

**"TGF- β /Smad signaling in hepatic stellate cells and
during liver fibrogenesis"**

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To my parents

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1. Summary

Transforming growth factor (TGF)- β is a key mediator of hepatic stellate cell (HSC) activation and extracellular matrix accumulation leading to fibrosis. Aim of the present study was to perturb the TGF- β pathway in this process by overexpression of Smad7, an intracellular antagonist of TGF- β signaling. Moreover, a new TGF- β response gene was identified, which participates in the control of profibrogenic transdifferentiation to myofibroblast-like cells.

Ligation of the common bile duct was used to induce liver fibrosis in rats. Animals received injections of an adenovirus overexpressing Smad7. The effect of Smad7 on TGF- β signaling and activation of HSC was further analyzed in primary cultured cells. AdSmad7 infected rats displayed reduced collagen deposition and hydroxyproline content in the liver, when compared with animals administered AdLacZ. Such a beneficial effect was also observed, when Smad7 was expressed in animals with established fibrosis. Accordingly, Smad7 arrested transdifferentiation of primary cultured HSC. AdSmad7 infected cells remained in a quiescent stage and retained storage of vitamin A droplets. Smad7 expression totally blocked TGF- β signal transduction, shown by inhibiting Smad2/3 phosphorylation.

In an attempt to elucidate TGF- β target genes responsible for fibrogenesis, an analysis of Smad7-dependent mRNA expression profiles in HSC was performed, resulting in identification of the inhibitor of differentiation 1 (Id1) gene. Ectopic Smad7 expression reduced Id1 expression. Conversely, Id1 overexpression in HSC enhanced cell activation and circumvented Smad7-dependent inhibition of transdifferentiation. Moreover, knock-down of Id1 in HSC interfered with the transdifferentiation process, indicating a pivotal role of Id1 for fibrogenesis. Additionally, treatment of HSC with TGF- β led to increased Id1 protein expression, which was dependent on activation of the novel ALK1/Smad1/5/8 pathway. Knocking-down expression of ALK1 in HSC interfered with TGF- β dependent Smad1 phosphorylation and Id1 upregulation, but did not block the activation of the Smad2/3 pathway.

In summary, gene transfer of Smad7 inhibits experimental fibrogenesis *in vivo*. Studies with isolated HSC suggest that the underlying mechanisms involve inhibition of TGF- β signaling and HSC transdifferentiation. Moreover, a novel TGF- β pathway involving ALK1 and Smad1 was shown to mediate HSC activation via Id1 upregulation. These new findings

represent valuable information for the development of more efficient antifibrotic strategies to treat liver damage in the future.

1a. Zusammenfassung

Der transformierende Wachstumsfaktor β (Transforming growth factor β ; TGF- β) spielt eine wichtige Rolle in der Aktivierung von hepatischen Sternzellen (hepatic stellate cells, HSCs) und der Freisetzung von extrazellulärer Matrix während der Leberfibrose. Ziel der vorliegenden Studie war durch Expression des intrazellulären Antagonisten, Smad7, den TGF- β Signalweg zu blockieren. Darüber hinaus wurde ein neues TGF- β Zielgen identifiziert, das an der Transdifferenzierung von HSCs beteiligt ist.

Die *in vivo* Fibrogenese wurde in einem Galengangsligatur-Model in der Ratte untersucht. Die Tiere wurden mit Smad7 codierenden Adenoviren infiziert. Die Kollagen-Expression und der Hydroxyprolin-Gehalt war in den Lebern von Smad7 behandelten Ratten verglichen mit Kontrollratten stark reduziert. Smad7 zeigte auch starke hemmende Wirkung auf die Transdifferenzierung von HSCs *in vitro* und blockiert den TGF- β Signalweg in den Zellen. Die Phosphorylierung von Smad2/3 ist runterreguliert und Smad7-exprimierende Zellen verloren die Vitamin A Tröpfchen nicht.

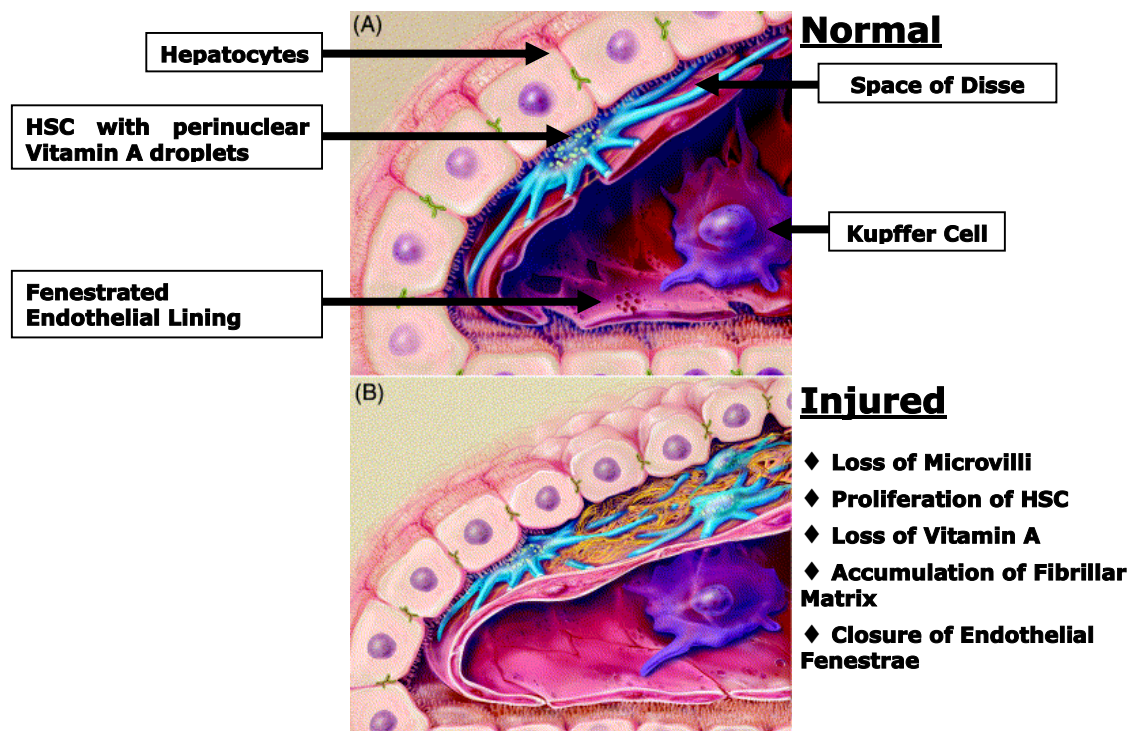
Die Rolle von TGF- β bei der Transdifferenzierung von HSCs wurde in einer Smad7 abhängigen Microarray-Analyse untersucht, was zur Identifizierung des Differenzierungsinhibitors 1 (inhibitor of differentiation; Id) führte. Smad7 führte zu starker Reduktion der Id1 Expression, wohingegen Id1 Überexpression Aktivierung von HSCs verstärkt und hinreichend ist, um den Smad7-Effekt zu überkommen. Si-RNA abhängiger Id1 *knock down* interferiert mit HSC-Aktivierung, was eine zentrale Rolle von Id1 während der Fibrogenese unterstreicht. Ich klärte weiter auf, dass die TGF- β -abhängige Expression von Id1 in HSCs über einen neuen ALK1/Smad1/5/8 Signalweg reguliert wird. Ein ALK1 si-RNA *knock down* blockierte die TGF- β induzierte Smad1-Phosphorylierung und Id1-Expression, hatte jedoch keinen Einfluss auf den ALK5/Smad2/3 Signalweg.

Zusammenfassend hemmt adenoviraler Transfer von Smad7 die Leberfibrose *in vivo*. Für den Effekt ist wahrscheinlich die Blockade TGF- β induzierter HSC-Aktivierung verantwortlich. Zusätzlich wurde ein neuer TGF- β Signalweg über ALK1 und Smad1 untersucht, der zu Id1 Expression. Diese neuen Erkenntnisse sollen dazu beitragen in Zukunft Leberfibrose besser behandeln zu können.

2. Introduction

2.1. Liver fibrogenesis

The normal liver is composed of an epithelium containing hepatocytes, an endothelial lining, tissue macrophages (Kupffer cells) and the perivascular stellate cells. Activation of hepatic stellate cells (HSC) is the key step in liver fibrogenesis (Gressner et al., 1995).



(Modified from Friedman et al., 2003)

Fig. 1. Subendothelial changes during liver fibrogenesis. A) Normal liver: Quiescent Hepatic Stellate Cells containing vitamin A droplets. Low density matrix allows normal function of the liver. B) Liver injury: transdifferentiation of HSC, loss of fat droplets, proliferation and remodeling of extracellular matrix; replacement of low density matrix with fibril matrix diminishes liver function.

They are situated in the subendothelial space of Disse, separating hepatocytes from the sinusoidal endothelium and are the principle storage site for retinoids. Space of Disse contains low density 'basement membrane-like' extracellular matrix (ECM) providing cellular support while allowing unimpeded transport of solutes. During liver injury the ECM composition becomes scar-like, and hepatocellular function deteriorates (Freedman et al., 2003) (Fig. 1).

The hepatic response to injury represents a paradigm for wound healing compared to other tissues including skin, lung and kidney, as it involves many of the same cell types and mediators (Li et al., 1999). A controlled healing response results in minimal scarring and in preservation of normal structural and functional abilities. Deregulation of this complex process at any stage may lead to extensive tissue scarring, with loss of physiological functions (Azouz et al., 2004).

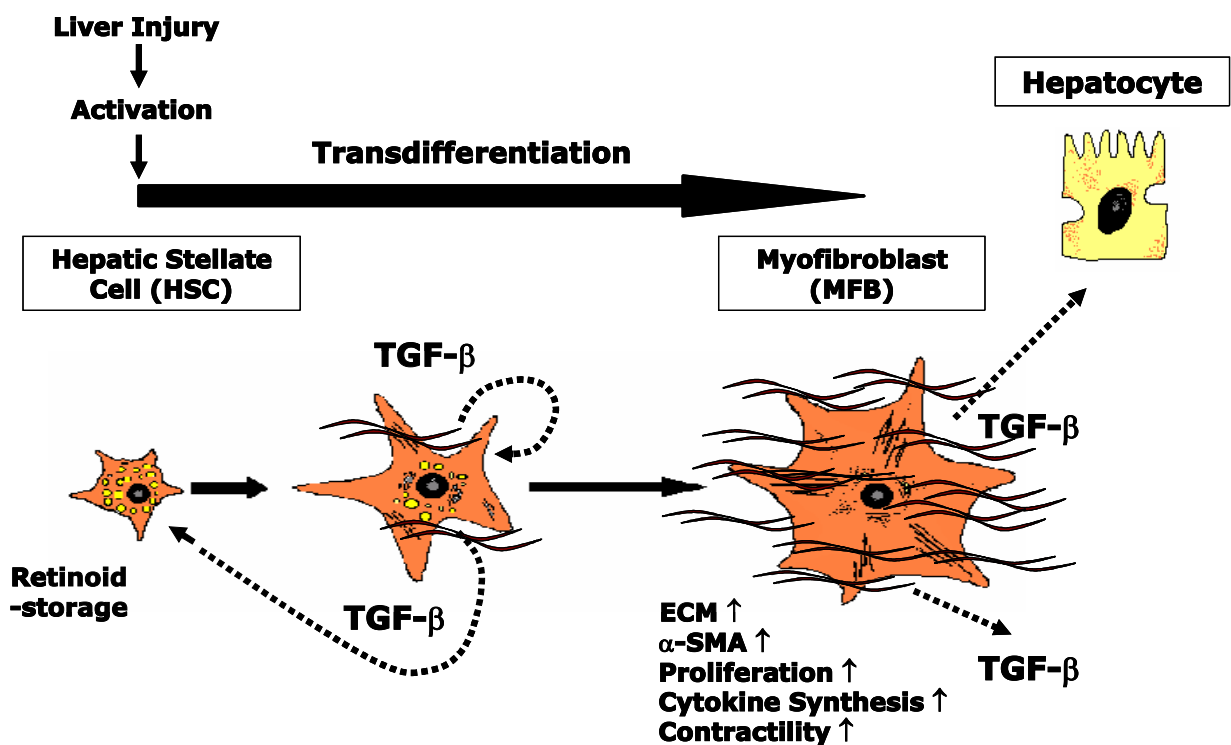
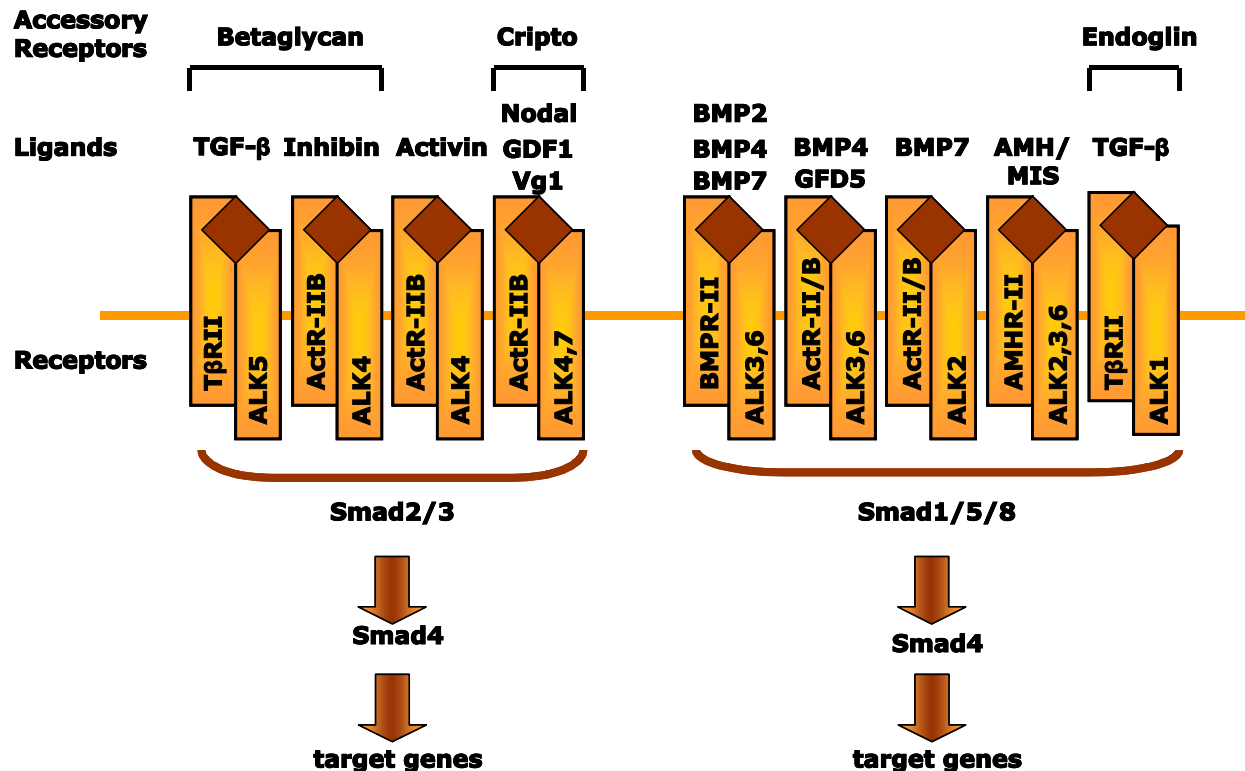


Fig. 2. Chronic liver damage leads to activation of HSC that lose fat droplets and start to produce extracellular matrix. They are also the major source for TGF- β in injured liver and can therefore mediate activation of quiescent HSC in a paracrine route and stimulation of matrix component expression in an autocrine manner. Released TGF- β can also have an effect on other cell types, e.g. hepatocytes.

Activated stellate cells are responsible for production of fibrotic ECM components like collagen types I and II, sulfated proteoglycans and glycoproteins (Knittel et al., 1996) (Freedman et al., 2003) (Garcia-Trevijano et al., 1999). Numerous studies implicate the crucial role of TGF- β in the activation of HSC and fibrogenesis (Bissel et al., 2001). Once activated, HSC start to produce and secrete TGF- β , which act in a paracrine or autocrine manner, promoting the transdifferentiation of HSC (Fig. 2).

2.2. TGF- β signal transduction pathways

TGF- β 1 belongs to the TGF- β family of cytokines, which includes three isoforms of TGF- β , activins and bone morphogenic proteins (BMPs) (ten Dijke et al., 2000). They regulate a wide range of cellular responses, such as proliferation, migration, differentiation, apoptosis, and extracellular matrix production (ten Dijke et al., 2002) (Shi et al., 2003). Signaling pathways of these cytokines are similar.

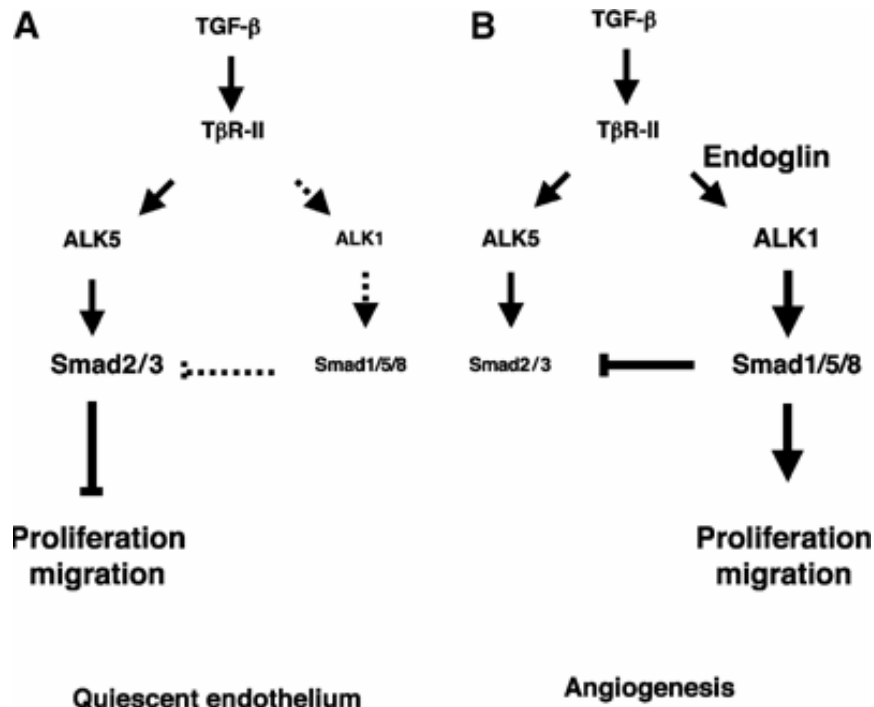


(Modified from Shi et al., 2003)

Fig. 3. TGF- β superfamily ligands signal through miscellaneous type I and II receptors and recruit different Smad proteins. In vertebrates there are also some accessory receptors taking part in signaling of TGF- β superfamily. GDF – growth and differentiation factor (Vg1 in *Xenopus*), MIS – Muelerian inhibiting substance (also known as AMH – anti-Muelerian hormone).

The initial step bases on binding of the ligand and inducing formation of heteromeric complexes of specific type I and type II receptor serin/threonin kinases on the cell surface (Shi et al., 2003). In this complex constitutively phosphorylated receptor type II phosphorylates receptor type I at a highly conserved intracellular domain called GS domain (Massague et al., 1998). The TGF- β family of receptors includes five receptors type II and seven receptors type I, which differ in their affinities to specific ligands, as well as in the

choice of Smads, specific intracellular molecules, triggering a diversity of cellular responses (Fig. 3).



(Lebrin et al., 2004)

Fig. 4. Endoglin modulates the TGF-β signaling pathway in endothelial cells, favoring TGF-β/ALK1 signaling and indirectly inhibiting the TGF-β/ALK5 pathway. In the absence of endoglin, TGF-β/ALK5 signaling is predominant and leads to inhibition of proliferation and migration of endothelial cells. High endoglin expression stimulates angiogenesis via activating the ALK1 pathway.

Moreover, accessory transmembrane receptors have been shown to participate in TGF-β signaling. Betaglycane (TGF-receptor type III) promotes binding of TGF-β to its type II receptor (TβRII), which is a critical event for TGF-β2 signaling. In contrast to TGF-β1 and TGF-β3, TGF-β2 binds TβRII only with low affinity and requires betaglycane for high-affinity binding (ten Dijke et al., 2004). A betaglycane-related protein, endoglin only binds ligand while in association with TβRII and displays affinity for TGF-β1 and TGF-β3, but not for TGF-β2.

In most cell types, TGF-β signals through TβRII and receptor type I (ALK5, also known as TβRI) complex. In endothelial cells, however, exists an alternative TGF-β pathway involving ALK1 (Goumans et al., 2002). The two pathways have opposite effects. ALK5

promotes Smad2/3 activation and inhibits endothelial cell migration and proliferation, whereas ALK1 induces Smad1/5 phosphorylation and stimulates both processes. ALK1 activation requires the presence of phosphorylated ALK5 (Goumans et al., 2003). Furthermore, ALK5 recruits ALK1 to the receptor complex with T β RII. ALK1 cannot build complexes with T β RII in cells lacking ALK5, leading to abrogation of TGF- β /ALK1 responses in these cells. Moreover, ALK1 inhibits ALK5 signaling downstream of Smad via inducing heteromeric complex formation between Smad2/3 and Smad1/5, which interfere with DNA binding and recruitment of cofactors.

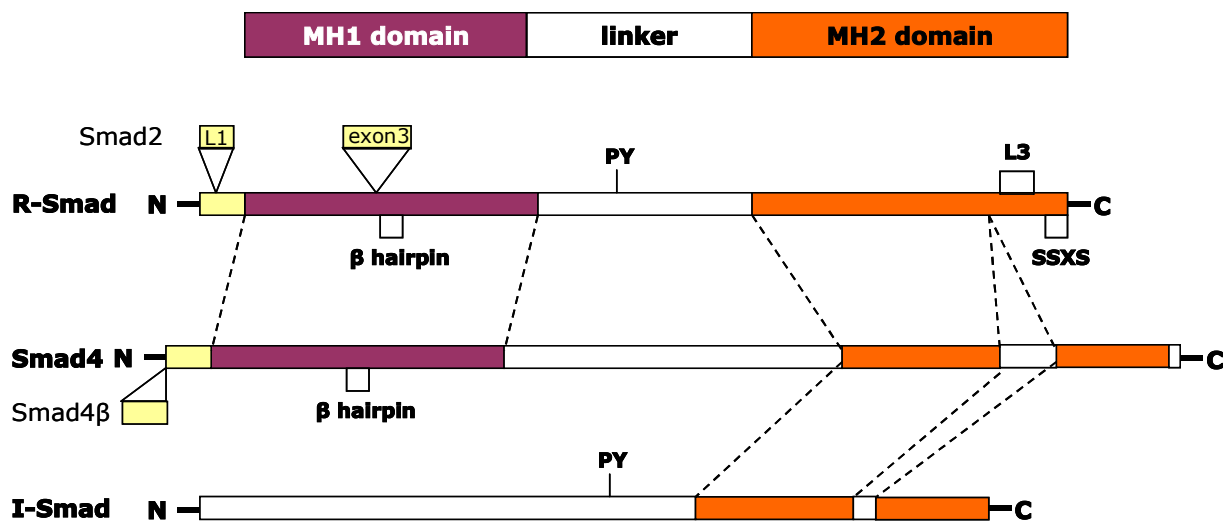
Endoglin is a necessary component of ALK1 signaling (Lebrin et al., 2004) modulating the balance between TGF- β /ALK1 and TGF- β /ALK5 signaling in endothelial cells and stimulating ALK1-induced Smad1/5 responses. Moreover, the ALK5 pathway is potentiated after knockdown of endoglin expression, implicating that endoglin indirectly inhibits the ALK5 signaling (Fig. 4). Interestingly, mice lacking endoglin produce decreased ALK5 levels; possible representing an adaptation process to overcome the ALK5 induced inhibition of angiogenesis (Lebrin et al., 2005).

Mutations in the endoglin or ALK1 genes cause hereditary hemorrhagic telangiectasia (HHT1 or HHT2 respectively), an autosomal dominant vascular dysplasia (Abdalla et al., 2005), underlining the close conjunction between ALK1 and endoglin in the endothelium. Moreover, mice lacking endoglin die at day 11.5 from defective vascular development (Li et al., 1999) and ALK1 knock-out mice exhibit lethality at E10.5-11.5 with dilation of blood vessels and failure in branching capillary network (Goumans et al., 2000).

2.2.1. Smad proteins

There are eight Smad proteins identified in vertebrates and subdivided into three subgroups (Massague et al., 2000). Receptor-regulated Smads (R-Smads) bind to the receptor complex and become phosphorylated (Smad1, 2, 3, 5 and 8). A second group is known as Co-mediator Smads (Co-Smads) and has only one member, Smad4, in vertebrates and its splice variant Smad4 β in *Xenopus* (Shi et al., 2003). Smad4 forms heteromers with phosphorylated R-Smads and these complexes act as transcription factors in the nucleus. The third group of Smad proteins (Inhibitory Smads; I-Smads) includes two members: Smad6 and Smad7 (Nakao et al., 1997). These molecules compete with R-Smads for binding to receptor complexes and act as intracellular inhibitors of TGF- β signaling. R-Smads and Smad4 share two conserved domains: the N-terminal Mad Homology 1 (MH1) domain and the C-terminal

MH2 domain (ten Dijke et al., 2004). The N-terminal domain of I-Smads shows only weak sequence homology to MH1 domain of R-Smads, thus I-Smads are not able to bind DNA. In contrast, the MH2 domain is highly conserved among all Smads and is responsible for the receptor interaction (Shi et al., 2003). The R-Smads are phosphorylated by a specific type I receptor on a carboxy-terminal SSXS motif (Macias-Silva et al., 1996) (see also Fig. 5).



(Modified from ten Dijke et al, 2004)

Fig. 5. Schematic structure of Smad proteins. The MH1 and MH2 domains are conserved among Smads. Non-conserved domains are shown in yellow (N-terminal domain of R-Smads and Smad4, *Xenopus* splice variant Smad4β or insertion in MH1 domain of Smad2) or white (linker region). SSXS – C-terminal phosphorylation site of R-Smads. PY – phosphorylation site in the linker region of R-Smads and I-Smads. L3 – loop3 of R-Smads interacts with receptor type I. β-hairpin – Smad3 and Smad4 DNA binding site.

Smad4 and all R-Smads, except Smad2, bind to DNA in a sequence-specific manner, although their affinity for DNA is very low and requires cooperation with other sequence-specific-factors for efficient binding to promoters of target genes (ten Dijke et al., 2004). Smad2 contains a unique 30 residue insertion responsible for a disruption of the DNA binding domain (Shi et al., 2003). The minimal Smad binding element for Smad3 and Smad4 contains four base pairs 5'-AGAC-3'. This sequence is known as Smad Binding Element (SBE) and is present in promoter regions of TGF-β and activin target genes (Miyazawa et al., 2002).

More than 30 different transcription factors (e.g. FAST1, TFE3, c-Jun, Sp1) and co-activating proteins (e.g. CBP/p300) have been reported to interact with Smad proteins, leading to different transcriptional responses in target cells (Pouponnot et al., 1998) (Miyazono et al., 2003).

Smads bind transcriptional co-repressors c-Ski and SnoN leading to complex formation with histone deacetylases, which causes repression of promoters of target genes (Miyazono et al., 2003). Activation of Smad3 by TGF- β directly induces expression of ATF3, which then in complex with Smad3 represses Id1 promoter activity in epithelial cells (Kang et al., 2003).

Felici et al. described a novel modulator of the TGF- β pathway, Trap-1-like-protein (TLP) (Felici et al., 2003). TLP associates with TGF- β /activin type II receptors and is involved in endocytic trafficking, due to the presence of a clathrin heavy-chain repeat domain. Interestingly, TLP abrogates Smad3/Smad4 signaling via binding to the Smad complex without abrogating Smad3 phosphorylation. Smad2 signaling is not affected by TLP indicating that TLP is a modulator of the balance of Smad2/Smad3-specific TGF- β responses.

Cross-talks with other signaling pathways are further modulators of TGF- β responses. R-Smads are targets for the mitogen-activated protein kinase (MAPK) pathway. MAP kinases phosphorylate R-Smads at the PXSP sequence in the linker region (Massague et al., 2003) (Attisano et al., 2002) and prevent nuclear localization of Smads, but not receptor-mediated Smad activation. However, R-Smads phosphorylated at both sites are not able to translocate into the nucleus. Moreover, activation of Ca²⁺/calmodulin-dependent protein kinase II (CamKII) leads to phosphorylation of R-Smads and inhibition of nuclear transport and transcription (Wicks et al., 2000). Phosphorylation of MH1 domain of Smad3 by protein kinase C (PKC) abrogates Smad3 DNA binding ability (Derynck et al., 2003). Fibroblast growth factor (FGF) and insulin-like growth factor (IGF) induce MAPK dependent phosphorylation of the Smad1 linker-region and promotes neural differentiation in *Xenopus* (Pera et al., 2003). When this phosphorylation is prevented, Smad1 becomes an efficient antineural agent.

2.2.2. Smad7

Smad7 was shown to inhibit TGF- β , activin and BMP signaling. In contrast to that, Smad6 specific abrogates only the BMP pathway with high efficiency (Imamura et al., 1997).

Smad7 is an early response gene to TGF- β ; its mRNA expression shows a peak 30-90 minutes after stimulation. The promoter region of Smad7 contains highly conserved elements within the mouse, rat and human genome, e.g. a palindromic Smad Binding Element (SBE) with adjacent E-box and AP1 site. Mutations in any of these recognition sites lead to a dramatic decrease in promoter activity (Stopa et al., 2000). These data suggest interactions between Smads and other transcription factors for highly efficient Smad7 expression. Studies on the human Smad7 promoter revealed that Smad3 and Smad4, but not Smad2 are absolutely required for Smad7 promoter activation by TGF- β (von Gersdorff et al., 2000). Furthermore, other independent pathways including activin, BMP-7, IFN- γ , shear stress and NF- κ B, were shown to induce Smad7 expression (Afrakhte et al., 1998) (Ulloa et al., 1999) (Topper et al., 1997). Smad7 stably interacts with the activated ALK5 and blocks binding and phosphorylation of R-Smads. Studies with a mutated form of Smad2, which can not be phosphorylated, showed stable binding of this mutant to receptor complex and abrogation of TGF- β signaling (Macias-Silva et al., 1996). These findings provide a good model for the action of Smad7 as a TGF- β antagonist. Smad7 binds efficiently to the activated receptor, but cannot be phosphorylated, therefore does not dissociate from the complex. The MH2 domains of both Smad6 and Smad7 are essential for the inhibition of BMP and TGF- β signaling (Hanyu et al., 2001). Furthermore, the N domain of Smad7, which determines the subcellular localization of I-Smads, is required for efficient binding to ALK5. The N domain of Smad7 interacts with the MH2 domain of this protein and facilitates the interaction with the receptor leading to enhanced inhibitory activity of Smad7.

Kavsak et al. showed that Smad7 recruits Smurf2, a novel C2-WW-HECT domain ubiquitin ligase, to the receptor complex (Kavsak et al., 2000). This induces an ubiquitination cascade and finally leads to degradation of the whole complex. In the absence of stimuli Smad7 and Smurf2 accumulate in the nucleus (Itoh et al., 1998) (Kavsak et al., 2000). TGF- β stimulation leads to Smad7/Smurf2-complex export from the nucleus and recruitment to activated receptors. Thus, Smurf2/Smad7-dependent receptor turnover provides a mechanism facilitating reset of the pathway and preparing for new stimuli.

Recent studies suggested that Smad7 may also have a direct effect on the regulation of transcription (Pulaski et al., 2001). Phosphorylation of Smad7 at Ser-249 in the C-terminal part of the linker region was shown to play a crucial role in Smad7 dependent SV40 minimal promoter activity. This is in agreement with previously reported Smad7 nuclear localization (Imamura et al., 1997) (Itoh et al., 1998). Moreover, Smad7 phosphorylation does not affect Smad7 ability to abrogate TGF- β signaling indicating independency of both processes.

2.3. The basic Helix-Loop-Helix transcription factor family

The helix-loop-helix (HLH) proteins form a large family of transcription factors involved in a multitude of developmental processes, such as hematopoiesis, pancreatic and heart development, sex determination, neurogenesis and myogenesis (Massari et al., 2000) (Jones et al., 2004). Based on their sequence and cellular function they can be divided into 7 subgroups (Table 1).

Phylogenetic class	HLH family	Example mammalian protein	Function
I	E	E12, E47, HEB	Many different tissues and cell types
II	MyoD	Myf4	Myogenic: initiates myogenic program in many cell types
	NeuroD	Neurogenic differentiation factor NDF2	Neurogenic: involved in terminal neuron differentiation
	SCL	Tal1	Hematopoietic: essential for primitive hematopoiesis and invokes enhanced proliferation and differentiation during erythroid development
	Hand	dHand	Cardiogenic: regulates the morphogenetic events of asymmetric heart development
III	Myc	c-Myc	Cell proliferation and differentiation; oncogenic
	SREBP	SREBP-2	Cholesterol metabolism
IV	Mad	Mad1	Regulation of cell proliferation
V	Emc	Id1	Myogenic and neurogenic; negative inhibition of DNA binding
VI	Hairy	Hes1	Neurogenic: restricts differentiation of neurons from neural precursor cells
VII	Sim	Single-minded 1 (Sim1)	Neurogenic: regulation of midline cell lineage in the central nervous system

Table 1. Functional classes of HLH proteins (Jones et al, 2004) (Massari et al., 2000).

Over 240 proteins belong to the HLH transcription factor family. Most of them possess the basic domain (basic-HLH-proteins; bHLH) involved in DNA binding ability of the protein. The first discovered recognition site was the E-box, CANNTG, originally found as a binding site for E47 protein (CACCTG) (Massari et al., 2000). E47 protein belongs to a group of bHLH proteins that are expressed in a virtually ubiquitous pattern in many different cell and tissue types, termed E proteins. These proteins form homodimers or heterodimers with tissue and cell specific bHLH proteins (Massari et al., 2000). So called tissue-restricted bHLH

are incapable to form homodimers, preferentially dimerize with E proteins and bind canonical and noncanonical E-box recognition sites.

Myogenic regulatory factors (MRFs), e.g., MyoD, MRF-4, Myf-5, and myogenin play a pivotal role in myogenesis and convert mesodermal cells into muscle progenitor cells, so called myoblasts. Interestingly, HSC express elevated levels of MyoD protein whereas RNA expression is not increased. In HSC, specific MyoD does not display α -SMA-E-box binding activity, but is able to bind to a mE-box sequence present in the promoter region of the pyrimidine biosynthetic enzyme carbonyl phosphate synthase, which is required for the S phase of the cell cycle (Vincent et al., 2001). In contrast, the c-Myb HLH protein binds the E-box of the α -SMA promoter and thus contributes to the upregulation of α -SMA gene expression during HSC activation caused by oxidative stress (Lee et al., 1995).

The Myc family, SREBP and TFE3 contain a leucine zipper (bHLHZ) which stabilizes the dimer. The Myc family of proteins is broadly expressed during embryogenesis (Grandori et al., 2000). c-Myc is a proto-oncogene that regulates cell cycle progression. About 15% of all human tumors are correlated with c-Myc upregulation (Yagi et al., 2002). TGF- β is the most potent inhibitor of c-myc expression, leading to growth arrest (Kowalik et al., 2002). TGF- β mediated repression of the c-Myc promoter occurs within 1 hour after stimulation and requires formation of a complex containing Smad3, E2F-4 or -5 and retinoblastoma (Rb)-related factor p107 (Frederick et al., 2004).

The next group of HLH proteins lacks the basic domain (Id family in mammals and emc in *Drosophila*) and therefore cannot bind to DNA (Massari et al., 2000).

bHLH proteins containing proline in their basic region (e.g. Hairy) and a WRPW motif at the C terminus (required for the recruitment of the co-repressor *groucho*) belong to the VI class (Ruzinova et al., 2003). They bind to a so called N-box sequence (CACNAG) and inhibit promoter activity (Hollenberg et al., 1995).

Finally, the class VII HLH proteins include the Sim family, HIF-1 α and HIF-2 α , which possess an additional dimerization PAS domain (bHLH-PAS) (Carmeliet et al., 1999).

2.3.1. Id1

Four proteins belong to the Id family (Id1, 2, 3, 4) (Skider et al., 2003). They differ in their efficiency to bind diverse HLH proteins and in their expression patterns in various cell types. It was shown, that Id proteins intercept bHLH proteins into inactive heterodimers,

which are incapable of binding to DNA due to the absence of the basic region in the Id proteins. This leads to expression changes of genes that depend on HLH transcription factors and consequently determine distinct cellular responses. Ids were shown to interact with E proteins, e.g., E2A and E2-2 (Ruzinova et al., 2003), abrogating the dimerization of these factors with tissue specific bHLH proteins (Carmeliet et al., 1999). Recently, Id proteins were found to bind other transcription factors, including MIDA1 and Rb (Norton et al., 2000). From the group of myogenic regulatory factors (MRF), MyoD and Myf-5 form complexes with Id1 and Id2, whereby the interaction with myogenin and Myf-6 is very weak. Id3 displays only weak binding activity to all four MRF (Langlands et al., 1997).

Id1 gene expression is stimulated by BMPs (Korchynskyi et al., 2002). The promoter regions of the mouse and human Id1 gene contain three conserved regions, two Smad binding elements (SBE) and a GC-rich region that includes the palindromic sequence GGCGCC flanked by two CAGC and two CGCC motifs. These elements are recognition sites for Smad proteins and the synergistic cooperation between them is required for specific BMP2 responsiveness (Lopez-Rovira et al., 2002). Recent studies implicate Id1 as a target gene of TGF- β signaling, due to TGF- β property to induce the Smad1/5/8 pathway (Goumans et al., 2002). Furthermore, protein degradation plays an important role in regulating the cellular level of Id protein. Ids are short-lived proteins with varying half-life from 20 minutes to 1 hour (Norton et al., 2000). Id1 degradation is mediated via the COP9 signalosome, a protein complex sharing homologies with the proteasome (Berse et al., 2004). The process takes place in the nucleus and is modulated by proteins interacting with Id1 (Trausch-Azar et al., 2004).

High expression of Id proteins is connected with undifferentiated, proliferative cells, which are characteristic features for progenitor cells in developing tissues. Id proteins are upregulated in different human tumors, e.g., breast, prostate, colorectal, pancreatic and ovarian cancer (Skider et al., 2003) and the expression level correlates with clinical grading (Yokota et al., 2002). *In vivo* knocking down of Id1 with an antisense construct in human metastatic breast cancer cells significantly reduces invasiveness, probably due to downregulation of MT1-MMP (membrane-type matrix metalloproteinase 1) (Fong et al., 2003). MT1-MMP is degrading extracellular matrix and activates other matrix metalloproteinases, e.g. MMP2 (Ruangpanit et al., 2001). In line with these results, tumor xenografts implanted to Id1^{+/+}Id3^{-/-} mice display reduced growth and metastatic potential when compared with wild type mice presumably as a result of abrogated expression of $\alpha\beta\gamma$ 3-integrin and MMP2 in these knockout mice (Lyden et al., 1999). On the other hand, Id2 seems to be an activator of mammary epithelial cell differentiation. When introduced into aggressive

breast cancer cells, Id2 is able to ameliorate their highly proliferative and invasive phenotype (Itahana et al., 2003). Ectopic expression of Id1, but not other members of the Id family, in primary fibroblasts, leads to centrosomal localization of the protein and results in accumulation of cells with abnormal centrosome numbers, suggesting a role in the control of mitosis (Hasskarl et al., 2004).

Moreover, Id proteins are involved in angiogenesis. BMP2 induces endothelial cell migration and tube formation via Id1 stimulation. Ectopic expression of Id1 was sufficient to mimic BMP2 mediated activation of the endothelium (Valdimarsdottir et al., 2002). Id1 also downregulates expression of trombospondin-1, an extracellular matrix glycoprotein acting as an inhibitor of angiogenesis (Volpert et al., 2002). High levels of Id1 upregulate the expression of $\alpha\beta\gamma$ 3-integrin, which binds MMP2 leading to breakdown of the basement membrane and allowing migration of activated endothelial cells (Carmeliet et al., 1999).

Another cellular action of Id1 is influencing the cell cycle. Immortalized mammary epithelial cells and breast cancer cells ectopically expressing Id1, but not Id2, continue to proliferate under low serum conditions (Swarbrick et al., 2005). Id1 action in these cells mimics that of c-Myc. Furthermore, overexpression of c-Myc leads to upregulation of Id1, while downregulation of c-Myc expression results in decreased Id1 expression, suggesting that Id1 is a downstream factor of c-Myc.

Id proteins may also promote differentiation in some cellular contexts or support survival of finally differentiated cells. Astrocytes differentiation is not inhibited by Id proteins, unlike that of neurons and oligodendrocytes (Wang et al., 2001), because it is not promoted by specific bHLH proteins dimerizing with E proteins (Ross et al., 2003). In Id2 knockout mice, Sertoli cells, unique somatic cells constituting the epithelial lining of the seminiferous tubules of the testis, whose proliferation stops at puberty, undergo apoptosis, indicating that Id2 is a strong survival factor allowing them to survive in adulthood without dividing (Yokota et al., 2002).

2.4. Anti TGF- β strategies for the treatment of fibrosis (based on Breitkopf et al., in press).

Qi et al. were successful in delivering a dominant negative (dn) T β RII to the liver of rats (Qi et al., 1999). Overexpression of dnT β RII attenuated dimethylnitrosamine (DMN) induced liver fibrosis as was determined by histological examinations, measurement of hepatic hydroxyproline content and serum levels of hyaluronate. Moreover, an adenovirus

expressing an entire ectodomain of human T β RII fused to the Fc portion of human IgG (AdT β -ExR) was similarly efficient in the DMN model without apparent systemic or local side effects (Ueno et al., 2000). This result indicates that transient systemic presence of soluble T β RII is sufficient to inhibit TGF- β 1 action and might therefore be a useful approach for the treatment of different diseases that involve TGF- β signaling without necessity to directly target the affected organ.

The most progressed approach in regard to a usage in human disease is the application of neutralizing antibodies against TGF- β . A human monoclonal antibody directed against TGF- β 2 significantly suppressed kidney fibrosis in streptozotocin-induced diabetic rats (Hill et al., 2001). In another study, a mouse monoclonal antibody against all TGF- β isoforms (1D11) led to reduced fibrosis of the vasa recta bundles in the kidney (Dahly et al., 2002). The same antibody was used in a combined therapy with angiotensin II inhibition in a model of glomerulonephritis (Yu et al., 2004), with maximal reduction of glomerular matrix accumulation from approximately 59% (enalapril alone or 1D11 alone) to 80% using enalapril plus 1D11.

Another strategy of TGF- β neutralization is targeting the activation process of latent TGF- β . Thus, the serine protease inhibitor camostat mesilate was used and attenuated hepatic fibrogenesis at least in part by inhibiting plasmin activity (Okuno et al., 2001). Interestingly, camostat mesilate also blunted the effect of recombinant, active TGF- β itself, suggesting an additional antagonizing mechanism of this drug or plasmin. This is confirmed by the observation that the phenotype of plasmin knock out animals is different from that of TGF- β 1 knock-out mice (Carmeliet et al., 1994) (Shull et al., 1992). Further, thrombospondin-1 (TSP-1), an ECM protein involved in the regulation of cellular responses to injury (Reed et al., 1995) displays an increasingly important role as activator of the small latent TGF- β complex (Crawford et al., 1998) (Murphy-Ullrich et al., 2000) (Poczatek et al., 2000). TSP-1 expression is increased in livers from patients who suffer from congenital hepatic fibrosis (El-Youssef et al., 1999). A peptide derived from LAP, Leu-Ser-Lys-Leu (LSKL), inhibits activation of TGF- β 1 by blunting the LAP-TSP-1 interaction, which is necessary for the TSP-1 mediated activation mechanism (Kondou et al., 2003). LSKL peptides prevented progression of hepatic damage and fibrosis in a DMN model through inhibition of TGF- β 1 activation and subsequent signal transduction *in vivo*.

Interfering further downstream with the TGF- β signaling pathway may lead to a more specific blunting, since not all TGF- β effects would be abrogated. Delivery of CMV-Smad7

adenovirus to C57BL/6 mice 8-12 hours after initiation of bleomycin-induced lung fibrosis led to inhibition of profibrogenic TGF- β signaling and subsequent responses in the animals lungs. Smad7 was also provided as anti-fibrotic tool in several models of kidney fibrosis. Lan and coworkers (Lan et al., 2003) transferred a Smad7 gene into rat kidneys after ureter ligation, which resulted in complete inhibition of Smad2/3 nuclear translocation and tubulointerstitial fibrosis. Similarly, decreased fibrosis was found when Smad7 was introduced one day after ureter ligation by injection of an adenoviral CMV-Smad7 expression construct into the pelvic space followed by electroporation (Terada et al., 2002).

Another promising TGF- β antagonist is BMP7, a member of the TGF- β superfamily of cysteine-knot growth factors. While TGF- β mainly signals via activation of Smad2 and 3, TGF- β receptors activated by BMPs phosphorylate Smad1, 5 and 8. Treatment of cultured mesangial cells with BMP7 is blunting TGF- β dependent inhibition of matrix degradation and thus is able to block profibrogenic activities of TGF- β . In mouse distal tubular epithelial cells, incubation with TGF- β 1 induced epithelial to mesenchymal transition (EMT), a process which was reversed by addition of BMP7 (Zeisberg et al., 2003a) (Zeisberg et al., 2003b). In the same study, BMP7 was administered to mice with nephrotoxic serum nephritis (NTN), a model of progressive renal injury that leads to high mortality due to end stage renal failure and excessive fibrosis. When BMP7 was provided intraperitoneally after 1 week of NTN, it prevented progression of the disease and even if it was given after 3 weeks of NTN it still led to significant improvement of renal function.

2.5. Aim of the study

In the present study, a Smad7 overexpressing adenovirus was used to inhibit profibrogenic TGF- β signaling and experimentally induced fibrosis *in vivo*. Bile duct ligation (BDL) in rats was used, establishing fibrosis based on cholestasis. To investigate the influence of Smad7 from the beginning of injury and on established fibrosis, the treatment started at the day of ligation or one week after ligation, respectively. Collagen expression and deposition were measured as fibrogenesis markers and α -SMA expression as feature of HSC activation. Intracellular mechanisms of TGF- β signaling after ectopic Smad7 expression in HSC were elucidated. Smad2 and Smad3 phosphorylation and nuclear translocation were assayed to control the activation status of the TGF- β pathway. TGF- β /Smad3/4 specific promoter reporter gene activity was used to ensure proper TGF- β signaling.

Based on micro-array data achieved from Smad7 overexpressing HSC versus a LacZ control, the Id1 gene was found to be downregulated by Smad7. Id1 is a regulator of helix-loop-helix transcription factors and was recently identified as TGF- β /BMP2 target gene. Therefore, the role of Id1 in transdifferentiation of HSC was studied. Since α -SMA fiber formation is one typical feature of transdifferentiation, the influence of Id1 expression on this marker was analyzed. In the next step, the intracellular TGF- β pathway leading to Id1 upregulation was elucidated. TGF- β was recently shown to mediate Id1 expression via ALK1/Smad1 pathway in endothelial cells and the presence of this pathway in HSC was proven.

3. Materials and Methods

3.1 Buffers, media, markers and chemical reagents

3.1.1. Cell culture reagents

DMEM, Dulbecco's Modified Eagle's Medium with 25 mM Hepes and 4.5 g glucose (Bio Whittaker Europe, Verviers, Belgium).

Trypsin, 10 x (2.5 % in PBS w/o Calcium and Magnesium) (PAA Laboratories, Linz, Austria).

Accutase (PAA Laboratories).

PBS Dulbecco's w/o Calcium, Magnesium, Sodium bicarbonate (Life Technologies, Karlsruhe, Germany).

Additives

L-Glutamine, final conc. 4 mM (Stock solution 200 mM) (ICN Biomedicals, Aurora, USA).

Penicillin/Streptomycin, final conc. 100 U/ml, 100 μ g/ml (Stock solution 10000 U/ml, 10000 μ g/ml), respectively (Biochrom KG, Berlin, Germany).

FCS (Fetal Calf Serum) (PAA Laboratories).

MEM Non-Essential Amino Acids Solution 10 mM (100X) (Invitrogen, Carlsbad, CA).

HEPES-buffered saline (HBSS), 2 x concentrated

11.9 g HEPES

16.4 g NaCl

0.74 g KCl

0.21 g Na₂HPO₄

pH adjusted to 7.05 with 0.1 M NaOH

H₂O added to 1000 ml

3.1.2. Cytokines

TGF-β, recombinant human Transforming Growth Factor β1 (rh TGFβ1; R&D Systems, Minneapolis, USA).

BMP2, recombinant human Bone Morphogenetic Protein 2 (rh BMP2; R&D Systems).

3.1.3. Buffers for Western blot

RIPA – cell lysis buffer – 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 % Nonidet P-40 (NP-40), 0.5 % sodium deoxycholate, 0.1 % SDS, Proteases Inhibitor Cocktail (Roche, Mannheim, Germany), Phosphatases Inhibitor Cocktail II (Sigma, München, Germany).

MOPS – SDS PAGE running buffer – fresh prepared by diluting 25 ml of NuPAGE MOPS SDS Running Buffer (20 x) (Invitrogen GmbH) in 475 ml distilled water.

Transfer buffer – 750 ml distilled water, 200 ml methanol, 50 ml NuPAGE Transfer Buffer (20 x) (Invitrogen GmbH).

10xTBS – Tris-buffered– saline – 12.10 g Tris-base (Merck, Darmstadt, Germany), 87.66 g NaCl (Merck) dissolved in 800 ml distilled water. pH adjusted to 7.6 with 1 M HCl, water added to 1 liter.

TBST - 100 ml of 10 x TBS and 10 ml of 10 % Tween20 solution (Bio-Rad Laboratories, Hercules, CA) adjusted with distilled water to 1 liter.

Blocking solution – 5 g of BLOT-QiuckBlocker (Oncogene, Darmstadt, Germany), Top-Block (Fluka, Steinheim, Germany) or skimmed milk powder dissolved in 100 ml TBST.

1M DTT – 154 mg dithiothreitol (Sigma) dissolved in 1 ml distilled water, aliquots were kept in -20°C till use.

Protein standard – Bovine albumin, Fraction V, (Sigma) was diluted in RIPA buffer in concentration 4.0; 3.0; 2.0; 1.0 and 0.5 µg/µl and used as a standard for protein concentration determination.

Protein Marker – See Blue 2 Plus (Invitrogen GmbH).

3.1.4. Buffers for immunfluorescent staining of monolayer cell culture

Fixative solution – 4 % paraformaldehyde in PBS pH 7.4; add 50 ml hot water to 4 g paraformaldehyde, add NaOH dropwise under agitation (Paraformaldehyde dissolved in neutral pH), add 10 ml 10 x PBS, adjust pH 7.4 using phosphoric acid, add water to 100 ml end volume. Use immediately after preparation or store at -20°C for no longer than 2 weeks.

Permeabilization solution – 0.1 % Triton X-100 (Serva, München, Germany) in 0.1 % sodium citrate (Merck).

PBS (10 x) – 1.37 M NaCl (Sigma), 27 mM KCl (Sigma), 100 mM Na₂HPO₄ (Sigma), 15 mM KH₂PO₄ (Sigma), pH 7.4.

PBS pH 7.4 – 100 ml 10 x PBS + 900 ml water.

1 % (w/v) BSA in PBS pH 7.4 – 1 g albumin RIA grade (Sigma) in 100 ml PBS pH 7.4.

50 % (v/v) FCS; 0.1 % (w/v) BSA in PBS pH 7.4 – 50 ml FCS + 10 ml 1 % BSA in PBS + 9 ml 10 x PBS adjust to 100 ml with water.

0.1 % (v/v) fish gelatin; 0.5 % (w/v) BSA in PBS pH 7.4 – 50 ml 1 % BSA in PBS + 50 ml PBS + 100 µl fish gelatin (Sigma).

Antifade – 2 g DABCO (Sigma) dissolved in 90 ml glycerol at 60-70°C in water bath, add 10 ml 1 M Tris-HCl pH 7.0 and 100 µl 20 % (w/v) natriumazide, store aliquots at +4°C.

3.2. Eukaryotic cells and their culture

3.2.1. Cells

Hepatic stellate cells were isolated from male Sprague-Dawley rats by the pronase/collagenase method with single-step density gradient centrifugation with Nycodenz (Nyegaard Co. AS, Oslo, Norway) as previously described (Schäfer et al., 1987). In detail, Sprague-Dawley rats (weight > 500 g) were anesthetized; the abdominal cavity was opened, followed by *in situ* perfusion of the liver via the *vena portae* with HBSS buffer w/o Ca^{2+} and Mg^{2+} . During perfusion the liver was taken out and placed on the funnel and the perfusion with pronase for 10 min took place. After that liver was perfused with collagenase for 30 min with recirculation. At the end the liver was fragmented and cells were isolated. Cell suspension was gradient centrifuged with Nycodenz, followed by washing steps and plating. The mean purity was higher than 95 %, and the yield ranged from 30 to 50 x 10⁶ cells/liver.

HEK293A, a permanent line of human embryonic kidney cells transformed by sheared human adenovirus type 5 (Ad 5) DNA. The cells express the transforming gene of adenovirus 5 (Invitrogen GmbH).

CFSC, cell line derived from CCl₄-induced cirrhotic rat HSC. (Greenwel et al., 1991).

3.2.2. Cell culture

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), 4 mM L-glutamine, including 10, 5 or 0.5 % FCS. Additionally, all culture media were supplemented with penicilin/streptomycin. Cells were maintained at 37°C, 5 % CO₂ in humidified atmosphere. CFSC culture medium was supplemented with MEM Non-Essential Amino Acids Solution (Invitrogen), final conc. 0.1 mM.

3.3. Adenoviruses

3.3.1. Virus amplification

Human recombinant E1-deleted adenoviral vectors carrying a DNA construct of interest were amplified in HEK293A cells. For each preparation five T175 flasks with low-passage HEK293A cells were used. Hek293A cells were cultured till the density of 80 % in DMEM with 10 % FCS. Cells were infected with 3×10^6 ifu (infection units) per 10^6 cells after 2 hours incubation in DMEM with 5 % FCS. The cytopathic effect was usually completed after 2-4 days of incubation. Cells and medium were harvested and adenovirus purification was performed using BD Adeno-X Virus Purification Kit (BD Biosciences Clontech, Palo Alto, CA) following the instruction manual.

3.3.2. Constructs

No.	Name	Description	Source
1	AdLacZ	Adenovirus expressing bacterial β -galactosidase under the control of a CMV promoter (constitutively active promoter comprising a cytomegalovirus enhancer)	Biosciences Clontech, Palo Alto, CA
2	AdSmad7	Mouse Smad7 cDNA fused with the Flag epitope sequence under the control of a CMV promoter	Kindly provided by C. H. Heldin, Department of Medical Sciences, Clinical Chemistry, Uppsala, Sweden
3	AdALK1	Adenovirus expressing mouse constitutively active TGF- β receptor type I (ALK1) under the control of a CMV promoter	Kindly provided by P. ten Dijke, LUMC, Leiden, The Netherlands

4	AdALK5	Adenovirus expressing mouse constitutively active TGF- β receptor type I (ALK5) under the control of a CMV promoter	Kindly provided by C. H. Heldin, Department of Medical Sciences, Clinical Chemistry, Uppsala, Sweden
5	AdALK3	Adenovirus expressing mouse constitutively active bone morphogenetic protein receptor type IA (ALK3) under the control of a CMV promoter	Kindly provided by P. ten Dijke, LUMC, Leiden, The Netherlands
6	AdId1	Adenovirus expressing mouse Id1 under the control of a CMV promoter	Kindly provided by P. ten Dijke, LUMC, Leiden, The Netherlands
7	AdsiId1	Adenovirus expressing siRNA for mouse Id1 under the control of a CMV promoter	Kindly provided by P. ten Dijke, LUMC, Leiden, The Netherlands
8	Ad (CAGA)₉-MLP-Luc	Adenovirus expressing firefly luciferase under the control of an artificial promoter containing 9 repeats of Smad Binding Elements (SBE – CAGA) upstream of a minimal adenoviral major late promoter (MLP)	Cloned from a plasmid kindly provided by J. M. Gauthier, Hybrigenics, Paris, France
9	AdSmad3	Adenovirus expressing mouse Smad3 under the control of a CMV promoter	Kindly provided by C. H. Heldin, Department of Medical Sciences, Clinical Chemistry, Uppsala, Sweden

10	AdSmad4	Adenovirus expressing mouse Smad4 under the control of a CMV promoter	Kindly provided by C. H. Heldin, Department of Medical Sciences, Clinical Chemistry, Uppsala, Sweden
11	Adsi-ALK1	Adenovirus expressing interfering RNA for mouse ALK1 under the control of a CMV promoter	Kindly provided by P. ten Dijke, LUMC, Leiden, The Netherlands

Tab. 2. List of adenoviral constructs used in this study.

3.3.3. Infection of cell cultures

Cells were incubated for 2-3 hours in DMEM with 5 % FCS. For optimal infection conditions $0.5 \times 10^7 - 10^7$ ifu of purified virus per 10^5 cells was used (50 – 100 moi). Medium change was performed after 2 hours of incubation.

3.3.4. Determination of virus titer

To determine the titer of the purified virus preparation, the “Adeno-XTM Rapid Titer Kit” (BD Bioscience) was used. 0.5 ml of healthy HEK293 cells (2.5×10^5 cells/ml) were plated in each well of a 24-well plate using standard growth medium (DMEM + L-glutamine + antibiotics + 10 % FCS). 10-fold dilutions of viral samples were prepared ($10^{-1} \div 10^{-6}$). 50 µl of viral dilution was added dropwise to each well (each dilution was assayed in duplicate). After 48h incubation at 37°C at humified atmosphere cells were fixed with methanol and stained with antibodies against viral hexon protein. Positive-brown cells were counted under the microscope (x 20) and infectious units (ifu/ml) were calculated using the following formula:

$$[(\text{infected cells} / \text{field}) \times (\text{fields} / \text{well})] / [(\text{volume of virus [ml]}) \times (\text{dilution factor})] = [(\text{infected cells} / \text{field}) \times 247] / [0.05 \text{ ml} \times (\text{dilution factor})] = [\text{ifu} / \text{ml}]$$

The yield ranged between 1 and 5×10^{10} ifu/ml. Representative results are shown in figure 6.

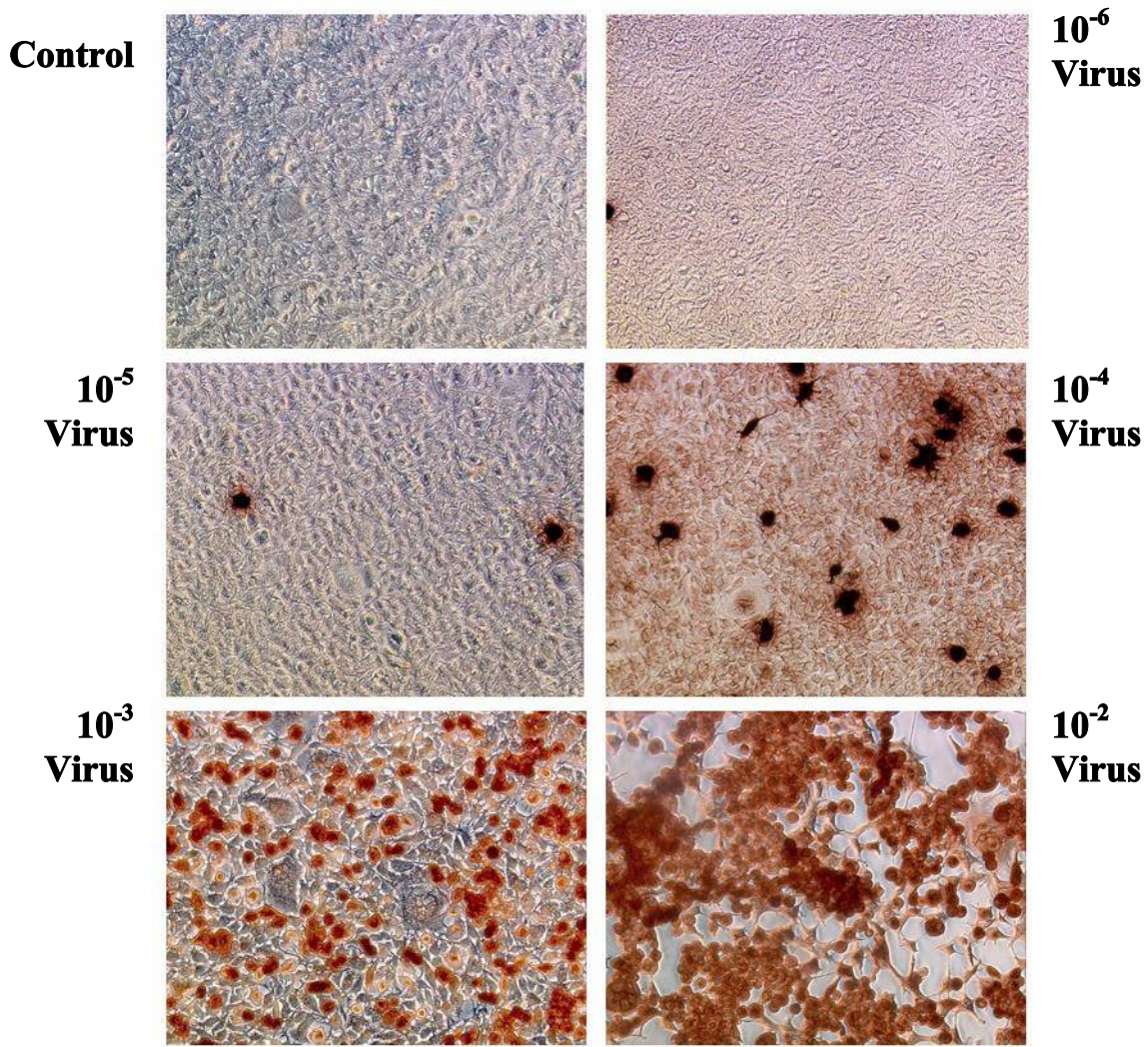


Fig. 6. Determination of virus titer (representative results). Control – cells not infected with virus, 10^{-2} – 10^{-6} – 10 fold virus dilutions added to the cells. Brown cells – positive for virus. Virus titer = $21 \times 247 / (0,05 \times 10^{-4}) \cong 10^{10}$ ifu/ml.

3.3.5. Determination of virus functionality

After determination of virus titer HSC or/and CFSC cells were infected with 0.5; 1.0; 1.5; 2.0×10^7 ifu per 10^5 cells followed by 24-hour incubation. Cell lysates were tested in Western Blot (using specific antibody, e.g. anti-Smad7 when the cells were infected with AdSmad7) or luciferase activity was measured (e.g. Ad(CAGA)₉-MLP-Luc). For further experiments the smallest amount of virus still causing strong expression of the transgene was used.

3.3.6. Testing of cell vitality after virus infection

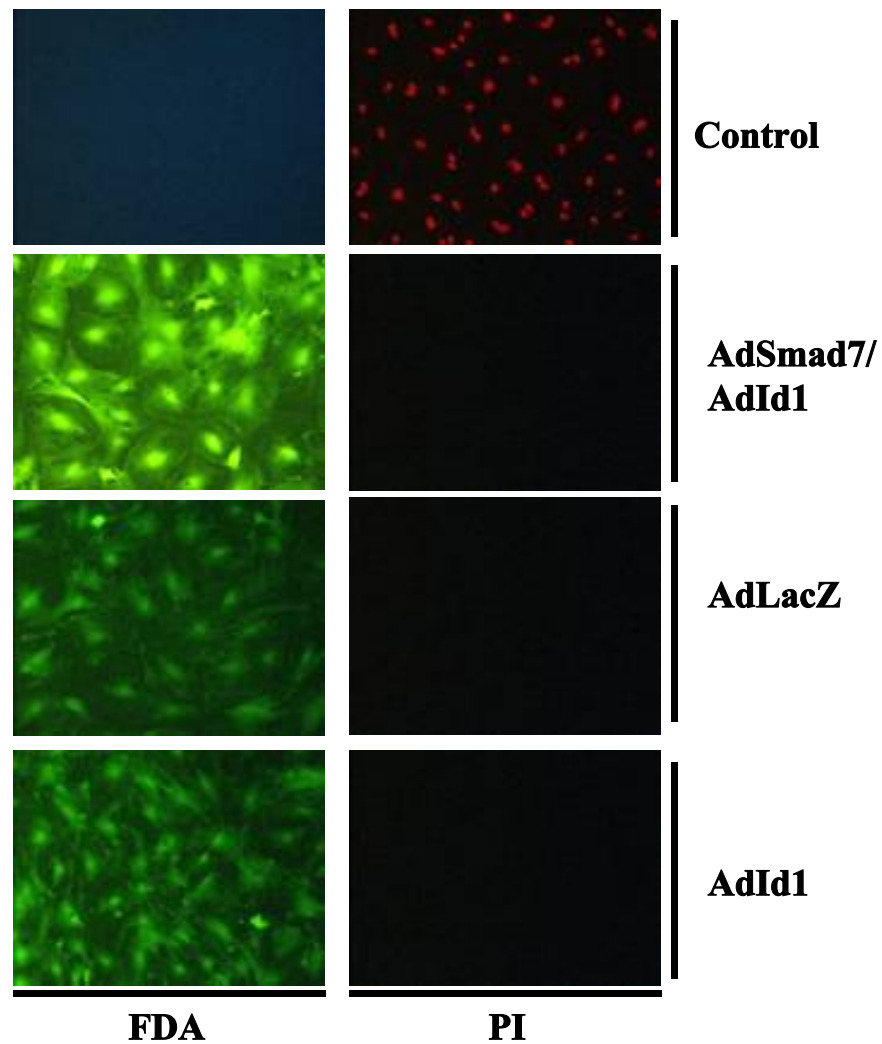


Fig. 7. HSC were infected with normal amount of virus, with 2, 5 and 10 fold more and also in combination used in other experiments at the second day after plating. At day 5 and day 7 cells were stained with Propidiumiodid (PI) and Fluorescein diacetat (FDA) to test cell toxicity of the virus infection. Representative results are shown.

HSC were infected with each viral construct using the normal amount of virus and 2, 5 and 10 times more virus then usually used. After 3 and 5 days incubation cells were stained with Fluorescein Diacetat (FDA, Sigma) and Propidiumiodid (PI, Sigma) at a final concentration of 2 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ respectively. Positive control for cell death was obtained by incubation with 0.1 % (v/v) Triton-X 100 in 0.1 % (w/v) sodium citrate for two minutes. Living cells stain green and the nuclei of dead cells stain red. Pictures were taken using a fluorescent microscope, with 485 nm excitation filter and 515 nm bandpass filter for FDA, and

with 546 nm excitation filter and 590 nm bandpass filter for PI. Representative results are shown in figure 7.

None of the adenoviral constructs used cause cell death of HSC. Also all used combinations of viruses had no effect on cell vitality. Only AdALK5 in the highest concentration (ten fold more than normally used) causes death of 2 % of infected cells after 5 days of incubation.

3.4. Animal experiments

Bile duct ligation (BDL) in rats was used as a well established model for *in vivo* studies of liver fibrogenesis based on cholestasis (Kountouras et al., 1984). All animal protocols were in full compliance with the guidelines for animal care and were approved by the Animal Care Committee from the German government.

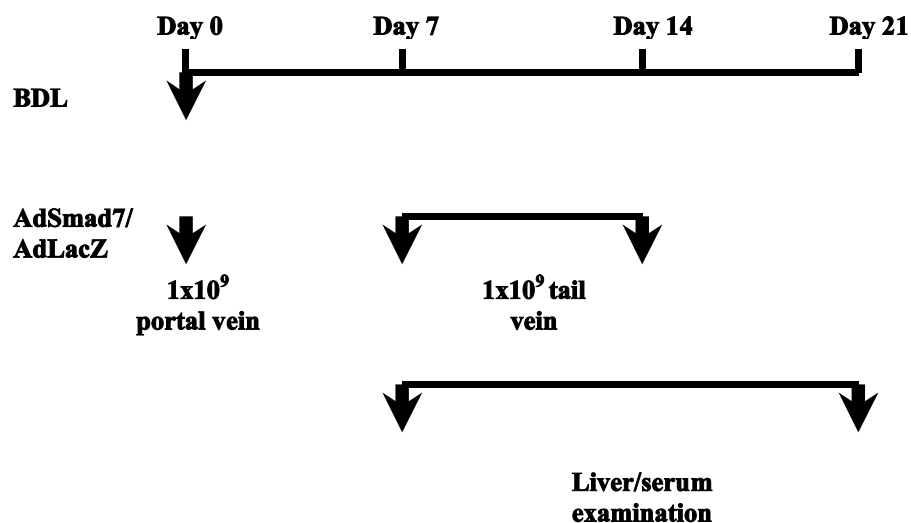


Fig. 8. Time schedule of BDL, virus administration, and sample withdrawal. Virus (AdSmad7 or AdLacZ) was given immediately before BDL via the portal vein and then again after 1 and 2 weeks via the tail vein to examine the effect of Smad7 on fibrogenesis. Alternatively, AdSmad7 was delivered after 1 week for the first time to investigate its impact on established fibrosis. Blood and liver samples were taken at the indicated time points (1 and 3 weeks after surgery).

In detail, Sprague-Dawley rats (weight: ~250g) were anesthetized, the abdominal cavity was opened, and the bile duct was ligated with two knots and cut between them, followed by closure of the abdominal cavity, waking up and recovery from surgery. Animals were infected

with 0.5 ml (1×10^{10} or 1×10^9 ifu of viruses) AdSmad7, AdLacZ or saline, either via the portal vein at the time of surgery or via the tail vein 1 and 2 weeks after ligation, as shown in Fig. 8. At least 8 animals were used for each treatment.

3.4.1. Blood tests

Blood tests were performed every week during the experiment. 0.6 ml of blood was taken from the tail vein from each rat. Bilirubin concentration and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl-transferase (γ -GT) activities were determined using standard clinical chemical assays.

3.4.2. Tissue samples

Liver and other tissue (e.g. lung, colon, heart) samples were collected and fixed in 4 % buffered formaldehyde for immunostaining or snap frozen in liquid nitrogen for protein lysate, hydroxyproline determination and RNA preparation.

3.4.3. Hydroxyproline assay

Hydroxyproline content was measured as described before (Jamall et al., 1981) (Boigk et al., 1997). In detail, 100 mg of liver tissue was homogenized in 4 ml 6 N HCl and hydrolyzed by incubation at 110°C for 16 hours. Lysates were filtered and centrifuged. 30 μ l Aliquots were mixed with 10 μ l methanol (Sigma) and dried at 60°C for 20 min under nitrogen. Crystallite was dissolved in 1 ml 50 % isopropanol (Sigma) and vortex-homogenized. 200 μ l of 0.84 % chloramin-T solution was added, followed by vortex-homogenization and incubation at room temperature for 10 minutes. 1 ml freshly prepared Ehrlich's Reagent solution was added, vortex-homogenized and incubated in 50°C for 90 minutes.

Absorption was measured by 558 nm and the hydroxyproline content was calculated using the following formula:

$$(\text{Absorption/ coefficient of the standard curve}) \times 1333.3 = \mu\text{g/g tissue}$$

Hydroxyproline solution: 1 mg/ml (Fluka) – hydroxyproline stock solution in 50 % isopropanol (HP*).

1:100 diluted HP* – ‘working’-HP-solution (10 µg/ml)

Hydroxyproline amount [µg]	Dilution of ‘working’-HP-solution
0.0	1000 µl 50 % isopropanol
0.2	20 µl HP + 980 µl 50 % isopropanol
0.4	40 µl HP + 960 µl 50 % isopropanol
0.6	60 µl HP + 940 µl 50 % isopropanol
0.8	80 µl HP + 920 µl 50 % isopropanol
1.0	100 µl HP + 900 µl 50 % isopropanol
1.4	140 µl HP + 860 µl 50 % isopropanol
1.8	180 µl HP + 820 µl 50 % isopropanol
2.2	220 µl HP + 780 µl 50 % isopropanol
2.6	260 µl HP + 740 µl 50 % isopropanol
3.0	300 µl HP + 700 µl 50 % isopropanol
3.4	340 µl HP + 660 µl 50 % isopropanol

Tab. 3. Standard series for hydroxyproline assay.

Solutions:

6 N HCl – 49.7 ml 37 % HCl (Sigma) + 50.3 ml water.

Chloramin-T solution – 84 mg Chloramin-T (Sigma) + 6.05 ml Chloramin-T buffer + 3.95 ml isopropanol.

Chloramin-T buffer – 42 mM sodium acetate (2.86 g/ 500 ml buffer) (Sigma) + 26 mM citric acid (Sigma) (2.73 g/ 500 ml buffer) – pH 6 with 1 N NaOH (Sigma).

Ehrlich’s reagent solution – 248 mg Ehrlich’s reagent (Fluka) + 270 µl 60 % perchlor acid + 730 µl isopropanol.

3.5. Western blot

3.5.1. Protein lysates

Tissue – 10 mg of tissue was snap frozen in liquid nitrogen, powdered, dissolved in 0.5 ml RIPA buffer, incubated 1 hour on ice, and centrifuged 10 min at 15.000 g. The supernatant was collected and the protein concentration determined.

Cell culture – Cells growing in monolayer in 10 cm dishes were washed twice with PBS and then 200 µl of RIPA buffer was added. Cells were scraped off and solutions were collected in 1.5 ml tubes followed by 10 min centrifugation at 15.000 g. The supernatant was collected and protein concentration was determined.

3.5.2. Protein concentration

Protein concentration was determined using D_C Protein Assay (Bio-Rad Laboratories) based on the Lowry assay. Each probe was piped threefold into a microtiterplate and measured. The absorbance was read at 690 nm using a spectrophotometer (Wallac 1420 Victor, Wallac, Turku, Finland). Protein concentration was calculated for the average value of three counts and related to the protein standard curve and blank value of RIPA.

3.5.3. Preparation of samples for western blot

20 µg of protein sample was piped into a 1.5 ml tube. 10 µl of NuPage LDS Sample Buffer (4x) (Invitrogen GmbH) and 2.5 µl of 1 M DTT were added. Samples were adjusted to 40 µl with sterile water. After 10 min of incubation at 70°C the samples were briefly centrifuge at 14.000 g and loaded onto a gel.

3.5.4. SDS – polyacrylamide gel electrophoresis

NuPAGE 4-12 % Bis-Tris gel (1.5 mm x 10 wells) (Invitrogen GmbH) and MOPS buffer were used as a standard protocol. Electrophoresis was carried out in an XCell II Mini Cell apparatus (Invitrogen GmbH) for about 1 hour at a constant voltage of 150 V.

3.5.5. Western transfer

Protein transfer was performed using an XCell II blotting apparatus with an XCell II Blot Module (Invitrogen GmbH). Chromatography Paper (3MM Chr, Whatman, Maidstone, England) and Nitrocellulose Membrane Sheets (0.45 micron, Pierce, Rockford, IL) were used

for preparing the gel-membrane sandwich. Blotting pads and the gel-membrane sandwich were placed in the XCell II Blot Module according to the manufacture's instruction. Blotting was performed for 1.5 hours at a constant current of 200 mA.

3.5.6. Antibodies

Antibody against:	Species	Dilution used for western blot	Source
Flag	Mouse	1:1000	Sigma
HA	Mouse	1:500	Sigma
Smad7	Rabbit	1:500	Abcam, Cambridge, UK
p-Smad1	Rabbit	1:750	Cell Signaling, Beverly, MA
p-Smad2	Rabbit	1:1000	Cell Signaling
p-Smad2	Rabbit	1:1000	Serum, Kindly provided by C. H. Heldin, Department of Medical Sciences, Clinical Chemistry, Uppsala, Sweden
p-Smad3	Rabbit	1:1000	Serum, Kindly provided by C. H. Heldin, Department of Medical Sciences, Clinical Chemistry, Uppsala, Sweden
Smad2/3	Rabbit	1:1000	Santa Cruz Biotechnology, Santa Cruz
Smad1	Rabbit	1:1000	Cell Signaling
Smad2	Rabbit	1:1000	Zymed, Karlsruhe, Germany
β -actin	Mouse	1:5000	Sigma
α -SMA	Mouse	1:5000	Sigma
rabbit	Mouse	1:5000	Pierce
mouse	Goat	1:5000	Pierce
endoglin	Rabbit	1:500	Serum, kindly provided by A. Lux, Fachhochschule Mannheim, Germany
ALK5	Rabbit	1:1000	Santa Cruz Biotechnology

Tab. 4. Antibodies and their dilutions used for western blot analysis.

3.5.7. Immunodetection of proteins

The blotting membrane was incubated with blocking solution for 1 hour at room temperature, followed by 1 hour incubation with the first antibody. Afterwards, the membrane was washed three times for 5 min with TBST and incubated with second antibody for 1 hour. After three additional wash steps the membrane was incubated with SuperSignal West Dura Extended Duration Substrate (Pierce) following the manufacture's instructions and the proteins were detected using a Lumi-Imager (Roche, Mannheim, Germany).

3.6. Immunostaining of monolayer cells

3.6.1. Preparation of glass slides and cell culture

- Incubate slides for 8 hours at 37°C in 25 % HCl under agitation.
- Wash 10 min with flowing tap water.
- Wash 3 x with a large volume of deionized water.
- Wash 3 x with large volume of sterile water.
- Wash 2 x with 70 % ethanol.
- Keep in 70 % ethanol at 4°C till use.

For cell culture: Single glass slides were taken from the 70 % ethanol bath, air-dried and placed in a well of a 6-well-plate. 2 ml DMEM with 10 % FCS was added followed by incubation at 37°C, 5 % CO₂ in humidified atmosphere for 12-48 hours. After that the medium was removed and cells were seeded in new medium.

3.6.2. Staining

Cells were washed 3 times with 2 ml PBS and fixed with 2 ml 4 % paraformaldehyde for 15 min at room temperature. After 3 wash steps, cells were permeabilized with ice cold permeabilization solution for exactly 2 min on ice, followed by 3 wash steps. Unspecific binding sites were blocked with 2 ml 50 % (v/v) FCS; 0.1 % (w/v) BSA in PBS pH 7.4 for 30 minutes at 37°C and with 2 ml 0.5 % (w/v) BSA; 0.1 % (v/v) fish gelatin in PBS pH 7.4 for

30 minutes at 37°C. After another 3 wash steps, cells were incubated with 200 µl of the first antibody against α -SMA (dilution 1:100), Smad4 (dilution 1:100), Smad 2/3 (Santa Cruz Biotechnology) (dilution 1:100), phospho-Smad2 (dilution 1:50) or Smad7 (dilution 1:100) for 1 hour at 37°C in a humid chamber. After three wash steps, cells were incubated with the secondary antibody, Cy3-conjugated goat anti-mouse or goat anti-rabbit (Santa Cruz Biotechnology).

For double staining of Smad7 and α -SMA, mouse anti- α -SMA and rabbit anti-Smad7 were detected using Cy3-conjugated goat anti-mouse immunoglobulin G and fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology), respectively.

3.7. Detection of mRNA expression

3.7.1. RNA isolation from monolayer cells

RNA isolation was performed using the RNeasy kit (Qiagen, Hilden, Germany) according to the instruction manual. Cells growing in 10 cm Petrie dishes were lysed with 600 µl RLT buffer, homogenized and adjusted with ethanol for proper binding conditions. Samples were pipetted into a spin column for adsorption. After centrifugation, followed by 3 washing steps, the RNA was eluted with water. RNA concentration was determined using a spectrophotometer. The integrity of the isolated RNA was examined by formaldehyde agarose gel.

3.7.2. RNA electrophoresis

0.5 g agarose diluted in 35 ml H₂O was boiled and placed on a magnetic stirrer; 10 ml 5 x MOPS (0.01 M NaOAc pH 7.0; 1 mM EDTA pH 8.0; 0.02 M MOPS; incubation over night with 0.1 % DEPC followed by autoclaving) and 4 ml formaldehyde were added. The gel was poured and allowed to polymerize. 200 ng RNA was separated for 1-2 hours to prove the integrity of the RNA samples and to demonstrate accuracy of the quantity-determination.

3.7.3. Reverse transcription

Reverse transcription reaction was performed using the Omniscrypt Reverse Transcription Kit (Qiagen) according to the instruction manual. 1 µg total RNA was reverse transcribed into 20 µl cDNA and used for standard PCR.

3.7.4. Polymerase chain reaction (PCR)

1 µl of cDNA was used in standard PCR amplification to prove the expression of TGF-β receptor genes in hepatic stellate cells. The following primers were used: ALK1 (GI: 11967972): forward 5' – GTC AAG AAG CCT CCA GCA AC – 3'; reverse 5' – CAT CAA CTC AGG CTT CGG G – 3'; ALK3 (GI: 13540660): forward 5' – AGA TTA CTG GGA GCC TGT CT – 3'; reverse 5' – ACG TCA CTC CAT TTT CCG GC – 3'; ALK5 (GI: 416397): forward 5' – CGT CTG CAT TGC ACT TAT GC – 3'; reverse 5' – CTG TGG CAG AAT CAT GTC TC – 3'; betaglycane (GI: 8394445): forward 5' – GCC TTG ATG GAG AGC TTC AC – 3'; reverse 5' – CTT GCT ATC TTG AGT TCG GTG – 3'; endoglin (GI: 45550266): forward 5' – CTG GGC ATC ACC TTT GGT GC – 3'; reverse 5' – GCC ATG CTG CTG GTG GAG – 3'; rS6 (GI: 37194797): forward 5' – GAC TGA CAG ATA CCA CTG TGC CT – 3'; reverse 5' – TTA TTT TTG ACT GGA CTC AGA T – 3'. Samples were initially denaturized at 95°C for 5 minutes and then subjected to 36 cycles (ALK3, ALK5, betaglycane), 30 cycles (ALK1) or 25 cycles (endoglin, rS6) of amplification. PCR products were separated on 1 % or 1.5 % agarose gels.

3.7.5. Real-time PCR quantification

Using gene-specific primers, a cDNA fragment was amplified and column-purified using a QIAquick PCR purification kit (Qiagen). The following primer sequences were used: ALK5 (GI: 416397): forward 5' – CGT CTG CAT TGC ACT TAT GC – 3', reverse 5' – AGC AGT GGT AAA CCT GAT CC – 3'. Gene-specific standards were produced as previously reported (Wickert et al., 2002) and subsequent purification was confirmed by sequencing on a semiautomatic sequencer (310A, Applied Biosystems, Foster City, USA). Thereafter, serial ten times logarithmic dilutions of the cRNA were used for reverse transcription, which was performed at 42°C for 60 min using the Expand reverse transcription kit (Roche). In order to generate cDNA from cell culture samples, 1 µg of total RNA was reverse transcribed. Real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturers' protocol and the following rat Id1 primers were used

(GI: 516116): Forward 5' - TGG ACG AAC AGC AGG TGA AC – 3', reverse 5' - TCT CCA CCT TGC TCA CTT TGC – 3'. The chosen light cycler conditions were: Initial denaturation at 95°C for 600 s, followed by 40 cycles with denaturation at 95°C for 10 s, annealing at 60°C for 10 s and elongation at 72°C for 15 s. By comparing to the ALK5 standard curve, relative quantities of target mRNA in the unknown samples were determined.

3.8. Promoter-reporter assays

3.8.1. Transfection of monolayer cells

A mouse Id1-promoter-luciferase construct (pId1-Prom-Luc) containing the fragment -1231/+88 from the mouse genomic region, that has been subcloned into *KpnI* and *XmaI* sites upstream of a minimal adenoviral major late promoter (MLP) reporter construct was used. 1.2×10^5 cells were plated onto a well in a 6-well-plate. They were transfected with 0.8 µg of this construct and 0.2 µg of a control plasmid pSV40-β-gal (Promega, Mannheim, Germany), for normalization of the transfection efficiency. FuGENE 6 transfection reagent (Roche) was used according to the manufactures' protocol. 1 day after transfection, cells were serum-starved for 16 hours with medium containing 0.5 % FCS and stimulated with 1ng/ml TGF-β or 10 ng/ml BMP2.

3.8.2. Luciferase assay

After transfection with the pId1-Prom-Luc construct or infection with Ad(CAGA)9-MLP-Luc adenovirus, cells were lysed in 100 µl Reporter-lysis buffer (Promega). 50 µl of the lysate was used for luciferase measurement and 50 µl for the β-galactosidase assay (normalization of the transfection efficiency). Assays were performed using kits from Promega following the instruction manuals.

4. Results (based on (Dooley et al., 2003) and (Wiercinska et al., submitted))

4.1. Expression of the Smad7 transgene in rat liver

BDL animal model was used for study Smad7 influence on liver fibrogenesis. Infection of rats with 1×10^9 pfu AdSmad7 resulted in specific expression of exogenous Smad7 protein in the liver and other organs (heart, kidney, lung, muscle and spleen) did not show detectable expression of the transgene as demonstrated in western blot analysis of protein lysates of different tissues (Figure 9A). These data were also confirmed on the RNA level and in immunostaining of Smad7 in liver tissue (results of our group, data not shown).

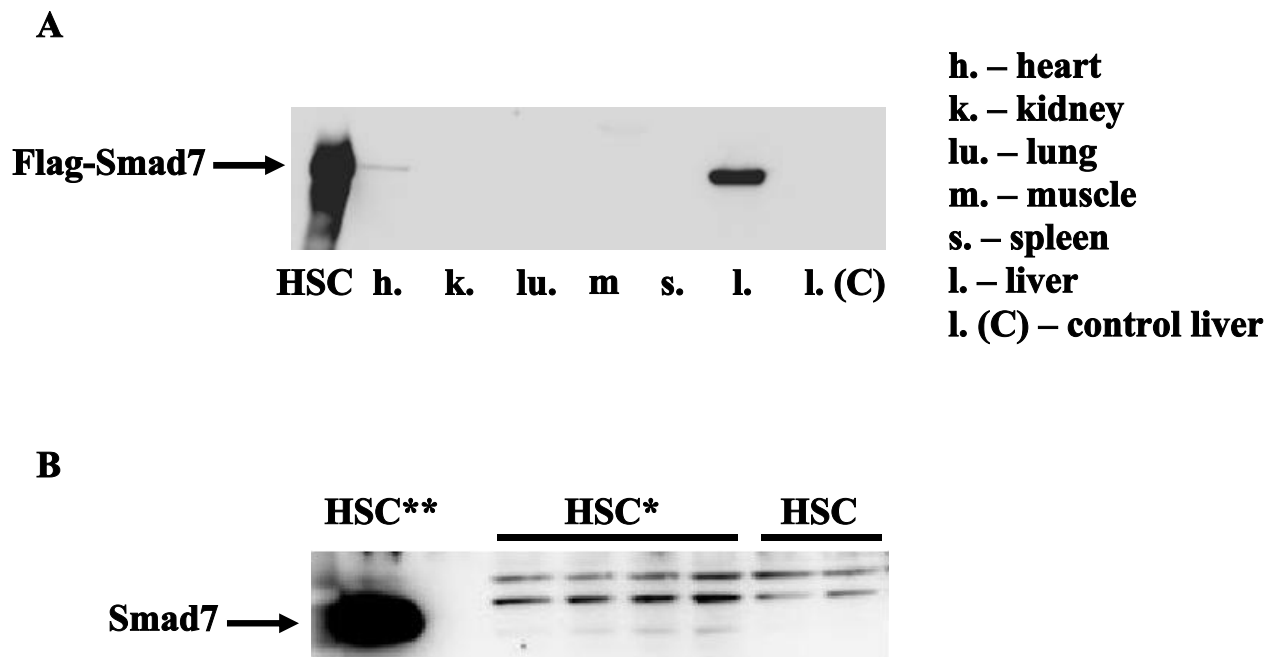


Fig. 9. Expression of the Smad7 transgene in rat. (A) Smad7 protein expression was investigated in different rat tissues 7 and 14 days subsequent to infection with 1×10^9 pfu adenoviral Smad7 expression construct (injection via the tail vein) or in tissue from untreated controls (liver (C)). Smad7 protein expression was analyzed by anti-Flag antibody. HSC, which were transfected with a Smad7 expression construct, were used as a positive control. (B) Lysates of purified HSC from 4 different AdSmad7 infected rats were investigated for Smad7 expression in Western blots (HSC*) with anti-Flag antibody. HSC, isolated from 2 untreated rats (HSC) and those infected with AdSmad7 in culture (HSC**), were used as controls.

Expression levels of the transgene in the liver peaked at day 2 after adenoviral infection and persisted at least for 2 weeks (results of our group, data not shown). The procedure of BDL decreased expression of virus encoded Smad7, but basal amounts were still detectable at all time points investigated (results of our group, data not shown).

Ectopic expression of Smad7 in HSC was shown by Western blot analysis in primary cultured cells, which were isolated from AdSmad7-infected rats (Figure 9B). These results demonstrate efficient transgene expression specifically in the liver of AdSmad7 infected animals.

4.2. Effect of Smad7 expression on collagen synthesis and activation of HSC in liver fibrogenesis

To determine the impact of Smad7 on liver fibrogenesis, AdSmad7 and mock virus (AdLacZ) were injected as indicated in the scheme shown in Figure 8.

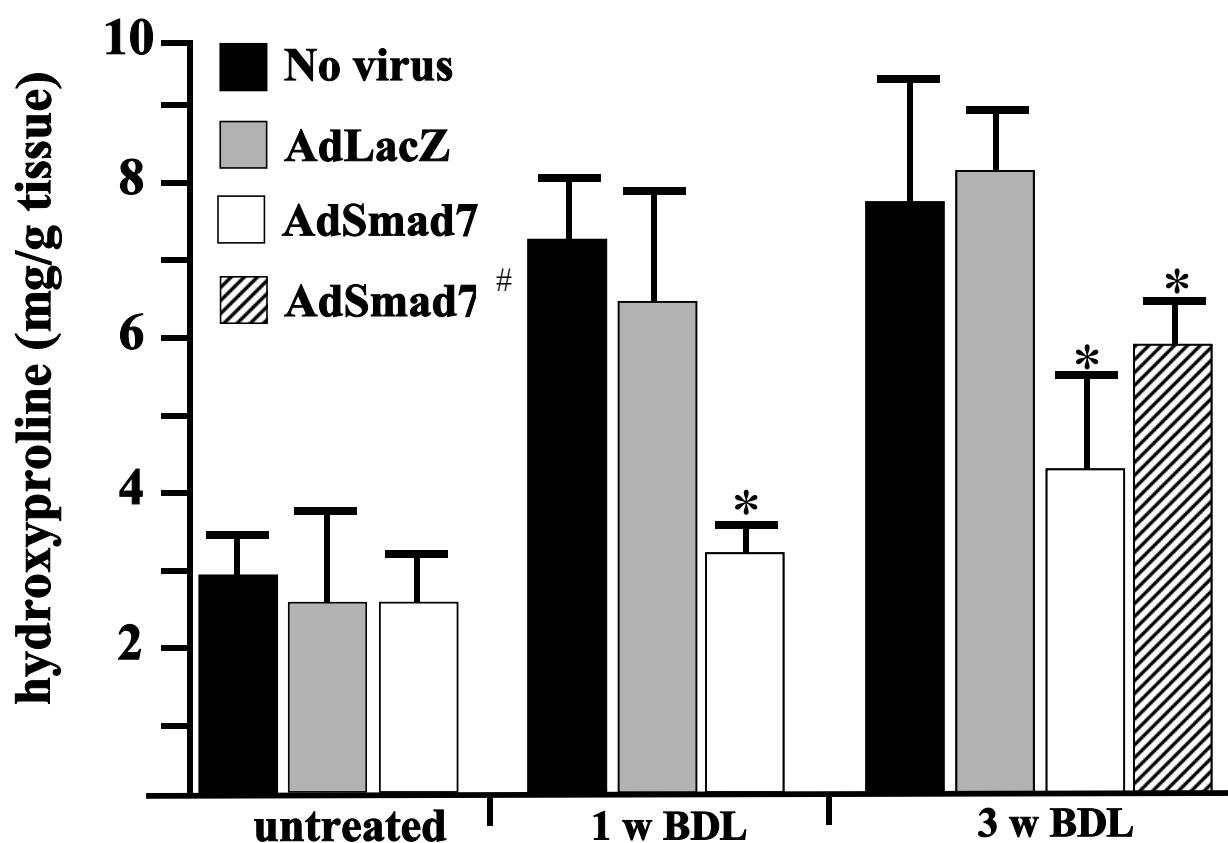
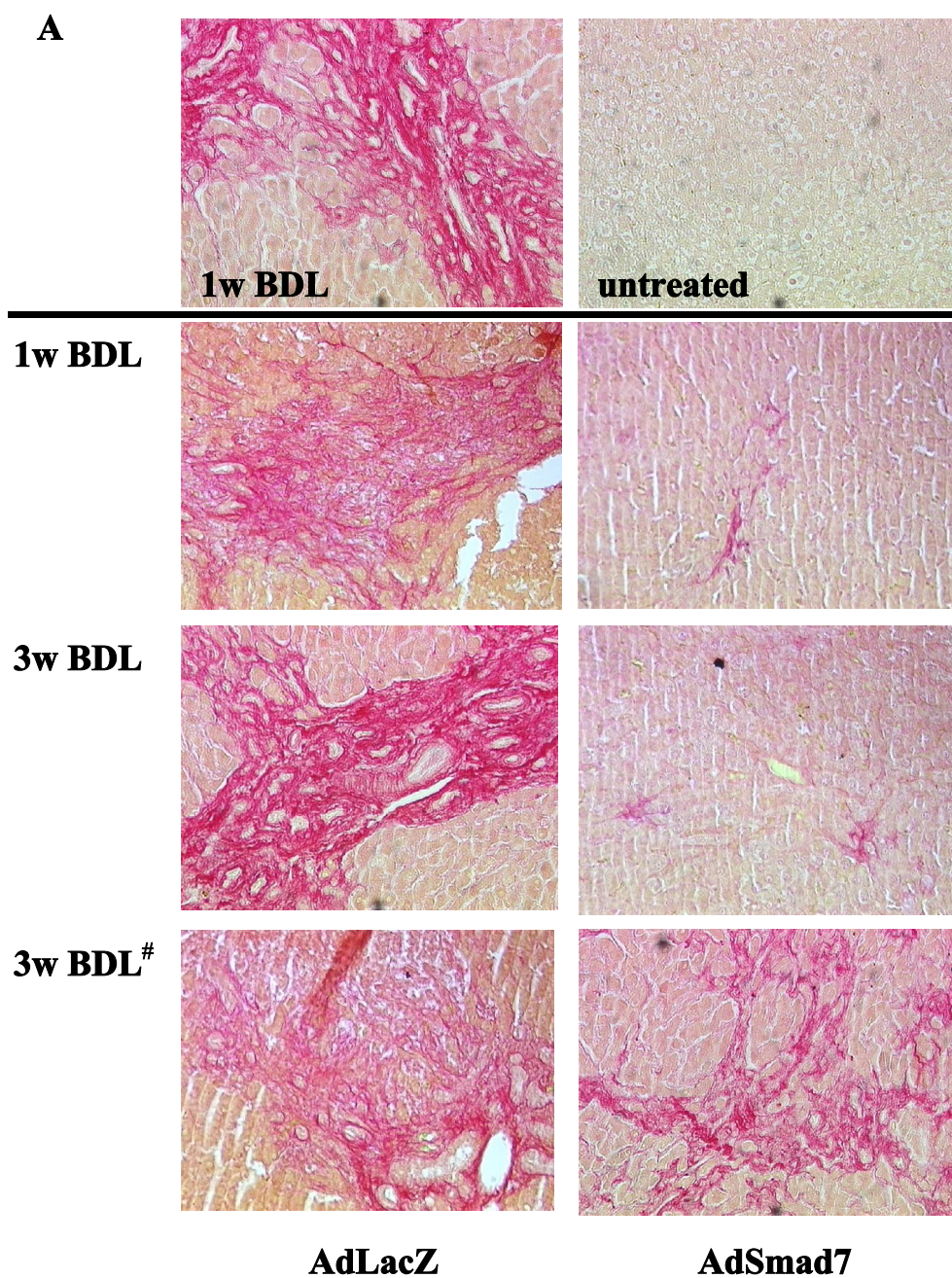


Fig. 10. Smad7 reduces hydroxyproline content in experimental fibrosis. Rats were treated with BDL for 1 to 3 weeks and infected as indicated in Fig. 8. The hydroxyproline content was measured spectrophotometrically and is shown as mean $\pm$ SD (n = 8). Three samples were analyzed from each rat liver. *Indicates $P < 0.01$ versus BDL control or mock infected animals. # indicates animals infected with AdSmad7 1 week subsequent to BDL.

Serum and tissue samples were taken as indicated and the amount of hydroxyproline, as an estimate of total liver collagen contents, was assessed. The amount of hydroxyproline in total liver extracts from BDL rats was about 3 times higher than in those from healthy rats (Figure 10). In accordance with mRNA data for type I collagen (data of our group, not shown), hydroxyproline was downregulated in BDL rats treated with AdSmad7, when compared with the AdLacZ controls.



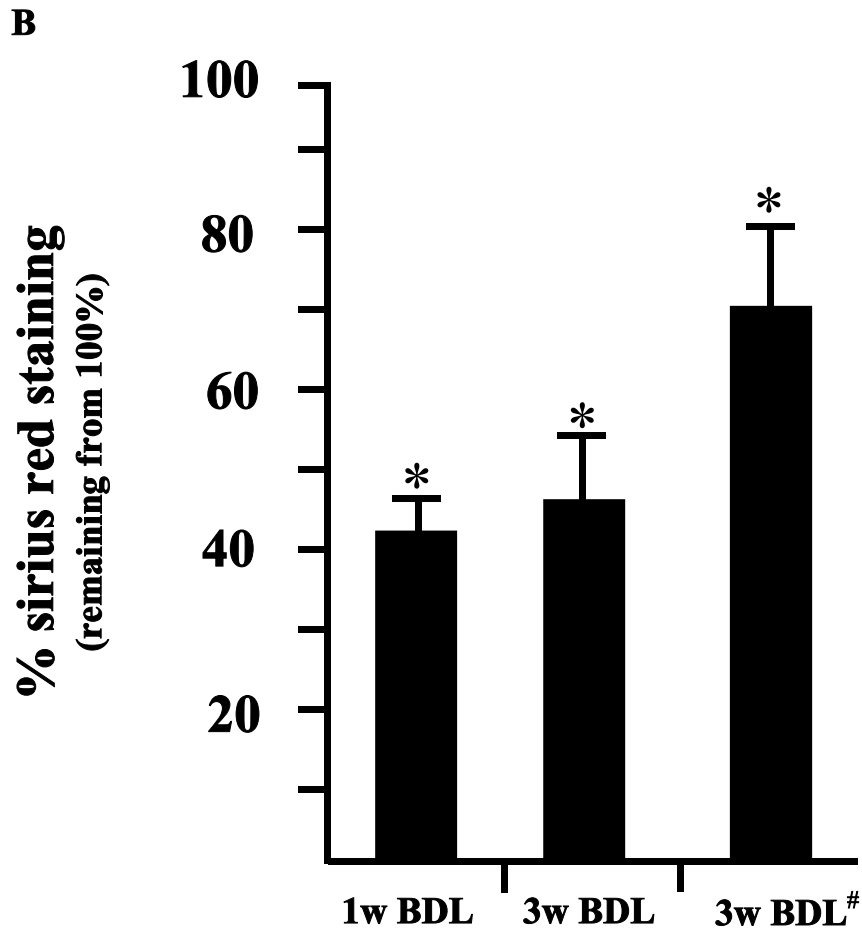


Fig. 11. Smad7 abrogates fibrogenesis-dependent collagen expression. One or 3 week(s) after BDL and gene transfer, livers were resected and examined with sirius red staining. A representative histology of the different treatments is shown in part (A) of the figure (magnification 100 x). The histology of untreated BDL rats was virtually the same as that of AdLacZ infected BDL rats, whereas AdSmad7- infected animals exhibited a significantly reduced staining. [#]Animals that received adenovirus 1 week after BDL. (B) Morphometric quantitation of sirius red staining as a measurement of collagen deposition and fibrosis. Ten fields were selected randomly from every section of 8 rats from each group; OPENLAB improvision software (U.K.) was used. The graph shows the remaining percentage of collagen staining in AdSmad7-treated animals in relation to the corresponding AdLacZ-infected rats.

Collagen protein deposition, as assessed with sirius red dye, also showed that ectopic overexpression of Smad7 in the liver of BDL rats potently reduced the number of collagen fibrils compared to controls receiving AdLacZ (Figure 11A).

Semiquantitative analysis of fibrotic areas 1 week after BDL showed that collagen deposition was reduced to 43 % $\pm$ 4.1 % (n = 8) after AdSmad7 infection in relation to those

rats infected with AdLacZ (n = 8). A similar Smad7-dependent effect was found in animals that were analyzed after 3 weeks of treatment (n = 10, 46 ± 7.4 %) (Figure 11B).

In the case in which AdSmad7 was given 1 week subsequent to BDL, a beneficial effect was also found (Figure 10 and Figure 11) that, however, was lower in comparison to animals that received adenovirus treatment from the time point of BDL (n = 8, 69 % ± 9.8 %). These findings indicate that overexpression of Smad7 suppressed production of type I collagen and hydroxyproline content in the liver of BDL rats during fibrogenesis.

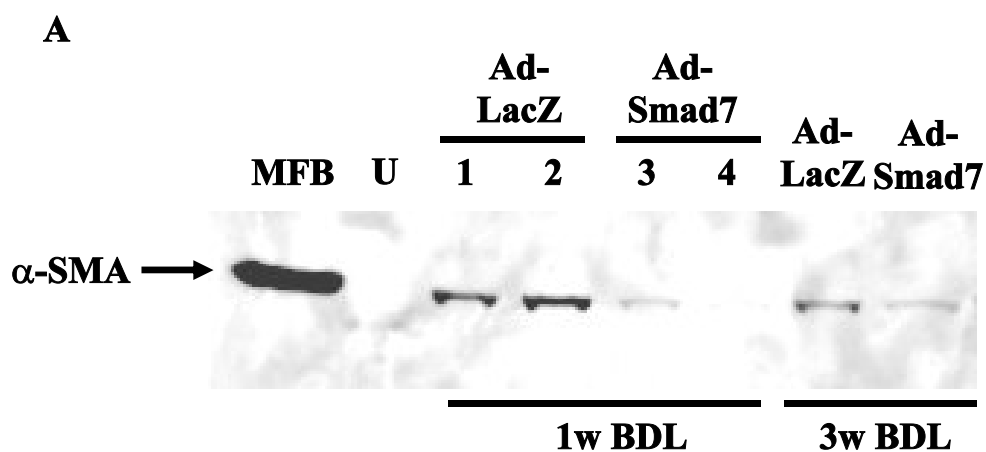
AST, ALT, and bilirubin, used as serum markers for liver damage, were increased similarly in BDL-treated rats, either with or without injecting AdLacZ simultaneously (Table 5). AdSmad7-infected animals showed a lower increase of these markers after 1 week (n = 8) that, however, was only significant (P < 0.05) for AST. After 3 weeks, serum values for liver enzymes and bilirubin displayed a high variance for all animals without differences for AdSmad7-infected animals. Because of the fact that BDL was used as an experimental model, an overall healing effect on liver injury can probably not be expected.

	Bilirubin, mg/dL (1wk)	AST, U/L (1 wk)	ALT, U/L (1 wk)	Bilirubin mg/dL (1 wk)	AST, U/L (3 wk)	ALT, U/L (3 wk)
Untreated	0.065	42 ± 5	32 ± 5	0.1 ± 0.05	45 ± 4	24 ± 4
BDL	10.9 ± 3.7	355 ± 187	153 ± 127	14.2 ± 4.6	275 ± 32	56 ± 38
BDL- AdLacZ	8.6 ± 6	328 ± 33	91 ± 48	344 ± 33	144 ± 105	52 ± 32
BDL- AdSmad7	6.5 ± 5	140 ± 69	57 ± 30	88 ± 14	149 ± 112	66 ± 38

Table 5. Measurements of serum bilirubin and hepatocytes enzymes.

To investigate the influence of Smad7 on activation of HSC *in vivo*, we analyzed the expression of α -SMA in total liver lysates (Figure 12A). BDL-treated rats, with AdLacZ infection, displayed similarly strong α -SMA signal in western blot analyses. In animals treated with AdSmad7, α -SMA expression was impressively reduced. Lysate achieved from myofibroblasts (MFB) served as a positive control.

To investigate the cell type specific impact of Smad7 *in vivo*, we purified HSC from the livers of AdSmad7-infected animals and analyzed the transdifferentiation process *in vitro*. Immunostainings indicated that about 70 % of the cells were positive for Smad7, which was localized predominantly in the nucleus (Figure 12B). Furthermore, Smad7-expressing cells displayed no or significantly reduced α -SMA staining 7 days after seeding. In contrast, Smad7-negative cells displayed strong α -SMA fiber formation.



B

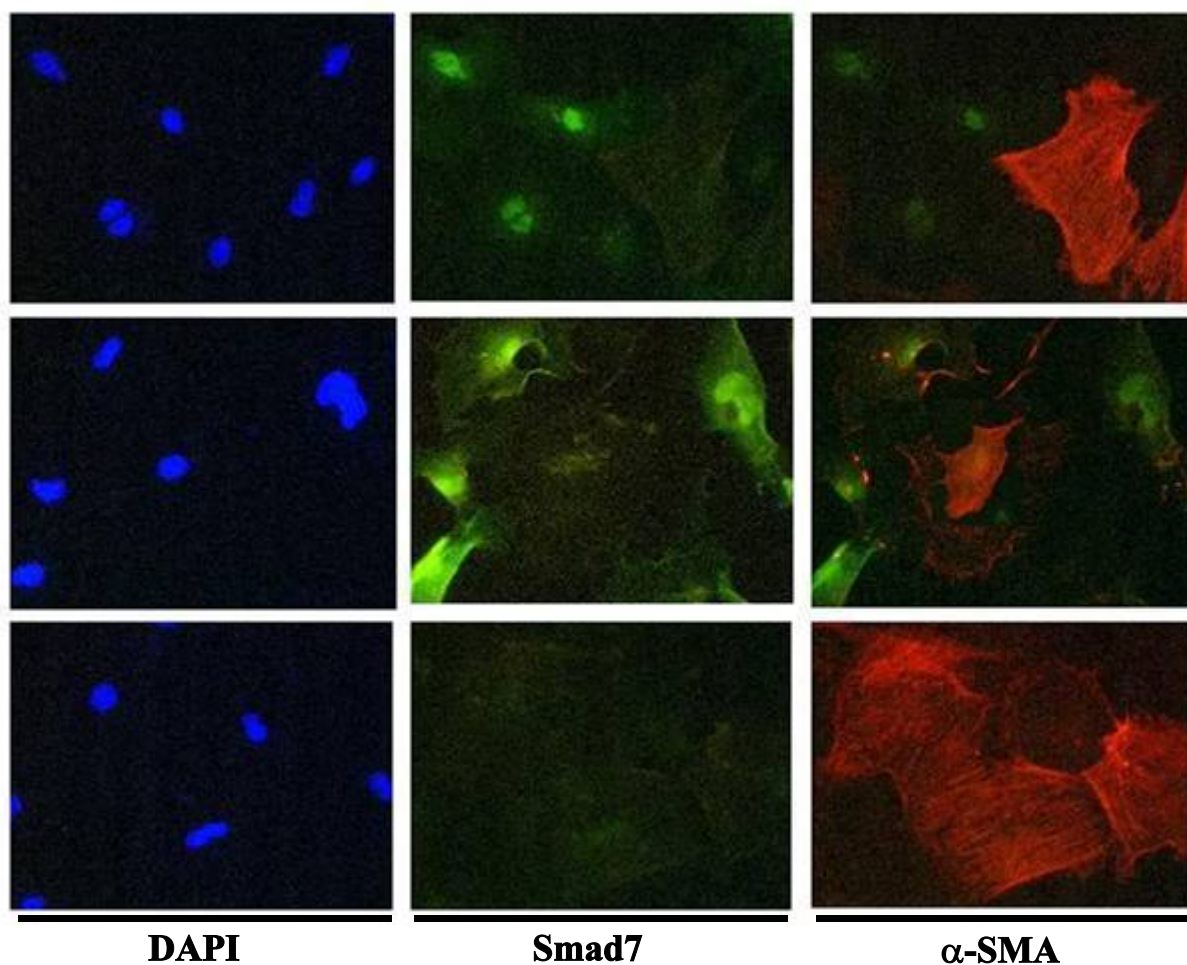


Fig. 12. (A) Equal amounts of protein lysate were examined for α -SMA expression in Western blots. Smad7 abrogates α -SMA expression *in vivo*. A representative blot containing different samples as indicated is presented (AdLacZ/ 1 week BDL – treated rats: 1, 2, AdSmad7/ 1 week BDL – treated rats: 3, 4). Lysate from transdifferentiated HSC (myofibroblasts, MFB) was used as a positive control. (B) Smad7-expressing HSC isolated from AdSmad7-infected rat remain in a quiescent stage and are α -SMA negative. Seven days after seeding, HSC were stained for Smad7 (*green*) and α -SMA (*red*). Nuclei were stained with 4,6,- diamidino-2-phenylindole (*blue*). Ectopically expressed Smad7 is mainly located within the nuclei.

4.3. Ectopic expression of Smad7 inhibits HSC activation

HSC were isolated from rat liver, and morphological changes during transdifferentiation to myofibroblast-like cells were monitored by phase contrast and fluorescence microscopy (retinoid autofluorescence). Freshly isolated cells showed a compact spherical configuration until day 3. During the next 3 to 4 days, the cells flattened, spread, developed long cytoplasmic extensions, and increased their diameter 3- to 5-fold.

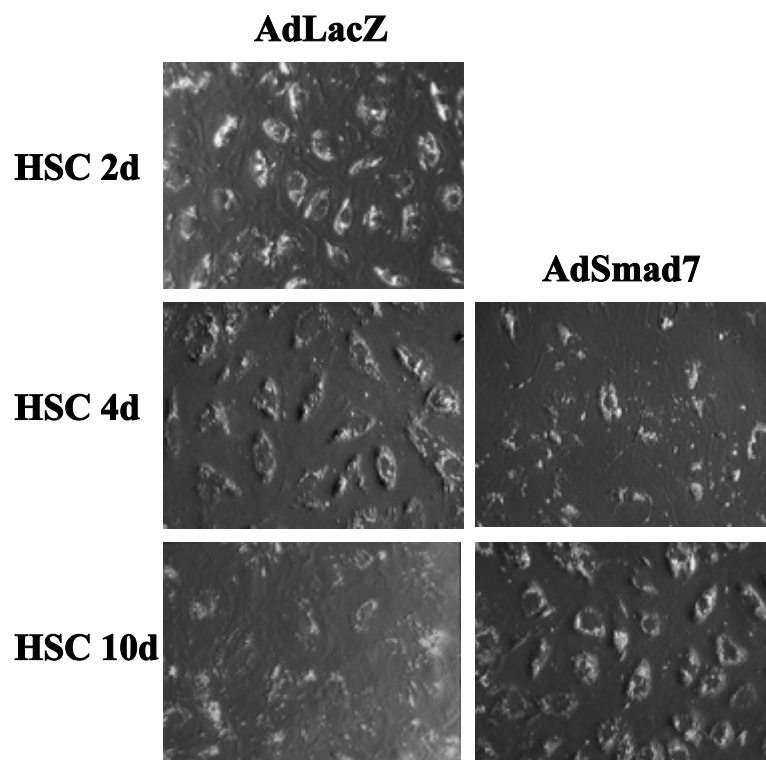


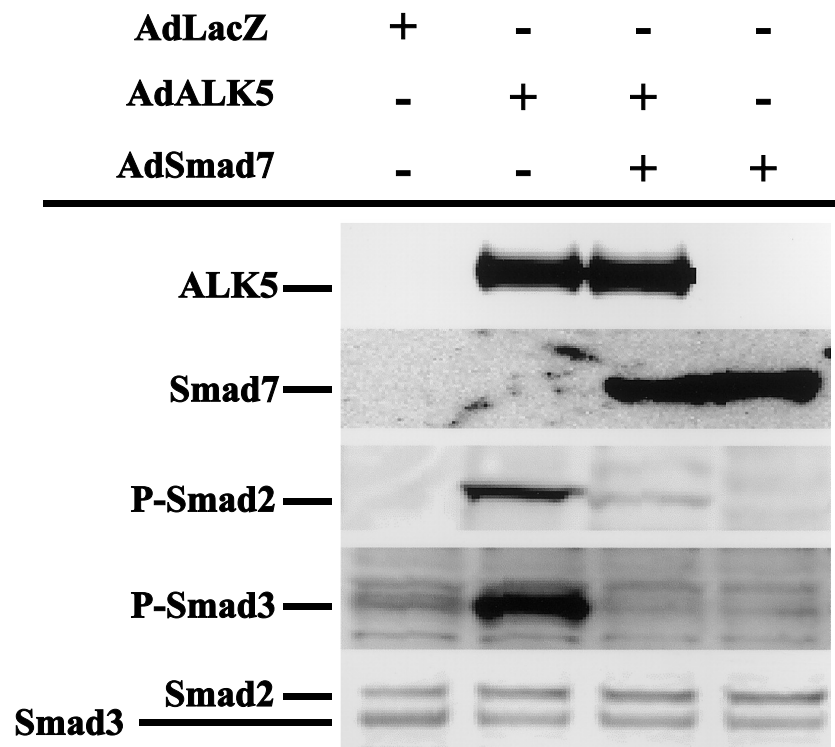
Fig. 13. Smad7 inhibits transdifferentiation of primary-cultured HSC. Nomarski differential interference contrast micrograph of rat liver HSC and myofibroblast-like cells during culture on plastic dishes. Two days after seeding, cells were infected with the indicated adenoviruses (infection rate > 90 %). Transdifferentiation was monitored by storage of lipid droplets and the shape of the cells.

Additionally, the cells lost fat droplets and displayed characteristic biochemical features, associated with the transdifferentiated phenotype (e.g., increased expression of TGF- β , α -SMA, type I collagen, fibronectin) (Friedman SL, 2000) (Gressner AM, 2002). Spontaneous activation through contact with plastic and transdifferentiation was uninfluenced by infection with 50 moi AdLacZ (Figure 13). Overexpression of Smad7 completely abolished morphological transdifferentiation of HSC, leaving the cells in a compact spherical configuration and sustaining retinoid storage at least until day 10 of cultivation.

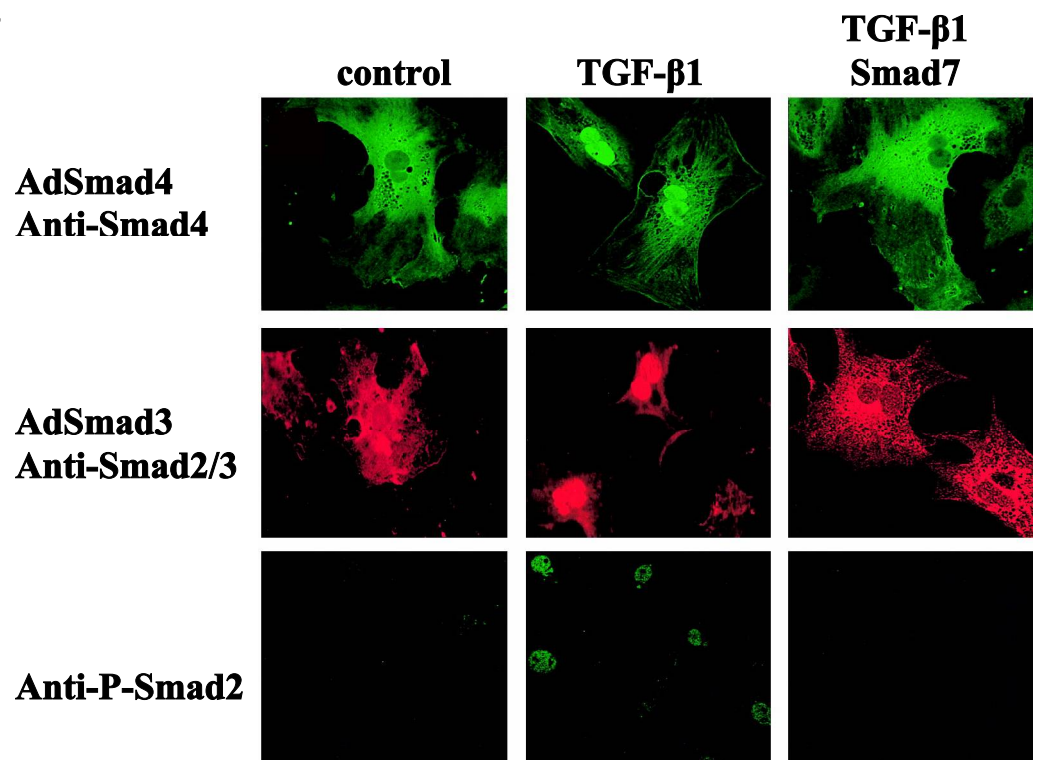
4.4. Ectopic expression of Smad7 blocks paracrine and autocrine TGF- β signal transduction in HSC

To find out if the observed Smad7 effects in HSC are due to an inhibition of TGF- β signaling or to TGF- β -independent functions, we investigated TGF- β signal transduction in Smad7-overexpressing HSC. Infection of HSC with an adenovirus expressing a constitutively active TGF- β receptor type I (AdALK5) led to phosphorylation of Smad2 and Smad3. Simultaneous infection with AdSmad7 resulted in significant inhibition of Smad2/3 phosphorylation (Figure 14A). These data were confirmed by immunostainings of cultured HSC showing that Smad7 interferes with TGF- β signaling by preventing nuclear translocation of activated Smad complexes (Figure 14A). Adenoviral overexpressed pathway specific Smads are mostly localized in the cytoplasm of serum-starved HSC. After exposure to TGF- β 1 for 1 hour nuclear staining of Smad2/3/4 was predominant in 60 % to 90 % of the cells. Infection with AdSmad7 before adding TGF- β 1 suppressed TGF- β signaling and abrogated nuclear accumulation of Smad2/3/4 (Figure 6B). To study the effect of Smad7 on autocrine TGF- β signaling in activated HSC, primary-cultured cells were coinfecting with Ad(CAGA)₉-MLP-Luc and with AdLacZ, AdSmad7, or AdALK5 at day 3 after seeding. Twenty-four hours later, lysates were prepared to measure luciferase activity (Figure 14C). Autocrine stimulation by endogenously produced TGF- β family growth factors is demonstrated by luciferase activity in AdLacZ-infected cells. Overexpression of ALK5 or treatment with TGF- β 1 increased “basal” luciferase activity. Ectopic expression of Smad7 very efficiently inhibited autocrine signaling, squeezing down luciferase activity nearly to the baseline and it significantly reduced the TGF- β or ALK5 mediated effects (Fig. 14C).

A



B



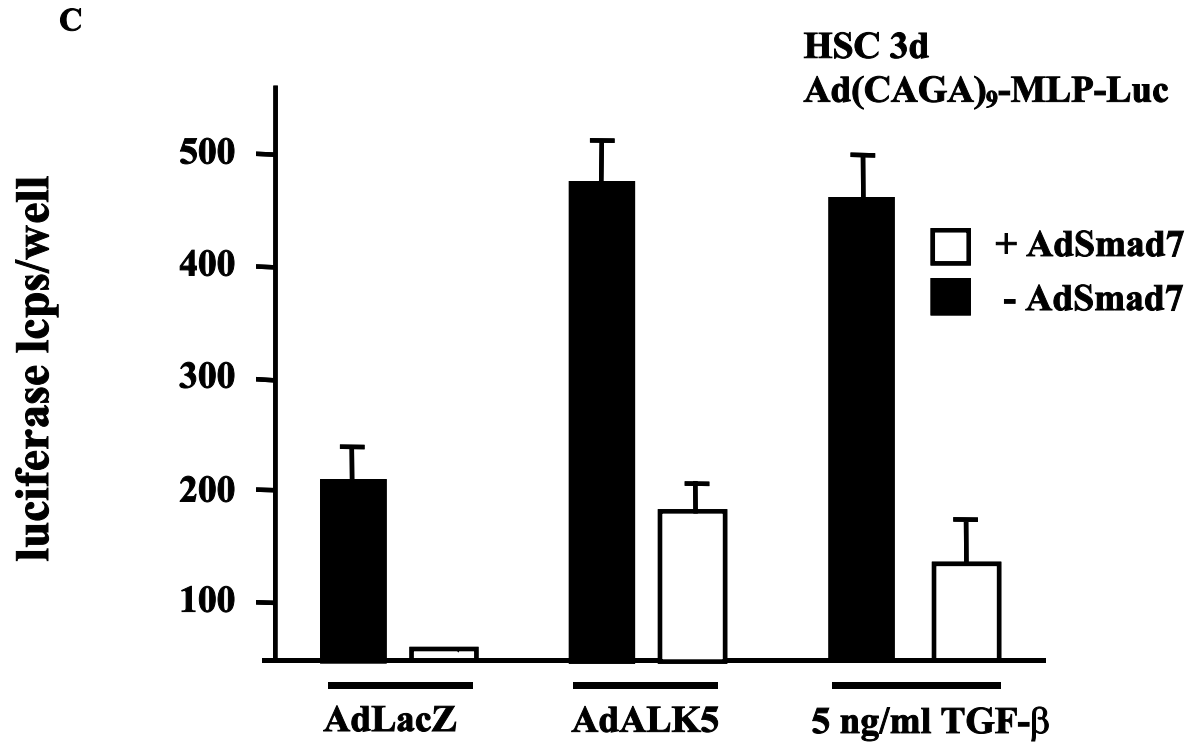
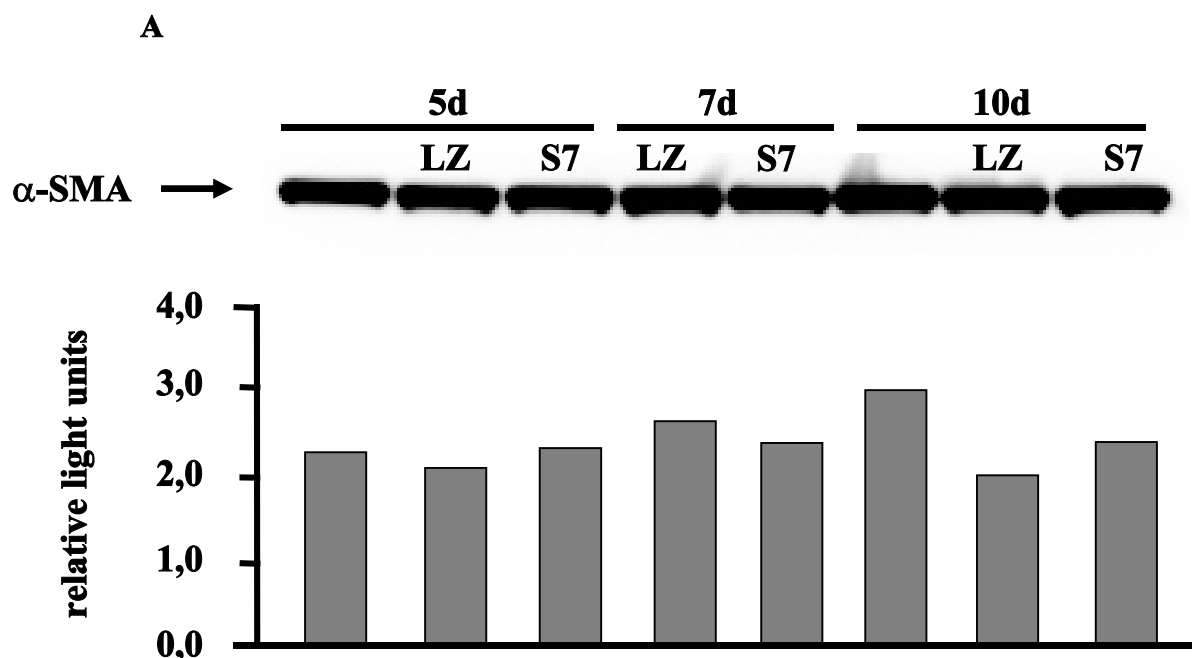


Fig. 14. Smad7 prevents TGF- β signaling in HSC. (A) HSC were infected with AdLacZ, AdALK5, and AdSmad7 as indicated (+), and protein lysates were used for Western blot analysis. Ectopic protein expression was detected with an anti-Flag antibody (Smad7) or with an antibody against ALK5. To prove the biological effects of ectopically expressed proteins, specific antisera against phosphorylated Smads 2 and 3 (PS2, PS1 (Dooley S, 2001)) were used. Parallel blots were probed with a polyclonal Smad2/3 specific antibody to confirm equal protein expression. (B) Smad7 blocks nuclear translocation of Smads complexes. HSC were subjected to adenoviral infections as indicated. Ectopically expressed Smad3/4 and phosphorylated endogenous Smad2 were detected with specific antisera as indicated. TGF- β 1 induced nuclear translocation of activated Smad complexes. Nuclear staining was abolished by overexpression of Smad7. (C) Analysis of the Smad7 effect on autocrine TGF- β signal transduction in HSC. Primary-cultured HSC were infected with 100 moi of Ad(CAGA)₉-MLP-Luc and AdLacZ or AdSmad7 as indicated at day 2 after seeding (infection rate >90%). Lysates were prepared 24 hours later. Luciferase light counts per second (LCPS) are shown. Similar luciferase activity was obtained, when the cells were treated with 5 ng/mL TGF- β 1 instead of AdALK5. The presented data are representative for three independent experiments (n = 4, $\pm$ SD).

4.5. Effects of Smad7 on α -SMA expression during culture activation of HSC

α -SMA is the most pronounced marker for activated HSC. Blocking TGF- β signaling by Smad7, which leads to inhibition of HSC activation (see above), did not result in decreased α -SMA expression in initially activated cells. Western blot analysis of lysates showed that there was no difference in the level of total α -SMA in activated or LacZ-infected cells compared to Smad7-overexpressing HSC (Figure 15A). However, culture-activated, AdLacZ-infected HSC showed prominent actin fibers after 7 days of culture, whereas AdSmad7-infected cells completely lost actin fiber organization. In these cells α -SMA staining was detected as a diffuse cytoplasmic staining (Figure 15B). These results suggest that Smad7 expression in culture-activated HSC may modulate alterations in the actin cytoskeleton and morphology via interfering with organization and polymerization of α -SMA while leaving the expression levels unaffected.



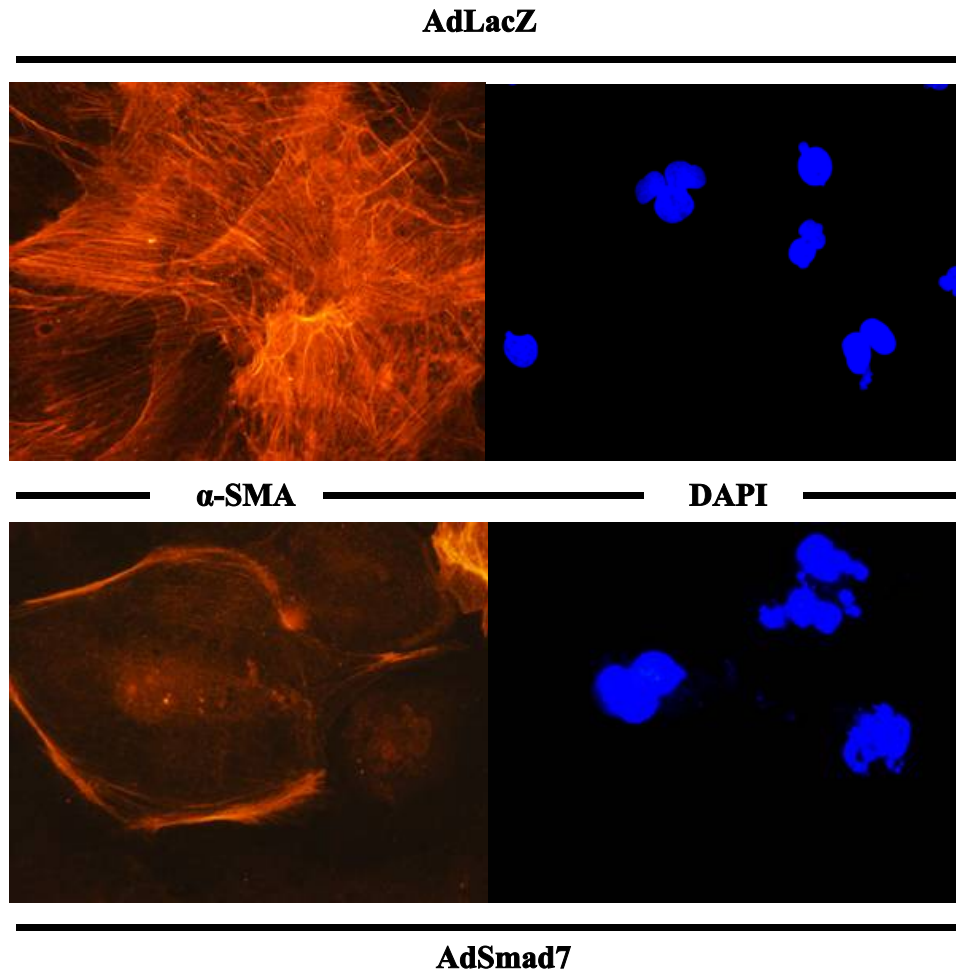
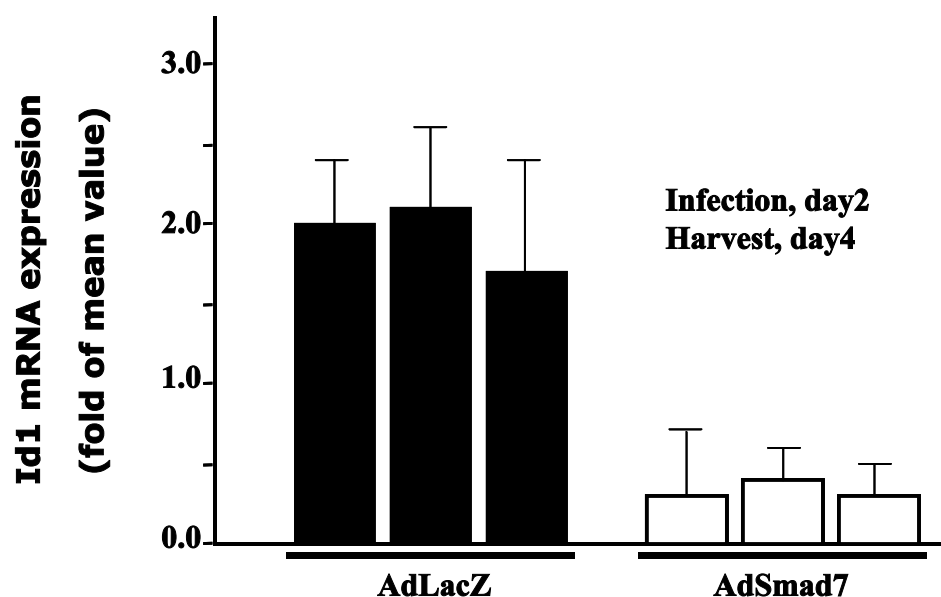
B

Figure 15. (A) α -SMA expression in activated HSC is not influenced by Smad7. α -SMA expression was analyzed in Western blots using 25 μ g total lysate from primary-cultured HSC, infected or not with AdLacZ (LZ) or AdSmad7 (S7) at day 2 and cultured for different times as indicated. A representative experiment is shown, which was performed in triplicate. α -SMA signal intensity was evaluated densitometrically using Lumi Imager software (Roche). The data indicate that the expression is essentially unaltered. (B) Smad7 induces morphological changes of actin fiber organization in primary-cultured HSC. Immunostaining was performed with a monoclonal anti α -SMA antibody. Representative fluorescence microscopy images of primary-cultured HSC, infected with AdLacZ or AdSmad7, are shown. AdLacZ-infected cells display actin stress fibers; AdSmad7-infected cells, in contrast, have completely lost the ability to establish a fibrillar organization and display focal or diffuse staining within the cytoplasm (magnification 400x).

4.6. Smad7 interferes with Id1 expression in activated HSC

To identify Smad7-dependent gene responses, HSC were infected at day 2 after seeding with AdSmad7 and total RNA was purified two days later, when the stress response caused by viral infection was no longer predominant. Transcript profiles were generated in comparison to AdLacZ infected cells using the Affymetrix rat genome RG-U34 microarray (about 8.800 probe sets). A total of 377 genes were found to be differentially regulated by Smad7 with a change of at least 2-fold ($p < 0.001$). Among these, 230 were identified with informative annotations. The affected genes were subsequently clustered into 7 categories according to their biological characteristics and functions, namely cell adhesion/ECM, membrane proteins, receptors, 7TM receptors - RGS proteins, intracellular signaling, Ca²⁺ binding-transport, electron transport (manuscript in preparation). Among the most intensely regulated genes was a recently identified TGF- β /BMP2 target gene (Goumans M-J, 2002), Id1, whose known functions include cell cycle regulation and/or inhibition of epithelial to mesenchymal transition (Kondo M, 2004) (Kowanetz M, 2004). Smad7-dependent downregulation of endogenous Id1 mRNA levels in HSC was confirmed by semiquantitative RT-PCR analysis using rat Id1 specific primers and revealed a decrease of message levels by 70 to 80 percent (Figure 16A). Id1 protein expression during transdifferentiation was completely undetectable after overexpression of Smad7 (Figure 16B).

A



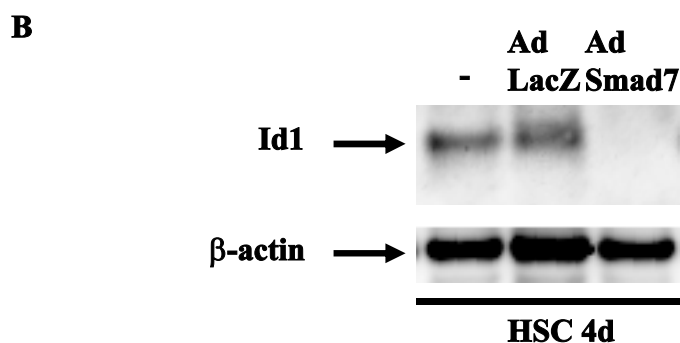
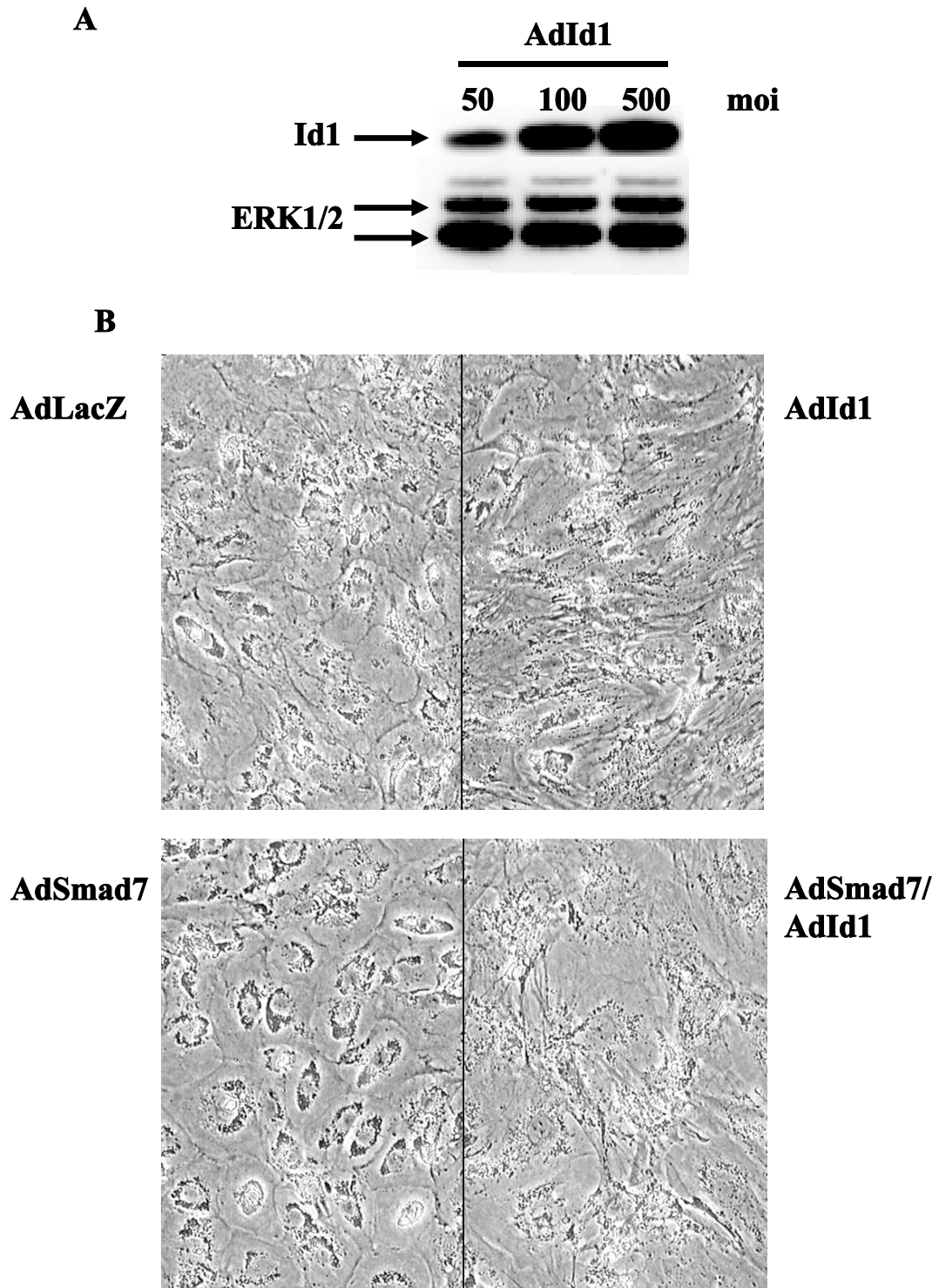


Fig. 16. Smad7 abrogates Id1 expression during transdifferentiation of HSC. HSC were infected with adenoviruses (AdLacZ, AdSmad7) as indicated (50 and 100 moi, respectively) at day 2 after plating. (A) Total RNA was analyzed by SYBR green I-based real-time PCR with rat Id1 specific primers as indicated in Materials and Methods. Three different preparations of rat HSC were investigated independently and in triplicates. (B) Cell lysates for total protein were prepared at day 4. Western blot analysis was performed using specific antibody. As loading control, the same blot was probed with an anti-β-actin antibody.

4.7. Ectopic Id1 expression enhances activation of HSC and abrogates inhibitory effects of Smad7

To assess the effect of Id1 on the HSC phenotype and on Smad7 activities, adenoviruses carrying cDNA for either Id1 or Smad7 were introduced into 2 days-old HSC. Ectopic expression of Id1 was confirmed by Western blot analysis (Figure 17A). Morphological changes were monitored by phase contrast and fluorescence microscopy until day 7 of culture (Figure 17B and C). As expected, Smad7 expressing HSC remained in a quiescent stage with compact spherical shape and sustained retinoid storage. In contrast, HSC expressing Id1 alone or together with Smad7 showed characteristic transdifferentiation dependent morphological changes that are loss of fat droplets, flattened appearance with spreading and cytoplasmic extensions representing MFB phenotype. In comparison to untreated (not shown) or Ad-LacZ infected controls, Id1 overexpressing cells were more progressed in the transdifferentiation process. Since α-SMA fiber formation is one typical feature of transdifferentiation which is potently abrogated by Smad7, the influence of ectopic Id1 expression on α-SMA fiber formation was examined. HSC were cultured on glass coverslips, infected with adenoviruses expressing Id1 and/or Smad7 and cultured until day 7,

when α -SMA staining was performed. Ectopic expression of Id1 eliminated the effect of co-expressed Smad7 and resulted in polymerization of α -SMA into fiber structures (Figure 17C). These data imply that ectopic Id1 expression suffices to overcome inhibitory Smad7 effects on HSC transdifferentiation.



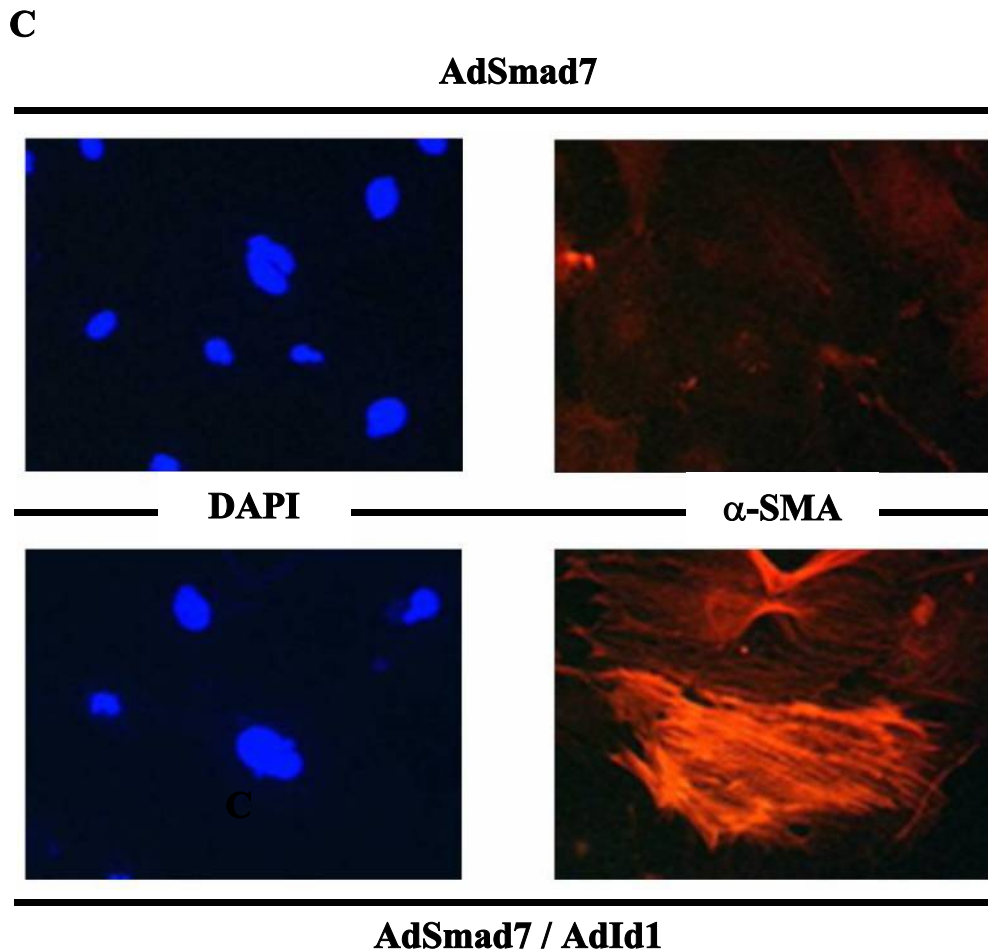


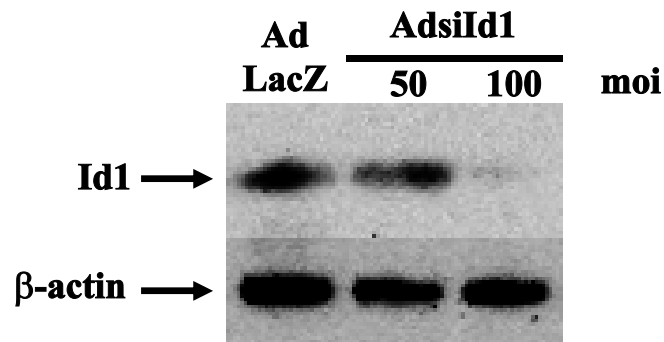
Fig. 17. Id1 expression enhances activation of HSC and is required for α -SMA fiber formation. HSC were infected with adenoviruses (AdLacZ, AdSmad7, AdId1) as indicated at day 2 after plating (50, 100 and 100 moi respectively). The representative images were taken on day 7 of culture. (A) Titration of virus and corresponding ectopic Id1 protein expression was monitored by Western blot analysis. Expression of ERK1/2 was determined to assure equal loading. (B) Morphological changes were monitored by phase contrast microscopy after virus infections as indicated. (C) Immunofluorescent staining for α -SMA (right). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; left). Staining was documented by fluorescence microscopy with an LSM 510 laser scanning microscope; magnification: 200x.

4.8. RNA interference-mediated knock down of Id1 expression inhibits transdifferentiation of HSC

To conversely examine the effect of depleted Id1 expression on in vitro transdifferentiation of primary cultured HSC, small interfering RNA targeting Id1 expression was designed and introduced by means of adenoviral infection. Efficacy of Id1 knock down was demonstrated by immunoblotting, showing approximately 90% down-regulated Id1

protein levels at 100 moi (Figure 18A). Concurrently with inhibited Id1 expression, immunostaining for α -SMA fibers were reduced in 7 days old HSC (Figure 18B, middle), when compared to AdLacZ infected (Figure 18B, top) or Id1 overexpressing cells (Figure 18B, bottom).

A



B

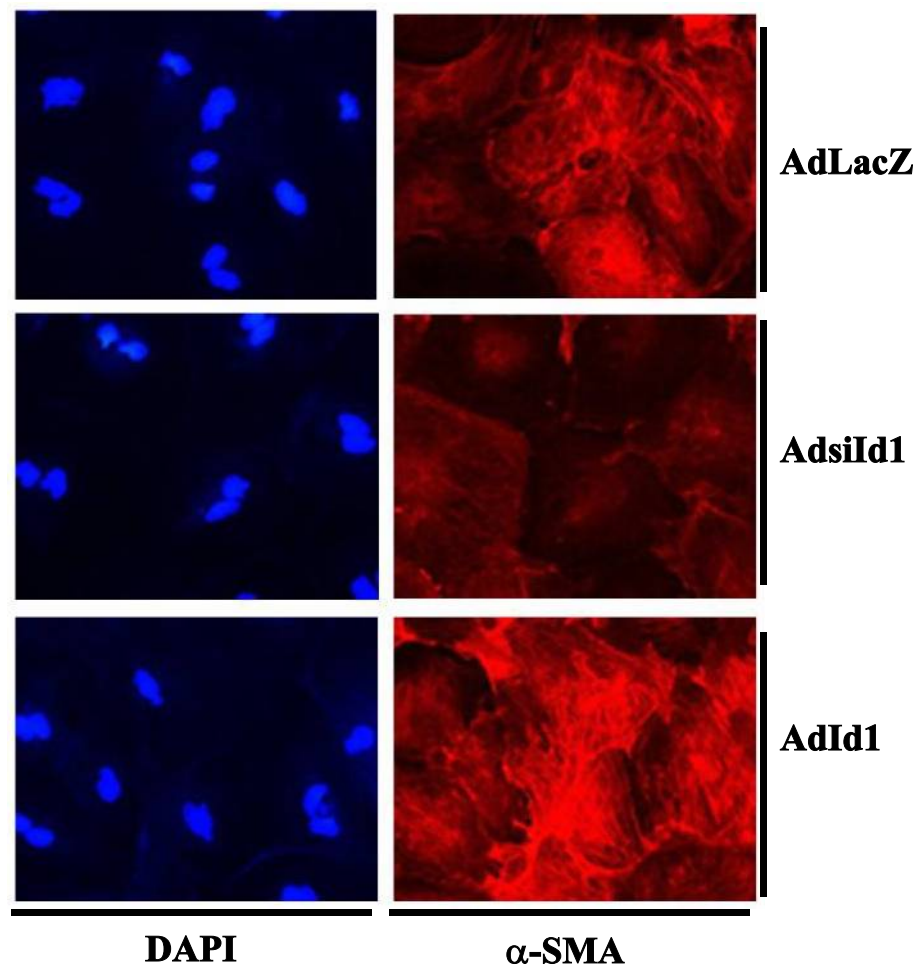
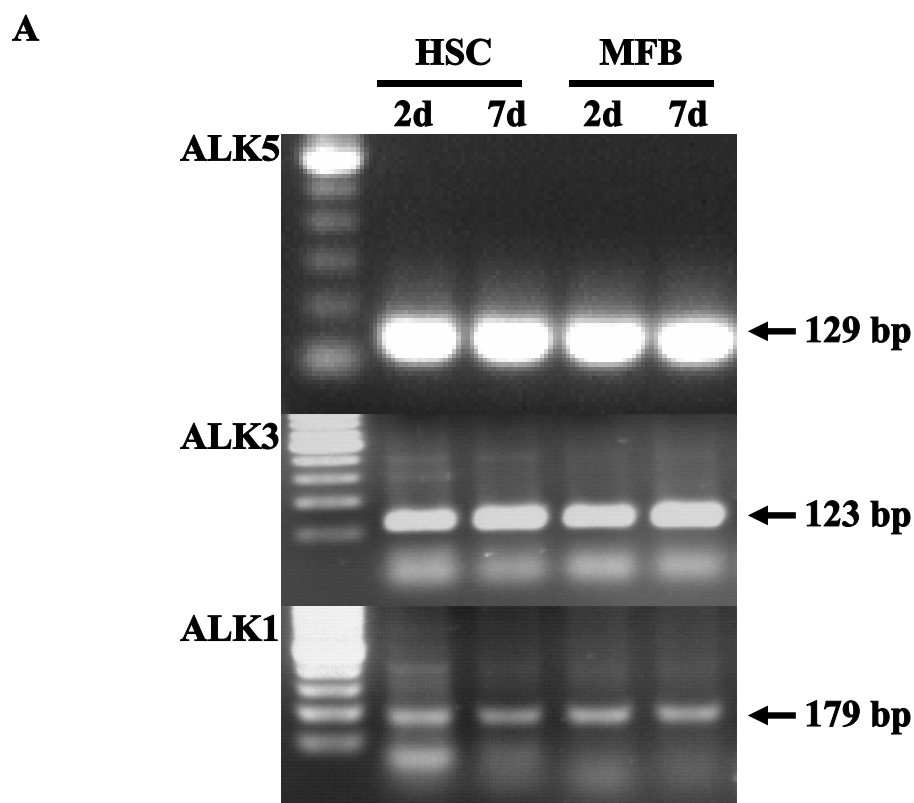


Fig. 18. Overexpression of Id1 enhances and knock down of Id1 abrogates transdifferentiation of HSC. HSC were infected with adenoviruses (AdLacZ, AdId1, Adsild1) as indicated (50, 100 and 100 moi, respectively) at day 2 after plating. (A) Titration of Adsild1 effectively downregulates Id1 in Western blot analysis. β -actin was assayed as loading control. (B) Representative immunofluorescent images from day 7 of cell culture are shown.

4.9. TGF- β induces expression of Id1 in HSC via the ALK1 but not the ALK5 pathway

ALK1 was recently identified as TGF- β type I receptor in endothelial cells (Goumans et al., 2002). In this cell type, TGF- β was shown to regulate the activation state via balancing ALK5 and ALK1 signaling and Id1 was found to mediate an ALK1 specific response. Therefore, we examined the TGF- β type I receptor expression pattern in HSC by reverse transcription PCR analysis. As shown in Figure 19A, ALK1 mRNA was detected in HSC, as were ALK3 and ALK5. Furthermore, the two known TGF- β type III receptors, betaglycan and endoglin, are also expressed in activated HSC exhibiting diverse profiles. While T β RIII is upregulated around days 2 to 4, endoglin mRNA levels increase from day 3 on and remain at elevated levels at least up to day 8 (Figure 19B). The upregulation of endoglin expression during activation of HSC on mRNA level is in line with western blot analysis of endoglin protein expression (Figure 19C).



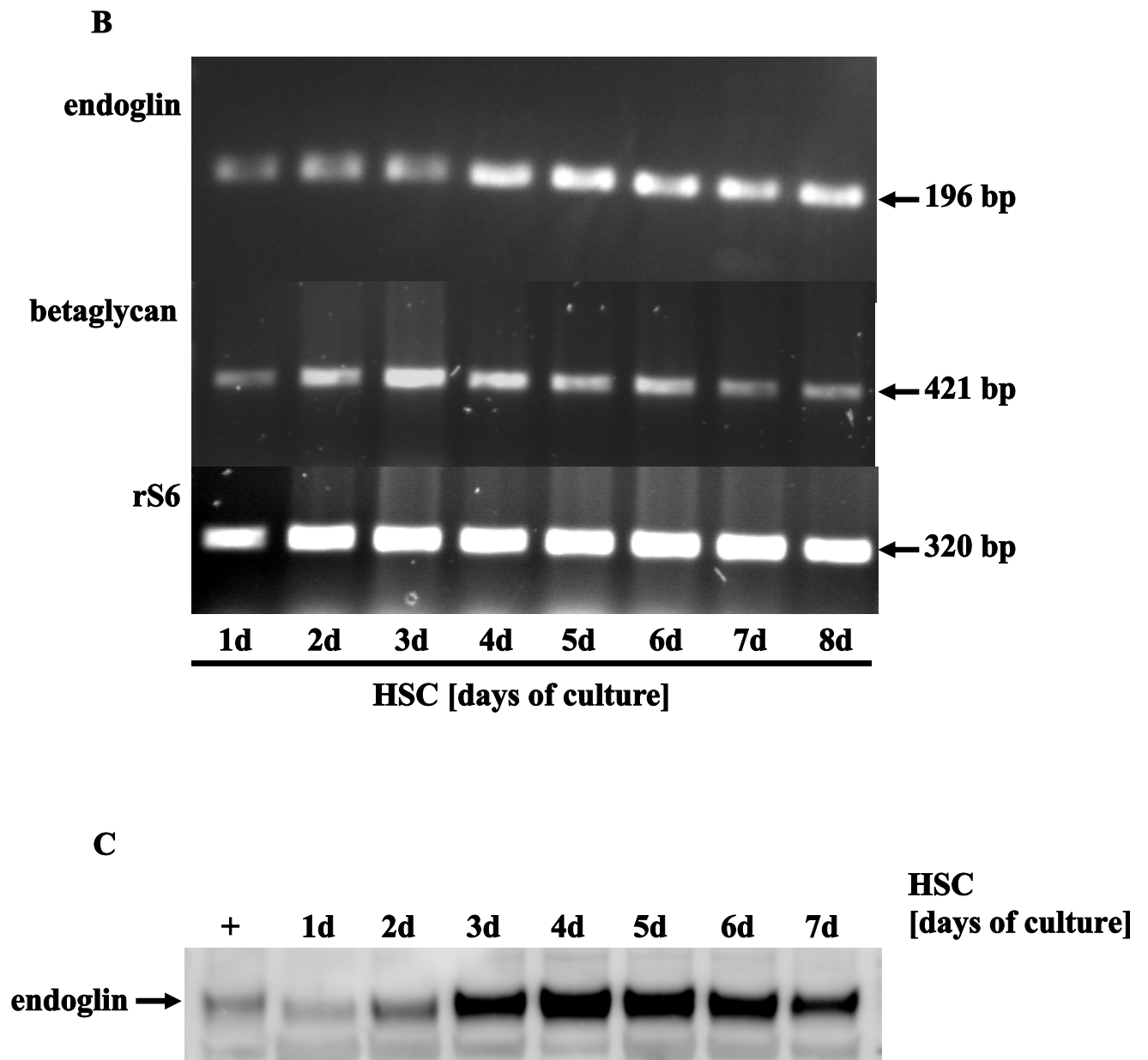


Fig. 19. TGF- β type I and III receptor expression patterns in HSC. (A) Total RNA samples were processed by RT-PCR and ALK1, 3 and 5 mRNA expression of 2- or 7 days old HSC and MFB were investigated by PCR as indicated. Identity of amplification products was ascertained by sequencing. (B) Betaglycan and endoglin mRNA expression was investigated in 1 to 8 days old HSC. Expression of rS6 is included as positive control. (C) Endoglin protein expression in 1 to 7 days old HSC was assayed in western blot using specific antibody.

Stimulation of 4-days-old HSC with 5 ng/ml TGF- β 1 strongly induced Id1 protein expression within 1 hour of stimulation (Figure 20). Expression thereafter decreased to basal levels within 6 hours. TGF- β 1 induced phosphorylation of both, Smad2 and Smad1 (Figure 20).

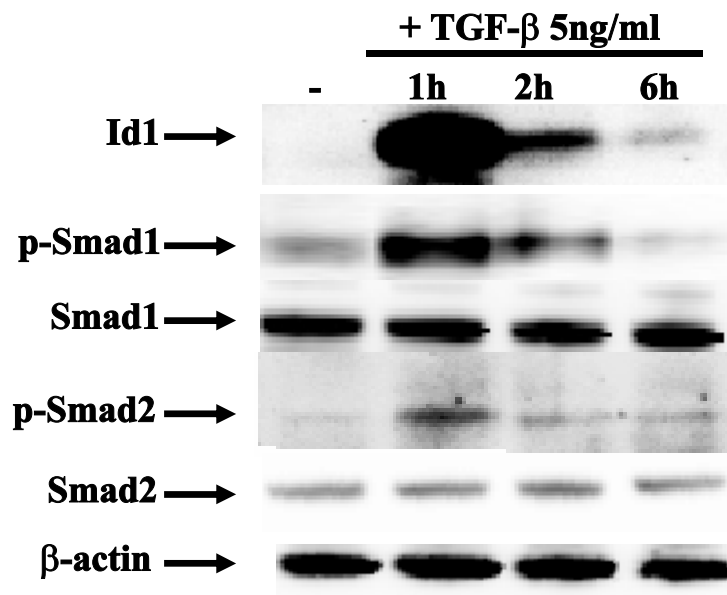
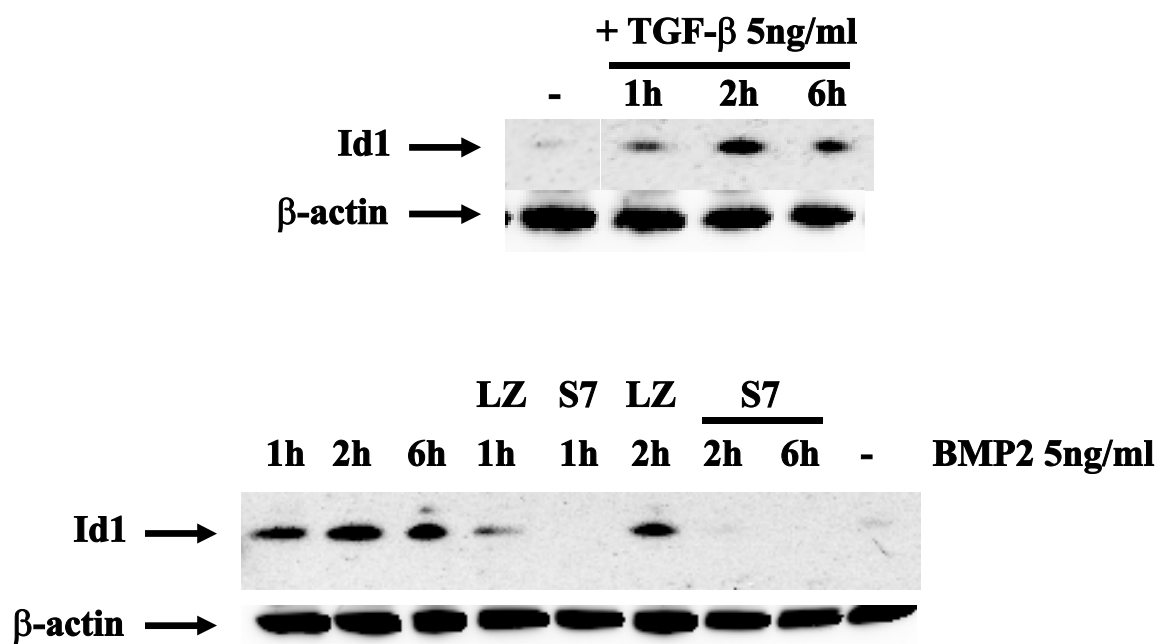


Fig. 20. TGF- β induces Id1 expression and Smad1/2 phosphorylation in HSC. At day 4, after 16 hours of serum starvation in medium containing 0.5 % FCS, HSC were stimulated with 5 ng/ml TGF- β as indicated. Cells were lysed after 1, 2 and 6 hours, as indicated followed by Western blot analysis of Id1, phospho-Smad1/2, total Smad1/2 and β -actin expression.

Further studies were performed with a cell line derived from cirrhotic rat liver, CFSC that represents activated HSC. Treatment of these cells with TGF- β 1 or BMP2 also resulted in upregulation of Id1 protein expression (Figure 21A). Overexpression of Smad7 inhibited BMP2 induced Id1 expression in CFSC (Figure 21A). To further investigate if TGF- β induces Id1 expression via activation of ALK1, small interfering RNA targeting ALK1 expression was designed and introduced into CFSC by means of adenoviral infection. The results show strongly reduced Id1 expression and Smad1 phosphorylation with increasing amounts of virus in TGF- β treated cells (Figure 21B).

A



B

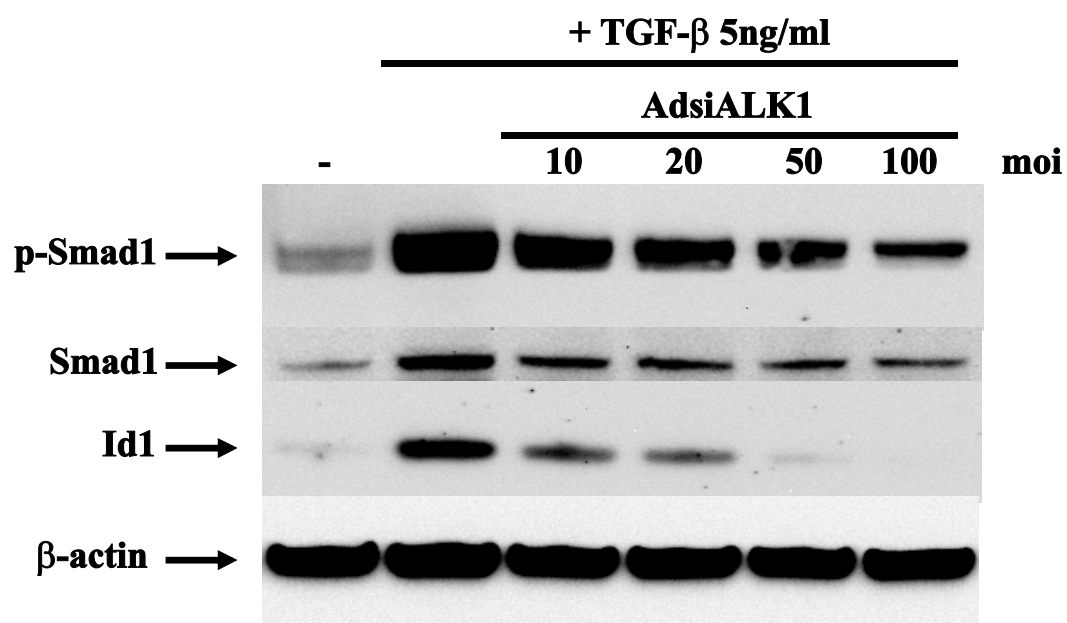


Fig. 21. TGF- β induced Id1 expression is mediated via ALK1/Smad1 in CFSC. (A) After a period of 16 hours of serum starvation, CFSC were stimulated with 5 ng/ml TGF- β or BMP2 and lysed after 1, 2 and 6 hours. Western blot analysis was performed using an anti-Id1 antibody. Infection with AdSmad7 ("S7"; 100 moi) abrogated Id1 induction by BMP2. (B) TGF- β stimulated CFSC (5ng/ml, 1h) were pretreated with increasing amounts of adenoviruses expressing si-RNA for ALK1 (AdsiALK1) as indicated. Expression of Id1, phosphorylation and total amount of Smad1 were analyzed by Western blot.

Constitutively active (ca) ALK3, which is the BMP2 receptor type I, provides exactly the same downstream signaling as caALK1, namely Smad1/5/8 phosphorylation, and thus also induces Id1 expression in CFSC or HSC (Figure 22). Expression of caALK5 did not influence Id1 expression levels and selectively led to Smad2 phosphorylation (Figure 22).

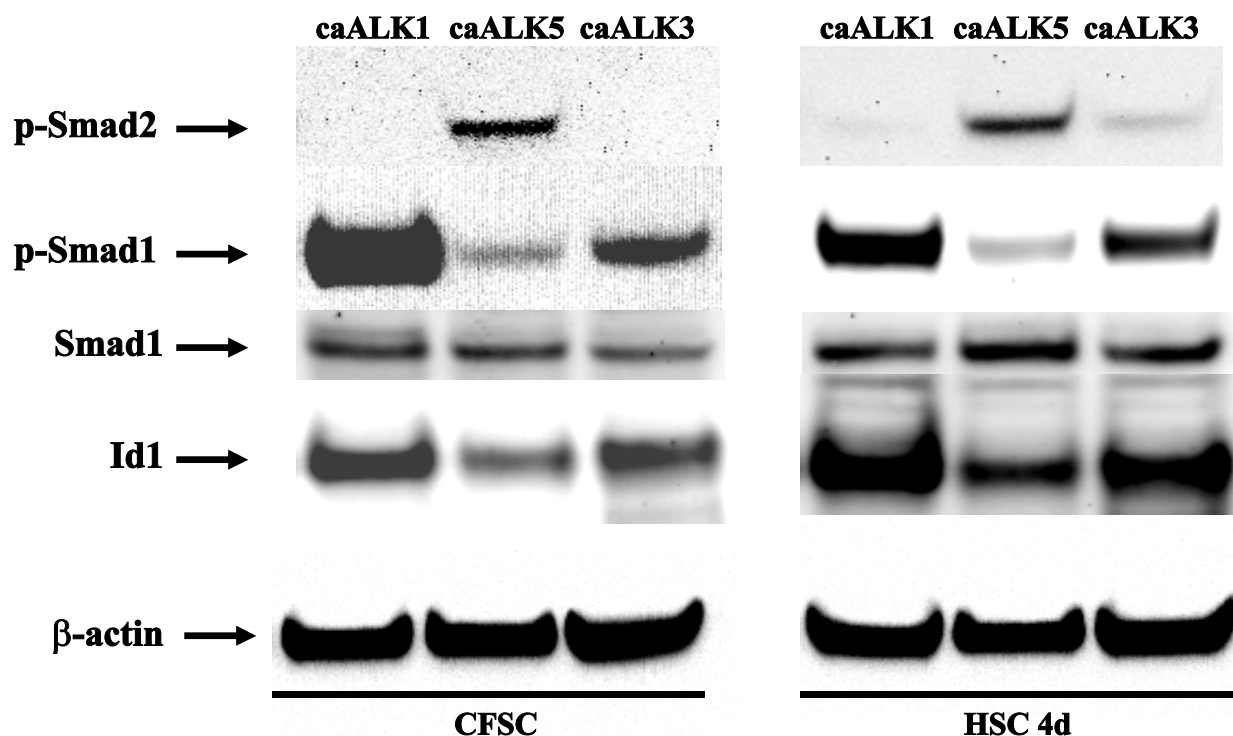


Fig. 22. Id1 induction can be mediated by ALK1 or ALK3 but not by ALK5 in CFSC and HSC. Cell lysates were prepared from CFSC and HSC after adenoviral overexpression of constitutively active ALK1, -3 or 5 and expression of Id1 and phosphorylation of Smad1 and 2 were analyzed by Western blot.

In summary, these data implicate that in HSC, TGF- β signaling via ALK5 activates the Smad2/3 pathway while in parallel signaling via ALK1 leads to phosphorylation of Smad1. Furthermore, TGF- β /ALK1/Smad1 but not TGF- β /ALK5/Smad2/3 pathways stimulate Id1 expression in HSC.

To investigate induction of Id1 directly at the level of transcription, a mouse Id1-promoter luciferase reporter construct (-1231/+88) (Korchynskyi O, 2002) was transiently introduced into CFSC, followed by stimulation with TGF- β or BMP2. In accordance with the protein expression data, both cytokines displayed comparable promoter activation (Figure 23).

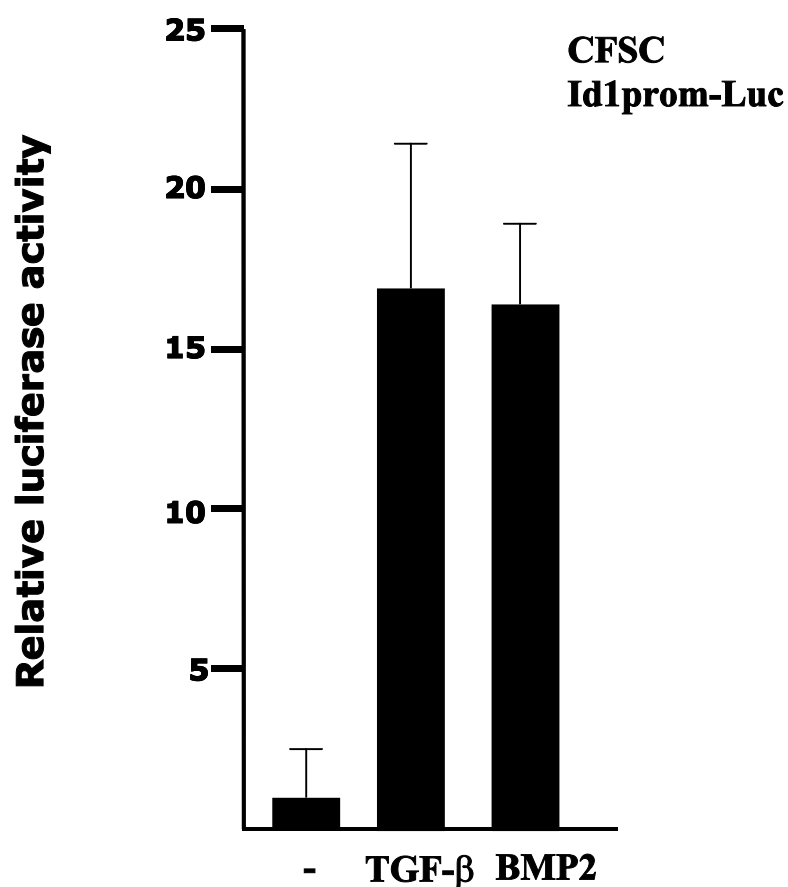


Fig. 23. TGF- β and BMP2 activate an Id1-promoter luciferase construct. CFSC were transiently transfected with an Id1-promoter reporter plasmid followed by 8 hours of serum starvation. Cells were then stimulated with 1 ng/ml TGF- β or 10 ng/ml BMP2 for 14 hours. Lysates were prepared and luciferase activity was measured. Each assay was performed in quadruplicates and repeated in three independent experiments. One representative experiment $\pm$ standard deviation is shown.

4.10. Id1 protein expression is upregulated during activation of HSC

To assess the role of Id1 during HSC activation, lysates from 1 to 7 days old HSC were analyzed in western blot using specific antibody against Id1 (Figure 24A). The Id1 protein level is increasing during transdifferentiation of HSC, supporting the hypothesis that Id1 induction plays an important role in this process. In contrast, Id1 mRNA expression decreases during HSC activation (Figure 24B). These controversial results can be explained by the fact, that Id1 protein expression is strongly controlled by the degradation via proteosome. Thus, Id1 seems to get more stable and to accumulate in the cells during transdifferentiation of HSC.

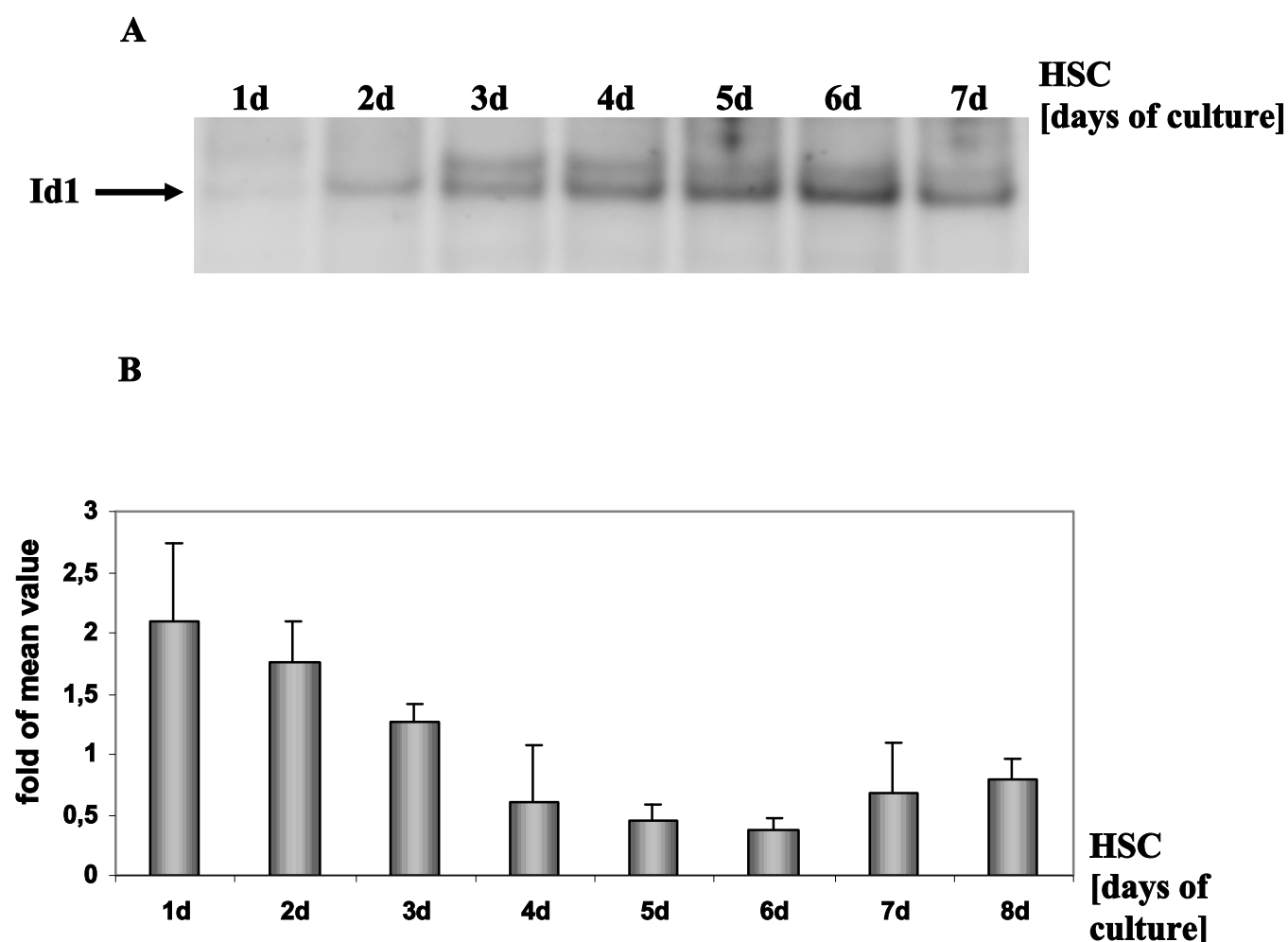


Fig. 24. Id1 expression during transdifferentiation of HSC. (A) lysates from 1 to 7 days old HSC were assayed in western blot using specific antibody. (B) Total RNA from 1 to 8 days old HSC was analyzed by SYBR green I-based real-time PCR with rat Id1 specific primers as indicated in Materials and Methods.

5. Discussion

TGF- β hyperactivity is recognized as the driving force of the fibrogenic response to injury and anti TGF- β strategies were successfully used to counteract this process in different tissues, including liver, lung and kidney (Nakao et al., 2002). Smad7 is a major inhibitory regulator of TGF- β superfamily signal transduction and recent studies have implicated Smad7 as an important molecule for regulating TGF- β activity in human and experimental disease. Smad7 dependent inhibition of TGF- β signaling was shown to be important for the chronic production of pro-inflammatory cytokines in inflammatory bowel disease (Monteleone et al., 2001) and deficient Smad7 expression, resulting in enhanced TGF- β signaling was reported in skin lesions of skleroderma (Dong et al., 2002). Two recent approaches used adenoviral infection and overexpression of Smad7 to intervene profibrogenic TGF- β effects *in vivo*. Intratracheal injection of AdSmad7 was able to block bleomycin dependent Smad2 phosphorylation, resulting in suppression of collagen I mRNA, reduced hydroxyproline content and missing fibrotic responses in the lungs (Nakao et al., 1999), suggesting that Smad7 may have potential to treat pulmonary fibrosis. The antifibrotic potential of Smad7 was further confirmed by a combination of adenoviral infection and *in vivo* electroporation on unilateral ureteral obstructed kidney, which demonstrated a significant Smad7 dependent suppression of renal fibrosis (Terada et al., 2002).

In the present study, overexpression of Smad7 in primary cultured HSC and in BDL rats, respectively, using adenoviral gene transfer, was used to find out whether Smad7 can regulate activation of HSC and *in vivo* fibrogenesis in rat liver. Results of this study show, that Smad7 prevents BDL dependent activation of HSC and liver fibrosis. Collagen I deposition and hydroxyproline content were significantly reduced in Smad7 overexpressing liver when compared with AdLacZ infected animals. Expression of α -SMA, a marker for HSC transdifferentiation, was strongly reduced in Smad7 rats indicating that, HSC remained in a quiescent stage. Furthermore, Smad7 was also useful to prevent progression of established fibrosis, when introduced to the animals one week after bile duct ligation. Thus, Smad7 has a similar effect on *in vivo* fibrogenesis as the truncated versions of T β RII, which have been previously used to treat BDL or DMN induced liver fibrosis in rats (George et al., 1999) (Nakamura et al., 2000). In contrast to the antifibrotic approaches using dominant negative T β RII in rat (Qi et al., 1999) (Nakamura et al., 2000) or a soluble T β RII in mice (Yata et al., 2002), in the present study it could not be find a significant downregulation of

clinical chemical parameters for liver damage (AST, ALT, bilirubin) in serum after delivery of Smad7. However, this discrepancy may depend more on using a different method of treatment, BDL versus DMN or CCl₄, than on the effect of Smad7 itself.

From *in vitro* data with primary cultured HSC, I conclude that Smad7 completely inhibits autocrine and paracrine TGF- β signaling in this cell type, leading to abrogation of Smad2/3 activation and subsequent downstream events. TGF- β induced phosphorylation of Smad2 and nuclear deposition of Smad2/3 and Smad4 were abolished in cells overexpressing Smad7. Accordingly, activity of a specific Smad3/4 promoter reporter gene construct was significantly reduced in cells infected with AdSmad7 when compared with LacZ control. The fact that Smad7 overexpressing HSC remain in a quiescent stage, confirms the important role of TGF- β during the early phase of activation. Collagen expression was significantly downregulated, but not completely inhibited in HSC infected with AdSmad7. Similar data were reported for culture activated HSC from Smad3 knock out mice, in which collagen expression was downregulated to 73% in comparison to cells from wt mice (Schnabl et al., 2001). The effect of Smad7 on collagen expression in primary cultured cells is more dramatic than Smad3 deficiency, indicating that pathways negatively regulated by Smad7 and not involving Smad3 are participating in this process, e.g. TGF- β signal transduction via other Smads.

Surprisingly, but in line with the studies from Smad3 knock out mice and a previous report describing abrogation of TGF- β signaling with a soluble T β RII (George et al, 1999), Smad7 did not decrease α -SMA expression in primary cultures of HSC as shown in the western blot analysis of total cell lysates, indicating that α -SMA expression itself is independent from TGF- β pathway. Interestingly, immunostaining of α -SMA in cultured cells showed abolished fiber organization in cells infected with AdSmad7. In line with these findings, recent review from Wells et al. showed that α -SMA expression is dependent from the matrix, on which HSC are cultivated. Cells cultured on Matrigel remain in quiescent stage when compared with cells cultivated on plastic due to many orders of magnitude lower elastic modulus of the Matrigel than tissue culture plastic. It was also shown that TGF- β signaling affects only stress fiber formation in primary cultured cells but not α -SMA expression itself (Wells et al., 2005).

In contrast, α -SMA was strongly downregulated *in vivo* in livers of Smad7 treated animals as shown in western blot analysis. These discrepancies between *in vivo* and *in vitro* data can be due to different environmental circumstances in which HSC are situated in these

approaches, underlying the importance of extracellular matrix composition, as mentioned before. Furthermore, these results are in line with the findings of George et al. (George et al., 1999), where α -SMA was strongly downregulated *in vivo* in livers of animals treated with a soluble T β RII. Interestingly, Smad3 knock out mice treated with CCl₄ did even express higher levels of α -SMA compared to wild type animals, suggesting that abrogation of Smad3 releases other cellular responses than overexpression of Smad7. Smad7 is a TGF- β intracellular antagonist, which interferes with phosphorylation of distinct Smads and is not specific for Smad3 activation (Massague et al., 1998). Also other not Smad dependent TGF- β pathways were shown to be affected by Smad7 (Lindert et al., 2005). Thus, Smad7 has broader inhibitory effect than Smad3 knock out. Therefore, in Smad3 knock out mice other Smad pathways may be used for TGF- β signaling in damaged liver.

One impact of Smad7, which may be important for „freezing“ HSC in a quiescent stage could be its influence on α -SMA organization. The typical prominent α -SMA fibers, which are observed in transdifferentiating HSC, are completely lost after overexpression of Smad7. This points to a possible role of TGF- β signal transduction for mobilization of actin cytoskeleton in HSC via signaling by small GTPases, as previously reported for PC-3U human prostate carcinoma cells (Edlund et al., 2002). In these cells, long term stimulation with TGF- β resulted in an assembly of stress fibers, a response, which required signaling via Cdc2 and RhoA as well as via Smad proteins and could be abrogated by inhibitors for these pathways. Furthermore, Cdc2 and RhoA, both were directly activated by TGF- β . Whether this pathway is functional in transdifferentiating HSC and whether Smad7 has inhibitory effects in this context will be an interesting topic for future studies. Furthermore, investigating α -SMA organization in Smad3 knock out mice would be useful to determine if TGF- β dependent Smad3 activation is necessary for fiber formation.

In summary, our data indicate that transient gene transfer by adenoviral vectors and expression of exogenous Smad7 in the liver may prevent BDL induced liver fibrosis, suggesting a therapeutic potential of Smad7. Furthermore, we could show that Smad7 is able to block activation of HSC in primary culture, which are the major fibrogenic cells in the liver. In line with the profibrogenic role of TGF- β , this seems to be based primarily on blocking paracrine and autocrine TGF- β signaling in these cells. Beyond this, it is possible that specific functions of Smad7, which are not yet determined, may be involved, particularly in regulating α -SMA assembly and fiber formation.

Although our approach to block profibrogenic effects of TGF- β by overexpressing Smad7 is effective in experimental models and targets activation of HSC, its efficacy and specificity needs to be improved. Adenoviral gene delivery systems are difficult to use in treatment of human disease due to strong immunogenicity of these viruses. They can infect many kinds of cells in different organs and the CMV promoter used in this study shows also no cell specific activity. This problem could be overcome by usage of modified adenoviruses, expressing Smad7 under the control of a HSC specific promoter. Currently, we do not know the (long term) consequences of Smad7 overexpression in hepatocytes. Overexpression of Smad7, especially in hepatocytes, may have tumorigenic potential and could lead to oncogenic transformation. Surprisingly, ongoing studies of our group show beneficial effects of targeting TGF- β signaling in hepatocytes on fibrogenesis. For this study transgenic mice with specific inducible Smad7 overexpression in hepatocytes in a CCl₄ fibrosis model were used. These findings indicate complicity of TGF- β action in liver fibrogenesis and open new opportunities to trigger this process. Moreover, more detailed future studies need to take into consideration that the effect of Smad7 is broad in that it may modulate signaling by different members of the TGF- β family, including BMPs and activins.

To better understand the role of Smad7 on activation of HSC Smad7-dependent expression profiling analysis was performed, resulting in identification of the inhibitor of differentiation 1 (Id1) gene. Following studies gave evidence that Id1 is a target gene of TGF- β signaling in HSC. Id1 exhibits functional properties that make it a central mediator of transdifferentiation of this cell type. Furthermore, underlying signaling events are elucidated, namely that TGF- β signaling through ALK1/Smad1, but not ALK5/Smad2/3, triggers Id1 expression in HSC. However, it can not be excluded that ALK5 kinase activity is a prerequisite for ALK1 signal transduction, as was shown to be the case in endothelial cells (Goumans et al., 2003).

Id proteins are important modulators of signaling pathways involved in development, cell cycle progression and tumorigenesis (Skider et al., 2003) (Swarbrick et al., 2004). They act as dominant negative antagonists of the basic helix-loop-helix (bHLH) family of transcription factors, which regulate differentiation of many cell types. Id proteins, among others associate with Ets transcription factors and the Rb family of tumor suppressor proteins and are downstream targets of TGF- β and BMP signaling (Ruzinova et al., 2003). Target genes for Id proteins have been identified largely based on the knowledge of promoters activated by bHLH-proteins. In embryo-fibroblasts from wild-type and Id1 knockout mice, a differential gene expression profiling system displayed targets involved in diverse biological

functions such as matrix remodeling, intracellular signaling pathways and angiogenesis (Ruzinova et al., 2003). In a TGF- β dependent global expression profiling with fibroblasts Id1 was found as early response gene, followed by upregulation of smooth muscle cell marker genes such as α - and γ -smooth muscle actin, basic calponin, smooth muscle myosin heavy chain and transgelin (SM22) (Chambers et al., 2003). In promoter regions of a number of smooth-muscle specific genes E-box sequences were found, which represent binding sites for bHLH transcription factors. Thus, our data indicate that upregulation of genes favoring a myofibroblast phenotype represents profibrogenic TGF- β effects in HSC that are provided by initiation of a cascade involving ALK1 and Smad1 activation and subsequent induction of Id1 expression, an important regulator of E-box sequences.

The function of Id1 in HSC is poorly understood. Mesenchymal cells have the propensity to transdifferentiate into smooth muscle α -actin positive contractile MFB during tissue repair and fibrogenesis in various organ systems. These cells are highly activated and synthesize ECM proteins and fibrogenic cytokines. MFB are the predominant fibroblast phenotype present within fibrotic lesions in a number of organs such as liver, lung, kidney and heart. The cellular conversion of mesenchymal cells to MFB during development, injury and disease is mediated by cytokines, with TGF- β being crucial. Nevertheless, molecular details underlying the profibrogenic TGF- β action in injured liver are not fully delineated. The finding that Id1 overexpression enhances transdifferentiation of HSC by means of polymerization of α -SMA, together with the observation that knock down of Id1 expression is sufficient to antagonize activation of HSC, suggests a central role for Id1 in this process. In most cell types, TGF- β binds and activates T β RII/ALK5 receptor complex which subsequently leads to phosphorylation of Smad2/3, while BMPs have been described to signal via ALK1/Smad1/5 (Miyazawa et al., 2002). In endothelial cells (ECs), TGF- β signaling is mediated via two different type I receptors with opposing effects. Activation of ALK5 leads to inhibition of migration and proliferation, whereas ALK1 signaling has the converse effects. From these findings it has been concluded that the activation state of ECs depends on the balance between both pathways (Goumans et al., 2002). Interestingly, although ALK1 directly antagonizes ALK5/Smad signaling in ECs, ALK5 kinase activity is necessary for optimal TGF- β /ALK1 signal transduction (Goumans et al., 2003). Furthermore, TGF- β /ALK1 signaling in ECs leads to Id1 gene expression (Goumans et al., 2003).

Previously published data of our group suggests that quiescent HSC display Smad2/3 activation, SBE-binding activity and stimulation of luciferase activity that is driven by a (CAGA)₉-MLP-luciferase reporter gene after TGF- β stimulation (Dooley et al., 2000)

(Dooley et al., 2001) (Lindert et al., 2005), indicating ALK5 activity. Further, quiescent cells do not migrate and are sensitive to TGF- β dependent inhibition of DNA synthesis. Inhibition of ALK5/Smad2/3 activation is blunting upregulation of ECM components, while it has no effect on α -SMA expression (Lindert et al., 2005). In contrast, phosphorylation of Smad1 (with induction of Id1) seems to be indispensable for upregulation and polymerization of α -SMA, the second typical feature of fibrogenesis.

In transdifferentiated HSC (MFB), the TGF- β signaling pathway is modulated and TGF- β has no longer an inhibitory effect on proliferation. Additionally, activated HSC lack ligand-dependent Smad2/3 phosphorylation or induction of Smad7 gene expression. Furthermore, transdifferentiated HSC display significant migration and proliferation activities. It is hypothesized that in HSC, a differential usage of TGF- β receptors ALK5 and ALK1 similar to that in endothelial cells exists. This assumption is supported by the findings that (i) ALK1 and ALK5 are expressed in HSC and (ii) ALK1/Smad1 signaling but not ALK5/Smad2/3 signaling induces Id1 expression in HSC. Increased expression and phosphorylation of Smad1 during activation of HSC has been reported previously (Shen et al., 2003), pointing to a significant role of Smad1/5/8 signaling during transdifferentiation of HSC. The existence of two TGF- β /Smad pathways in HSC, via ALK1 and ALK5, enables a careful fine-tuning of TGF- β -induced biological responses. The mechanism controlling signaling activity of ALK5 versus ALK1 in HSC is not clear and may depend on concentrations of active TGF- β and the amounts of the different receptors present on the cell surface within a given situation. This may trigger the cellular response in opposite directions, as was proposed for endothelial cells (Goumans et al., 2003) (Goumans et al., 2002) (Goumans et al., 2003). Thus, the counteractive interplay between ALK5 and ALK1 provides a more general, intricately regulated TGF- β -controlled switch, which subsequently may change the cellular fate and therefore may be an important prerequisite for HSC activation.

The accessory receptor endoglin may also be involved in targeting of TGF- β signaling. Endoglin expression was found to counteract the inhibitory effect of TGF- β on endothelial cell migration (Li et al., 1999). Ectopic expression of endoglin in cell lines modulates TGF- β signaling by favoring usage of ALK1 as receptor kinase (Letamendia et al., 1998). Furthermore, endothelial cells lacking endoglin are growth-arrested since TGF- β /ALK1 signaling is reduced and the TGF- β /ALK5 pathway is enhanced, indicating a pivotal role for endoglin in the balance of ALK1 and ALK5 signal transduction (Lebrin et al., 2004). In line with this, increased expression levels of endoglin were observed in transdifferentiating HSC, pointing to a yet unidentified pathway by which endoglin may mediate profibrogenic

responses in the liver. This assumption is supported by data showing endoglin exposed at the plasma membrane of activated HSC (Meurer et al., 2005).

In contrast to our findings and the results from ECs, Id proteins can also antagonize a mesenchymal phenotype. Thus, TGF- β induced transdifferentiation of NMuMG mammary epithelial cells to a fibroblastic phenotype is mediated via E2A proteins. In this cell type, TGF- β decreases Id1 expression, thereby increasing the amount of functional E2A and antagonizing transdifferentiation (Kondo et al., 2004). Another complex regulatory mechanism was described for Id2 and Id3 expression in the same cell type. Like Id1, both Id proteins display long term repression by TGF- β and sustained induction by BMP, whereupon a converse regulation of Id genes by different TGF- β family members is critical for proliferative and differentiation responses (Kowanetz et al., 2004). We conclude that the outcome of Id protein expression in a specific cell type is determined by the function of the diverse bHLH proteins that can interact with Id proteins.

Id1 protein level is upregulated during activation of HSC. Interestingly Id1 mRNA expression decreases during this process. This can be due to Id1 protein stabilization. Since Id proteins are short-lived and the degradation via COP9 signalosome was shown to play a pivotal role in controlling their cellular level (Berse et al., 2004), the Id1 degradation process during activation of HSC seems to be abrogated leading to accumulation of Id1 in these cells. Further studies will explain Id1 protein stabilization process in HSC. Moreover, it will be interesting to elucidate, which bHLH transcription factors are building not functional complexes with Id1, leading to target genes expression changes and transdifferentiation of HSC.

In conclusion, Smad7 prevents experimental induced fibrosis in rat BDL model. Collagen deposition as well as hydroxyproline content was reduced in rats treated with Smad7 when compared with LacZ control rats. α -SMA western blot implicates reduced activation of HSC in the tissue of treated rats. Moreover, TGF- β intracellular pathway in HSC was abolished after Smad7 overexpression, as shown by reduced Smad2/3 phosphorylation and inhibition of the activity of a specific promoter reporter construct. α -SMA fiber formation, an indicator of HSC transdifferentiation process, was abrogated in cells overexpressing Smad7.

Id1 was shown as a potent mediator of HSC transdifferentiation. Overexpression of Id1 abrogates beneficial effects of Smad7 on activation of HSC, leading to enhanced α -SMA fiber formation. TGF- β stimulation leads to Smad1 phosphorylation and Id1 expression. This process was abrogated by overexpression of siRNA for ALK1, indicating that ALK1/Smad1

pathway is involved in TGF- β dependent Id1 upregulation. Id1 was shown to be upregulated during activation of HSC, suggesting that Id1 may play a pivotal role during fibrogenesis and TGF- β pathway via ALK1/Smad1 may be involved in this process.

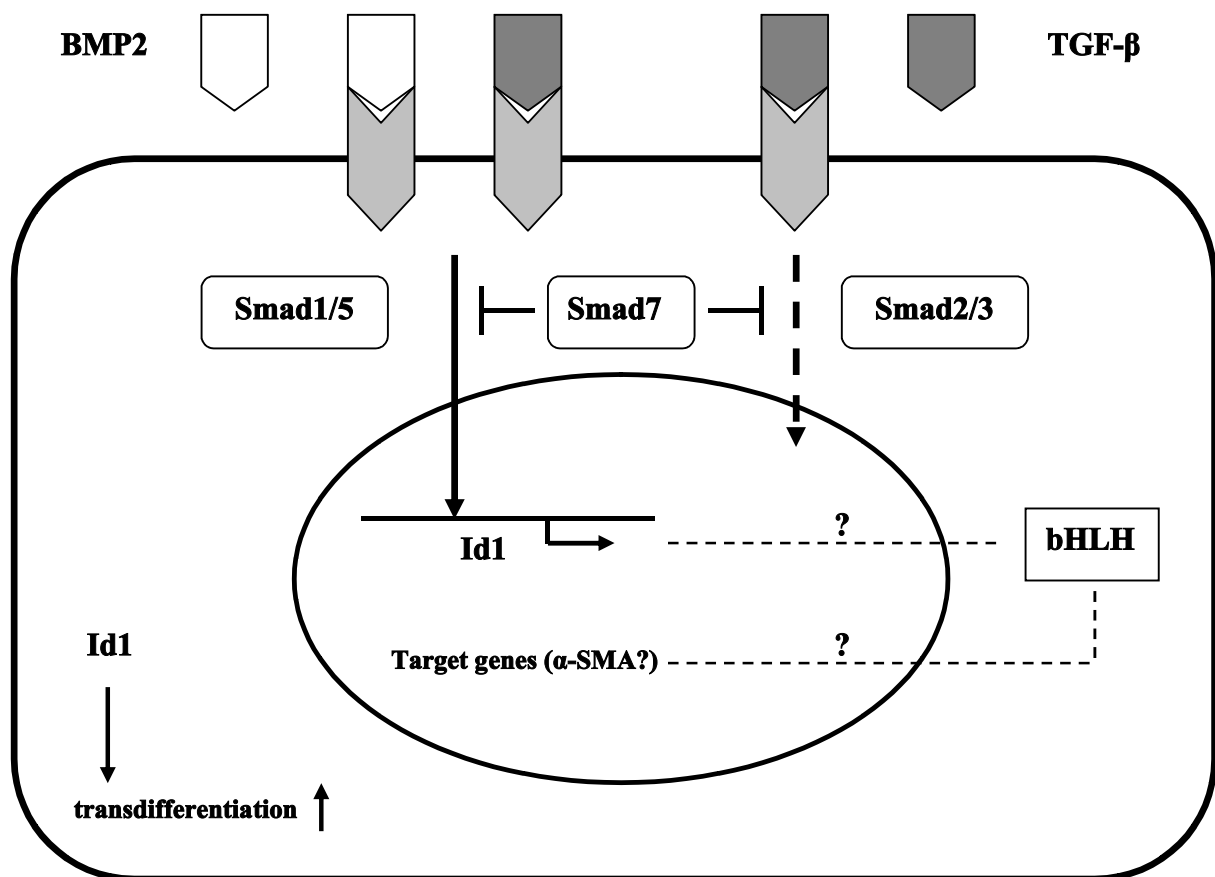


Fig. 25. Scheme of TGF- β family signaling in HSC. TGF- β /ALK1 and BMP2/ALK3 signaling lead to induction of Id1 expression in HSC, whereas TGF- β /ALK5 signaling targets different genes in the same cell type. Subsequently, Id1 enhances activation of HSC by increasing α -SMA fiber formation. Id1 interacting bHLH transcription factors and regulated target genes in HSC remain to be determined.

6. References

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7. Abbreviation

Ad	Adenovirus
ALK	activin like kinase
ALT	Alanine aminotransferase
AMH	Anti-Muellerian hormone
AMP	Adenosine-mono-phosphate
AP1	activator protein 1
AST	Aspartate aminotransferase
ATF3	Activating transcription factor 3
BDL	Bile duct ligation
bHLH	Basic-helix-loop-helix
BMP	Bone morphogenic protein
ca	constitutively active
CamKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CBP	CREB binding protein
CCl ₄	carbon tetrachloride
Cdc2	Cell division cycle 2
c-Jun	from Japanese ju-nana for 17
CMV	Cytomegalovirus
COP9	Constitutive photomorphogenesis complex
CREB	Cyclic AMP response element binding protein
c-Ski	Sloan-Kettering viral oncogene homolog
DMN	Dimethylnitrosamine
dn	Dominant negative
DTT	Dithiotreitol
ECM	Extracellular matrix
ECs	endothelial cells
Emc	Extra-macrochaetae protein
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-related protein kinase
Ets	erythroblast transformation specific
FAST1	Forkhead activin signal transducer-1
Fc	Antibody fragment c
FGF	Fibroblast growth factor
GDF	Growth and differentiation factor
HA	Hemagglutinin antigen
Hand	Heart-and neural crest derivatives-expressed protein
Hes	Hairy and enhancer of split
HHT	Hereditary hemorrhagic telangiectasia
HIF	Hypoxia inducible factor
HLH	Helix-loop-helix
HSC	Hepatic stellate cells
Id	Inhibitor of differentiation (Inhibitor of DNA binding)
IFN	Interferon
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IGHM	Immunoglobulin heavy constant mu
LAP	Latency-associated peptide
Luc	Luciferase
Mad	mitotic arrest deficient protein
MAP	Mitogen-activated protein
MFB	Myofibroblast
MH	Mad homology domain
MIDA	Mouse Id Associate protein
MIS	Muellerian inhibiting substance
MLP	Adenoviral major late promoter
MMP	Matrix metalloproteinase
MRF	Myogenic regulatory factor

Myc	Myelocytomatosis oncogene
Myf	Myogenic factor
MyoD	Myoblast determination protein
NDF	Neu differentiation factor
NeuroD	Neurogenic differentiation protein
NF- κ B	Nuclear factor-kappaB
NTN	Nephrotoxic serum nephritis
PAS	Per (period circadian protein), Arnt (Ah receptor nuclear translocator protein), Sim (single-minded protein) - domain
PKC	Protein kinase C
Rb	Retinoblastoma
RGS	Regulator of G Protein Signaling
RhoA	Ras homolog gene family member A
rS6	30S ribosomal protein S6
SBE	Smad binding element
SCL	Stem cell leukemia
Sim	Single-minded
siRNA	small interfering RNA
SMA	Smooth muscle actin
Smurf	Smad ubiquitination regulatory factor 1
SnoN	Ski-related novel gene
Sp1	specificity protein 1
SREBP	Sterol regulatory element-binding protein 1
SV40	Simian Virus 40
TaI	T-cell acute lymphocytic leukemia-1 protein homolog
TFE3	Transcription factor binding to IGHM enhancer 3
TGF- β	Transforming Factor beta
TLP	Trap-1-like-protein
TM	transmembrane
TSP	Thrombospondin
T β R	TGF- β receptor
β -gal	beta-galactosidase
γ -GT	gamma-glutamyl-transferase

8. Curriculum Vitae

Name: Eliza Wiercinska
Date and place of birth: 04.11.1977 in Gdansk (Poland)
Address: Steenstraat 43, 2312 BV Leiden, the Netherlands
Nationality: polish

Education:

1984-1985 First class of primary school (Aachen, Germany)
1985-1996 Primary and secondary school (Gdansk, Poland)
1996-2001 Studies on the Technical University of Gdansk, Faculty of Chemistry, branch of study: Biotechnology (Gdansk, Poland)
26. Juni 2001 Master of Science degree

Professional life:

Sep.1998 - June 2001 Training and Master of Science thesis at the Inst. of Microbiology on the Technical University of Gdansk
Aug. 2001 - July 2003 Scientific employee at the Inst. of Clinical Chemistry and Pathobiochemistry, University Hospital, RWTH-Aachen, Germany
Aug. 2003 - July 2003 Heinz-Breuer-Research-Fellowship for Clinical Chemistry founded by Pathobiochemistry and Molecular Diagnostic Foundation
Aug. 2004 – Oct. 2005 Scientific employee at the Medical Department II, Division of Molecular Alcohol Research in the Gastroenterology, University Hospital Mannheim, University Heidelberg
Since Oct. 2005 Scientific employee at the Molecular Cell Biology Department, Leiden University Medical Center, Leiden University, The Netherlands

Affiliations:

Dec.1999-Oct. 2001	Member of Polish Association of Biotechnology Students
Jan. 2000-Jan. 2001	Member of the Chemical Association at the Technical University of Gdansk
Jan. 2000-Oct. 2001	Member and president of the Association for Biotechnology Students at the Technical University of Gdansk
Aug. 2000-Oct. 2001	President of the Polish Association of Biotechnology Students

Awards:

Aug. 2000	Award for Young Scientist on the 36. Annual Meeting of the Polish Biochemistry Association
Apr. 2002	Bursary for Young Scientist at the Annual Meeting of European Association for the Study of the Liver (EASL)
Jan. 2004	Poster award at the 20. Annual Meeting of the German Association for the Study of the Liver (GASL)

Publications:

Dooley S, Hamzavi J, Breitkopf K, Wiercinska E, Said HM, Lorenzen J, Ten Dijke P, Gressner AM. Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology*. 2003 Jul;125(1):178-91.

Lindert S, Wickert L, Sawitza I, Wiercinska E, Gressner AM, Dooley S, Breitkopf K. Transdifferentiation-dependent expression of alpha-SMA in hepatic stellate cells does not involve TGF-beta pathways leading to coinduction of collagen type I and thrombospondin-2. *Matrix Biol*. 2005 May;24(3):198-207.

Breitkopf K, Haas S, Wiercinska E, Singer MV, Dooley S. Anti-TGF- β strategies for the treatment of chronic liver disease. Review. *Alcoholism: Clinical and Experimental Research* (in press)

Wiercinska E, Breitkopf K, Wickert L, Denecke B, Said H, Hamzavi J, Gressner AM, Thorikay M, ten Dijke P, Mertens PR, Dooley S. Id1 is a critical mediator in TGF- β -induced transdifferentiation of rat hepatic stellate cells. (submitted)

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