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# Transdifferentiation-dependent expression of α-SMA in hepatic stellate cells does not involve TGF-β pathways leading to coinduction of collagen type I and thrombospondin-2

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**Abstract**

Hepatic stellate cells (HSC) cultured on plastic spontaneously transdifferentiate to a myofibroblast-like cell type (MFB). This model system of hepatic fibrogenesis is characterized by phenotypic changes of the cells and increased matrix synthesis.

Here, we analyzed if transdifferentiation-dependent induction of ECM components, e.g., collagen type I and thrombospondin-2 (TSP-2), and phenotypic changes are coregulated events and if both processes are mediated via TGF-β pathway(s).

Blocking the TGF-β-dependent p38 MAPK pathway in HSC with the specific inhibitor SB203580 strongly reduces collagen I and TSP-2 mRNA expression without inhibiting upregulation of the typical MFB-marker, α-smooth-muscle actin (α-SMA). Similarly, interference with the Smad2/3/4 pathway using dexamethasone also heavily decreased expression of collagen type I and TSP-2 whereas transdifferentiation of HSC to the typical morphology of MFB with loss of fat droplets and increasing α-SMA was unchanged. Further, p38 MAPK mediated induction of collagen I and TSP-2 expression by TGF-$\beta_1$ was still achieved in the presence of dexamethasone, showing that dexamethasone does not block p38 while it delays Smad2 phosphorylation and antagonizes stimulation of a Smad3/Smad4 dependent TGF-β reporter construct. Interestingly, in contrast to SB203580 and dexamethasone, overexpression of the TGF-β antagonist Smad7 reduced ECM expression and simultaneously inhibited morphologic transdifferentiation, indicating that Smad7 fulfills additional features in HSC.

In conclusion, our data show that phenotypic changes of transdifferentiating HSC and induction of matrix synthesis are independent processes, the latter being stimulated by both, Smad dependent and MAPK dependent TGF-β signaling.
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*Keywords:* Dexamethasone; Liver; p38; Smads; Fibrosis

## 1. Introduction

A common feature of diverse fibroproliferative diseases is generation of a myofibroblastoid cell type (MFB) that produces extracellular matrix (ECM) proteins as part of wound healing and scar formation processes. During liver fibrogenesis, hepatic stellate cells (HSC) undergo such process and transdifferentiate into MFB. When isolated from healthy rat liver and cultured on plastic, HSC display

*Abbreviations:* TSP, thrombospondin; TGF-β, transforming growth factor-β; HSC, hepatic stellate cells; MFB, myofibroblast-like cells; HC, hepatocytes; KC, Kupffer cells; SEC, sinusoidal endothelial cells; MAPK, mitogen activated protein kinase; FCS, fetal calf serum; ECL, enhanced chemiluminescence; α-SMA, α-smooth-muscle actin; ECM, extracellular matrix.

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a very similar behavior in vitro thereby representing a well-accepted model system to study molecular details of fibrogenesis. Quiescent HSC are small cells with short extensions harboring large amounts of retinol droplets, whereas MFB are enlarged in size, possess an extended α-SMA cytoskeleton and have lost fat droplets. Expression and synthesis of ECM components, e.g., collagens, fibronectin, decorin and others, are strongly upregulated during transdifferentiation. However, the molecular mechanisms that initiate and perpetuate in vitro transdifferentiation of HSC remain unclear or are under controversial discussion. Transdifferentiation of cells to ECM producing MFB has been described for a wide variety of non-hepatic fibroproliferative disorders as well, e.g., for proximal tubular epithelial cells (Zhang et al., 2004; Yang et al., 2003), pancreatic stellate cells (Jaster, 2004) and normal or keloid-derived dermal fibroblasts (Shephard et al., 2004; Ehrlich et al., 1994).

TGF-$\beta_1$ is an important fibrogenic cytokine due to its collagen inducing function in many cell types and because it inhibits matrix degradation (Border and Noble, 1994; Gressner et al., 2002). The main signal transducing molecules upon TGF-β stimulation are the Smad proteins. Upon ligand binding to the cell surface, the activated receptor complex phosphorylates receptor Smads which then associate with Smad4, translocate into the nucleus and positively or negatively regulate expression of TGF-β target genes (Miyazawa et al., 2002). TGF-$\beta_1$ synthesis increases during transdifferentiation of HSC (Breitkopf et al., 2001) implicating autocrine stimulation.

The synthetic glucocorticoid dexamethasone was previously described to antagonize TGF-β effects, especially collagen inducing properties (Gras et al., 2001; Cockayne et al., 1986) and it was recently demonstrated in HSC that glucocorticoids reduce the bioavailability of TGF-β directly (Bolkenius et al., 2004).

TSP-2 knock-out mice show abnormally long and flexible collagen fibers (Kyriakides et al., 1998), indicating that the presence of TSP-2 may be a prerequisite for the correct formation of a collagen-rich matrix. This hypothesis is further supported by subcutaneous implantation of sponges in TSP-2 knock-out mice that display, besides increased angiogenesis and hemorrhage, the development of significant fibrosis after 21 days (Kyriakides et al., 2001).

The aim of the present study was to investigate if increased ECM expression and morphological transdifferentiation, e.g., de novo synthesis of α-SMA are coregulated events in HSC and if TGF-β signaling is essential for both processes.

Our results demonstrate that collagen I expression in activated HSC is mediated via p38 MAPK and Smad pathways. However, blocking these pathways did not abrogate in vitro transdifferentiation of HSCs that was examined as α-SMA expression or loss of fat droplets, indicating that phenotypic transformation and ECM expression are independent processes.

Furthermore, we are first to show that TSP-2 is induced during transdifferentiation of HSC and thereby represents a new marker for HSC activation probably participating in ECM formation.

## 2. Materials and methods

Recombinant human TGF-$\beta_1$ was purchased from R&D Systems (Minneapolis, USA), dexamethasone from Sigma, SB203580 from Calbiochem and normal IgG from Santa Cruz, CA, USA.

### 2.1. Isolation and culture of cells

KC and SEC were isolated by centrifugal elutriation. HC were isolated from male Sprague–Dawley rats (strain Han:SPRD from the Institute of Versuchstierkunde, RWTH Aachen; body weight about 250 g) by the two-step collagenase method of Seglen (1993). HC were further purified by centrifugal elutriation, resulting in a final contamination by nonparenchymal cells of <3% (Kountouras et al., 1984). Primary HSC were isolated from rat livers by the pronase–collagenase perfusion technique followed by single-step density gradient centrifugation with Nycodenz as described earlier (Breitkopf et al., 2001). The cells were seeded on day 0 with a density of $4 \times 10^4$ cells/cm$^2$ and cultured in Dulbecco's modification of Eagle's medium (Bio Whittaker Europe, Verviers, Belgium) containing 4 mM L-glutamine (Flow Laboratories, Bonn, Germany), penicillin (100 IU/ml)/streptomycin (100 μg/ml) (Biochrom KG, Berlin, Germany) and 10% FCS (Seromed, Berlin, Germany) under humidified atmosphere of 5% $CO_2$/95% air at 37 °C. HSC were kept in primary culture for up to 10 days and were then passaged once. Secondary cultures were always fully transdifferentiated cells and were therefore termed myofibroblasts (MFB). Medium was changed every 2–3 days.

### 2.2. RT-PCR

Total RNA was isolated using the "high pure RNA isolation kit" from Roche (Mannheim, Germany) and under standard conditions equal amounts of each sample were reverse transcribed into cDNA by action of the AMV reverse transcriptase (Roche). The following primers were used in subsequent PCR (36 cycles): α-SMA: for. 5′-GTGATGGTGGGAATGGGC-3′; rev. 5′-CAATGAAGGAAGGCTGGAAC-3′. TSP-2: for. 5′-GGATGTACGTGGCCAAGGG-3′; rev. 5′-CTGGGTCCCAGAGCCACA-3′. rS6: for. 5′-GACTGACAGATACCACTGTGCCT-3′; rev. 5′-TTATTTTTGACTGGACTCAGAT-3′; Specificity of PCR-products was verified by sequencing (see below).