JBC Papers in Press. Published on April 10, 2006 as Manuscript M600711200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M600711200

Transcriptional profiling reveals novel markers of liver fibrogenesis: Gremlin and Insulin-like Growth Factor Binding Proteins

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Running Title: Gremlin and IGFBPs in liver fibrosis

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ABSTRACT

Activated hepatic stellate cells (HSC) that transdifferentiate to myofibroblasts in the injured liver are responsible for scar formation that leads to fibrosis and eventually cirrhosis. To investigate the gene expression profile during different stages of this process, we performed serial analysis of gene expression (SAGE), representing a quantitative and qualitative description of all expressed genes. Stellate cells were isolated from human livers and cultured. SAGE was performed on RNA isolated from quiescent, activated and transdifferentiated HSC. Comparison of the three resulting transcriptomes showed that less than 5% of all genes changed significantly in expression. Established markers of liver fibrosis showed enhanced expression in accordance with the transdifferentiation process. In addition, induction was seen for several genes not yet recognized to be involved in liver fibrosis, such as insulin-like growth factor binding proteins (IGFBP) and antagonists of bone morphogenic proteins: follistatin and gremlin. The induction of these genes was validated *in vivo* in mice developing liver fibrosis. The expression of IGFBPs and gremlin was measurable in the livers of these mice while it was low or undetectable in control mice without liver fibrosis. Since gremlin modulates the activity of bone morphogenic growth factors, it may represent a novel pathway and a target for therapeutic intervention and together with IGFBPs as a specific marker of liver fibrosis. In conclusion, the comparison of the three transcriptomes of (activated) stellate cells reveals novel genes involved in fibrogenesis and provides an appreciation of the sequence and timing of the fibrotic process in liver.

INTRODUCTION

Hepatic fibrosis is the common response to most chronic liver injuries like viral hepatitis, parasitic infection, metabolic or autoimmune diseases, congenital abnormalities and drugs or alcohol abuse (1,2). It is characterized by increased production of components of the extracellular matrix (ECM) like fibril-forming collagens I and III, proteoglycans, fibronectins and hyaluronic acid (3). The hepatic stellate cells (HSC) are the primary source of excess ECM accumulation in liver fibrosis. HSC in normal human liver comprise roughly one third of the non-parenchymal cell population or about 5-8% of total liver cells (4). HSC are the main storage site for retinoids (5) and are situated in the sinusoid within the subendothelial space of Disse in close contact with the hepatocytes. During the development of liver fibrosis, HSC undergo a process of activation resulting in a reduced retinoids storage capacity and transdifferentiation to a myofibroblast-like phenotype that is characterized by expression of α-smooth muscle actin (α-SMA). Actually, this myofibroblast-like phenotype is not limited to the liver but also is a prominent feature of fibrosis in other tissues including pancreas, kidney, lung, and skin (6).

The HSC activation process is the result of a complex interplay between the different hepatic cell types through cytokines, growth factors and oxidative stress signals (7). The cellular

transformation that develops gradually *in vivo* can be mimicked *in vitro* by short-term culture of HSC on plastic, providing a model to study the intra- and extracellular determinants that regulate the transformation/activation process. The hallmarks of activation include excessive cellular proliferation and an abundant ECM protein production that is not counteracted by increased ECM degradation (8). Another major feature of the activation process is the responsiveness of stellate cells to cytokines, resulting in expression of the PDGF receptor (9,10) and members of TGF-ß receptor family. PDGF is a major mitogen for stellate cells (11) and TGF-ß appears to be the primary fibrogenic cytokine (12,13). Although several genes play a role in the activation of HSC, it is yet not clear which specific genes are responsible for the initiation and perpetuation of the fibrotic response. To identify genes involved in the transformation of HSC into myofibroblasts we performed transcriptional profiling during different stages of activation. For this purpose, we have chosen to use the technique of serial analysis of gene expression (SAGE) (14), which allows the effective monitoring of tens of thousands genes without prior knowledge of the genes and their expression. This technology provides both a quantitative and qualitative measurement of gene transcription by any cell type or tissue. Under the auspices of the Cancer Genome Anatomy Project (CGAP) an analysis of differential gene expression by SAGE in normal and tumor tissues and normal and malignant cell types has been applied (http://www.ncbi.nlm.nih.gov/SAGE/ and http://cgap.nci.nih.gov/SAGE/). This project represents over $1.2x10^6$ unique SAGE tags from more than 300 libraries of human origin and has been designed to assist in unraveling the molecular basis of cancer. A similar analysis of quiescent and activated HSC might provide insight into the development of hepatic fibrosis and could be used as a paradigm for fibrotic processes in other tissues (1,2,6). Here, we present a global gene expression pattern in human hepatic stellate cells in three stages: the resting, quiescent phase, activated HSC and the fully transdifferentiated form, the hepatic myofibroblasts. Subsequently, we have validated the induction of several genes by real time PCR and in addition we confirmed the induction of these genes *in vivo* in an animal model of liver fibrosis.

METHODS

Animals. *Mdr2* (-/-) mice (FVB strain) (15), 4 months old, and congenic control mice were fed with purified control diet (Hope Farms, Woerden, The Netherlands) supplemented with 0.03% sodium cholate (Merck, Darmstadt, Germany). Food and water were supplied *ad libitum.* After 6 weeks the mice were sacrificed, blood was collected and livers resected for further analysis. All animal experiments were performed under approved protocols of the AMC Committee on Animal Research.

Cells and culture. Human hepatic stellate cells (HSC) were isolated from wedge sections of normal human liver unsuitable for transplantation or from tumor-free human liver after partial hepatectomy as previously reported (12,16). Briefly, after a combined digestion with collagenase/pronase, HSC were separated from other liver nonparenchymal cells by ultracentrifugation over gradients of Larcoll (Sigma, Zwijndrecht, Netherlands). The percentage of HSC in these isolates was > 90% as assessed by transmission microscopy, autofluorescence of vitamin A and immunofluorescence of vimentin (16). Immediately after isolation RNA was extracted for the construction of the SAGE library of quiescent HSC. To obtain activated stellate cells, these cells were cultured for 15 days on plastic culture dishes in modified Dulbecco's medium (DMEM) supplemented with 0.6 U/ml insulin, 2.0 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, antibiotic antifungal solution (Gibco, Breda, Netherlands) and 20% fetal bovine serum. To obtain fully transdifferentiated hepatic stellate cells, or myofibroblasts, the cells were cultured until they had reached passage 6 to 7. Myofibroblast phenotype was confirmed by detection of vimentin and α-smooth muscle actin (α-SMA) using immunofluorescence with monoclonal antibodies from Dako (Heverlee, Belgium) and Sigma, respectively (not shown). Medium was refreshed twice a week and at the indicated period RNA was extracted using Trizol (Quiagen, Venlo, Netherlands).

SAGE procedure. The SAGE libraries were obtained essentially following the SAGE protocol (14). Additional information, including graphical presentation of the SAGE technique can be found

at http://www.sagenet.org. In short, using 100 µl of oligo-dT biotinylated beads (Dynabeads, Invitrogen, Paisly, UK) mRNA was isolated from 5 to 20 µg total RNA derived from freshly isolated, 15 days cultured human hepatic stellate cells or completely transdifferentiated human hepatic stellate cells. First and second strand cDNA synthesis were performed using Super Script II (Invitrogen). The double stranded cDNA was digested with *NlaIII* for one hour at 37^0 C, washed and divided into two pools. Using T4 ligase both pools were ligated to two different linkers both containing the *BsmF1* recognition site. Subsequently, both pools were digested with *BsmF1* for one hour at 65^0 C. Upon removal of the beads both pools were mixed and ligated. The resulting ligation product was amplified with 28 cycles of PCR and amplicons were digested with *NlaIII* and separated on a 12% polyacrylamide (PA) gel to isolate the 28 bp ditags. The ditags were ligated to form concatemers. Concatemers between 400 and 1000 bp were isolated from an 8% PA-gel, cloned into the *SphI* site of pZero (Invitrogen) and transformed into TOP 10F' electrocompetent cells (Invitrogen). Clones containing inserts between 400 and 1000 bp were selected using colony-PCR with M13 primers and analyzed with *NlaIII* digestion for the presence of concatemers.

Sequencing and data analysis. DNA sequencing was done on an ABI 377XL automatic sequencer (Perkin Elmer, Boston, MA) using a DYEnamic ET-T7 primer (Amersham Pharmacia Biotech, Uppsala, Sweden), and analyzed using Sequence Analysis Software v.3.4. SAGE data analysis was performed using USAGE V2 software, a web-based application developed in the Bioinformatics Laboratory of our Institute (17), for extraction of single tags from sequence data and subsequent identification on the NCBI human gene database. The three obtained non-normalized SAGE libraries were evaluated using SAGEstat (18) and differences were evaluated as being statistically significant with *P* values less than 0.01. To further study tag identification and expression, NCBI/CGAP/SAGEMAP program was used at http://www.ncbi.nlm.nih.gov/SAGE/ or http://cgap.nci.nih.gov/SAGE/. The normal liver library was obtained from these web sites at http://cgap.nci.nih.gov/SAGE/SAGELibInfo?LID=135&ORG=Hs.

Reverse Transcriptase quantitative PCR. RT-qPCR analysis on various stages of stellate cell activation was performed on cDNA prepared for the SAGE analysis using real-time PCR (Light Cycler (Roche, Almere, Netherlands)) with SYBR Green. The primers used are shown in Table 1A. To allow comparison with the SAGE data, mRNA expression levels were normalized to the copy number of GAPDH.

RT-qPCR to detect fibrosis markers in the Mdr2 (-/-) mice. Total RNA was isolated from mouse livers using Trizol reagent (Quiagen) and transcribed to cDNA using SuperScript II reverse transcriptase. Subsequently the mRNA copy number of several fibrotic markers was determined using real-time PCR (Light Cycler (Roche)) with SYBR Green; the primers used are listed in Table 1B. To correct for differences in RT efficiency, all data were normalized to the copy number of 18S RNA.

RESULTS

SAGE tag expression in human hepatic stellate cells.

The tag distribution for the three stages of trans-differentiation is presented in Table 2. Total tags represent the entire number of tags sequenced for each stage. The unique tags represent the number of the different, distinct and exclusive tags in the total library. In principal, each unique tag should identify a single gene. However, due to internal priming and variable polyadenylation more tags can identify the same gene. Most unique tags were detected less than 5 times, indicating that the expression of the genes recognized by these tags is low. Only a small percentage of the tags was detected more than 10 times: 2.3% in quiescent, 3.6% in partially activated and 2.8% in the fully transformed cells. The relative distribution of the tags was independent of the magnitude of the sample.

Analysis of SAGE libraries of human HSC

An important issue is the purity of the freshly isolated HSC. Careful examination of the SAGE data revealed no markers for hepatocytes, endothelial cells, Kupffer cells or cholangiocytes. The presence of 4 tags for CD14 (Table 3B, supplemental data) at this stage suggests some minor contamination; any effect of this on the transcriptome will be negligible.

Statistical analysis of the three non-normalized SAGE libraries revealed that no significant difference was seen for more than 95% of the unique tags ($p < 0.01$). This implicates that during activation of human stellate cells and subsequent transformation into myofibroblast, less than 5% of all expressed genes do show a significant change in expression. In the library of the quiescent human stellate cells, 98 genes were identified with a significant higher expression when compared to both other libraries. Tags representing 28 different genes were only detected in freshly isolated stellate cells (Table 3A, supplemental data). Their absence in both later stages indicates that the expression of these genes seems to be lost rapidly during stellate cell activation. Six genes are unique for quiescent stellate cells since these tags were not present in a public SAGE library of a normal liver. For 62 genes the expression seems to decrease more slowly during stellate cell activation. The number of tags of these genes was reduced in partially activated cells compared to that in quiescent cells while no tags were present in the fully transdifferentiated cells (Table 3B, supplemental data). All genes of which the expression is lost during activation seem to be suitable as a marker of quiescent stellate cells. Some of them may play a role in determining the resting state in stellate cells. In the partially activated stellate cells, 135 genes were significantly higher expressed compared to the quiescent cells. Of these, 31 genes were completely absent in the quiescent state suggesting that their expression is linked to stellate cell activation (Table 4A, supplemental data). 220 genes were significantly higher expressed in the partially activated HSC than in the fully transformed cells; 96 were completely absent in the myofibroblasts (Table 4B, supplemental data). In the partially activated cells 19 genes were identified, which were absent both in resting and in completely transdifferentiated stellate cells (Table 4C, supplemental data). Their increased expression during the transition state indicates that they could play a role in the transdifferentiation of stellate cells. 5 tags identified in the partially activated stellate cells were not present in a public SAGE library of a normal liver, suggesting that they are specific for this cell type. Finally, in the fully transformed HSC expression of 83 genes was significantly different from that in both other stages. Of these, 49 tags (Table 5A, supplemental data) were only detected in the myofibroblasts. Apparently, the myofibroblast have a particular phenotype since 37 tags present in these cells were not found in a SAGE database of a normal liver. Tags representing 19 different genes were not expressed in quiescent stellate cells but had a low expression in the partially activated stellate cells as shown in Table 5B (supplemental data). The gradual upregulation of these genes suggest they may have a role in the activation of these cells. 12 of these tags were not found in normal liver, again indicating the particular character of hepatic myofibroblasts. All genes expressed exclusively in the myofibroblasts could play a role in phenotyping these fully transdifferentiated stellate cells.

Markers of activation of HSC

To validate our SAGE libraries we evaluated the expression of known markers of activated stellate cells including collagens, enzymes involved in matrix remodeling like uPA and its inhibitor PAI-1, metalloproteinases and their inhibitors, and genes coding for cytoskeletal proteins like actin β and γ, vimentin and α smooth muscle actin. As shown in Table 6, the expression of most of the established markers of fibrosis did increase upon activation, in particular the matrix-metalloproteinases MMP-1 and –3, and their inhibitors TIMP-1 and –2, whereas MMP-2 did not display a difference in expression. The upregulation in the myofibroblasts of procollagen-proline 4-hydroxylase (P4HB), an enzyme involved in the synthesis of collagens, is in agreement with the increased matrix production occurring in fibrotic liver. In a SAGE library of a normal human liver composed of total 66.308 tags of which 15.496 are unique, the expression of all these genes was much lower (see Table 6). Even when taking into account that only a few percent of mRNA from total liver will be derived from stellate cells, this small number of tags indicates that the expression of these genes is low in non-activated stellate cells present in normal liver. The expression of GAPDH, which as a housekeeping gene is used as a reference, is upregulated 2 fold upon activation.

Potential novel markers of HSC transformation

Genes of which the expression was statistically different and which could be identified as new markers of HSC transformation are listed in Table 7. Interesting is the difference in expression of several insulin-like growth factor binding proteins (IGFBP) and IGFBP-related Proteins (IGFBP-rP).

IGFBP-4, -5 and –6 were all upregulated during transformation of HSC and the expression of IGFBP-5 in the hepatic myofibroblasts is extremely high. IGFBP-rP1 (also known as IGFBP-7) showed the highest expression in the mid-phase of the transdifferentiation process. Other IGFBPs demonstrated a relatively low expression level that did not differ significantly between the three stages (IGFBP-3) or were below detection limit (like IGFBP-1 and–2).

Other genes that are induced and seem to be of interest are two members of the TGF-β antagonists, gremlin and follistatin. Gremlin is not present in normal stellate cells while in myofibroblasts 322 tags were found. This strong induction suggests a potential role of this member of the cysteine knot superfamily in stellate cell activation. Finally, two TGF-β induced genes are upregulated during the transdifferentiation process: TGF-β induced 68 kD (BIGH3) and TGF-β induced transcript 1 (TGFB1I1). The latter is not detected in the SAGE libraries of quiescent and partially activated HSC.

To identify potential markers for liver fibrosis we compared our SAGE data for these novel genes with a public SAGE library of a normal human liver. In this library no tags were present for several markers (see Table 6 and 7) including collagen Iα2, collagen VIα3, MMP-1, MMP-3, IGFBP-6, gremlin, follistatin and TGF-β induced transcript 1, indicating that these proteins are specific for (activated) stellate cells and could be suitable markers for liver fibrosis.

Confirmation of SAGE data by quantitative PCR

We selected 8 genes (1 housekeeping gene, 3 genes that are well-known markers of HSC activation and 4 genes that could be classified as putative new markers), for validation by reverse transcriptase quantitative PCR (RT-qPCR) analysis using the SYBR Green I based LightCycler method. The results of the SAGE data are summarized in Fig.1 giving the relative expression as found in the 3 SAGE libraries from quiescent HSC, partially activated HSC and fully transdifferentiated HSC, respectively. To be able to compare SAGE data (Fig. 1) with the RT-qPCR data (Fig. 2) both are normalized to the relative expression of GAPDH. However, since our SAGE data indicate that the GAPDH expression is induced 2 fold during stellate cell activation, this normalization results in a 2-fold underestimation of the actual effect. The expression of collagen Iα1 (Col1α1) and TIMP-1 was highly upregulated in the hepatic myofibroblasts. The increases by RT-qPCR and SAGE were similar. For all putative new markers tested, IGFBP-5, gremlin, follistatin and TGF-β induced 68 kD (BIGH3), the relative expression measured by RT-qPCR was comparable to that as measured by SAGE.

Expression in vivo of putative new markers of fibrosis

The novel markers of liver fibrosis identified by SAGE were present in mRNA isolated from human stellate cells cultured and activated *in vitro*. To investigate the potential of these novel markers *in vivo*, we decided to study their induction in an established animal model for fibrosis. We chose the *Mdr2 (-/-)* mouse model that develops a fibrotic liver upon a cholate containing diet (15). Histochemical analysis using Sirius Red demonstrated that after six weeks of cholate feeding, all *Mdr2 (-/-)* mice had developed moderate fibrosis. In contrast, the liver histology of wild type mice fed the same cholate diet was normal. To follow the development of fibrosis in these animals we selected collagen Iα1 and TIMP-1 as established markers of liver fibrosis. In addition, we determined the induction of three potential novel markers, gremlin, IGFBP-5 and IGFBP-7. The expression of all 5 genes was measured by RT-qPCR in total liver RNA. The expression of ColIα1 was upregulated 10 times in the livers of the *Mdr2 (-/-)* mice ($P< 0.005$) (Fig. 3A). The expression of TIMP-1 was not detectable in normal livers but in the fibrotic livers a faint but clear band was visible after PCR amplification (fig 3D). Quantification of TIMP-1 mRNA showed that in the fibrotic liver this gene was expressed at about 1 copy per 10^{8} copies of 18S

The three potential novel markers were clearly induced in the fibrotic livers. For two novel markers, IGFBP-5 and –7, we were able to determine the extent of induction in fibrotic liver, which were 9 fold ($P <0.005$) and 3 fold ($P <0.01$), respectively (fig 3 B and C) .No gremlin expression was detectable in all but one of the normal livers while a clear band was present upon PCR amplification of cDNA from the fibrotic livers (fig. 3 D). Similar to TIMP-1, the extremely low or no expression in normal liver does not allow quantification of the extent of induction of gremlin. However, using RT-qPCR quantification we found that in the fibrotic livers about 10 copies of gremlin

mRNA was present per 10 ^{8} copies of 18S. The upregulation of these three genes *in vivo* indicates that they seem suitable as novel markers for hepatic fibrogenesis.

DISCUSSION

The term transcriptome is used to describe the identity and multiplicity of mRNA expressed by a population of specific cells (19). The object of this study was to define such a profile for hepatic stellate cells during and after *in vitro* transformation from quiescent to partially activated and ultimately, completely transdifferentiated liver myofibroblasts. For this purpose we made use of RNA from human HSC directly after isolation, 15 days in culture and after 6 to 7 passages i.e. 5 to 6 weeks. The resulting expression profiles contain more than 10.000 genes and give a reliable and overall representation of the genes involved in the activation process. We limit the discussion to those genes of which the expression changes significantly. It seems evident that this group will contain genes that play an important role in stellate cell activation. Analysis of the transcriptome of the activated HSC shows a cell that is highly involved in synthesis of components of the extracellular matrix as illustrated in Table 6. Markers for transformation from quiescent HSC to activated myofibroblasts are α-smooth muscle actin and vimentin as previously identified by immunohistochemistry (20,21). This is in accordance with the relative high expression of these genes in the transcriptome of activated human HSC. Also, the induced expression level of genes involved in collagen synthesis, I > VI ≥ III, in the transcriptome of activated stellate cells is as expected. During fibrosis the synthesis and deposition of these fibril-forming collagens is strongly increased compared to that of collagen IV (22). The relative high expression of procollagen proline 4-hydroxylase, an enzyme involved in collagen synthesis, is also in accordance with an enhanced formation of collagen matrix. At the same time, the induced expression of inhibitors of metalloproteinases, TIMP-1 and –2, indicates that matrix degradation will be reduced. The enhanced expression of TIMPs will result in the inhibition of MMP-1 that, due to its interstitial collagenase activity, plays a pivotal role in the degradation of collagen –1 and –3. Furthermore, the induction of plasminogen activator inhibitor type 1 (PAI-1) during transdifferentiation indicates that matrix degradation will be reduced. Increased PAI-1 levels will impair the activation of plasminogen and will as such reduce the conversion of proMMP-1 into active MMP. The induction of these three inhibitors will result in a diminished breakdown of the fibril-forming collagens III and I (23) and thus lead to an increased deposition of these matrix components After six weeks of cholate feeding the expression of TIMP-1 is still low in the liver of the *Mdr2 (-/-)* mice, 1 copy of mRNA per 10^{8} copies of 18S. This relatively low expression seems to correlate with the increase seen in the intermediate state of activation: HSC cultured for 15 days (Table 6). This suggests that after 6 weeks of cholate feeding the stellate cells in the fibrotic liver have not yet completely transdifferentiated into myofibroblasts. In this context it is noteworthy that our SAGE data reveal that culturing HSC for 15 days results in an (partially) activated state. Culturing these cells for a longer period further enhanced especially the expression of the profibrogenic genes.

A novel finding in our SAGE is the relative high expression of insulin-like growth factor binding proteins upon stellate cell activation, especially in the end-stage. These IGFBPs modulate IGF-I and II activity. *In vitro*, the proliferation of HSC is enhanced by IGF-I (24,25). *In* vivo however, production of IGF-1 by HSC has recently reported to attenuate liver fibrosis (26). Furthermore, IGF-1 replacement therapy seems to benefit patients with liver cirrhosis (27). Since IGFBPs modulate IGF-I and II activity, the induction of these binding proteins may affect fibrosis. So far, six distinct IGFBPs have been identified, which differ in molecular mass and binding affinities for IGF-I and –II (28). In addition, low-affinity IGF binders termed IGFBP-related proteins (IGFBP-rP) have been found. IGFBP-7, also called IGFBP-rP1, is one of these low affinity binders of IGF-1. In the transcriptome of activated human HSC expression of five IGFBP family members is detected. Most prominent is the extremely high expression of IGFBP-5 that by far is the most abundant tag in the myofibroblasts. Also IGFBP-7 is highly expressed in these cells while a smaller induction is seen for IGFB-4, 6 and 3. No tags were found for IGFBP-2 and –1, which are secreted from hepatocytes (29). A similar expression pattern of these IGFBPs has been reported for rat HSC (30). In activated human HSC, Gentilini *et al* detected expression of IGFBP-1 through 6 using an RNAse protection assay (31).

These authors also reported excretion of IGFBP-2 through –6 to be differentially regulated by IGF-I and TGF-β. The discrepancy for IGFBP-1 and 2 expressions most likely is due to the purity of the stellate cell preparation or some cross-reactivity of the antibody. The modulation of IGF activity by IGBFPs is very complex. For instance soluble IGFBP-5 inhibits IGF-I induced mitogenesis through the IGF-1 receptor by forming inactive complexes with IGF-I (32). In contrast, ECM associated IGFBP-5 has a decreased affinity for IGF-I, resulting in enhanced bioavailability and mitogenic action of IGF-1 (33). The different induction patterns of the various IGFBPs will affect the ratio between these factors and IGF-1. Since the various factors have different affinities for IGF-I, these differences may be an important factor in determining the local autocrine and paracrine actions of IGF-I and –II.

In a gene expression profile (8,596 unique tags) of a normal human liver the expression of IGF-binding proteins could not be detected (34). However, in a public SAGE library of a normal human liver compromising 15.496 unique tags, 27 per 100.000 tags for IGFBP-2 are present. Since we do not detect any tags in our HSC, it seems evident that other liver cells, most likely the hepatocytes (29), produce this protein. The relatively low number of tags for IGFBP- 3 and 4 in HSC in comparison to normal liver indicates that other liver cells produce these factors too. In contrast, the low level of IGFBP 5, 6 and 7 in total liver compared to HSC, indicates that stellate cells are the main or even the only source of these factors in liver. The striking upregulation of IGFBP-5 and –7 observed in the SAGE shows that both are good markers for stellate cell activation. Furthermore, because both appeared specific for stellate cells, they seem promising markers for liver fibrosis. Our subsequent *in vivo* studies in the *Mdr2 (-/-)* mice, confirmed the upregulation of both factors upon the development of liver fibrosis thereby validating their potential as a novel *in vivo* marker.

Another original finding is that two genes coding for transforming growth factor superfamily antagonists, gremlin and follistatin, are strongly upregulated in fully transdifferentiated myofibroblasts. Both are not detected in the SAGE library of a normal human liver and may therefore be novel and specific markers for fibrosis (Table 7). Furthermore, because both are secreted proteins they may be suitable as serum markers of fibrogenesis.

Gremlin is a 184 amino acid member of the cysteine knot superfamily. By binding to bone morphogenic proteins (BMPs) gremlin inhibits their activity and as such plays a role in growth and differentiation during embryonic development (35). Recently, increased expression of this embryonic factor was also reported in several models of diabetic nephropathy (36). We showed induction of gremlin in activated human stellate cells and subsequently in the fibrotic livers of *Mdr2 (-/-)* mice. Administrating BMP-7 inhibits the development of kidney fibrosis and may even reduce pre-existing fibrosis in the kidney (37). This suggest that induction of gremlin may have a pro-fibrotic effect, indicating that inhibition of gremlin expression could be beneficial for patients suffering from liver- or kidney fibrosis

Follistatin mainly binds activin but can also bind several BMPs, albeit with a much lower affinity. It has been reported earlier that follistatin is induced both in patients with hepatocellular carcinoma and in patients with alcoholic cirrhosis (38). Activin A was shown to activate cultured rat hepatic stellate cells increasing the expression of α-SMA and collagen (39). In that study follistatin blocked not only the effect of activin A but also the effect of TGF-β on the expression of type I collagen. Similarly, follistatin inhibited TGF-β induced secretion of collagen from HSC. In that respect, the increased expression of follistatin could have an inhibitory effect on stellate cell activation. A recent paper showed that follistatin reduced fibrosis in CCl_4 exposed rats with 32%. Follistatin not only caused a dose dependent decrease in HSC proliferation but also reduced apoptosis of hepatocytes with 87% (40).

Finally, increased expression of two genes coding for TGF-β inducible proteins, i.e. TGF-β induced 68kD (BIGH3) and TGF-β induced transcript I (TGFB1I1), was observed in fully transdifferentiated myofibroblasts. In normal liver expression of these genes is not detected or only at a low level. BIGH3 has been identified after treatment of an adenocarcinoma cell line with TGF-β and thus has been associated with processes involving TGF-β. However, the physiological role of BIGH3 and its encoded protein keratoepithelin is still unknown. Mutations in the gene are linked with corneal dystrophies and in the mouse embryonic expression of BIGH3 is observed in the mesenchyme of

numerous tissues throughout all the development stages (41). TGFB1I1, also known as HIC-5, was first isolated as a TGF-β inducible gene in mouse osteoblastic cells and belongs to the paxillin family. Its localization at focal adhesions and structural features suggests that HIC-5 plays some role as an adaptor molecule in integrin signaling thereby modulating cellular phenotypes especially cells of mesenchymal origin as fibroblasts, osteoblasts and myoblasts (42). In this context, the relative high expression of Disabled-2 (DAB2) found only in the fully transdifferentiated HSC is also remarkable (Table 7). DAB2 is an essential component of the TGF-β signaling pathway involved in transmission of TGF-β signaling from the TGF-β receptors to the Smad family of transcriptional activators (43). The induction of these two factors may therefore also have a role in modulating the transdifferentiation of stellate cells.

In conclusion, we constructed SAGE libraries of human hepatic stellate cells in three stadia of activation and identified the corresponding characteristic gene expression profile. In addition to transcripts encoding proteins known to be induced in hepatic fibrosis, a number of transcripts were identified that were not yet associated with HSC transformation. Subsequently we showed that the induction of both established and novel factors also occurs *in vivo*. The identification of these novel factors may not only provide new insights into the development of liver fibrosis but may also offer new serum markers for liver fibrogenesis. Finally, the comparison of the transcriptomes of quiescent, partially activated HSC and fully transdifferentiated myofibroblasts provides an appreciation of the sequence and timing of the fibrotic process in general.

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FOOTNOTES

We wish to thank Dr. D. Geerts and Dr. P.H. Reitsma for helpful discussions. This work was partially supported by grants from The Dutch Digestive Diseases Foundation.

ABBREVIATIONS

HSC, hepatic stellate cells; SAGE, serial analysis of gene expression; IGFBP, insulin-like growth factor binding protein; ECM, extracellular matrix; α-SMA, alpha smooth muscle actin; PDGF, platelet derived growth factor; TGF-β, transforming growth factor beta; CGAP, Cancer Genome Anatomy Project; *Mdr2* (-/-), disrupted *Mdr2* P-glycoprotein gene; DMEM, modified Dulbecco's medium; RT-qPCR, Reverse

Transcriptase quantitative Polymerase Chain Reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; P4HB, procollagen proline 4-hydroxylase; IGF, insulin-like growth factor; IGFBP-rP, insulin-like growth factor binding protein related protein; BIGH3, transforming growth factor beta induced 68 kD; TGFB1I1, transforming growth factor beta induced transcript; Col1α1, collagen Iα1; BMP, bone morphogenic protein; DAB-2, Disabled 2.

FIGURE LEGENDS

Fig. 1. Relative expression profile of standard and putative novel markers for transdifferentiation of hepatic stellate cells to myofibroblasts as measured by serial analysis of gene expression.
At the indicated stages of transdifferentiation RNA was isolated from the cultured cells and analyzed by serial analysis of gene expression (SAGE). The expression of the different genes is shown relative to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following genes were analyzed as standard markers of transdifferentiation: alpha-smooth muscle actin (α-SMA), collagen I alpha 1 (Col1α1) and tissue inhibitor of metalloproteinases 1 (TIMP-1). As putative novel markers of transdifferentiation the following genes were analyzed: insulin-like growth factor binding protein 5 (IGFBP-5), gremlin, follistatin (FST) and TGF-β induced 68kD (BIGH3).

Fig. 2. Relative expression profile of standard and putative novel markers for transdifferentiation of hepatic stellate cells to myofibroblasts as measured by quantitative real-time PCR.
At the indicated stages of transdifferentiation mRNA was isolated from the cultured cells, transcribed to cDNA and analyzed by quantitative real-time PCR. All points were measured in quadruplicate with the corresponding standard deviation. The expression of the different genes is shown relative to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following genes were analyzed as standard markers of transdifferentiation: alpha-smooth muscle actin (α-SMA), collagen I alpha 1 (Col1α1) and tissue inhibitor of metalloproteinases 1 (TIMP-1). As putative novel markers of transdifferentiation the following genes were analyzed: insulin-like growth factor binding protein 5 (IGFBP-5), gremlin, follistatin (FST) and TGF-β induced 68kD (BIGH3).

Fig. 3 Expression of collagen Iα1, IGFBP-5, IGFBP-7, tissue inhibitor of metalloproteinases-1 (TIMP-1) and gremlin in RNA isolated from livers of FVB *Wild type* mice (n=6) and FVB mice with a disrupted *Mdr2* P-glycoprotein gene [*Mdr2 (-/-)*] (n=6), fed a 0.03% cholate diet for 6 weeks.
(A) Collagen Iα1 expression relative to 18S ($x10^{-6}$) as measured by Reverse transcriptase quantitative Polymerase Chain Reaction. *Wild type*: 2.1 vs *Mdr2 (-/-)*: 21.2, # $p<0.005$
(B) IGFBP-5 expression relative to 18S ($x10^{-6}$) as measured by Reverse transcriptase quantitative Polymerase Chain Reaction. *Wild type*: 1.7 vs *Mdr2 (-/-)*: 15.6, # $p<0.005$
(C) IGFBP-7 expression relative to 18S ($x10^{-6}$) as measured by Reverse transcriptase quantitative Polymerase Chain Reaction. *Wild type*: 91 vs *Mdr2 (-/-)*: 293, * $p<0.01$
(D) Agarose gel analysis of RT-qPCR products of TIMP-1 and gremlin in 6 individual *Wild type* mice (1-6) and 6 *Mdr2 (-/-)* mice (1-6). Amplified products were separated using 2% agarose gels and then visualized with ethidium bromide and UV.

Table 1A. Sequences of primers used for quantitative real-time PCR of cDNA from human stellate cells and myofibroblasts.

Gene	Accession nr.	Primer sequence

glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	M33197	Forward: CCGTCTAGAAAAACCTGCC Reverse: AGCCAAATTCGTTGTCATACC
α-smooth muscle actin (α-SMA)	NM_001613	Forward: CTGTTCCAGCCATCCTTCAT Reverse: CGGCTTCATCGTATTCCTGT
collagen Iα1 (COLIα1)	NM_000088	Forward: GAACATCACCTACCACTGCA Reverse: GTTGGGATGGAGGGAGTTTA
tissue inhibitor of metalloproteinases 1 (TIMP-!)	A10416	Forward: AGACGGCCTTCTGCAATTCC Reverse: GAAGCCCTTTTCAGAGCCTTG
insulin-like growth factor binding protein 5 (IGFBP-5)	NM_000599	Forward: GAAGCAGTGAAGAAGGACCG Reverse: GAATCCTTTGCGGTCACAAT
gremlin (GREM 1)	AF110137	Forward: GCAAATACCTGAAGCGAGAC Reverse: CGATGGATATGCAACGACAC
follistatin (FST)	NM_013409	Forward: TGTGGTGGACCAGACCAATA Reverse: CCGAAATGGAGTTGCAAGAT
transforming growth factor beta induced 68 kD (BIGH3)	M77349	Forward: ATCTCCACCATCACCAAC Reverse: TCCAGAGAGATGATTGCC

Table 1B. Sequences of primers used for quantitative real-time PCR of cDNA from mouse liver.

Gene	Accession nr.	Primer sequence
ribosomal protein 18S (18S)	AY248756	Forward: TTCGGAACTGAGGCCATGAT Reverse: CGAACCTCCGACTTTCGTTCT
collagen Iα1 (COL1α1)	NM_007742	Forward: ACCTCAAGATGTGCCACTC Reverse: TGCTCTCTCCAAACCAGAC
Insulin-like growth factor binding protein 5 (IGFBP-5)	NM_010518	Forward: AAACAGCAAGGCAGAAAGGA Reverse: TCTAGCCATCCACACTGCTG
Insulin-like growth factor binding protein 7 (IGFBP-7)	NM_008048	Forward: GGAAAATCTGGCCATTCAGA Reverse: ATTTCATGGAGGGCATCAAC
gremlin (GREM 1)	NM_011824	Forward: CCCCAGGCACATCCGAA Reverse: CAAGTCGATATGCAACG
tissue inhibitor of metalloproteinases 1 (TIMP-1)	X69413	Forward: TGCCTGCTGCGATTACAACC Reverse: GGAATGGTGTGGTGATGCATGG

Table 2. Tag distribution in the 3 stages of transdifferentiation of hepatic stellate cells to myofibroblasts as measured by serial analysis of gene expression.

	HSC fresh	%*	HSC 15 days	%*	Myofibroblasts	%*
Total	16187		28486		13353	
Unique	7775	100%	10188	100%	6305	100%

count 1	6105	78,52%	7248	71,14%	4898	77,68%
count 2-5	1236	15,90%	2118	20,79%	1038	16,46%
count 5-10	252	3,24%	456	4,48%	195	3,09%
count 10-20	114	1,47%	188	1,85%	105	1,67%
count >20	68	0,87%	178	1,75%	69	1,09%

* percentage relative to unique tags

Table 6. Relative expression of GAPDH and standard markers for transdifferentiation of hepatic stellate cells to myofibroblasts. Relative expression in 3 stages of transdifferentiation and in a normal liver as measured by serial analysis of gene expression. The relative expression levels of all genes are based on number of tags per 100.000.

	Expression level*			
GENE	**HSC fresh**	**HSC 15 days**	**Myofibroblasts**	**Liver**
GAPDH	465	843	764	170
collagen I α1	227	503	853	3
collagen I α2	104	176	778	0
collagen III α1	55	49	436	6
collagen VI α1	55	98	327	14
collagen VI α2	0	4	366	3
collagen VI α3	0	0	22	0
TIMP-1	31	18	412	2
TIMP-2	73	141	90	5
MMP-1	0	0	112	0
MMP-2	37	25	24	3
MMP-3	0	0	22	0
P4HB	0	18	157	40

uPA	0	0	22	0
PAI	43	137	12	12
α-SMA	73	155	142	2
vimentin	86	60	157	2
actin β	98	221	562	20
actin γ1	116	109	277	12
actin γ2	6	46	0	2

*normalized to number of tags per 100.000

Table 7. Relative expression of putative novel markers for transdifferentiation of hepatic stellate cells to myofibroblasts. Relative expression in 3 stages of transdifferentiation and in a normal liver as measured by serial analysis of gene expression. The relative expression levels of all genes are based on number of tags per 100.000.

	Expression level*			
GENE	**HSC fresh**	**HSC 15 days**	**Myofibroblasts**	**Liver**
IGFBP-1	0	0	0	2
IGFBP-2	0	0	0	27
IGFBP-3	12	14	22	5
IGFBP-4	80	137	240	116
IGFBP-5	61	112	2635	5
IGFBP-6	0	11	127	0
IGFBP-7	1096	1994	419	70
Gremlin	0	18	322	0
Follistatin	0	7	112	0
TGF-β induced 68kD	110	134	299	9
TGF-β induced transcript I	0	0	30	0
Disabled 2	0	0	37	0

*normalized to number of tags per 100.000

Fig. 1

SAGE normalized GAPDH

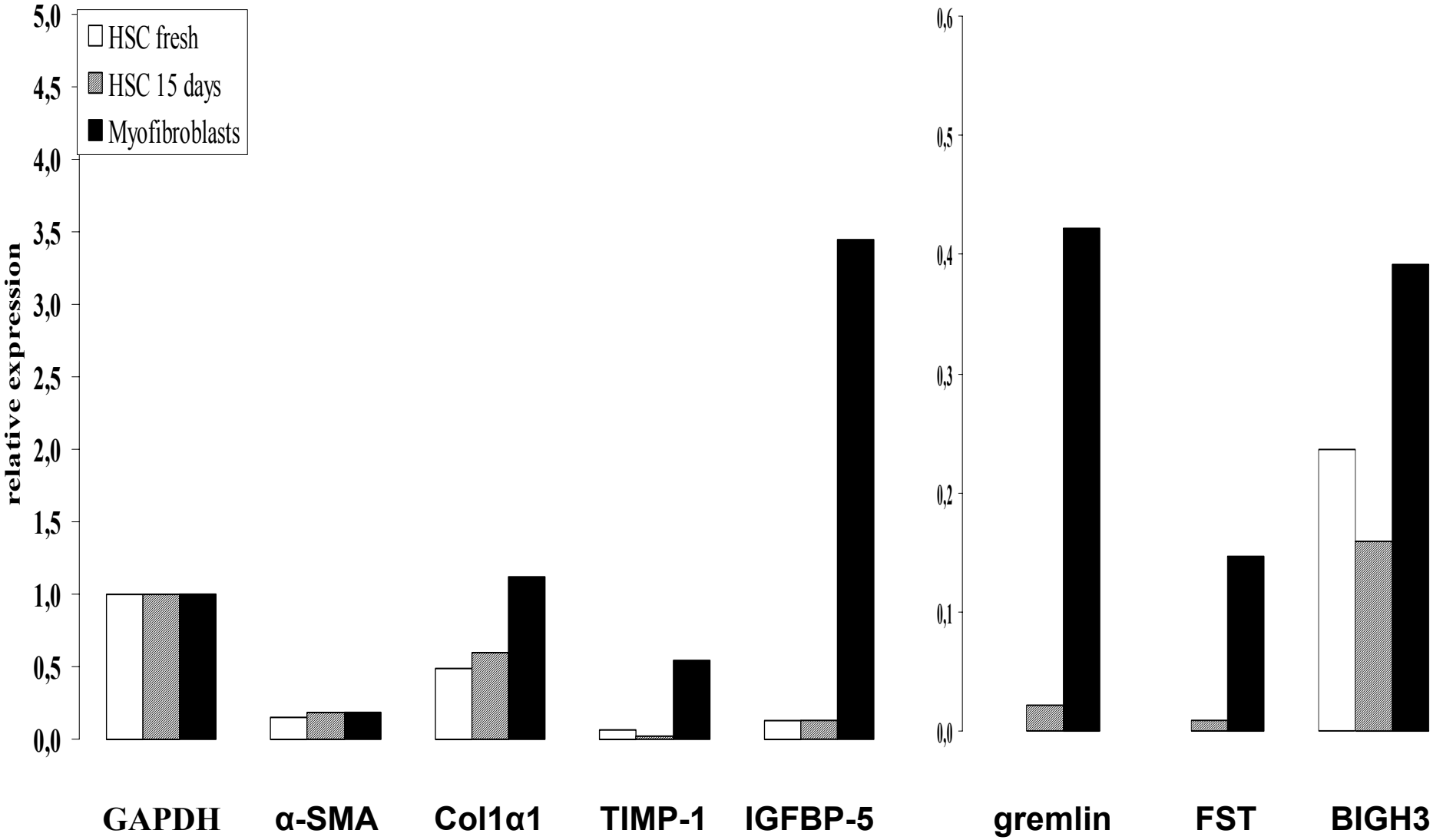


Fig. 2

RT-qPCR

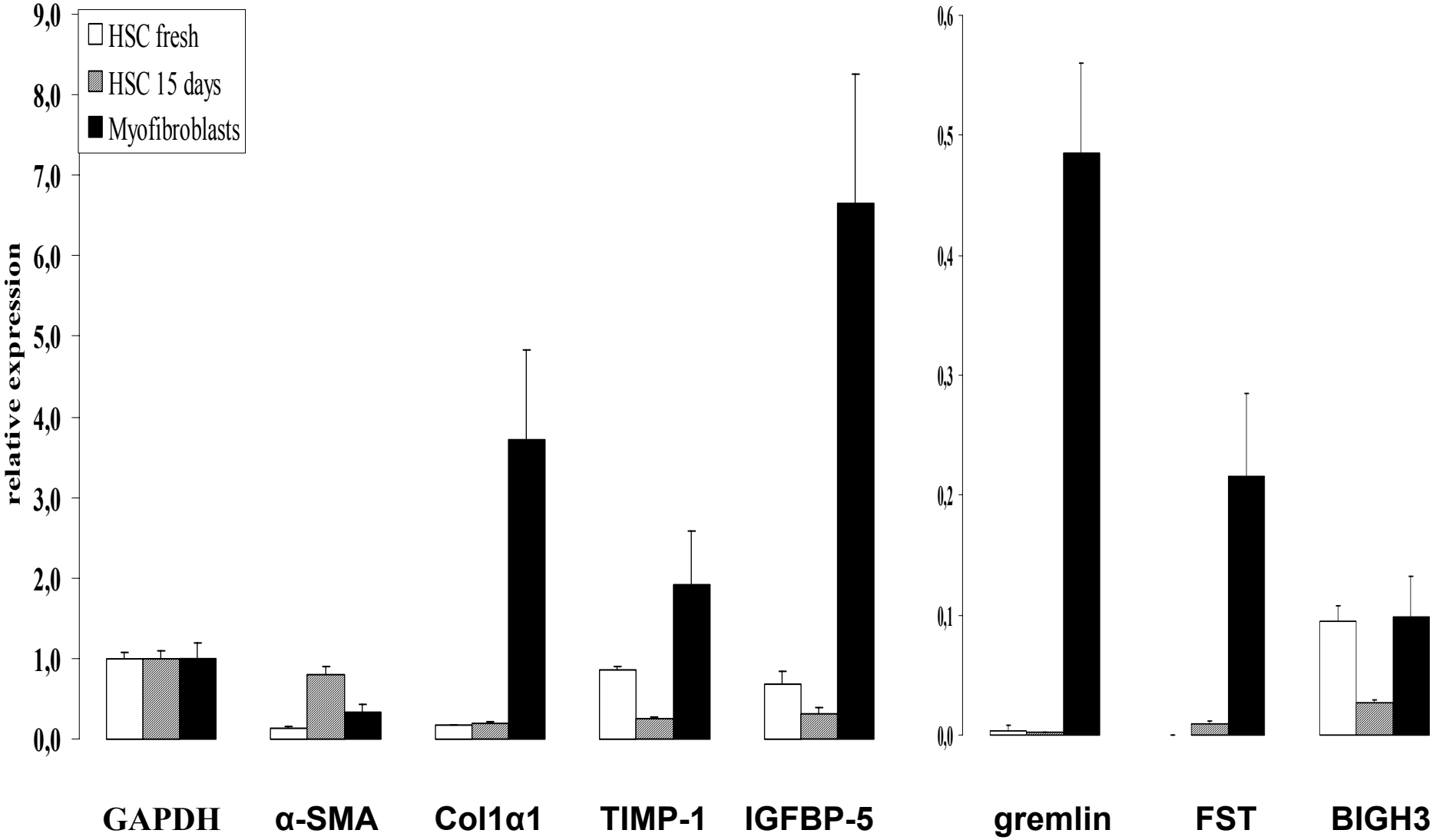
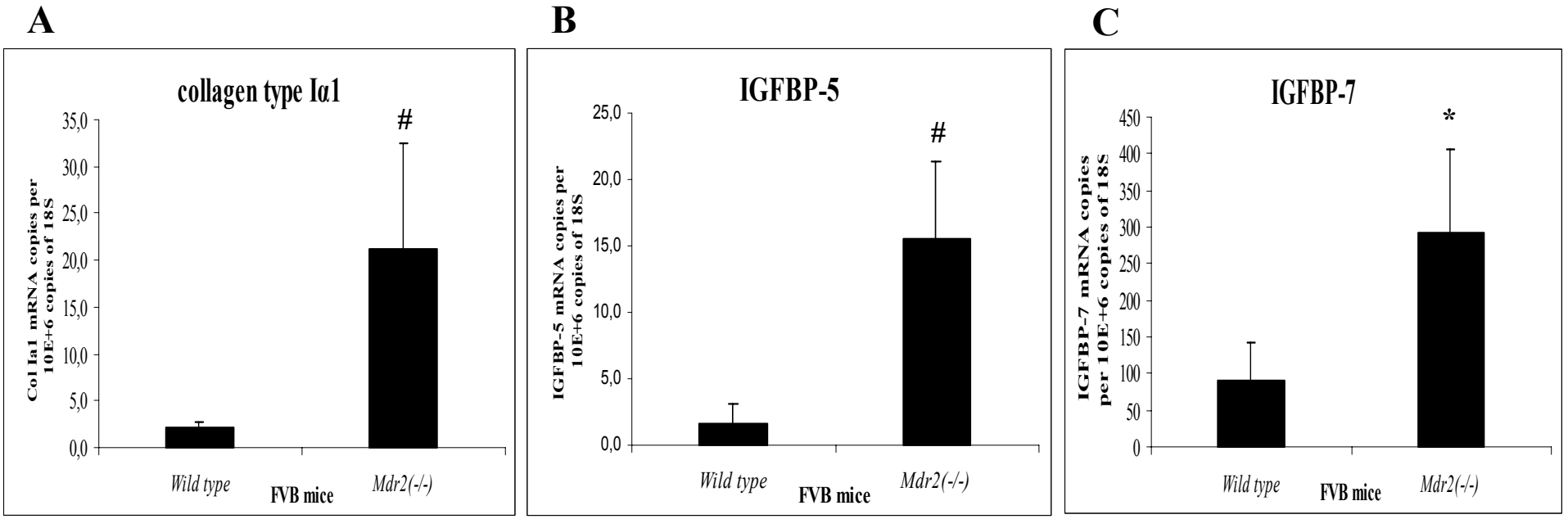
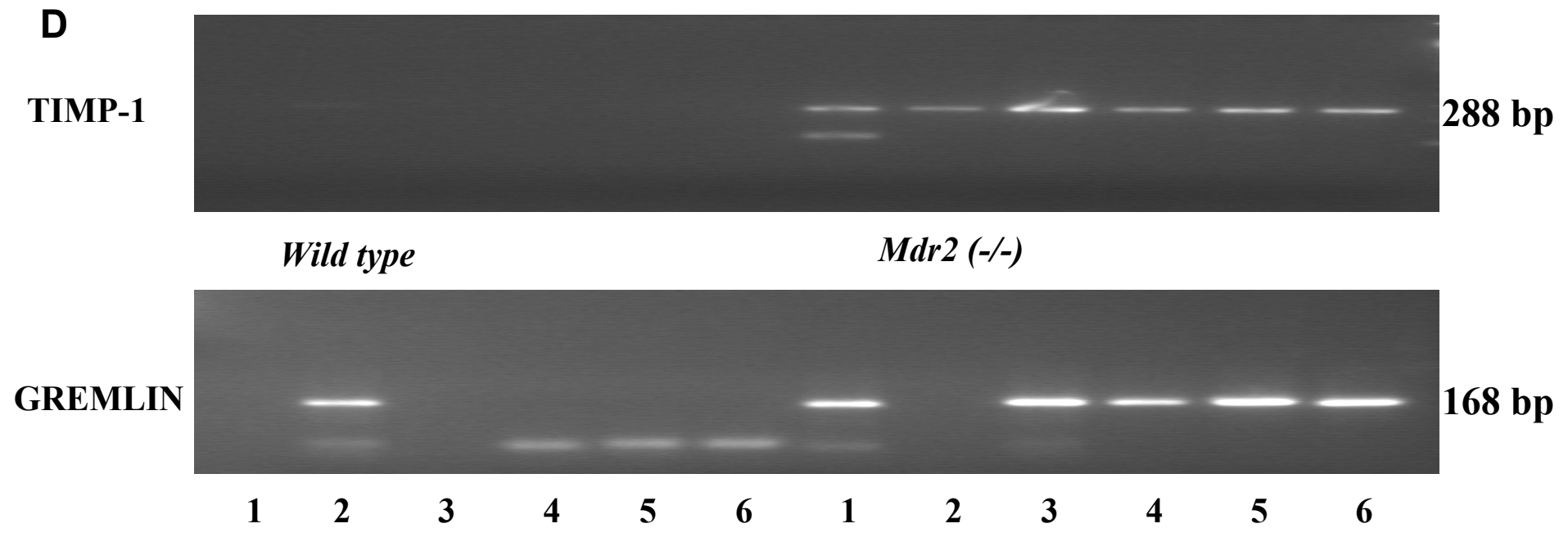

HSC fresh
HSC 15 days
Myofibroblasts
relative expression
9,0
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0,6
0,5
0,4
0,3
0,2
0,1
0,0
GAPDH
α-SMA
Col1α1
TIMP-1
IGFBP-5
gremlin
FST
BIGH3

Fig 3


A
collagen type Iα1
Col Ia1 mRNA copies per 10E+6 copies of 18S
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
#
Wild type
Mdr2(-/-)
FVB mice
B
IGFBP-5
IGFBP-5 mRNA copies per 10E+6 copies of 18S
25,0
20,0
15,0
10,0
5,0
0,0
#
Wild type
Mdr2(-/-)
FVB mice
C
IGFBP-7
IGFBP-7 mRNA copies per 10E+6 copies of 18S
450
400
350
300
250
200
150
100
50
0
*
Wild type
Mdr2(-/-)
FVB mice


D
TIMP-1
288 bp
Wild type
Mdr2 (-/-)
GREMLIN
168 bp
1 2 3 4 5 6 1 2 3 4 5 6

Transcriptional profiling reveals novel markers of liver fibrogenesis: Gremlin and insulin-like growth factor binding proteins

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J. Biol. Chem. published online April 10, 2006
