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TGF-β up-regulates serum response factor in activated hepatic stellate cells

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Abstract

In differentiated smooth muscle cells (SMC) the regulation of SMC marker genes (e.g. α-smooth muscle actin) is mainly conducted by the serum response factor (SRF) and accessory co-factors like myocardin. A number of SMC markers are also expressed in activated hepatic stellate cells which are the main cellular effectors in liver fibrogenesis. In the present study we found that during cellular activation and transdifferentiation the SRF transcription factor is up-regulated by transforming growth factor-β, accumulated in the nucleus, and exhibited increased DNA-binding activity. These observations were accompanied by a forced expression of the SRF co-activator myocardin. Specific targeting of SRF by small interference RNA resulted in diminished contents of α-smooth muscle actin. Therefore, we conclude that hepatic stellate cells retain differentiation capacity to evolve characteristics that are typical for cells of the cardiac and smooth muscle lineages.

Keywords: Serum response factor; Hepatic stellate cell; Transdifferentiation; Smooth muscle cell; Transforming growth factor-β; Myocardin; Gene regulation; siRNA; α-Smooth muscle actin; Liver fibrogenesis

1. Introduction

The serum response factor (SRF) is ubiquitously expressed and a founding member of the MADS-box containing transcription factor family. It is predominantly localised in the nucleus and binds to a specific sequence in the promoters of diverse target genes, called CArG-box. Thus, it was found that SRF is mainly involved in differentiation of cardiac, skeletal, or smooth muscle cells (SMC) by regulation of genes controlling cell growth, cytoskeletal organisation, cell contractility, and cellular motility [1]. Comprehensive research in vascular SMC and myofibroblasts revealed the relevance of SRF in regulating marker genes of cellular differentiation and of the contractile apparatus, e.g. α-smooth muscle actin (α-SMA), SM22α (transgelin), or smooth muscle-myosin heavy chain (SM-MHC) [2]. Expression of SRF is inducible by the transforming growth factor-β1 (TGF-β1) [3] and its transcriptional activity depends on phosphorylation of distinct serine or threonine residues [4–7]. It has been demonstrated that TGF-β-induced differentiation of embryotic 10T1/2 mesenchymal cells into a SMC phenotype was necessarily mediated by a strong up-regulation of SRF accompanied by enhanced DNA-binding activity [8]. The SRF-mediated control of myogenic genes is influenced by TGF-β-dependent RhoA/Rho kinase pathway and cytoplasmic G-actin polymerisation [9,10]. Contrarily, it was shown that the platelet-derived growth factor-BB (PDGF-BB) suppressed α-SMA expression by cellular redistribution of SRF out of the nucleus in rat aortic vascular SMC [11]. Since SRF binds to several promoters and controls disparate programs of gene expression, the specificity in activation requires different accessory co-activators, whereas SRF-mediated regulation of SMC differentiation marker genes is essentially supported by myocardin [12,13].

Chronic liver injury induces activation of hepatic stellate cells (HSC) that transit from a quiescent, fat-storing phenotype into a proliferative, extracellular matrix-producing myofibroblastic cell type (MFB) [14]. This differentiation process is accompanied amongst others by up-regulation of SMC marker genes [15,16] and enhanced susceptibility for TGF-β, the main profibrogenic cytokine during liver fibrogenesis [14]. To date, the molecular

Abbreviations: α-SMA, α-smooth muscle actin; ALK5, activin receptor-like kinase 5; DMEM, Dulbecco's modified Eagle medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; HSC, hepatic stellate cell(s); IgG, immunoglobulin G; MFB, myofibroblast-like cell(s); siRNA, small interference RNA; SMC, smooth muscle cell(s); SRE, serum response element; SRF, serum response factor; STR, soluble TGF-β type II receptor; TGF-β, transforming growth factor-β

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mechanisms involved in TGF-β-directed SMC marker gene expression in activated HSC are only partially known. It has been demonstrated that antagonising of TGF-β signalling by a soluble TGF-β type II receptor (STR), antisense RNA or transient over-expression of the TGF-β antagonist Smad7 influences α-SMA expression and cytoskeletal organisation [17–19]. Blocking of the p38 mitogen activated protein kinase (MAPK) had a repressive impact on α-SMA expression by influencing the expression of the transcription factor myocyte enhancer factor 2 (MEF2) [20]. The suppression of RhoA activity in trichostatin A-treated HSC reduced the content of α-SMA and impaired the formation of actin filament formation that was also observed when the cells were treated with the Rho kinase inhibitor Y-27632 [21,22]. However, the expression and functional role of SRF in liver is only sparely investigated. A very recent paper addressed the relevance of SRF for liver regeneration after partial

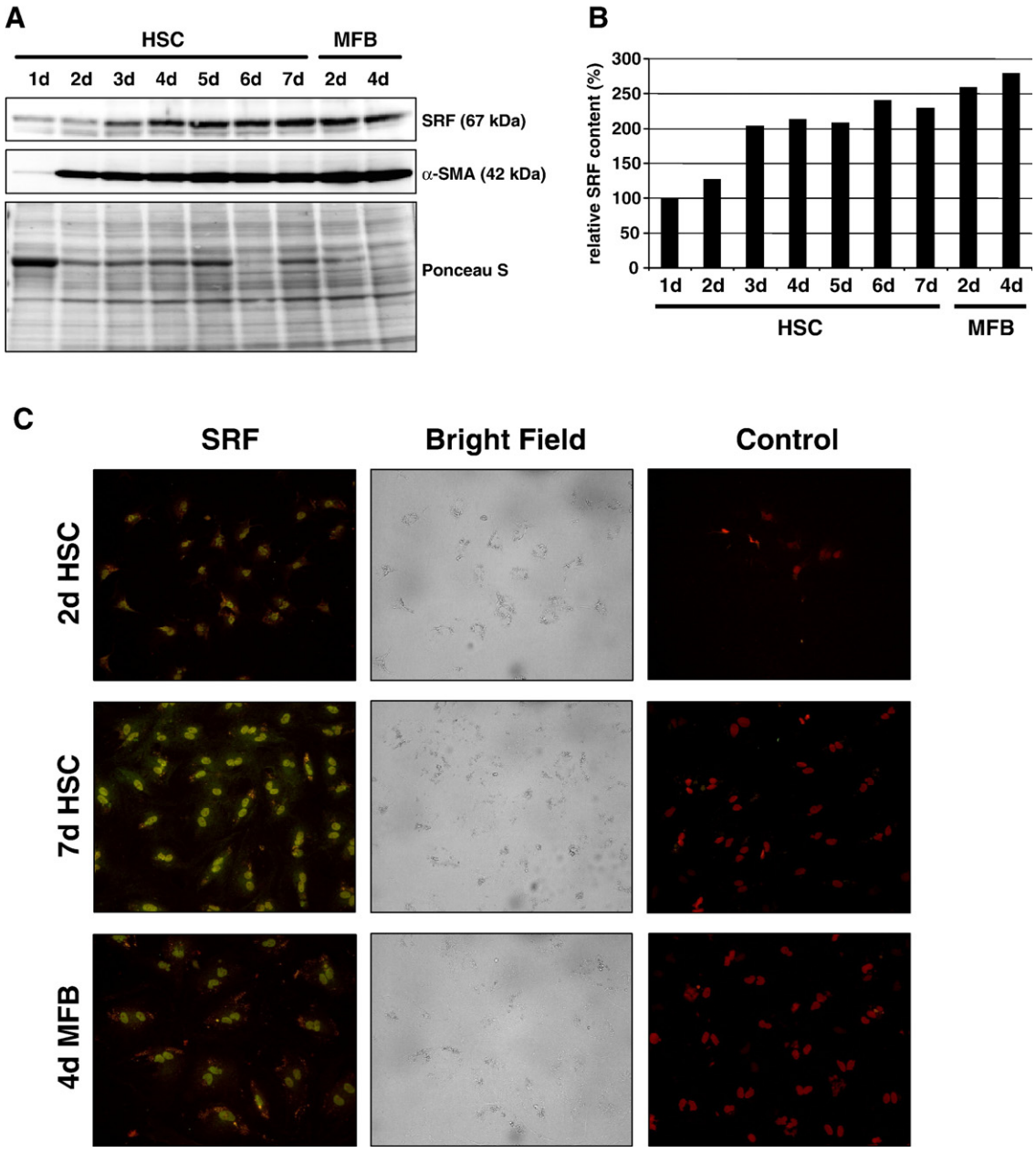

Fig. 1. *Expression and cellular localisation of SRF in activated HSC*. (A) Increased SRF and α-SMA expression during transdifferentiation. A representative Western blot of cell lysates (60 μg each) taken from HSC or MFB cultured for indicated time points is shown. Ponceau S staining served as loading control. (B) Densiometric analysis of SRF expression in activated HSC and MFB. The contents of SRF and whole protein in Ponceau S stain from (A) were semi-quantitatively measured by densiometry and the SRF content was normalised against the whole protein data in each sample. The determined value for SRF at day 1 of cultivation was set as 100%. (C) SRF accumulates in the nucleus of activated HSC but is found to a lesser extent in nuclei of MFB. Representative immunofluorescence stainings of SRF in activated HSC at day 2 or day 7 of culturing and in 4 days cultured MFB are shown. Nuclei were counterstained with propidium iodide. The left panel represents overlays of SRF (green) and nuclear stains (red) resulting in a yellow dyeing that is most prominent in HSC cultured for 7 days. In the middle panel, the same fields are shown in bright field. Negative controls (right panel) showed only nuclear staining. Both, the Western blots and immunofluorescent cytochemistry experiments were performed in triplicate with cells taken from three independent HSC preparations.

hepatectomy, which was delayed in mice conditionally deleted for SRF in hepatocytes [23].

The aim of the present study was to analyse the expression of SRF and its function for SMC marker gene activity in activated HSC and transdifferentiated MFB. We found that (i) the expression and activity of SRF increased during cellular activation, (ii) concomitantly the expression of the accessory SRF co-factor myocardin elevates, (iii) SRF expression is controlled by TGF-β1, merely partial *via* the ALK5 signalling pathway, and (iv) SRF is a critical mediator in the control of SMC marker gene expression in HSC. These results support previous studies demonstrating that SRF essentially contributes to the emergence of myofibroblasts during injury of lung and oesophagus [24,25]. Moreover, these findings underpin the notion that HSC imply high cellular plasticity allowing them to adopt of a SMC phenotype.

2. Materials and methods

2.1. Isolation and culture of HSC

HSC were isolated from male Sprague–Dawley rats by the pronase–collagenase method, followed by centrifugation in a Nycodenz gradient as described [26]. Cells were seeded in Dulbecco's modified Eagle medium (DMEM) (Bio Whittaker Europe, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (FCS) (Hyclone, Perbio, Bonn, Germany), and 4mM L-glutamine (PAA Laboratories, Linz, Austria), 100IU/ml penicillin and 100μg/ml streptomycin (PAA Laboratories). MFB were prepared from HSC by trypsinization at day 7 of primary culture. For induction, freshly isolated HSC were cultured 1day in medium containing 10% FCS, starved for 24h in medium containing 0.2% FCS, then preincubated for 1h in serum free medium and subsequently stimulated with 1ng/ml recombinant human TGF-β1 (R&D Systems, Wiesbaden, Germany) for additional 4h or left untreated. The experiments for antagonizing TGF-β signalling were performed with 1μg/ml soluble TGF-β type II receptor (STR) or 5μM ALK5 inhibitor SB-431542 (Sigma, Taufkirchen, Germany) as described [27]. Specific down-regulation of SRF in rat HSC was achieved by addition of 10nM siRNA (Rn_LOC301242_2_HP) and the HyPerFect transfection reagent (Qiagen, Hilden, Germany) 1day after seeding. The study as presented was approved by the local committee for care and use of laboratory animals, and was performed according to strict governmental and international guidelines on animal experimentation.

2.2. Adenoviral infection of HSC

Primary HSC were seeded in 6-well dishes at 3×10^5 cells/well and infected at day 1 of culturing with 2×10^8pfu adenoviral constructs Ad5-CMV-GFP, Ad5-SM22α-GFP, or Ad5-TIMP-1-GFP as described previously [16]. The expression of GFP was monitored by fluorescence microscopy.

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

Cell lysates were quantified by Bradford assay and 60 to 80μg total protein (as indicated in the respective figure legends) were resolved on a 4–12% (w/v) Bis–Tris gel (Invitrogen, Carlsbad, CA) in SDS-PAGE under reducing conditions in MOPS buffer and proteins were electroblotted onto a Protran membrane (Schleicher & Schuell, Dassel, Germany) according to standard procedures. After blocking, the membranes were incubated with primary antibodies against SRF (HM1350; Hypromatrix, Worcester, MA), myocardin (sc-21559; Santa Cruz Biotech., Santa Cruz, CA), SM22α (ab10135; Abcam, Cambridge, UK), or α-SMA (CBL171; Cymbus Biotechnology Ltd., Chandlers Ford, UK) in 1:1000 dilutions. The formed immunocomplexes were subsequently detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG in a 1:5000 dilution. The detection of the ribosomal protein S6 (54D2) (#2317; Cell Signaling Technology, Beverly, MA) was used as loading control. In addition, equal loading of the individual lanes was further confirmed by densiometric analysis of Ponceau S staining. Densiometric analysis was done using the LumiAnalyst 3.0 software (Roche Diagnostics, Mannheim, Germany).

2.4. Electrophoretic mobility shift assay (EMSA)

Whole cell lysates (15μg) were incubated with 35fmol ^{32}P-labeled, double-stranded consensus SRE probes (sc-2523; Santa Cruz) in binding buffer [10mM Tris (pH 7.5), 50mM NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, 4% (v/v) glycerol, 1:500 Protease-Inhibitor Cocktail (Sigma), 1:100 Phosphatase-Inhibitor Cocktail 2 (Sigma)] and in the presence of 1μg poly[d(I–C)] (Roche Diagnostics). Competitor oligonucleotides [consensus- or mutant-SRE (sc-2524; Santa Cruz)] were included in a 40-fold molar excess. Supershift analyses were performed with 200ng anti-SRF antibody (sc-335; Santa Cruz) or normal rabbit IgG (sc-2027; Santa Cruz). Samples were resolved on 6% DNA retardation gels

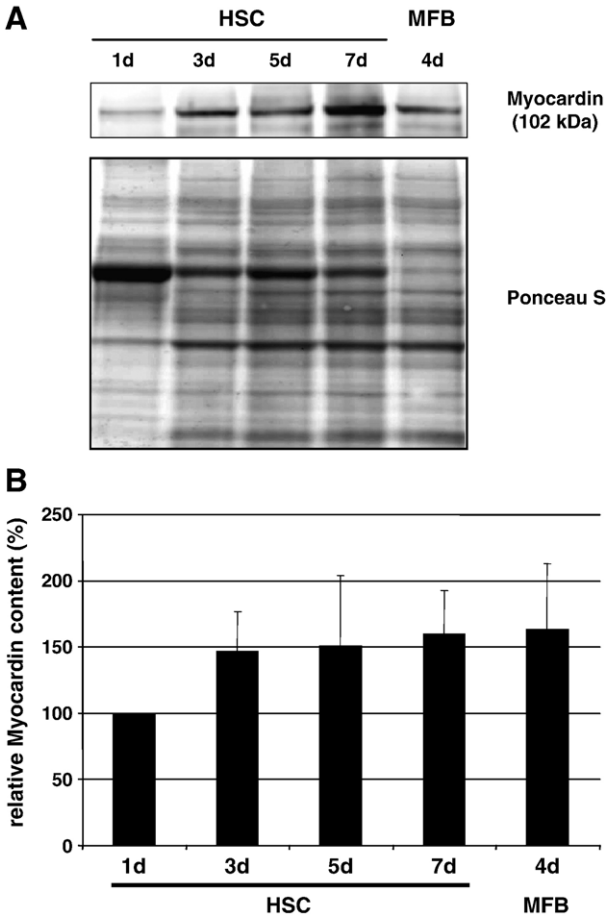

Fig. 2. *Expression of myocardin during transdifferentiation*. (A) Myocardin contents are increased in activated HSC. Total proteins extracts from HSC at indicated time points were isolated and analysed (80 μg each) by Western blot. As shown in this representative experiment, the expression of myocardin is elevated during HSC transdifferentiation peaking at day 7 of culturing and followed by a slight down-regulation in MFB cultured for 4 days. Ponceau S staining was used as loading control. The detection of myocardin was performed in triplicate with cells taken from three independent HSC preparations each. (B) Densiometric analysis of myocardin expression in activated HSC and MFB. The individual myocardin contents of the 3 independent Western blot analyses with cell lysates taken from 3 individual HSC preparations were normalised against the respective amounts of total protein in each sample as depicted by Ponceau S staining. In this analysis, the determined value for myocardin at day 1 of cultivation in the 3 individual cell preparations was set as 100% and the calculated averages with the respective standard deviations are shown.

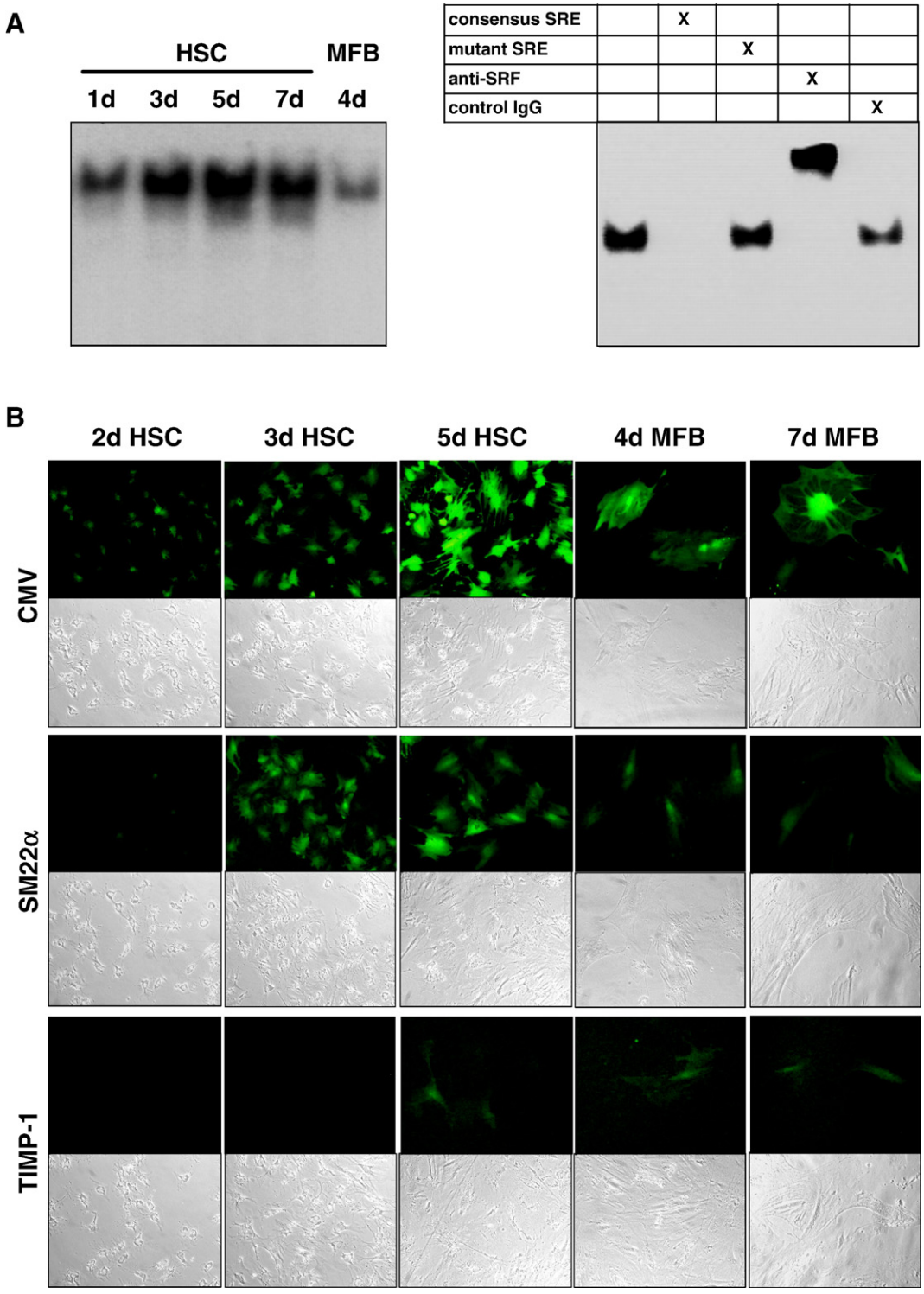

Fig. 3. *DNA-binding activity of SRF and SM22α reporter gene expression during transdifferentiation*. (A) Enhanced DNA binding of SRF in activated HSC is diminished in MFB. In the left panel, the DNA-binding capacity of SRF during HSC transdifferentiation was analysed by EMSA. Therefore, whole cell lysates were taken from cultured HSC/MFB at indicated time points and incubated with a radiolabeled oligonucleotide containing the SRE consensus sequence. In the right panel, a 40-fold excess of unlabeled SRE consensus or a mutant-SRE served as a control for specificity. In addition, a supershift was induced with an antibody directed against SRF. In this analysis, a control IgG served as a further control. The extracts used in this EMSA were taken from HSC that were cultured for 7 days. The presented EMSAs reveal representative data of three independent experiments. (B) SRF target gene expression correlates with its DNA-binding activity. One day cultured HSC were infected with the adenoviral constructs Ad5-CMV-GFP (upper panel), Ad5-SM22α-GFP (middle panel), or Ad5-TIMP-1-GFP (lower panel), respectively. The expression of the GFP reporter gene was visualized at indicated time points by fluorescence microscopy. Respective bright field views are depicted in parallel.

(Invitrogen) in 0.5× TBE buffer [67mM Tris, 23mM boric acid, 1.7mM EDTA] that were subsequently dried under vacuum and exposed to Kodak X-OMAT AR films at − 80°C using intensifying screens.

2.5. Immunofluorescent cytochemistry

Approximately 3×10^5 freshly isolated rat HSC were seeded on coverslips mounted in 6-well dishes. Two- or 7-day cultivated HSC as well as MFB cultured for 4days were fixed in paraformaldehyde [4% in phosphate buffered saline (PBS) pH 7.4], permeabilized on ice in 0.1% sodium citrate containing 0.1% Triton X-100, and blocked against endogenous biotin in Biotin Blocking Reagent (X0590; DAKO, Hamburg, Germany) followed by unspecific blocking in PBS (pH 7.4) supplemented with 50% FCS and 0.5% bovine serum albumin. The cells were then incubated with 10μg/ml anti-SRF and subsequently with a secondary biotinylated swine anti-rabbit IgG (E0353; DAKO). Negative controls were treated with the secondary antibody as described [28]. The immunocomplexes were visualized by addition of FITC-labeled streptavidin (F0422; DAKO) in laser-scanning microscopy (LEICA DM LB 100W; Leica Microsystems, Wetzlar, Germany). Nuclei were counterstained with propidium iodide.

2.6. Statistical analysis

Results are presented as the mean of 3 independent experiments (± SD). Statistical analysis was performed with a Student's *t*-test and differences were considered as significant (*) at $p < 0.01$.

3. Results and discussion

3.1. SRF and myocardin are expressed in activated HSC

We comparatively analysed the expression of SRF and α-SMA in primary HSC undergoing differentiation at different culture days by Western blot (Fig. 1A). The content of both proteins gradually increased during conversion from quiescent to activated HSC and at last fully differentiated MFB. We observed that SRF was already present at low levels in HSC cultured for 1 and 2days, while it increased rapidly at day 3 of cultivation resulting in a doubling of SRF contents and followed by a further increase in MFB (Fig. 1B). In contrast to the kinetics of SRF expression, the amounts of α-SMA climaxed at day 2 of culturing reflecting the transcriptional potency of SRF (Fig. 1A). Next we determined the cellular localisation of SRF by immunocytochemistry revealing that the transcription factor was already present in HSC cultured for 2days and accumulated in the nuclei of HSC that were cultured for 7days (Fig. 1C). Interestingly, the amount of nuclear SRF was diminished in MFB. Because SRF-mediated transcriptional regulation of SMC marker genes is further dependent on specific co-factors, we next analysed the expression of the accessory SRF co-activator myocardin in lysates taken from culture-activated HSC and MFB (Fig. 2A). Myocardin was initially up-regulated early during cellular activation and peaked in 7days cultured HSC. However, we found a slight decrease in myocardin expression in MFB (Fig. 2A and B). Interestingly, another SRF co-factor, the LIM domain protein CRP2 that was shown to be a potent SMC marker gene co-activator [29], exhibits a similar expression pattern in activated HSC [30]. The simultaneous activation of these genes previously shown to be SMC marker genes suggests that the differentiation from HSC to MFB is a well orchestrated process in which HSC might acquire some characteristics of SMC resulting in the expression of typical cytoskeletal compounds.

3.2. SRF DNA-binding activity is enhanced in activated HSC

To prove if the expressed SRF is functionally active in HSC, we performed EMSAs using the consensus CArG-box binding motif (Fig. 3A). We found that the fraction of formed complexes was highest at day 5 of culturing which was in agreement with the result of the Western blot showing that SRF was highly expressed at this time point. Contrarily, we observed a strong reduction of DNA-binding capacity in fully differentiated MFB. To analyse if the lowered SRF DNA binding caused changes in target gene expression, we performed a reporter gene assay in which HSC at day 1 were infected with the adenoviral constructs Ad5-CMV-GFP, Ad5-SM22α-GFP, or Ad5-TIMP-1-GFP. The expression of GFP driven by the constitutive active CMV promoter was already detectable 1day after infection, increased at later time points and stayed at high level in MFB (Fig. 3B). In contrast, GFP expression regulated by a SM22α promoter fragment harbouring CArG-boxes was hardly detectable 1day after

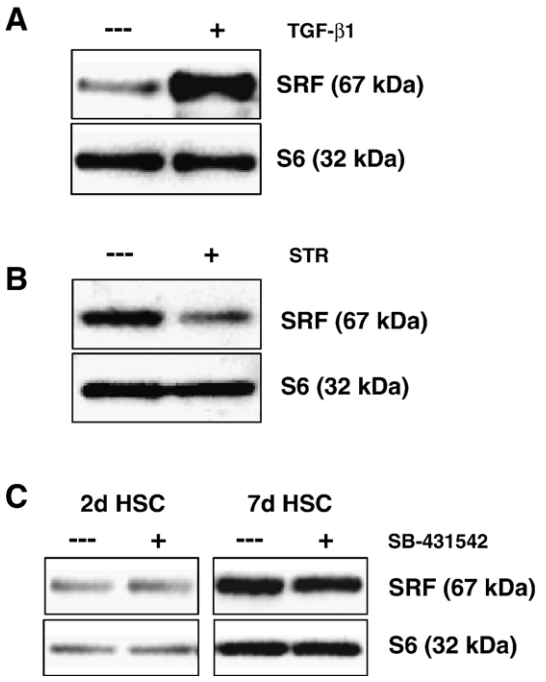

Fig. 4. *SRF expression is TGF-β-dependent in activated HSC*. (A) Culture-activated HSC were treated with 1 ng/ml TGF-β1 under starvation conditions as described in Materials and methods. Western blot analysis (60 μg total protein/lane) revealed that the SRF content increased rapidly in cells that were treated with TGF-β1 for 4 h. Detection of the ribosomal S6 protein served as loading control. (B) Freshly isolated HSC were seeded in the presence of 1 μg/ml soluble TGF-β type II receptor (STR). The cells were harvested at day 7 of culturing and lysates taken from treated or untreated cells (60 μg each) were analysed for SRF expression by Western blotting. The result as shown in the representative immunoblot supports the observation presented in A that TGF-β is mainly involved in SRF up-regulation during HSC transdifferentiation. The ribosomal S6 protein served as an internal loading control. (C) Primary rat HSC were incubated at day 1 of culturing with 5 μM ALK5 inhibitor SB-431542. Cell extracts (60 μg each) were prepared at indicated time points and SRF expression was detected by Western blot. Inhibition of the classic TGF-β signalling pathway *via* Smad2/3 resulted in an only marginal down-regulation of SRF that was observable in HSC that were cultured for 7 days. Detection of the ribosomal S6 protein served as loading control. All presented Western blot results are representative results of 3 independent experiments.

infection, peaked in 5days HSC, and decreased during phenotypic transition to MFB indicating that the transcriptional activity of SRF was reduced at later stages of differentiation. The TIMP-1 promoter fragment without any SRF binding element directed very weak expression of GFP that was hardly detectable in HSC at day 5 of culturing. However, comparable to the expression obtained with the CMV promoter this expression level stayed constant in MFB.

The discrepancy between total SRF protein content and transcriptional activity in MFB might be the result of a partial redistribution of SRF from the nucleus into the cytoplasm. In line with this hypothesis, we demonstrated that nuclei of MFB contained less SRF compared to HSC that were cultured for 7days (cf. Fig. 1C). Such an extra-nuclear redistribution of SRF was previously shown in serum-deprived tracheal myocytes in which the SRF transcriptional activity is regulated through reversible translocation between cytoplasm and nucleus [31].

3.3. The increase of SRF protein in activated HSC is TGF-β-dependent

TGF-β is the most prominent profibrogenic factor for HSC activation and liver fibrogenesis and a powerful inducer of SMC marker gene expression. Therefore, we tested if the expression of SRF in HSC is influenced by this cytokine. Treatment of HSC with TGF-β1 under starvation conditions resulted in a strong up-regulation of SRF (Fig. 4A). In an opposing experimental approach the sequestering of active TGF-β by a soluble TGF-β receptor type II (STR) led to a distinct reduction of SRF (Fig. 4B). Blockade of the Smad2/Smad3 signalling pathway by the TGF-β type I receptor ALK5 inhibitor SB-431542 had only minor effects on SRF expression levels during HSC transdifferentiation suggesting that this pathway might have only accessory relevance in TGF-β mediated regulation of the *srf* gene (Fig. 4C). Likewise, previous studies have shown that SRF expression in 10T1/2 cells and canine tracheal SMC is influenced by TGF-β1 [3,32] but the responsible intracellular mediators are still unknown. Our results indicate that the classical TGF-β signalling cascade that is mediated by Smad proteins [33] might play an only ancillary role in the regulation of SRF expression in HSC. In general, SRF-dependent SMC marker gene activation might be principally regulated by two other mechanisms, (i) the transcriptional up-regulation of SRF and (ii) the increase/decrease of SRF activity by reversible shuttling between cytoplasm and nucleus that is mediated by the RhoA/Rho kinase pathway [34]. The latter mechanism might also contribute to SRF activity in activated HSC because the selective GTPase Rho/Rho-associated kinase inhibitor Y-27632 was shown to attenuate α-SMA expression and contractility in HSC [22,35]. Previous reports have revealed that MFB are insensitive towards the activity of TGF-β1 [36]. Possibly this insensitivity might be a reasonable explanation why the overall SRF activity is decreased in MFB, while the SRF protein contents remained constant (see above). It is also known that the interaction of SRF and Smad3 is important for the regulation of *SM22α* gene expression in TGF-β-stimulated 10T1/2 cells [3]. Likewise, this cooperative effect was inhibited by over-expression of the Smad3-antagonist Smad7 in canine tracheal SMC [32]. Another study described that the TGF-β-induced differentiation of the neural crest stem cell line Monc-1 into a SMC-like phenotype was attributed by a modulation of Smad signalling by the RhoA/Rho kinase pathway [37]. All these findings indicate that the classical TGF-β signalling pathway *via* activation of Smad2 and Smad3 is potentially able to directly affect SRF activity in other cellular systems.

3.4. SRF directs SMC marker gene expression in activated HSC

Next, we examined the influence of SRF for the regulation of SMC marker genes in activated HSC. Therefore, we transfected HSC at day 1 in primary culture with siRNA directed against SRF mRNA and analysed the expression of SRF, α-SMA, and SM22α after a further 2-day culture period. The analysis of 3 independent experiments revealed that the application of the siRNA (i.e. siSRF) decreased the SRF content significantly (up

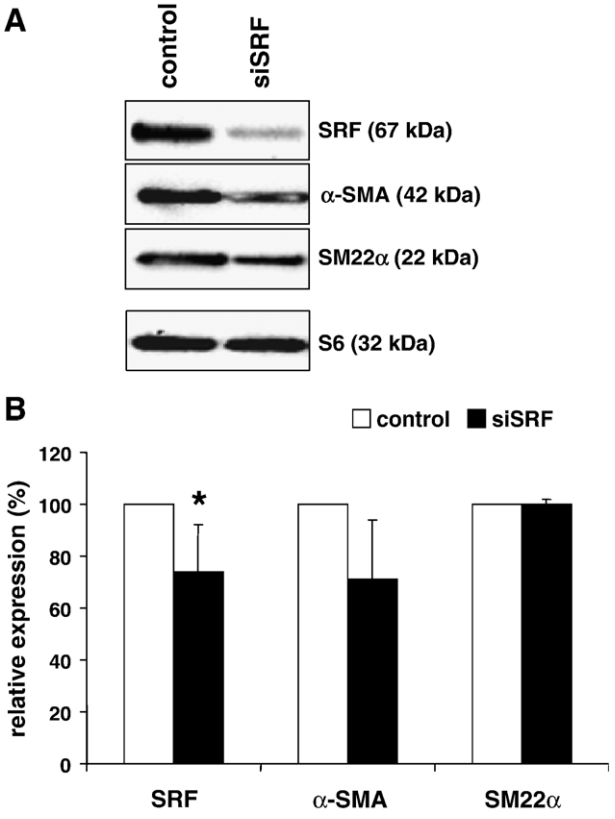

Fig. 5. *SMC marker gene expression in activated HSC is influenced by SRF.* Primary HSC were transfected with siRNA directed against SRF (siSRF) or a scrambled control siRNA at day 1 of culturing. Two days after transfection the cells were harvested and protein expression levels were detected by Western blotting (60 μg total protein/lane). (A) A representative Western blot analysis of SRF, α-SMA and SM22α contents in whole cell lysates taken from HSC treated with siRNA. SRF was markedly reduced, while the protein contents of the SMC markers α-SMA or SM22α were decreased at lower levels. Detection of the ribosomal S6 protein served as loading control. (B) Densiometric analyses of SRF, α-SMA, and SM22α contents were performed with Western blot results taken from three independent experiments. Data were normalised against S6 protein contents and statistical relevance was determined by the Student's *t*-test (* = $p<0.01$).

to averaged 74%) and subsequently the expression of α-SMA without any significance as a result of a higher inter-experimental variance, while the SM22α expression remained unaffected (Fig. 5A and B). We were not able to further increase the expression of SRF by higher amounts of siSRF or other siRNAs targeting endogenous SRF mRNA (data not shown). The suppression of α-SMA expression demonstrated that SRF indeed possesses a regulatory function in control of this SMC marker gene in activated HSC. However, we were not able to induce a relevant reduction of SM22α expression by the siSRF. Possibly, the down-regulation achieved by the siRNA targeting SRF expression was not sufficient to affect SM22α expression or alternatively, the regulation of the α-SMA gene is more reliant on SRF in HSC.

In conclusion, we have shown for the first time that SRF and myocardin, which are essential regulators of SMC differentiation, become expressed during the transition of quiescent HSC into proliferative, fibrogenic, and contractile MFB. The potential of HSC to differentiate into SMC of the endothelial lineage was recently suggested for HSC carrying the CD133 progenitor cell marker [38] indicating the high degree of plasticity of this cells. However, the data presented in this study imply that this characteristic is a common feature of all HSC.

Future experiments investigating the activity and fine tuning of the transcriptional machinery containing SRF, myocardin, and other SRF accessory co-factors (e.g. CRP2) might lead to fundamental insights in understanding the biology behind cellular differentiation from HSC into its fibrotic counterpart (i.e. MFB).

Acknowledgments

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