Transforming growth factor beta 1 induces Snail transcription factor in epithelial cell lines.

Mechanisms for Epithelial Mesenchymal Transitions.

Hector Peinado, Miguel Quintanilla and Amparo Cano#

Instituto de Investigaciones Biomédicas “Alberto Sols” (CSIC-UAM) and Departamento de Bioquímica (UAM), Arturo Duperier 4, 28029 Madrid. Spain.

# Corresponding Author:

Dr. Amparo Cano
Tel. 34-91-585.4597.
Fax. 34-91-585.45.87.
E-mail: acano@iib.uam.es

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SUMMARY

The Snail transcription factor has been recently described as a strong repressor of E-cadherin in epithelial cell lines, where its stable expression leads to the loss of E-cadherin expression and induces Epithelial-Mesenchymal-Transitions (EMTs) and an invasive phenotype. The mechanisms regulating Snail expression in development and tumor progression are not yet known. We show here that transforming growth factor beta-1 (TGFβ1) induces Snail expression in MDCK cells and triggers EMT, by a mechanisms dependent of the MAPK signaling pathway. Furthermore, TGFβ1 induces the activity of Snail promoter, while fibroblast growth factor-2 (FGF2) has a milder effect but cooperates with TGFβ1 in the induction of Snail promoter. Interestingly, TGFβ1-mediated induction of Snail promoter is blocked by a dominant negative form of H-Ras (N17Ras), whereas oncogenic H-Ras (V12Ras) induces Snail promoter activity and synergistically cooperates with TGFβ1. The effects of TGFβ1 on Snail promoter are dependent of MEK1/2 activity but are apparently independent of Smad4 activity. In addition, H-Ras-mediated induction of Snail promoter, alone or in the presence of TGFβ1, depends on both MAPK and PI3K activities. These data support that MAPK and PI3K signaling pathways are implicated in TGFβ1-mediated induction of Snail promoter, probably through Ras activation and its down-stream effectors.
INTRODUCTION

The molecular mechanisms underlying local invasion and metastasis are still poorly understood, but evidence accumulated in the last years indicates the existence of common cellular mechanisms for the local invasive process which represent the first stage into the metastatic cascade of carcinomas (1, 2). Among those, loss of expression or function of the E-cadherin cell-cell adhesion molecule has emerged as an important event for local invasion of epithelial tumor cells, leading to the consideration of E-cadherin as an invasion-suppressor gene (3-5). The process of invasion is frequently associated with the loss of other epithelial markers, and the acquisition of mesenchymal markers and a migratory and motility behavior, collectively known as epithelial-mesenchymal transitions (EMTs)\(^1\) (see ref. 6, for a recent review). EMTs also occur during normal embryonic development in a strict spatio-temporal control and they are required at specific stages, such as during gastrulation, formation of the neural crest cells and other morphogenetic processes (6-8). These developmental EMTs are always accompanied by the loss of functional E-cadherin mediated cell-cell adhesion (9, 10).

The molecular mechanisms underlying downregulation of E-cadherin during EMTs and tumor progression are starting to be uncovered. Genetic alterations of the E-cadherin loci have been found in a scarce number of tumors, particularly, in lobular breast carcinomas and diffuse gastric carcinomas (3, 11, 12), while the majority of carcinomas with downregulated E-cadherin maintain an intact E-cadherin locus. Hypermethylation of the E-cadherin promoter and transcriptional alterations have emerged as the main mechanisms responsible for E-cadherin downregulation in most carcinomas (5, 13). Several transcriptional repressors of E-cadherin have been recently isolated, including the zinc finger factors Snail (14, 15) and Slug (16, 17), the two-handed zinc factor ZEB-1 and SIP-1 (18, 19) and the bHLH factor E12/E47 (20). Snail family factors are in fact involved in EMTs when overexpressed in epithelial cell lines (14, 15, 17) as well as in embryonic development (reviewed in ref. 21) and proposed to act as inducers of the invasion process (14, 22).
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Generation of Snail knock out mice has further established the role of this factor in EMT and as E-cadherin gene repressor. The null Snail embryos die at gastrulation as they fail to undergo a complete EMT process, forming an altered mesodermal layer which maintains the expression of E-cadherin (23). Nevertheless, the mechanisms that regulate the expression of Snail factors are still poorly understood (6, 21).

Different growth factors and cytokines have also been implicated in the process of EMTs both in epithelial cell systems and in embryonic development. Studies on development have indicated the participation of several members of the transforming growth factor/bone morphogenetic (TGFβ/BMP) family of growth factors in specific EMT processes in different species (24, 25), while fibroblast growth factor (FGF) signaling has been recently reported as a determinant for mesoderm cell fate specification in the mouse embryo (26). Several studies have also indicated that a multiple cross talk between TGFβ/BMPs, FGF, and Wnt signals could be required for some EMTs in development (26-28). In epithelial cell systems, several growth factors have been widely studied and reported to induce a scattering phenotype or a complete EMT depending of the specific cell system analyzed (reviewed in refs. 6, 29). Among them, TGFβ has been identified as an important molecular player of EMT both in vitro and in vivo (30-34). In some cell systems, a synergistic cooperation between H-Ras activation and TGFβ signaling appears to be required for induction of a complete EMT (33, 35, 36). Recently, TGFβ has been reported to induce the expression of Snail in fetal and in immortalized murine hepatocytes and in human mesothelial cells (37-39), but whether this is a direct or indirect effect has not yet been established.

The participation of specific signaling pathways activated by TGFβ and/or H-Ras-activation in EMTs has been previously analyzed with somewhat contradictory results as regard to the specific implication of Smad, mitogen activated protein kinase (MAPK) and/or phosphatidylinositol 3-OH kinase (PI3K) pathways (36, 40-43). The issue has been recently unraveled in the EpRas model with the implication of MAPK in TGFβ-induced EMT,
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tumorigenesis and metastasis, while PI3K is involved in cell scattering and resistance to TGF-β induced apoptosis (36). It remains to be established, however, if the same situation applies to other systems and, more importantly, the identification of the target genes involved in the specific growth factor signaling leading to EMTs.

We have used the prototypic epithelial MDCK cells to further analyze the process of EMT induced by TGFβ and FGF. We have previously used this cell system to show that Snail overexpression leads to the full repression of E-cadherin expression and induction of a complete EMT (14). In the present work we have investigated the ability of TGFβ1 and FGF2 to induce an EMT in MDCK cells and ask whether Snail is a target gene of this process. We present evidence that TGFβ1 treatment induces an EMT process linked to Snail induction in MDCK cells. Analysis of the mouse Snail promoter indicates that it is directly induced by TGFβ1, and that FGF2 and activated H-Ras cooperate with TGFβ1 in induction of the Snail promoter. Our results also indicate that the MAPK and PI3K pathways are involved in the TGFβ1- and H-Ras-mediated induction of Snail promoter. These results strongly support that Snail is a direct target of TGFβ1 and oncogenic H-Ras, and open the way for future studies on the molecular mechanisms and targets of EMTs and the invasion process.
**EXPERIMENTAL PROCEDURES**

**Cell culture and treatments.** MDCK-II cells were grown in DMEM medium and MCA3D and PDV cells in Ham’s F12 medium, in the presence of 10% FBS, 10 mM glutamine (Gibco BRL) and 100 µg/ml ampicilin, 32 µg/ml gentamicin (Sigma Chemical Co). Cells were grown at 37°C in a humidified CO2 atmosphere. All the transfections and treatments were done in FBS-free culture medium. For the indicated treatments, 10 µg/ml stocks of recombinant TGFβ1 (BioNova Corp.) and 100 µg/ml of FGF2 (Peprotech) were prepared according to manufacturer’s instructions and added to the indicated concentrations. The PI3K and MEK1/2 inhibitors, LY294002 and PD98059 (Calbiochem), respectively, were kept as 30-10 mM stocks in DMSO, that was used as vehicle control in all the inhibitor treatments.

**RT-PCR analyses.** Total RNA was isolated from the different cell lines and RT-PCR analyses were carried out as previously described (14, 17, 20). Canine PCR products, were obtained after 30-35 cycles of amplification with an annealing temperature of 60-65°C. Primer sequences were as follows. For canine *E-cadherin* (sequence kindly provided by Y. Chen, Harvard Medical School, USA), forward: 5' GGAATCTTTGGAGGGATCCTC 3'; reverse: 5' GTCGTCCTCGCCACCGCCGTACAT 3' (amplifies a fragment of 560 bp). For canine *Snail*, forward: 5’ CCAAGGGCCAGCCGATGAG 3'; reverse: 5’ CTTGGCCACGGAGAGCCC 3' (amplifies a fragment of 200 bp). For canine *glyceraldehyde-3-phosphatedehydrogenase* (GADPH), forward: 5’TGAAGGGTCGCTG- GTGAACGGATTTGGC3'; reverse: 5’ CATGTAGGCCATGAGGTCCACAC 3' (amplifies a fragment of 900 bp).

**3TP-Lux, E-cadherin and Snail promoter analyses.**

For 3TP-Lux assays a reporter construct containing the TPA and TGFβ response elements fused to the *Luciferase* reporter gene (44) was used. The generation of mouse *E-cadherin* promoter constructs containing -178/+92 sequences in its wild type or mutant Epal (mE-pal) fused to *Luciferase*, has been previously reported (17). Generation of full length mouse *Snail* promoter
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construct (-900 bp) has been also recently described (17). Deletion constructs of the Snail promoter mutants were obtained by PCR amplification from the full length –900 bp promoter using appropriate primers containing BamHI and KpnI restriction sites, and the corresponding PCR products cloned into the same restriction sites in the pXP1-Luciferase vector.

To determine the activity of 3TP-Lux and the Snail promoter 2x10⁵ cells grown in twenty-four well plates were transiently transfected with 200 to 500 ng of the indicated reporter constructs and 20 ng of TK-Renilla construct (Promega) as a control of transfection efficiency. Luciferase and renilla activities were measured using the Dual-Luciferase Reporter assay kit (Promega) and, after normalization, the results were referred to the wild type promoter activity detected in mock-transfected cells. Results represent the mean ± s.d. of at least two independent experiments performed in duplicate samples.

For the cotransfection experiments 500 ng of the following plasmids were used: pSmad4 DN (1-514) in pCMV5 vector (provided by J. Massagué, Sloan-Kettering Center, USA)(44); pLXSNHRasV12, pLXSNHRasN17 and the different mutants of HRasV12 (pLXSNHRasV12S35, pLXSNHRasV12C40, pHrasLXSNRasV12G37) in the pLXSN vector (a gift of P. Rodriguez-Viciana, USCF, USA) (45); β-catenin S33Y (provided by A. Ben-Ze’ev, Weizmann Institute, Israel) and Lef-1 (provided by H. Clevers, Utrecht University Hospital, The Netherlands) cloned in pcDNA3. The corresponding empty vectors, pLXSN, pCMV5 or pcDNA3 were used in control transfections and for normalization of the total amount of DNA.

**Immunofluorescence and western blot analyses.** For immunofluorescence staining cells grown on coverslips were fixed in methanol (-20º C, 30 sec) and stained for E-cadherin, vimentin, cytokeratin-8 and fibronectin, as previously described (14, 17, 20). For F-actin stain, cells were fixed in 3.7% formaldehyde, 0.5% Triton X-100 for 30 min at room temperature, stained with TRICT-conjugated phalloidin (Sigma Chemical Co.) and washed (4X) in PBS. The cells were mounted on Mowiol and the preparations were visualized using a Leica confocal TCS SP2
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microscope. For western blot, whole cell extracts of control and treated cells were obtained in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 5% Deoxycholate, 0,1% SDS) and analyzed for the indicated molecules by western blot and ECL detection as previously described (14, 17, 20). Primary antibodies included: rat monoclonal anti-E-cadherin ECCD-2 (1:100) (provided by M. Takeichi, Kyoto University, Japan); mouse monoclonal anti-vimentin (1:200) (Dako); mouse monoclonal anti-cytokeratin 8 (1:200) (Progen); rabbit polyclonal anti-fibronectin (1:100) and mouse monoclonal anti-α-tubulin (1:1000) (Sigma Chemical Co.). For cell signaling analysis, western blots were carried out on cell extracts obtained by lysis in Buffer A (20 mM Hepes pH 7.5, 10 mM EGTA, 40 mM β-glycerolphosphate, 2.5 mM MgCl2, 1% NP-40, 1 mM DTT), containing the apropiate protease and phosphatase inhibitors, during 30 min. at 4ºC. Primary antisera included: goat anti-AKT (1:1000) (Santa Cruz Biotech.); rabbit anti-phospho (Ser473)-AKT (1:500); rabbit anti-ERK1/2 and anti-phospho (Thr202/Tyr204)-ERK1/2 (1:1000) (Cell Signalling Tech.); rabbit anti-Smad2/3 and rabbit anti-phospho (Ser-465/467)-Smad2/3 (1:500) (Upstate). Secondary antibodies were BODIPY-conjugated goat anti-rat; anti-mouse and anti-rabbit IgG (Molecular Probes), and HRP-conjugated sheep anti-mouse (1:1000) (Amersham); donkey anti-goat (1:1000) (Santa Cruz Biotech); goat anti-rat (1:10.000) (Pierce); and goat anti-rabbit (1:4000) (Nordic) IgG.

Cell proliferation assays. The indicated number of cells (2.5x10^5 or 5x10^5) were seeded in triplicate samples in ninety-two plates and grown in complete medium for 3h. After washing in PBS, TGFβ1 (10 ng/ml) in FBS-free medium was added and the cells were grown for additional 24h. ³H-thymidine was added during the last 5h of treatment. The cells were collected using a cell harvester device and ³H-thymidine incorporation was determined in a scintillation counter. The values, representing the mean± s.d., were normalized to those obtained in control untreated cells.

Migration assays. The migratory/motility behaviour of MDCK cells was analysed in in vitro wound healing assays as previously described (14, 17). Monolayers of confluent cultures were
lightly scratched with a Gilson pipette tip and, after washing to remove detached cells, treated with TGFβ1 (10 ng/ml) and/or PD98059 (10 μM), as indicated. Cultures were observed at timely intervals for up to 36h post-incision.
RESULTS

TGFβ1 induces cell scattering and increased motility in MDCK cells dependent on MEK1/2 activity.

Some previous studies have related the TGFβ/BMP signaling pathway to the regulation of EMT both during embryonic development (24, 25) and in some epithelial cell lines (30, 32, 33, 37), while others have potentially implicated a similar function for FGFs (46-48). To get further insights into the regulation of EMT by both kinds of growth factors, we choose the prototypic epithelial MDCK cell line. Twenty-four hours treatment with TGFβ1 (10 ng/ml) (Fig. 1A, b) or a combination of TGFβ1 (10 ng/ml) and FGF2 (100 ng/ml) (Fig. 1A, c) induced a dramatic change of the cellular phenotype: MDCK cells became dissociated with reduced cell-cell contacts and acquired a more spindle phenotype. Lower concentrations of TGFβ1 (1-5 ng) induced a milder effect and treatment with FGF2 alone did not affect the phenotype of MDCK cells (data not shown). The phenotypic changes induced by TGFβ1 were also associated to increased cell motility, as ascertained by in vitro wound healing assays (Fig. 1B). Eight hours after incision of the wound, MDCK cells growing in the presence of TGFβ1 started to colonize the wound surface, while control untreated cells hardly started to migrate (data not shown). The differences in cell motility were evident 24h after incision when TGFβ1-treated cells colonized about 70-80% of the wound surface in a random fashion (Fig. 1B, g), in contrast to untreated cells that had only colonized 20-30% of the wound surface by unidirectional migration (Fig. 1B, e). The increased motility induced by TGFβ1 treatment is not due to increased cell proliferation. Analysis of 3H-thymidine incorporation showed that MDCK cells treated with TGFβ1 exhibited an 80% reduction of their proliferation potential, as compared with control untreated cells (Fig. 2 A). After 3-4 days of TGFβ1 treatment MDCK cells started to show signs of apoptosis, and most cells died after 7 days of treatment (data not shown). The sensitivity of MDCK cells to TGFβ1 was also evidenced by the quick induction of the responsive 3TP-Lux promoter in the presence of the growth factor (Fig. 2B).
We then analysed the implication of MAPK and PI3K pathways in the phenotypic effects induced by TGFβ1 in MDCK cells, since they have been previously implicated in epithelial cell scattering induced in MDCK and in other cell systems by several growth factors (33, 35, 36, 40, 42, 43). One-hour pretreatment with the specific MEK1/2 inhibitor PD98059 (10 µM) abolished the cell dissociation and scattering induced by TGFβ1 (Fig. 1A, e) or by the combination of TGFβ1 and FGF2 (Fig. 1A, f) treatments. No significant effect of PD98059 on the cell phenotype was observed in control untreated (Fig. 1A, d) or FGF2-treated cells (data not shown), although increased intracellular vacuolization was observed in all PD98059 treated samples. In agreement with those observations, PD98059 pretreatment also blocked the TGFβ1-induced migration of MDCK cells (Fig. 1B, h), but did not have any effect on the migration of untreated cells (Fig. 1B, f). Pretreatment with the PI3K inhibitor LY294002 (30 µM) followed by TGFβ1 treatment caused cell disintegration (data not shown), thus precluding further studies on the implication of PI3K in the phenotypic or migratory effects of TGFβ1.

Activation of the MAPK and PI3K pathways following TGFβ1 treatment of MDCK cells was confirmed by western blot analyses of phosphorylated ERK1/2 and AKT, respectively, using phospho-specific antibodies to both effectors. As shown in Fig. 3, increased levels of P-ERK2 (Pp-42) were detected after 30 min. of TGFβ1 treatment, peaking after 1h and slowly decreasing thereafter (Fig. 3, upper panels). A similar kinetics was observed in the levels of P-ERK1 (Pp-44), although of a lesser extent. Interestingly, increased levels of P-ERK1/2 were detected even after 6h of TGFβ1 treatment, indicating a sustained response of the MAPK pathway. Activation of PI3K followed a slower kinetics in response to TGFβ1, increased levels of P-AKT were firstly detected after 1h of TGFβ1 treatment, peaked by 3h and decreased thereafter (Fig. 3, middle panels). TGFβ1 treatment also induced a fast and sustained activation of the Smad pathway in MDCK cells, since P-Smad2 was detected after 15 min. and was maintained up to 3h of TGFβ1 treatment (data not shown).
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These results indicate that TGFβ1 induces a scattering and motile phenotype in MDCK cells, apparently depending on MAPK signaling, and suggest that activation of the PI3K pathway might be required for survival in the presence of TGFβ1. FGF2 by itself does not have a significant effect on the MDCK phenotype, although it can potentially collaborate with TGFβ1.

**TGFβ1 treatment induces EMT associated to Snail induction and E-cadherin repression in MDCK cells.**

The phenotypic changes and increased motility observed in MDCK cells after TGFβ1 treatment were reminiscent of those observed after stable transfection of MDCK cells with the Snail repressor (14), and suggested that Snail might be induced by TGFβ1. To analyze this hypothesis, the endogenous levels of Snail transcripts in MDCK cells following TGFβ1 treatment were analyzed by RT-PCR (Fig. 4A). Twenty-four hours treatment with TGFβ1 led to a 2 to 3-fold induction of Snail mRNA over the basal levels; the level of Snail transcripts decreased thereafter but remained above the basal levels, at least up to 72h of treatment. Analysis of E-cadherin mRNA levels showed no significant changes after 24h of TGFβ1 treatment, but a marked decrease was detected after 48h and 72h of TGFβ1 treatment, when E-cadherin mRNA was almost undetectable (Fig. 4A). Densitometric analyses showed that by 72h of TGFβ1 treatment the Snail and E-cadherin transcripts were present at levels representing 150% and 20%, respectively, of those detected in control untreated cells. In agreement with those data, analysis of an exogenous mouse E-cadherin promoter (17) by transient transfection showed a 50-60 % inhibition after 48 to 72h of TGFβ1 treatment treatment (Fig. 4B, left panel). Furthermore, E-cadherin promoter inhibition by TGFβ1 depends on the presence of Snail-binding site, the E-pal element (14, 17), since its mutation fully abolished the TGFβ1 effect (Fig. 4B, right panel). These data support that the TGFβ1-induced repression of E-cadherin can be mediated by Snail expression. Western blot analyses showed a
TGFβ induces Snail transcription factor moderate decrease (around 35% of control cells) in the total level of E-cadherin, but strong reduction of other epithelial markers, such as cytokeratin 8 (more than 50% of control levels) after 72h of TGFβ1 treatment (Fig. 4C). The inhibition of E-cadherin promoter activity and decreased mRNA levels detected between 48-72 h of TGFβ1 treatment contrast with the levels of E-cadherin protein detected at this time point. This apparent discrepancy has also been observed in other cell systems (47) and can be explained by the long half-live of the E-cadherin protein, estimated in more than 40h in other cell systems (49). The above described results suggest that Snail induction, even at moderate levels, could be required to trigger the repression of E-cadherin and, potentially, of other epithelial genes that eventually lead to the EMT induced by TGFβ1 treatment in MDCK cells.

To further investigate if TGFβ1 indeed induces a full EMT in MDCK cells, we analyzed the expression and localization pattern of E-cadherin, cytokeratin 8, as well as that of vimentin and fibronectin as prototypic markers of epithelial and mesenchymal cells, respectively. Confocal immunofluorescence analysis showed that 24 to 48h treatment with TGFβ1 led to a redistribution of E-cadherin from the cell-cell contacts to the cytoplasm (data not shown). By 72h of TGFβ1 treatment almost complete disappearance of E-cadherin at cell-cell interactions was observed (Fig 5A, b), as compared with control untreated cells (Fig. 5A, a). Cotreatment with TGFβ1 and FGF2 induced a similar redistribution of E-cadherin (Fig. 5A, d). In agreement with the lack of phenotypic effects, FGF2 treatment alone did not produce redistribution of the E-cadherin molecules (Fig. 5A, c). The TGFβ1-induced redistribution of E-cadherin was fully abolished by pretreatment of MDCK cells with PD98059 (Fig 5A, e), which showed a similar E-cadherin stain as control cells pretreated with PD98059 (Fig. 5A, f). Forty-eight hours treatment of MDCK cells with TGFβ1 also induced a marked decrease and disorganization of cytokeratin 8 stain (Fig. 5B, b) also confirmed by western blot (Fig. 4C), and increased staining of vimentin (Fig.5B, e) and fibronectin (Fig.5B, h) which were organized in clear intermediate filaments and apparently
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secreted matrix respectively, although no changes in total vimentin levels were detected (Fig. 4C). Staining for F-actin also showed a marked reorganization of the microfilament network with appearance of stress fibres and membrane protrusions, resembling lamellipodia and filopodia, in TGFβ1-treated MDCK cells (Fig. 5B, k, arrows), in contrast to untreated control cells that showed a more defined cortical actin filaments (Fig. 5B, j). These results, together with those shown in Fig. 1, indicate that TGFβ1 induces a full EMT in MDCK cells. Furthermore, the multiple changes detected in the different markers and in cytoskeleton organization after TGFβ1 treatment were fully abolished by pre-treatment of MDCK cells with PD98059 (Fig. 5B, c, f, i, l), as well as E-cadherin redistribution (Fig. 5A, e), indicating that the MAPK activity is necessary for TGFβ1-induced EMT in this cell line.

TGFβ1 and FGF2 signaling pathways collaborate in Snail promoter induction and depend on MAPK activity.

To investigate if the observed induction of Snail expression is a direct effect of TGFβ1, we analyzed the effect of the growth factor on the mouse Snail promoter (17) by transient transfection assays. As shown in Fig. 6A, TGFβ1 treatment of MDCK cells induced the Snail promoter activity in a dose dependent manner. TGFβ1 at 10 ng/ml was able to induce the promoter activity by 3-fold, while treatments with lower concentrations of 1 and 5 ng/ml induced Snail promoter activity by 1.3- and 2-fold, respectively. To determine if this effect was restricted to MDCK cells, we analyzed two other epithelial cell lines, the mouse epidermal keratinocyte MCA3D and PDV cells, representing immortalized and transformed stages of the mouse skin carcinogenesis model, respectively (49, 50). TGFβ1 (10 ng/ml) treatment induced the Snail promoter activity about 2-fold in both MCA3D and PDV cell lines (Fig. 6B). These results indicate that Snail promoter could in fact be controlled by signals downstream of TGFβ1 in epithelial cell lines. Although the level of
Snail promoter induction by TGFβ1 in the analyzed cell lines is only moderate, it is consistent with the induction of Snail mRNA level detected in MDCK cells (see Fig. 4A).

As previously indicated, FGF signaling has been recently implicated in the regulation of Snail expression during embryonic development (26), and previous work in epithelial NBT-II cells also suggested its involvement in the regulation of Slug (a closely related homolog of Snail) (51). We, therefore, analyzed the effect of FGF2, alone or in combination with TGFβ1, on the Snail promoter activity in MDCK cells (Fig. 6C). FGF2 (100 ng/ml) treatment induced a slight activation of the Snail promoter (1.5-fold), lower than that induced by TGFβ1 at 10 ng/ml (3-fold activation), but an additive effect on the Snail promoter activity (4.5-fold induction) was observed by the combination of both FGF2 and TGFβ1 (Fig. 6C). The collaboration between both factors has also been observed in other contexts, such as in embryonic development where this synergism is necessary for the subsequent correct development of the EMTs areas, together with others signals, such as Wnt (28). However, the canonical Wnt signaling pathway seems not to play a significant role in the regulation of Snail expression in MDCK cells, as no effect on the Snail promoter activity was observed by the treatment with TGFβ1 in the presence of activated β-catenin and Lef-1 factor (data not shown). These latter results are also in agreement with a previous report showing that ILK-induced activation of the human Snail promoter in colon cancer cells is independent of β-catenin/Tcf complex (52).

We next investigated the TGFβ1 and FGF2 signaling pathways involved in the regulation of Snail promoter. Cotransfection of a dominant negative version of Smad4 (1-514) that blocks the classical TGFβ-Smad signaling pathway (44), as confirmed here by its action on the responsive 3TP-lux promoter (Fig. 6E), did not significantly change the TGFβ1-mediated induction of the Snail promoter activity, and even increased the combined effect of TGFβ1 and FGF2 on the promoter activity (Fig. 6D). These results indicated that Smad4 signaling is not directly involved in the regulation of Snail promoter activity by TGFβ1. We then analyzed the participation of the
MAPK pathway in the regulation of *Snail* promoter by TGFβ1 and FGF2, since it has been recently implicated in TGFβ signaling in other contexts (36, 53). To that end, the activity of the *Snail* promoter was analyzed in MDCK cells pretreated with the MEK1/2 inhibitor PD98059 (10 µM) before treatment with TGFβ1 and/or FGF2. Pre-treatment with PD98059 decreased the basal activity of *Snail* promoter to about 60% (Figs. 6C, 7A). More significantly, PD98059 pretreatment fully blocked the *Snail* promoter induction observed by treatment with TGFβ1, FGF2 or the combination of both factors (Fig. 6C). These results strongly suggest that MAPK signaling is one of the pathways implicated in the TGFβ1-mediated regulation of *Snail* expression in MDCK cells.

**TGFβ1 and Ras pathways collaborate in Snail induction.**

Several recent works have shown the requirement of Ras downstream signaling in the process of EMT in different epithelial cell systems, in some cases in cooperation with TGFβ (33, 35, 36). It was, therefore, important to determine the potential contribution of Ras, either by itself or in cooperation with TGFβ1, to the regulation of the *Snail* promoter. Cotransfection of a dominant active version of Ras (HRasV12) induced a 3- to 4-fold activation of the *Snail* promoter activity (Fig. 7A, C), similar to that observed in the presence of TGFβ1 (Fig. 7B, D), while a dominant negative version of Ras (HRasN17) did not have any significant effect on the *Snail* promoter (Fig. 7A). Pretreatment with the MEK1/2 inhibitor PD98059 or the PI3K inhibitor LY294002 resulted in the total blockade of *Snail* promoter induction after HRasV12 cotransfection (Fig. 7A), indicating that both MAPK and PI3K signaling pathways are involved in *Snail* promoter induction by activated H-Ras. In contrast to PD98059, the LY294002 inhibitor did not have a significant effect on the basal non-induced *Snail* promoter (Fig. 7A). Interestingly, activated H-Ras seems to be required for, and synergistically cooperates with, TGFβ1-mediated *Snail* induction. Cotransfection with HRasV12 and TGFβ1 treatment induced a much stronger activation of the *Snail* promoter (about 8 to 12-fold) than that induced separately by the growth factor or HRasV12.
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(Fig. 7B, D). Furthermore, cotransfection with the dominant negative HRasN17 resulted in a 60% reduction of the TGFβ1-mediated induction of Snail promoter (Fig. 7B).

The above results indicated the participation of activated H-Ras and its cooperation with TGFβ1 in the regulation of Snail induction, with the involvement of both MAPK and PI3K signaling pathways. To confirm these results we used different mutants of activated HRasV12 that are able to transduce signals by specific pathways (45). We cotransfected the mutants RasV12C40 (activated PI3K pathway), RasV12S35 (activated MAPK pathway) and RasV12G37 (activated Ral-GDS) and analyzed the induction of the Snail promoter in the absence or presence of TGFβ1 treatment. Results indicate that both V12S35 and V12C40 mutants maintain high levels of Snail promoter activity both in the absence (Fig. 7C) and presence of TGFβ1 (Fig. 7D), accounting for about 70% of the level obtained by HRasV12 in both situations. In contrast, the V12G37 mutant had a lower activity, accounting for only about 50% of the level obtained with HRasV12 mutant. Of note, under TGFβ1 treatment, the V12G37 mutant did not show any significant Snail promoter induction as compared with the TGFβ1 treatment alone (Fig. 7D).

Taken together, these results indicate that both MAPK and PI3K pathways are required for the H-Ras and TGFβ1/H-Ras mediated induction of Snail promoter, while the Ral-GDS pathway might play a more modest role in Snail induction by activated H-Ras alone.

**Characterization of the Snail promoter regulatory elements.**

Finally, to get further insights into the regulation of Snail promoter activity by TGFβ1 and H-Ras signals, we have performed initial studies on the putative regulatory elements implicated. *In silico* analysis of the cloned mouse Snail promoter region (–900 bp) indicated the presence of several putative interaction sites for different transcriptional regulators, including AP-4, AP-1, STAT, MZF-1 or MyoD consensus sites (Fig. 8A). The organization of this promoter led us to generate several deletion mutants containing the different control elements, as indicated in the
schematics of Fig. 8B. Particularly, we were interested in the AP-1 site located at the –23/-33 position (from the ATG start codon), since AP-1 sites are highly sensitive to downstream signals generated in response to TGFβ and RasV12 pathways (54).

Transfection of MDCK cells with the different Snail promoter mutants showed that the –900 bp construct exhibited the highest activity, and decreased activities (40-25%) were detected in most of the other constructs (results not shown). The –100 bp construction has not significant activity as compared with the other mutants or the full length –900 bp construct, and could, therefore, be considered as a minimal basal promoter region (data not shown). The effect of TGFβ1 and HRasV12 was analyzed on the different Snail promoter constructs, and the activities were normalized to that of the basal activity of each promoter construct (Fig. 8B). Surprisingly, the –100 bp promoter region was enough to respond to HRasV12 cotransfection which induced a 3.6-fold activation of this basic Snail promoter (Fig. 8B). The other Snail promoter constructions showed a similar sensitivity to HRasV12 cotransfection, with exception of the –575 bp construct that exhibited a stronger activation (5.8-fold) (Fig. 8B). These results suggested that the proximal AP-1 site could be the main regulatory element implicated in H-Ras induction of Snail promoter, and point to the potential involvement of negative regulatory elements for H-Ras signals located between –900 and –575 position of the Snail promoter.

Analyses of the various Snail promoter constructs in response to TGFβ1 treatment showed that the –900 bp construct exhibited the stronger induction (3-fold activation over basal non-stimulated control) (Fig. 8B). Deletion of sequences from –575 bp position greatly reduced TGFβ1 activation, and no response to TGFβ1 was achieved with the –100 bp construct (Fig. 8B). Two additional constructs containing –675 and –200 bp sequences showed the same induction by TGFβ1 as the –575 and –300 bp constructs, respectively (data not shown). These results suggest that the TGFβ1 response elements are located between –675 and –900 bp positions, correlating with all the experiments done with the full-length construction. Several putative binding regions for
different regulatory transcription factors (AP-4, MZF-1) are present between $-675$ and $-900$ bp region that could be responsible of the TGFβ1 activation of the Snail promoter.
DISCUSSION

The process of EMT is essential for certain morphogenetic movements within the embryo and is strongly associated with the pathological process of tumor invasion (6, 7). The molecular mechanisms and signals involved in EMTs have been previously studied by different groups, with particular interest in the implication of several growth factors, such as FGF and TGFβ family factors (6, 29) and transcription factors of the Snail family (21). One of the hallmarks of EMTs in both normal and pathological situations is the lost of expression or function of the E-cadherin molecule. In this context, the identification of Snail as a strong E-cadherin repressor in normal and epithelial tumor cells (14, 15, 22), has reinforced the essential role of Snail family factors in EMTs (21, 23, 55). Despite this increased understanding, the link between the signals required for EMT and the direct target genes is still missing, although some recent studies in mouse development have started to address this important issue (26). In the current work, we present evidence for a direct link between TGFβ1 signaling and induction of Snail expression during EMT in MDCK cells.

TGFβ1 induces a scattering phenotype in MDCK cells characterized by the quick internalization and further loss of E-cadherin from the cell surface, decreased expression of cytokeratins, induction/reorganization of mesenchymal markers, reorganization of the actin cytoskeleton and increased cell motility. The phenotypic and differentiation markers changes observed here are consistent with some of the operational criteria recently proposed for the definition of a complete EMT process (36). Nevertheless, it should be kept in mind that the EMT process induced by TGFβ1 in MDCK cells occurs concomitantly to a growth inhibitory response, in agreement with previous reports on MDCK cells and other non-transformed epithelial cell types (30, 35, 50, 53). The phenotypic changes induced by TGFβ1 in MDCK cells are similar to those observed in other epithelial cell systems (37, 40, 56), but differ in the extent of E-cadherin repression observed in the different systems. They also differ from those observed in the mammary...
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EpH4 cells in which TGFβ is not able to induce by itself an EMT process (33), indicating that the sensitivity and phenotypic response to TFGβ can be modulated by the specific epithelial cell type.

Our present results provide several evidences in support that Snail is mediating the EMT-triggered by TGFβ1 in MDCK cells. a) Twenty-four hours treatment of MDCK cells with TGFβ1 leads to a 2 to 3-fold induction of Snail mRNA level and the Snail transcripts are maintained above the basal levels after 72h of treatment. b) The Snail promoter activity is induced to a similar level after 24h of TGFβ1 treatment. c) The increase and maintenance of Snail transcripts after TGFβ-1 treatment, is correlated with the reduction of E-cadherin mRNA levels and promoter activity detected between 48h and 72h of treatment and with the overall changes in the cell phenotype. The partial repression of the exogenous mouse E-cadherin promoter and the fact that total E-cadherin protein levels are only slightly decreased after 72h of TGFβ1 treatment might argue against a direct repression of E-cadherin by Snail. However, the repression of the exogenous E-cadherin promoter activity after TGFβ-1 treatment is dependent on the Snail-interacting promoter sequences (Fig 4B) located in the E-pal element (14, 17). Furthermore, a strong repression of E-cadherin mRNA is indeed observed after 72h of TGFβ-1 treatment. The partial repression of the E-cadherin promoter observed here might reflect intrinsic differences between endogenous and exogenous E-cadherin promoter regulation in MDCK cells, or be explained by the moderate levels of Snail induction under those conditions. On the other hand, the slow turnover of E-cadherin protein (49) might well explain the moderate decrease in E-cadherin protein levels observed after 72h of TGFβ1 treatment. Despite this fact, the strong redistribution of E-cadherin induced by TGFβ1 in MDCK cells suggests that perturbation of the functional localization of E-cadherin at cell-cell contacts should be enough to initiate the EMT, that could be latter sustained by effective repression of E-cadherin mRNA following TGFβ1 treatment. Furthermore, Snail might regulate other genes required, in conjunction with E-cadherin downregulation, for the EMT process. Indeed, Snail-mediated repression of cytokeratin 18 has been recently reported in colon carcinoma HT29 cells (57), and our
ongoing studies on Snail target genes indicate that besides *E-cadherin*, expression of genes coding for several cytokeratins, desmogleins and desmoplakins are strongly repressed in Snail-expressing MDCK cells (H. Peinado, A. Fabra, J. Palacios, and A. Cano, manuscript in preparation).

A direct effect of TGFβ1 signaling in *Snail* expression is supported by our analysis of the mouse *Snail* promoter, since the growth factor consistently induced the promoter activity by 3-5-fold over the basal levels in MDCK cells, and also induced the *Snail* promoter in other epithelial cell lines. In contrast, FGF2 had a milder effect on the *Snail* promoter activity, but a cooperation between FGF2 and TGFβ1 was clearly detected (Fig. 6C). These results are also in agreement with the phenotypic effects observed in MDCK cells in the presence of these two factors, since FGF2 alone was unable to induce significant phenotypic changes or decreased E-cadherin organization at the cell-cell contacts (Figs. 1 and 5). Interestingly, activated H-Ras is also able to induce the *Snail* promoter activity and, more significantly, synergistically cooperates with TGFβ1 (Fig. 7). These results might explain the apparently increased induction of *Snail* mRNA levels observed by TGFβ treatment in murine hepatocytes after H-Ras transformation (35). The cooperation between TGFβ and activated H-Ras has been previously reported to be required for a complete EMT in some cell systems, such as in EpRas cells where indeed both signals participate into the invasive and metastatic phenotype (33, 36).

The specific signaling pathways involved in EMT mediated by TGFβ and activated H-Ras have been also addressed in the present study. Our results do not support a direct involvement of the Smad pathway in *Snail* promoter regulation, although an indirect involvement can not be presently discarded, since the Smad pathway is activated by TGFβ1 in MDCK cells (data not shown). In fact, the cooperation between FGF2 and TGFβ1 in *Snail* promoter induction is magnified by a dominant negative version of Smad4 (Fig. 6D), suggesting a potential cross talk between Smad and growth factor signals, as reported in other cell systems (50). In contrast, the MAPK pathway appears to be directly involved in the EMT process driven by TGFβ1 in MDCK
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cells. This conclusion is supported by the strong and sustained activation of ERK1/2 after TGFβ1 treatment, the blockade of the phenotypic effects of the growth factor by the MEK1/2 inhibitor PD98059 and, more significantly, from the studies on the Snail promoter. Even the basal activity of the Snail promoter is inhibited by PD98059, suggesting the requirement of active MAPK for expression of Snail promoter in MDCK cells. An active MAPK pathway is also required for the induction of Snail promoter by activated H-Ras, as deduced from the studies with PD98059 and specific RasV12 mutants (Figs. 6 and 7). The PI3K pathway, although apparently not required for the activity of the basal Snail promoter, it is needed for Snail promoter activation by oncogenic H-Ras, alone or in cooperation with TGFβ1(Figs. 6 and 7). These results are in agreement with the observed activation of the PI3K pathway after TGFβ1 treatment (Fig.3), and with recent findings indicating that PI3K activity is necessary for cell scattering and survival after TGFβ1 treatment in other cell systems (36, 38), and for the maintenance of the fibroblastic phenotype in H-Ras transformed murine hepatocytes (35). Taken together, our results support a major role for the MAPK pathway in TGFβ1-mediated induction of Snail promoter, and the cooperation between MAPK and PI3K pathways in the synergistic induction of Snail mediated by TGFβ1 and activated H-Ras. The participation of MAPK pathway into the EMT and invasive phenotype of MDCK cells has been previously reported either in stable transfectants with an activated MEK1 version (42) or by using an inducible form of c-Raf (Raf-ER) which also led to the autocrine production of TGFβ (53). This latter report established a strong link and synergism between TGFβ and the Raf-MAPK pathway in the promotion of invasiveness and in vivo malignancy. The requirement of TGFβ signaling for invasiveness and metastasis has also been previously established in the EpRas cell system (58).

A large body of evidence strongly supports that TGFβ acts as stimulator of malignant progression in late stages of carcinogenesis (reviewed in refs. 59, 60). The results presented here provide the first evidence to link TGFβ1 signaling to Snail repressor and EMTs, further reinforcing
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the important role of this growth factor into the malignant progression. Furthermore, the cooperation between H-Ras and TGFβ1 in Snail promoter induction reported here can be of biological significance, since activating mutations of H-Ras are present in a high number of tumors and can eventually contribute, together with acquired resistance to the anti-proliferative effects of TGFβ, to the malignant conversion. Interestingly, H-Ras activation can lead to the autocrine production of TGFβ in various cell systems (35, 53). These findings, together with the overproduction of TGFβ observed in a high percentage of human tumors (61) and the fact that most tumors maintain a functional TGFβ signaling system (59, 60), further reinforce the cooperation between H-Ras and TGFβ signals in malignancy. Our present results add a further step into the mechanisms of tumor progression, linking TGFβ signaling and oncogenic Ras activation to induction of the promoter of invasion Snail.

The promoter region of Snail transcription factor contains several potential control elements for H-Ras and TGFβ downstream signals. The signals that are highly induced by H-Ras seem to activate the minimal promoter region of Snail near the initiation site. In contrast, this proximal region is not sensitive for TGFβ1 signals, indicating that transduction of the different signals could require the coordination of several response elements in the Snail promoter. On other hand, the central region of Snail promoter (from –900 to –575 bp) appears to negatively regulate its basal expression and the signal-mediated induction, suggesting the presence of negative regulators in this region. It is tempting to speculate that those putative control elements can be involved in the fine regulation of Snail expression in normal biological process. Although further studies are clearly required to characterize the specific control elements and transcription factors responsible of Snail expression in different biological situations, the results reported here can contribute to a better understanding of the molecular mechanisms of malignant progression, involving some relevant regulators, such as H-Ras, TGFβ, and Snail. They also open the way to future studies in which positive regulators of EMT should be considered as promising targets for new anti-tumor therapies.
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REFERENCES

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ABBREVIATIONS

The abbreviations used are: AP-1, activator protein 1; EMTs, epithelial-mesenchymal transitions; ERK, extracellular regulated kinase; FGF, fibroblast growth factor; GADPH, glyceraldehyde-3-phosphate dehydrogenase; H-Ras, Harvey-ras; MAPK, mitogen-activated protein kinase; MDCK, Mardin-Darby canine kidney; MEK, mitogen-activated protein kinase kinase; mRNA, messenger ribonucleic acid; PI3K, phosphatidylinositol 3OH-kinase; RT-PCR, reverse transcription-polymerase chain reaction; TGFβ, Transforming growth factor beta; Wnt, wing signalling pathway.

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FIGURE LEGENDS

Figure 1. TGFβ1 induces cell scattering and increased cell motility in MDCK cells. (A). (a-c) Phase-contrast images of living cultures of (a) control untreated MDCK cells and MDCK cells treated for 24 h with (b) TGFβ1 (10 ng/ml) or (c) TGFβ1 (10 ng/ml) and FGF2 (100 ng/ml). (d-f) Phase contrast images of live MDCK cells after 1 h treatment with PD98059 (10 µM) (d), and pretreated with PD98059 1 hour before addition of (e) TGFβ1 (10 ng/ml) or (f) TGFβ1 (10 ng/ml) and FGF2 (100 ng/ml). (B). (a-h) Phase-contrast images of living cultures of a MDCK cells in a wound healing assay performed on cells grown in (a, b, e, f) the absence and (c, d, g, h) presence of TGFβ1 (10 ng/ml). Immediately after incision of the culture, cells were pretreated with (b, d, f, h) PD98059 (10 µM) or (a, c, e, g) DMSO vehicle for 1h before addition of TGFβ1. Photographs were taken just after incision (a-d) (0h) and (e-g) 24 h post-incision (24 h).

Figure 2. TGFβ1 treatment induces proliferation arrest and transcriptional responses in MDCK cells. (A). 3H-Thymidine incorporation assay of MDCK cells grown in the absence (-) and presence of TGFβ1 (+). The indicated number of cells were treated with TGFβ1 (10 ng/ml) for 24 h and incubated with 3H-Thymidine during the last 5h. 3H-Thymidine incorporation is represented as the relative proliferation index referred to control untreated MDCK cells. (B). Transcriptional response of MDCK cells to TGFβ1 treatment. Cells were transiently cotransfected with 500 ng of the 3TP-Lux reporter plasmid and 20 ng of TK-renilla plasmid in T24 well plates. Cells were grown in FBS-free medium during 24h after transfection, and then treated with TGFβ1 (10 ng/ml) for 6 or 20h. Luciferase and renilla activities were determined, and the promoter activity represented as the relative activity detected in control untreated cells.

Figure 3. Activation of the MAPK and PI3K signaling pathways by TGFβ1 treatment of MDCK cells. MDCK cells were treated with TGFβ1 (10 ng/ml) for the indicated time periods. Whole cell extracts were obtained and analyzed for total protein levels and phosphorylated forms of
ERK1/2 and AKT using appropriated specific antibodies. α-tubulin levels were also determined as a loading control. p44 and p42, ERK1 and ERK2, respectively; P-p44 and P-p42, phospho-ERK1 and phospho-ERK2, respectively; P-AKT, phospho-AKT.

**Figure 4. TGFβ1 induces Snail expression and represses E-cadherin in MDCK cells.** (A). RT-PCR analysis of the levels of endogenous canine Snail and E-cadherin transcripts in untreated MDCK cells (0h) and after the indicated time points of TGFβ1 treatment. The expression of GAPDH transcripts was analyzed in the same samples as a control for the amount of cDNA present in each sample. (B). MDCK cells were transiently cotransfected with 200 ng of the proximal mouse E-cadherin promoter (-178 bp) wt (left panel) or mutated (mut) in E-pal element (right panel) fused to the Luciferase reporter gene (17) and 20 ng of TK-renilla plasmid in T24 well plates. Cells were grown in FBS-free medium during 24h after transfection and were treated with 10 ng/ml TGFβ1 for 24, 48, and 72h. Luciferase and renilla activities were determined and the promoter activity was normalized to that obtained in the absence of treatment. (C). Western blot analysis of epithelial and mesenchymal protein markers in MDCK-treated cells with TGFβ1 (10 ng/ml) at the indicated time points. α-tubulin was used as loading control.

**Figure 5. TGFβ1 induces EMT in MDCK cells concomitantly with the loss of epithelial markers, and expression of mesenchymal markers.** (A). (a-d), Immunofluorescence images of MDCK cells showing the localization and organization of E-cadherin in (a) control untreated cells, and (b-e) cells treated for 72h with (b) TGFβ1 (10 ng/ml), (c) FGF2 (100 ng/ml), (d) TGFβ1 (10 ng/ml) and FGF2 (100 ng/ml), (e) pretreated with PD98059 (10 µM) 1 h before addition of TGFβ1 (10 ng/ml), and (f) treated with PD98059. (B). (a-l), Immunofluorescence images of MDCK cells showing the localization and organization of the indicated markers, before (a, d, g, j), and after (b, e, h, k) 48 h treatment with TGFβ1 (10 ng/ml). Cells shown in panels c, f, i and l were pretreated 1h with PD98059 before TGFβ1 addition. CK8; cytokeratin-8, Vim; vimentin, Fn; fibronectin and F-
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act; Fibrillar-actin. Arrows in panel k indicate reorganization of F-actin at apparent stress fibres and lamelipodia.

**Figure. 6.** Growth factor-mediated induction of *Snail* promoter is blocked by MEK 1/2 inhibitor but not by a negative dominant mutant of Smad4. (A) MDCK, or (B) PDV and MCA3D cells were transiently cotransfected with 200 ng of the full length *Snail* promoter construct (-900 bp) fused to the *Luciferase* reporter gene (17) and 20 ng of TK-*Renilla* plasmid in T24 well plates. Cells grown in FBS-free medium during 24 h after transfection were treated with the indicated amounts of TGFβ1 for additional 24 h. Luciferase and renilla activities were determined and the promoter activity was normalized to that obtained in the absence of treatment. (C). MDCK cells were transiently transfected with the –900 bp *Snail* promoter construct and treated with TGFβ1 (10 ng/ml) and/or FGF2 (100 ng/ml) for additional 24h; when indicated cells were pretreated for 1h with PD98059 (10 μM). (D). MDCK cells cotransfected with the -900 bp *Snail* promoter construct and 500 ng of Smad4DN (1-514) expression vector, and treated with TGFβ1 (10 ng/ml), or TGFβ1 (10 ng/ml) plus FGF2 (100 ng/ml), as indicated. (E). Cells were cotransfected with 500 ng of the 3TP-Lux reporter plasmid and 500 ng of Smad4DN (1-514) expression vector, and treated with TGFβ1 (10 ng/ml), as indicated. Luciferase and Renilla activities were determined 24h after growth factor treatment. The activity of the promoter is expressed relative to that obtained in the presence of empty control plasmid and/or in the absence of treatment.

**Figure. 7.** HRasV12 and TGFβ1 cooperate in the induction of the *Snail* promoter via PI3K and MAPK pathways. (A, B) The activity of the -900 bp *Snail* promoter was measured in MDCK cells after cotransfection with 500 ng of HRasV12 or H-RasN17 expression vectors without (A), and with (B) cotreatment with TGFβ1 for 24h. When indicated, cells were pretreated for 1h with PD98059 (10 μM) or LY294002 (30 μM) inhibitors before TGFβ1 treatment. (C, D) The activity of the -900 bp *Snail* promoter was measured in MDCK cells after cotransfection of 500 ng of...
HRasV12 or the indicated HRasV12 effector mutants without (C) and with (D) cotreatment with TGFβ1 (10 ng/ml). Cells were transiently cotransfected in FBS-free medium with the indicated HRasV12 mutants; 24h after transfection and when indicated were treated with TGFβ1 (10 ng/ml) for additional 24h. Luciferase and renilla activities were determined 24h after the growth factor treatment. The activity of the promoter is expressed relative to that obtained in the presence of empty control plasmid and in the absence of treatment (Mock).

**Figure. 8.** HRasV12 and TGFβ1 response elements in Snail promoter. (A). Schematic representation of the mouse *Snail* promoter indicating the position of potential regulatory control elements. (B). Left side, schematics of the deletion mutant constructs generated; right side, diagram showing the relative promoter activity of the different constructs detected in control non-stimulated cells (basal, black bars), in the presence of TGFβ1 treatment (TGFβ, light grey bars) or after HRasV12 cotransfection (RasV12, white bars). Cells were transiently cotransfected with 200 ng of the indicated *Snail* promoter constructs and 20 ng of pTK-renilla. When indicated cells were either treated with TGFβ1 (10 ng/ml) for 24h, or cotransfected with 500 ng of pLXSNHRasV12 vector. Luciferase and renilla activities were determined as in Figs. 6 and 7. The activity of each promoter construct is represented relative to that obtained in the presence of empty control plasmid and in the absence of TGFβ1 treatment.
Fig. 1. Peinado et al. TGFβ induces Snail transcription factor.
Figure 2. Peinado et al.

TGF-β1 (10 ng/ml) affects proliferation index (3H-thymidine incorporation).

3TP-lux promoter

Peinado et al.
TGFβ induces Snail transcription factor.
Fig. 4. Peinado et al.

E-cadherin promoter activity (RLU) in LAR/RAR cells treated with TGFβ1 (10 ng/ml) for 24, 48, and 72 hours. E-cadherin expression is reduced in the presence of TGFβ1 compared to controls.

- GTPase
- Vimentin
- Cytokeratin-8

E-cadherin
Snail

TGFβ induces Snail transcription factor.
Fig. 5. Peinado et al.

TGFβ induces Snail transcription factor
Fig. 6. Peinado et al.

TGFβ1 (10 ng/ml) decreases Snail promoter activity.

A.

B.

C.

Snail promoter activity

RLU (LAR/RAR)

TGFβ1 (10 ng/ml)

Snail promoter activity

RLU (LAR/RAR)

Snail promoter activity

RLU (LAR/RAR)

TGFβ induces Snail transcription factor.
Snail promoter activity
RLU (LAR/RAR)

TGFβ1 (10 ng/ml)
FGF2 (100 ng/ml)

Smad-4 DN

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+
-

+++
Fig. 7. Peinado et al.

TGFβ induces Snail transcription factor activity.

Snail promoter activity (RLU [LAR/RAR])

- Mock
- TGFβ
- HRas V12
- HRas N17
- HRas V12 + PD
- HRas V12 + LY

Increased Snail promoter activity in response to TGFβ.
Fig. 7. Peinado et al.

Snail promoter activity

RLU (LAR/RAR)

Mock

+TGFβ

Snail promoter activity

RLU (LAR/RAR)

Mock

HRas

V12

S35

G37

C40

TGFβ induces Snail transcription factor
Fig. 8. Peinado et al.