Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail

Stefan Grotegut¹, Dietrich von Schweinitz², Gerhard Christofori^{1,*} and François Lehenbre¹

¹Department of Clinical-Biological Sciences, Institute of Biochemistry and Genetics, Center of Biomedicine, University of Basel, Basel, Switzerland and ²Department of Pediatric Surgery, Ludwig-Maximilians University, Munich, Germany

Hepatocyte growth factor/scatter factor (HGF) exerts several functions in physiological and pathological processes, among them the induction of epithelial cell scattering and motility. Its pivotal role in angiogenesis, tumor progression, and metastasis is evident; however, the underlying molecular mechanisms are still poorly understood. Here, we demonstrate that HGF induces scattering of epithelial cells by upregulating the expression of Snail, a transcriptional repressor involved in epithelial-mesenchymal transition (EMT). Snail is required for HGF-induced cell scattering, since shRNA-mediated ablation of Snail expression prevents this process. HGF-induced upregulation of Snail transcription involves activation of the mitogen-activated protein kinase (MAPK) pathway and requires the activity of early growth response factor-1 (Egr-1). Upon induction by Egr-1, Snail represses the expression of E-cadherin and claudin-3 genes. It also binds to the Egr-1 promoter and represses Egr-1 transcription, thereby establishing a negative regulatory feedback loop. These findings indicate that Snail upregulation by HGF is mediated via the MAPK/Egr-1 signaling pathway and that both Snail and Egr-1 play a critical role in HGF-induced cell scattering, migration, and invasion.

The EMBO Journal (2006) 25, 3534–3545. doi:10.1038/sj.emboj.7601213; Published online 13 July 2006

Subject Categories: signal transduction; molecular biology of disease

Keywords: cancer; c-Met; E-cadherin; Egr-1; Snail

Introduction

Hepatocyte growth factor/scatter factor (HGF) is a mesenchyme-derived multifunctional molecule that elicits mitogenic and morphogenic activities in developmental as well as in many pathophysiological processes (Weidner *et al*, 1993a; Jeffers *et al*, 1996). Its receptor has been identified as the

c-Met tyrosine kinase, a product of the proto-oncogene *c-met* (Bottaro *et al*, 1991; Naldini *et al*, 1991). Upon HGF binding, c-Met undergoes autophosphorylation on several tyrosine residues and constitutes a unique multisubstrate docking site for SH2-containing adaptor proteins, including Grb2 (growth-factor-receptor-bound protein 2) and Gab1 (Grb2-associated binder 1) (Ponzetto *et al*, 1994; Furge *et al*, 2000). Recruitment of these molecules results in the activation of protein tyrosine phosphatase 2 (SHP2), Src tyrosine kinase, and several downstream signaling cascades, such as 'mitogen-activated protein kinase' (MAPK), 'phosphatidylinositol 3'-kinase' (PI3K), and 'Janus kinase-signal transducer and activator of transcription' (Jak/STAT) pathways (Birchmeier *et al*, 2003; Jiang *et al*, 2005). All these pathways have been shown to be essential for HGF-induced cellular changes, including proliferation, migration, invasion, and anchorage-independent growth during branching morphogenesis (Boccaccio *et al*, 1998; Birchmeier *et al*, 2003).

A large body of experimental evidence has demonstrated that HGF and c-Met play a critical role in the invasive growth of tumor cells, a hallmark of metastatic cancers (Comoglio and Trusolino, 2002). Malignant tumor progression involves multiple steps, including the loss of cell-cell adhesion, cell migration, invasion through the basement membrane into connective tissue, survival in the bloodstream, and colonization of distant tissues (Thiery, 2002). HGF-induced signaling could contribute to these steps through its pleiotropic effects on cell scattering, production of (metallo-)proteinases, induction of anti-apoptotic signals, and induction of cellular proliferation and angiogenesis (Weidner *et al*, 1993b; Vande Woude *et al*, 1999). Accordingly, high levels of HGF or overexpression of c-Met or both are frequently found in many cancer types and often correlate with poor prognosis (for a comprehensive list, see www.vai.org/vari/metandcancer; Birchmeier *et al*, 2003).

Loss of cell-cell adhesion followed by the dissociation of epithelial structures is a prerequisite for increased cell motility and tumor invasion. Accordingly, E-cadherin-mediated cell-cell adhesion is frequently lost during malignant tumor progression by gene mutation, transcriptional repression, or protein degradation (Gumbiner, 2000; Thiery, 2002; Cavallaro and Christofori, 2004). Recently, it has been shown that downregulation of E-cadherin expression is mediated by the zinc-finger transcription factor Snail (reviewed in Nieto, 2002; Huber *et al*, 2005). By binding to specific DNA sequences in the E-cadherin promoter, named E-boxes, Snail represses E-cadherin transcription, leading to the disruption of adherens junctions (Battle *et al*, 2000; Cano *et al*, 2000). Snail-deficient mouse embryos die during gastrulation unable to undergo epithelial-mesenchymal transition (EMT; Carver *et al*, 2001). Notably, Snail appears to contribute to the development of malignant carcinomas,

*Corresponding author. Department of Clinical-Biological Sciences, Institute of Biochemistry and Genetics, Center of Biomedicine, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland. Tel.: +41 61 267 3562; Fax: +41 61 267 3566; E-mail: Gerhard.Christofori@unibas.ch

Received: 8 February 2006; accepted: 6 June 2006; published online: 13 July 2006

where loss of E-cadherin expression is frequently observed (Cano *et al*, 2000; Sugimachi *et al*, 2003).

Snail protein function and gene expression are regulated by various mechanisms. For example, Snail expression in epithelial cells can be induced by TGF β , oncogenic Ras, or activated integrin-linked kinase signaling (Tan *et al*, 2001; Gotzmann *et al*, 2002; Peinado *et al*, 2003). Moreover, Snail's subcellular localization and its protein levels as well as its repressor functions are regulated by the activity of p21-activated kinase 1 (Yang *et al*, 2005) and glycogen synthase kinase-3 β (GSK-3 β) (Zhou *et al*, 2004; Bachelder *et al*, 2005). Active GSK-3 β counteracts Snail's transcriptional repressor activity by promoting nuclear export and proteasomal degradation of Snail. GSK-3 β -mediated phosphorylation of Snail's consensus site II promotes nuclear export, and subsequent phosphorylation of site I by cytoplasmic GSK-3 β . This reveals a destruction box recognized by β -TrCP and leads to Snail ubiquitylation and proteasomal degradation (Zhou *et al*, 2004). Interestingly, two members of the lysyl-oxidase gene family (LOXL-2 and -3) stabilize Snail protein by attenuating the GSK-3 β -dependent phosphorylation, thus leading to a cooperative downregulation of E-cadherin (Peinado *et al*, 2005).

Since HGF is well known for its ability to induce cell motility in various epithelial cell types, where loss of tight and adherens junctions is a requirement, we investigated the possible link between HGF-induced cell scattering, Snail function, and junctional protein expression. Here, we demonstrate that Snail plays a critical role in HGF-mediated downregulation of E-cadherin and claudin-3 expression, thereby promoting cell scattering, migration, and invasion. HGF activates the MAPK signaling pathway to employ the early growth response-1 (Egr-1) transcription factor for the induction of Snail gene expression. In turn, Snail represses its target genes, among them E-cadherin and claudin-3 but also Egr-1, thus mediating a negative feedback loop. These results provide new insight into the factors and molecular pathways underlying HGF-induced cell scattering and tumor progression.

Results

The Snail gene is a direct target of HGF-c-Met signaling

HGF induces cell scattering in a number of epithelial cells and epithelial cancer cell lines, including HepG2 cells (von Schweinitz *et al*, 2000). However, the mechanisms by which HGF induces scattering, and whether Snail plays a critical role in this process, have remained elusive.

Snail is an extremely labile protein with a half-life of only 25 min, and thus difficult to detect by conventional immunoblotting methods. We employed the GSK-3 β inhibitor lithium chloride (LiCl) and the proteasome inhibitor MG132 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 1A), whereas treatment with MG132 or LiCl or both combined led to a slight stabilization of the protein. Notably, when HGF was added to inhibitor-treated cells, Snail protein levels were markedly elevated. In contrast, treatment of cells with HGF in the absence of inhibitors did not result in increased Snail protein levels. These experiments indicate that HGF is able to induce the expression of Snail in HepG2

cells, but that increased Snail protein levels are only detectable by preventing proteasomal degradation.

We next determined whether HGF directly affected Snail gene expression. HepG2 cells were treated with HGF in the presence or absence of LiCl and MG132 for 8 h. Quantitative RT-PCR (qRT-PCR) analysis revealed that Snail mRNA levels were approximately 8-fold (± 3.1) upregulated in HepG2 cells treated only with HGF (Figure 1B). Interestingly, when HepG2 cells were preincubated with proteasome and GSK-3 β inhibitors, Snail mRNA levels increased up to 40-fold (± 1.4) and co-incubation with inhibitors and HGF elevated Snail mRNA levels up to 50-fold (± 1.8). These results suggest that a proteolytically sensitive protein, possibly Snail itself, cooperated with HGF to induce Snail gene transcription and/or to stabilize its mRNA.

Similar experiments in MDCK cells, which are known to readily respond to HGF treatment by cell scattering (Weidner *et al*, 1993b), revealed comparable results: HGF by itself moderately increased Snail mRNA expression (2.2-fold ± 0.8 ; Figure 1C). HGF also significantly increased Snail mRNA levels in the presence of LiCl and MG132. Thus, HGF treatment of epithelial cells induced Snail expression not only at the protein but also at the mRNA level. qRT-PCR analysis revealed that HGF treatment did not affect Twist and Slug expression (data not shown), suggesting that Snail was the most prominent E-cadherin transcriptional repressor activated by HGF in HepG2 and MDCK cells.

We next asked whether the Snail gene is a direct downstream target of the c-Met pathway. HEK293 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 treatment, Snail promoter activity was found elevated by 1.8-fold (± 0.09) (Figure 1D). These results indicate that HGF 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.

Next, we investigated whether HGF-induced upregulation of Snail had an effect on E-cadherin expression. HepG2 cells were treated with HGF alone or in combination with MG132 and LiCl and analyzed for E-cadherin mRNA levels by qRT-PCR. When cells were stimulated with HGF alone, a 20% decrease of E-cadherin mRNA was apparent (Figure 1E). Cells treated with the proteasome inhibitor MG132 and the GSK-3 β inhibitor LiCl showed a 25% decrease in E-cadherin mRNA levels. Combined treatment of HepG2 cells with HGF and inhibitors resulted in a reduction in E-cadherin mRNA levels by 70% as compared to untreated cells.

HGF-induced Snail stabilization is independent of GSK-3 β activity

Previously, it has been reported that GSK-3 β activity could be inhibited by HGF signaling (Papkoff and Aikawa, 1998), whereas GSK-3 β -mediated phosphorylation of Snail leads to its nuclear export and degradation by the proteasome (Zhou *et al*, 2004; Bachelder *et al*, 2005). We therefore assessed whether GSK-3 β is involved in HGF-induced Snail upregulation. Immunoblot analysis revealed an increase in phosphorylation of GSK-3 β in HepG2 cells and MDCK cells after treatment with LiCl compared to untreated cells

Figure 1 HGF upregulates Snail mRNA and protein levels and downregulates E-cadherin expression. (A) HepG2 cells were incubated with 10 ng/ml HGF for 8 h in the presence or absence of GSK-3 β inhibitor LiCl (40 mM) and proteasome inhibitor MG132 (10 μ M). Cell lysates were subjected to immunoblotting using an anti-Snail antibody. Equal loading of proteins was confirmed by Ponceau S staining. (B) HepG2 and (C) MDCK cells were incubated with HGF 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. (D) A Snail-promoter luciferase-reporter construct was transiently transfected into HEK293 cells. To normalize transfection efficiency, cells were cotransfected with a *Renilla* luciferase reporter construct. Cells were incubated with HGF for 24 h, before luciferase activities were determined. RLU represent firefly luciferase (LAR) normalized against *Renilla* luciferase activity (RAR). Values given represent means and standard deviations of triplicate measurements. * $P=0.0031$; unpaired t -test. (E) HepG2 cells were treated with HGF in the presence or absence of MG132 and LiCl. E-cadherin mRNA levels were quantified by qRT-PCR. Values shown are the averages and standard deviations of triplicate measurements. * $P=0.0204$, ** $P=0.0003$, *** $P=0.0059$; unpaired t -test.

(Figure 2A and B). However, HGF treatment did not lead to further GSK-3 β phosphorylation, suggesting that GSK-3 β activity was not involved in HGF-mediated Snail expression.

To evaluate whether HGF-mediated signaling could stabilize Snail protein independent of GSK-3 β activity, HEK293 cells were transiently transfected with Flag-tagged Snail-8SA, a mutated version of Snail that cannot be phosphorylated by GSK-3 β (Zhou *et al*, 2004). Immunoblot analysis with an anti-FLAG antibody revealed that HGF treated cells exhibited higher levels of FLAG-tagged Snail-8SA, indicating that Snail protein was stabilized upon HGF treatment independently of GSK-3 β -mediated phosphorylation of Snail (Figure 2C).

Snail is required for HGF-induced cell scattering

To assess whether Snail upregulation was required for HGF-induced cell scattering, we generated stably transfected HepG2 cell lines expressing small hairpin RNA (shSnail) to ablate Snail expression. 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. Clones S-1 and S-3 showed up to 80% downregulation of Snail mRNA levels, whereas control clones did not exhibit any significant reduction in Snail mRNA (Figure 3A). Immunoblotting analysis also revealed a decrease in Snail protein levels in shSnail-expressing clones S-1 and S-3 compared to control shRNA-expressing clones (Figure 3B).

Figure 2 HGF induces Snail expression independently of GSK-3β activity. (A) HepG2 and (B) MDCK cells were preincubated with LiCl and MG132 for 30 min and then stimulated with HGF for further 30 min. Whole-cell lysates were analyzed by immunoblotting with specific antibodies against phosphorylated (p)-GSK-3β and total (t)-GSK-3β. (C) HEK293 cells were transiently transfected with a plasmid encoding Flag-tagged Snail-8SA, and 24 h later stimulated with HGF. After an additional 24 h, cells were analyzed by immunoblotting with anti-Flag-tag antibodies. Immunoblotting for β-actin was used as loading control.

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 start of HGF treatment. Control shRNA clones showed normal scattering behavior as observed with untransfected HepG2 cells (Figure 3C and D). In contrast, shSnail-expressing clones S-1 and S-3 exhibited a discernible reduction in HGF-induced cell scattering, indicating that Snail function is required for this process (Figure 3E–H). HGF-induced Snail protein levels and the extent of cell scattering also correlated in the hepatoblastoma cell lines HepT1 and HuH6 (Supplementary Figure 1). Noteworthy, forced expression of Snail by itself was not sufficient to induce a scattering phenotype in the absence of HGF, as, for example, tested in HEK293 expressing Snail-8SA and in HepG2 cells transiently transfected with Snail-8SA (data not shown). In conclusion, while Snail appears to be required for HGF-induced cell scattering, it is not sufficient to replace HGF's scattering activity.

HGF-induced Snail expression depends on the MAPK pathway

Binding of HGF to c-Met results in the activation of several downstream signaling pathways. To identify the pathways involved in Snail upregulation, various c-Met effector path-

ways were repressed by the use of specific inhibitors. The MAPK pathway inhibitor PD98059 completely blocked HGF-induced Snail expression, while the PI3K inhibitor wortmannin (Wn) had no effect on Snail mRNA expression in HepG2 cells (Figure 4A). These results indicate that the MAPK pathway plays a critical role in HGF-induced Snail expression, whereas PI3K is not involved. This notion was further confirmed by cell scattering assays. HepG2 cells that were incubated with both HGF and Wn were still able to scatter, whereas PD98059 completely repressed HGF-mediated cell scattering without affecting cell morphology or other cellular parameters (Figure 4B, data not shown). Together, these results demonstrate that HGF induces cell scattering and increased motility by utilizing the MAPK signal transduction pathway and not the PI3K pathway.

HGF activates Egr-1 to induce Snail expression

To further elucidate the molecular mechanisms by which HGF-stimulated MAPK signaling induced Snail expression, we investigated the potential involvement of candidate transcription factors. Several recent studies have shown that HGF 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 manner through early growth response factor-1 (Egr-1). Egr-1 is a nuclear, zinc-finger transcription factor capable of binding to specific GC-rich DNA sequences containing the consensus binding site GCG(G/T)GGGCG (Sukhatme *et al*, 1988). We investigated the potential role of Egr-1 in the regulation of HGF-mediated Snail gene expression. A survey of the Snail promoter sequence using MatInspector software (Cartharius *et al*, 2005) identified four putative Egr-1 binding sites between –450 and –50 upstream of the Snail gene transcriptional start site (Figure 5A).

We next assessed whether Egr-1 was expressed in HepG2 cells and whether its expression was affected by HGF. Analysis by qRT-PCR revealed a 12-fold (± 4.7) transient increase in Egr-1 mRNA levels by HGF, with maximum levels after 1 h and a decline to basal levels within 3 h of HGF stimulation (Figure 5B). Immunoblotting analysis showed a slightly delayed kinetics of the upregulation of Egr-1 protein levels, which peaked after 2–3 h and declined afterwards (Figure 5C). Snail mRNA levels steadily increased beginning at 2 h, right after the first appearance of Egr-1 protein (Figure 5B), and increased Snail protein levels could be detected after 5 h, yet only in the presence of proteasome inhibitors (Figure 5C). The early increase in Snail expression already correlated with the downregulation of Snail target genes, such as E-cadherin or claudin-3 during HGF treatment, with a first decline in mRNA levels after 3 h and further reduction during 8 h of HGF incubation time (Figure 5D). These results suggest an order of events in HGF-induced Snail expression: HGF-mediated MAPK activation induces the expression of Egr-1, which in turn may activate Snail gene expression and the subsequent repression of E-cadherin and claudin-3 gene expression.

The Snail gene is a target of Egr-1

To investigate a direct involvement of Egr-1 in Snail expression, HepG2 cells were transfected with a plasmid encoding a dominant-negative version of Egr-1 (dnEgr-1; Al-Sarraj *et al*, 2005), and HGF-induced Snail expression was monitored

Figure 3 Snail is required for HGF-induced scattering. (A) 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'-UTR of Snail, respectively. Values are the averages and standard deviations of triplicate measurements. * $P < 0.03$, ** $P < 0.01$; unpaired *t*-test. (B) HepG2 clones expressing shSnail or a control shRNA were incubated with LiCl, MG132, and HGF for 8 h. Snail and β-actin protein levels were analyzed by immunoblotting. (C–H) shSnail-expressing (S-1, S-3) and control shRNA-expressing (C-2) HepG2 clones were incubated with or without HGF. Cell scattering was observed by light microscopy after 72 h of HGF treatment (magnification: $\times 200$).

Figure 4 HGF-induced scattering requires MAPK activity. **(A)** HepG2 cells were incubated with HGF for 8 h in the presence or absence of the MEK inhibitor PD98059 (20 μ M) or the PI3K inhibitor Wn (1 μ M). Human Snail mRNA levels were determined by qRT-PCR. Values given represent the means and standard deviations of triplicate measurements. $*P < 0.0001$; unpaired *t*-test. **(B)** HepG2 cells were preincubated with or without PD98059 or Wn. After 1 h, HGF was added, and cell scattering was observed over a period of 72 h by light microscopy (magnification: $\times 200$).

(Figure 6A). In these experiments, the presence of dnEgr-1 completely abolished HGF-induced Snail expression, indicating that Egr-1 is required for HGF-mediated Snail expression.

It has been previously reported that Egr-1 induces the expression of its co-repressor Nab2 (Houston *et al*, 2001), thereby establishing a negative feedback loop and preventing a direct analysis of the overexpression of wild-type Egr-1 (Kumbrink *et al*, 2005). To test whether Egr-1 is sufficient to induce Snail expression, we transfected HEK293 cells with a plasmid encoding the Egr-1 DNA binding domain fused to the CREB-2 activation domain (nabR-Egr-1), a construct that is insensitive to Nab2's repressive activity (Al-Sarraj *et al*, 2005). Luciferase reporter assays revealed a dose-dependent activation of the Snail promoter by nabR-Egr-1, indicating that Egr-1 is sufficient for activating Snail gene expression (Figure 6B).

We next sought to determine whether Egr-1 could directly bind to the Snail promoter by chromatin immunoprecipita-

tion (ChIP) experiments with anti-Egr-1 antibodies followed by a PCR with primers specific for the Snail promoter. Egr-1 specifically bound to the Snail promoter 1 h after HGF stimulation, while no binding occurred in the absence of HGF (Figure 6C). 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 6D). Altogether, these results indicate that the Snail promoter is a direct target of Egr-1 upon HGF stimulation.

Kinetics of Snail expression

To elucidate the long-term kinetics of HGF-mediated gain of Snail expression, and loss of adherens and tight junction functions, HepG2 cells were treated with HGF for 10 days. To avoid cell death by prolonged exposure, the proteasome inhibitor MG132 and the GSK-3 β inhibitor LiCl were added 8 h prior to cell lysis for each time point. Analysis of Snail mRNA expression revealed that HGF-induced Snail

Figure 5 Egr-1 expression precedes HGF-induced Snail expression. (A) Putative Egr-1 binding sites within 650bp 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, 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 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. (D) Kinetics of HGF-mediated downregulation of E-cadherin and claudin-3 mRNA levels in HepG2 cells in the presence of MG132 and LiCl. Values represent means of triplicate measurement ± standard deviations in comparison to untreated control samples.

expression reached its maximum 8 h after HGF stimulation and declined with longer treatment (Figure 7A). Immunoblot analysis showed that HGF-induced Snail protein expression followed a similar kinetic (Figure 7B). As expected, with increased Snail expression, E-cadherin (Figure 7C) and claudin-3 (data not shown) mRNA levels declined, yet remained low at time points where Snail expression had already returned to basal levels.

The observed kinetics in Snail and Egr-1 expression suggest a feedback loop mechanism, where Snail would repress its own activation. Interestingly, 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

Figure 6 Snail is an Egr-1 target gene. (A) 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 for 8 h, before Snail mRNA levels were measured by qRT-PCR. ****P* = 0.0018; unpaired *t*-test. (B) 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 (nabR-Egr-1), 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 for 24 h, before luciferase activities were determined. RLU represent firefly luciferase (LAR) normalized against *Renilla* luciferase activity (RAR). Values are means of induction ± standard deviations of triplicate measurement in comparison to untreated control samples. **P* = 0.0145, ***P* < 0.001; unpaired *t*-test. (C) ChIP analysis of Egr-1 binding to the Snail promoter. HEK293 cells were stimulated with HGF for 1 h before being subjected to ChIP. 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. (D) Quantitative PCR analysis of the Snail promoter region immunoprecipitated with antibodies against Egr-1, as described in (C). Results indicate amplified Snail promoter levels normalized against PCR reactions detecting an unrelated promoter (NCAM). Bar graphs represent the means and standard deviations. **P* = 0.0432; unpaired *t*-test.

Figure 7 Kinetics of HGF-mediated Snail expression. (A) HepG2 cells were treated with HGF 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 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. (C) HepG2 cells were incubated with HGF for the time points indicated, and E-cadherin mRNA levels were analyzed by qRT-PCR. Values represent the means and s.d. of triplicate measurements.

sites for Snail (Figure 8A). To test whether overexpression of Snail inhibited HGF-induced Egr-1 activation, we generated HEK293 cells expressing Snail under the control of the tetracycline-inducible system (HEK293-FlpInTRex-Snail-8SA; Supplementary Figure 2). These cells were cultured with and without doxycycline before stimulation with HGF. Analysis of Egr-1 mRNA expression by qRT-PCR was then performed. Indeed, when Snail expression was induced, the amounts of Egr-1 mRNA declined in the presence and even in the absence of HGF (Figure 8B). 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 8C). These results suggest that Snail directly represses Egr-1 expression, thus exerting a negative feedback regulation on its own activator.

We next examined whether upregulated expression of Snail, Egr-1 and HGF, and c-Met also correlated in human cancer biopsies concomitantly with reduced expression of E-cadherin and claudins. Computer-assisted analysis of 30 data sets of specific human cancer types using the public database 'Oncomine' (www.oncomine.org) revealed a significant correlation between the expression of these genes

Figure 8 Egr-1 is downregulated by Snail via a negative feedback loop. (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 $\pm$ s.d. of triplicate measurement in comparison to untreated control samples. * $P=0.007$, ** $P=0.0021$; unpaired t -test. (C) ChIP analysis of Snail binding to the Egr-1 promoter. HepG2 cells were incubated with HGF for 1 h before proteins were crosslinked 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.

in glioblastoma multiforme and in clear cell renal carcinoma (Supplementary Figure 3). These results indicate that the HGF/MAPK/Egr-1/Snail axis may also be functional in inducing progression to tumor malignancy in patients.

Discussion

To infiltrate the surrounding tissues, single motile tumor cells leave the tumor mass by breaking cell-cell contacts, known as tight and adherens junctions (Friedl and Wolf, 2003). Snail family proteins have emerged as major transcriptional repressors of E-cadherin expression, the prototype mediator of epithelial adherens junctions. Here, we have used three independent cellular systems to analyze the mechanisms

underlying HGF-mediated cell scattering and migration. We demonstrate that HGF requires Snail to repress E-cadherin expression and to induce cell scattering. Furthermore, we have unraveled major parts of the HGF-mediated signaling pathway leading to Snail expression and thus to cell scattering, migration, and invasion.

HGF induces 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 is observed upon HGF treatment. A similar correlation has been previously reported in hepatocellular carcinomas (HCC) and in colon epithelial cells, where Snail expression and E-cadherin downregulation is associated with higher invasiveness (Sugimachi *et al*, 2003; Boon *et al*, 2005). In contrast, recent experiments with MDCK cells have demonstrated that the early events of HGF-induced scattering do not involve a loss of E-cadherin protein and that a disruption of junctions by traction forces allows cell scattering and migration to occur (de Rooij *et al*, 2005). Yet, the Snail shRNA knockdown experiments presented here clearly indicate that Snail is required for HGF-mediated cell scattering. Hence, HGF-induced Snail expression leads to repression of E-cadherin function. 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).

Employing a combination of specific inhibitors and biochemical analyses, we have found that HGF-induced Snail upregulation and cell scattering depend on the MAPK pathway but not on the PI3K or GSK-3 β pathways, consistent with previous reports demonstrating that the MAPK pathway is required for HGF-induced scattering of various epithelial cell types (Tanimura *et al*, 1998; Maina *et al*, 2001; Janda *et al*, 2002; Abella *et al*, 2005). 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-induced Snail expression. Unfortunately, forced expression of wild-type 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 (Houston *et al*, 2001; Kumbrink *et al*, 2005). Circumventing this problem, expression of a mutant Egr-1, which is resistant to the Nab co-repressors (Al-Sarraj *et al*, 2005), has resulted in a dose-dependent upregulation of Snail promoter activity. Interestingly, our data suggest that upon induction by Egr-1, Snail binds to the Egr-1 promoter and represses its transcription and, recently, Snail has been also 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.

HGF has been shown to induce the expression of a number of pro-angiogenic factors by activating Egr-1 (Recio and Merlino, 2003; Day *et al*, 2004; 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 (Riggs *et al*, 2000; Abdulkadir *et al*, 2001). '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 in glioblastoma multiforme and in clear cell renal carcinoma. Consistent with this observations, *in vivo* inhibition of the c-Met pathway has resulted in a decrease of glioblastoma growth by 60% (Brockmann *et al*, 2003).

The results presented here show that HGF 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-mediated scattering involves in addition to Egr-1/Snail a hitherto unknown pathway that remains to be elucidated. Contrary to our result, Snail has been previously shown to induce EMT in other epithelial cell lines (Zhou *et al*, 2004; Bachelder *et al*, 2005). 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). However, analysis of HGF-treated HepG2 cells has not revealed any upregulation of mesenchymal markers (data not shown), suggesting that Snail-dependant scattering is a distinct mechanism from Snail-induced EMT. Based on our and others' findings, it is conceivable that Snail plays a critical role in the early stages of cell scattering and EMT, by making tumor cells receptive for further EMT stimuli and setting the stage for invasive tumor progression (Huber *et al*, 2005).

Also highlighting their importance in tumor progression, HGF and c-Met have been recently proposed as potential targets for chemotherapeutic intervention (Christensen *et al*, 2005; Corso *et al*, 2005). We describe here a novel mechanism by which HGF induces expression of the transcriptional repressor Snail and, with it, cell scattering, migration and invasion: stimulation of cells with HGF 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 9). Although further studies are warranted to characterize the molecular details of HGF-mediated signal transduction and Egr-1 function, the results reported here contribute to a better understanding of the molecular mechanisms underlying the progression to tumor malignancy.

Materials and methods

Cells lines

HepG2, HepT1, and HuH6 cells were cultured in RPMI1640 medium supplemented with 2 mM glutamine and 10% FCS. MDCK cells were grown in MEM medium containing 10% FCS and 2 mM glutamine.

To generate HEK293 cells stably expressing Snail, human Snail-8SA cDNA (a gift from M-C Hung, University of Texas, Houston) was subcloned into pcDNA5/FRT/TO and transfected into HEK293-FlpInTRex. Stable clones were selected with hygromycin B and blasticidin. Snail-8SA-transfected HEK293 cells were kept in FlpInTRex Medium (DMEM medium supplemented with 10%

Figure 9 Molecular mechanisms underlying HGF-induced cell scattering. Binding of HGF 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-3 β provides a nuclear export signal by phosphorylating Snail on its phosphorylation consensus site II. Once in the cytoplasm, Snail is phosphorylated by GSK-3 β on consensus site I, leading to its ubiquitilation and proteasomal degradation, a process repressed by LiCl and MG132.

FCS, 2 mM glutamine, 15 μ g/ml blasticidin, 100 μ g/ml hygromycin B), and Snail expression was induced with 1 μ g/ml doxycycline.

To generate pSUPER-Snail-shRNA and pSUPER-Control-shRNA constructs, oligonucleotides encoding human Snail-specific and murine NCAM-specific shRNA, respectively (Supplementary Table I), were ligated into pSUPER-retro-puro (OligoEngine). HepG2 cells were transfected with Fugene (Roche Diagnostics) and kept under selection with 150 μ g/ml puromycin. HepG2 and HEK293 were transiently transfected with a plasmid encoding a murine dominant-negative (dn) Egr-1-GST fusion protein (pEBGN-Egr-1), empty vector (pEBGN; Al-Sarraj *et al*, 2005) or wild-type Egr-1 (pCMVzif; Thiel *et al*, 1994).

CD4⁺ HepG2 cells were generated by transient co-transfection of the eukaryotic vector pMACS 4.1, and subsequent magnetic separation on MACS CD4 MicroBeads was performed according to the manufacturer's instructions (Miltenyi Biotec).

Reagents and antibodies

Antibodies were anti-Snail (Abgent), anti- β -actin (Abcam), anti-E-cadherin (BD Transduction Laboratories), anti-FLAG (Stratagene), anti-phospho-GSK-3 β and anti-GSK-3 β (Cell Signaling Technology), and anti-Egr-1 (Santa Cruz Biotechnology). The following concen-

trations were used in all experiments: MG132 (z-Leu-Leu-Leu-CHO; Calbiochem): 10 μ M; LiCl: 40 mM; HGF (R&D Systems): 10 ng/ml.

Scattering assay

Cells were seeded in 12-well tissue culture dishes at a density of 1×10^5 per well. After 24 h, cells were incubated with HGF (5–20 ng/ml) or PBS. When inhibitors were used, cells were preincubated with inhibitors for 1 h before addition of HGF. After 72 h, phase-contrast pictures were taken.

Immunoblotting analysis

Cell lysate preparation and immunoblotting analysis were performed as described (Zhou *et al*, 2004; Bachelder *et al*, 2005). The immunoblots were visualized by chemiluminescence detection with ECL+ (Amersham Biosciences) or UptiLight (Interchim).

Quantitative real time RT-PCR

Total RNA was prepared using Trizol, and reverse transcribed with random hexamer primers using M-MLV reverse transcriptase RNase (H-) (Promega). Transcripts of various genes were detected by quantitative RT-PCR (qRT-PCR) on an ABI Prism 7000 TaqMan using SYBR green PCR MasterMix (both Applied Biosystems) with the primers listed in Supplementary Table I. Relative mRNA expression was calculated of the mean value with the comparative C_t method ($\Delta\Delta C_t$). Human ribosomal protein L19 (hRPL19) primers were used for normalization.

Reporter gene assays

HEK293 or HepG2 cells were transiently transfected with a mouse-Snail promoter firefly luciferase reporter construct (Peinado *et al*, 2003). For normalization, the pRenilla luciferase reporter construct (Promega) was co-transfected. Dual-Luciferase reporter assays were performed as directed by the manufacturer (Promega), and luciferase activities were determined with a Mithras LB 940 luminometer (Berthold Technologies). Relative light units (RLU) represent firefly luciferase normalized against Renilla luciferase activity.

ChIP assay

ChIP assays were performed using a ChIP assay kit according to the manufacturer's instructions (Upstate Biotechnology). Rabbit anti-Egr-1, anti-Snail or rabbit IgG were used to immunoprecipitate DNA-containing complexes. PCR was performed with primers complementary to the Snail or to the Egr-1 promoter region. The primer sequences are given in Supplementary Table I.

Statistical analysis

All experiments were performed in duplicates or triplicates at least three times. Values in the figures are given as means $\pm$ s.d. Statistical analysis was performed using GraphPad Prism 4.0c.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We would like to thank M-C Hung (University of Texas, Houston) for providing the Snail-8SA construct, G Thiel (University of Saarland, Homburg, Germany) for the wt- and dnEgr-1 constructs, T Pietsch (University of Bonn, Germany) for the hepatoblastoma cell lines, and A Cano, H Peinado (CSIC-UAM, Madrid) and Advanced *In vitro* Cell Technologies (Barcelona, Spain) for the Snail-promoter reporter construct and critical input. We are grateful to T Schlange (Friedrich-Miescher-Institute, Basel) for technical help and M Hoefer, M Cabrera and A Fantozzi for comments. This work was supported in part by the Novartis Foundation and Swiss National Science Foundation Grant No. 3100-063744 (DvS), and the EU-FP6 framework programme BRECOSM LSHC-CT-2004-503224 and the Swiss Bridge Award (GC).

References

Abdulkadir SA, Carbone JM, Naughton CK, Humphrey PA, Catalona WJ, Milbrandt J (2001) Frequent and early loss of the EGR1 corepressor NAB2 in human prostate carcinoma. *Hum Pathol* **32**: 935–939

Abella JV, Peschard P, Naujokas MA, Lin T, Saucier C, Urbe S, Park M (2005) Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. *Mol Cell Biol* **25**: 9632–9645

- Al-Sarraj A, Day RM, Thiel G (2005) Specificity of transcriptional regulation by the zinc finger transcription factors Sp1, Sp3, and Egr-1. *J Cell Biochem* **94**: 153–167
- Bachelder RE, Yoon SO, Franci C, de Herreros AG, Mercurio AM (2005) Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. *J Cell Biol* **168**: 29–33
- Barrallo-Gimeno A, Nieto MA (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* **132**: 3151–3161
- Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* **2**: 84–89
- Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF (2003) Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* **4**: 915–925
- Boccaccio C, Ando M, Tamagnone L, Bardelli A, Michieli P, Battistini C, Comoglio PM (1998) Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature* **391**: 285–288
- Boon EM, Kovarikova M, Derksen PW, van der Neut R (2005) MET signalling in primary colon epithelial cells leads to increased transformation irrespective of aberrant Wnt signalling. *Br J Cancer* **92**: 1078–1083
- Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA (1991) Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* **251**: 802–804
- Brockmann MA, Papadimitriou A, Brandt M, Fillbrandt R, Westphal M, Lamszus K (2003) Inhibition of intracerebral glioblastoma growth by local treatment with the scatter factor/hepatocyte growth factor-antagonist NK4. *Clin Cancer Res* **9**: 4578–4585
- Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* **2**: 76–83
- Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21**: 2933–2942
- Carver EA, Jiang R, Lan Y, Oram KF, Gridley T (2001) The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol* **21**: 8184–8188
- Cavallaro U, Christofori G (2004) Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer* **4**: 118–132
- Christensen JG, Burrows J, Salgia R (2005) c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Lett* **225**: 1–26
- Comoglio PM, Trusolino L (2002) Invasive growth: from development to metastasis. *J Clin Invest* **109**: 857–862
- Corso S, Comoglio PM, Giordano S (2005) Cancer therapy: can the challenge be MET? *Trends Mol Med* **11**: 284–292
- Day RM, Thiel G, Lum J, Chevere RD, Yang Y, Stevens J, Sibert L, Fanburg BL (2004) Hepatocyte growth factor regulates angiotensin converting enzyme expression. *J Biol Chem* **279**: 8792–8801
- de Rooij J, Kerstens A, Danuser G, Schwartz MA, Waterman-Storer CM (2005) Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol* **171**: 153–164
- Eid MA, Kumar MV, Iczkowski KA, Bostwick DG, Tindall DJ (1998) Expression of early growth response genes in human prostate cancer. *Cancer Res* **58**: 2461–2468
- Friedl P, Wolf K (2003) Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* **3**: 362–374
- Furge KA, Zhang YW, Vande Woude GF (2000) Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene* **19**: 5582–5589
- Gaggioli C, Deckert M, Robert G, Abbe P, Batoz M, Ehrenguber MU, Ortonne JP, Ballotti R, Tartare-Deckert S (2005) HGF induces fibronectin matrix synthesis in melanoma cells through MAP kinase-dependent signaling pathway and induction of Egr-1. *Oncogene* **24**: 1423–1433
- Gashler A, Sukhatme VP (1995) Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. *Prog Nucleic Acid Res Mol Biol* **50**: 191–224
- Gotzmann J, Huber H, Thallinger C, Wolschek M, Jansen B, Schulte-Hermann R, Beug H, Mikulits W (2002) Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-beta1 and Ha-Ras: steps towards invasiveness. *J Cell Sci* **115**: 1189–1202
- Gumbiner BM (2000) Regulation of cadherin adhesive activity. *J Cell Biol* **148**: 399–404
- Houston P, Campbell CJ, Svaren J, Milbrandt J, Braddock M (2001) The transcriptional corepressor NAB2 blocks Egr-1-mediated growth factor activation and angiogenesis. *Biochem Biophys Res Commun* **283**: 480–486
- Huber MA, Kraut N, Beug H (2005) Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* **17**: 548–558
- Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H, Grunert S (2002) Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J Cell Biol* **156**: 299–313
- Jeffers M, Rong S, Woude GF (1996) Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. *J Mol Med* **74**: 505–513
- Jiang WG, Martin TA, Parr C, Davies G, Matsumoto K, Nakamura T (2005) Hepatocyte growth factor, its receptor, and their potential value in cancer therapies. *Crit Rev Oncol Hematol* **53**: 35–69
- Kaufmann K, Bach K, Thiel G (2001) The extracellular signal-regulated protein kinases Erk1/Erk2 stimulate expression and biological activity of the transcriptional regulator Egr-1. *Biol Chem* **382**: 1077–1081
- Kaufmann K, Thiel G (2002) Epidermal growth factor and thrombin induced proliferation of immortalized human keratinocytes is coupled to the synthesis of Egr-1, a zinc finger transcriptional regulator. *J Cell Biochem* **85**: 381–391
- Kumbrink J, Gerlinger M, Johnson JP (2005) Egr-1 induces the expression of its corepressor nab2 by activation of the nab2 promoter thereby establishing a negative feedback loop. *J Biol Chem* **280**: 42785–42793
- Maina F, Pante G, Helmbacher F, Andres R, Porthin A, Davies AM, Ponzetto C, Klein R (2001) Coupling Met to specific pathways results in distinct developmental outcomes. *Mol Cell* **7**: 1293–1306
- Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, Narsimhan RP, Hartmann G, Zarnegar R, Michalopoulos GK, Birchmeier W, Comoglio P (1991) Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J* **10**: 2867–2878
- Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* **3**: 155–166
- Papko J, Aikawa M (1998) WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells. *Biochem Biophys Res Commun* **247**: 851–858
- Peinado H, Del Carmen Iglesias-de la Cruz M, Olmeda D, Csiszar K, Fong KS, Vega S, Nieto MA, Cano A, Portillo F (2005) A molecular role for lysyl oxidase-like 2 enzyme in Snail regulation and tumor progression. *EMBO J* **24**: 3446–3458
- Peinado H, Quintanilla M, Cano A (2003) Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem* **278**: 21113–21123
- Peiro S, Escrivá M, Puig I, Barbera MJ, Dave N, Herranz N, Larriba MJ, Takkunen M, Franci C, Munoz A, Virtanen I, Baulida J, Garcia de Herreros A (2006) Snail1 transcriptional repressor binds to its own promoter and controls its expression. *Nucleic Acids Res* **34**: 2077–2084
- Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, Graziani A, Panayotou G, Comoglio PM (1994) A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* **77**: 261–271
- Recio JA, Merlino G (2003) Hepatocyte growth factor/scatter factor induces feedback up-regulation of CD44v6 in melanoma cells through Egr-1. *Cancer Res* **63**: 1576–1582
- Riggs PK, Rho O, DiGiovanni J (2000) Alteration of Egr-1 mRNA during multistage carcinogenesis in mouse skin. *Mol Carcinog* **27**: 247–251
- Sugimachi K, Tanaka S, Kameyama T, Taguchi K, Aishima S, Shimada M, Sugimachi K, Tsuneyoshi M (2003) Transcriptional repressor snail and progression of human hepatocellular carcinoma. *Clin Cancer Res* **9**: 2657–2664

- Sukhatme VP, Cao XM, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PC, Cohen DR, Edwards SA, Shows TB, Curran T, Le Beau MM, Adamson ED (1988) A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell* **53**: 37–43
- Tan C, Costello P, Sanghera J, Dominguez D, Baulida J, de Herreros AG, Dedhar S (2001) Inhibition of integrin linked kinase (ILK) suppresses beta-catenin-Lef/Tcf-dependent transcription and expression of the E-cadherin repressor, snail, in APC^{-/-} human colon carcinoma cells. *Oncogene* **20**: 133–140
- Tanimura S, Chatani Y, Hoshino R, Sato M, Watanabe S, Kataoka T, Nakamura T, Kohno M (1998) Activation of the 41/43 kDa mitogen-activated protein kinase signaling pathway is required for hepatocyte growth factor-induced cell scattering. *Oncogene* **17**: 57–65
- Thiel G, Schoch S, Petersohn D (1994) Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. *J Biol Chem* **269**: 15294–15301
- Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2**: 442–454
- Vande Woude G, Jeffers M, Cortner J, Alvord G, Tsarfaty I, Resau J (1999) Met-HGF/SF: tumorigenesis, invasion and metastasis. *Ciba Found Symp* **212**: 119–130
- von Schweinitz D, Faundez A, Teichmann B, Birnbaum T, Koch A, Hecker H, Gluer S, Fuchs J, Pietsch T (2000) Hepatocyte growth-factor-scatter factor can stimulate post-operative tumor-cell proliferation in childhood hepatoblastoma. *Int J Cancer* **85**: 151–159
- Weidner KM, Hartmann G, Naldini L, Comoglio PM, Sachs M, Fonatsch C, Rieder H, Birchmeier W (1993a) Molecular characteristics of HGF-SF and its role in cell motility and invasion. *Exs* **65**: 311–328
- Weidner KM, Sachs M, Birchmeier W (1993b) The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J Cell Biol* **121**: 145–154
- Worden B, Yang XP, Lee TL, Bagain L, Yeh NT, Cohen JG, Van Waes C, Chen Z (2005) Hepatocyte growth factor/scatter factor differentially regulates expression of proangiogenic factors through Egr-1 in head and neck squamous cell carcinoma. *Cancer Res* **65**: 7071–7080
- Yang Z, Rayala S, Nguyen D, Vadlamudi RK, Chen S, Kumar R (2005) Pak1 phosphorylation of snail, a master regulator of epithelial-to-mesenchyme transition, modulates snail's subcellular localization and functions. *Cancer Res* **65**: 3179–3184
- Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC (2004) Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* **6**: 931–940