Snail1 suppresses TGF-β-induced apoptosis and is sufficient to trigger EMT in hepatocytes

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Summary

Although TGF-β suppresses early stages of tumour development, it later contributes to tumour progression when cells become resistant to its suppressive effects. In addition to circumventing TGF-β-induced growth arrest and apoptosis, malignant tumour cells become capable of undergoing epithelial-to-mesenchymal transition (EMT), favouring invasion and metastasis. Therefore, defining the mechanisms that allow cancer cells to escape from the suppressive effects of TGF-β is fundamental to understand tumour progression and to design specific therapies. Here, we have examined the role of Snail1 as a suppressor of TGF-β-induced apoptosis in murine non-transformed hepatocytes, rat and human hepatocarcinoma cell lines and transgenic mice. We show that Snail1 confers resistance to TGF-β-induced cell death and that it is sufficient to induce EMT in adult hepatocytes, cells otherwise refractory to this transition upon exposure to TGF-β. Furthermore, we show that Snail1 silencing prevents EMT and restores the cell death response induced by TGF-β. As Snail1 is a known target of TGF-β signalling, our data indicate that Snail1 might transduce the tumour-promoting effects of TGF-β, namely the EMT concomitant with the resistance to cell death.

Key words: EMT, TGF-beta, Apoptosis

Introduction

Transforming growth factor beta (TGF-β) is an important suppressor factor in the adult liver, inhibiting hepatocyte DNA synthesis and inducing active cell death (Rossmanith and Schulte-Hermann, 2001). However, TGF-β overexpression is frequently observed in human hepatocellular carcinomas, suggesting that liver tumour cells, as with many other tumour cells, can overcome the suppressive effects of TGF-β (Breuhan et al., 2006; Massagué, 2008). Indeed, in foetal rat hepatocytes and hepatoma cells, TGF-β induces both cell death through a mitochondrial-dependent mechanism, and a survival response through the activation of the c-Src and epidermal growth factor receptor (EGFR) pathways (Herrera et al., 2001; Park et al., 2004; Murillo et al., 2005; Caja et al., 2007). Interestingly, cells that survive the apoptotic effects of TGF-β undergo epithelial-to-mesenchymal transition (EMT) (Valdés et al., 2002; Caja et al., 2007). EMT is an important physiological process during embryogenesis and wound healing by which epithelial cells lose their polarity and cell-cell adhesion molecules, subsequently expressing mesenchymal markers and becoming motile with scattering properties (Thiery et al., 2009). A closely related phenotypic conversion is also detected in fibrosis and neoplasia, which is associated with disease progression (Lopez-Novoa and Nieto, 2009). Members of the TGF-β family can initiate and maintain EMT in a variety of biological systems and pathophysiological situations, particularly through activation of major signalling pathways and transcriptional regulators integrated in extensive signalling networks (Zavadil and Bottinger, 2005; Heldin et al., 2009). Indeed, evidence points to the crosstalk between the genetic programs that control TGF-β-induced growth arrest and/or apoptosis and those that regulate EMT, as once the cell has adopted a mesenchymal phenotype, it does not respond to the TGF-β suppressor effects (Valdés et al., 2002; Gal et al., 2008).

The Snail family of zinc-finger transcription factors are excellent candidates to mediate the escape from the tumour suppressor effects of TGF-β as they are very potent inducers of EMT and well-known targets of TGF-β signalling in many cell types, including hepatocytes (Spagnoli et al., 2000; Gotzmann et al., 2002; Valdés et al., 2002; Thiery et al., 2009). In addition, Snail1 also confers resistance to the cell death induced either by the withdrawal of survival factors or pro-apoptotic stimuli (Barrallo-Gimeno and Nieto, 2005). Thus, we decided to analyze the role of Snail1 in the activity of TGF-β in the liver in order to assess its contribution to the loss of TGF-β tumour-suppressor effects.

Results

TGF-β-induced Snail expression in hepatocytes and hepatoma cells requires the activation of the TGF-β receptor 1 and the NF-kappaB pathway

It is known that TGF-β transiently induces *Snail1* expression in primary cultures of foetal rat hepatocytes (Valdés et al., 2002) (Fig. 1A). This effect can be impaired in the presence of SB431542 (Fig. 1B), a specific TGF-β receptor 1 (TGFβR1) inhibitor (Fig. 1C), confirming the implication of this receptor in *Snail1* induction. TGF-β-induced intracellular signals appear to act directly on the *Snail1* promoter as the induction of *Snail1* transcription did not require de novo protein synthesis (Fig. 1D). Many of the intracellular pathways examined did not appear to be implicated in this process, including the phosphatidylinositol-3 kinase, MEK/ERK, p38 and c-Jun-N-kinase pathways, as their inhibitors had no effect on TGF-β-induced *Snail1* upregulation

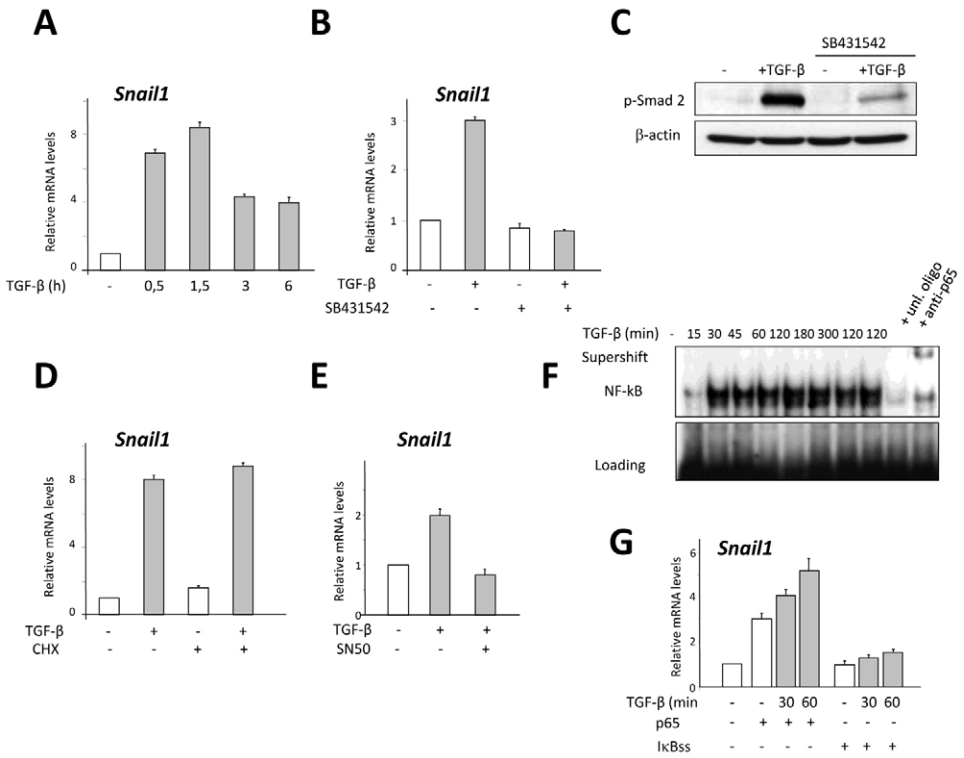

Fig. 1. TGF-β-induced Snail expression in hepatocytes requires the activation of TGF-β receptor 1 and the NF-κB pathway. (**A**) TGF-β induces *Snail1* expression in foetal rat hepatocytes analyzed by real time RT-PCR. Cells were maintained in the presence or absence of TGF-β (2 ng/ml) for the times indicated. (**B**) Expression of *Snail1* analyzed by real time RT-PCR in hepatocytes incubated in the presence or absence of TGF-β (2 ng/ml, 3 hours) and either untreated or pretreated (1 hour before TGF-β addition) with 10 μM SB431542. (**C**) Phosphorylation of Smad2 was analyzed in the presence or absence of TGF-β (2 ng/ml, 1 hour) and/or 10 μM SB431542 (TGF-β receptor 1 inhibitor). Protein extracts were collected to analyze pSmad2 in western blots. β-actin was used as a loading control. (**D**) As in B, but treated with 0.5 μg/ml of the protein synthesis inhibitor cycloheximide (CHX). (**E**) Inhibition of NF-κB activity by incubation with SN50 impairs the activation of *Snail1* transcription by TGF-β. Foetal rat hepatocytes were pretreated with SN50 (50 μM, 1 hour) and then stimulated with TGF-β (2 ng/ml) for 3 hours. *Snail1* mRNA levels were analyzed by real time PCR. (**F**) TGF-β activates NF-κB in foetal rat hepatocytes. EMSA analysis with nuclear protein extracts shows NF-κB DNA binding activity in cells maintained in the presence or absence of TGF-β (2 ng/ml). (**G**) Overexpression of p65 induces *Snail1* upregulation, which is prevented by the IκB super-repressor. A CMV-p65 expressing vector, ssIκB vector or an empty vector (pcDNA3) was transfected into foetal rat hepatocytes. After 36 hours, hepatocytes were stimulated with TGF-β (2 ng/ml) for the times indicated. *Snail1* mRNA levels were analyzed by quantitative RT-PCR and the transcription was normalized to *Gapdh* mRNA levels. Bars represent the s.e.m.

(supplementary material Fig. S1). However, SN50, a specific inhibitor of the nuclear translocation of p65, a functional subunit of the nuclear factor-kappaB (NF-κB) pathway, abolished the TGF-β-induced increase in *Snail1* transcripts (Fig. 1E). Interestingly, TGF-β activated NF-κB in foetal rat hepatocytes (Fig. 1F) and overexpression of p65 induced *Snail1* transcription, whereas abrogation of the NF-κB pathway by the IκB super-repressor prevented *Snail* induction by TGF-β (Fig. 1G).

As we have previously published (Caja et al., 2007), TGF-β can also induce a full EMT in liver tumour cells. Indeed, EMT occurs in FaO rat hepatoma cells concomitant with *Snail1* induction, involving the downregulation of E-cadherin expression and the induction of vimentin expression and that of other cytoskeletal markers (Fig. 2A; supplementary material Fig. S2). As in foetal rat hepatocytes, TGF-β-induced *Snail1* transcription does not appear to involve the phosphatidylinositol-3 kinase, MEK/ERK, p38 and c-Jun-N-kinase pathways (data not shown) and SN50 prevented the increase in *Snail1* expression, confirming that NF-κB is required (Fig. 2B). In agreement with this, knockdown of p65 with specific siRNA (*sip65-1*) abolished TGF-β-induced *Snail1* upregulation (Fig. 2C). We used a second sequence to interfere with p65 expression, obtaining similar results (*sip65-2*; data not shown). Because NF-κB appeared to be necessary for TGF-β-mediated *Snail1* induction in hepatocytes, we assessed whether it directly regulated *Snail1* transcription. Two putative NF-κB binding sites were found in the *Snail1* promoter that are conserved in the rat and human genomes (supplementary material Fig. S3) and, hence, we generated luciferase reporter constructs carrying the wild-type mouse *Snail1* promoter (–1000 pb) or a modified promoter in which each or both of the two κB-boxes was deleted (–672: κB1 and –162: κB2). When these constructs were transfected into FaO rat hepatoma cells along with a CMV-p65 expression vector, it was evident that although the κB1 box was dispensable, the κB2 was required for p65 to regulate *Snail1* promoter activity (Fig. 2D). Chromatin immunoprecipitation analysis carried out with an anti-p50 antibody confirmed that upon TGF-β stimulation, transcriptionally active NF-κB (p65/p50) is recruited to the κB2 box of the *Snail1* promoter (Fig. 2E).

Snail1 downregulation enhances the apoptotic response to TGF-β in immortalized murine hepatocytes

To explore the contribution of Snail1 to the effects of TGF-β in hepatocytes, we used specific siRNAs to knockdown *Snail1* expression. To favour the transfection efficiency, rather than using

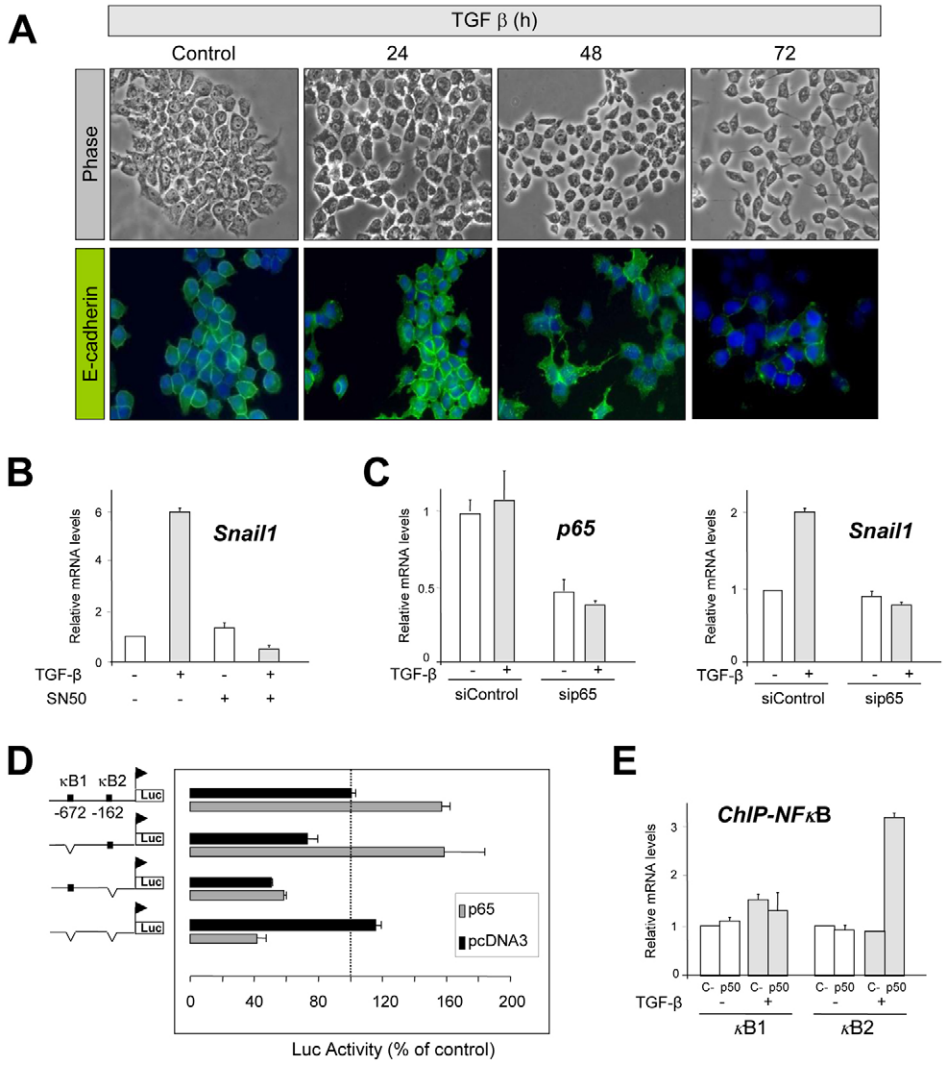

Fig. 2. *Snail1* transcription requires NF-κB binding to the *Snail1* proximal promoter in FaO rat hepatoma cells. (**A**) Phase-contrast images and immunofluorescence detection of E-cadherin in FaO cells treated with TGF-β (2 ng/ml). (**B**) RT-PCR analysis of endogenous *Snail1* expression in FaO cells, which were either untreated or pretreated with SN50 (50 μM, 1 hour) and then stimulated with TGF-β (2 ng/ml; T) for 3 hours. The expression of *Gapdh* was used as a control for the amount of cDNA template in each sample. (**C**) Knockdown of p65 with specific siRNA (rat *sip65-1*) inhibits TGF-β-induced Snail1 upregulation. Cells were transfected with either an unspecific siRNA (siControl) or the sip65-1 (100 nM) and then treated with TGF-β (2 ng/ml), as described in the Materials and Methods. Graphs represent real-time PCR for the analysis of *p65* (left) and *Snail1* (right) transcript levels. (**D**) NF-κB regulates the activity of the *Snail1* promoter. Luciferase reporter constructs carrying the wild-type mouse *Snail1* promoter (–1000 pb) or deletions in the κB-boxes were transfected into FaO cells together with a CMV-p65 expression vector or empty vector (pcDNA3) as a control. Luciferase activity was assayed 40 hours after transfection. Activity is expressed relative to that of the wild-type construct. (**E**) NF-κB is recruited to the *Snail1* promoter following TGF-β administration. FaO cells were maintained in the presence or absence of TGF-β (2 ng/ml) for 72 hours and processed for chromatin immunoprecipitation (ChIP) analyses as described in the Materials and Methods. P50 refers to the anti-p50 antibody. The experiment was repeated three times with similar results.

primary cultures, we used an immortalized cell line obtained from neonatal murine hepatocytes (González-Rodríguez et al., 2008) that responds to TGF-β in a very similar way to that found in foetal rat hepatocytes (our unpublished results) (supplementary material Fig. S4). As expected, the induction of *Snail1* by TGF-β was significantly reduced in the presence of siRNA for *Snail1* (Fig. 3C), the downregulation of E-cadherin expression was impaired and the EMT was prevented after 30 hours of treatment (Fig. 3A). We used a second sequence to interfere with Snail1 expression, obtaining similar results (si*Snail-3′*; data not shown; see Materials and Methods). TGF-β-treated control cells depicted a clear apoptotic process (Fig. 3D,E) that produced cell death at later times (Fig. 3B,F). Upon Snail downregulation, treatment with TGF-β induced a higher level of caspase 3 activity and apoptotic nuclei (Fig. 3D,E) that gave rise to a massive cell death after 48 hours (Fig. 3B,F). Interestingly, at this time, the only hepatocytes that survived were those showing a mesenchymal phenotype, indicative of those that, albeit probably reduced, still had enough levels of Snail and had finally undergone EMT (Fig. 3F; blue arrows in Fig. 3B). To confirm that the massive cell death observed in the presence of *Snail1* siRNA was specifically due to Snail1 downregulation and to show that Snail1 could prevent the death induced by TGF-β, we carried out a rescue experiment by transfecting cells with a construct containing the Snail1 coding region (Snail1CD). This construct was able to prevent the cell death induced by TGF-β (Fig. 3G). We then designed a *Snail1* siRNA specific for 3′ untranslated sequences (si*Snail1-3′*) so that it could not target Snail1CD. si*Snail-3′* reduced *Snail1* transcript levels to around 50% (Fig. 3H). When Snail1CD was expressed together with si*Snail1-3′*, it very significantly attenuated the action of the siRNA (Fig. 3G). This experiment (Fig. 3G,H) was carried out cotransfecting Snail1CD and/or si*Snail1-3′* together with a construct encoding GFP and all the calculations were made based on green fluorescent cells to avoid the interference that untransfected cells might have caused. These data confirm the specificity of the Snail1 knockdown experiments and indicate that Snail1 is sufficient to prevent the death induced by TGF-β in cultured hepatocytes.

In the presence of siRNA for *Snail1*, in addition to an increase in cell death, we observed an earlier and stronger upregulation of genes previously related to the apoptosis induced by TGF-β, such as the BH3-only genes *Bim* (*Bcl2l11*) and *Bmf* (Ramjaun et al., 2007) (Fig. 4A,B). Interestingly, Snail1 interference is sufficient to upregulate *Bim* expression, a BH3-only protein that responds to the absence of survival signals in general, whereas the expression of *Bmf* is significantly upregulated in response to TGF-β when

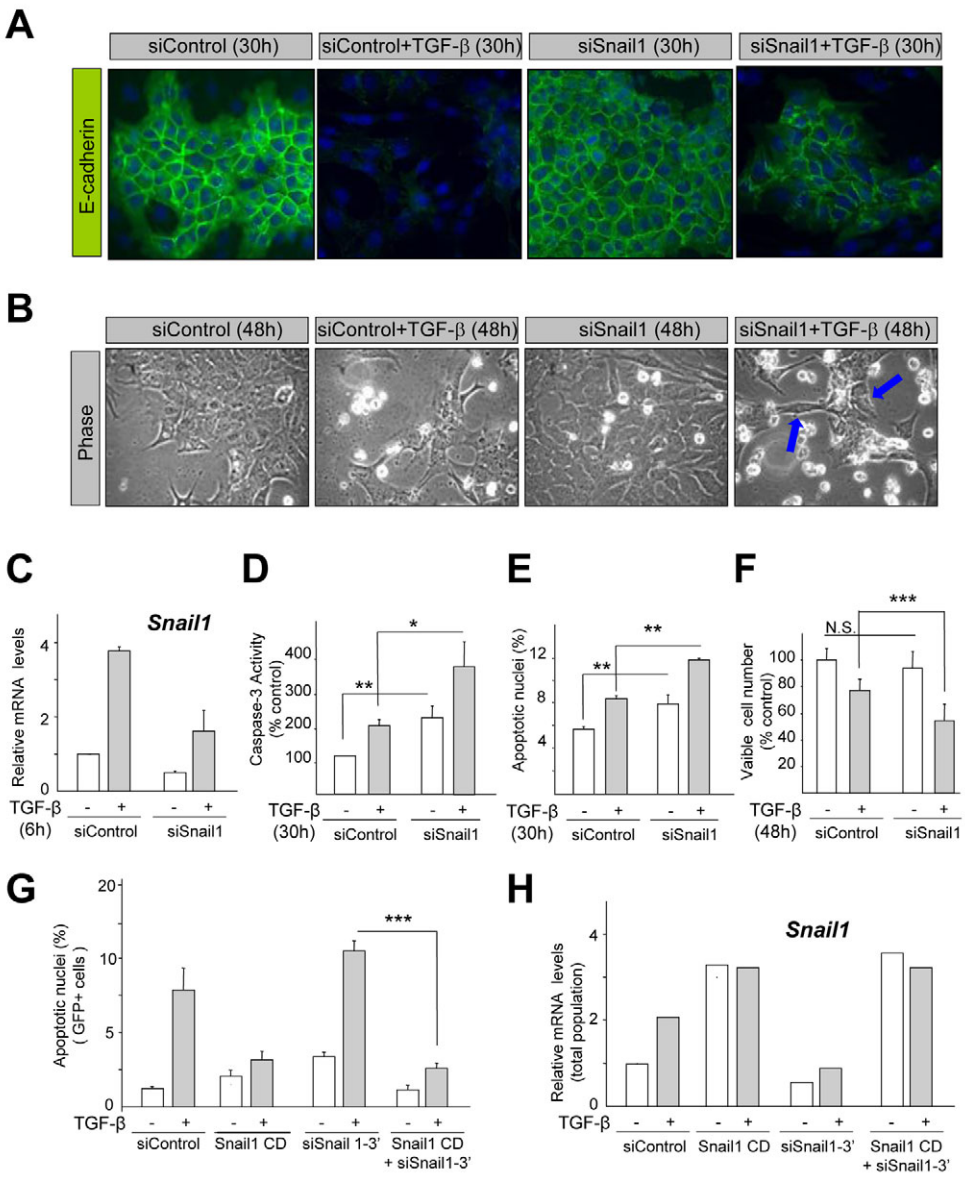

Fig. 3. Snail1 downregulation enhances the apoptotic response to TGF-β in immortalized murine hepatocytes. Cells were transfected with either an unspecific siRNA (siControl) or a *Snail1* siRNA (mouse si*Snail1-1*) and then treated with TGF-β (2 ng/ml) as described in the Materials and Methods. (**A**) E-cadherin expression in cell cultures fixed after 30 hours of TGF-β treatment. (**B**) Micrographs of similar cultures after 48 hours of TGF-β treatment. Arrows indicate examples of cells that had undergone EMT. (**C**) *Snail1* transcription levels, analyzed by real-time PCR after 6 hours of TGF-β treatment. (**D**) Caspase 3 activity 30 hours after TGF-β treatment, analyzed by fluorimetry and presented as a percentage of the control. (**E**) Apoptotic nuclei 30 hours after TGF-β treatment (percentage of condensed and/or fragmented nuclei assessed by Hoechst staining). (**F**) Cell viability after 48 hours treatment, analyzed by Crystal Violet staining and expressed as a percentage of the control. (**G**) A plasmid containing the coding region of Snail1 (Snail1CD) was able to prevent TGF-β-induced cell death and was also able to rescue the cell death observed after Snail1 downregulation (si*Snail1-3′*). (**H**) *Snail1* transcription levels, analyzed by real-time PCR after 6 hours of TGF-β treatment in the presence or in the absence of si*Snail3′* and/or Snail1CD. Data are means ± s.e.m. of at least three independent experiments. In E, 10-15 different fields per condition were counted in each experiment (n=3). (Student's t-test: *P<0.05; **P<0.005; ***P<0.0005.)

Snail is downregulated. TGF-β can also induce anti-apoptotic signals in foetal hepatocytes and liver tumour cells (Murillo et al., 2005; Caja et al., 2009), mediating an increase in the intracellular content of anti-apoptotic proteins of the Bcl2 family, BclxL and Mcl1. A similar response was observed in this murine neonatal liver cell line (Fig. 4C), a response that was impaired when Snail1 levels were decreased with si*Snail1* (Fig. 4C). Indeed, *Snail1* downregulation provokes changes in the TGF-β-mediated regulation of BclxL and Mcl1, favouring the increase in the ratio of the pro-apoptotic versus anti-apoptotic members. Coincident with these changes in gene expression, the percentage of cells exposed to TGF-β with activated Bax or Bak significantly increased in the presence of *Snail1* siRNA, indicating that Snail1 can counteract the mitochondrial-dependent apoptosis induced by TGF-β in hepatocytes (Fig. 4D).

Having confirmed that Snail1 plays an important role in the resistance to apoptosis induced by TGF-β, we examined whether it might also protect hepatocytes from death by anoikis. Indeed, a decrease in *Snail1* expression in immortalized murine hepatocytes impaired the TGF-β-induced cell adhesion in the absence of substrate. The number of adherent cells, reflecting their capacity to attach to untreated plastic, only increased on exposure of control hepatocytes to TGF-β but not in those with diminished *Snail1* expression (Fig. 5, left). Furthermore, when the cells that did not adhere were plated on cell culture dishes to analyze their capacity to survive for 48 hours in the absence of substrate, those cells transfected with specific *Snail1* siRNA were more susceptible to die than control cells, both in the presence or absence of TGF-β (Fig. 5, right).

Snail1 activation induces EMT and prevents the apoptotic effects of TGF-β in adult hepatocytes

TGF-β does not provoke an anti-apoptotic response in adult rat hepatocytes and it fails to induce *Snail1* expression and EMT in these cells (Caja et al., 2007). Thus, we assessed whether the lack of Snail1 activation was responsible for the deficient anti-apoptotic response to TGF-β by forcing *Snail1* expression in adult hepatocytes, in which it is normally silenced. Because adult

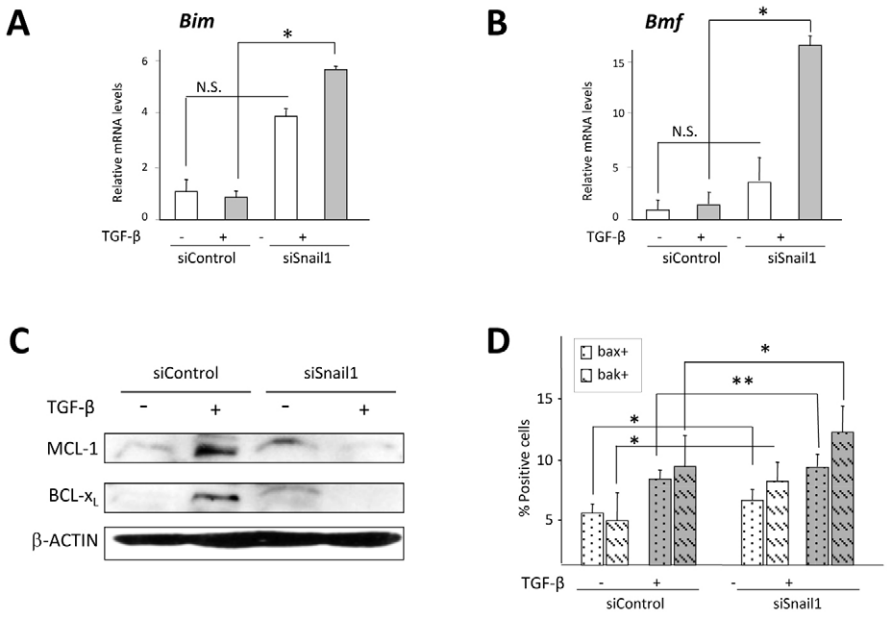

Fig. 4. Snail1 downregulation increases the mitochondrial-dependent apoptotic events. Cells were treated as described in Fig. 3. (**A**,**B**) Analysis of the levels of pro-apoptotic *Bim* and *Bmf* transcripts by real time RT-PCR after 3 hours of TGF-β treatment. Data correspond to five independent experiments. (**C**) Mcl1 and BclxL protein levels after 24 hours of TGF-β treatment. (**D**) Percentage of cells expressing the active form of Bax or Bak after 6 hours of TGF-β treatment and analyzed by immunofluorescence as described in the Materials and Methods. Data are means ± s.e.m. of a representative experiment (n=3), where 25 independent fields per condition were counted. (Student's t-test: *P<0.05; **P<0.005; N.S., non-significant.)

hepatocytes are very difficult to transfect in culture, we took advantage of a conditional transgenic mouse line in which Snail1 could be activated by tamoxifen administration (Snail-ER^{T2}) (De Frutos et al., 2007; De Frutos et al., 2009) (see Materials and Methods). We selected a line that expressed the transgenic protein in the liver (Fig. 6A) enabling us to study a virtually homogeneous population of hepatocytes that constitutively express exogenous Snail1 protein. In these cells, the protein is sequestered in the cytoplasm and it is thus inactive as a transcription factor. However, it becomes active after nuclear translocation upon tamoxifen administration (De Frutos et al., 2007; De Frutos et al., 2009; Feil et al., 1996).

Hepatocytes obtained from these Snail1-ER transgenic mice that were treated in vivo with tamoxifen adopted a mesenchymal phenotype (not shown), as did primary hepatocyte cultures established from untreated transgenic mice that were subsequently exposed to tamoxifen (Fig. 6B). This phenotypic change indicates that these cells had undergone a process of EMT following the nuclear translocation of Snail1 in the presence of tamoxifen (Fig. 6B). The Snail1-induced EMT was confirmed by the downregulation of E-cadherin expression both at the mRNA and the protein levels and by the reorganization of the F-actin filaments to form lamellipodia and ruffles even in the absence of TGF-β (Fig. 6C,D). We then wondered whether these hepatocytes were still able to respond to TGF-β in terms of growth arrest by examining the expression of the cell cycle inhibitor p21. We found that TGF-β can induce *p21* transcription regardless of the presence of activated Snail1, indicating that Snail1 overexpression is not interfering with TGF-β signalling and that TGF-β can still induce growth arrest independent of Snail1 expression (Fig. 6E). Furthermore, TGF-β was still able to interfere with the increase in cell number mediated by a mitogenic stimulus (EGF plus insulin) even in the presence of Snail1, although in this case, cell numbers were maintained at the control level owing to the effect of Snail1 in preventing cell death (Fig. 6F). The observation that TGF-β can still induce growth arrest in FaO rat hepatoma cells in the presence of *Snail1* siRNA as assessed in studies of BrdU incorporation (not shown) also reinforces the idea that TGF-β can induce growth arrest independent of Snail1. Conversely, although adult hepatocytes resist EMT in response to TGF-β (Caja et al., 2007), Snail1 activation is sufficient to elicit this

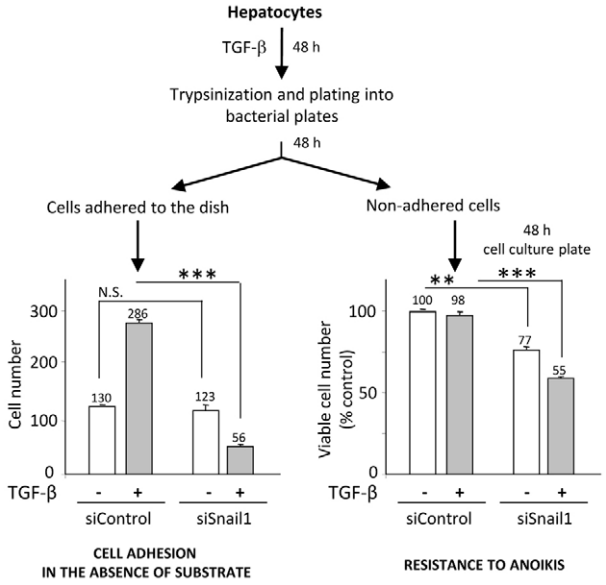

Fig. 5. Snail1 downregulation sensitizes immortalized murine hepatocytes to cell death by anoikis. Cells were transfected with either a non-specific siRNA (siControl) or a *Snail1* siRNA (si*Snail*) as described in the Materials and Methods and were then treated with TGF-β (2 ng/ml) for 48 hours. Cells were subsequently trypsinized and plated on bacteria dishes for 48 hours. Left: Number of cells that adhered to the bacteria dishes (counted after Crystal Violet staining), reflecting their capacity to attach on non-treated plastic. Data are means ± s.e.m. of a representative experiment (n=3; 25 independent fields). Right: Cells that did not adhere to the bacteria dishes were later plated at identical cell density on cell culture dishes. Viable cells were quantified after 48 hours (Crystal Violet staining and spectrophotometric analysis). si*Snail* cells were sensitized towards apoptosis in the absence of cell adhesion (anoikis). Data are means ± s.e.m. of a representative experiment (n=3). (Student's t-test: **P<0.005; ***P<0.0005; N.S., non-significant.)

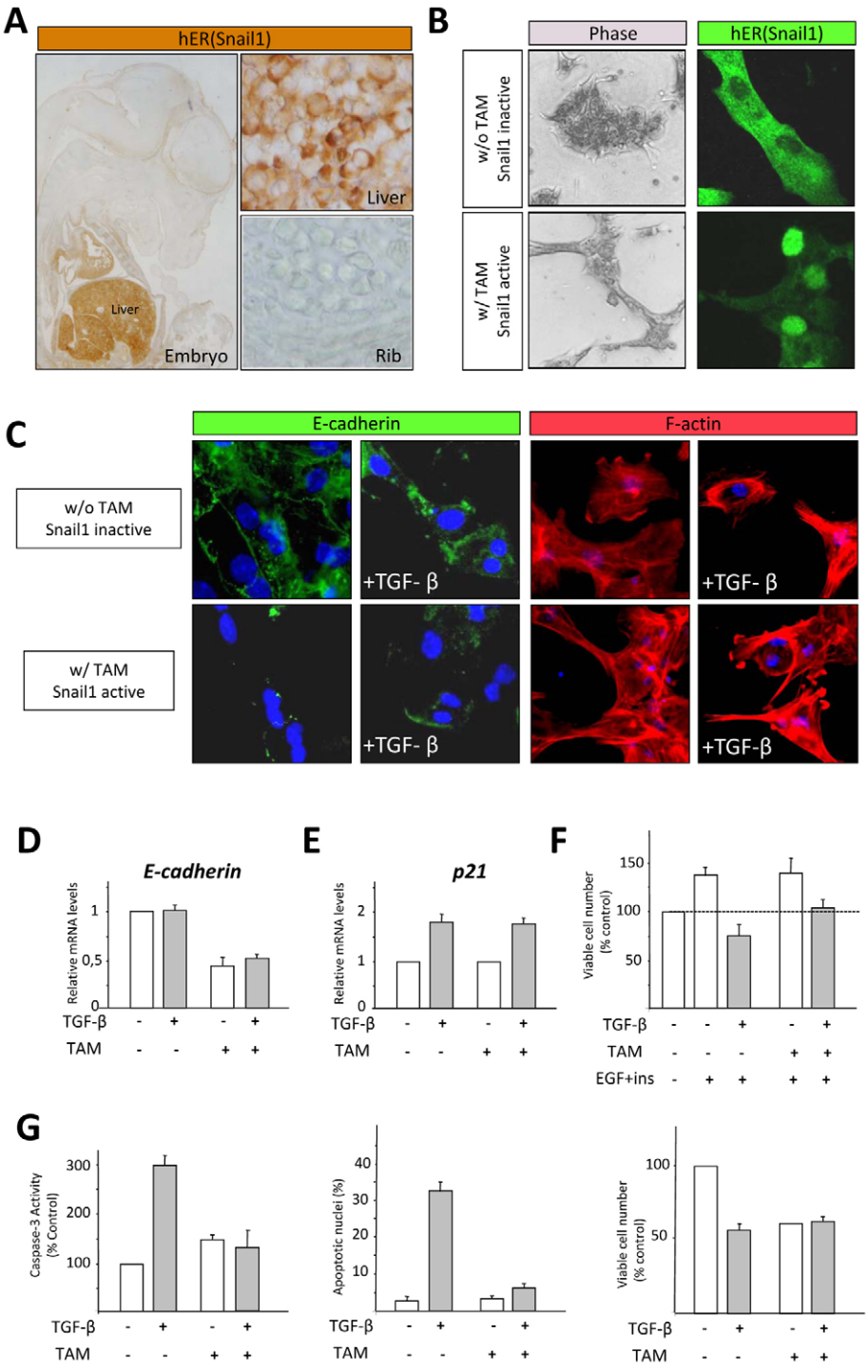

Fig. 6. Snail1 activation induces EMT and blocks the apoptotic effects of TGF-β in adult hepatocytes. (**A**) Snail1-ER fusion protein detected in a section of embryo at 14.5 days post-conception. High-power images of the liver show the presence of Snail1-ER transgenic protein in the cytoplasm of transgenic livers, evident as brown staining of the anti-human estrogen receptor antibody (hER). A photograph of a rib is shown as a negative control of non-expressing tissues. (**B**) Hepatocytes isolated from 2-month-old Snail1-ER transgenic mice were either untreated or treated with tamoxifen (TAM) for 72 hours and their response analyzed after a further 48 hours in the presence of TGF-β (2 ng/ml). Phase-contrast images showing the epithelial (parenchymal) or mesenchymal phenotype of hepatocytes, either treated or untreated with TAM. TAM treatment was accompanied by an efficient nuclear translocation of the Snail1-ER transgenic protein as assessed by immunoflourescence analysis with the same anti-hER antibody as in A. (**C**) TGF-β induces the loss of E-cadherin expression and the reorganization of the F-actin to form lamellipodia and ruffles only in TAM-pretreated hepatocytes and independent of TGF-β administration. (**D**,**E**) Analysis of *E-cadherin* and *p21* transcript levels by real time RT-PCR. (**F**) TGF-β impairs the mitogenic effect of epidermal growth factor (EGF) even in the presence of high levels of Snail1 expression. Ins, insulin. (**G**) Effect on apoptosis after 48 hours of treatment. Left: Caspase 3 activity analyzed fluorimetrically. Results are expressed as the mean ± s.e.m. percentage of their respective controls from five independent experiments. Middle: Percentage of apoptotic nuclei analyzed by microscopy observation of condensed and/or fragmented nuclei (Hoechst staining). A representative experiment is shown and the mean ± s.e.m. of ten different fields is represented. Right: Cell viability analyzed by Crystal Violet staining and expressed as a percentage of their respective control. Data are the mean ± s.e.m. of four independent experiments, in duplicate.

response and induce the morphological and gene expression changes associated with EMT. In addition to the EMT, striking changes were also observed in the apoptotic response. As such, caspase 3 activation was blocked when Snail1 was activated, concomitant with the absence of apoptotic nuclei, and TGF-β was unable to induce cell death although tamoxifen affected the basal levels of viable cells (Fig. 6G). These results indicate that Snail1 is sufficient to protect hepatocytes from cell death induced by TGF-β even in the context of normal adult hepatocytes, which are refractory to express Snail1.

Snail1 impairs the apoptotic effects of TGF-β in hepatocellular carcinoma cells

Once shown that Snail1 activation is sufficient to induce EMT and protect adult hepatocytes from TGF-β-mediated cell death, we wondered whether Snail1 could be responsible for the resistance to TGF-β-induced apoptosis in human hepatocellular carcinoma cells, which might be relevant for the progression of human liver tumourigenesis (Fabregat, 2009). We used the Hep3B cell line, which although maintaining the epithelial phenotype, respond much less to TGF-β in terms of apoptosis than adult hepatocytes (Caja

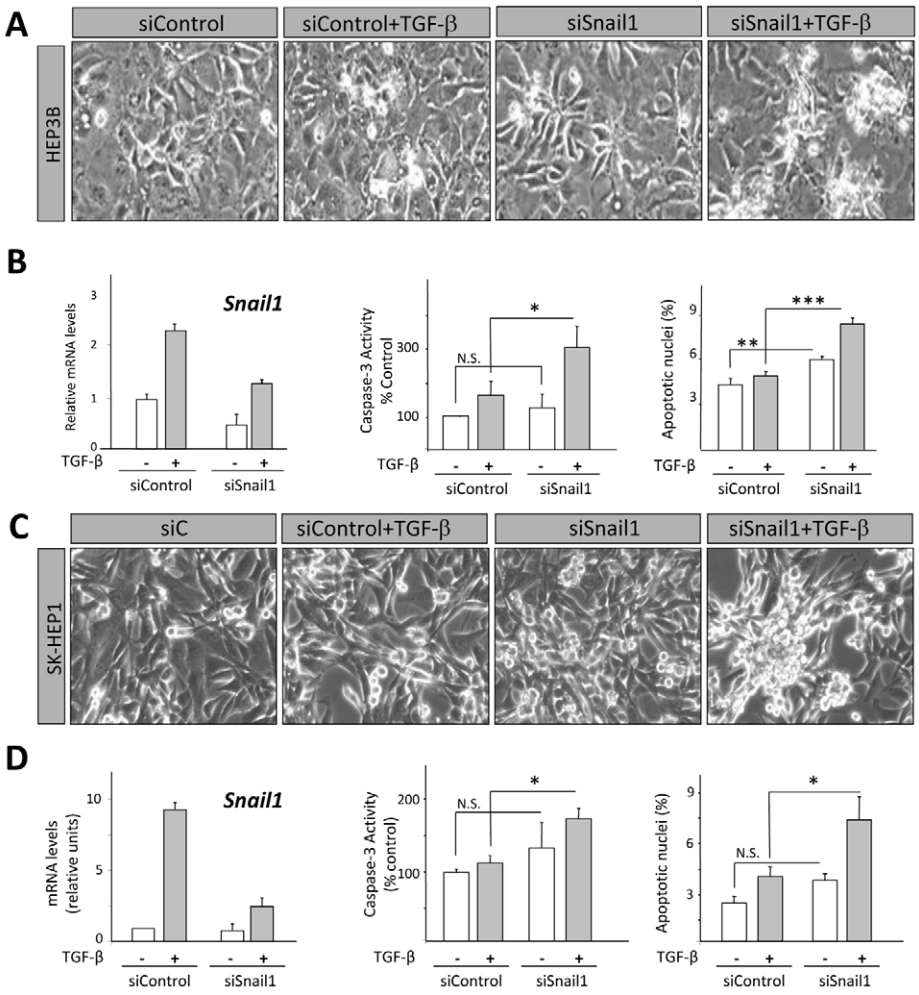

Fig. 7. Snail1 downregulation restores the apoptotic response to TGF-β in human liver tumour cells. Hep3B or SK-Hep1 cells were transfected with either an unspecific siRNA (siControl) or the specific *Snail1* siRNA (human si*Sna1-1*), as described in the Materials and Methods, and then were treated with TGF-β (2 ng/ml). (**A**,**C**) Photographs of Hep3B, SK-Hep1 cell cultures (36 hours of TGF-β treatment) from one representative experiment of three. (**B**,**D**) In all cases, cells were treated with TGF-β over 24 hours. Left: *Snail1* transcript levels analyzed by real time RT-PCR after a 1-hour treatment with TGF-β. Middle: Caspase 3 activity was analyzed by fluorimetry and presented as the percentage of the controls. Right: Percentage of apoptotic nuclei (microscopy observation of condensed and/or fragmented nuclei after Hoechst staining; 15 independent fields/dish). Data are the means ± s.e.m. of at least three independent experiments and they were compared as indicated in the figure. (Student's *t*-test: **P*<0.05; ***P*<0.005; ****P*<0.0005; N.S., non-significant.)

et al., 2009). They are also susceptible to express Snail1 and undergo EMT in a similar way to foetal hepatocytes upon TGF-β exposure (our unpublished data). Without serum, Hep3B cells show autocrine growth as they express survival signals that attenuate TGF-β-induced cell death (Fig. 7A,B). By contrast, when Snail1 was downregulated in these cells, TGF-β was able to efficiently induce cell death (Fig. 7A) as also assessed by the increased levels of caspase 3 activation and the percentage of apoptotic nuclei (Fig. 7B), which correlated with diminished levels of *Snail1* induction after TGF-β administration (Fig. 7B). We then tested SK-HEP1, a liver adenocarcinoma cell line that depicts a late TGF-β signature (Coulouarn et al., 2008), showing a fibroblastic-like appearance, autocrine production of TGF-β and resistance to TGF-β-induced cell death (Fig. 7C,D). In these cells, Snail1 downregulation promoted apoptotic features that were enhanced in the presence of additional extracellular TGF-β. Altogether, these results indicate that malignant cells can circumvent the death-inducing effect of TGF-β by directing it towards the induction of *Snail1* expression, which promotes survival and EMT.

Discussion

The complex and sometimes contrasting signals induced by TGF-β in epithelial cells (Massagué, 2008; Zavadil and Bottinger, 2005; Heldin et al., 2009) make it difficult to understand its specific role in tumour progression. Indeed, its influence might even be cell-context-specific and dependent on the extracellular environment. In the case of the liver, recent findings have offered new insights into the role of TGF-β in human hepatocarcinogenesis. Indeed, its clear tumour suppressor role evident at early stages is converted to a tumour promoter function at advanced stages (Massagué, 2008). Liver cancer cells become resistant to TGF-β-mediated cell death, and they become capable of undergoing EMT and acquiring invasive properties. Accordingly, liver tumours expressing an early TGF-β signature (suppressor genes) have a less invasive phenotype and tumour recurrence when compared with those that express late TGF-β-responsive genes (anti-apoptotic and metastatic) (Coulouarn et al., 2008). Thus, it is crucial to understand the mechanisms that permit liver cancer cells to escape from the suppressive effects of TGF-β, both to understand tumour progression and to design therapies to block the pro-tumourigenic effects of TGF-β in human hepatocellular carcinoma. Here, we show that, as well as inducing EMT, Snail1 overcomes the death-inducing effects of TGF-β in liver cells.

With respect to the induction of *Snail1* expression by TGF-β, we show that it requires the nuclear translocation of the NF-κB transcription factor in foetal or tumoural hepatocytes and its binding to the NF-κB box2 (–162) in the proximal mouse *Snail1* promoter. This confirms the connection between NF-κB and Snail upregulation described in several cell lines (Barberá et al., 2004;

Julien et al., 2007; Kim et al., 2007) and during development (Zhang et al., 2006), and it is reminiscent of the recently described binding of p65 to the *snail1a* promoter in zebrafish embryos (Liu et al., 2009). Interestingly, other intracellular signals appear to be dispensable, such as MEK/ERKs or PI3-K.

Snail1 is required for TGF-β-mediated EMT in hepatocytes

As mentioned above, in addition to becoming refractory to the tumour-suppressor effects that lead to decreased cell proliferation and cell death, tumour cells also respond to TGF-β by undergoing EMT, a phenotypic change associated with tumour invasion and metastatic potential (Massagué, 2008; Thiery et al., 2009). Conversely, blocking TGF-β signalling upregulates E-cadherin, reducing cell migration and the invasion of hepatocellular carcinoma cells (Fransvea et al., 2008), indicative of a reversion of the EMT process. Because Snail1 actively contributes to tumour progression by inducing EMT and is a well-known target of TGF-β signalling (Thiery et al., 2009), we examined whether Snail1 could provoke the EMT response observed in liver tumour cells upon exposure to TGF-β. Our data indicate that Snail1 does indeed play an important role in regulating this phenotypic transition as its silencing prevents the TGF-β-mediated E-cadherin loss and EMT. Furthermore, forced activation of Snail1 is sufficient to induce EMT in non-transformed adult hepatocytes that are otherwise extremely refractory to such a transition, even in the continued presence of TGF-β. The failure to undergo EMT is compatible with the failure of TGF-β to induce *Snail1* expression (Caja et al., 2007). Thus, our data are consistent with adult tumour cells being able to respond to TGF-β by undergoing EMT as it occurs in foetal hepatocytes (Valdés et al., 2002) and suggest that a reactivation of developmental genes such as *Snail1* in liver tumour cells contributes to the pro-tumourigenic role of TGF-β. This is reminiscent of the EMT process that accompanies the switch from the anti-inflammatory to the profibrogenic role of TGF-β observed during the progression of organ fibrosis. Interestingly, this switch is also associated with the reactivation of *Snail1* expression and, likewise, forced *Snail1* expression in normal kidneys is sufficient to generate EMT and fibrosis, leading to organ failure in transgenic mice (Boutet et al., 2006; López-Novoa and Nieto, 2009). It will be interesting to define the mechanism that prevents *Snail1* upregulation in non-transformed adult hepatocytes. Perhaps the susceptibility of cancer cells is related to the presence of activated Ras, which is crucial for the TGF-β-mediated induction of *Snail1* expression and EMT in renal cells as well as in hepatic and pancreatic cell lines (Gotzmann et al., 2002; Peinado et al., 2003; Grande et al., 2009; Horiguchi et al., 2009).

Snail1 confers resistance to TGF-β-induced cell death in hepatocytes

In addition to preventing EMT, our data show that Snail1 downregulation significantly enhances the apoptotic response to TGF-β of liver cells. Conversely, Snail activation confers full resistance to TGF-β-induced apoptosis in immortalized mouse hepatocytes and in adult hepatocytes obtained from transgenic mice. These results indicate that Snail1 coordinates the EMT with cell survival signals in the liver, which is reminiscent of situations taking place during embryonic development (Barrallo-Gimeno and Nieto, 2005). Snail1 can also decrease cell death in cultured liver cells not treated with TGF-β when compared with control cells, indicating that Snail not only protects from the death induced by TGF-β. Indeed, Snail family members are known to confer resistance to the cell death induced by the removal of survival signals, apoptotic stimuli, gamma radiation and genotoxic stress (Inoue et al., 2002; Vega et al., 2004; Pérez-Losada et al., 2003; Kajita et al., 2004; Vitali et al., 2008; Martínez-Álvarez et al., 2004). Importantly, we show that Snail is required to overcome the cell death induced by TGF-β.

We also show that Snail1 downregulation induces an earlier and stronger activation of Bim and Bmf by TGF-β, two pro-apoptotic BH3-only members of the Bcl2 family, and it impairs the TGF-β-induced increase in the anti-apoptotic BclxL and Mcl1. This indicates that Snail1 might antagonize TGF-β-induced apoptosis by repressing pro-apoptotic genes of the Bcl-2 family that are essential for TGF-β to induce an efficient mitochondrial-mediated apoptosis (Ramjaun et al., 2007) and by upregulating the anti-apoptotic members of the family. As Snail factors have been described as strong transcriptional repressors, the positive action of Snail on the expression of anti-apoptotic factors is very likely to be indirect, although the possibility of Snail acting as a direct activator cannot be formally excluded. These effects might be connected with the known function of another Snail family member, Snail2, in antagonizing p53-mediated apoptosis of haematopoietic progenitors by repressing *Puma* transcription (Wu et al., 2005). Indeed, the effects of Snail downregulation that we have seen in hepatocytes are similar to those observed after Puma expression in myeloid progenitor cells, including Bax activation (Jabbour et al., 2009). Additionally, our results showing that Snail1-targeted knockdown impairs the TGF-β-mediated increase in BclxL and Mcl1, indicate that Snail1 can indirectly activate intracellular survival signals, perhaps because the increase in their levels in hepatocytes exposed to TGF-β occurs concomitant with the activation of the PI3-K/Akt pathway (Valdés et al., 2004). These data are also compatible with the effects of Snail1 transfection in epithelial kidney MDCK cells, where it increases the expression of BclxL and the activity of the PI3-K/Akt and MEK/ERK survival pathways (Vega et al., 2004). In addition, we show that Snail1 can also control hepatocyte adhesion and confer resistance to the apoptosis induced by loss of contact (i.e. anoikis). This effect confirms and expands previous data showing that Snail1 regulates cell attachment to the extracellular matrix (Haraguchi et al., 2008) and with recent data supporting a role for a Twist-Snail axis in the TrkB-induced EMT and anoikis resistance in rat intestinal epithelial cells (Smit et al., 2009).

Snail1 overcomes the death-inducing effects of TGF-β in liver cancer cells

As a consequence of the failure of liver carcinoma cells to respond to the death-promoting effect of TGF-β, and given their ability to respond in terms of undergoing EMT, hepatoma cells overcome the TGF-β tumour-suppressor effects. We show here that, in human hepatocellular carcinoma cell lines, Snail1 downregulation restores the capacity of cells to respond to TGF-β by undergoing apoptosis, the most important role of TGF-β in controlling hepatocellular carcinoma progression. Indeed, Smad3, a physiological mediator of TGF-β tumour-suppressor activity, functions by repressing Bcl2 expression and inducing apoptosis (Yang et al., 2006). Interestingly, Snail1 acts in an opposite way by increasing BclxL and Mcl1 expression. As Smad3 is also necessary for the TGF-β-induced Snail1 expression (Sato et al., 2003), the final cellular response and perhaps hepatocellular carcinoma progression might depend on the equilibrium between the activation of these two Smad3

targets, Bcl2 and Snail1. As Snail1 can recruit Smad3 to repress the promoters of several epithelial genes, including that of *E-cadherin* (Vincent et al., 2009), it would be interesting to investigate whether, by sequestering Smad3, Snail1 could also prevent Smad3 from repressing Bcl2, switching the response to TGF-β from cell death to survival and EMT.

In summary, we show here that Snail1 overcomes the apoptosis induced by TGF-β, as occurs in immature liver and hepatocarcinoma cells that respond to this factor, by expressing Snail1 and undergoing EMT. Furthermore, forced Snail1 expression is sufficient to induce EMT and protection from undergoing apoptosis in non-transformed adult hepatocytes. It is worth noting here that adult hepatocytes are normally refractory to activate Snail1 and undergo EMT even in the presence of TGF-β. These data indicate that the reactivation of developmental genes in the adult impinges on their response to cytokines that might initially be released as a healing response, such as TGF-β. This has important implications in degenerative organ disease, and in particular in tumour cells, where TGF-β activates Snail1 to switch the response towards the induction of EMT, thereby promoting invasive and metastatic properties. As Snail1 has also been associated with resistance to conventional chemotherapy (Thiery et al., 2009), its role in preventing cell death in the liver might have important implications in cancer progression and treatment. Together, our work points to a role of Snail1 in overcoming TGF-β tumour-suppressor effects in hepatocytes, and particularly in liver cancer cells, switching the response from tumour suppression to tumour progression, making them resistant to cell death and prone to undergo EMT and acquire invasive properties.

Materials and Methods

Materials

Human recombinant TGF-β1 was obtained from Calbiochem (La Jolla, CA) and foetal bovine serum (FBS) was obtained from Sera Laboratories International (Cinder Hill, UK). The primary monoclonal anti-vimentin and anti-β-actin antibodies, as well as Rhodamine-conjugated phalloidin, were from Sigma-Aldrich (St Louis, MO). Antiphospho-Smad2 (Ser465/467) was from Cell Signalling Technology (Beverly, MA) and the monoclonal anti-E-cadherin, anti-Bax antibody 6A7 clone and anti-Bak G317-2 clone were from BD Transduction Laboratories (Erembodegem, Belgium). The anti-human oestrogen receptor (hER), the polyclonal anti-Mcl1 (S-19) and anti-BclX (S-18) were from Santa Cruz Biotech. Inc (Santa Cruz, CA). The Cy3-conjugated anti-rabbit and Green Oregon-conjugated anti-mouse secondary antibodies were from DAKO (Glostrup, Denmark). Alexa Fluor-488-conjugated anti-rabbit and anti-mouse immunoglobulin was from Molecular Probes (Eugene, OR). Cycloheximide, rapamycin, SB431542 and SB203580 were all obtained from Sigma-Aldrich, whereas LY294002 and SP600125 were from Alexis Biochemicals (Lausen, Switzerland). AG1478, PD98059 and SN-50 were purchased from Calbiochem. The ssIκB expression vector carrying mutated Ser 32 and Ser 36 to prevent phosphorylation and proteolysis of IκB was generously provided by Dra Mónica A. Costas (Werbajh et al., 2000).

Cell isolation and culture

Foetal rat hepatocytes were obtained by collagenase disruption of 20-day-old foetal Wistar rat liver and they were cultured in uncoated plastic dishes with arginine-free, ornithine-supplemented M 199 medium as described previously (Roncero et al., 1989). Adult murine hepatocytes were isolated by collagenase perfusion of mouse livers as described elsewhere (Kao et al., 1996). Briefly, anesthetized mice were subjected to liver perfusion with 50 ml of a 0.02% (w/v) collagenase in Hanks Balanced Salt Solution through the vena cava at a rate of 2.5 ml/minute using a peristaltic pump. The liver tissue was processed mechanically, passed through a 70 μm filter and resuspended in an isotonic Percoll solution. After centrifugation, the cells were seeded at 0.25×10^6 cells in 6-well dishes in DMEM/F:12 (1:1) supplemented with 10% FBS. Immortalized neonatal murine hepatocytes were generously provided by Angela M. Valverde (Madrid, Spain), obtained as previously described (González-Rodríguez et al., 2008). In primary cultures, the serum was removed after cell attachment and 12–14 hours later, TGF-β was added. In the case of immortalized neonatal hepatocytes, TGF-β treatment was initiated after 4 hours serum starvation. In all cases, inhibitors were added 30 minutes before TGF-β. FaO rat hepatoma cells and Hep3B human negroid hepatocyte carcinoma cells were obtained from the European Collection of Cell Cultures (ECACC), cultured in DMEM supplemented with 10% FBS and maintained in a humidified atmosphere of 37°C, 5% CO_2. For experiments, cells at 70% confluence were serum-starved for 8–12 hours before treatments.

Primary cultures from transgenic mice

The Snail-ER^{T2} construct (De Frutos et al., 2007; De Frutos et al., 2009) was microinjected into fertilized C57×CBA hybrid eggs to generate transgenic mice according to Hogan et al. (Hogan et al., 1994). A line expressing the transgenic protein in the liver was selected (Fig. 6A). Two-month-old mice were sacrificed by cervical dislocation and their livers were dissected out and cultured as described above. Cells were cultured in the presence or absence of 200 nM 4OH-tamoxifen.

Real-time PCR

Total RNA was obtained using the RNeasy Kit (Qiagen, Hilden, Germany) and complementary DNA was generated by the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) using oligo (dT) as the primer. Quantitative RT-PCRs were carried out by two different methods. (1) Step One Plus (Applied Biosystems, Carlsbad, CA) sequence detection system, using the SYBR Green method. RNA expression was calculated using the comparative Ct method normalized to GAPDH. For cell culture experiments, data were normalized using the $C_0 \pm$ s.d. formula to compare the relative expression between the different genes. The data are represented as the mean ± s.d. of the different experiments, each examined in triplicates (n=3). Specific primers for mouse and rat sequences used were as follows: *Gapdh* 5′-CTGAGCAAGAGAGGCCCTATCC-3′ (forward), 5′-CTCCCTAGGCCCCTCCTGTT-3′ (reverse); *Snail1* 5′-CCACACTGGTGAGAAGCCATTC-3′ (forward), 5′-TCTTCACATCCGAG-TGGGTTTG-3′ (reverse); *p21* 5′-AGGAGCCAGGCCAAGATGGT-3′ (forward), 5′-GCTTTGACACCCACGGTATTCA-3′ (reverse); *Ecdh* 5′-ACCTCCGTGATGAAGGTCTC-3′ (forward), 5′-CCGGTGTCCCTATTGACAGT-3′ (reverse). (2) ABIPrism7700 System, following the manufacturer's protocol and using pre-designed Taqman primers: Rat – *p65* Rn01502266_m1, *Gapdh* Rn99999916_s1; Mouse – *18S* Hs03003631_g1, *Bcl2l11* Mm00437796_m1 (Bim), *Bmf* Mm00506773_m1; Human – *Snai1* Hs00195591_m1; *18S* Hs03003631_g1.

Western blot analysis

Total protein extracts and western blot analysis were performed as described previously (Murillo et al., 2005).

Electromobility shift assay (EMSA)

A specific probe for DNA-protein interaction analysis was used, containing a nuclear factor-kappaB (NF-κB) binding element. The sequence that corresponded to the NF-κB motif in the iNOS promoter was as follows: 5′-tcgaCCAACTGGGACTCTCCCTTTGGGAACA-3′ (forward), 5′-tcgaTGTTCCCAAAGGGAGAGTCCCAGTTGG-3′ (reverse). After annealing each forward and reverse oligonucleotide, 0.5 μg were radiolabelled using the Klenow enzyme (Roche, Mannheim, Germany) and (α^{32}P) dCTP (GE Healthcare, Chalfont St. Giles, UK). Radiolabelled oligonucleotides were purified on MicroSpin SC-200 HR columns (GE Healthcare) and used as probes. Radiolabelling was measured by liquid scintillation counting. Nuclear extracts and EMSA experiments were performed as described previously (Murillo et al., 2007). For specificity controls, 10 μg of protein extract were incubated for 15 minutes at 4°C with an anti-p65 antibody (2 μg) or with unlabelled oligonucleotide (100 ng), as indicated. Gels were dried and the complexes were visualized by autoradiography.

Immunohistochemistry and immunofluorescence microscopy studies

Fluorescence microscopy studies were performed as described previously (Caja et al., 2007). To stain F-actin, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature and incubated with Rhodamine-conjugated phalloidin (1:500) diluted in 0.1% BSA for 1 hour. To detect E-cadherin and vimentin, the cells were fixed with cold methanol for 2 minutes. Primary antibodies (1:50) were diluted in 1% BSA and incubated for 2 hours at room temperature. After several washes with PBS, the samples were incubated with fluorescent-conjugated secondary antibodies for 1 hour at room temperature (1:500 for Cy3 conjugated anti-rabbit, 1:200 for Alexa-Fluor-488-conjugated anti-rabbit and 1:200 for Oregon-Green-conjugated anti-mouse immunoglobulins) and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). The blue signal represents the nuclear DNA stained with DAPI. Representative images were taken with a Spot 4.3 digital camera and software and edited in Adobe Photoshop. For analysis of apoptotic nuclei after DAPI staining, fragmented and/or pyknotic nuclei were counted. Cells were visualized in an Olympus BX-60 or a Leica DMR microscope with the appropriate filters.

Histological sections were obtained from embryos fixed in 4% paraformaldehyde in PBS and embedded in paraffin. The presence of the human estrogen receptor was detected with an α-hER antibody (1:200; Santa Cruz). Immunodetection in embryo sections was carried out either with the biotin-streptavidin system (ABC kit, Pierce) or with an anti-rabbit Alexa-Fluor-488 (1/5000; Molecular Probes) in hepatocytes in culture.

Transient transfection and promoter analysis

Foetal hepatocytes

Foetal rat hepatocytes were cotransfected with 2 μg of total DNA (400 ng of CMV-Rel A or 400 ng of ssIκB plasmid or an empty pcDNA3 vector and 800 ng of pmSnail-Luc vector). A Renilla luciferase plasmid was also cotransfected as a control of efficiency. Transient transfection were performed with JET PEI (Genycell Biotech, Granada, Spain) following the manufacturer's instructions in DMEM/F:12 (1:1) supplemented with 5% FBS overnight. The medium was replaced by 2% FBS DMEM/F:12 and 8 hours later was replaced by a serum-free medium. Twelve hours later, hepatocytes were stimulated with TGF-β (2 ng/ml). Luciferase and Renilla activities were assayed using a Dual-Luciferase Reporter Assay System Kit (Promega Biotech Iberica, Madrid, Spain) according to the manufacturer's instructions. The results are presented as a percentage of luciferase activity relative to controls (luciferase values in cells cotransfected with empty vectors). Three independent experiments were carried out.

Cell lines

The mouse *Snail* promoter sequence containing 1 Kb upstream of the ATG was amplified by PCR from genomic mouse DNA using the following primers: 5′-CCGGTACCTGTGAACGTTCCAACACGAT-3′ (forward; containing the *Kpn*I site), 5′-CCAAGCTTGTTGGCCAGAGCGACCTAGG-3′ (reverse; containing the *Hin*dIII site). The purified PCR product was subcloned into the pGL3-basic vector (Promega Biotech Iberica) and the Quickchange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, Texas) was used to delete the κb-boxes present in the mouse *Snail1* promoter. FaO cells were transfected with 50 ng of CMV-Rel A or an empty pcDNA3 vector and 400 ng of pmSnail1-Luc vector with control or mutated κb sites. A Renilla luciferase plasmid was also cotransfected as a control of efficiency. Transfections were carried out using Lipofectamin (Roche Diagnostics, Barcelona, Spain) and JET PEI (Genycell Biotech., Granada, Spain). The medium was replaced 24 hours after transfection by a serum-free F12 Coon's Modified Medium and 12 hours later the cells were stimulated with TGF-β (2 ng/ml). Luciferase and Renilla activities were assayed as described above. The results are presented as a percentage of luciferase activity relative to controls (luciferase values in cells cotransfected with empty vectors).

ChIP assays

FaO cells (8×10^6) were exposed to TGF-β (2 ng/ml) for 72 hours or they were left untreated, then were crosslinked with formaldehyde and washed twice with PBS. The cell pellet was lysed and sonicated to shear the chromatin to an average length of 0.5–1 kb. After centrifugation, 1% of the extract was aliquoted and used as the total input control. The remaining extracts were precleared with protein A-agarose and divided equally between three tubes containing agarose-conjugated p50; agarose-conjugated normal goat IgG, used as a negative control; and anti-acetylated H3, used as a positive control (Santa Cruz Biotechnology). Samples were incubated overnight at 4°C with rotation. The purified DNA was suspended in 30 μl of water. A 200 pb fragment of the rat *Snail* promoter containing the conserved κB-box was amplified with the primers: 5′-CTATTGGCGCAATCTTGA-3′ (forward) and 5′-CGCAA-GGTCGGTAGACAACTC-3′ (reverse). A more distal 200 pb fragment of the promoter carrying the non-conserved κB-box was amplified with the primers 5′-CACCAACCTCATCCTGGG-3′ (forward); 5′-ACAGCTGTGACCGTCAATGGA-3′ (reverse).

Apoptosis studies

Cell protein extraction and fluorimetric analysis of caspase 3 activity were performed on 10–20 μg protein as described previously (Murillo et al., 2005). Fluorescence was measured with an Optima Fluostar Microplate Fluorescence Reader. Final caspase 3 activity was expressed as fluorescence units/hour/mg protein. Protein concentration was measured using the Bio-Rad (Hercules, CA) protein reagent. After DAPI staining, apoptotic nuclei were quantified via those with a fragmented and/or pyknotic appearance. To analyze cell attachment on plastic and for anoikis studies, immortalized murine hepatocytes were transfected with either a non-specific siRNA or the specific *Snail1* siRNA and they were treated with TGF-β (2 ng/ml) for 48 hours. Cells were subsequently trypsinized and plated on bacterial dishes for 48 hours. After this time, analysis of the number of cells that adhered to the bacterial dishes reflected their capacity to attach to untreated plastic. Cells that did not adhere to the bacteria dishes were later plated, at identical cell density, on cell culture dishes. Only the cells surviving after 48 hours in the absence of adhesion could attach to the dish. The viable cell number was quantified by Crystal Violet (0.2% in 2% ethanol) staining and spectrophotometric analysis of cell lysates, as described previously (Valdés et al., 2002). The percentage of viable cells that remained was calculated from the absorbance relative to that of control cells (incubated in the absence of growth factors). To determine the percentage of cells containing active Bax or Bak, cells were plated on gelatin-coated glass coverslips and the monolayer was washed with PBS before the cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. The cells were incubated for 2 minutes with 0.1% Triton X-100 and then with anti-Bax 6A7 clone and anti-Bak G317-2 clone antibodies (1:50), diluted in 1% BSA and incubated overnight at 4°C. After several washes with PBS, the samples were incubated with fluorescent-conjugated secondary antibodies (1:200 for Alexa-Fluor-488-conjugated anti-rabbit) for 1 hour at room temperature and embedded in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). The cells were visualized on an Olympus BX-60 microscope with the appropriate filters and the DAPI nuclear DNA staining was apparent in blue. Representative images were taken with a Spot 4.3 digital camera and the images processed according to the instructions provided by the journal. The results are shown as the percentage of positive cells relative to the total cell number.

Snail1 interference assays

For transient siRNA transfection, cells at 30% confluence were transfected over 18 hours using TransIT-siQuest (Mirus, Madison, WI) at a 1:300 dilution in complete medium according to the manufacturer's recommendation. After a further 24 hours of incubation in complete medium, the FBS concentration was decreased to 2% and TGF-β was added 4 hours later. Interference oligonucleotides were obtained from Sigma-Genosys (Suffolk, UK) and assessed as previously described (de Frutos et al., 2007). After control experiments with different siRNAs that gave similar results, we chose the next sequences: Mouse si*Snail1-1* 5′-CAAACCCA-CUCGGAUGUGAAGAGAU-3′ (used at 50 nM); Mouse si*Snai1-3′* 5′-CAGCUGCUUCGAGCCAUAGAACUAA- 3′ (used at 200 nM); Human si*Sna1-1* 5′-UCCCAGAUGAGCAUUGGCAGCGAGG-3′ (used at 50 nM); Human si*Sna-2* 5′-CCACAGGACUUUGAUGAAGACCAUU-3′ (used at 100 nM); Rat *sip65-1* 5′-CUCAAGAUCUGCCGAGUAA-3′ (used at 100 nM); Rat *sip65-2* 5′-CGC-AAAAGGACCUACGAGA-3′ (used at 100 nM). Control siRNA was as previously described (Sancho et al., 2006).

Rescue experiments

Cells were transfected using the Magnet Assisted Transfection System (IBA BioTAGnology, Göttingen, Germany), either with a pEGFP-C3 plasmid alone or in combination with a pcDNA3 plasmid containing the mouse Snail1 coding region (CD), following manufacturer's instructions. Briefly, a mixture of MATra A Reagent and DNA in a ratio 1 μl reagent:1 μg DNA was added to the cell culture and incubated for 15 minutes on a magnet plate. After 8 hours, Snail interference was performed as described above.

Statistical analysis

ANOVA and Student's *t*-test were used to analyze the differences between two groups of data.

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Supplementary material available online at
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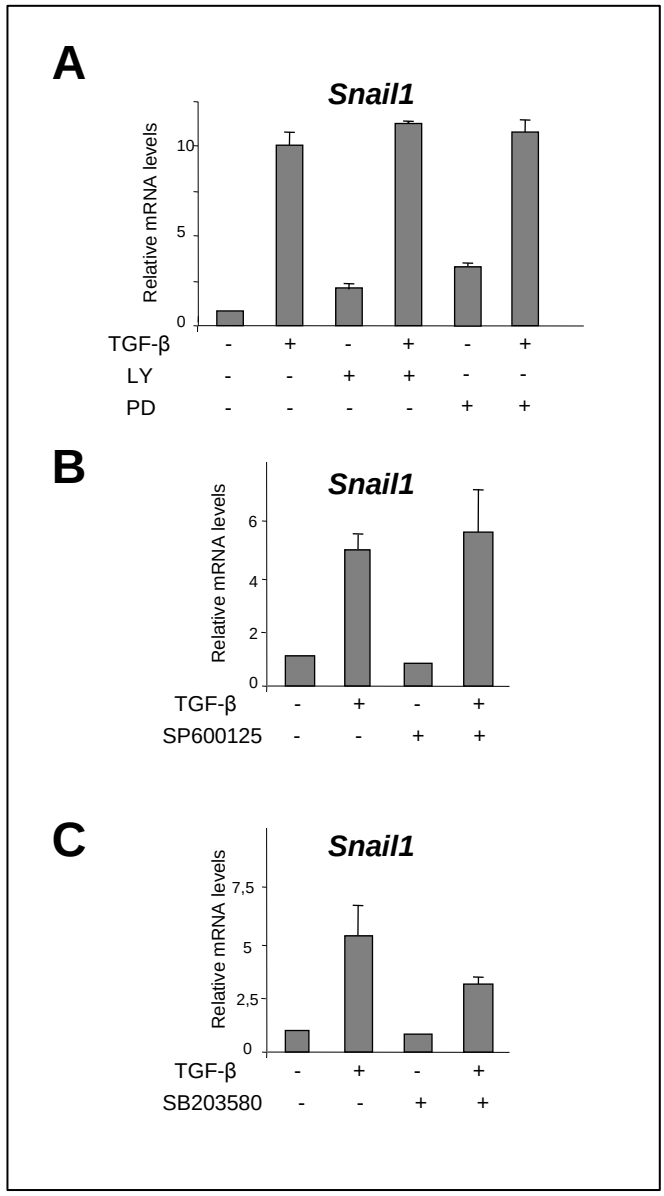
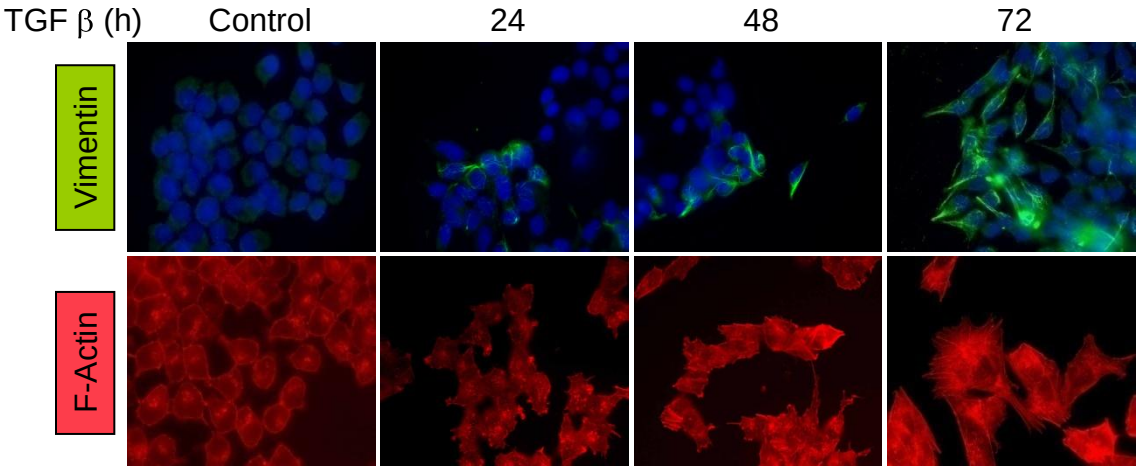


Suppl. Fig. 1. TGF-β-induced *Snail1* transcription in fetal rat hepatocytes does not require activation of the PI 3-K, ERKs, c-Jun-N-kinase and p38 pathways. *Snail1* expression, analyzed by real time RT-PCR, in hepatocytes incubated in the presence or absence of TGF-β (2 ng/ml, 3h) and either pre-treated or not (1 h before TGF-β addition) with: (A) 20 μM LY294002 or 50 μM PD98059 (PI3-K and MEK inhibitors, respectively); (B) 40 μM SP600125 (c-Jun-N-kinase inhibitor); (C) 5 μM SB203580 (p38-MAPK inhibitor). *GAPDH* expression was used as a control of the cDNA templates. –RT lane, amplification in the absence of template. Concentrations of the different inhibitors were chose according to previous studies: LY294002 - Valdés et al. Exp Cell Res. 292:209-18 (2004) . PD98059 : Roncero et al., Biochim Biophys Acta. 1402:151-64 (1998). SP600125: Carmona-Cuenca et al. J Cell Physiol. 207: 322-30 (2006). SB203580: Bragado et al. Apoptosis. 12:1733-42 (2007).



Suppl. Fig. 2. Immunofluorescence detection of Vimentin and F-actin filaments (Phalloidin) in FaO cells treated with TGF-β (2 ng/ml). See Fig. 2 and Materials and Methods for details.

NFKB binding site in *Snail1* promoter

Alignment and position (from ATG) of first nucleotide of KB1 site
Mouse: -672, Rat: -667; Human: -957

```
Mouse   cagaagttactgattcttaccccgggcctttccctcggtgcttcttcacttcc
Rat     ctgaaattactgactctaacctcgggcctttccctccgtgcttcttcacttcc
Human   aagcagttgccacttcttccctcgggccttttccttgataattcttcacttcc
```

Alignment and position (from ATG) of first nucleotide of KB2 site
Mouse: -162, Rat: -163; Human: -171

```
Mouse   cagccattggctcagccttgacaaaggggcgtgaccaacagtacggtcacgccc
Rat     cagctattggcgcaatcttgacaaaggggcgtgatcgtagccgaggccacgccc
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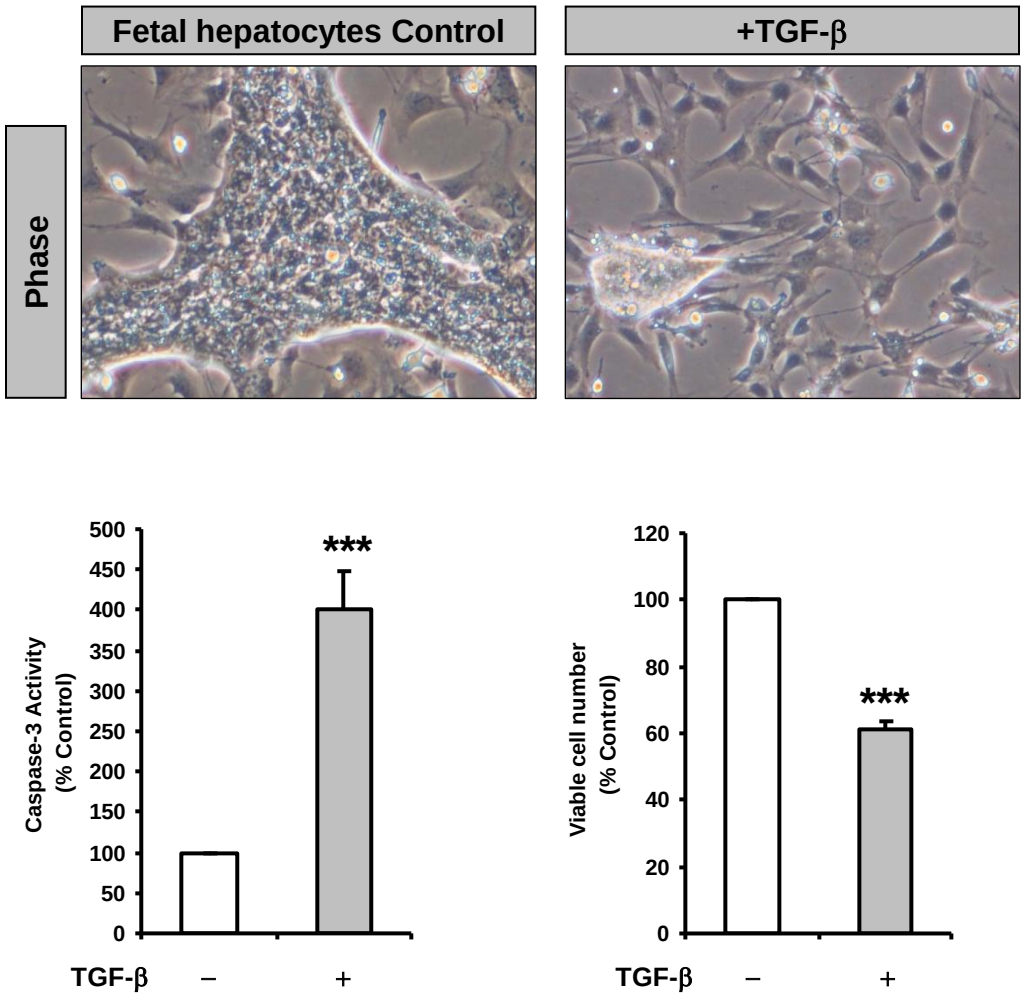

Suppl. Fig. 4. TGF-β (2 ng/ml) induces EMT and cell death in rat fetal hepatocytes. See Materials and Methods for details. We have extensively studied this transition previously (Sánchez et al., Exp Cell Res., 1999; Valdés et al., Mol Cancer Res., 2002; Del Castillo et al., Exp Cell Res., 2006).