The Epithelial Mesenchymal Transition Confers Resistance to the Apoptotic Effects of Transforming Growth Factor β in Fetal Rat Hepatocytes

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Abstract

Fetal rat hepatocytes treated with transforming growth factor β (TGF-β) die by apoptosis. However, a subpopulation of them survives and undergoes an epithelial mesenchymal transition (EMT). This transition also occurs upon incubation with fetal bovine serum. We have isolated the subpopulations that undergo EMT (TGF-β-treated-fetal hepatocytes: TβT-FH; serum-treated-fetal hepatocytes: ST-FH) and show that they present high levels of vimentin and Snail expression and lack cytokeratin 18 and E-cadherin. Both TβT-FH and ST-FH cells require mitogens to grow and maintain the response to TGF-β in terms of growth inhibition. However, they lack differentiation markers such as the liver-enriched transcription factors hepatocyte nuclear factor 4 (HNF-4) or HNF-1α and express the progenitor marker OV-6. Interestingly, the EMT process confers them resistance to the apoptotic effect of TGF-β, with cells showing higher levels of active AKT and Bcl-x_L than fetal hepatocytes. In summary, these cells are refractory to the apoptotic effects of TGF-β, showing characteristics of liver progenitors and of some hepatocellular carcinoma cells.

Introduction

Apoptosis is an important mechanism in multicellular organisms to eliminate cells that are either in excess or potentially dangerous. As such, and in conjunction with cell division and migration, apoptosis regulates cell number and tissue size. During the initial stages of hepatocarcinogenesis in the rat, a gradual increase in apoptotic activity is seen during the formation of preneoplastic foci (1). Preneoplastic liver cells are more susceptible than normal hepatocytes to cell death stimuli, which suggests that tumor initiation, at the organ level, can be reversed by preferential elimination of initiated cells. Indeed, tumor promoters and nongenotoxic carcinogens inhibit active cell death, thereby increasing the accumulation of preneoplastic cells and accelerating the development of cancer (2, 3).

The transforming growth factor β (TGF-β), a member of a family of structurally related polypeptide factors important in growth control, development, and differentiation (4), has multiple and somehow contradictory roles in liver development and carcinogenesis. On the one hand, TGF-β secretion inhibits proliferation (5), suppresses transformation (6), and induces apoptosis (7) during liver carcinogenesis, and disruption of TGF-β signaling can deregulate apoptosis in hepatocellular carcinomas (HCCs) (8). On the other hand, its activation has been associated with the progression of hepatocarcinogenesis. Indeed, human HCC strongly expresses TGF-β mRNA and protein *in vivo* (9), an elevated urinary TGF-β level has been related to poor survival in cirrhotic HCC (10), and constitutive expression of mature TGF-β in the liver accelerates hepatocarcinogenesis in transgenic mice (11). Furthermore, TGF-β promotes spontaneous transformation of cultured rat liver epithelial cells (12).

We have previously shown that TGF-β inhibits growth (13) and induces apoptosis in fetal hepatocytes (14), by a mechanism dependent on oxidative stress, and that involves *bcl-x_L* down-regulation and mitochondrial release of cytochrome *c* (15). However, a subpopulation of these cells survives, concomitant with changes in morphology and phenotype, reminiscent of an epithelial mesenchymal transition (EMT) (16). Cells that overcome apoptosis show a fibroblastic appearance, a replacement of the cytokeratin network by vimentin, lower expression of liver-specific genes, and higher levels of proto-oncogenes (16). Similar results have been obtained in rat neonatal hepatocytes (17). Whether TGF-β promotes morphological and phenotypic changes in fetal hepatocytes that allow them to impair apoptosis is an unsolved question yet.

Loss of cell adhesion and the acquisition of migratory and invasive properties are characteristic of EMT both during embryonic development and tumor progression (18–21). As a result of the down-regulation of E-cadherin expression, a cell-cell adhesion molecule considered an invasion-suppressor gene, EMT involves the loss of the ability of cells to recognize and adhere to their neighbors. Among the transcription factors that

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have been implicated in the direct repression of E-cadherin transcription (20, 22–24), it is noteworthy that Snail is active at the invasive front of mouse skin tumors and in human breast carcinomas (23, 25, 26).

In this work, we show that TGF-β induces EMT in fetal hepatocytes and that this EMT confers cell resistance to apoptosis. We have isolated and subcultured cells that suffered this EMT process and analyzed their properties in terms of growth, liver-specific gene expression, and response to TGF-β. We propose that these cells are refractory to the apoptotic effects of TGF-β, showing characteristics of liver progenitors and of some HCC cells. These cells may constitute a model to study liver differentiation and the pathological progression to hepatocarcinomas.

Results

TGF-β and Fetal Bovine Serum Induce EMT in Fetal Hepatocytes: Isolation Cells with Mesenchymal Phenotype

Fetal hepatocytes were cultured in Arg-free medium 199 [selective for parenchymal hepatocyte survival and growth... (27)], where they form small clusters of parenchymal cells (control cultures; Fig. 1A). When cells were cultured in the absence of serum but in the presence of TGF-β (2 ng/ml) for 24 h, 40–50% of the cells detached and died through apoptosis, whereas the majority of cells that survived acquired a fibroblastic morphology (Fig. 1A, *middle*). A similar progressive acquisition of fibroblastic phenotype, but in the absence of cell death, was observed in fetal hepatocytes cultured in the presence of 10% fetal bovine serum (FBS) for 24–48 h (Fig. 1A, *right*). In both cases (TGF-β or FBS treatment), the cytokeratin network was progressively replaced by vimentin, characteristic of a fibroblast cell and absent in parenchymal hepatocytes (Fig. 1B). Furthermore, some cells coexpressed vimentin and albumin (a plasma protein secreted exclusively by hepatocytes), indicating that the cells that suffered these morphological and phenotypic changes were indeed hepatocytes (Fig. 1B). The actin cytoskeleton was also affected because both the cells that survived TGF-β-induced apoptosis and those subjected to FBS treatment presented abundant stress fibers (Fig. 1B). In contrast, epithelial cells either in the process of dying (induced by TGF-β) or organized in clusters (in the presence of serum) showed a peripheral distribution close to the plasmalemma.

We decided to isolate and characterize these cells that had undergone an EMT (see Fig. 2 for the isolation procedure). Morphologically, serum-treated-fetal hepatocytes (ST-FH) and TGF-β-treated-fetal hepatocytes (TβT-FH) were similar, although not identical (Fig. 3A). In both cases, the cultures were best maintained in 50% conditioned medium, were capable of completing at least 30 passages, and could be stored frozen in FBS with 10% DMSO although they have not been immortalized. Flow cytometry analysis revealed that both TβT-FH and ST-FH were larger than the original hepatocytes (Fig. 3B). The cells expressed vimentin but not cytokeratin 18, and showed low levels of albumin expression (Fig. 3A), indicating that they are hepatocytes that have suffered a transdifferentiation process. Western blot analysis of the intermediate filament pattern (vimentin and cytokeratin 18) corroborated the immunofluorescence studies in both TβT-FH and ST-FH cells (Fig. 4A).

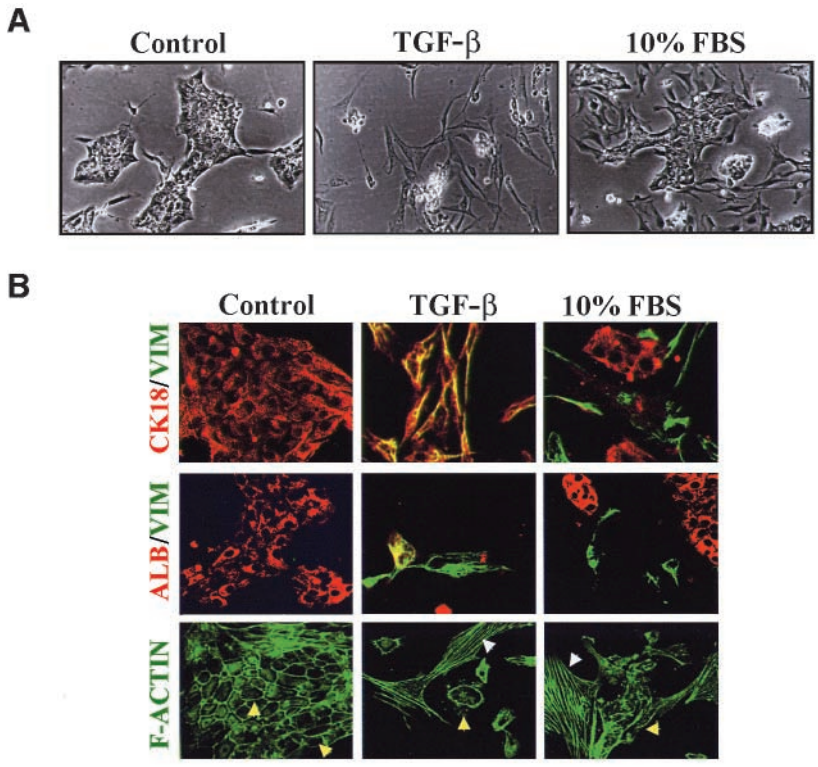

FIGURE 1. TGF-β and FBS induce phenotypic changes in fetal rat hepatocytes reminiscent of EMT. **A.** Light microscopy photographs of cells incubated for 24 h in the absence (*Control*) or presence of 2 ng/ml TGF-β or 10% FBS. **B.** Immunofluorescence detection of vimentin, cytokeratin 18, albumin, and F-actin in the fibroblastoid cells that appeared after treatment with TGF-β or FBS during 24 h. Cells were double immunostained with either mouse anti-vimentin and rabbit anti-cytokeratin 18 or mouse anti-vimentin and rabbit anti-albumin. Fluorescent secondary antibodies were TRITC-conjugated goat anti-rabbit immunoglobulins (in *red*, CK-18 and albumin) or FITC-conjugated goat anti-mouse immunoglobulins (in *green*, vimentin). Actin filaments were stained with rhodamine-conjugated phalloidin. A typical epithelial actin distribution is found in parenchymal hepatocytes (*yellow arrow*), whereas stress fibers (*white arrow*) can be observed in the fibroblastic-like cells that appear after TGF-β or FBS treatments. Representative images of more than 10 different preparations are shown.

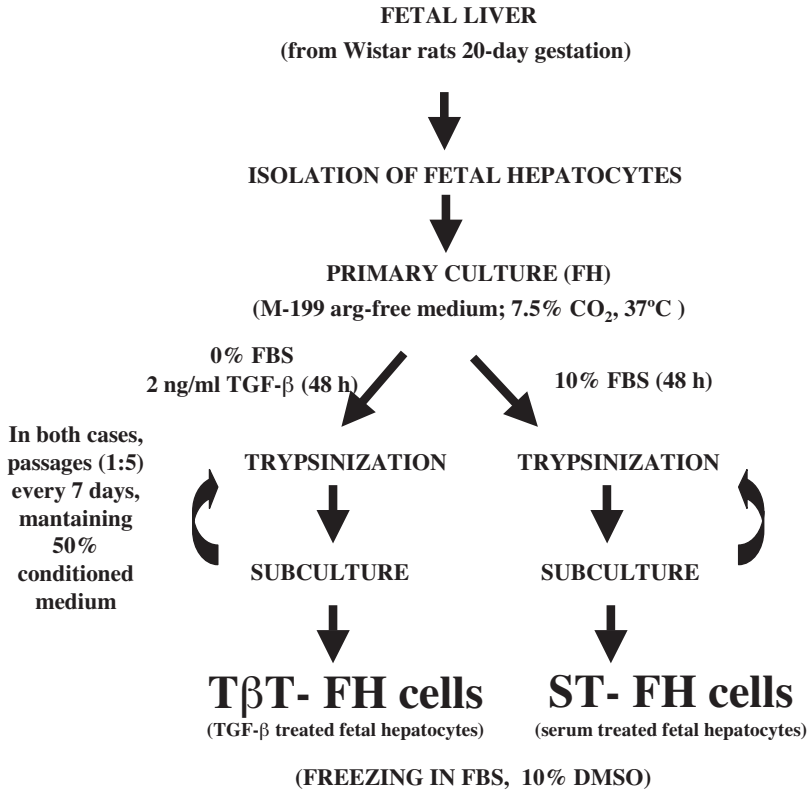

FIGURE 2. Isolation of TβT-FH and ST-FH cells: subculture of fetal hepatocytes previously treated with TGF-β or FBS. After treatment with either 2 ng/ml TGF-β or 10% FBS, the cells were trypsinized, pelleted, and plated. Following trypsinization, DMEM medium supplemented with 10% FBS was always used. In successive passages, 50% conditioned medium was used for plating.

We have previously shown that hepatocytes that survive treatment with TGF-β express low levels of liver-specific genes and higher levels of proto-oncogenes (16). Indeed, expression of the liver-enriched transcription factors hepatocyte nuclear factor 4 (HNF-4) and HNF-1α and β was barely detected in TβT-FH and ST-FH cells (Fig. 4B), whereas HNF-3α and HNF-3β mRNA levels remained unaffected, indicating that these cells are less differentiated than fetal hepatocytes. In agreement with this finding, high levels of OV-6 protein were detected in both TβT-FH and ST-FH cells, OV-6 being a protein expressed in hepatocyte progenitors (28) which is completely absent from fetal hepatocytes (Fig. 4A). Fibronectin expression was also analyzed because it is up-regulated during EMT and, as expected, fibronectin mRNA levels were higher in TβT-FH and ST-FH cells than in fetal hepatocytes (Fig. 4B). Another feature of cells undergoing EMT is the loss of cell-cell adhesion molecules. High levels of E-cadherin mRNA were detected in fetal hepatocytes, whereas its expression was barely detected in TβT-FH and ST-FH cells (Fig. 4B).

TGF-β and FBS Induce Snail Expression in Fetal Hepatocytes and Its Expression Is Maintained in TβT-FH and ST-FH Cells

The transcription factor Snail has been shown to induce EMT by repressing the expression of E-cadherin. Thus, we examined the expression of Snail in TGF-β- and serum-treated primary fetal hepatocytes and in TβT-FH and ST-FH cells. Exposure of fetal hepatocytes to TGF-β was sufficient to detect the onset of Snail expression and the disappearance of E-cadherin transcripts, which indicates that the TGF-β-induced EMT involves Snail activation (Fig. 5A). A detailed analysis of timing revealed that TGF-β induced Snail expression as soon as 5 h after treatment, which was followed by E-cadherin repression, because its transcripts disappeared by 15 h later (Fig. 5B). Hepatocytes cultured with 10% serum (FBS) constituted a heterogeneous population composed of both epithelial and fibroblastic cells that express both Snail and E-cadherin transcripts (Fig. 5A). FBS effects could be due to TGF-β itself, which is known to be present in serum, but also to other factors, since epidermal growth factor and hepatoctye growth factor were also able to induce Snail under our experimental conditions (results not shown). When purified, both TβT-FH and ST-FH cells expressed high levels of Snail, in the absence of E-cadherin transcripts.

Because the changes in morphology and gene expression profiles suggested that those cells had undergone EMT, we performed a cell migration experiment. Both TβT-FH and ST-FH cells presented a migratory phenotype (Fig. 5C). This was demonstrated by incising a wound into the central area of a 70% confluent culture and then incubating the cells for 24 h. Both TβT-FH and ST-FH cells formed a homogeneous population of cells that progressively occupied the open space. When a similar wound was made in primary cultures of fetal hepatocytes, parenchymal cells did not migrate from the clusters. However, when the wound was made in the middle of a parenchyma, some fibroblast-like cells did appear in the open area, indicating the phenotypic instability of fetal hepatocytes in primary cultures (Fig. 5C).

Growth Properties of TβT-FH and ST-FH Cells

The cells were characterized in terms of their growth properties. Flow cytometry revealed that, in the absence of any proliferative stimuli, both ST-FH and TβT-FH cells presented a similar proportion of cells in the S and G_2-M phases of the cell cycle, a proportion that was lower than that of the control preparations of fetal hepatocytes (Table 1). Furthermore, serum and/or mitogens were required to induce proliferation of these cells. In the presence of either 10% FBS or a mitogen mix [20 ng/ml epidermal growth factor (EGF) + 100 nM insulin], the percentage of cells in the S and G_2-M phases of the cell cycle only increased from 15–18% to 22–25%, whereas fetal hepatocytes responded to EGF + insulin by increasing the percentage of cells in S + G_2-M from 20–25% to 40–45% (results not shown). These results indicate that TβT-FH and ST-FH cells are not more proliferative than fetal hepatocytes.

We were interested to determine what would be the response of the homogeneous populations of TβT-FH and ST-FH cells to a further treatment with TGF-β. Cells were maintained for 3 days in the presence of 5%, 7.5%, and 10% FBS (Fig. 6A), or mitogens (EGF and insulin; Fig. 6B). The serum-dependent increase in cell number was similar in both cell types. However, TβT-FH cells appeared to be more responsive to the mitogen mixture than the ST-FH cells. Exposure to TGF-β attenuated the response to FBS and insulin + EGF in both ST-FH and TβT-FH cells (Fig. 6), although the treatment of TβT-FH with TGF-β had little effect in the presence of 10% FBS compared to that of an identical treatment in ST-FH. These experiments were carried out with cells that had not undergone more than 15 passages as prolonged culture of cells induced a loss of the antiproliferative response to TGF-β (results not shown).

Response of TβT-FH and ST-FH Cells to TGF-β in Terms of Apoptosis

As may be expected from the criteria for selection, apoptosis was not observed when TβT-FH cells were exposed to TGF-β (Fig. 7). Interestingly, neither was apoptosis observed in ST-FH cells, even at high concentrations of TGF-β (20 ng/ml). Indeed, in the absence of serum and conditioned medium, the cell number (Fig. 7A) and the percentage of hypodiploid cells (Fig. 7B) in the cultures was maintained, as was the absence of caspase-3 activation (Fig. 7C). In addition, apoptosis was not induced in TβT-FH and ST-FH cells by serum depletion, whereas fetal hepatocytes died within 3 days of serum removal. In these conditions, both ST-FH and TβT-FH cells could be maintained in culture for up to 10 days, although they did not proliferate (results not shown). These results indicate that both ST-FH and TβT-FH cells are insensitive to the TGF-β-induced apoptotic signals and are less sensitive to other proapoptotic stimuli such as serum depletion. However, this is not due to the absence of the intracellular apoptotic machinery, because treatment with 1 μM staurosporine for 6 h induced the death of 50–55% of both fetal hepatocytes and ST-FH or TβT-FH cells. In addition, caspase-3 was activated in both fetal hepatocytes and ST-FH cells (8- and 5-fold, respectively) and hypodiploid cells appeared in both cases (results not shown).

TβT-FH and ST-FH Cells Show High Levels of Active AKT and Bcl-x_L

Because both TβT-FH and ST-FH were resistant to the apoptotic effect of TGF-β, we analyzed the expression pattern and/or activation of proteins implicated in survival processes. Initially, we studied the levels of active ERKs (p-p42 and p-p44) and AKT (p-AKT). Whereas in TβT-FH and ST-FH cells the levels of active ERKs (either in the absence or in the presence of FBS) were lower than in fetal hepatocytes, p-AKT levels were slightly higher in the cells that underwent EMT (Fig. 8). We also analyzed the expression of Bcl-x_L, an anti-apoptotic member of the Bcl-2 family capable of preventing cytochrome *c* release and inhibiting cell death. Both TβT-FH and ST-FH cells expressed much higher levels of Bcl-x_L protein (Fig. 8) and mRNA (results not shown). In contrast, X-IAP, an IAP family member that inhibits caspase activity, was not overexpressed in TβT-FH and ST-FH cells. Thus, TβT-FH and ST-FH cells present high levels of two molecules proposed to act as survival signals in the apoptosis induced by TGF-β in hepatocytes (15, 29).

Discussion

Different experimental approaches have established that the progressive loss of the epithelial character and the

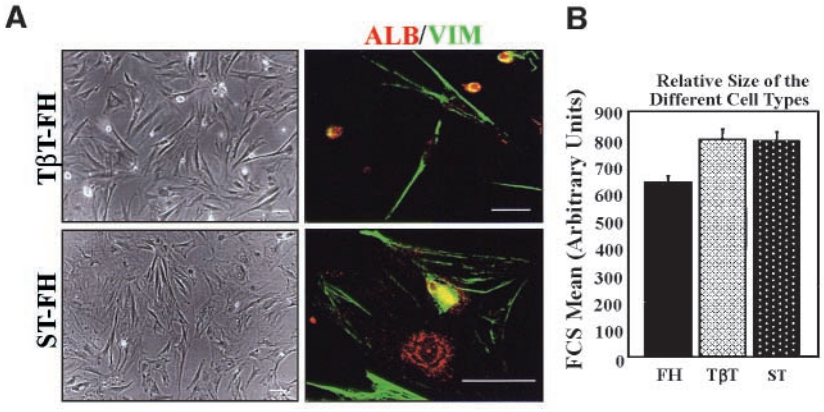

FIGURE 3. Vimentin and albumin immunofluorescence and size analysis of TβT-FH and ST-FH cells. **A.** *Left,* phase-contrast photographs (passage 3) of confluent cultures. *Right,* immunofluorescence detection of vimentin (*green*) and albumin (*red*) (passages 3–5) by confocal microscopy (fields are different from those shown in phase-contrast micrographs). *Scale bar,* 25 μM. **B.** Relative size of the different cell types analyzed by flow cytometry.

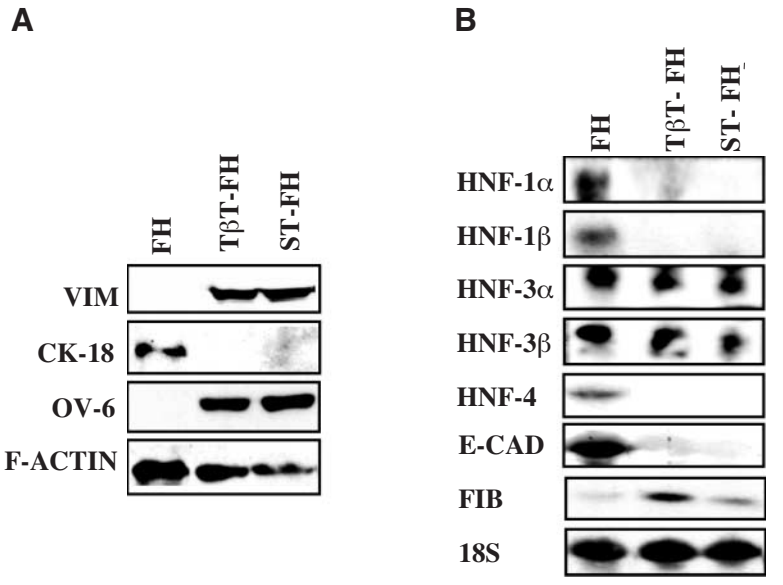

FIGURE 4. Phenotypic properties of TβT-FH and ST-FH cells. **A.** Western blot analysis for cytokeratin 18 (*CK 18*), vimentin (*VIM*), and OV-6 in fetal hepatocytes (*FH*, 24 h in culture), TβT-FH, and ST-FH (passages 3–15; 70% confluence). Thirty to 50 μg of protein were separated by SDS-PAGE and Western blotted as described in "Materials and Methods." A representative blot is shown ($n = 4$). F-actin levels were analyzed as a cytoskeletal control protein whose levels were not expected to change during EMT. **B.** Analysis of gene expression in TβT-FH and ST-FH cells and comparison with fetal hepatocytes. RNA (20 μg) isolated from fetal rat hepatocytes (24 h in culture), TβT-FH, and ST-FH (passages 3–15; 70% confluence) was used in Northern blot experiments to visualize mRNA levels as described in "Materials and Methods." A representative experiment is shown ($n = 3$). *HNF,* hepatocyte nuclear factor; *E-CAD,* E-cadherin; *FIB,* fibronectin. 18S ribosomal probes were used to show RNA loading.

acquisition of a spindle-shaped cell morphology (EMT) are major events during development and carcinogenesis (18). In cancerous cells, EMT reflects a failure in the mechanisms that mediate cell-cell recognition and adhesion and its coupling to maintenance of cell shape and polarity. Therefore, understanding the molecular events that provoke EMT during development or cell differentiation might provide an insight into the regulatory events at play during the invasive process.

Here, we describe the process of EMT that fetal hepatocytes undergo when they are cultured in the presence of TGF-β or 10% FBS. Under these conditions, cells adopt a fibroblast-like appearance, replace their cytokeratin intermediate filament network with vimentin, and assemble actin microfilaments in stress fibers. In the case of TGF-β-treated hepatocytes (TβT-FH cells), virtually 100% of surviving cells adopt this fibroblastic phenotype. In the case of serum-treated hepatocytes (ST-FH cells), the cells with mesenchymal phenotype coexist with clusters of parenchymal hepatocytes, but following trypsinization and passage of cells, the fibroblast-like cells predominate. This kind of phenotypic transition also occurs in neonatal liver cells *in vitro* (30) and could be addressed by the presence of mitogens that act as scatter factors (such as EGF or HGF) and/or TGF-β in the serum (17). Apart from the high levels of vimentin and the lack of cytokeratin 18, both ST-FH and TβT-FH cells show increased levels of Snail transcripts and down-regulate E-cadherin expression, indicating that they have undergone EMT. Previous works have related TGF-β to the induction of EMT (31–36), TGF-β receptors and Smad proteins being involved in the response (31, 37, 38). However, these experiments were carried out either in tumor or immortalized cell lines overexpressing different proto-oncogenes (such as *ras*, *raf*, or *met*). Here, we show that TGF-β alone is able to induce EMT in primary cultures of parenchymal nontransformed hepatocytes, activating Snail and down-regulating E-cadherin expression. Preliminary results in our laboratory indicate that regenerating (48 h after two-thirds partial hepatectomy), but not adult hepatocytes, are also able to undergo this response (unpublished observations). Fetal (and regenerating) hepatocytes are proliferating cells that are not terminally differentiated, and thus, plasticity could explain the transdifferentiation from an epithelial to a fibroblast-like phenotype. Unexpectedly, these fibroblast-like cells can be subcultured for several passages and frozen with almost a 100% recovery, despite not being immortalized. Maintenance of 50% conditioned medium was convenient, although not indispensable, for culture progression. Cells could be producing TGF-β and other factors that could be acting in an autocrine loop. The possibility of maintaining these cells for long periods in culture provides a powerful tool to study the molecular mechanisms that control EMT in the liver.

A detailed analysis of the growth properties of TβT-FH and ST-FH cells showed that they do not proliferate at a higher rate than fetal hepatocytes, require mitogens to grow, and maintain the response to TGF-β in terms of growth inhibition. These results indicate that they do not possess any proliferative advantage against fetal hepatocytes. Nevertheless, they are much less differentiated. The two major transcription factors enriched in liver cells, HNF-4 or HNF-1α, are not expressed by TβT-FH and ST-FH cells, but interestingly, they do express OV-6. The OV-6 protein is not found in hepatocytes but is present in liver cell progenitors, oval cells, as well as in different lines of HCCs (28). It is generally accepted that specific combinations of liver-enriched transcription factors control critical steps in liver differentiation, and that the HNF-4/HNF-1α couple plays a key role in this process (39). Hepatoma cells fail to express liver-specific genes and HNF-4 expression overcomes the repression of the hepatic phenotype and induces the expression of epithelial markers (39, 40). Interestingly, the phenotypic transition that we observed here is also reminiscent of that seen by Spagnoli *et al.* (41) in the palmate cell, an immortalized nontransformed bipotential cell that does not express the liver-enriched transcription factors and is a precursor of the

epithelial-hepatocyte in Met murine hepatocyte (MMH) cell lines. In these cells, TGF-β stabilizes the palmate phenotype and provokes epithelial cells to acquire palmate-like morphological characteristics, in parallel with down-regulation of expression of HNF-4 and HNF-1α and activation of Snail transcripts (35). Therefore, the phenotypic transition from fetal hepatocytes to TβT-FH and ST-FH cells is reminiscent of that seen in liver cell progenitors and in some HCCs.

In addition to dedifferentiation, EMT confers resistance to TGF-β-induced apoptosis. This response is not exclusive to TGF-β, because the cells could survive in the absence of serum for more than 10 days (data not shown). Interestingly, the apoptotic machinery of these cells is not altered, as they are able to respond to staurosporine (data not shown). This is again reminiscent of certain tumors, including hepatocarcinoma cells, which become insensitive to certain effects of TGF-β, and in particular, to its ability to cause growth arrest and apoptosis, concomitant with the loss of cell surface receptors and/or Smads (8, 42). However, mutations in the TGF-β receptor II or the Smad 2 and 4 genes, frequently observed in other human

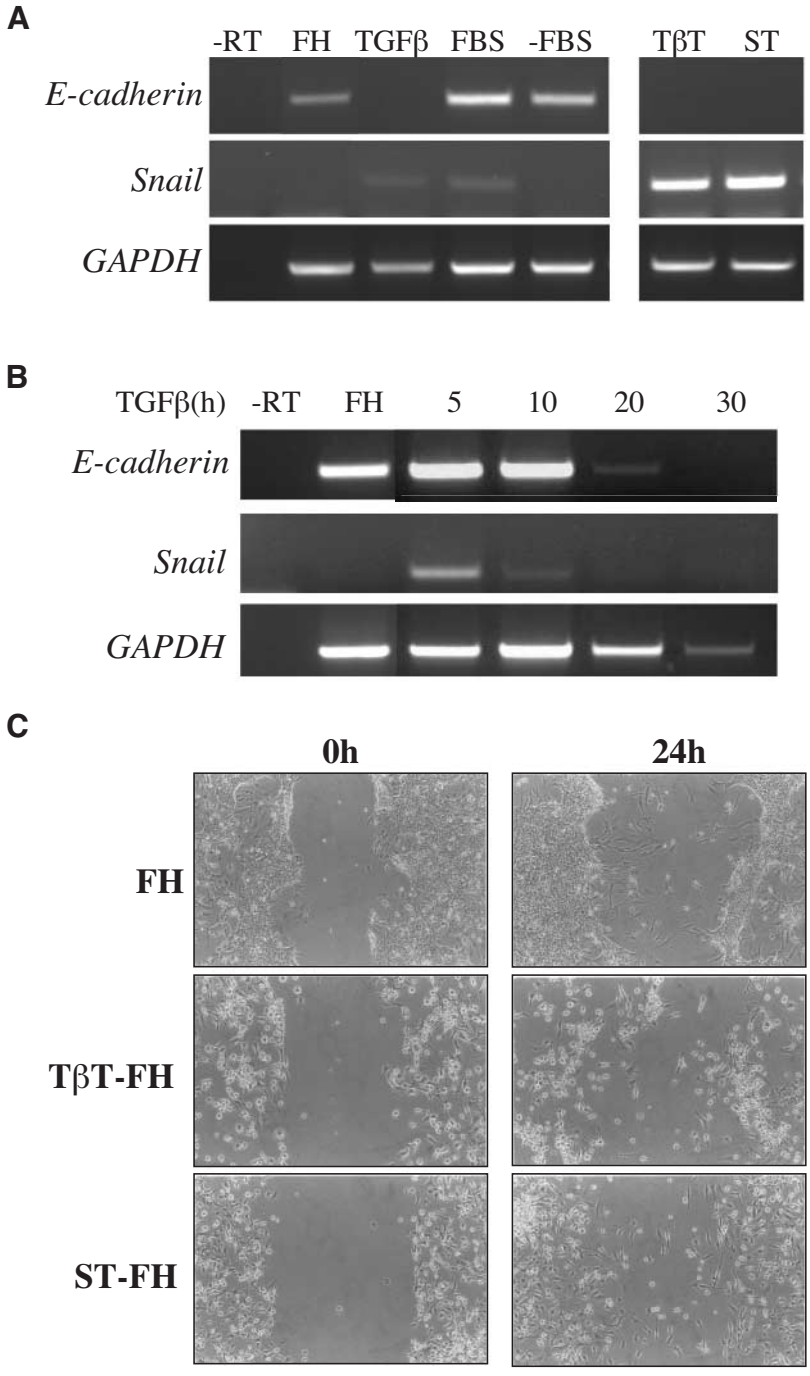

FIGURE 5. TGF-β and FBS induce Snail expression and migratory properties in fetal hepatocytes. **A.** Expression of Snail and E-cadherin was analyzed by RT-PCR in fetal hepatocytes after 24 h (*FH*) or 72 h in culture in the absence (*-FBS*) or presence of 2 ng/ml TGF-β (*TGF*β) or 10% FBS (*FBS*) for the last 48 h. Expression of Snail is maintained in the subcultured TβT-FH (*7*β*T*) and ST-FH (*ST*) cells (passage 12). GAPDH expression was analyzed as a control of the cDNA template in each sample. The *-RT* lane shows the results of amplification in the absence of reverse transcriptase. **B.** Time course analysis of the effect of TGF-β on Snail and E-cadherin expression. **C.** Fetal hepatocytes (*FH*, 48 h in culture) and TβT-FH and ST-FH cells (passages 3–5) were subjected to an *in vitro* wound analysis. Cultures were gently scratched with a pipette tip to produce a wound. Photographs were taken immediately (t = 0) and 24 h after the incision.

Table 1. FACScan Analysis of the Percentage of Cells in Each Phase of the Cell Cycle: Comparison Between Fetal Primary Hepatocytes (FH) and the TβT-FH and ST-FH Cells, Cultured in the Absence of Any Mitogenic Stimuli (Serum or Growth Factors) for 24 h

	Cell Cycle Phase		
	G_1	S	G_2-M
FH	76.5 ± 2.8	15.2 ± 2.3	8.1 ± 0.9
TβT-FH	83.2 ± 1.9	10.4 ± 4.1	6.4 ± 2.2
ST-FH	82.7 ± 3.1	10.4 ± 0.7	6.9 ± 2.4

cancers, have only rarely been observed in HCCs (9, 42). The results presented here indicate that both TβT-FH and ST-FH cells maintain their sensitivity to the inhibition of cell proliferation mediated by TGF-β, demonstrating that the receptors and intracellular signaling pathways associated with TGF-β remain effective. In contrast, some intracellular survival signals appear to be altered in these cells, because higher levels of active AKT and Bcl-x_L were detected. In relation to this, we have recently shown that growth factors, such as EGF, impair the apoptotic effect of TGF-β in fetal hepatocytes by a mechanism independent of ERKs, but that is associated with phosphatidylinositol 3-kinase/AKT activation and Bcl-x_L up-regulation (29). Phosphatidylinositol 3-kinase also appears to be required for TGF-β-mediated EMT and cell migration in mammary epithelial cells (33, 36). Interestingly, Shin *et al.* (43) have recently observed that TGF-β may induce cytoplasmic retention of FKHRL1 through the activation of AKT and thus, act as an antiapoptotic factor in epithelial cells. This indicates that the same intracellular signals responsible for the EMT could be involved in the promotion of survival mechanisms. Despite the fact that the MEK-ERK signaling cascade appears to be essential for TGF-β-induced EMT in pancreatic cells (44), both TβT-FH and ST-FH cells show lower levels of active p42 and p44 mitogen-activated protein kinases (MAPKs) than fetal hepatocytes. This result indicates that MAPKs are not responsible for the resistance to TGF-β-mediated apoptosis in these cells. However, Ras/Raf/MAPK pathway could be required for EMT, as has been recently suggested (45).

In summary, the results presented here demonstrate that TGF-β and other stimuli present in FBS are able to induce EMT in fetal rat hepatocytes in primary culture. Upon undergoing EMT, the cells maintain a regulated response in terms of growth, but dedifferentiate and become resistant to the apoptotic effect of TGF-β. Moreover, these cell lines can be maintained in culture up to 30 passages, whereas primary fetal hepatocytes proliferate only within the first several days/weeks after isolation and die after trypsinization. Therefore, taking together the results of proliferation and apoptosis, these cells do possess a growth advantage against fetal hepatocytes. The isolation of fetal rat hepatocytes that have suffered an EMT process and the possibility of maintaining them in culture for several passages provide an important tool for the future analysis of the mechanisms that allow these liver cells to

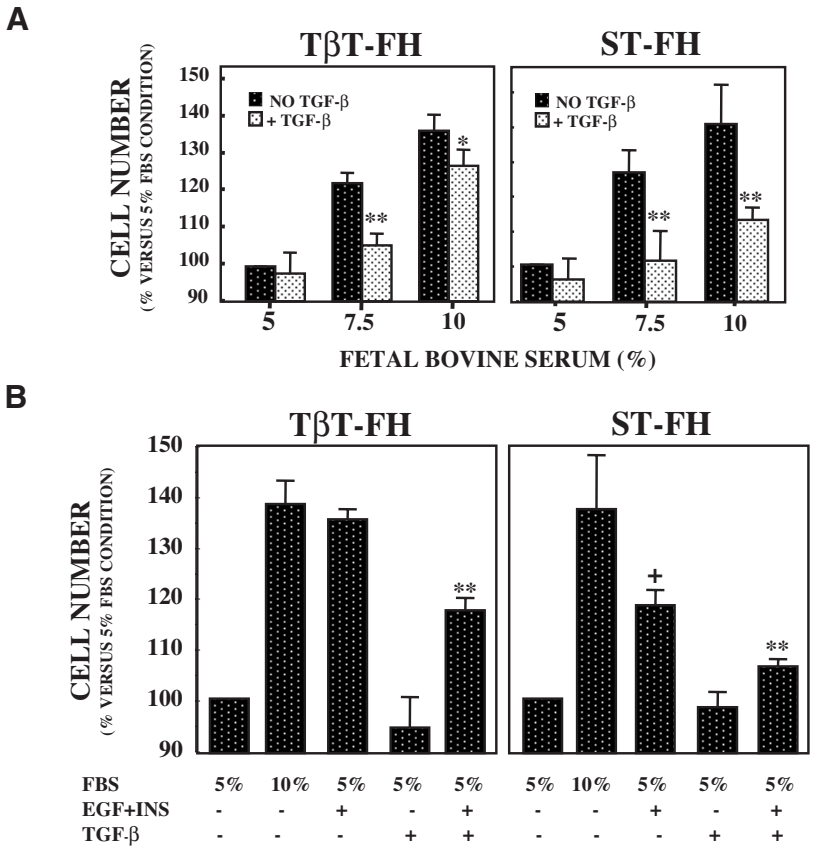

FIGURE 6. Anti-proliferative response of TGF-β in both ST-FH and TβT-FH cells. **A.** TβT-FH and ST-FH cells (passages 3–15) were incubated for 72 h with different concentrations of FBS and in the absence or presence of 2 ng/ml TGF-β. Viable cells were analyzed by crystal violet staining as described in "Materials and Methods." For each concentration of FBS, data from TGF-β-treated cells were compared *versus* untreated cells by the Student *t* test. *, $P < 0.05$; **, $P < 0.01$. **B.** Cells were incubated for 72 h with 5% FBS alone or supplemented with EGF (20 ng/ml) + insulin (100 nM), TGF-β (2 ng/ml), or EGF + insulin + TGF-β. Viable cell number was analyzed by crystal violet staining. Cell number in 10% FBS is also included in the figure to evaluate the mitogenic capacity of EGF + insulin. Data from EGF + INS-treated cells were compared *versus* 10% FBS-treated cells by the Student *t* test; +, $P < 0.05$. Data from EGF + INS + TGF-β-treated cells were compared *versus* EGF + INS treatment by the Student *t* test; **, $P < 0.01$. In **A** and **B**, the results are expressed as the percentage of cells relative to those present in cultures containing 5% FBS. *Columns,* means of values from three independent experiments with triplicate dishes; *bars,* ±SE.

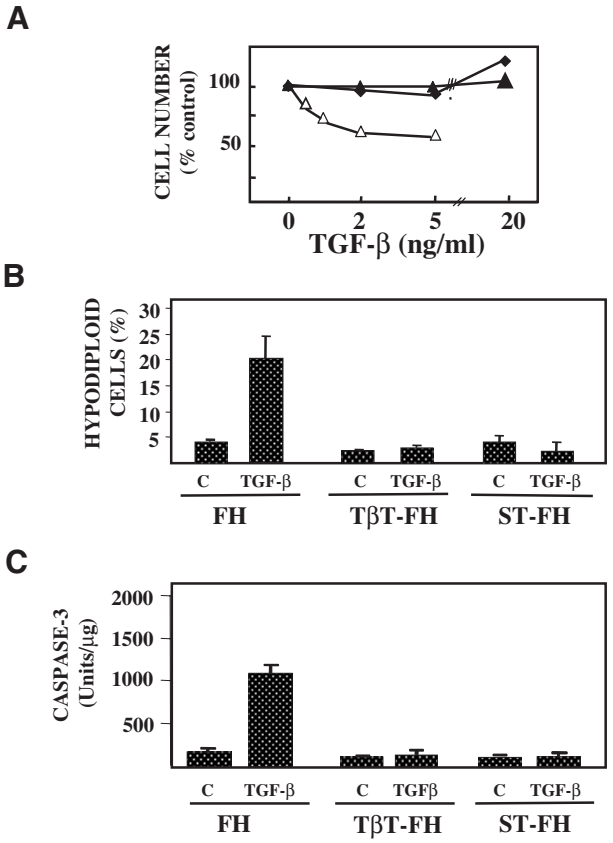

FIGURE 7. Lack of apoptotic response to TGF-β in TβT-FH and ST-FH cells. **A.** Cells were incubated for 48 h in the absence of serum (no conditioned medium) and in the presence of increasing concentrations of TGF-β. Cell number was analyzed by crystal violet staining. The results represent the percentage of remaining cells as compared to control (untreated) cells. A representative experiment is shown (n = 3; passages 3–15) (△, FH; ▲, TβT-FH; ◆, ST-FH). **B.** The DNA content was determined in fetal hepatocytes (*FH*, 24 h in culture) and TβT-FH and ST-FH cells (passages 3–15; 70% confluence) incubated for 24 h in the absence of serum (Control, *C*) or in the absence of serum but in the presence of 2 ng/ml TGF-β. The results are expressed as the percentage of hypodiploid cells (apoptotic cells). *Columns,* means of values from three independent experiments with duplicate dishes; *bars,* ±SE. **C.** Cells were also processed to analyze caspase-3 activity. The results are expressed as units/μg protein. *Columns,* means of values of at least three independent experiments with duplicate dishes; *bars,* ±SE.

overcome the TGF-β-mediated apoptotic effects. Furthermore, the low levels of HNF-1α and HNF-4 observed in TβT-FH and ST-FH and the accumulation of OV-6 suggest that these cells may behave as progenitors of liver cell lineages. Finally, TGF-β overexpression is frequently observed in several tumors, including human HCCs (9). We propose that the mesenchymal transition in response to TGF-β and other scatter factors during spontaneous transformation *in vivo*, might recapitulate the phenotypic effects that we have observed after the isolation of the liver cells that have survived TGF-β exposure.

Materials and Methods

Materials

Human recombinant TGF-β1 was obtained from Calbiochem (La Jolla, CA). Culture media, and fetal and neonatal calf sera were from Imperial Laboratories (Hampshire, UK). The primary antibodies used were: anti-active MAPK, phospho-p44/phospho-p42 (V6671) (Promega, Madison WI); anti-Bcl-x polyclonal antibody (sc-634) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-active AKT (New England Biolabs, Beverly, MA); anti-β-actin and anti-F-actin (Sigma-Aldrich, Madrid, Spain); anti-X-IAP (BD Transduction Laboratories, Lexington, KY); monoclonal anti-vimentin (clone V9) (Boehringer Mannheim, Mannheim, Germany); and anti-rat albumin polyclonal antibody (Nordic Immunological Laboratories, Tilburg, the Netherlands). Polyclonal anti-cytokeratin 18 (CK-18) and monoclonal anti-rat OV-6 were kindly provided by Drs. Bachs and Bastos (Spain) and Drs. Yin, Sell, and Leffert (USA), respectively. The secondary antibodies included FITC-conjugated sheep anti-mouse immunoglobulins and TRITC-conjugated goat anti-rabbit immunoglobulins (DAKO Corp., Santa Barbara, CA). Radiochemicals were from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and other reagents were supplied by Sigma-Aldrich or Roche Molecular Biochemicals (Barcelona, Spain).

Cell Isolation and Culture

Pregnant Wistar rats were used in this study. Hepatocytes from 20-day-old fetal rats were isolated by collagenase disruption as previously described (2.5×10^6 cells/fetus:16). The cells were plated on noncoated plastic dishes in Arg-free medium 199, supplemented with ornithine (200 μM), FBS (10%), penicillin (120 μg/ml), and streptomycin (100 μg/ml). The cells were incubated in 7.5% CO_2, at 37°C to facilitate attachment and the medium was changed after 4 h.

Light Microscopy

Following treatment, the cells were twice washed with PBS, and fixed in BOUIN (71% picric acid/24% formaldehyde/5% acetic acid) at room temperature for 30 min. The fixative was removed and the cells were rinsed extensively with 70% ethanol and PBS before being visualized with a Nikon Eclipse TE 300 microscope and photographed with Kodak Elite film.

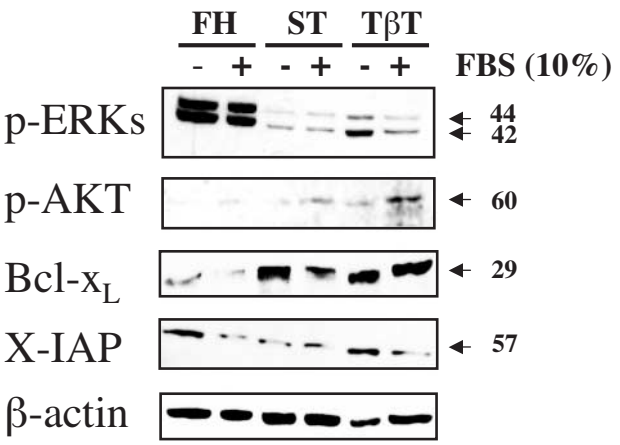

FIGURE 8. TβT-FH and ST-FH cells show high levels of p-AKT and Bcl-x_L. Fetal hepatocytes (*FH*, 24 h in culture) and TβT-FH and ST-FH cells (passages 3–5), 70% confluence in all the cases, were incubated for a further 24 h in the absence (−) or presence (+) of 10% FBS. The content of active ERKs (p-ERKs), active AKT (p-AKT), Bcl-x_L, and X-IAP was analyzed by Western blot using β-actin as a control for gel loading. A representative experiment of three is shown.

Confocal Microscopy Studies

For immunofluorescence detection of intermediate filaments (vimentin and cytokeratins) and albumin, cells were washed twice with PBS, fixed in methanol (−20°C) for 2 min, and processed for double immunofluorescence, as previously described (16). Cells were exposed to the primary antibodies for 1 h at 37°C, washed four times in PBS (5 min), incubated for 45 min with fluorescent-conjugated secondary antibodies, and finally washed in PBS as above. Actin filaments were visualized in paraformaldehyde-fixed cells with TRITC-conjugated phalloidin (5 ng/ml). Cells were visualized after coverslipping using immunofluorescence medium and examined with an MRC-1024 confocal microscope (Bio-Rad, United Kingdom) adapted to an inverted Nikon Eclipse TE 300 microscope. Images were acquired at 488 nm laser excitation for FITC-conjugated antibodies and 514 nm for TRITC-conjugated antibodies. Fluorescence emissions were detected through a 513/24-nm bp filter for FITC and a 605/15-nm bp filter for TRITC.

Western Blots

Cells were extracted in cytoskeleton buffer containing Triton X-100 following a modified method of Pagan *et al.* (17) and Franke (46) to detect vimentin, cytokeratins, and F-actin. To detect phospho-MAPKs, phospho-AKT, Bcl-x_L, and X-IAP, the cells were scraped off the plates after washing with cold PBS and lysed at 4°C as previously described (29). Proteins were separated by SDS electrophoresis on 12% polyacrylamide gels, transferred to membranes that were then blocked in TTBS containing 5% nonfat dried milk. Membranes were incubated for 2 h with the corresponding primary antibody at 37°C in TTBS containing 0.5% nonfat dried milk (all antibodies diluted 1:1000, except for cytokeratin 18 antibody, 1:200). After washing, the membranes were incubated with peroxide-conjugated anti-mouse immunoglobulin for 2 h at room temperature (1:5000 in TTBS 0.5% nonfat dried milk). Antibody binding was visualized using enhanced chemiluminescence (Amersham).

RNA Isolation for Northern Blot Analysis

Total RNA was isolated as described by Chomczynski and Sacchi (47). For each assay, RNA was extracted from the cells pooled from two 92-mm dishes. cDNA probes and hybridization conditions were as described previously (48). Fibronectin and 18S ribosomal cDNAs were labeled with [α-^{32}P]dCTP by nick translation, whereas all the HNF probes were labeled by random priming reaction. Serial hybridizations with the different probes were performed successively.

Reverse Transcription-PCR Analysis

Polyadenylated + RNA was isolated from the different hepatocyte cell lines using the Microfast Track isolation kit (Invitrogen, Barcelona, Spain). RT was carried out with oligodeoxythymidylate primer, whereas PCR reactions were performed using mouse specific primers for Snail, E-cadherin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follows: Snail forward, GCAGCTGGCCAGGCTCTCGGTGGC; Snail reverse, GTAGCTGGGTCAGCGAGGGCCTCC; E-cadherin forward, CGTGATGAAGGTCTCAGCC; E-cadherin reverse, ATGGGGGCTTCATTCAC; GAPDH forward, TGAAGGTCGGTGTGAACGGATTTGGC; and GAPDH reverse, CATGTAGGCCATGAGGTCCACCAC. PCR products were obtained after 30–35 cycles of amplification at annealing temperatures of 62–65°C.

Wound Healing Assay

Cells were seeded in 60-mm culture dishes and 48 h later (or 70% confluence, in the case of ST-FH and TβT-FH cells), a wound was incised in the central area of the culture. After washing to remove detached cells and addition of fresh medium without serum (in the absence of arginine for fetal hepatocytes), the cultures were incubated for a further 24 h. Photographs were taken at t = 0 and 24 h in an inverted Zeiss Axiovert microscope.

Analysis of Viable Cells

Viable adherent cells were stained with crystal violet (0.2% in 2% ethanol) for 20 min, as previously described (14). 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).

Analysis of Cell DNA Content by Flow Cytometry

Cells were detached from dishes with 0.25% trypsin/0.02% EDTA, fixed in 70% ethanol (−20°C) for 1 min, and treated with RNase (10 μg/ml) for 30 min at 37°C. After propidium iodide staining (0.05 mg/ml, 15 min at room temperature in the dark), the cellular DNA content was evaluated in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). For computer analysis, only signals from single cells were considered (10,000 cells/assay).

Analysis of Caspase 3 Activity

Cells were lysed at 4°C in 5 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100. A reaction mixture containing 25 μl cell lysate, 325 μl assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiotreitol), and 20 μM caspase 3 substrate (Ac-DEVD-AMC) (29) was incubated for 2 h in the dark. Enzymatic activity was measured in a Luminescence Spectrophotometer (Perkin-Elmer LS-50, Shelton, CT) (λ excitation, 380 nm; λ emission, 440 nm). A unit of caspase 3 activity was defined as the amount of active enzyme necessary to produce an increase of 1 arbitrary luminescence unit after the 2-h incubation. Subsequently, the protein concentration of the cell lysates was determined with the Bio-Rad protein assay kit and the results were presented as units of caspase 3 activity per μg of protein.

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