The Snail transcription factor has been described recently 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 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 β-1 (TGFβ1) induces Snail expression in Madin-Darby canine kidney cells and triggers epithelial-mesenchymal transitions by a mechanism dependent on the MAPK signaling pathway. Furthermore, TGFβ1 induces the activity of Snail promoter, whereas fibroblast growth factor-2 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 phosphatidylinositol 3-kinase activities. These data support that MAPK and phosphatidylinositol 3-kinase signaling pathways are implicated in TGFβ1-mediated induction of Snail promoter, probably through Ras activation and its downstream effectors.

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 that 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 conformation 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 down-regulation 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), whereas the majority of carcinomas with down-regulated 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 down-regulation in most carcinomas (5, 13). Several transcriptional repressors of E-cadherin have been isolated recently, including the zinc finger factors Snail (14, 15) and Slug (16, 17), the two-handed zinc factors 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 are proposed to act as inducers of the invasion process (14, 22). Generation of Snail knockout mice has further established the role of this factor in EMT and as the 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 that 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 in both epithelial cell systems and in embryonic development. Studies on development have indicated the participation of several members of the transforming growth factor (TGFβ)/bone morphogenetic family of growth factors in specific EMT processes in different species (24, 25), whereas fibroblast growth factor (FGF) signaling has been reported recently (26) as a determinant for mesoderm cell fate specification in the mouse embryo. Several studies have also indicated that a multiple cross-talk among TGFβ/bone morphogenetics, FGF, and Wnt signals could be required for some EMTs in development (26–28). In epithelial cell systems, several
eral growth factors have been widely studied and reported to induce a scattering phenotype or a complete EMT depending on the specific cell system analyzed (reviewed in Refs. 6 and 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 analyzed previously with somewhat contradictory results as regard to the specific implication of Smad, mitogen-activated protein kinase (MAPK) and/or phosphatidylinositol 3-kinase (PI3K) pathways (36, 40–43). The issue has been unraveled recently (36) in the EpRas model with the implication of MAPK in TGFβ-induced EMT, tumorigenesis, and metastasis, whereas PI3K is involved in cell scattering and resistance to TGFβ induced apoptosis. 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 Dulbecco’s modified Eagle’s medium and MCA3D and PDV cells in Ham’s F-12 medium, in the presence of 10% FBS, 10 mM glutamine (Invitrogen), and 100 μg/ml of ampicillin, 32 μg/ml of gentamicin (Sigma). 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 of stock of recombinant TGFβ1 (BioNovo Corp.) and 100 μg/ml of FGF2 (Pepro Tec) were prepared according to manufacturer’s instructions and added to the indicated concentrations. The PI3K and MEK1/2 inhibitors, LY294002 and PD98059 (Calbiochem), were used as vehicle control in all the inhibitor treatments.

**Western Blots**—Whole cell extracts of control and treated cells were obtained by lysis in radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM dithiothreitol), containing the appropriate protease and phosphatase inhibitors, by Western blot and enhanced chemiluminescence detection as described previously (14, 17, 20). Antibodies included goat anti-AKT (1:1000) (Santa Cruz Biotechnology, Inc.), rabbit anti-phospho (Ser-465/Ser-467)-Smad2/3 (1:500) (Upstate Biotechnology), anti-ERK1/2 and anti-phospho (Thr-202/Tyr-204)-ERK1/2 (1:1000) (Cell Signaling Technology), and rabbit anti-Smad2/3 and rabbit anti-Phospho Smad2/3 (Ser-466/467) (1:200) (Upstate Biotechnology). Secondary antibodies were goat anti-rabbit IgG (1:2000) (Progen), rabbit polyclonal anti-fibronectin (1:100) (Dako), mouse monoclonal anti-cytokeratin 8 (1:200) (Dako), rat monoclonal anti-E-cadherin ECCD-2 (1:100) (provided by A. Ben-Ze’ev, Weizmann Institute) and Lef-1 (provided by H. Clevers, Utrecht University Hospital) 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 (–20°C, 30 s) and stained for E-cadherin, vimentin, cytokeratin-8, and fibronectin as described previously (14, 17, 20). For F-actin staining, cells were fixed in 3.7% formaldehyde, 0.5% Triton X-100 for 30 min at room temperature, stained with tetramethyl rhodamine isothiocyanate-conjugated phallidin (Sigma) and washed four times in phosphate-buffered saline. The cells were mounted on Mowiol, and the preparations were visualized using a Leica confocal TCS SP2 microscope. For Western blot, whole cell extracts of control and treated cells were obtained in radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM dithiothreitol), containing the appropriate protease and phosphatase inhibitors, by Western blot and enhanced chemiluminescence detection as described previously (14, 17, 20). Primary antibodies included rat monoclonal anti-E-cadherin ECD-2 (1:100) (provided by M. Takeichi, Kyoto University), 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). For cell signaling analysis, Western blots were carried out on cell extracts obtained by lysis in Buffer A (20 mM Heps, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM MgCl2, 1% Nonidet P-40, 1 mM dithiothreitol), containing the appropriate protease and phosphatase inhibitors, during 30 min, at 4°C. Primary antisera included goat anti-AKT (1:1000) (Santa Cruz Biotechnology, Inc.), rabbit anti-phospho (Ser-473)-AKT (1:500), rabbit anti-ERK1/2 and anti-phospho (Thr-202/Tyr-204)-ERK1/2 (1:1000) (Cell Signaling Technology), and rabbit anti-Smad2/3 and rabbit anti-Phospho Smad2/3 (Ser-466/467) (1:200) (Upstate Biotechnology). Secondary antibodies were goat anti-rabbit IgG (1:1000) (Amersham Biosciences), donkey anti-goat (1:1000) (Santa Cruz Biotechnology, Inc.), goat anti-rat (1:1000) (Pierce), and goat anti-rabbit (1:4000) (Nordic) IgG.

**Cell Proliferation Assays**—The indicated number of cells (2.5 × 105 or 5 × 105) were seeded in triplicate in 96-well plates in Ham’s F-12 medium in complete medium for 3 h. After washing in phosphate-buffered saline, TGFβ1 (10 ng/ml) in FBS-free medium was added, and the cells were grown for an additional 24 h. [3H]Thymidine was added during the last 5 h of treatment. The cells were collected using a cell harvester device, washed with Tris-acetate and TGFβ-tetradecanoylphorbol-13-acetate 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 Epa1 fused to Luciferase has been reported previously (17). Generation of full-length mouse Snail promoter construct (~900 bp) has also been described recently (17). Deletion constructs of the Snail promoter mutants were obtained by PCR amplification from the full-length (~900 bp promoter) with appropriate primers containing BamHI and KpnI restriction sites and the corresponding PCR products cloned into the same restriction sites in the pGL3-Luciferase vector.

To determine the activity of 3TP-Lux and the Snail promoter 2 × 105 cells 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 a dual-luciferase reporter assay kit (Promega). 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: pSmd4 DN (1–514) in pCMV5 vector (provided by J. Massagué, Sloan-Kettering Memorial Cancer Center) (44); pLSXSNHRasV12, pLSXSNHRasV17, and the different mutants of the E-cadherin promoter. Our recent results indicate that it is directly induced by TGFβ1 and that FGF2 and activated H-Ras cooperate with TGFβ1 in induction of the Snail promoter. The corresponding empty vectors, pLXSN, pCMV5, or pcDNA3 were used in control transfections and for normalization of the total amount of DNA.

**Migration Assays**—The migratory/motility behavior of MDCK cells was analyzed in in vitro wound healing assays as described previously (14, 17). Monolayers of confluent cultures were lightly scratched with a
phenotypic changes induced by TGF-α affect the phenotype of MDCK cells (data not shown). The treatment with TGF-α induced a milder effect, and treatment with FGF2 alone did not have any effect on the migration of untreated cells (Fig. 1B, f). Pretreatment with the PI3K inhibitor LY294002 (30 μM) followed by TGF-β1 treatment caused cell disintegration (data not shown), thus precluding further studies on the implication of PI3K in the phenotypic or migratory effects of TGF-β1.

Activation of the MAPK and PI3K pathways following TGFβ1 treatment is not because of 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. 2A). 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 analyzed the implication of MAPK and PI3K pathways in the phenotypic effects induced by TGFβ1 in MDCK cells, because they have been implicated previously (33, 35, 36, 40, 42, 43) in epithelial cell scattering induced in MDCK and in other cell systems by several growth factors. A 1-h 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

Fig. 1. TGFβ1 induces cell scattering and increased cell motility in MDCK cells. A, a–c, phase-contrast images of living cultures of control untreated MDCK cells (a) and MDCK cells treated for 24 h with TGFβ1 (10 ng/ml) (b) or TGFβ1 (10 ng/ml) and FGF2 (100 ng/ml) (c). d–f, phase contrast images of live MDCK cells after 1 h treatment with PD98059 (10 μM) (d) and treated with PD98059 1 h before addition of TGFβ1 (10 ng/ml) (e) or TGFβ1 (10 ng/ml) and FGF2 (100 ng/ml) (f). B, a–h, phase-contrast images of living cultures of MDCK cells in a wound healing assay performed on cells grown in the absence (a, b, c, and f) and presence (c, d, g, and h) of TGFβ1 (10 ng/ml). Immediately after incision of the culture, cells were pretreated with PD98059 (10 μM) (b, d, f, and h) or Me2SO vehicle (a, c, e, and g) for 1 h before addition of TGFβ1. Photographs were taken just after incision (0 h) (a–d) and 24 h post-incision (24 h) (e–g). 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 36 h 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/bone morphogenetic signaling pathway to the regulation of EMT both during embryonic development (24, 25) and in some epithelial cell lines (30, 32, 33, 37), whereas 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 h of 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 h after incision of the wound, MDCK cells growing in the presence of TGFβ1 started to colonize the wound surface, whereas control untreated cells hardly started to migrate (data not shown). The differences in cell motility were evident 24 h 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

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TGFβ1 treatment of MDCK cells was confirmed by Western blot analyses of phosphorylated ERK1/2 and AKT, respectively, using phosphospecific antibodies to both effectors. As shown in Fig. 3, increased levels of phospho-ERK2 (P-p42) were detected after 30 min of TGFβ1 treatment, peaking after 1 h and slowly decreasing thereafter (Fig. 3, upper panels). A similar kinetics was observed in the levels of phospho-ERK1 (P-p44), although to a lesser extent. Interestingly, increased levels of phospho-ERK1/2 were detected even after 6 h 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 first detected after 1 h of TGFβ1 treatment, peaked by 3 h, and decreased thereafter (Fig. 3, middle panels). TGFβ1 treatment also induced a fast and sustained activation of the Smad pathway in MDCK cells, because P-Smad2 was detected after 15 min and was maintained up to 3 h of TGFβ1 treatment (data not shown).

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 with 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 h of 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 72 h of treatment. Analysis of E-cadherin mRNA levels showed no significant changes after 24 h of TGFβ1 treatment, but a marked decrease was detected after 48 and 72 h of TGFβ1 treatment, when E-cadherin mRNA was almost undetectable (Fig. 4A). Densitometric analyses showed that by 72 h 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 72 h of TGFβ1 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), because 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 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 72 h of TGFβ1 treatment (Fig. 4C).
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-life of the E-cadherin protein, estimated in more than 40 h 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 whether 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 48 h of treatment with TGFβ1 led to a redistribution of E-cadherin from the cell-cell contacts to the cytoplasm (data not shown). By 72 h 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 h of 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 interme-
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 indicated previously (26), FGF signaling has been implicated recently in the regulation of Snail expression during embryonic development, and previous work (51) in epithelial NBT-II cells also suggested its involvement in the regulation of Slug (a closely related homolog of Snail). 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 (52) showing that integrin linked kinase-induced activation of the human Snail promoter in colon cancer cells is independent of the β-catenin/Tcf complex.

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 involved directly 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, because it has been implicated recently (36, 53) in TGFβ signaling in other contexts. 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% (see Fig. 6C and Fig. 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...
FIG. 6. Growth factor-mediated induction of Snail promoter is blocked by MEK1/2 inhibitor but not by a dominant negative mutant of Smad4. MDCK (A) or PDV and MCA3D (B) 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 TR-Nulla plasmid in 24-well plates. Cells grown in FBS-free medium during 24 h after transfection were treated with the indicated amounts of TGFβ1 for an 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 an additional 24 h; when indicated cells were pretreated for 1 h 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 24 h 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.

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. 7, A and C), similar to what was observed in the presence of TGFβ1 (Fig. 7, B and D), whereas 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 (Fig. 7, B and 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 pro-
moter, whereas 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~/933 position (from the ATG start codon), because 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 the −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 the −675- and −900-bp positions of the Snail promoter.

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 studied previously 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.

**DISCUSSION**

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 proposed recently (36) for the definition of a complete EMT process. 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 (30, 35, 50, 53) on MDCK cells and other non-transformed epithelial cell types. 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 EpH4 cells in which TGFβ is not able to induce by itself an EMT process (33), indicating that the sensitivity and phenotypic response to TGFβ can be modulated by the specific epithelial cell type.

Our present results provide the following evidence supporting that Snail is mediating the EMT triggered by TGFβ1 in MDCK cells. (a) 24-h 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 72 h of treatment. (b) The Snail promoter activity is induced to a similar level after 24 h of TGFβ1 treatment. (c) The increase and maintenance of Snail transcripts after TGFβ1 treatment is correlated with the reduction of E-cadherin mRNA levels and promoter activity detected between 48 and 72 h 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 72 h 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 TGFβ1 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 72 h of TGFβ1 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 72 h 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, which could be later sustained by effective repression of E-cadherin mRNA following TGFβ1 treatment. Furthermore, Snail might regulate other genes required, in conjunction with E-cadherin down-regulation, for the EMT process. Indeed, Snail-mediated repression of cytokeratin 18 has been reported recently 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.

A direct effect of TGFβ1 signaling in Snail expression is supported by our analysis of the mouse Snail promoter, because 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, because FGF2 alone was unable to induce significant phenotypic changes or decreased E-cadherin organization at the cell-cell contacts (see 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 reported previously (33, 36) 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.

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 cannot be presently discarded, because 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 crosstalk 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 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 (see Figs. 6 and 7). The PI3K activity, although apparently not required for the activity of the basal Snail promoter, is needed for Snail promoter activation by oncogenic H-Ras, alone or in cooperation with TGFβ1 (see 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 reported previously 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 established previously (58) in the EpRas cell system.

A large body of evidence strongly supports that TGFβ acts as stimulator of malignant progression in late stages of carcinogenesis (reviewed in Refs. 59 and 60). The results presented here provide the first evidence to link TGFβ1 signaling to Snail repressor and EMTs, further reinforcing the important role of

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this growth factor into the malignant progression. Furthermore, the cooperation between H-Ras and TGFβ in Snail promoter induction reported here can be of biological significance, because 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 in fibrosarcoma lead to the autocrine production of TGFβ in various cell systems (35, 53). These findings, together with the over-production 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 TGFβ signals, indicating that transduction of the different signals could require the coordination of several response elements in the Snail promoter. On the 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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