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CSRP2, *TIMP-1*, and *SM22α* promoter fragments direct hepatic stellate cell-specific transgene expression *in vitro*, but not *in vivo*

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Abstract: *Background/Aims:* The activation of hepatic stellate cells (HSC) and their transdifferentiation into myofibroblasts (MFB) is the key step for development of liver fibrosis. Over the past several years, significant progress has been made in the understanding of the critical pathways involved in cells undergoing activation. Cellular activation in the course of transdifferentiation involves, among other biochemical modifications, functionally relevant changes in the control of gene expression. These include the up-regulation of transcription factors, different extracellular matrix proteins, cell adhesion molecules, smooth muscle specific genes, and proteins involved in matrix remodelling, or cytoskeletal organization. The corresponding regulatory elements of these genes have afforded us the opportunity to express transgenes with antifibrotic potential in a cell type- and/or transdifferentiation-dependent manner. *Methods:* In the present study, we have tested several promoters for their ability to mediate cell-specific expression, including those for *CSRP2*, *SM22α*, and *TIMP-1* (*CSRP2*, gene encoding the LIM domain protein CRP2; *SM22α*, smooth muscle-specific gene encoding a 22-kDa protein; *TIMP-1*, gene encoding the tissue inhibitor of metalloproteinases-1), which in liver are specifically expressed in HSC or become strongly activated during the acute remodelling into MFB. We constructed adenoviral reporter vectors in which relevant portions of the promoters were fused to the green fluorescent protein. *Results and Conclusion:* Our experiments demonstrate that each of these promoters is sufficient to achieve strong or partially selective expression *in vitro* but none is able to direct a specific or inducible expression of transgenes in HSC/MFB *in vivo*.

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Key words: adenovirus (Ad) – cysteine- and glycine-rich protein 2 – gene transfer – hepatic stellate cell – liver fibrosis – myofibroblast – smooth muscle specific gene – tissue inhibitor of metalloproteinases-1

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Hepatic stellate cells (HSC) play a pivotal role in liver fibrogenesis. They are located in the sub-endothelial space of Disse and store retinoids (1). During conditions of chronic liver injury as well as after prolonged culturing on uncoated plastic, they transit from a quiescent to a proliferative extracellular matrix producing myofibroblast (MFB)-like cell type (2). This process of transdifferentiation is the key event in development and maintenance of liver fibrosis. Once activated, HSC significantly increase the expression of transforming growth factor-β (TGF-β) acting as an autocrine positive regulator for assembly of extracellular matrix. This profibrogenic cytokine directly increases the synthesis of collagens resulting in perpetuation of liver fibrosis. Additionally, transdifferentiation into MFB involves an increased expression of the intracellular microfilament protein α-smooth muscle actin (α-SMA) and the tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibiting matrix degradation by matrix metalloproteinases. For this reason, the major players of fibrogenesis, i.e. activated HSC and

Abbreviations: Ad5, adenovirus (serotype 5); bp(s), basepair(s); *CSRP2*, gene encoding the LIM domain protein CRP2; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HSC, hepatic stellate cell(s); Luc/luc, luciferase; MFB, myofibroblast(s); pfu, plaque-forming units; α-SMA, α-smooth muscle actin; SMC, smooth muscle cell; *SM22α*, smooth muscle-specific gene encoding a 22-kDa protein; *TIMP-1*, gene encoding the tissue inhibitor of metalloproteinases-1; TGF-β, transforming growth factor-β; utr, untranslated region.

TGF-β are targets for an antifibrotic gene therapy in liver. In experimental models of liver fibrogenesis, decreased contents of extracellular matrix proteins in HSC/MFB were observed after administration of a soluble type II TGF-β receptor, other TGF-β scavenger proteins, TGF-β1 antisense RNA, or by blocking TGF-β function by expressing downstream inhibitors of the intracellular TGF-β signalling pathways (3–5). However, these intervention strategies are able to suppress TGF-β function *per se*, but are not feasible to specifically antagonize profibrotic events in HSC.

A well-established method to target transgene expression and to achieve selective expression in tissues, cell types or differentiation states is the delivery of transgenes under specific promoters. Gene targeting to activated HSC is potentially possible by promoters up-regulated in the course of transdifferentiation or expressing transgenes under transcriptional control of regulatory units exclusively expressed in this liver cell subpopulation. *TIMP-1* gene expression is increased in culture-activated rat HSC, if they have been cultured for longer than 3 days (6). The proximal *TIMP-1* gene promoter containing base pair (bp) −255 to bp +96 is sufficient to drive transdifferentiation-dependent expression in HSC (7). Comparably, the promoter of α-SMA, a marker protein for smooth muscle cell (SMC) differentiation, is strongly stimulated during the activation process. Previous studies have suggested that SM22α, a 22-kDa protein (also denoted as transgelin) with structural homology to the thin filament myofibrillar regulatory protein calponin, is expressed exclusively in smooth muscle-containing tissues (8–10). It was previously found that *SM22α* gene expression is responsive to TGF-β in the murine neural crest-derived TC-1S cell line, in multipotent mouse embryonic 10T1/2 cells, and in human diploid fibroblasts (11–13). In liver, we recently demonstrated that endogenous *SM22α* is exclusively expressed by stellate cells, while no transcripts are detectable in hepatocytes, sinusoidal endothelial cells (SEC), and Kupffer cells (KC) (14). Another gene specifically expressed in HSC is the cysteine- and glycine-rich LIM domain protein gene (*CSRP2*) encoding CRP2. During the initiation phase of HSC-activation *CSRP2* is transcriptionally activated and suppressed during later stages of transdifferentiation (14, 15).

The aim of this study was to evaluate potential candidate promoters for their capability to direct HSC-specific expression *in vitro* and *in vivo*. Therefore, we constructed adenoviral vectors expressing the green fluorescent protein (GFP) under control of the *CSRP2*, *SM22α*, and *TIMP-1* promoters, respectively. We found that the different promoters are sufficient to mediate targeted transgene expression in cultured liver cells. However, any cell type specificity was not observed *in vivo*.

Material and methods

Isolation and culture of liver cells

Male Sprague–Dawley rats had free access to altromin chow and water. HSC, KC, and SEC were isolated by liver perfusion with pronase and collagenase (16, 17). The resulting cell suspension was filtered through a nylon mesh, centrifuged and washed in ice-cold Hanks buffered standard saline (PAA Laboratories GmbH, Linz, Austria) containing 0.25% (w/v) BSA. KC and SEC were collected by centrifugal elutriation in a JE-5.0-type rotor equipped with a standard separation chamber in a Beckman centrifuge (Avanti™ J-20; Beckman Instruments Inc, Palo Alto, CA, USA). HSC were further purified by a density gradient centrifugation with 8.25% (w/v) Nycodenz® (Nycomed Pharma AS, Oslo, Norway) as described in detail elsewhere (18, 19). Purified HSC were seeded in Dulbecco's modified Eagle medium (DMEM; Bio Whittaker Europe, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (FCS; Seromed, Biochrom KG, Berlin, Germany), and 4 mM L-glutamine (ICN Biomedicals Inc., Aurora, OH). Additionally, the culture medium was supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml). MFB were prepared from HSC by trypsinisation at day 7 of primary culture. Rat hepatocytes were isolated following a slightly modified version of Seglen's collagenase method (20). Briefly, animals were anaesthetized and the liver was preperfused *in situ via* the portal vein followed by recirculating perfusion *ex situ*. Thereafter, the liver was perfused with collagenase and the capsule was gently removed. The tissue was dissected under constant swirling and the obtained cell suspension was subsequently filtered through a nylon mesh, centrifuged, washed three times, and seeded in DMEM supplemented with 10% FCS, 4 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.02 U/ml bovine pancreas insulin. The study as presented was proved by the local committee for care and use of laboratory animals, and was performed according to strict governmental and international guidelines on animal experimentation.

RNA isolation and Northern blot analysis

Isolation and Northern blot analysis of total cellular RNA was carried out as described

previously (17, 21). Purified RNA was resuspended in water and the concentration was determined by UV absorbance. Equal amounts of total RNA were separated by electrophoresis on a 1.2% (w/v) denaturing agarose gel, transferred to a Hybond-N membrane (Amersham Pharmacia, Freiburg, Germany), and fixed by baking for 2 h at 80 °C. Blots were hybridized with [α-^{32}P]-dCTP-labeled multiprimed probes (Amersham Pharmacia), washed and exposed to Kodak X-OMAT AR films at -80 °C using intensifying screens. As an internal standard for equal gel loading the blots were rehybridized with a cDNA specific for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Twenty microgram whole liver lysate or 10 µg cell lysate per lane was resolved on a 12% (w/v) Tris-glycine gel (Novex, Groningen, The Netherlands) by SDS-PAGE under reducing conditions. For immunoblotting, proteins were electroblotted onto a Protran membrane (Schleicher & Schuell, Dassel, Germany) according to standard procedures. The membrane was then blocked 1.5 h with 1 × Rotiblock (Roth, Karlsruhe, Germany) and incubated 1 h with an 1:1000-diluted mixture of two mouse monoclonal antibodies (clones 7.7 and 13.1) raised against GFP (Roche, Mannheim, Germany). The primary antibodies were visualized using 1:5000-diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotech., Santa Cruz, CA) for 1 h and the supersignal chemiluminescent substrate (Pierce, Rockford, IL). β-actin was detected as a loading control by a 1 h incubation with a 1:10000-diluted monoclonal mouse antibody (clone AC-15) (Sigma, Taufkirchen, Germany).

Construction of recombinant adenoviruses

The construction of the recombinant replication-deficient reporter adenovirus (serotype 5) (Ad5)-cytomegalovirus (*CMV*)-GFP has been described previously (22). For construction of the adenoviral shuttle vector pΔE1sp1A-*CSRP2*-GFP the blunted 871-bp *Stu*I/*Xho*II fragment of clone ICRFP700-M03112Q06 (23) was cloned into the *Nhe*I/*Asn*I opened and Klenow filled-in vector pEGFP-C1 (BD Biosciences, Clontech, Heidelberg, Germany) harboring the GFP. From this construct, the blunted *Ppu*10I/*Ssp*I *CSRP2* promoter/GFP-cassette was transferred into the filled-in *Eco*RI site of shuttle vector pΔE1sp1A. For cloning of the shuttle vector pΔE1sp1A-*SM22*α-GFP a 1690-bp *Bam*HI/*Bsa*I fragment containing the *SM22*α promoter and parts of the 5′-untranslated region (5′ utr) was released from a clone harboring a 5.2-kbp *Bam*HI fragment containing the 5′ end of the rat *SM22*α gene (24). This fragment was filled-in by Klenow DNA polymerase and cloned into the *Asn*I/*Nhe*I digested and filled-in vector pEGFP-C1 exchanging the *CMV* promoter. Then the *SM22*α-GFP cassette was released with *Ppu*10I/*Ssp*I and cloned blunt ended into the *Eco*RI site of pΔE1sp1A. To generate shuttle vector pΔE1sp1A-*TIMP-1*-GFP a *Pst*I/*Nco*I fragment of reporter clone *TIMP-1*-CAT (25) containing 1012 bp of human *TIMP-1* gene promoter and part of the 5′ utr was cloned into vector pUCBM20 (Roche Diagnostics, Mannheim, Germany). The 1125-bp *Hinc*II/*Hind*III fragment of this vector was then ligated into pΔE1sp1A, which was cut by *Nhe*I, filled-in by Klenow, and digested with *Hind*III. The 1108-bp GFP encoding sequence was cut with *Xba*I/*Cla*I and inserted behind the promoter. The integrity of all cloning boundaries was verified by sequencing and the integration of transgenic sequences into the Ad5-backbone vector pJM17 (26) was performed by *in vitro* homologous recombination in the human embryo kidney cell line 293 as described in detail elsewhere (22).

Construction of a SM22α-Luciferase (Luc) reporter gene vector

A 1570 bp fragment including the *SM22*α promoter and the 5′ utr was amplified by PCR using vector pΔE1sp1A-*SM22*α-GFP as template. The forward primer (5′-CCC AAG CTT GGG CCT TCA GAT GCC ACG AGG AG-3′) and the reverse primer (5′-CTA GCT AGC TAG AGC AAG CTA GAG GAG ACT GGA-3′) introduced additional restriction sites for *Hind*III and *Nhe*I, respectively. The resulting PCR fragment was ligated into vector pGEM-T easy (Promega, Mannheim, Germany) and subsequently transferred into vector pBluescript II KS+ (Stratagene, La Jolla, CA). The resulting construct contained the *SM22*α promoter fragment and the 5′ utr within several restriction sites. A *Kpn*I/*Nhe*I digestion led to the formation of a 1618 bp promoter/5′ utr fragment, which was ligated into the *Kpn*I/*Nhe*I opened reporter gene vector pGL3 Basic (Promega) generating vector *SM22*α-Luc.

Transfections and luc assays

HepG2 cells and a rat cirrhotic fat storing cell (CFSC) line (27) were plated onto six-well plates, grown for ~48 h to 70–80% confluency and cotransfected with 1 µg *SM22*α-Luc reporter gene vector and 40 ng control vector pRL-TK (Promega)

using the FuGENE™ 6 transfection reagent (Roche). Twenty hours later, the cells of one well were split into four wells of a 24-well plate in DMEM containing 0.5% FCS. Stimulation occurred after cell adhesion with indicated concentrations of recombinant human TGF-β1 (TEBU, Frankfurt, Germany) for 24 h. The luc assay was executed with the Dual-luciferax™ Reporter Assay System (Promega) using 20 µl of cell lysates. Luc activities were measured with a luminometer 1450 Microbeta Wallac Jet (Wallac, Turku, Finnland). The normalized firefly luc data are presented as an average of three independent experiments performed in duplicate.

Adenoviral infection of cultured liver cells

For adenoviral infection of rat liver cell subpopulations 3×10^5 cells were seeded in six-well dishes. Rat HSC/MFB were infected at day 2 of primary culture with 2×10^8 plaque-forming units (pfu)/ml Ad5-*CMV*-GFP, Ad5-*SM22α*-GFP, Ad5-*TIMP-1*-GFP, or Ad5-*CSRP2*-GFP in DMEM containing 5% heat inactivated FCS and 4 mM L-glutamine for 24 h. Two days after infection the relative GFP expression was monitored by fluorescence microscopy. Rat hepatocytes were infected 1 day after primary culture as described above and GFP expression was detected 24 h later.

Administration of adenoviruses to animals

Two hundred microlitres of saline, Ad5-*CMV*-GFP, Ad5-*SM22α*-GFP, Ad5-*TIMP-1*-GFP, or Ad5-*CSRP2*-GFP (1×10^9 pfu total) were injected into the tail vein of male Sprague–Dawley rats, weighing about 200 g. Fibrosis was induced 24 h after infection with bile duct ligation. At day 6 the rats were sacrificed and liver pieces were either fixed with 4% (w/v) buffered paraformaldehyde for immunohistological examination or homogenized for SDS-PAGE with an Ultra Turrax in 2 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 2.5 mM EDTA, 2% NP-40 (v/v), 0.1% SDS (w/v), 0.5% deoxycholic acid (w/v), 1:500 protease inhibitor cocktail (Sigma), 1:100 phosphatase inhibitor cocktail 2 (Sigma)].

Immunohistochemistry

Fixed liver pieces of Ad5-*CMV*-GFP-, Ad5-*CSRP2*-GFP-, or mock-infected rats were embedded with paraffin. Sections of 1.5 µm thickness were blocked against endogenous peroxidases using 3% H_2O_2. GFP was detected with a 1:100 diluted polyclonal rabbit anti-GFP antibody (Santa Cruz Biotech.) and a secondary biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), diluted 300-fold, followed by treatment with an avidin-conjugated peroxidase (Vectastain ABC-Elite Kit, Vector Laboratories). Peroxidase activity was detected with diaminobenzidine (DAKO, Hamburg, Germany) and the tissue sections were briefly counterstained with methyl green.

Statistical analysis

Results are presented as the mean of three independent experiments ($\pm$ SD). Statistical analysis was performed with a Student's *T*-test and differences were considered significant (*) or highly significant (**) at $P<0.05$ or $P<0.01$, respectively.

Results

Cellular selectivity of the *CSRP2*, *SM22α*, and *TIMP-1* promoters *in vitro*

In liver, endogenous *CSRP2* and *SM22α* mRNA are exclusively expressed by HSC/MFB, while no transcripts are detectable in hepatocytes, SEC, and KC (Fig. 1A). The expression of the *TIMP-1* in HSC is strongly increased in the course of cellular activation (Fig. 1B). The finding that the expression of *CSRP2* and *SM22α* is specific for HSC/MFB and that *TIMP-1* expression is strongly correlated to cellular activation of HSC, turns out the respective promoters as potential candidates for therapeutic interventions allowing specific targeting to this liver cell subpopulation. Previous studies in other SMC have suggested that *CSRP2* and *SM22α* are expressed solely in smooth muscle-containing tissues and may be one of the earliest markers of the SMC lineage (9, 28, 29). Therefore, both genes and their encoded proteins might be involved in ongoing transdifferentiation and fibrogenesis most likely by influencing the cytoskeletal reorganizations and phenotypic plasticity observed during the transdifferentiation process (9, 29). Notably, SM22α in HSC/MFB is also localized to the actin-containing filamentous network (data not shown), essentially, the location where CRP2 was found in vascular SMC (30).

To test if the promoters of *CSRP2*, *TIMP-1*, and *SM22α* alone are sufficient to direct transgene synthesis in HSC/MFB, we constructed reporters in which relevant portions were fused to GFP. Because cultured HSC/MFB are almost refractory to conventional gene transfer methods, we cloned the corresponding fusions into an adenoviral expression system (Fig. 2). As a positive control we used an adenovirus harboring

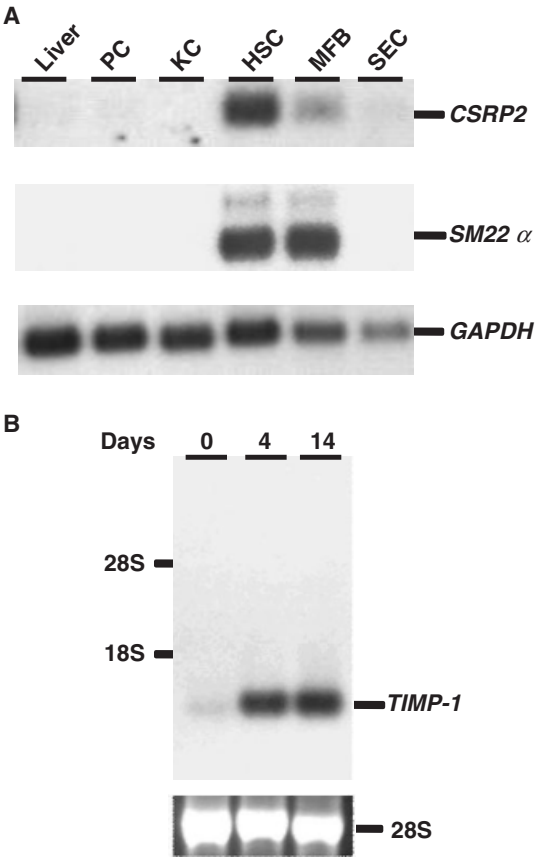

Fig. 1. Identification of HSC/MFB marker genes. (A) Expression of *CSRP2* and *SM22α* in isolated liver cell subpopulations. Total RNA of whole liver, hepatocytes (PC), Kupffer cells (KC), HSC, MFB and sinusoidal endothelial cells (SEC) was subsequently analyzed by Northern blot using cDNA probes specific for *CSRP2*, *SM22α*, and *GAPDH*. (B) Northern blot analysis of *TIMP-1* expression in HSC/MFB. Ten microgram aliquots of total RNAs from cultured HSC/MFB at days 0, 4, and 14 were hybridized with a *TIMP-1*-specific cDNA probe. As a loading control the 28S ethidium bromide staining was taken. HSC, hepatic stellate cells; MFB, myofibroblasts; *CSRP2*, gene encoding the LIM domain protein CRP2; *SM22α*, smooth muscle-specific gene encoding a 22-kDa protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *TIMP-1*, gene encoding the tissue inhibitor of metalloproteinases-1.

the GFP under transcriptional control of the constitutive *CMV* promoter. We used these reporter constructs to examine the transcriptional potency of the different promoters in cultured HSC, MFB, and hepatocytes. As expected, the Ad5-*CMV*-GFP-infected cells showed a strong, cell type-independent reporter gene expression detected by phase-contrast microscopy or Western blot analysis, respectively (Figs 3 and 4). In HSC all promoter fragments were able to direct GFP expression (Figs 3 and 4, upper panels), but only the *SM22α* promoter fragment was active in HSC and MFB (Figs 3 and 4, middle panels). Further, the *CSRP2* and *TIMP-1* promoter fragments were inactive in hepatocytes (Figs 3 and 4, lower panels). Consequently, the *CSRP2* promoter-directed reporter gene expression was in agreement with the observed endogenous *CSRP2* gene expression, whereas the activity of the *TIMP-1* promoter fragment differed from the endogenous expression pattern. Unexpectedly, we observed a low-expression activity of the *SM22α* promoter fragment in hepatocytes, which does not correspond to the endogenous *SM22α* gene expression in these cells (Figs 3 and 4, lower panels).

In several cell lines, the *SM22α* gene promoter is inducible by TGF-β (11–13), which is also a main effector in liver fibrogenesis and strongly induced upon culturing in hepatocytes. Thus, we tested if the unexpected activity of the *SM22α* reporter is due to an activation of this promoter by TGF-β1 in cultured cells. To perform our experiments more quantitatively, we fused the *SM22α* promoter to the luc reporter gene (Fig. 5A) and transfected HepG2 cells and an established rat CFSC line. In these cells, TGF-β1 was indeed able to increase the luc activity significantly (Figs 5B and C) showing that our promoter fragment is senstive for TGF-β1.

CSRP2, *TIMP-1*, and *SM22α* promoter regulated gene expression *in vivo*

To prove the transcriptional activity of our promoter reporters *in vivo*, we infected Sprague–Dawley rats *via* tail vein with the corresponding adenoviruses or saline as control. Six days after infection the promoter activities were estimated by the GFP expressed in whole liver lysates. In this Western blot analysis, all three promoters were able to direct the synthesis of GFP *in vivo* (Fig. 6A). In agreement with our *in vitro* data the amounts of protein were much less than those detected in livers infected with the constitutive active *CMV* promoter construct. We next examined if the activity of the promoter fragments is influenced by bile duct ligation, an established experimental model for liver injury and fibrosis. In injured livers, the expression of all reporters was significantly reduced (data not shown), and even the constitutive *CMV* promoter-directed GFP expression was decreased (Fig. 6B).

To detect the sources of GFP expression in the infected rats, hepatocytes of Ad5-*CMV*-GFP and Ad5-*SM22α*-GFP-treated animals were isolated. We observed a relevant GFP expression in freshly isolated hepatocytes, which was decreased in cells cultured for 2 days (Fig. 7A). In HSC there was only a low expression in Ad5-*CMV*-GFP-infected animals observable (data not shown). Comparable results were obtained by immunostaining of liver sections from Ad5-*CMV*-GFP- or Ad5-*CSRP2*-GFP-treated rats (Fig. 7B). Liver

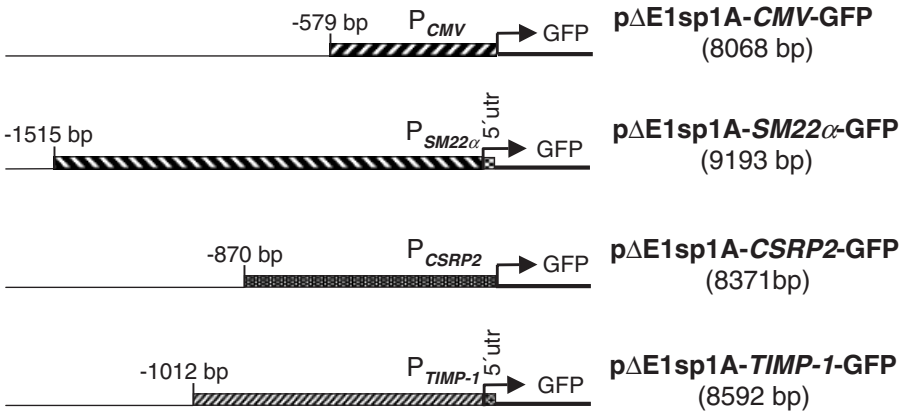

Fig. 2. Schema of adenoviral reporter gene vectors expressing GFP under transcriptional control of different promoters. The lengths (bp) of the promoter fragments and shuttle vectors are given. GFP, green fluorescent protein; bp, base pairs; P_{CMV}, promoter of the human cytomegalovirus immediate-early gene I; $P_{SM22}\alpha$, promoter of the rat *SM22*α gene; P_{CSRP2}, promoter of the human *CSRP2* gene; $P_{TIMP\text{-}1}$, promoter of the human *TIMP-1* gene; 5′ utr, 5′-untranslated region.

sections of animals infected with the *CMV*, or *CSRP2*, promoter reporters contained GFP-positive cells, which were identified as hepatocytes. In this analysis, the overall *CSRP2* promoter activity seems to be weaker than the *CMV* directed one and no cellular specificity was observed.

Discussion

Independent of the etiology of liver injury, HSC activation and transdifferentiation into MFB is the key step in liver fibrogenesis. Characteristic features of activated HSC are the stimulation of proliferation, matrix gene expression, and the acquisition of a contractile phenotype. Many of the morphological and metabolic changes, associated with the transition of these cells *in vivo*, are also observed with HSC grown on uncoated plastic, a widely accepted model for studying fibrogenesis at the cellular and molecular level. In our study, we used culture-activated HSC to examine the transcriptional potency and specificity of promoter fragments from genes that are exclusively expressed in HSC or become strongly activated during the acute remodelling into MFB. In normal liver, endogenous expression of *CSRP2* and *SM22*α genes is restricted to HSC/MFB, while *TIMP-1* activity is increased during transdifferentiation from HSC into MFB. *CSRP2*, encoding the cysteine- and glycine-rich LIM domain protein CRP2, was originally identified as a gene suppressed during cellular transformation and later found to be preferentially expressed in aortic SMC (31, 32). *SM22*α was shown to be expressed at high levels in differentiated smooth muscle tissue, but does not appear to be activated in other tissues (33). The demonstration that in liver both, *CSRP2* and *SM22*α, are expressed in HSC appears not to be contradictory to the suggested neural/neuroendocrine origin of these cells, because SMC is supposed to originate also from the neural crest.

To elucidate whether the corresponding regulatory elements of *CSRP2*, *SM22*α, and *TIMP-1* allow transcriptional targeting to HSC in a cell type- and/or transdifferentiation-dependent manner, we fused the GFP gene to these promoters originally cloned by different experimental strategies (23, 24, 34). These artificial fusions cloned in an adenoviral system allowed us to characterize the promoter activities in liver cells *in vitro* and *in vivo*.

The 1012-bp *TIMP-1* promoter fragment containing the entire non-coding exon 1 and part of intron 1 was tested for its ability to regulate transdifferentiation-dependent expression in HSC/MFB. The regulatory region include *cis*-acting elements like a serum response element (SRE), GC boxes, an AP1 site, and an upstream TIMP-1 element (UTE-1) necessary for TGF-β-, IL-6-, and OSM-induced *TIMP-1* expression during transdifferentiation (7, 25, 34–37). A minimal *TIMP-1* promoter containing these elements was found to direct transdifferentiation-dependent expression in HSC/MFB (7, 37). When we transfected HepG2 cells with a luc reporter gene driven by the 1012-bp *TIMP-1* promoter fragment, we found a 1.5- and 1.8-fold induction by TGF-β1 and OSM, respectively (data not shown). In CFSC the *TIMP-1* promoter fragment was more active than the *SM22*α promoter, but not inducible by TGF-β (data not shown). Infected HSC showed a moderate GFP expression, while the activity of the *TIMP-1* promoter was suppressed in MFB. One possible

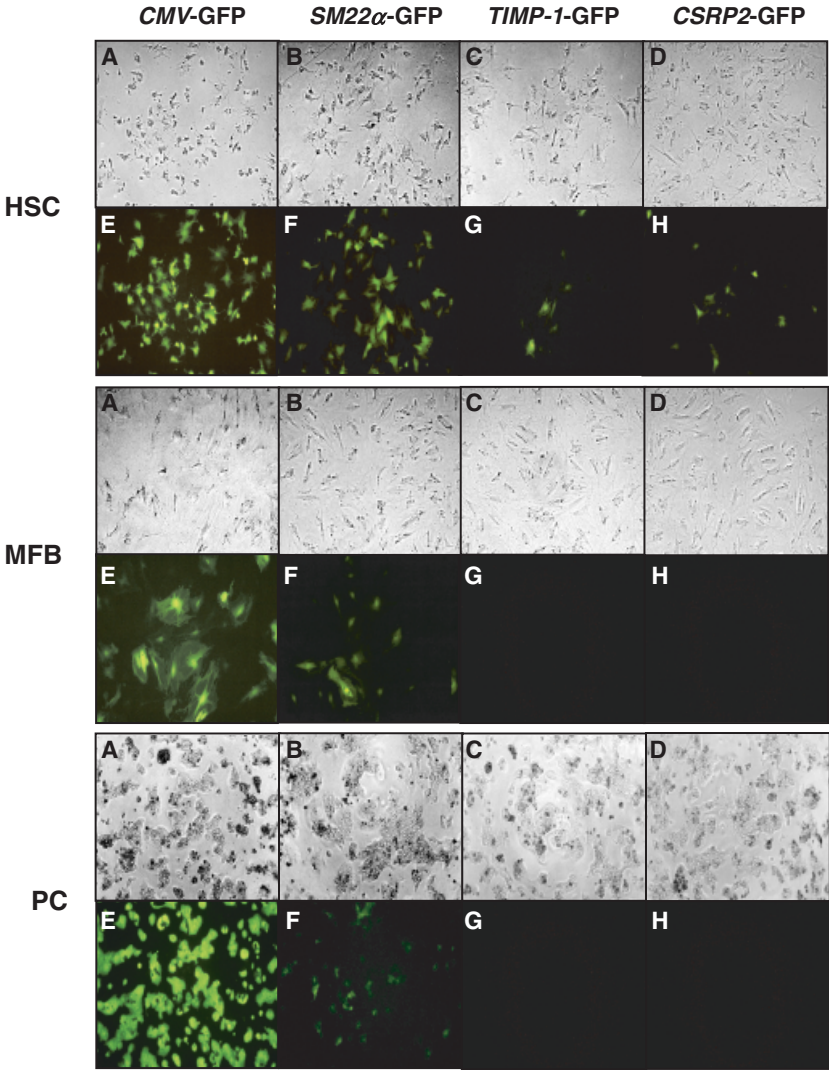

Fig. 3. GFP expression directed by *CSRP2*-, *SM22*α-, and *TIMP-1* promoters in infected liver cell subpopulations. Isolated rat HSC (upper panel), rat MFB (middle panel) and rat hepatocytes (PC) (lower panel) were infected with Ad5-*CMV*-GFP, Ad5-*SM22*α-GFP, Ad5-*TIMP-1*-GFP, or Ad5-*CSRP2*-GFP. Representative phase-contrast microscopy (A–D) and fluorescence microscopy (E–H) 48 h after adenoviral infection are shown. GFP, green fluorescent protein; *CSRP2*, gene encoding the LIM domain protein CRP2; *SM22*α, smooth muscle-specific gene encoding a 22-kDa protein; *TIMP-1*, gene encoding the tissue inhibitor of metalloproteinases-1; HSC, hepatic stellate cells; MFB, myofibroblasts; Ad5, adenovirus (serotype 5); *CMV*, cytomegalovirus.

explanation for this discrepancy might be the elongated size of our promoter fragment compared to the minimal promoter. Previously, it was demonstrated that a strong transcriptional activity is observable in activated HSC with promoter fragments of 255–162 bp in length. Fragments up to 736 bp showed a moderate but decreased activity (7, 34). In this study, we used a 1012 bp fragment to ensure that putative upstream control elements, necessary to drive transdifferentiation, were enclosed. However, these molecular manipulations resulted in a loss of transdifferentiation-dependent activity. *In vivo*, the *TIMP-1* promoter fragment directs only low level of GFP expression in normal liver, which is not increased during injury in the setting of our bile duct ligation model.

The 1515 bp fragment of the rat *SM22*α promoter was found to drive the reporter appropriately and is sufficient to direct reporter gene expression *in vitro* predominantly in HSC/MFB. SM22α is a marker protein of SMC and a 441 bp minimal promoter was necessary and sufficient to program high-level transcription of a luc reporter gene in primary rat aortic SMC, but it was inactive in NIH3T3, COS7, and HepG2 cells (38). This proximal rat *SM22*α promoter contains some muscle specific transcriptional regulatory elements like two CArG boxes, a CACC box, two GC boxes, an E-box, a potential MEF-2 binding site, and a TGF-β control element (24, 38–40). There are no additional regulatory elements included in the promotor (up to 2700-bp upstream) directing SMC-specific expression (10, 41). In liver, endogenous expression of *SM22*α is restricted to quiescent as well as activated HSC/MFB. In our study, we fused $\sim$1.5 kbp to the reporter gene expressing GFP and found high activity in activated HSC/MFB. However, the regulatory elements for HSC/MFB selective expression are not included in our promoter fragment, because we also observed a low expression in isolated hepatocytes. *In vivo*, GFP expression was noticed in hepatocytes of livers of

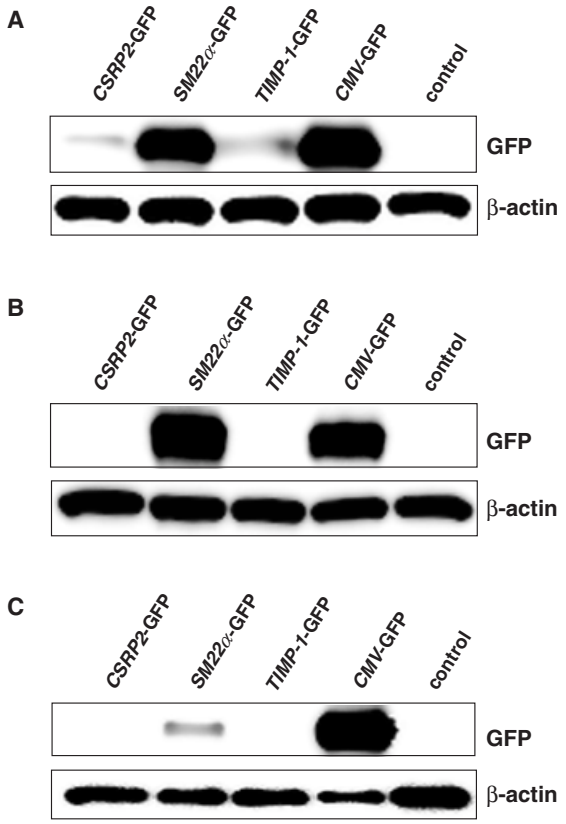

Fig. 4. Protein content of GFP expressed in liver cell subpopulations under transcriptional control of *CSRP2*-, *SM22*α and *TIMP-1* promoters. Western blot analysis was performed with protein lysates taken from isolated rat HSC (A), rat MFB (B), and rat hepatocytes (C), which were infected with Ad5-*CMV*-GFP, Ad5-*SM22*α-GFP, Ad5-*TIMP-1*-GFP, or Ad5-*CSRP2*-GFP. Non-infected cells were used as negative control. β-actin content was detected as a loading control. GFP, green fluorescent protein; *CSRP2*, gene encoding the LIM domain protein CRP2; *SM22*α, smooth muscle-specific gene encoding a 22-kDa protein; *TIMP-1*, gene encoding the tissue inhibitor of metalloproteinases-1; HSC, hepatic stellate cells; MFB, myofibroblasts; Ad5, adenovirus (serotype 5); *CMV*, cytomegalovirus.

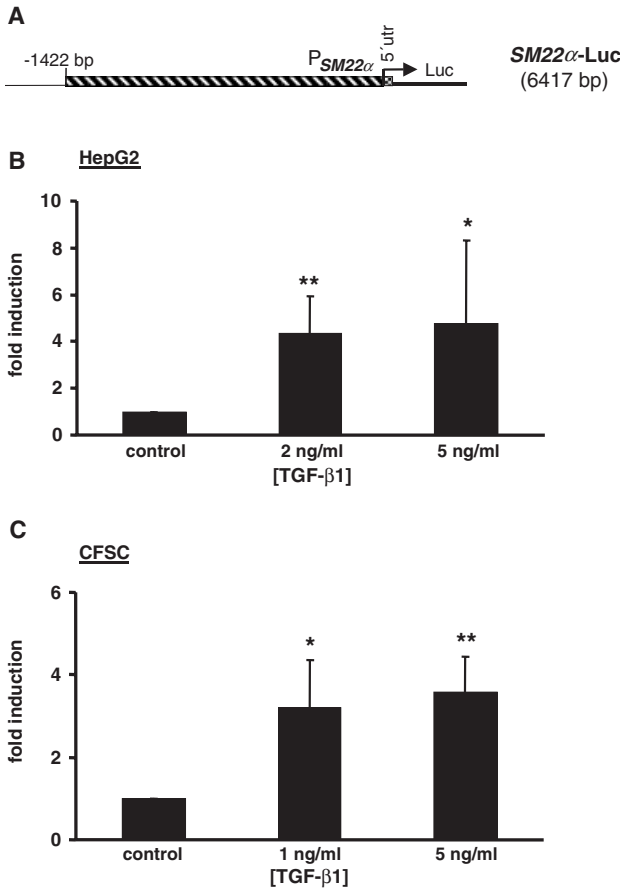

Fig. 5. TGF-β1-dependent induction of the *SM22*α gene promoter *in vitro*. (A) Schematic representation of the *SM22*α promoter-firefly luciferase (luc) reporter gene-construct. (B, C) HepG2 cells and CFSC were transfected with the *SM22*α-Luc reporter gene construct and stimulated for 24 h with indicated concentrations of recombinant TGF-β1. The relative luc values were measured in cell lysates and normalized to Renilla luc activities. The induction factors are means of three independent experiments and given as multiples of non-induced cells (control) which is set to 1. TGF-β1, transforming growth factor-β1; $P_{SM22\alpha}$, promoter of the smooth muscle-specific gene encoding a 22-kDa protein.

Ad5-*SM22á*-GFP-infected rats. However, the partially cellular selectivity observed *in vitro* was not found *in vivo*, suggesting that this *SM22*α promoter fragment lacks essential repressive elements necessary to regulate selective expression in liver. Expression of GFP in cultured hepatocytes isolated from Ad5-*SM22*α-GFP-infected animals was decreased after 2 days most likely to the induction of apoptosis within these cells (42).

The 870 bp fragment of the *CSRP2* promoter includes binding sites for basal transcription factors as well as for determining cellular specificity. We found that this promoter fragment-directed selective GFP expression to culture-activated HSC, but does not support HSC-selectivity *in vivo* indicating that the transcriptional machinery that functions on and dictate the activity of this promoter *in vitro* is different from the *in vivo* situation. Most likely, some separate regulatory elements of the *CSRP2* promoter necessary to drive HSC selectivity *in vivo* are not present in the chosen fragment. These sequences might harbor putative binding sites for specialized transcription factors allowing cell type specificity. In contrast to the *SM22*α and *TIMP-1* promoters there are no similar TGF-β response elements in the ~6 kbp upstream region of the *CSRP2* promoter. However, potential binding sites for MyoD/E47, estrogen receptor, STAT factors, or sterol regulatory factor-1 binding protein are located within the region of bp −2000 to −4000 upstream from the transcriptional start site as found by a computerized analysis of a 6600 bp fragment of the *CSRP2* gene using the software MatInspector 2.2 (43). Therefore, it is reasonable

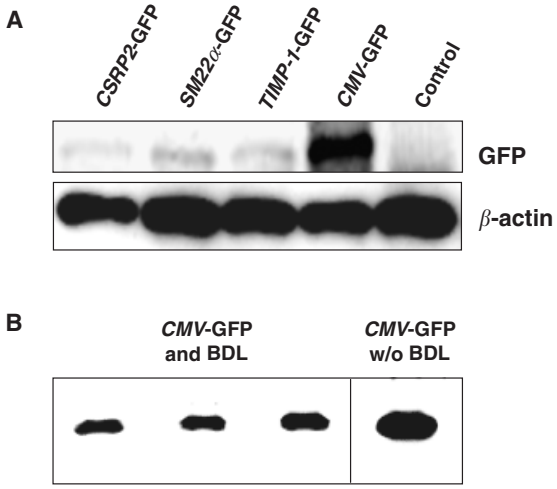

Fig. 6. *CSRP2*, *SM22α*, and *TIMP-1* promoters direct GFP expression *in vivo*. Whole liver lysates of adenoviral-infected rats were analyzed by Western blot. (A) The amount of GFP expressed by the *CSRP2*, *SM22α*, and *TIMP-1* promoters is compared with constitutive expressed GFP (*CMV*-GFP). Livers of non-infected rats were used as control. β-actin content was detected as a loading control. (B) Comparison of Ad5-*CMV*-GFP-directed GFP expression in three bile duct ligated (BDL) rats vs. an infected rat without receiving BDL. *CSRP2*, gene encoding the LIM domain protein CRP2; *SM22α*, smooth muscle-specific gene encoding a 22-kDa protein; *TIMP-1*, gene encoding the tissue inhibitor of metalloproteinases-1; GFP, green fluorescent protein; *CMV*, cytomegalovirus.

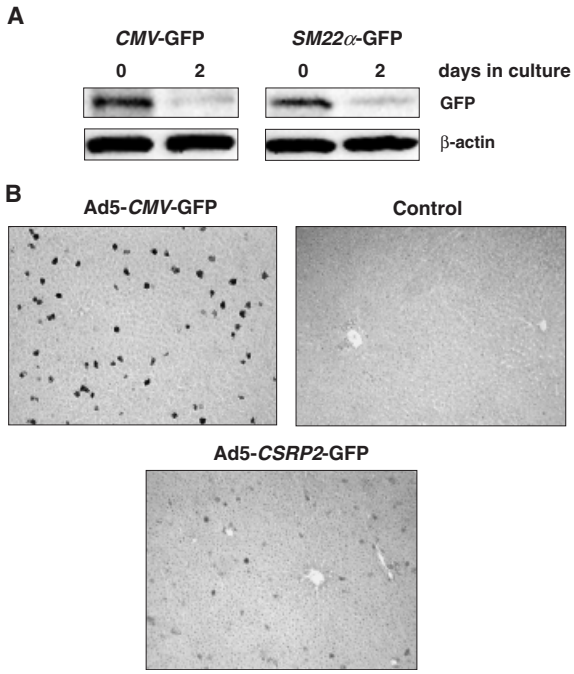

Fig. 7. *SM22α*- and *CSRP2*-promoter fragments drive expression in hepatocytes *in vivo*. (A) Western blot analysis of isolated hepatocytes from Ad5-*CMV*-GFP- or Ad5-*SM22α*-GFP-infected rats. Protein lysates were prepared from freshly isolated cells (0) or cells cultured for 2 days (2). β-actin content was detected as a loading control. (B) Immunostaining of liver sections of Ad5-*CMV*-GFP-, Ad5-*CSRP2*-GFP-, or saline-treated rats. GFP-positive cells are dark colored (magnification × 100). *SM22α*, smooth muscle-specific gene encoding a 22-kDa protein; *CSRP2*, gene encoding the LIM domain protein CRP2; Ad5, adenovirus (serotype 5); CMV, cytomegalovirus; GFP, green fluorescent protein.

that a prolongation of the *CSRP2* promoter might result in an extended specificity *in vivo*.

When we treated bile duct ligated rats with our reporter fusions, we observed that their hepatic expression was markedly reduced compared to normal rats, which is consistent with previous findings (44, 45). This phenomenon was independent of the promoter tested and was even observed with the constitutive active *CMV* promoter. Further, the decrease of expressed transgenic protein was independent of adenoviral uptake by liver cells, as discussed by Yu et al. (45), because we infected rats 24 h prior to the setting of bile duct ligature. One reason might be an enhanced cytotoxicity of the GFP in bile duct ligated rats (46, 47), but expression levels of other reporters (e.g. *LacZ*) were also reduced in ligated rats (data not shown). Another reason could be the environmental changes in injured livers (45).

Taken together, these studies demonstrate that the promoters of the *CSRP2* and *SM22α* genes are sufficient to mediate selective gene expression in HSC/MFB *in vitro*. However, an emerging problem arising from these studies is the following important question: How HSC specificity is mediated *in vivo*? In future studies we will isolate the relevant regulatory promoter elements responsible for selective expression of endogenous *CSRP2*, *SM22α*, or *TIMP-1*. Once characterized, these elements should provide fundamental insight into the molecular mechanisms that regulate HSC/MFB-specific transcription and will serve to develop new strategies for the prevention of liver fibrosis by allowing targeted gene transcription in these cells.

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