, and STAT6 homodimers, as well as STAT5:6 heterodimers upon IFN-α treatment (Figure 18). When HB and HCC cells were treated with IL-4 for 20 min, activation of STAT6 was determined. By the addition of an antibody against STAT6, the DNA/protein complex was supershifted, suggesting specific binding of STAT6 to the βCas probe. However, when cells were stimulated with HGF/SF for various time periods, no induction of STAT1, STAT5, or STAT6 were detected by EMSA analysis, suggesting that this pathway plays no or only a minor role in HGF/c-Met signal transduction (Figure 18). Interestingly, a small band at the height of STAT5 was observed in all HB and HCC cells, raising the question whether a constitutive activation

of STAT5 is present in these cells and whether this could contribute to increased cell proliferation.

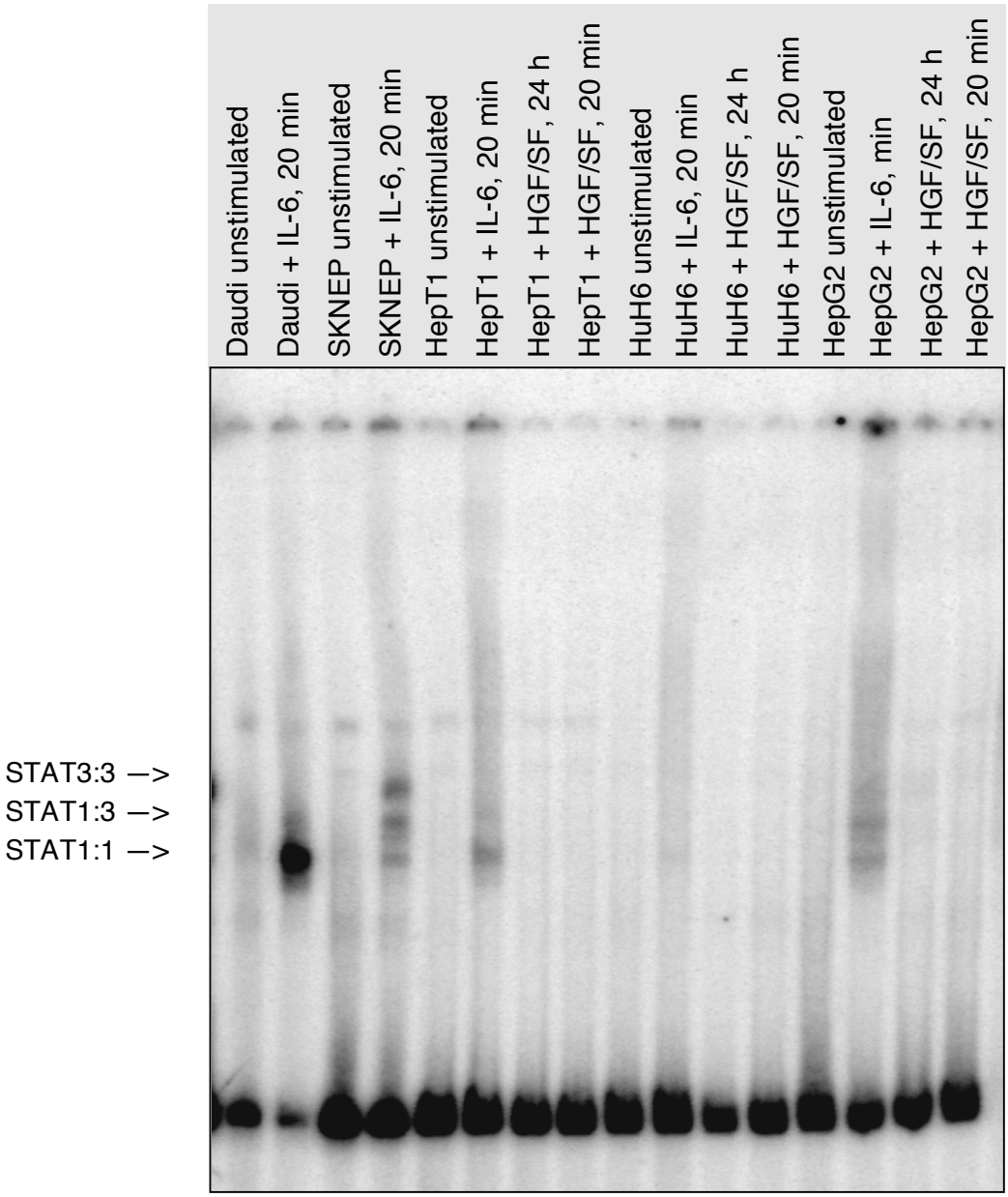

Figure 17: **STAT1 and STAT3 phosphorylation and subsequent binding to radiolabled probes.** Upon growth factor and cytokine stimulation STAT protein binding to DNA was analyzed by EMSA with the SIE-m67 probe in HB, HCC, B cell lymphoma Daudi, and Wilms' tumor SKNEP cell lines.

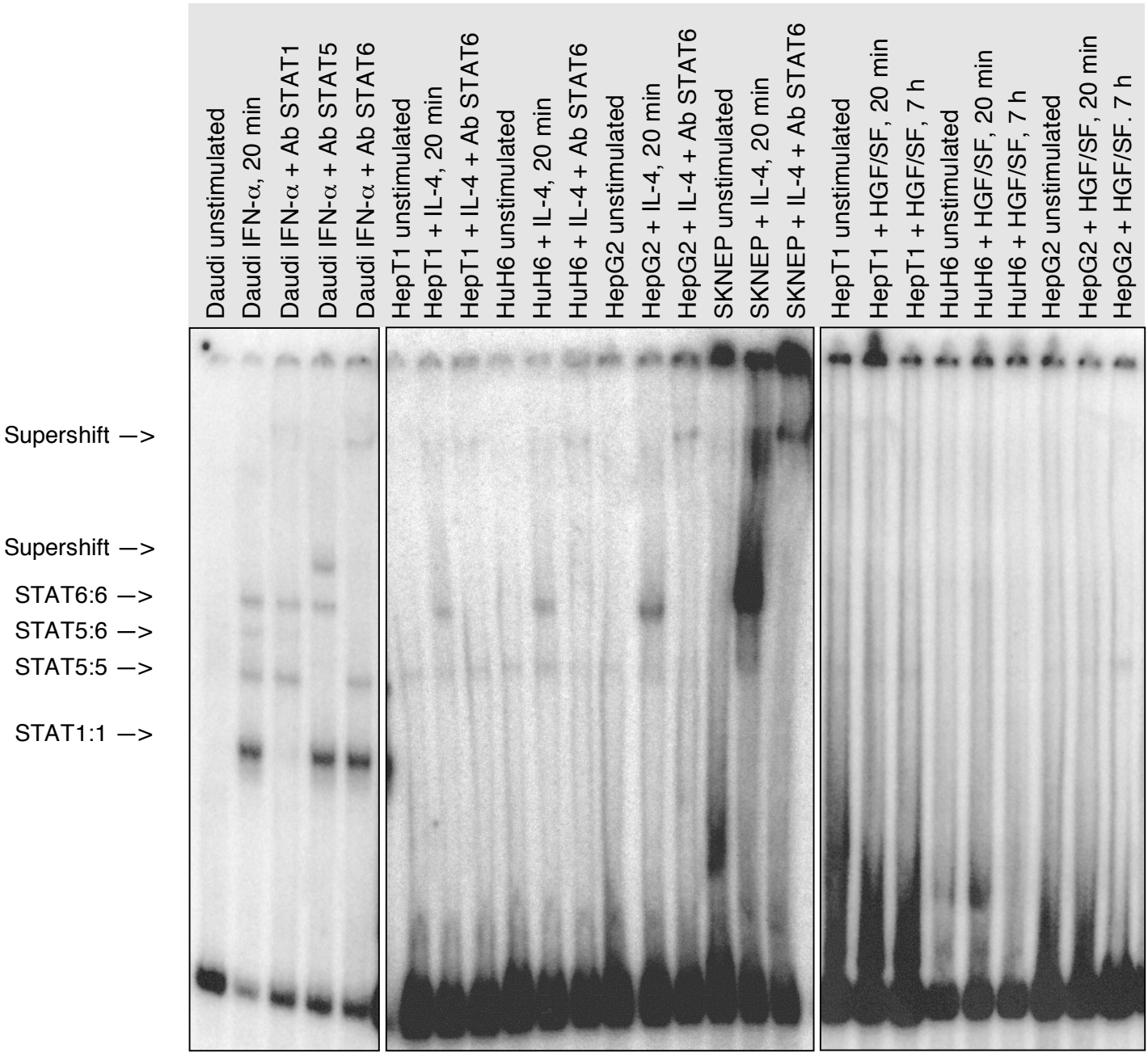

Figure 18: **STAT1, STAT5, and STAT6 phosphorylation and subsequent binding to radiolabeled probes**. Upon growth factor and cytokine stimulation STAT protein binding to DNA was analyzed by EMSA with the βCas probe in HB, HCC, Wilms' tumor SKNEP, and B cell lymphoma Daudi cell lines.

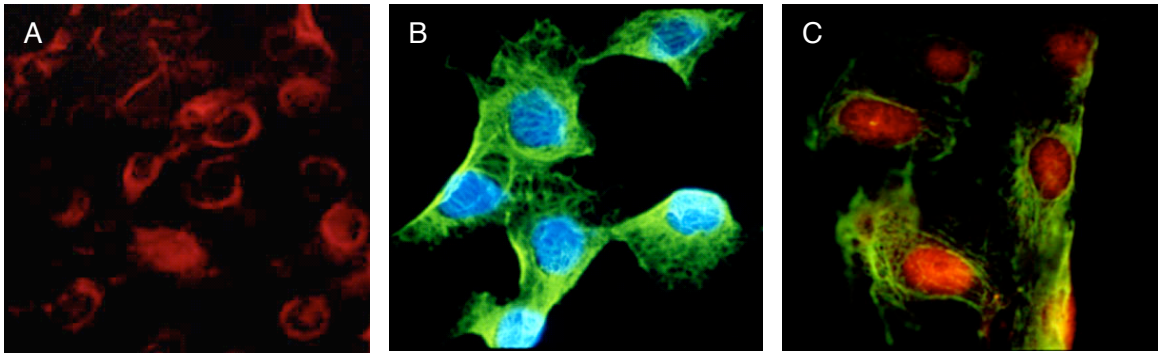

Figure 19: **Immunofluorescence stainings of HepT1 cells**.
(A) unstimulated: negative STAT1 staining in nuclei, (B) unstimulated, Hoechst and cytokeratin 18 staining, (C) nuclear localization of phosphorylated STAT1 after IFN-γ stimulation (500 U/ml) for 20 min and staining of cytoplasmic cytokeratin 18.

To test STAT activation by another assay system, translocations of phosphorylated STAT proteins to the nucleus were visualized by immunofluorescence staining. After IFN-γ treatment, cells were fixed and stained with antibodies specific for phosphorylated STAT proteins. Nuclear localization of phosphorylated STAT1 proteins could be detected upon IFN-γ treatment in HepT1 cells (Figure 19), but also in HuH6 and HepG2 cells (data not shown). However, no nuclear localization of STAT proteins were detected after HGF/SF, EGF, and SCF stimulation (data not shown).

Table 7: Summary of EMSA results

Cell line / GF, cytokine	HepT1	HuH6	HepG2
INF-α	-	-	STAT1
IFN-γ	STAT1	STAT1	STAT1
IL-4	STAT6	STAT6	STAT6
IL-6	STAT1	STAT1	STAT1, STAT3
EGF	-	-	-
HGF	-	-	-
SCF	-	-	-

In summary, these results show that IFN-γ activates STAT1 in HB and HCC cells whereas IFN-α activates STAT1 in HepG2 cells only. IL-6 was capable of activating STAT1 (and STAT3 in HepG2), and IL-4 activated STAT6 in all cell lines investigated. This indicates that the Jak/STAT pathway is functional in these cell lines. Upon stimulation of the cells with various HGF/SF, EGF or SCF concentrations for different time periods, no STAT activation could be detected, suggesting that these growth factors do not activate the Jak/STAT pathway in these cells.

From these experiments it appears that the functional integrity of pathways important for cell proliferation is given and that either these cells have lost their responsiveness to external growth stimulation, or other factors are responsible for HGF/SF not being able of inducing cell growth, *e.g.* culture conditions, the absence of the appropriate extracellular matrix or cell intrinsic growth stimulation.

4.3 HGF/SF as an anti-apoptotic factor

It was previously shown that HGF/SF also plays a pivotal role in inhibition of apoptosis (Fan *et al.*, 2000; Liu, 1999). Evasion from apoptosis is another key feature of tumor progression (Hanahan and Weinberg, 2000). Since many HB and HCC are resistant to chemotherapy, this resistance could be mediated by the presence of high HGF/SF levels that are frequently found.

To test the hypothesis whether HGF/SF is capable of preventing apoptosis in HB and HCC cells, thus leading to a higher survival rates of the tumor cells, apoptosis was induced with various known apoptosis inducers in the presence of absence of HGF/SF. Apoptosis was assessed using a fluorogenic DEVD-afc cleavage assay that measures caspase 3-like activity in the cells (Thornberry and Lazebnik, 1998).

4.3.1 Cisplatin

Cisplatin (CP) is used as standard treatment for many cancers including HB (Finegold, 2002; Fuertes *et al.*, 2003). The antitumoral activity of CP is in part due to its ability to form DNA adducts, mainly with the N7 of guanine and adenosine, which leads to the appearance of both intra- and interstrand crosslinks (Eastman, 1990). As a consequence, the platinum-DNA adducts in both the nuclear and mitochondrial compart-

ment inhibit transcription-dependent cell functions that finally lead to apoptosis (Eastman, 1990; Fuertes *et al.*, 2003).

To test whether HGF/SF has an inhibitory effect on CP-induced apoptosis, HB and HCC cells were pre-incubated in the presence or absence of 10 and 20 ng/ml HGF/SF for 24 h. Directly afterwards, various amounts of CP were added for additional 24 h before cells were lysed and subjected to the caspase 3-like activity assay (Figure 20). The results obtained from these experiments showed that apoptosis induction by CP is concentration-dependent. The caspase 3-like activity increased with the amount of CP added to the cells. Indeed, the cells that were pre-incubated with HGF/SF showed a reduced caspase 3-like activity, suggesting an anti-apoptotic effect of HGF/SF (Figure 20). HepG2 cells showed the strongest reduction in caspase 3-like activity after pre-incubation with HGF/SF.

The stronger effect of HGF/SF on HepG2 cell might be explained by the previously observed higher c-Met expression in those cells. Accordingly, in HuH6 cells, where the expression of c-Met protein was the lowest, the apoptosis reduction was also lower as compared to HepT1 and HepG2 cells.

4.3.2 Camptothecin

Camptothecin (CPT) and its derivatives (*e.g.* topotecan) are also frequently used for chemotherapeutic treatment of cancer. They are known to specifically target toposomerase I by binding non-covalently to DNA strand nicks in a so-called 'cleavable complex', stabilizing them and interfering with DNA religation (Fan *et al.*, 1998b). As a result, single- and double-strand DNA breaks occur when encountered by a replication fork, leading to inhibition of transcription (Bendixen *et al.*, 1990). Cells can repair DNA breaks caused by low concentrations of CPT, whereas higher concentrations lead to cell death (Darzynkiewicz *et al.*, 1996).

It was reasoned whether HGF/SF also exerts an anti-apoptotic effect on CPT-treated HB and HCC cells. Like in the previous experiments, the cells were pre-incubated for 24 h with various HGF/SF concentrations. After this time period, apoptosis was induced by various amounts of CPT for 24 h (Figure 21). As expected, CPT is a potent inducer of apoptosis in HB and HCC cells. Similar to the effects seen with CP treatment in the presence or absence of HGF/SF, CPT-induced caspase 3-like activity could be reduced by pre-treatment of the cells with HGF/SF. Notably, although the inducible caspase 3-like activity was lower in HepG2 than that of HB cells, pre-treatment with HGF/SF resulted in

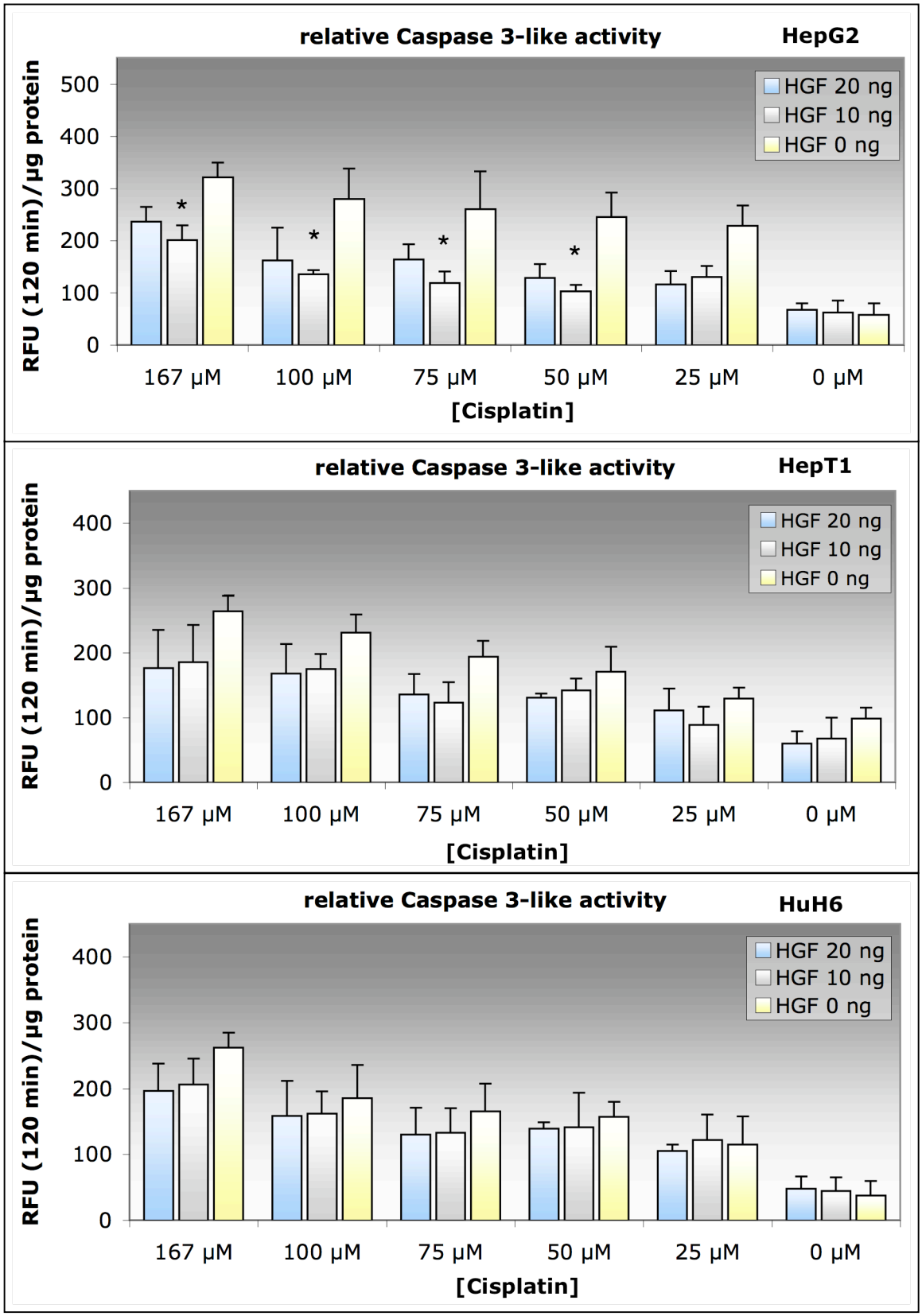

Figure 20: **Apoptosis induction with Cisplatin in HGF/SF-treated cells**.

HB and HCC cells were pre-incubated with indicated amounts of HGF/SF for 24 h and then apoptosis was induced by various amounts of CP for additional 24 h. Caspase 3-like activity was measured by the fluorogenic DEVD-afc cleavage assay. Values given represent means of triplicate measurement. *P<0.005, unpaired t-test.

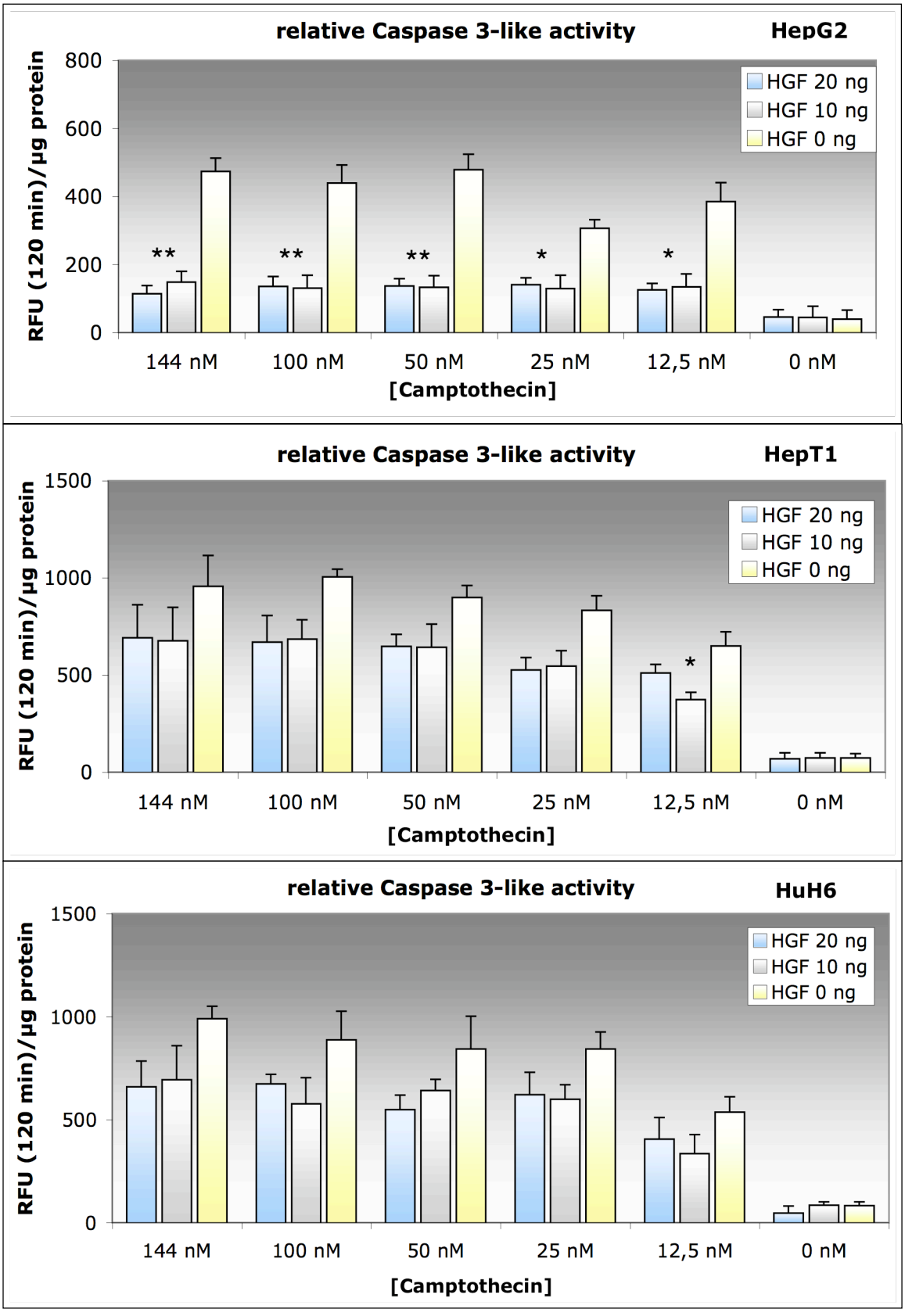

Figure 21: **Apoptosis induction with Camptothecin in HGF/SF-treated cells.**

HB and HCC cells were pre-incubated with indicated HGF/SF concentrations for 24 h before apoptosis was induced by various amounts of CPT for 24 h. Caspase 3-like activity was measured by the fluorogenic DEVD-afc cleavage assay. Values given represent means of triplicate measurement. *P<0.03, **P<0.001, unpaired t-test.

the strongest reduction of caspase 3-like activity in these cells, suggesting a higher resistance to CPT and higher sensitivity to HGF/SF.

4.3.3 Staurosporine

Cell permeable staurosporine (STS), isolated from *Streptomyces staurosporeus*, is one of the most potent and widely used inducers of apoptosis, which is achieved by inhibition of PKC and other protein kinases including PKA, PKG, S6K, and Src (Tamaoki *et al.*, 1986).

For assay optimization, several STS concentrations were tested. The optimal concentration to induce apoptosis in HB and HCC cells was found to be 0.5 µg/ml (data not shown). Next, the time-dependent activation of apoptosis by STS was assessed and the ideal time-point for apoptosis detection was found to be 5 h after stimulation (data not shown). To test the hypothesis whether HGF/SF is capable of preventing STS-induced apoptosis, HB and HCC cells were pre-incubated with various amounts of HGF/SF for 24 h before STS stimulation (Figure 22).

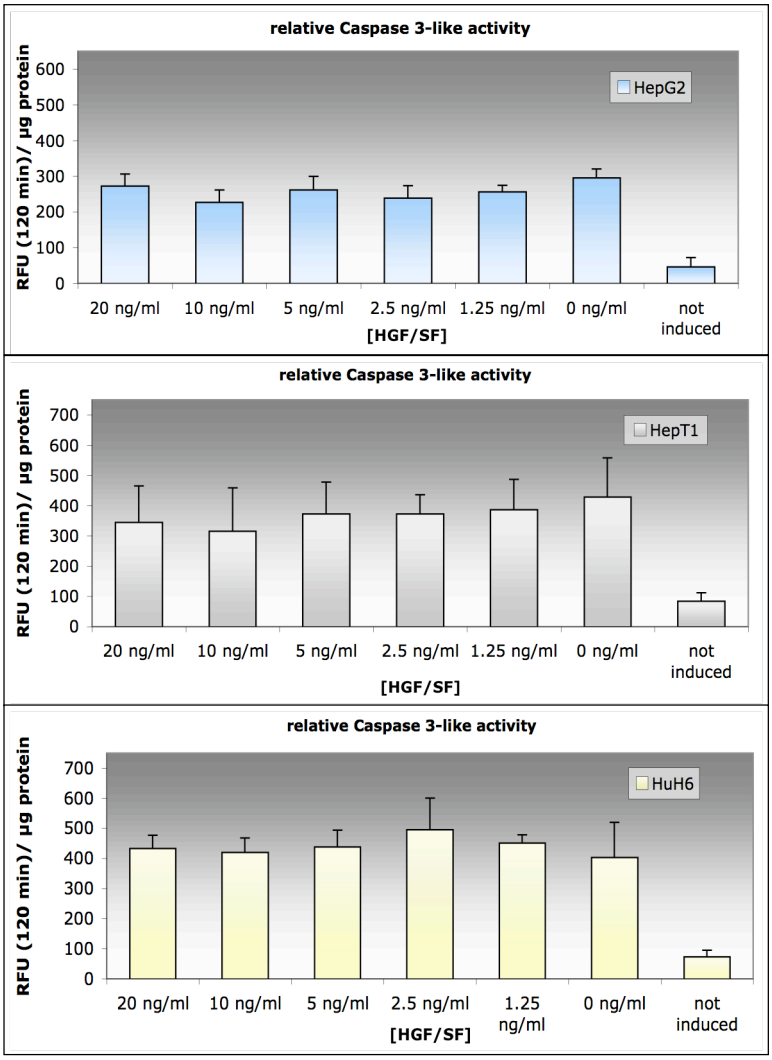

Figure 22: **Apoptosis induction with staurosporin in HGF/SF-treated cells** HB and HCC cells were pre-treated with indicated HGF/SF concentrations for 24 h and then apoptosis was triggered with 0.5 µg/ml staurosporine. Caspase 3-like activity was measured by the fluorogenic DEVD-afc cleavage assay. Values given represent means of triplicate measurement.

Repeated analysis of caspase 3-like activity revealed that pre-incubation of HB and HCC cells with various amounts of HGF/SF could not prevent STS-induced apoptosis,

suggesting that the inhibition of several protein kinases by STS might be too severe and causes cell death regardless of any survival signals.

4.3.4 Starvation

From the previous experiments it became evident that HGF/SF influences cell survival. Supply of oxygen and nutrients is a prerequisite for tumor survival.

To check whether HGF/SF can modulate the survival of starving cells, HB and HCC cells were incubated with starvation medium in the presence or absence of various HGF/SF concentrations (Figure 23). The starvation conditions used were not such a strong apoptosis stimulus as the tested apoptosis inducers. The apoptosis rate detected after 24 h was increased for untreated cells. When cells were incubated together with HGF/SF, a reduction of caspase 3-like activity could be observed. No significant differences between treated and untreated cells were detected at the 15 h time point. Of note, the HepT1 cell line was still able to proliferate under reduced serum conditions (data not shown).

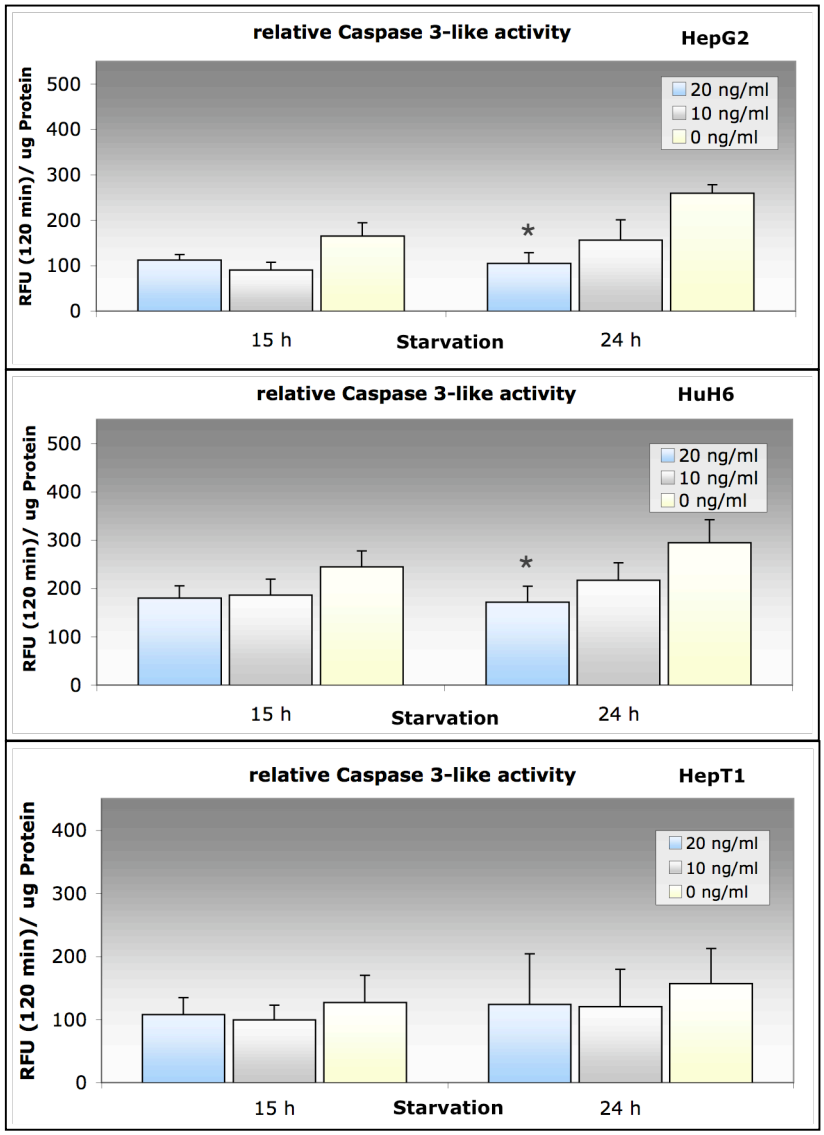

Figure 23: **Apoptosis induction by starvation in HGF/SF-treated cells.** HB and HCC cell lines were starved for the indicated time in the presence or absence of various HGF/SF concentrations. Caspase 3-like activity was measured by the fluorogenic DEVD-afc cleavage assay. Values given represent means of triplicate measurement. *P<0.03 vs. control; unpaired t-test.

4.3.5 Pathways involved in HGF/SF-mediated anti-apoptosis signaling

To better understand the molecular mechanisms underlying the anti-apoptotic effects exerted by HGF/SF, a detailed analysis of the involved pathways was performed. Signaling cascade-specific inhibitors were employed to unravel the responsible pathway for the observed phenotype. Wortmannin (Wn) was used for inhibition of the PI3K pathway (Walker *et al.*, 2000). For a potent inhibition of the MAPK kinase (MEK), PD98059 (2'-amino-3'-methoxyflavone) was employed (Alessi *et al.*, 1995).

In both CP- and CPT-induced HB and HCC cells, co-incubation with HGF/SF decreased caspase 3-like activity as previously observed (Figure 24 and Figure 25). When cells were pre-treated with Wn together with HGF/SF, apoptosis induction by CP resulted again in high caspase 3-like activity, suggesting an important role of PI3K. When cells were pre-treated with PD98059 together with HGF/SF, the caspase 3-like activity remained at the same reduced level as with HGF/SF pre-treatment alone (data not shown). The inhibitors by themselves had no or only minor effects on caspase 3-like activity in either induced and non-induced conditions. These experiments show that apoptosis inhibition via PI3K/Akt and not the MAPK/ERK-1/2 pathway may be a critical component of c-Met oncogenic pathway.

In conclusion, HGF/SF does not stimulate growth of HB and HCC cells, but increases their survival and resistance to apoptosis. Thus, in cultured HB and HCC cells, HGF/SF could be regarded as a survival factor rather than a growth factor. By serving as a survival factor, HGF/SF facilitates the cells to evade from apoptosis and decrease their sensitivity to chemotherapy. These results could have a strong impact for the clinical setting and might explain the poor prognosis of cancer patients with elevated HGF/SF serum levels.

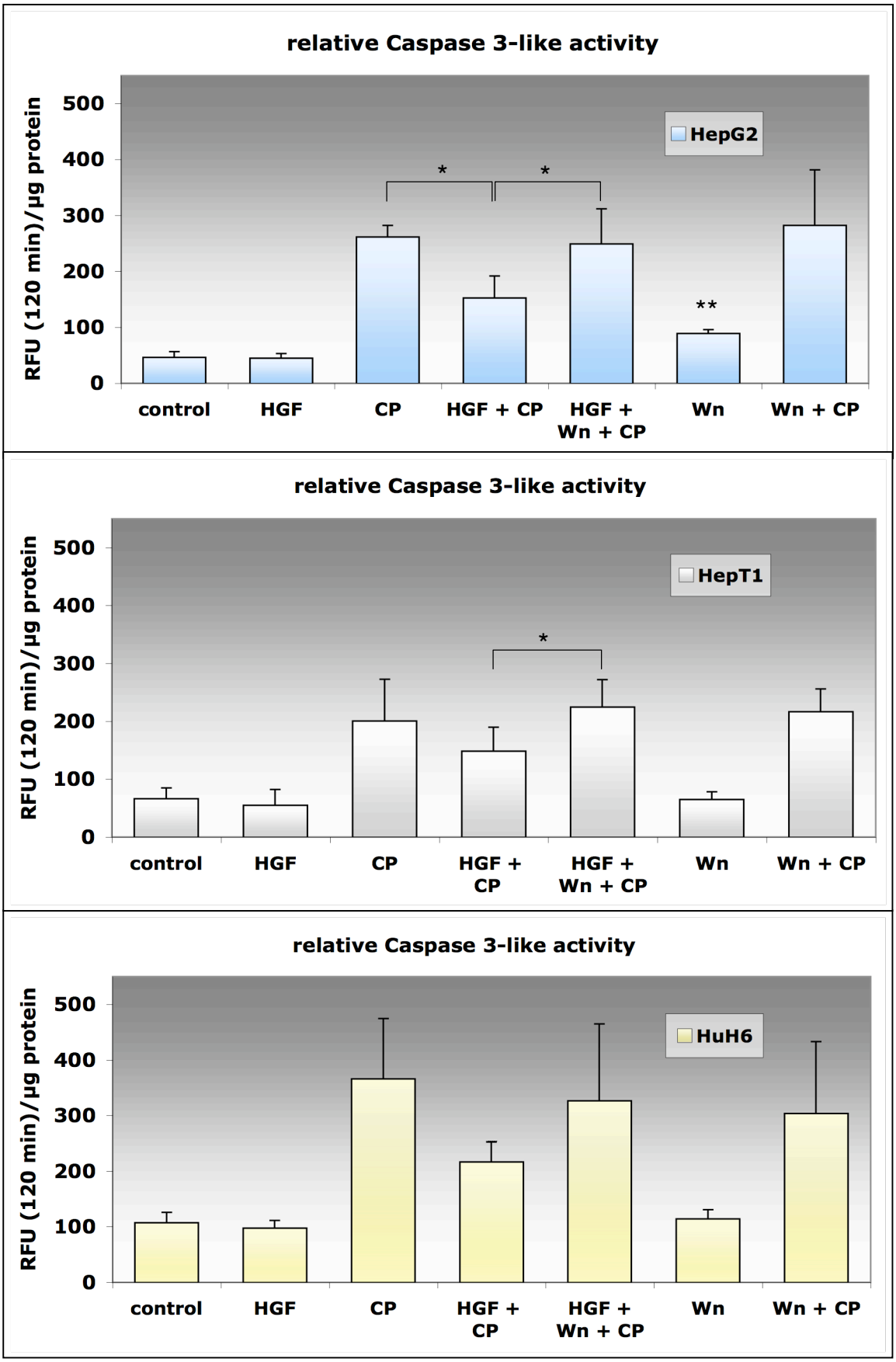

Figure 24: **Apoptosis induction by Cisplatin (CP) in PI3K-inhibited/HGF/SF-stimulated HB and HCC cells.** Cells were pre-incubated in the presence or absence of HGF/SF and PI3K inhibitor Wn for 24 h. Apoptosis was subsequently induced by 25 µM CP. Caspase 3-like activity was measured by the fluorogenic DEVD-afc cleavage assay. Values given represent the means of triplicate measurements. **P*<0.03, ***P*<0.003 vs. control; unpaired t-test.

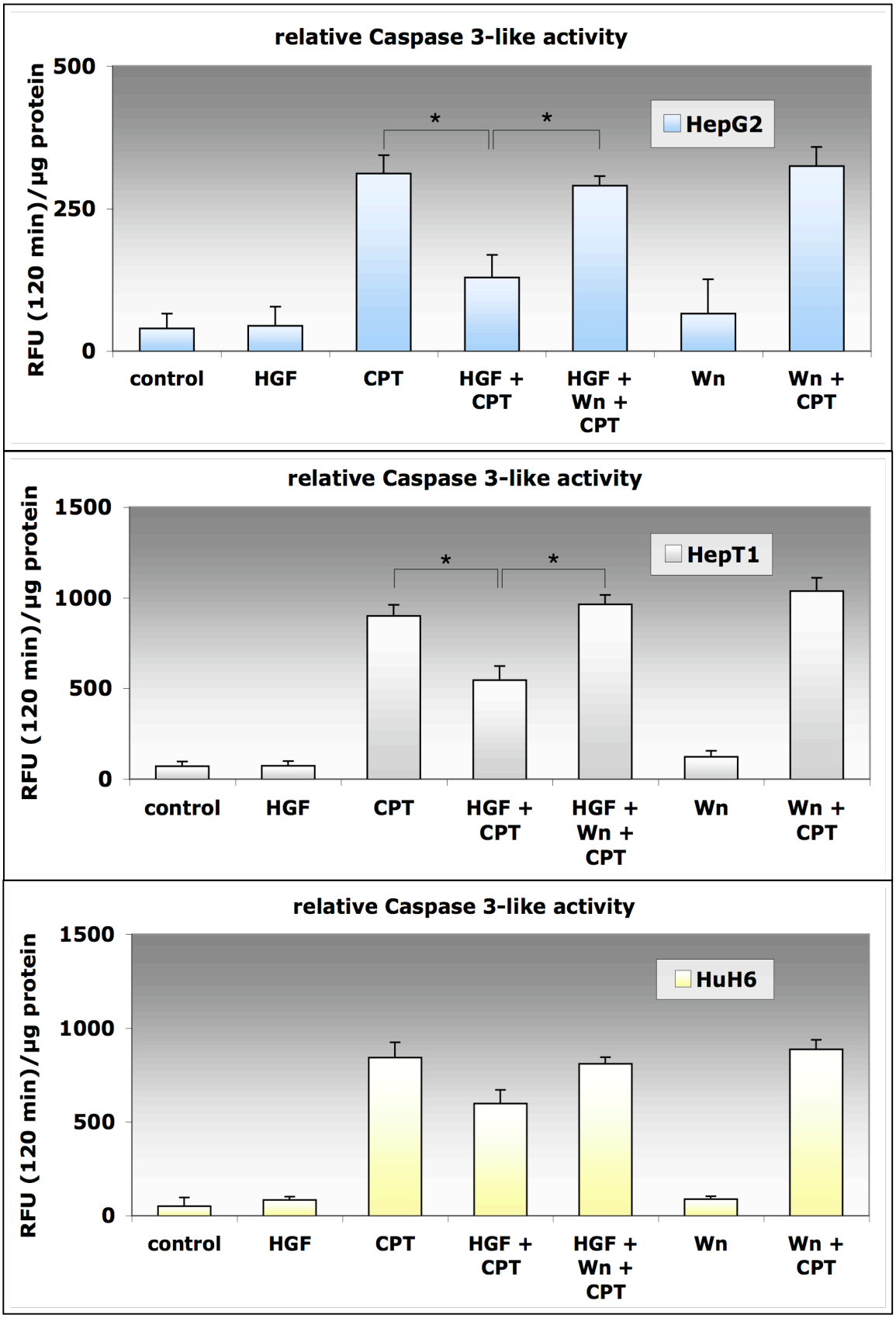

Figure 25: **Apoptosis induction by Camptothecin (CPT) in PI3K-inhibited/HGF/SF-stimulated HB and HCC cells.** Cells were pre-incubated in the presence or absence of HGF/SF and PI3K inhibitor Wn for 24 h. Apoptosis was subsequently induced by 25 nM CPT and caspase 3-like activity was measured by the fluorogenic DEVD-afc cleavage assay. Values given represent the means of triplicate measurements. *P<0.03; unpaired t-test.

4.4 HGF/SF as scatter factor for HB and HCC cells

HGF/SF was originally discovered not only as a liver cell mitogenic factor, but also as a motogenic factor (Stoker *et al.*, 1987; Weidner *et al.*, 1990). To date, several studies have shown the effect of HGF/SF on cell motility and invasion.

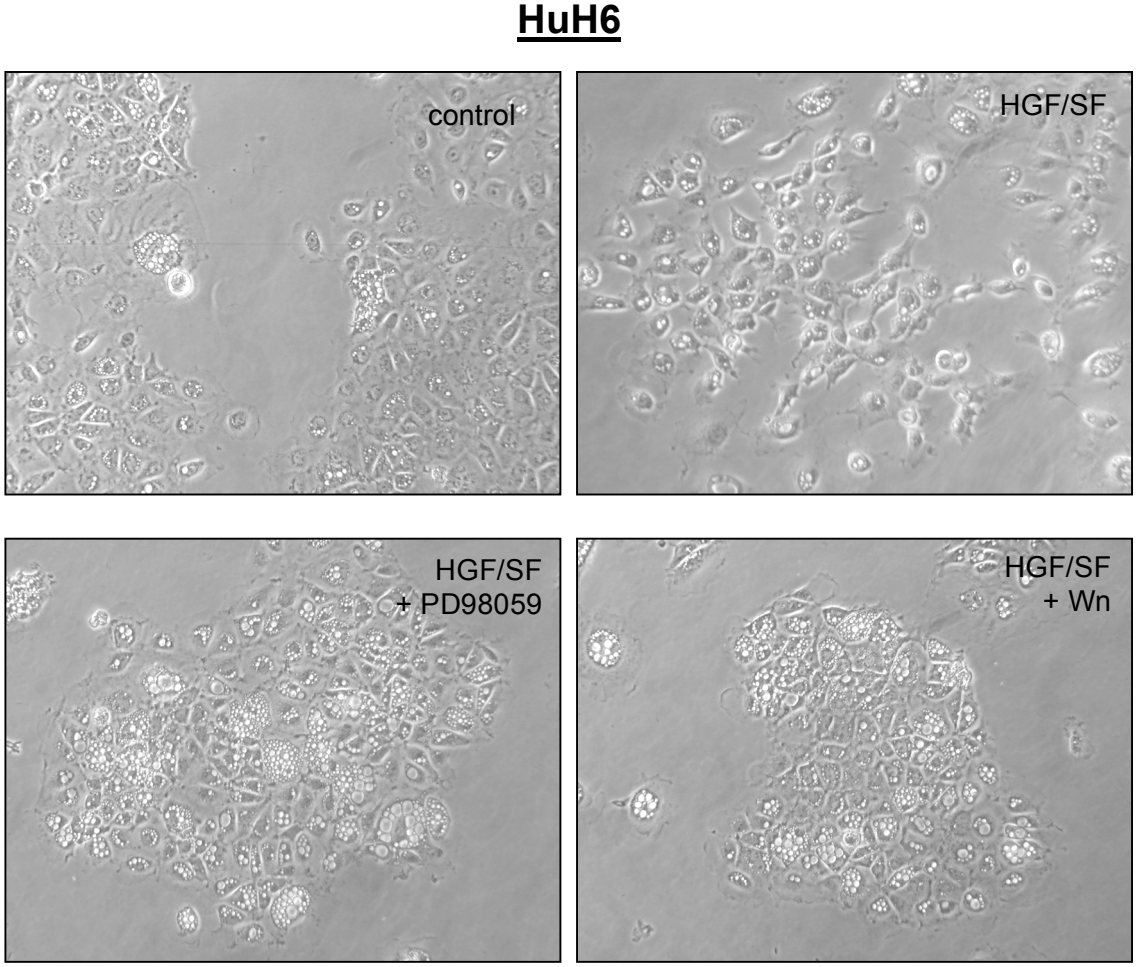

Figure 26: **Pathway-dependent induction of HuH6 cell scattering by HGF/SF**. Cells were plated on collagen I in the presence or absence of 10 ng/ml HGF/SF and pathway specific inhibitors. Microphotographs were taken after 72 h.

To investigate the role of HGF/SF on HB and HCC cell scattering, cells were plated on collagen I-coated tissue plates and incubated with 10 ng/ml HGF/SF for up to 72 hours. In addition, to find out which pathway might be involved, HB and HCC cells were also incubated with 10 ng/ml HGF/SF in the presence of Wn and PD98059 (Figure 26 - Figure 28).

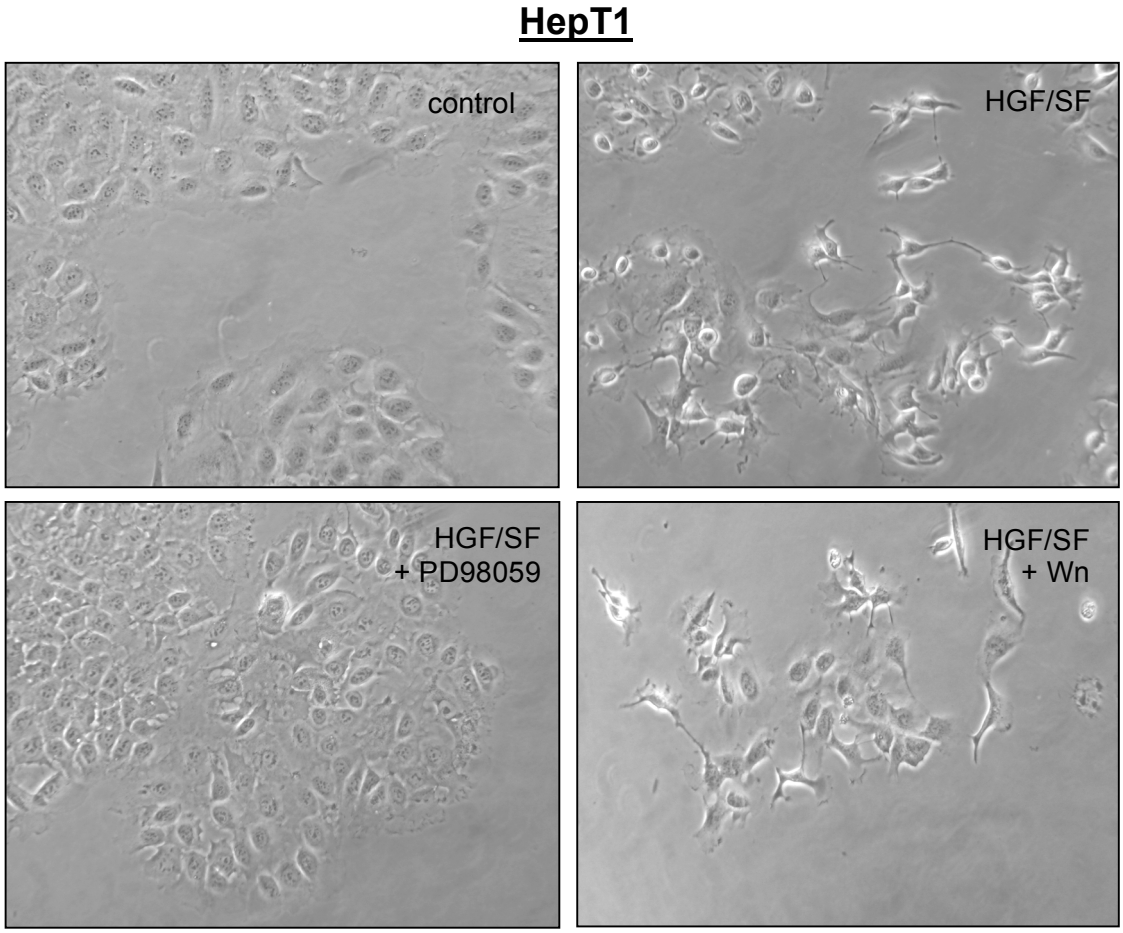

Figure 27: **Pathway-dependent induction of HepT1 cell scattering by HGF/SF**. Cells were plated on collagen I in the presence or absence of 10 ng/ml HGF/SF and pathway specific inhibitors. Microphotographs were taken after 72 h.

Indeed, HGF/SF induced scattering in all cell lines tested. The most dramatic changes in the cell morphology, however, were seen in HepG2 cells (Figure 28). These cells completely changed to a spindle-like, fibroblastic cell shape. Also the HB cell lines showed the distinct neurite outgrowth and filapodia formation. However, these alterations were stronger in HepT1 cells than in HuH6 cells.

Notably, co-incubation with the MEK inhibitor PD98059 almost completely blocked the scattering phenotype, whereas Wn had no such effect on HGF/SF-stimulated cells, suggesting a strong involvement of the MAPK pathway in HGF/SF-induced cell scattering and motility.

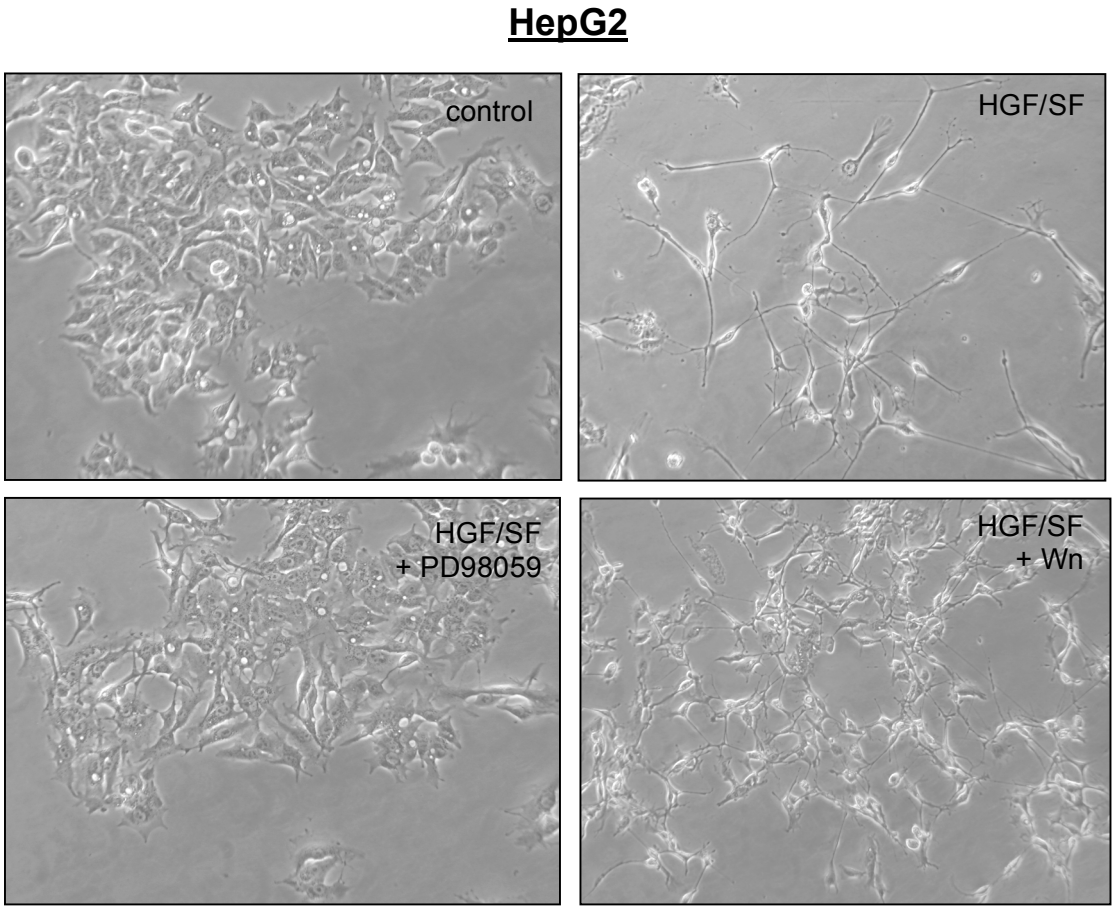

Figure 28: **Pathway-dependent induction of HepG2 cell scattering by HGF/SF**. Cells were plated on collagen I in the presence or absence of 10 ng/ml HGF/SF and pathway specific inhibitors. Microphotographs were taken after 72 h..

4.4.1 Involvement of HGF/SF in cell-cell adhesion regulation

During cell scattering the cell-matrix and cell-cell adhesion capacity of the cells are dramatically altered. To achieve an increase in cell motility, cells have to weaken or even completely loose their cell-cell contacts. In epithelial layers, adherens and tight junctions and desmosomes link cells to each other (Tsukita *et al.*, 2001). Loss of cell-cell adhesion followed by the dissociation of epithelial structures is a prerequisite for increased cell motility, but also for tumor invasion (Hanahan and Weinberg, 2000).

It was therefore analyzed whether HGF/SF-induced cell scattering is directly accompanied with downregulation of adherens and tight junctions.

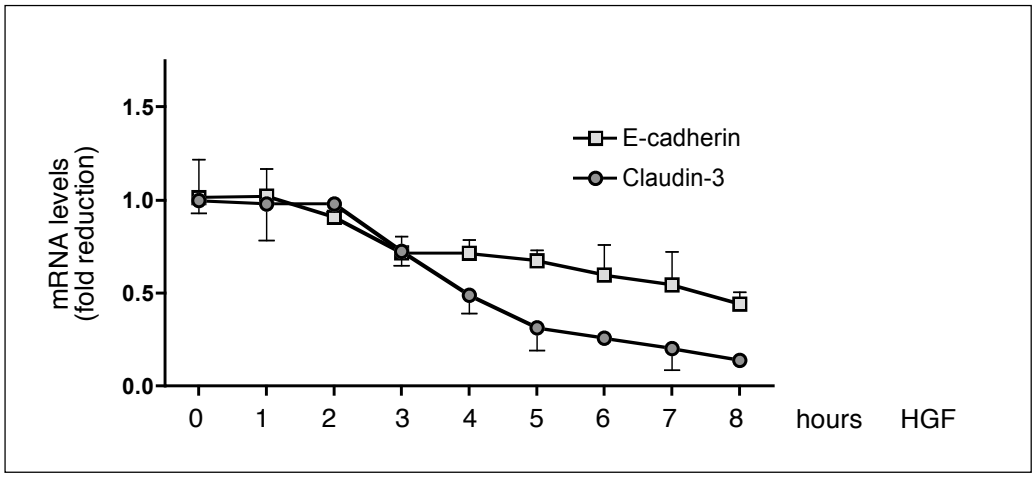

Figure 29: **Kinetics of HGF/SF-mediated downregulation of E-cadherin and claudin-3 mRNA**. HepG2 cells were incubated with 10 ng/ml HGF/SF in the presence of MG132 and LiCl. At indicated time points mRNA was analyzed by qRT-PCR. Values represent means of triplicate measurements ± standard deviations in comparison to untreated control samples.

Next it was checked by quantitative real-time RT-PCR (qRT-PCR), whether HGF/SF stimulation causes a change in the mRNA levels of the adherens junction protein E-cadherin and tight junction protein claudin-3 (Figure 29). It was observed that downregulation of junction proteins mRNA takes place at an early time point after HGF/SF stimulation. The first significant decrease could be detected as early as 3 h after the start of HGF/SF incubation. After 8 h, the mRNA levels were reduced by 80% for claudin-3 and by 60% for E-cadherin. These results indicate a strong influence of HGF/SF in regulating adherens and tight junctions.

4.4.2 Repressors of adherens and tight junctions

It has been reported that several transcriptional repressors are responsible for the downregulation of adherens and tight junctions, namely Snail, Slug, Twist, Smad-interacting protein (SIP)-1, E12/E47 (Grunert *et al.*, 2003; Huber *et al.*, 2005; Nieto, 2002; Savagner, 2001). By binding to specific DNA sequences in the promoter, termed E-boxes, these repressors prevent transcription, leading to the disruption of adherens and tight junctions. Cell-cell adhesion is frequently lost during malignant tumor progression by gene mutation, transcriptional repression or protein degradation (Cavallaro and Christofori, 2004; Gumbiner, 2000), therefore a pivotal role of any of these repressors in HGF/SF-mediated tight and adherens junctions loss was hypo-

thesized. To investigate whether these factors were also involved in HGF/SF-induced cell scattering and loss of cell-cell contacts, expression levels of Snail, Slug, and Twist were checked in HepG2 cells by qRT-PCR.

Preliminary experiments suggested that Snail mRNA was expressed in HepG2 cells and induced after 8 h HGF/SF stimulation. In contrast, Slug mRNA levels were low and not increased after HGF/SF stimulation. Similarly, Twist mRNA levels were rather low and insignificantly increased after HGF/SF treatment (data not shown), implicating a dominant role for Snail in HGF/SF-induced cell scattering. Therefore, further experiments were performed to characterize in more detail the role of Snail in HGF/SF-mediated cell motility.

Snail is an extremely labile protein with a half-life of only 25 minutes and difficult to detect by conventional immunoblotting methods. Thus, the GSK-3β inhibitor lithium chloride (LiCl) and the proteasome inhibitor MG132 were added to the culture medium to prevent the rapid degradation of Snail protein by the proteasomal pathway (Zhou *et al.*, 2004). In the absence of these inhibitors, Snail protein was barely detectable in HepG2 cells (Figure 30A), whereas treatment with MG132 or LiCl or both combined led to a slight stabilization of the protein. Notably, when HGF/SF was added to inhibitor-treated cells, Snail protein levels were markedly elevated. In contrast, treatment of cells with HGF/SF in the absence of inhibitors did not result in increased Snail protein levels. These experiments indicate that HGF/SF is able to induce the expression of Snail in HepG2 cells, but that increased Snail protein levels are only detectable by preventing proteasomal degradation.

A comparison of the Snail protein levels in HepG2 cells to the HB cells revealed also Snail protein induction in HuH6 and HepT1 cells, yet the strongest induction could be detected in HepG2 cells (Figure 30B). Interestingly, HepG2 cells showed the strongest scattering phenotype after HGF/SF treatment, suggesting a possible link between Snail expression levels and the degree of scattering. This observation could be based on the higher c-Met levels and c-Met and MAPK phosphorylation detected earlier in this study. For this reason, the following experiments were performed with HepG2 cells and not with HuH6 and HepT1 cells.

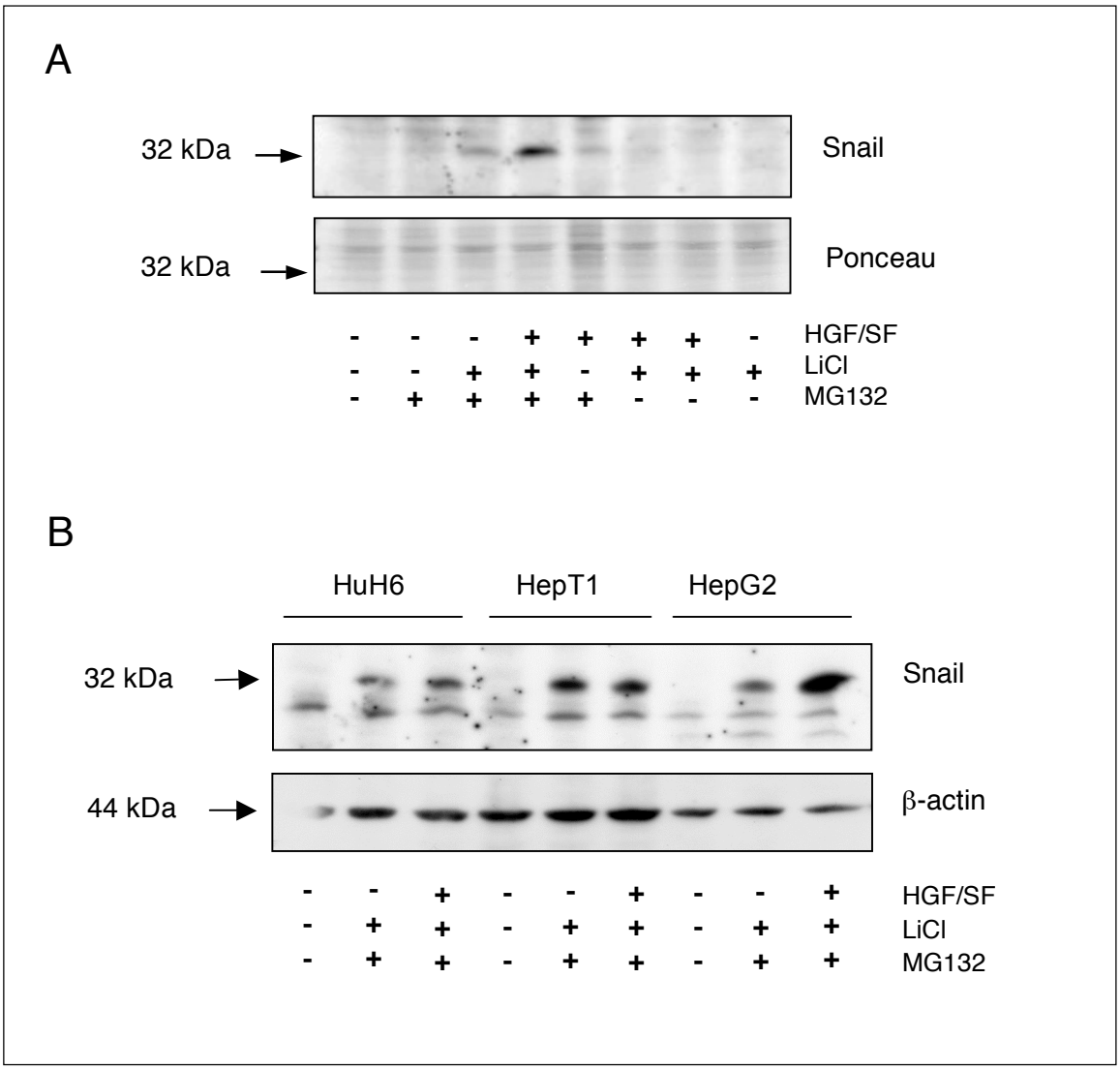

Figure 30: **Immunoblotting of Snail in HB and HCC cells**.

(A) HepG2 cells were incubated with 10 ng/ml HGF/SF for 8 h in the presence or absence of GSK-3β inhibitor LiCl (40 mM) and proteasome inhibitor MG132 (10 μM). Cell lysates were subjected to immunoblotting using an anti-Snail antibody. Equal loading of proteins was confirmed by Ponceau S staining.

(B) HuH6, HepT1 and HepG2 cells were incubated with 10 ng/ml HGF in the presence or absence of the indicated inhibitors. Immunoblot analysis with antibodies against Snail and β-actin reveal that HGF/SF-induced levels of Snail protein correlate with the degree of scattering.

Next, I determined whether HGF/SF directly affected Snail gene expression. For this, HepG2 cells were treated with HGF/SF in the presence or absence of LiCl and MG132 for 8 h. qRT-PCR analysis revealed that Snail mRNA levels were approximately 8-fold (±3.1) upregulated in HepG2 cells treated with HGF/SF (Figure 31). Interestingly, when HepG2 cells were pre-incubated with proteasome and GSK-3β inhibitors, Snail mRNA levels increased up to 40-fold (±1.4) and co-incubation with inhibitors and HGF/SF elevated Snail mRNA levels up to 50-fold (±1.8). These results suggest that a

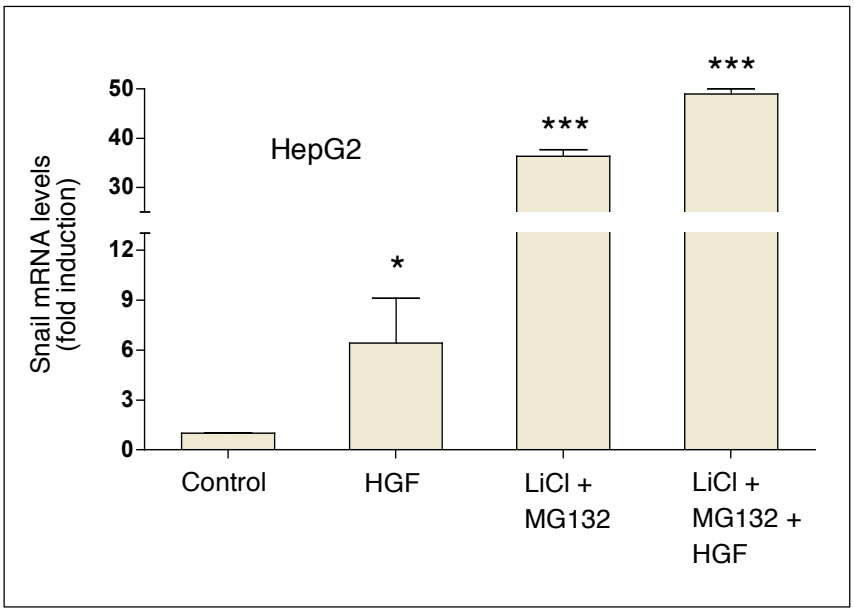

Figure 31: **Snail mRNA levels in HepG2 cells**. HepG2 cells were incubated with HGF/SF for 8 h in the presence or absence of MG132 or LiCl. Snail mRNA levels were analyzed by qRT-PCR. Values given are means and standard deviations of triplicate measurements. **P*=0.0358, ***P*=0.0007, ****P*<0.0001, unpaired t-test.

proteolytically sensitive protein, possibly Snail itself, cooperated with HGF/SF to induce Snail gene transcription and/or to stabilize its mRNA.

Similar experiments in MDCK cells revealed comparable results: HGF/SF by itself moderately increased Snail mRNA expression (2.2-fold ±0.8; data not shown). HGF/SF also significantly increased Snail mRNA levels in the presence of LiCl and MG132. Taken together, HGF/SF treatment of epithelial cells induced Snail expression not only at the protein level but also at the mRNA level.

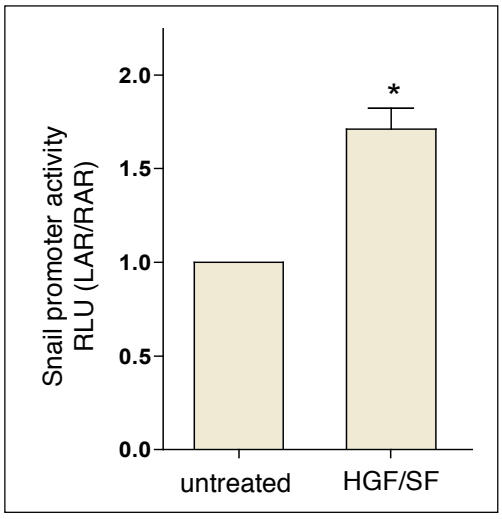

Figure 32: A Snail-promoter luciferase-reporter construct was transiently transfected into HEK293 cells. Cells were incubated with HGF/SF for 24 h, before luciferase activities were determined. Relative light units (RLU) represent firefly luciferase (LAR) normaliz * ainst *Renilla* luciferase activity (RAR). Values given represent means and standard deviations of triplicate measurements. **P*=0.0031; unpaired t-test.

4.4.3 Snail is a direct target of HGF/c-Met signaling

I then assessed whether the observed Snail upregulation is due to a directly activation of the Snail promoter. To address this question, human embryonal kidney (HEK) 293 cells were transiently transfected with a reporter construct in which the mouse Snail promoter sequence was cloned upstream of the firefly luciferase gene (Peinado *et al.*, 2003). Upon HGF/SF

treatment, Snail promoter activity was elevated by 1.8-fold (±0.09) (Figure 32). These results indicate that HGF/SF signaling directly induces the Snail promoter. The rather moderate induction is in agreement with previous reports using the same promoter-reporter construct (Peinado *et al.*, 2003) and is consistent with the levels of Snail mRNA upregulation detected in HepG2 and MDCK cells.

4.4.4 Generation of shSnail clones

Previous results indicated that Snail plays an important role in HGF/SF-induced cell scattering. To assess whether Snail upregulation was required for HGF/SF-induced cell scattering, stably transfected HepG2 cell lines expressing small hairpin RNA against Snail (shSnail) to ablate Snail expression were generated.

Various independent shSnail-expressing clones (clones S-1, S-2, S-3) and control shRNA clones (C-1, C-2) were analyzed for Snail mRNA and protein expression. Two different sets of primers were included in this experiment, one primer pair complementary to Snail and one pair complementary to the 3'-untranslated region (3'UTR) of Snail mRNA. Clones expressing shSnail (S-1, S-3) showed up to 80% repression of Snail mRNA and levels, whereas control clones did not exhibit any significant reduction in Snail mRNA (Figure 33). Immunoblotting analysis revealed a decrease in Snail

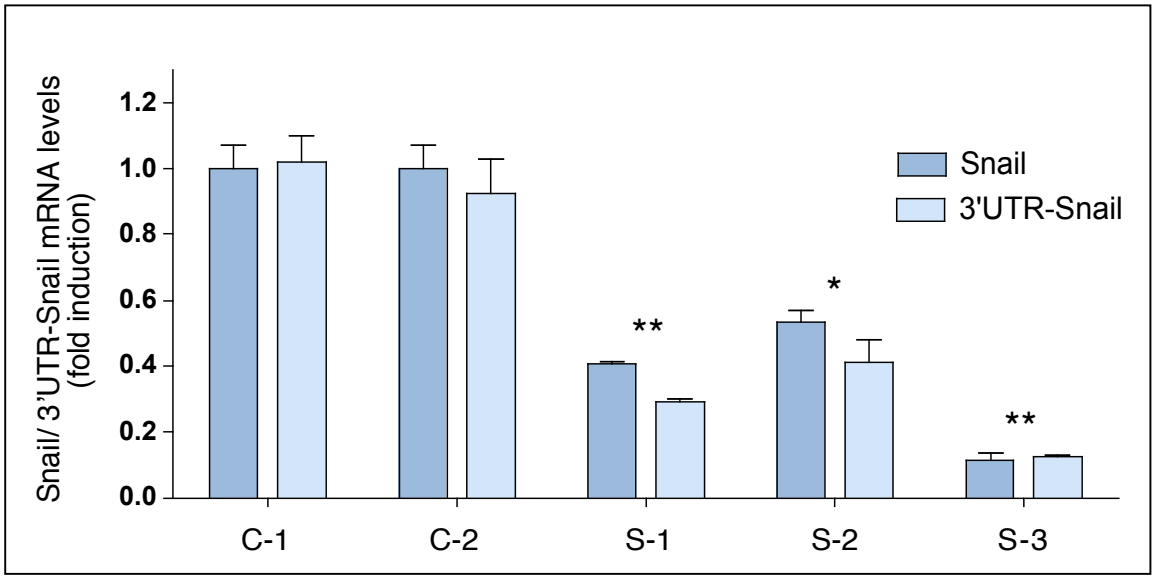

Figure 33: **Analysis of different shSnail and control shRNA expressing HepG2 clones**. shSnail-expressing (S-1, S-2, and S-3) and control shRNA-expressing (C-1, C-2) HepG2 clones were incubated with MG132 and LiCl for 4 h. mRNA expression was analyzed by qRT-PCR with specific primers against the coding region and the 3'-untranslated region (3'UTR) of Snail, respectively. Values are the averages and standard deviations of triplicate measurements. **P*<0.03, ***P*<0.01; unpaired t-test.

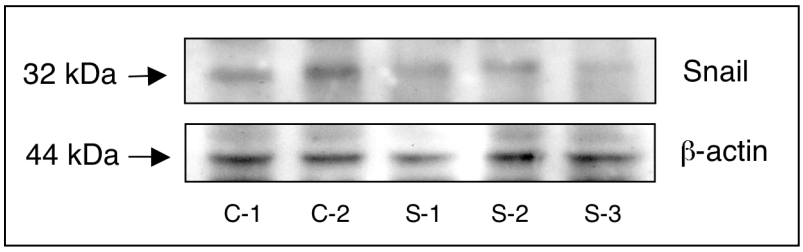

Figure 34: HepG2 clones expressing shSnail (S-1, S-2, S-3) or a control (C-1, C-2) shRNA were incubated with LiCl, MG132, and 10 ng/ml HGF/SF for 8 h. Snail and β-actin protein levels were analyzed by immunoblotting.

protein levels in the shSnail expressing HepG2 cells in comparison to control transfected HepG2 cells (Figure 34).

More important, however, was the question whether HepG2 cells with repressed Snail expression were still able to scatter after the stimulation with HGF/SF. Hence, the degree of scattering of shSnail-expressing clones S-1 and S-3 and control vector-transfected cells was observed over a time period of 72 h after the onset of HGF/SF treatment. Control shRNA clones showed normal scattering behavior as observed with untransfected HepG2 cells (Figure 35A - B). In contrast, shSnail-expressing clones S-1 and S-3 exhibited a discernible reduction in HGF/SF-induced cell scattering, indicating that Snail function is required for this process (Figure 35C - F). Interestingly, the Snail mRNA and protein levels correlate with the scattering behavior of shSnail-expressing HepG2 cells, indicating that the presence of Snail is essential for HGF/SF-induced cell scattering and increased cell motility. Notably, forced expression of Snail by itself was not sufficient to induce a scattering phenotype in the absence of HGF/SF, as for example tested in HEK293 cells expressing a stabilized form of Snail (Snail-8SA) and in HepG2 cells transiently transfected with Snail-8SA (data not shown). In conclusion, while Snail appears to be required for HGF/SF-induced cell scattering, it is not sufficient to replace HGF/SF's activity.

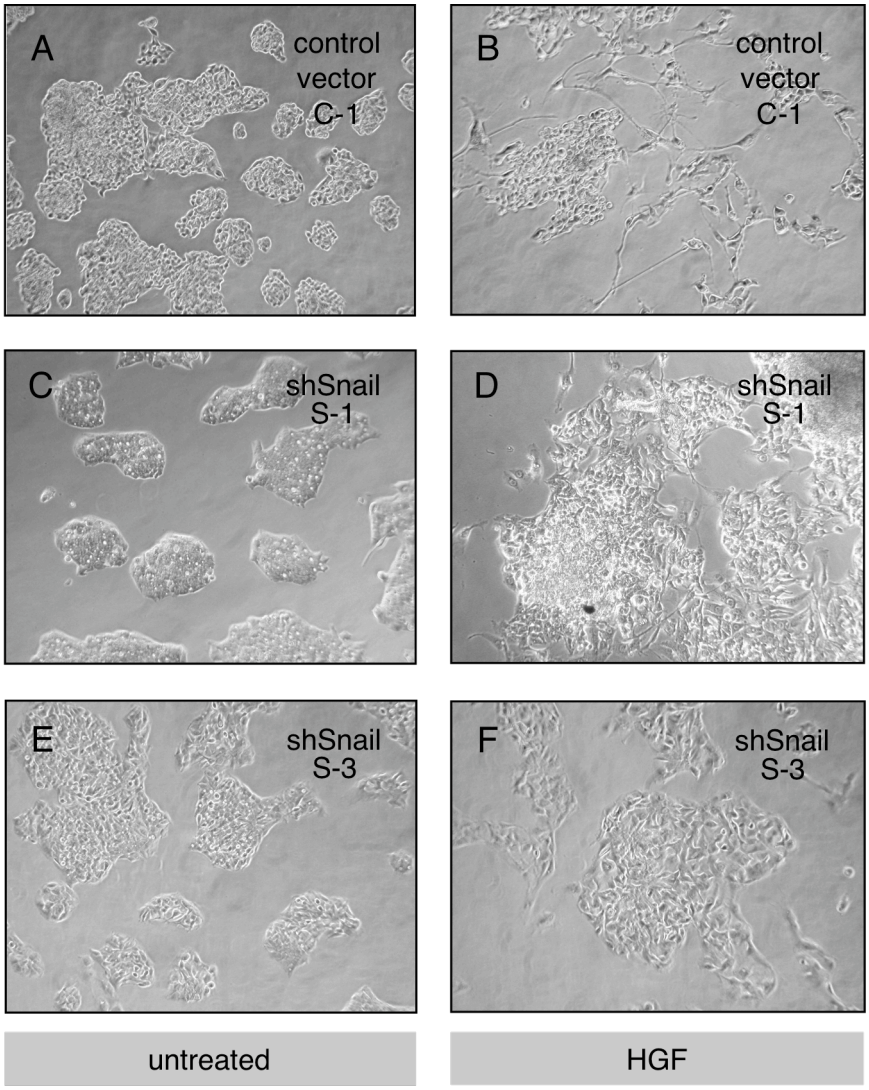

Figure 35: **shSnail prevents HGF/SF-induced cell scattering**.
shSnail-expressing (S-1, S-3) and control shRNA-expressing (C-2) HepG2 clones were incubated with or without 10 ng/ml HGF/SF. Cell scattering was observed by light microscopy after 72 h of HGF/SF treatment (Magnification: 200x).

4.4.5 Pathways involved in HGF/SF-induced Snail upregulation

Next, I investigated whether the regulation of Snail expression and Snail stabilization could depend on the activity of specific signaling cascades.

4.4.5.1 GSK-3β

The Snail protein exhibits two consensus sites for GSK-3β serine phosphorylation, leading to its nuclear export and degradation by the proteasome (Zhou *et al.*, 2004). In

addition, GSK-3β has been shown to directly inhibit Snail transcription (Bachelder *et al.*, 2005). It has been previously reported that GSK-3β activity could be inhibited by HGF/SF signaling (Papkoff and Aikawa, 1998), suggesting a possible link between HGF/SF signaling and increased Snail levels. Therefore, the involvement of GSK-3β in HGF/SF-induced Snail upregulation was analyzed in HepG2 and MDCK cells.

Immunoblot analysis revealed an increase in phosphorylation of GSK-3β in HepG2 cells and MDCK cells after treatment with LiCl compared to untreated cells (Figure 36). However, HGF/SF treatment did not lead to further GSK-3β phosphorylation, suggesting that GSK-3β activity was not involved in HGF/SF-mediated Snail expression.

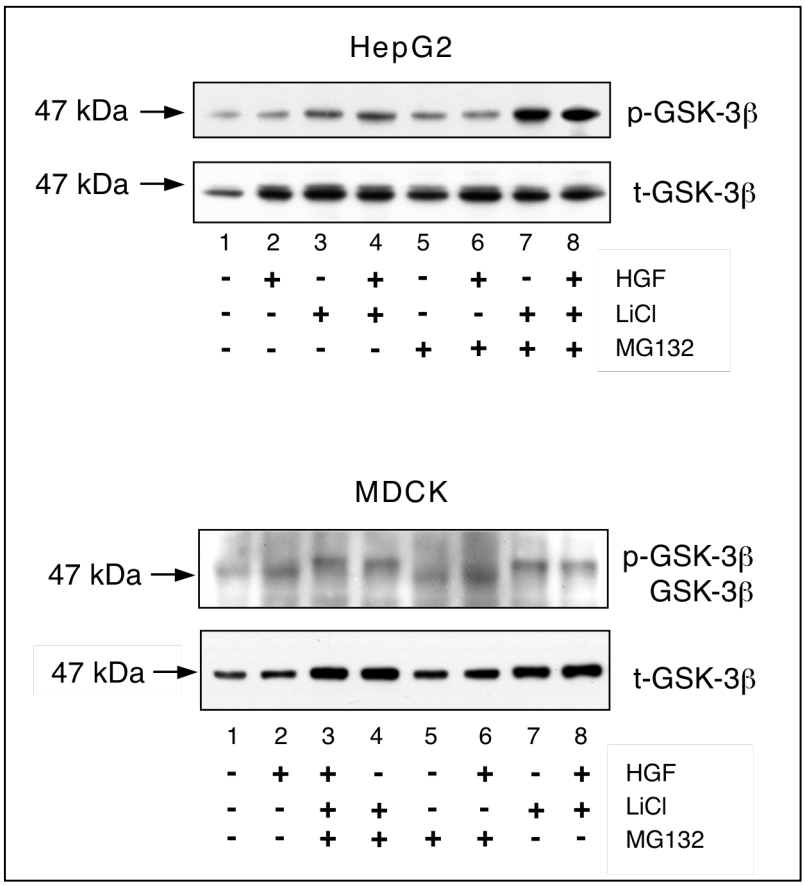

Figure 36: **Immunoblotting of GSK-3β phosphorylation.**
HepG2 and MDCK cells were pre-incubated with LiCl and MG132 for 30 min and then stimulated with 10 ng/ml HGF/SF for further 30 min. Whole cell lysates were analyzed by immunoblotting with specific antibodies against phosphorylated (p)-GSK-3β and total (t)-GSK-3β.

To evaluate whether HGF/SF-mediated signaling could stabilize Snail protein independent of GSK-3β activity, HEK293 cells were transiently transfected with a Flag-tagged Snail-8SA that cannot be phosphorylated by GSK-3β (Zhou *et al.*, 2004). Immunoblot analysis of HEK293 lysates with an anti-Flag antibody revealed that HGF/SF-treated cells exhibited higher levels of Flag-tagged Snail-8SA, indicating that the Snail protein was stabilized upon HGF/SF treatment independently of GSK-3β-mediated phosphorylation of Snail (Figure 37). Of note, the Flag-tagged Snail-8SA was

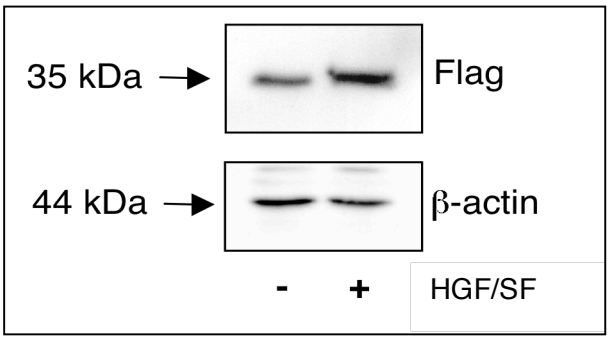

Figure 37: **HGF/SF-mediated Snail stabilization.**
HEK293 cells were transiently transfected with a plasmid encoding Flag-tagged Snail-8SA, and 24 h later stimulated with 10 ng/ml HGF/SF. After an additional 24 h, cells were analyzed by immunoblotting with anti-Flag-tag antibodies. Immunoblotting for β-actin was used as loading control.

under the control of the CMV promoter, which is not regulated by HGF/SF signaling, suggesting that other post-translational effects induced by HGF/SF lead to Snail stabilization.

4.4.5.2 PI3K and MAPK

As described above, specific inhibition of the MAPK pathway but not the PI3K pathway resulted in a repression of HGF/SF-induced cell motility (Figure 26 - Figure 28).

To identify if this pathway-specific effect could also be seen with HGF/SF-induced Snail upregulation, HepG2 cells were stimulated with HGF/SF in the presence or absence of the specific pathway inhibitors Wn and PD98059 (Figure 38). In the presence of the MAPK pathway inhibitor PD98059 the HGF/SF-induced Snail expression was prevented, while the PI3K inhibitor Wn had no effect on HGF/SF-induced Snail mRNA expression in HepG2 cells. These results further indicate that the MAPK pathway plays a critical role in HGF/SF-induced Snail expression, whereas the PI3K does not seem to be involved. The inhibitors themselves had no (PD98059) or only a minor (Wn) effect on Snail mRNA expression. Interestingly, Snail expression levels detected by qRT-PCR correlate with the scattering phenotype as observed earlier in this study (Figure 26 -Figure 28).

Taken together, these results demonstrate that HGF/SF induces cell scattering and increased motility by utilizing the MAPK signal transduction pathway, whereas the GSK-3β and PI3K pathways are not involved.

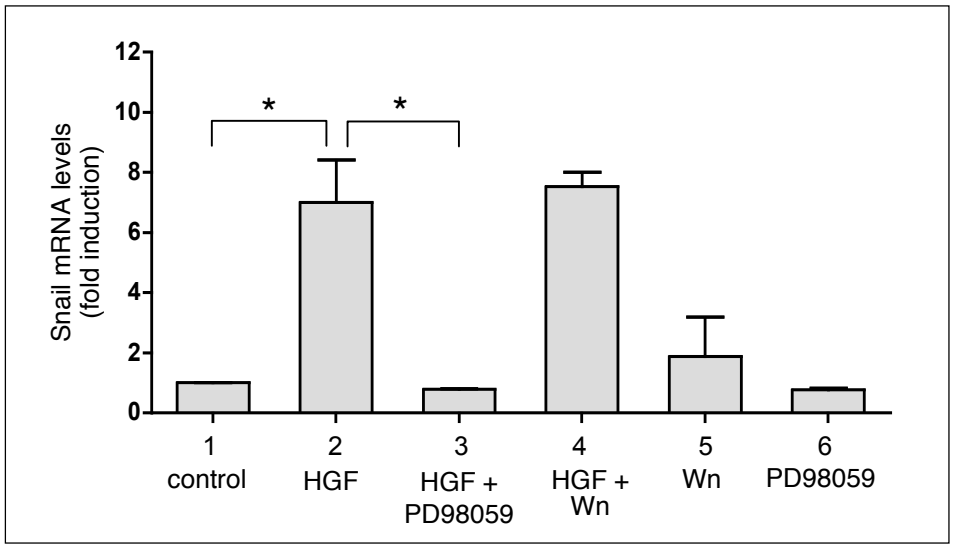

Figure 38: T**he MAPK pathway mediates Snail induction.** HepG2 cells were treated with 10 ng/ml HGF/SF for 8 h in the presence or absence of pathway specific inhibitors Wn (1 µM) and PD98059 (20 µM), respectively. Snail mRNA levels were determined by qRT-PCR. **P*<0.0001; unpaired t-test.

4.4.6 HGF/SF activates Egr-1 to induce Snail expression

To further elucidate the molecular mechanisms by which HGF/SF-stimulated MAPK signaling induced Snail expression, I investigated the potential involvement of candidate transcription factors. Several recent studies have shown that HGF/SF upregulates PDGF and VEGF (Worden *et al.*, 2005), CD44v6 (Recio and Merlino, 2003), angiotensin converting enzyme (ACE) (Day *et al.*, 2004), and fibronectin (Gaggioli *et al.*, 2005) in a MAPK-dependent and PI3K-independent manner through the early growth response factor (Egr)-1.

Thus, an *in silico* analysis of the Snail promoter sequence using the MatInspector software was applied (Cartharius *et al.*, 2005). The survey identified four putative Egr-1 binding sites between -450 bp and –50 bp upstream of the Snail gene transcriptional start site (Figure 39A). To assess whether Egr-1 was expressed in HepG2 cells and whether its expression was affected by HGF/SF, qRT-PCR analyses were performed. These revealed a twelve-fold (±4.7) transient increase in Egr-1 mRNA levels by HGF/SF, with maximum levels after 1 h and a decline to basal levels within 3 h of HGF/SF stimulation (Figure 39B). Immunoblotting analysis showed slightly delayed kinetics of the upregulation of Egr-1 protein levels, which peaked after 2 to 3 h and declined afterwards (Figure 39C).

Snail mRNA levels steadily increased beginning at 2 h, after the first appearance of Egr-1 protein (Figure 39B), and increased Snail protein levels could be detected after

5 h, again only in the presence of proteasome inhibitors (Figure 39C). The early increase in Snail expression already correlated with the downregulation of Snail target genes, such as E-cadherin or claudin-3 during HGF/SF treatment, with an initial decline in mRNA levels after 3 h and a further reduction during 8 h of HGF/SF incubation time (Figure 29).

These results suggest the following order of events in HGF/SF-induced Snail expression: (i) HGF/SF-mediated MAPK activation induces the expression of Egr-1, which in turn (ii) activates Snail gene expression and (iii) the subsequent repression of E-cadherin and claudin-3 gene expression, finally (iv) leading to cell scattering.

4.4.7 The Snail gene is a target of Egr-1

To validate a direct involvement of Egr-1 in Snail expression, the appropriate experiment would be to overexpress Egr-1 by transfection using a plasmid encoding for the wildtype Egr-1. Unfortunately, it has been previously reported that Egr-1 induces the expression of its co-repressor Nab2, thereby establishing a negative feedback loop and preventing a direct analysis of wildtype Egr-1 overexpression (Kumbrink *et al.*, 2005). To circumvent this problem, HEK293 cells were transfected with a plasmid encoding the Egr-1 DNA binding domain fused to the CREB-2 activation domain (nabR-Egr-1), a construct that is resistant to Nab2's repressive activity (Al-Sarraj *et al.*, 2005). Luciferase reporter assays revealed a concentration-dependent activation of the Snail promoter by nabR-Egr-1, indicating that Egr-1 is sufficient for activating Snail gene expression (Figure 40). Next, HepG2 cells were transfected with a plasmid encoding for a dominant-negative (dn) Egr-1 (Al-Sarraj *et al.*, 2005), and HGF/SF-induced Snail expression was monitored by qRT-PCR (Figure 41). In these experiments, the presence of dnEgr-1 completely abolished HGF/SF-induced Snail expression, further indicating that Egr-1 is indeed required for HGF/SF-mediated Snail expression. In summary, these results propose an Egr-1-controlled regulation of the Snail promoter.

Also, to determine whether Egr-1 could directly bind to the Snail promoter chromatin immunoprecipitation (ChIP) experiments were performed with anti-Egr-1 antibodies followed by PCR analysis with primers specific for the Snail promoter. Egr-1 specifically bound to the Snail promoter 1 h after HGF/SF stimulation, while no binding occurred in the absence of HGF/SF (Figure 42A).

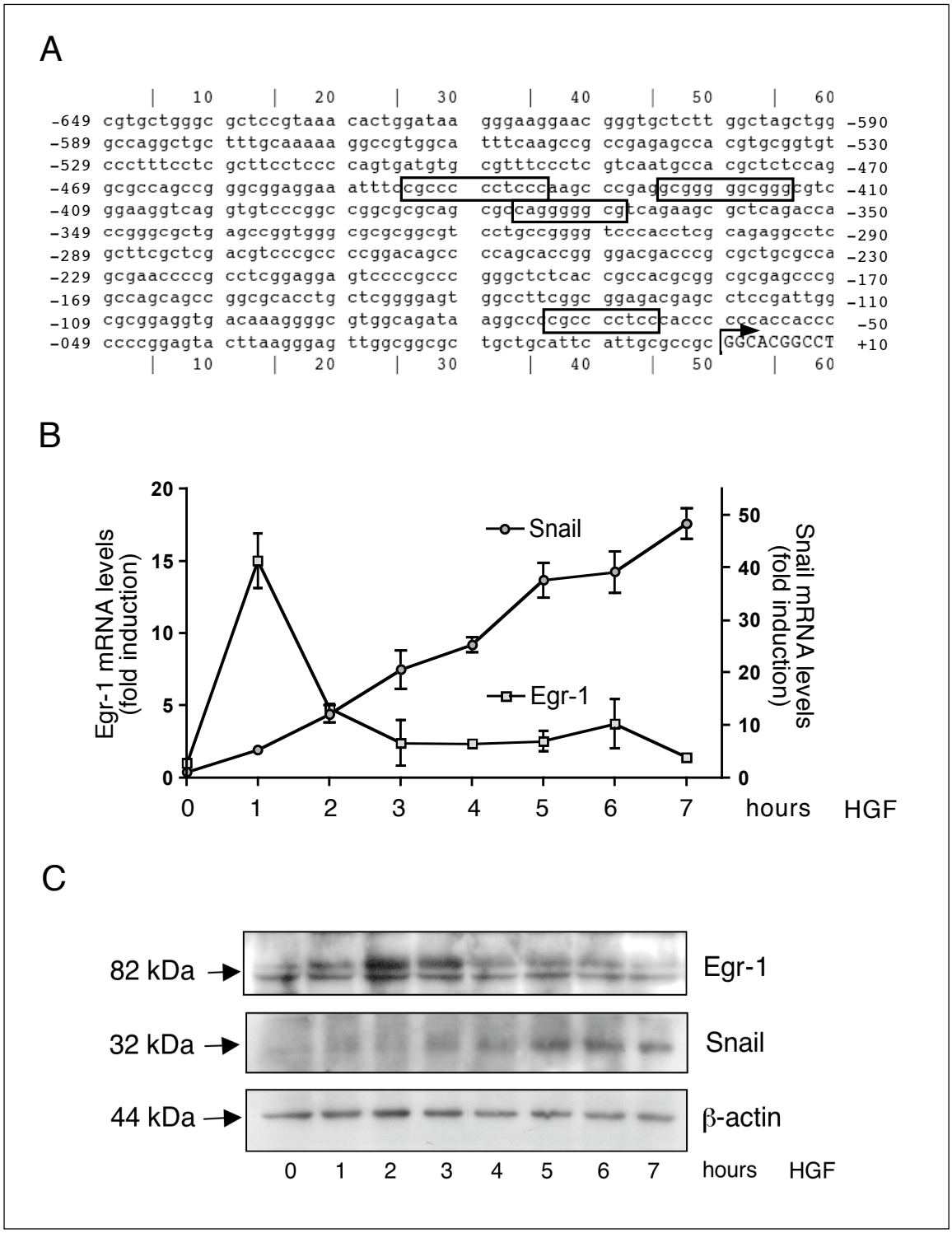

Figure 39: **Snail regulation by Egr-1**.

(A) Putative Egr-1 binding sites within 650 bp upstream of the transcriptional start site of the Snail gene (indicated by an arrow) are highlighted by boxes.

(B) HepG2 cells were incubated with HGF/SF, MG132, and LiCl, and Egr-1 and Snail mRNA levels were analyzed by qRT-PCR at the time points indicated. Values represent the averages and standard deviations of triplicate measurements.

(C) HepG2 cells were incubated with HGF/SF in the presence of MG132 and LiCl. Immunoblotting was performed with specific antibodies against Egr-1, Snail, and β-actin at the time points indicated.

Quantitative PCR analysis of anti-Egr-1-precipitated chromatin with primers specific for an Egr-1 binding site within the Snail promoter confirmed this observation (Figure 42B). Altogether, these results indicate that the Snail promoter is a direct target of the Egr-1 transcription factor upon HGF/SF stimulation.

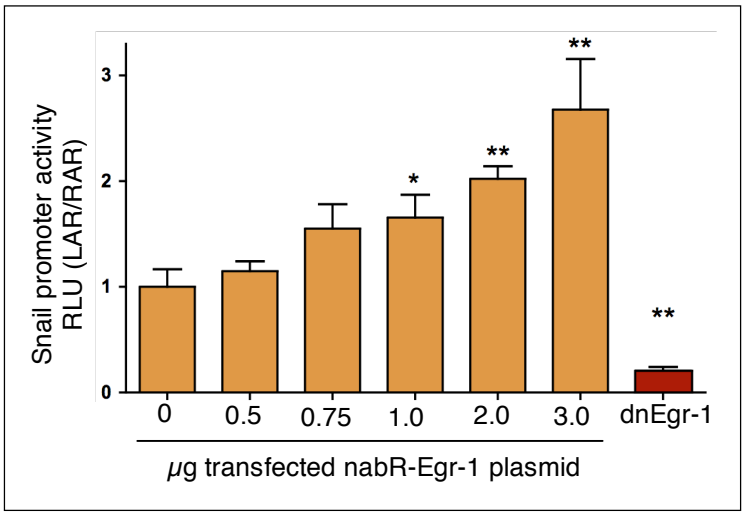

Figure 40: **Egr-1 activates the Snail promoter**.

HEK293 cells were transiently transfected with a plasmid encoding a constitutive-active Egr-1 construct, in which the Egr-1 DNA binding domain was fused to the CREB-2 activation domain, or a dominant-negative mutant (dnEgr-1). Snail-promoter luciferase-reporter and *Renilla* luciferase constructs were co-transfected with the Egr-1 plasmids. Cells were incubated with HGF/SF for 24 h, before luciferase activities were determined. Relative light units (RLU) represent firefly luciferase (LAR) normalized against *Renilla* luciferase activity (RAR). Values are means of induction ± standard deviations of triplicate measurements in comparison to untreated control samples. **P*=0.0145, ***P*<0.001; unpaired t-test.

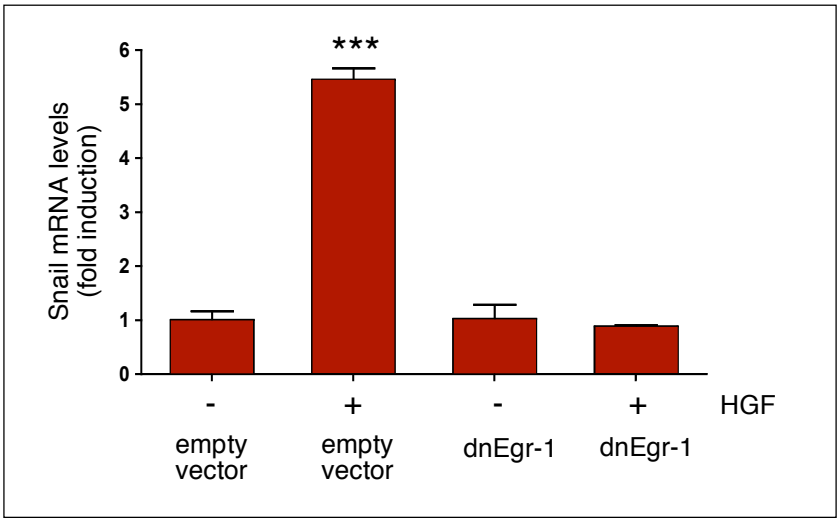

Figure 41: **Snail expression critically depends on Egr-1 function.**

HepG2 cells were transiently co-transfected with a dominant-negative version of Egr-1 (dnEgr-1) and truncated CD4. $CD4^+$-expressing cells were selected using MACS beads coated with anti-CD4 antibody, treated with HGF/SF for 8 h, before Snail mRNA levels were measured by qRT-PCR. ****P*=0.0018; unpaired t-test.

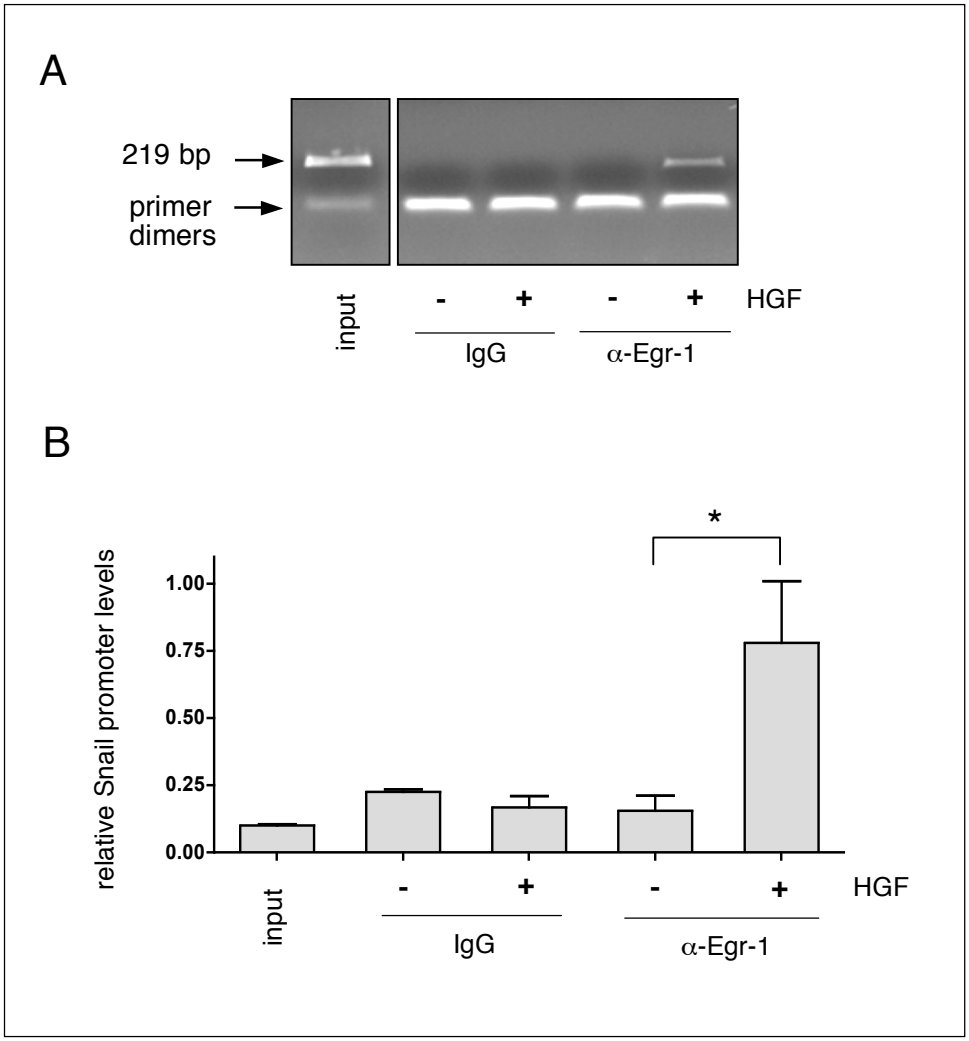

Figure 42: **Egr-1 directly binds to the Snail promoter.**

(A) Chromatin immunoprecipitation (ChIP) analysis of Egr-1 binding to the Snail promoter. HEK293 cells were stimulated with HGF/SF for 1 h before being subjected to ChIP analysis. Anti-Egr-1 antibodies or IgG were used to immunoprecipitate DNA-containing complexes. Subsequent PCR was performed with primers complementary to the Snail promoter region containing the Egr-1 binding sites. DNA samples were analyzed by agarose gel electrophoresis.

(B) Quantitative real time PCR analysis of the Snail promoter region immunoprecipitated with antibodies against Egr-1, as described in (A). Results indicate amplified Snail promoter levels normalized against PCR reactions detecting an unrelated promoter. Bar graphs represent the means and standard deviations. **P*=0.0432; unpaired t-test.

4.4.8 Transient upregulation of Snail

To analyze the long-term kinetics of HGF/SF-mediated gain of Snail expression, and loss of adherens and tight junction functions, HepG2 cells were treated with HGF/SF for 10 days. To avoid cell death by prolonged exposure to the proteasome inhibitor MG132 and the GSK-3β inhibitor LiCl, these were added 8 h prior to cell lysis for each time point. Analysis of Snail mRNA expression revealed that HGF/SF-induced Snail expression reached its maximum 8 h after HGF/SF stimulation and declined with longer treatment (Figure 43A). Immunoblot analysis showed that HGF/SF-induced Snail protein expression follows a similar kinetic (Figure 43B). As expected, with increased Snail

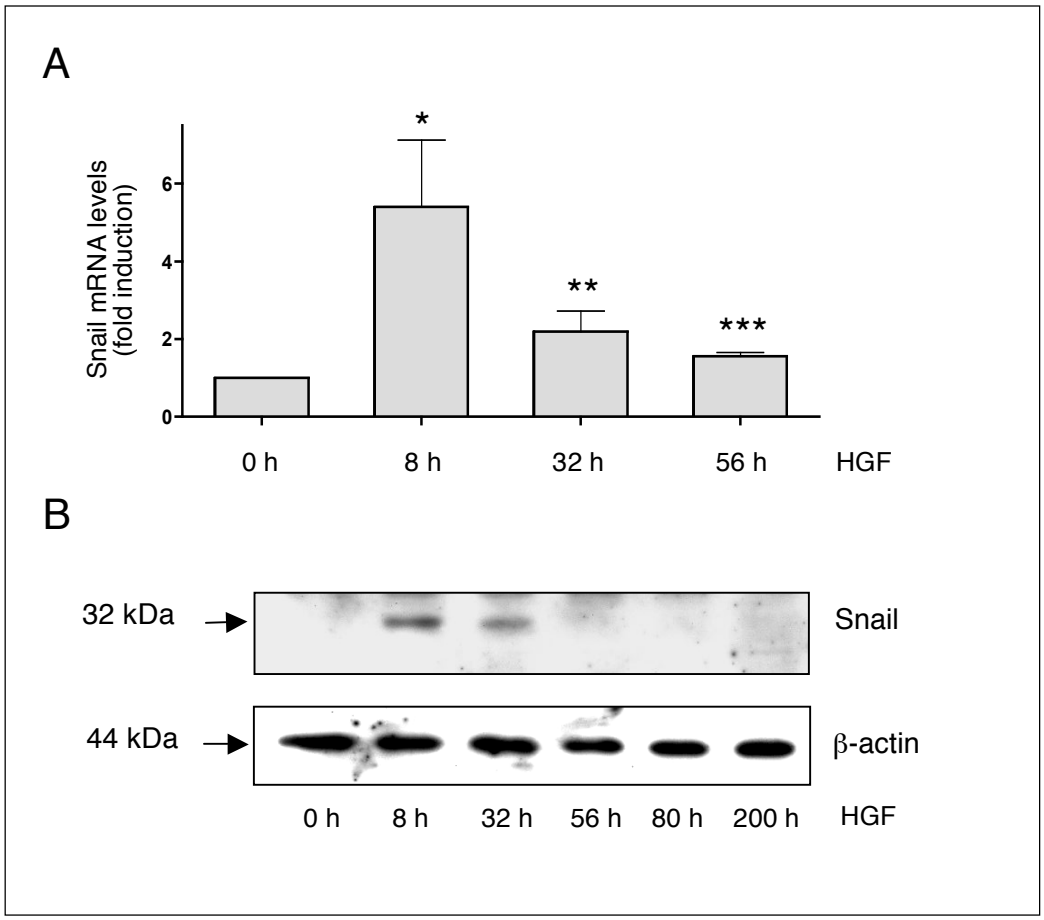

Figure 43: **HGF/SF induces transient upregulation of Snail.**

(A) HepG2 cells were treated with HGF/SF in the absence of MG132 and LiCl. Snail mRNA expression levels were determined by qRT-PCR at the time points indicated. Bar graphs represent the means and standard deviations of triplicate measurements. **P*=0.0023, ***P*=0.0013, ****P*<0.0001; unpaired t-test.

(B) HepG2 cells were incubated with HGF/SF for the time periods indicated. LiCl and MG132 were added 8 h before lysis of the cells. Snail protein levels and β-actin as loading control were analyzed by immunoblotting.

expression, E-cadherin (Figure 44A) and claudin-3 (data not shown) mRNA levels declined, yet remained low at time points where Snail expression had already returned to basal levels. Conversely, immunoblot analysis of whole cell lysates revealed that total E-cadherin protein levels remained unchanged after this time period (Figure 44B), suggesting a different regulation of the protein level or protein subcellular localization.

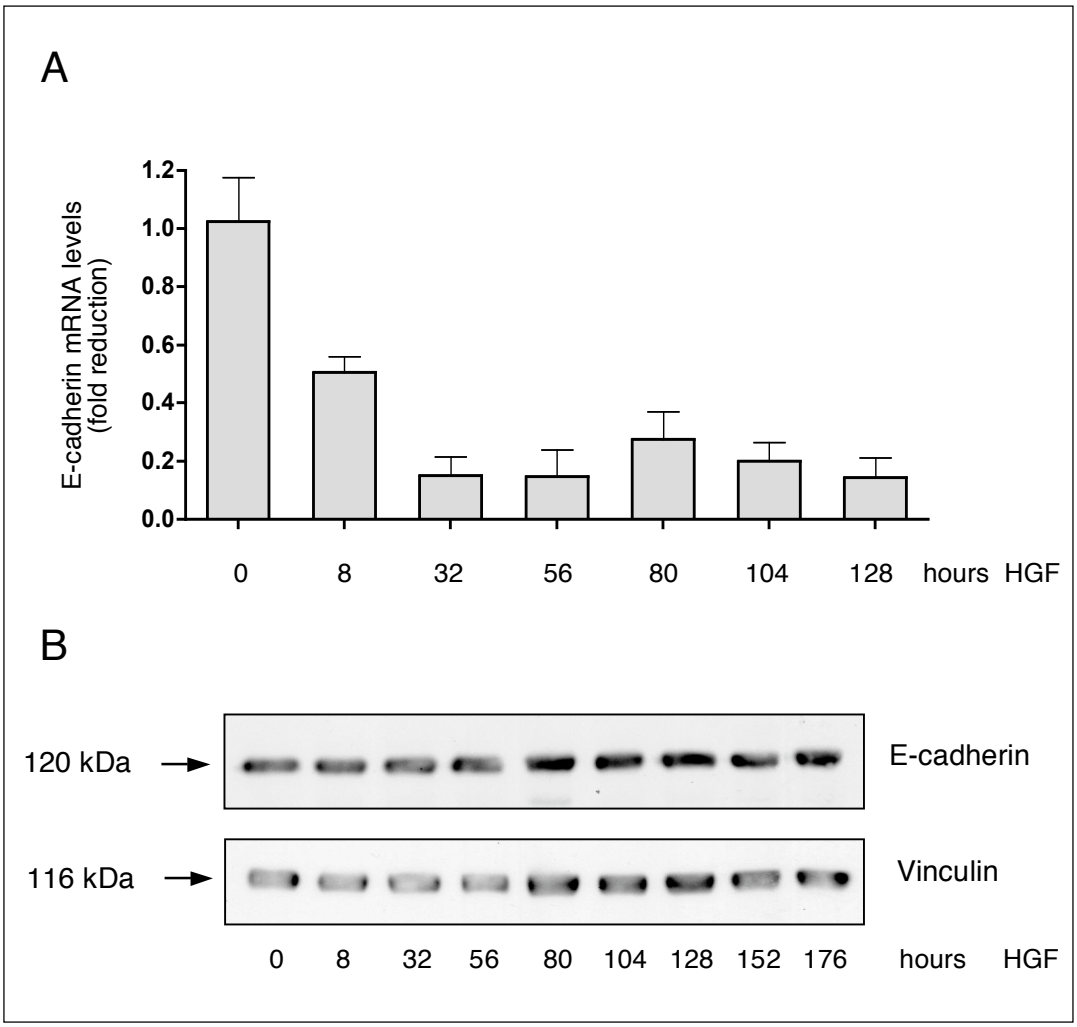

Figure 44: **HGF/SF induces downregulation of E-cadherin mRNA, but not protein**.

(A) HepG2 cells were incubated with HGF/SF for the time points indicated, and E-cadherin mRNA levels were analyzed by qRT-PCR. Values represent the means and standard deviations of triplicate measurements.

(B) HepG2 cells were incubated with HGF/SF for the time periods indicated. E-cadherin protein levels and vinculin as loading control were analyzed by immunoblotting.

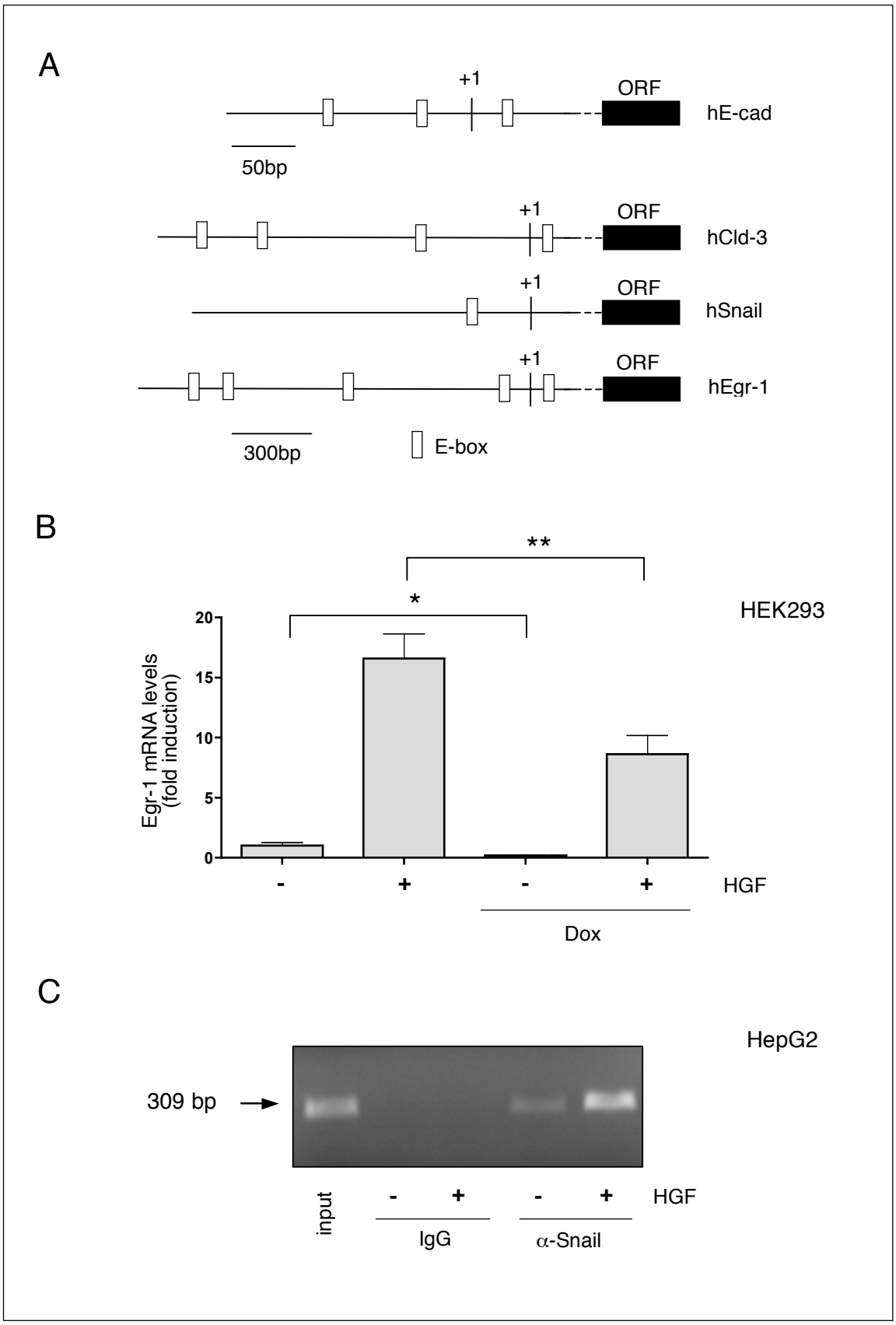
A
+1
ORF
hE-cad
50bp
+1
ORF
hCld-3
+1
ORF
hSnail
+1
ORF
hEgr-1
300bp
E-box
B
**
*
HEK293
Egr-1 mRNA levels (fold induction)
20
15
10
5
0
-
+
-
+
HGF
Dox
C
HepG2
309 bp
input
-
+
-
+
HGF
IgG
α-Snail

Figure 45: **Negative feedback loop: Snail represses its own activation**.

(A) Schematic representation of the promoter regions of the Snail target genes E-cadherin, claudin-3, Snail, and Egr-1. Open boxes = E-box; +1 = transcription start site; ORF = open reading frame.

(B) HEK293-FlpInTRex-Snail-8SA cells were cultured in the presence or absence of doxycycline for 24 h and then incubated with HGF for 1 h before subjected to qRT-PCR analysis for Egr-1 mRNA levels. Bar graphs represent means ± standard deviations of triplicate measurements.

(C) ChIP analysis of Snail binding to the Egr-1 promoter. HepG2 cells were incubated with HGF for 1 h before proteins were cross-linked to DNA, and chromatin-protein complexes were immunoprecipitated with an antibody against Snail. PCR was performed with primers specific for the Egr-1 promoter region containing E-box binding sites. DNA samples were analyzed by 2% agarose gel electrophoresis.

4.4.9 Signals for downregulating Snail

The observed kinetics in Snail and Egr-1 expression suggest a feedback loop mechanism, where Snail would repress its own activator. Recently, it has been reported that Snail is able to repress its own expression (Peiro *et al.*, 2006). Inspection of the Egr-1 promoter sequence also revealed the existence of five E-boxes, which could be potential binding sites for Snail (Figure 45A). To test whether overexpression of Snail inhibited HGF/SF-induced Egr-1 activation, HEK293 cells expressing Snail under the control of the tetracycline-inducible system were generated (HEK293-FlpInTRex-Snail-8SA). These cells were cultured with and without doxycycline before stimulation with HGF/SF and analysis of Egr-1 mRNA expression by qRT-PCR.

In the presence of high levels of induced Snail, the amounts of Egr-1 mRNA declined in the presence and even in the absence of HGF/SF (Figure 45B). Moreover, ChIP experiments in HepG2 cells with anti-Snail antibodies followed by PCR with primers complementary to the Egr-1 promoter region containing the E-boxes revealed specific Snail binding to the Egr-1 promoter (Figure 45C). These results suggest that Snail directly represses Egr-1 expression, thus exerting a negative feedback regulation on its own activator and on its own promoter (Peiro *et al.*, 2006).

5 Discussion

Hepatoblastoma (HB) is the most common pediatric malignant liver tumor, while hepatocellular carcinoma (HCC) is among the top five leading causes for cancer death. Elevated levels of HGF/SF can be found in HB and HCC cancer patients with a high tumor relapse rate and thus, are often associated with poor prognosis (Ueki *et al.*, 1997; von Schweinitz *et al.*, 1998). HGF/SF contributes to many physiological processes including organogenesis, wound healing, and tissue remodeling, but also participates widely in the metastatic process during tumor progression (Birchmeier *et al.*, 2003; Jiang *et al.*, 2005). Activation of the RTK c-Met by HGF/SF results in the recruitment of several signal transducers that regulate dissociation of adherens and tight junctions, and the stimulation of cellular motility, survival, proliferation, and morphogenesis (Martin *et al.*, 2004; Thiery, 2002; Weidner *et al.*, 1993).

Hence, in this study we investigated how HGF/SF contributes to the progression of HB and HCC and which underlying molecular mechanisms are involved. In a cell culture model of established HB and HCC cell lines, the various cellular responses evoked by HGF/SF were analyzed and quantitated. We have found that the highly differentiated HCC cell line expresses the highest levels of c-Met receptor when compared to the less differentiated HB cell lines. This corresponds to a higher sensitivity to HGF/SF stimulation. In consequence, these cells show stronger migratory behavior compared to HB cells. Also, as seen by apoptosis induction assays, these cells have a reduced sensitivity towards apoptotic stimuli and in the presence of HGF/SF caspase 3-like activities are reduced, suggesting a molecular mechanism for the higher malignancy observed in HCC.

5.1 HGF/SF as a proliferation factor for HB and HCC

Increased proliferation of tumor cells has been acknowledged as a key element for tumor growth and progression (Hanahan and Weinberg, 2000). HGF/c-Met signaling has been associated with higher proliferation rates, depending on the target cell (Jiang and Hiscox, 1997; Weidner *et al.*, 1993). We thus assessed whether HGF/SF could contribute to increased cell proliferation by [^{3}H]-thymidine incorporation, cell counts and MTT assays. In none of these assays an increased growth rate was observed upon treatment with HGF/SF. To test the possibility that the serum present in the culture medium could mask the effects elicited by HGF/SF stimulation, growth curves with

reduced serum conditions (2% FCS) were performed. Again, no increased growth rate could be detected in HB and HCC cell lines. Interestingly, when cells were grown under almost serum-free conditions (0.5% FCS), only the HepT1 cell line was able to proliferate, while the HuH6 and HepG2 cell lines underwent apoptosis, which could partially be inhibited by HGF/SF pre-incubation (see below).

It was previously reported that HB carried an activating mutation in β-catenin and displayed increased proliferation associated with cytoplasmic and nuclear β-catenin staining along with increased staining for c-Myc and cyclin D1 (Ranganathan *et al.*, 2005; Takayasu *et al.*, 2001). Aberrant Wnt/β-catenin activation has been observed in 70% of all HB and 20 – 40% of all HCC, which is one of the highest β-catenin mutation rates among solid tumors (Polakis, 2000). The HB and HCC cell lines used carry mutations in the β-catenin gene. In this respect, the cell lines even resemble the original tumors, since they carry the same mutations (Koch *et al.*, 1999). Hence, it might be possible that these cell lines already have high c-Myc and cyclin D1 protein levels and thus increased proliferation rates, which cannot be further stimulated by HGF/SF treatment. Detailed analysis of c-Myc and cyclin D1 mRNA and protein levels could provide an adequate answer to this question.

HGF/SF-stimulated recruitment of STAT3 to the c-Met receptor was previously shown as a crucial step in liver regeneration and tubule formation (Boccaccio *et al.*, 1998; Borowiak *et al.*, 2004). In addition, constitutive activation of STATs has been determined in a wide variety of human cancers. Therefore, this signaling pathway was examined. Given the shared common structural features of STAT proteins, such as the SH2-phosphotyrosine interaction domain and the central DNA-binding site (Becker *et al.*, 1998), all STAT members were analyzed.

Treatment of HB and HCC cells with IFN-γ, IL-4, and IL-6 resulted in normal STAT activation. However, upon stimulation of the cells with various HGF/SF, EGF or SCF concentrations for different time periods, no STAT activation could be detected, suggesting that these growth factors have no influence on the Jak/STAT pathway activation in these cells. Since no HGF/SF-mediated activation of STAT proteins has been shown in HB and HCC cells before, this behavior could be a cell type-specific effect. Interestingly, HepT1 was the only cell line that did not respond to IFN-α treatment with STAT phosphorylation. Defects in the Jak/STAT pathway of HepT1 may render these cells unresponsive to growth-inhibiting cytokines such as IFN-α and may impart a growth advantage. This was seen in the proliferation studies, where increased growth of

HepT1 cells was observed in comparison to other HB cells, even under serum-free conditions.

Cellular proliferation might be necessary, but not sufficient to obtain a transformed cellular phenotype. Due to the pleiotropic nature of HGF/SF and its ability to activate several signaling cascades, the maintenance of cell survival and induction of motogenic pathways may play an even more important role in HGF/SF's oncogenic signaling. Accordingly, HGF/SF-transfected Chinese hamster ovarian (CHO) cells showed increased chemoresistance to chemotherapeutic agents, albeit only a slight increase in cell proliferation (Meng *et al.*, 2000). Jiang and Hiscox also reported that an increased sensitivity to HGF/SF did not translate into a mitotic response, but instead led to increased invasion (Jiang and Hiscox, 1997).

5.2 HGF/SF as an antiapoptotic signal for HB and HCC cells

Perturbation of the balance between cell survival and apoptosis may lead to either excessive cell death or survival and plays a pivotal role in the pathogenesis of a large number of diseases, including cancer. Previous studies by others demonstrated that HGF/SF is a survival factor with potent ability to promote cell survival by inhibiting apoptosis (Fan *et al.*, 1998a; Liu, 1999; Yo *et al.*, 1998). Accordingly, dysregulation of c-Met and HGF/SF signaling has emerged as a crucial feature of many human malignancies (see Table 2). However, to date little is known about HGF/SF-induced cell survival in HB and HCC.

We found, that one of HGF/SF's critical roles in HB and HCC tumor progression is related to higher cell survival and enhanced resistance against apoptosis-inducers. Some of these apoptotic inducers are used for chemotherapy, thereby providing a possible rationale for the correlation between elevated HGF/SF serum levels and chemotherapy-resistant HB and HCC.

The underlying mechanisms of the observed effects could be explained by the activation of the PI3K/Akt pathway in HB and HCC. Inhibition of PI3K prevented HGF/SF-induced cytoprotection against apoptotic stimuli and HGF/SF treatment led to phosphorylation of Akt/PKB. PI3K/Akt-mediated serine residue phosphorylation of Bad leads to its dissociation from Bcl-x_L and binding to 14-3-3, thereby inactivating Bad function (Figure 46). Bcl-x_L is then free to bind to APAF-1 and block APAF-1's ability to activate the cell death protease caspase 9, which in turn activates the caspase 3 (Datta *et al.*, 1999; Kennedy *et al.*, 1999).

Changes of caspase 3-like activity upon HGF/SF pre-incubation were detected in this study. Indeed, as shown by others, overexpression of Bad increased apoptotic death in wild-type human proximal tubular epithelial (HKC) cells but not in HKC cells stably transfected with an expression vector containing human HGF/SF cDNA (H4 cells) (Liu, 1999). Immunoblotting confirmed that the Bad protein overexpressed in H4 cells was fully phosphorylated at the Ser^{136} site and thereby inactive. Interestingly, Wn was also shown to inhibit phosphorylation of Akt/PKB and Bad in HEK293 cells (Liu, 1999). Thus, the finding that HGF/SF inhibits apoptosis in a PI3K-dependent fashion is consistent with the notion that the PI3K signaling pathway plays an essential role in mediating growth factor-dependent cell survival (Datta *et al.*, 1999; Franke *et al.*, 1997a)

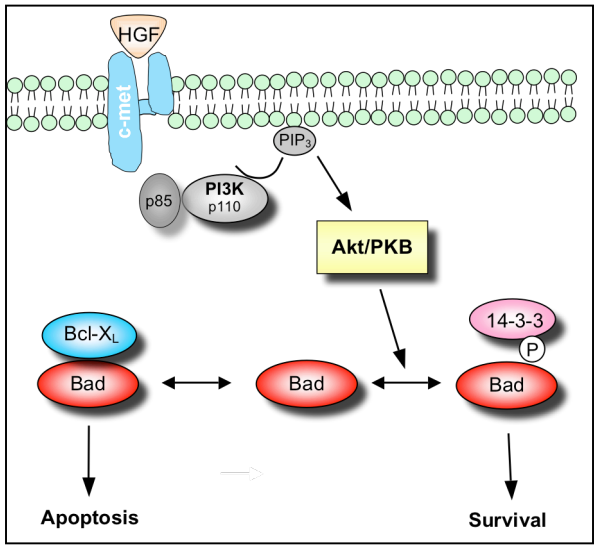

Figure 46: **Model of HGF/SF-mediated phosphorylation and inactivation of Bad**. HGF/SF activates Akt/PKB, which phosphorylates Bad, thus inactivation and cell survival. In the absence of survival factors and Akt/PKB activity, Bad is dephosphorylated. Dephosphorylated Bad interacts with pro-survival Bcl-2 family members, such as Bcl-x_L, and inactivates them, causing cell death. Modified from (Datta *et al.*, 1999).

A second possible mechanism would be a direct upregulation of the anti-apoptotic protein Bcl-x_L through Akt/PKB as was previously shown *in vitro* and *in vivo* (Dai *et al.*, 2003; Fan *et al.*, 2000; Liu, 1999). Prolonged HGF/SF pre-incubation blocked apoptosis induced by Camptothecin, Adriamycin, X-rays, and ultraviolet radiation (Fan *et al.*, 1998a; Meng *et al.*, 2000). In this instance, increased Bcl-x_L protein levels correlated with the degree of protection against apoptosis. Thus, the persistent presence of HGF/SF may contribute to the development of a chemoresistant phenotype in HB and HCC. However, our approaches to show increasing Bcl-x_L protein concentrations in HB and HCC failed for technical reasons. Further experiments to address this second possible mechanism, would include immunoblotting for the Bcl-x_L protein and the phosphorylated Ser^{136} residue of Bad in HB and HCC cells upon HGF/SF treatment. Such effects of HGF/SF on Akt/PKB phosphorylation and Bcl-x_L protein and mRNA levels upregulation were previously observed *in vivo* after partial hepatectomy (Hong *et al.*, 2000).

Transient Bad phosphorylation has a short-term cytoprotective effect, whereas upregulation of Bcl-x_L has a long-term cytoprotective effect, meaning that the HGF/SF-mediated protective machinery must be active within the cells *before* the cytotoxic damage occurs (Liu, 1999). As a universal mechanism, maintenance of HB and HCC cell survival by phosphorylation of Bad and upregulation of Bcl-x_L would serve to ensure the transformation potential of HGF/SF, finally leading to cancer. Notably, it was reported that upregulation of Bcl-x_L is not only mediated by Akt/PKB, but also by STAT3, thereby conferring resistance to apoptosis (Catlett-Falcone *et al.*, 1999; Nielsen *et al.*, 1999). However, in the HB and HCC cell lines tested, HGF/SF was not capable of activating STAT3, suggesting that this pathway is not involved in HB and HCC cytoprotection.

A third possibility would be that HGF/SF blocks the Bax translocation to the mitochondrial membrane, thereby inhibiting Bax pore formation and mitochondrial efflux of cytochrome c as previously reported (Liu, 1999; Nakagami *et al.*, 2002). Bax is transcriptionally controlled by the tumor suppressor protein p53. High levels of Bax oligomerize at the outer mitochondrial membrane to induce the mitochondrial permeability transition (MPT), followed by cytochrome c efflux. Cells overexpressing both Bax and Bcl-2 family members show no signs of caspase activation and survive with significant amounts of cytochrome c in the cytoplasm (Rosse *et al.*, 1998), suggesting that Bcl-2 family members control cell survival upstream and downstream of cytochrome c release (Hengartner, 1998; Hengartner, 2000). It would be interesting to investigate in more detail, whether HGF/SF is inducing one or more of the aforementioned effects in HB and HCC cells to further understand the molecular mechanisms leading to increased HB and HCC cell survival and thus tumor progression.

In summary, the regulation and function of the PI3K/Akt pathway and the downstream signals of Akt/PKB in HB and HCC cells were investigated in this study. It was demonstrated that the PI3K/Akt pathway acts as a survival (anti-apoptotic) signal and that HGF/SF activates this pathway in HB and HCC cells. These data further suggest that HGF/SF serves as a survival factor acting through the PI3K/Akt pathway, thereby contributing to carcinogenesis. Interestingly, HGF/SF also protects MDCK cells from apoptosis induced by disruption of cell detachment, a process termed *anoikis*, which is consistent with the scattering phenotype induced by HGF/SF as discussed below (Frisch and Francis, 1994).

5.3 HGF/SF as a motility factor

HGF/SF is particularly relevant to oncogenesis because not only does it enhance cell survival, but it also stimulates cell motility, invasive growth, and functions as a morphoregulatory agent. However, the knowledge about HGF/SF-induced signaling pathways involved in cell dissociation, motility, and invasion is restricted. Possible molecular mechanisms to explain the observed phenotypes were addressed in this study. To infiltrate the surrounding tissues, single motile tumor cells leave the tumor mass by breaking cell-cell contacts, known as tight and adherens junctions and break the basal lamina by activating MMPs (Friedl and Wolf, 2003; Kadono *et al.*, 1998). Snail family proteins have emerged as major transcriptional repressors of E-cadherin expression, the prototype mediator of adherens junctions, and claudins, which are critically involved in tight junction formation (Ikenouchi *et al.*, 2003). Here, three independent cellular systems were used to analyze the mechanisms underlying HGF/SF-mediated cell scattering and migration. When applying an shRNA approach, it was disclosed that HGF/SF requires Snail to repress E-cadherin expression and to induce cell scattering. Furthermore, the major parts of the HGF/SF-mediated signaling pathway leading to Snail expression and thus to cell scattering, migration and invasion have been unraveled.

We demonstrate that HGF/SF induced a rapid and transient increase in both Snail mRNA and protein levels. Thereby, an inverse correlation between E-cadherin mRNA and Snail mRNA and protein levels was observed upon HGF/SF treatment. A similar correlation has been previously reported in HCC and in colon epithelial cells, where Snail expression and E-cadherin downregulation is associated with higher invasiveness (Boon *et al.*, 2005; Sugimachi *et al.*, 2003).

In contrast, even after long-term treatment with HGF/SF we could not detect a clear downregulation of total E-cadherin protein in HepG2 cells. A long E-cadherin half-life and inconsistent mRNA and total protein levels have already been found in other cell systems (Maeda *et al.*, 2005; Peinado *et al.*, 2003; Strutz *et al.*, 2002), suggesting a redistribution from the adherens sites rather than degradation (Shibamoto *et al.*, 1994). Indeed, it has been previously reported that internalized E-cadherin is often recycled to sites of new cell-cell contacts (Gumbiner, 2000), or non-adhesion areas (Hiscox and Jiang, 1999). Furthermore, recent experiments with MDCK cells have demonstrated that the early events of HGF/SF-induced scattering do not involve a loss of E-cadherin protein and that a disruption of junctions by contractile forces induces cell scattering and

migration (de Rooij *et al.*, 2005). Thus far, the shRNA knock-down experiments presented here clearly indicate that Snail is required for HGF/SF-mediated cell scattering. Hence, HGF/SF-induced Snail expression and Snail-mediated repression of E-cadherin function must be considered as an order of events. Furthermore, Snail might regulate other genes required for the scattering process, in conjunction with E-cadherin downregulation. In fact, Snail-mediated repression of a number of components of adherens, tight, and desmosomal junctions has been recently reported (Barrallo-Gimeno and Nieto, 2005).

By employing a combination of specific inhibitors and biochemical analyses, the MAPK pathway was discovered to mediate HGF/SF-induced Snail upregulation and cell scattering. This finding was consistent with previous reports demonstrating that the MAPK pathway is required for HGF/SF-induced scattering of various epithelial cell types (Abella *et al.*, 2005; Janda *et al.*, 2002; Maina *et al.*, 2001; Tanimura *et al.*, 1998). Notably, the transcription factor Egr-1 appears to play a critical role in HGF/MAPK-mediated induction of Snail expression and cell scattering. In fact, expression of a dominant-negative version of Egr-1 abolished HGF/SF-induced Snail expression. Unfortunately, forced expression of wildtype Egr-1 induces the expression of its own repressor Nab2, thus excluding a direct test whether Egr-1 alone is sufficient to induce Snail expression (Kumbrink *et al.*, 2005). To circumvent this problem, expression of a mutant Egr-1, which is resistant to the Nab co-repressors (Al-Sarraj *et al.*, 2005), resulted in a concentration-dependent upregulation of Snail promoter activity. These data suggest that upon induction by Egr-1, Snail binds to the Egr-1 promoter and represses its transcription. In fact, Snail has recently been shown to repress its own transcription (Peiro *et al.*, 2006), thereby establishing a robust negative feedback loop that prevents sustained activation of Egr-1 and Snail. Hence, the stimulus-induced synthesis of Egr-1 and Snail is transient and might give a first signal to initiate a cascade leading to cell scattering.

5.3.1 Regulation of Snail mRNA and protein levels

The level of Snail in a cell is not only regulated at the transcriptional level, but also by protein stabilization. GSK-3β controls Snail's subcellular localization and activity by phosphorylation of consensus sites. Importantly, the results from our experiments suggest additional regulatory steps in Snail expression.

5.3.1.1 Stabilization of Snail mRNA

In our experiments we observed elevated mRNA levels of Snail upon exogenous expression of Snail protein (data not shown). At the same time, Snail promoter activity was not increased, suggesting a regulation of the Snail mRNA stability. Therefore, we performed an extensive search for known specific destructions sites, *e.g.* adenosine- and uridine-rich elements (ARE) (Espel, 2005) within the coding region of the mRNA, but did not detect any AREs within the Snail mRNA, suggesting other, yet unknown regulatory elements.

It will be of particular importance to identify such elements as well as proteins able to bind to and to stabilize the Snail mRNA, since this could play a critical role in tumor progression.

5.3.1.2 Alternative upregulation of translation

Increased Snail protein levels were detected after HGF/SF treatment of cells expressing a GSK-3β-resistant form of Snail, suggesting that augmented Snail protein levels could also be due to enhanced translation of its mRNA.

An alternative mode of translation initiation involves the recruitment of the translational initiation complex by an internal ribosome entry site (IRES). Translation by IRES was originally identified in picornavirus, but a number of cellular mRNAs have subsequently been found to contain IRES function, including basic FGF, VEGF, c-Myc, and HIF1-α (Lang *et al.*, 2002; Stein *et al.*, 1998; Stoneley *et al.*, 2000; Vagner *et al.*, 1995). The cellular IRES have been shown to be involved in preservation of translational efficiency during cellular stress, *e.g.* hypoxia, serum deprivation, heat shock, or apoptosis when protein synthesis is reduced. Structural characteristics of IRES are a 70% G + C-rich region in the 5'UTR of the mRNA and a 5'UTR length of at least 250 bp (Lang *et al.*, 2002).

When analyzing the originally published 5'UTR Snail sequence, we did not discover any characteristic IRES. However, an EST database search for extensions in the 5'UTR of Snail identified such a motif. This extended 5'UTR contains typical features of IRES, suggesting that Snail mRNA is a good candidate for being translated by alternative means and not being dependent on the cap-binding protein eIF4E, which is translation rate limiting. Further experiments, *e.g.* a dicistronic reporter assay, would be necessary to validate IRES-containing Snail mRNA and to check whether Snail translation is

increased under severe cellular conditions. This could be of particular importance since invading and metastasizing cells outlive several stressful environmental cues, such as lack of cell-cell and cell-matrix contacts, nutrients deprivation, and chemotherapeutic treatment. Increased translation of motility inducers like Snail could therefore help tumor cells in finding a new niche where they could rebuild the necessary microenvironment and reestablish the oxygen and nutrients' supply.

5.3.1.3 Increased Snail protein stability

Elevated protein levels do not only result from increased translation, but also from protein stabilization. Stabilization of the Snail protein has already been extensively studied. As previously described, Snail sublocalization and stability is tightly regulated by GSK-3β activity (Bachelder *et al.*, 2005; Zhou *et al.*, 2004). However, this pathway is not involved in HGF/SF-mediated Snail protein stabilization (Figure 36), suggesting another regulatory mechanism.

A mechanism that could contribute to the stabilization of Snail involves the protein kinase CKII (formerly known as casein kinase II). CKII is a ubiquitously expressed, pleitropic and constitutive-active serine/threonine kinase (reviewed in Faust and Montenarh, 2000). In most mammalian cells, the highly conserved holoenzyme is localized to both cytosol and nucleus. CKII consensus motifs (S/TxxE/D) are often found in close proximity to GSK-3β phosphorylation sites, *e.g.* in β-catenin and E-cadherin (Lickert *et al.*, 2000; Serres *et al.*, 2000). Interestingly, a potential CKII priming site exists in Snail, suggesting a role for CKII in Snail phosphorylation and increased degradation by the proteasome (see Figure 11). In fact, CKII has been found to associate with the proteasome complex, further suggesting that it targets or guides proteins to the proteasome and/or is degraded by the proteasomal pathway itself (Ludemann *et al.*, 1993).

CKII-mediated serine/threonine phosphorylation of β-catenin and E-cadherin leads to stabilization of adherens junctions, thereby promoting the maintenance of tissue homeostasis (Bek and Kemler, 2002; Lickert *et al.*, 2000). Consequently, Snail would be repressed in this scenario. Inhibition of CKII could then lead to weakened β-catenin and E-cadherin proteins and stabilized Snail protein. Interestingly, CKII was also found to associate with Egr-1 and thereby decreasing its DNA binding capacity (Jain *et al.*, 1996). Thus, we hypothesize that CKII, as a central effector, could regulate additionally to GSK-3β the stability of Snail and of cell-cell adhesions in general. Additional

experiments are necessary to evaluate the role of CKII in transforming processes and whether HGF/SF has an influence on its activity. Besides heparin, thus far no other negative, external regulatory mechanism of the constitutive-active CKII is known. However, further experiments to determine the role of CKII in HGF/SF-triggered cell morphology changes and increase in cell survival will be required.

5.3.1.4 Alternatives to Snail

In addition to Snail, we also tested for two other known transcription repressors of *E-cadherin*, Slug and Twist, in various cancer cell lines. Slug is mainly produced in chick gastrula as well as in the neural crest of *Xenopus* and chick. It has also been shown to be essential for EMT in the mesoderm formation in chick (Nieto, 2002). The basic helix-loop-helix (bHLH) transcription factor Twist was originally identified in the initiation of gastrulation in *Drosophila* (Thisse *et al.*, 1987) and is also known to be a key inducer of metastasis in some tumors (Yang *et al.*, 2004). In the qRT-PCR experiments performed, Slug and Twist mRNA was only detected in HEK293 cells, the breast cancer cell line MCF-7, and in the cervical HeLa cell line. In HepG2 cells, Slug and Twist mRNA levels were barely detectable. Upon HGF/SF treatment of the cell lines tested, only the HEK293 cells showed a slight increase in Twist and Slug mRNA levels. A similar finding correlating with our results was found in clinical data, where HCC were analyzed for E-cadherin, Snail and Slug mRNA levels. In tumors overexpressing Snail, a correlation between decreased E-cadherin levels and increased cell invasion was found (Sugimachi *et al.*, 2003). However, Slug mRNA levels were neither linked to E-cadherin expression nor to tumor invasiveness, suggesting Snail to be the most prominent repressor of E-cadherin. Notably, we cannot rule out that in other cell systems or upon other stimuli, *e.g.* by FGF, TGF-β, and bone morphogenetic protein (BMP), Snail function is replaced by other transcriptional repressors. An increasing number of zinc-finger transcription factors, including SIP-1 and E12/E47, have been described as E-cadherin repressors and have also been implicated in the regulation of the EMT phenomena (Comijn *et al.*, 2001). Accordingly, for some breast cancer cell lines it has been shown that SIP-1 mediates E-cadherin repression (Bindels *et al.*, 2006).

5.3.2 HGF/SF, Egr-1, and Snail: a novel network

Overall, the results presented here show that HGF/SF induces a transient expression of Snail, which is required for the induction of cell scattering. However, it is important to note that the forced expression of Snail by itself does not induce cell scattering in HepG2 or HEK293 cells (data not shown). This suggests that HGF/SF-mediated scattering involves in addition to Egr-1/Snail a hitherto unknown pathway. Contrary to our results, Snail overexpression has previously been shown to induce EMT in other epithelial cell lines (Bachelder *et al.*, 2005; Zhou *et al.*, 2004). EMT is a process, where cell-cell junctions are altered, epithelial polarity is lost, and the expression of mesenchymal markers, such as vimentin and smooth muscle actin, is gained (Thiery, 2002). Nonetheless, analysis of HGF/SF-treated HepG2 cells has not revealed any upregulation of mesenchymal markers (data not shown), suggesting that Snail-dependent scattering is distinct from Snail-induced EMT.

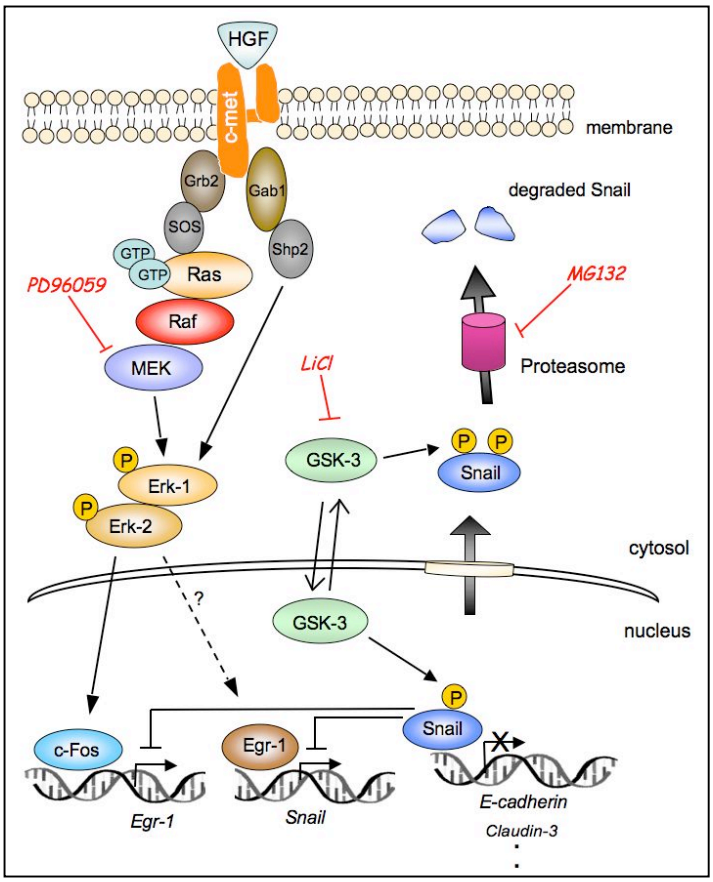

Figure 47: **Molecular mechanisms underlying HGF/SF-induced cell scattering.**
Binding of HGF/SF to its receptor leads to autophosphorylation of c-Met and via adaptor molecules to the activation of the Ras/Raf/MAPK pathway. Activated MAPK then phosphorylates and activates immediate early transcription factors, such as c-Fos and Elk, which bind the Egr-1 promoter and induce Egr-1 gene expression. In turn, Egr-1 induces Snail gene expression. Newly synthesized Snail then binds the promoter of its target genes, including E-cadherin and claudin-3, thereby repressing their transcription. In addition, Snail represses transcription of the Egr-1 and Snail genes, thus mediating a negative feedback loop. GSK-3β provides a nuclear export signal by phosphorylating Snail on its phosphorylation consensus site II. Once in the cytoplasm, Snail is phosphorylated by GSK-3β on consensus site I, leading to its ubiquitination and proteasomal degradation, a process repressed by LiCl and MG132.

Based on our findings and that of others', it is conceivable that Snail plays a critical role in the early stages of cell scattering and EMT, by making tumor cells sensitive for further EMT stimuli, *e.g.* TGFβ, and setting the stage for invasive tumor progression (Huber *et al.*, 2005).

Our experimental results show for the first time a mechanism by which HGF/SF induces expression of the transcriptional repressor Snail and, with it, cell scattering, migration and invasion: stimulation of cells with HGF/SF leads to (i) activation of the MAPK signaling pathway, (ii) upregulation of Egr-1, which in turn (iii) induces Snail transcription and, (iv) as a consequence results in the repression of Snail target genes, among them E-cadherin, claudins and Egr-1 itself (Figure 47).

Further studies are warranted to characterize the molecular details of HGF/SF-mediated signal transduction and Egr-1 function. HGF/SF has been shown to induce the expression of a number of pro-angiogenic factors by activating Egr-1 (Day *et al.*, 2004; Recio and Merlino, 2003; Worden *et al.*, 2005). In fact, induction of Egr-1 gene transcription is detected in many cell types in response to cytokines and mitogens (Gashler and Sukhatme, 1995; Kaufmann *et al.*, 2001; Kaufmann and Thiel, 2002). Egr-1 activity has also been implicated in the development of several human cancer types, in particular, in human prostate cancer (Eid *et al.*, 1998). Moreover, tumor progression in transgenic mouse models of prostate cancer is impaired when Egr-1 is lacking, and Egr-1 also appears to contribute to mouse skin carcinogenesis (Abdulkadir *et al.*, 2001; Riggs *et al.*, 2000).

Interestingly, public accessible database "Oncomine" analysis of gene expression data sets from patients' specimen has revealed a significant correlation between Snail, Egr-1, E-cadherin, claudins and c-Met and/or HGF/SF in glioblastoma multiforme (GBM), a highly invasive tumor, and in clear cell renal carcinoma (Grotegut *et al.*, 2006). Consistent with these observations, *in vivo* inhibition of the c-Met pathway has resulted in a decrease of glioblastoma growth by 60% (Brockmann *et al.*, 2003).

5.4 Conclusions

Although it is known that the majority of early-stage tumors are not life threatening, a small number of primary tumor cells will progress to metastatic tumors, which give rise to devastating and largely incurable disease. Tumor metastasis is comprised of multiple steps and thus tumor cells are required to express a variety of properties including altered adhesiveness, increased motility and invasive capacity to complete the

metastatic process (Hanahan and Weinberg, 2000). Consistent with previous studies demonstrating that HGF/SF is a potent promoter of the angiogenic and metastatic functions of tumor cells, this work shows that HGF/SF significantly enhanced cell survival, cell migration, and invasion capacity, this significantly contributes to a better understanding of the molecular mechanisms underlying the progression to tumor malignancy.

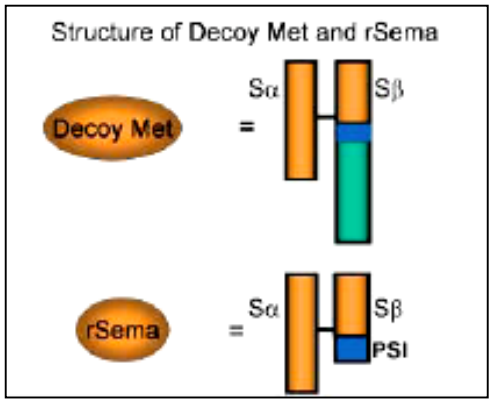

Figure 48: **Therapeutic strategies targeting the c-Met receptor.**
Soluble c-Met consisting of the entire extracellular domain (decoy Met) or recombinant Sema domain (rSema) inhibit HGF/SF binding and c-Met dimerization. Ligand-independent dimerization of c-Met is also blocked. PSI, plexin, semaphorin, and integrin domain (Zhang *et al*., 2004).

The work described here and that of others indicate that multiple pathways might be involved in the modulation of HGF/SF in tumor metastasis. First, it was found that HGF/SF caused higher resistance to chemotherapeutic agents and starvation. Second, HGF/SF-induced disturbance of tissue integrity through alterations of cell-cell and cell-matrix adhesion is a key event necessary for the progression of a variety of human carcinoma cells and the acquisition of an invasive phenotype. Third, HGF/SF-promoted cell motility is accompanied by activation of matrix metalloproteases (MMPs) and basement membrane breakdown. The functions of a family of structurally related MMPs and their inhibitors in tumor metastasis include not only a direct role in tumor invasion by facilitating extracellular matrix degradation, but also an important role in maintaining the tumor microenvironment, and thus promoting tumor progression (Curran and Murray, 1999). Fourth, HGF/SF induces angiogenesis at sites of metastasis, thereby promoting nutrient and oxygen supply essential for the tumor cells for colonizing new organs. However, further studies will be necessary to provide a comprehensive understanding of the role of HGF/SF in tumor progression and in metastatic processes.

c-Met inhibitor research stands at the doorstep of the oncology clinic. Neutralizing antibodies and the use of the HGF/SF antagonist NK4 to prevent ligand access to c-Met have successfully reversed cancer-associated phenotypes *in vivo* (Christensen *et al.*, 2005). New therapeutic strategies targeting the extracellular domain of c-Met such as soluble c-Met consisting of the entire extracellular region, called decoy Met, or the recombinant Sema domain that can inhibit ligand-dependent and -independent receptor

dimerization look highly promising (Figure 48)(Kong-Beltran *et al.*, 2004; Michieli *et al.*, 2004). Because c-Met is inappropriately expressed in a large number of human cancers (see Table 2), the HGF/c-Met signaling pathway should be an exceptional target for anticancer strategies and therapies (Corso *et al.*, 2005; Jiang *et al.*, 2005). These will eventually be used routinely for the benefit of cancer patients.

6 References

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7 Abbreviations

β-TrCP	β-transducin-repeat-containing protein
ACE	Angiotensin converting enzyme
APAF	Apoptotic peptidase activating factor
APC	Adenomatous polyposis coli
ARE elements	Adenosine- and uridine-rich elements
bHLH	Basic helix-loop-helix
bp	Basepair
c-fos	Cellular homologue to v-fos (FBJ murine osteosarcoma viral oncogene)
ChIP	Chromatin immunoprecipitation
CHO	Chinese hamster ovarian
c-jun	Cellular homologue to v-jun (avian sarcoma virus 17 oncogene)
CK	Casein kinase
CPT	Camptothecin
Crk	Chicken tumor virus no. 10 [CT10] regulator of kinase
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DOC	Sodium deoxycholate
DPM	Decays per minute
DTT	Dithiotreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamintetraacetate
EGF	Epidermal growth factor
Egr-1	Early growth response factor 1
EGTA	Ethylene glycol-bis(2-aminoethyl)-N, N, N', N'-tetraacetic acid
eIF4E	Eukaryontic translation initiation factor 4E
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase

EST	Expressed sequence tag
FAK	Focal adhesion kinase
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
FS-Fb-c Me	Human foreskin fibroblasts-conditioned medium
Gab1	Grb2-associated binder-1
GAS	Interferon-γ-activated sequence/ site
GEF	Guanine nucleotide exchange factor
Grb2	Growth factor receptor binding protein 2
GSK-3β	Glycogen synthase kinase 3β
GTP	Guanosin triphosphate
HB	Hepatoblastoma
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HEK293	Human embryonal kidney 293 cells
HEPES	N-(2-Hydroxyethyl)-piperazin-N'-2-ethansulfonic acid
HGF/SF	Hepatocyte growth factor/scatter factor
hRPL	Human ribosomal protein L19
IFN	Interferon
IL	Interleukin
ILK	Integrin-linked kinase
$InsP_3$	Inositol-1,4,5-triphosphate
IRES	Internal ribosome entry site
IRF9	Interferon regulatory factor 9
ISGF3	Interferon-stimulated gene factor 3
Jak	Just another kinase
Lef	Lymphoid enhancer factor-1
LOH	Loss of heterozygosity
LOI	Loss of imprinting
LOXL	Lysyl oxydase-like protein
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney

MDR-1	multidrug resistance gene
MEK	MAPK kinase
MMP	Matrix metalloproteinase
MPT	Mitochondrial permeability transition
MTA3	Metastasis-associated protein-3
mTOR	Mammilian target of rapamycin
Na_3VO_4	Sodium orthovanadate
Nab	NGF1A-binding protein
NaF	Sodium fluoride
NCAM	Neural cell adhesion molecule
$p130^{CAS}$	p130Crk-associated substrate
PAGE	polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PAK1	p21-activated kinase-1
PBS	Phosphate buffered saline
PD98059	2'-amino-3'-methoxyflavone
PDGF	Platelet-derived growth factor
PH	pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenyl methyl sulfonyl fluoride
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
qRT-PCR	Quantitative real time detection reverse transcription polymerase chain reaction
Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-ras (rat sarcoma viral oncogene)
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase

SCF	Stem cell factor
SDS	Sodiumdodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SH2	Src homology 2
Shp2	SH2 domain containing tyrosine phosphatase
shRNA	Small hairpin RNA
SIE	Serum-inducible element
SIP-1	Smad-interacting protein-1
SOS	Son of sevenless
Src	Homologue to v-src (sarcoma viral oncogene)
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TCF	T cell factor
TEMED	N, N, N', N'-Tetramethylethylenediamine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TKB	Tyrosine kinase binding
Tris	Tris(hydroxymethyl)aminomethan
Tween 20	Polyoxyethylensorbitanmonolaureate
VEGF	Vascular endothelial growth factor
Wn	Wortmannin
ZO-1	Zonula occludens (ZO)-1

8 Curriculum Vitae

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9 Publications

Grotegut S, von Schweinitz D, Christofori G, Lehembre F: “Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail.” The EMBO Journal 2006, 25, p. 3534 – 3545

Hoefer MM, Boneberg EM, Grotegut S, Kusch J, Illges H: “Possible tetramerization of the proteasome maturation factor POMP/proteassemblin/hUmp1 and its subcellular localization.” International Journal of Biological Macromolecules 2006, 38(3-5), p. 259 - 267

von Schweinitz D, Koch A, Grotegut S, Warmann S, Haeberle B, Bergmann F, Till H, Pietsch T: “Molekulargenetische und funktionelle Tumorzellveränderungen beim Hepatoblastom.” Monatsschrift Kinderheilkunde 2005, Volume 153, p. 1031 (abstract in german)

Grotegut S, Fasler-Kan E, Christofori G, von Schweinitz D: “HGF/SF downstream signaling in human hepatoblastoma and hepatocellular carcinoma cells.” Klinische Paediatrie 2004, Volume 216(3), p. 203 – 204

Grotegut S, Hochuli A, von Schweinitz D, Fasler-Kan E: "Activation of the Jak-STAT pathway in human hepatoblastoma cell lines." Signal Transduction 2002, 3-4, p. 160

Grotegut S, Fasler-Kan E, Koehler E, Berschtin S, Woodtli T, von Schweinitz D: "Anti-apoptotic effect of HGF/SF on hepatoblastoma and hepatocellular carcinoma cells." Klinische Paediatrie 2002, Volume 214(4), p. 261

von Schweinitz D, Koehler E, Grotegut S, Schnater M, Pietsch T: "Hepatocyte growth factor may promote postoperative growth of metastases in childhood hepatoblastoma", European Surgery ACA 2002, Vol. 34, Suppl. 181, p. 9

Talks/ Poster Presentations

Grotegut S, Fasler-Kan E, Christofori G, von Schweinitz D: "HGF/SF downstream signaling in human hepatoblastoma and hepatocellular carcinoma cells", 17th Meeting of the Kind-Philipp-Stiftung 2004, Wilsede, Germany

Grotegut S, Christofori G, von Schweinitz D: "The role of hepatocyte growth factor on hepatoblastoma progression." EMBL/Salk/EMBO Conference Oncogenes & Growth Control 2004, Heidelberg, Germany (poster)

Grotegut S, Fasler-Kan E, Hochuli A, von Schweinitz D: "Hepatocyte growth factor/scatter factor (HGF/SF): A key player for the invasiveness of hepatoblastoma and hepatocellular carcinoma cells?" 4th Symposium of the Upper Rhine University children's hospitals 2003, Freiburg, Germany

Grotegut S, Fasler-Kan E, von Schweinitz D: "The role of hepatocyte growth factor/scatter factor (HGF/SF) on hepatoblastoma cells." 2nd SPO Symposium 2002, Castelen, Switzerland

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Grotegut S, Koehler E, von Schweinitz D: "Anti-apoptotic effect of HGF/SF on hepatoblastoma and hepatocellular carcinoma cells" 15th Meeting of the Kind-Philipp-Stiftung 2002, Wilsede, Germany

Prizes/ Awards

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