

# Gene expression profiles during activation of cultured rat hepatic stellate cells by tumoral hepatocytes and fetal bovine serum

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## Abstract

**Purpose** Hepatic stellate cells (HSCs) transdifferentiate to become extracellular matrix-producing myofibroblasts during liver injury. Myofibroblasts can also promote invasion and metastasis of hepatocellular carcinoma (HCC). In this study, we determined gene expression changes in two different models of HSC activation, induction-activated HSCs (iHSCs) and culture-activated HSCs (cHSCs).

**Methods** Hepatic stellate cells were isolated by density centrifugation and exposed to conditioned medium (CM) from the rat HCC cell line C5F, and fetal bovine serum (FBS). Expression of 27,100 genes in quiescent HSCs, cHSCs and iHSCs was analyzed by microarray and was confirmed on a subset of genes by real-time RT-PCR and Western blot.

**Results** One thousand nine hundred sixty-seven genes were differentially expressed in cHSCs and iHSCs, including genes that encode proinflammatory factors, adhesion molecules, cell surface receptors, signaling transduction and immune factors such as *Il1a*, *Vcam1*, *Ccl6*, *Ilr7*, *PRAP*, osteopontin, *Gp39*, *Raf1*, *Rac2*, *Adam17*, *Wnt6*, *MMP-9*, and *Cfd*. C5F-CM-induced activation only partially

reproduced the gene expression changes observed during FBS culture activation. iHSCs showed specific gene expression, suggesting that HCC cells can specifically induce HSC activation.

**Conclusions** Induction-activated HSCs' gene expression patterns were partially similar to and different from that of cHSCs. iHSCs might play an important role in invasion and metastasis of HCC. This study provided theoretical foundations for investigating the biology of HSCs in HCC.

**Keywords** Hepatocellular carcinoma · Cultured cells · cDNA microarray · Gene expression profiles · Hepatic stellate cells

## Abbreviations

HSCs	Hepatic stellate cells
ECM	Extracellular matrix
$\alpha$ -SMA	$\alpha$ -Smooth muscle actin
FBS	Fetal bovine serum
qHSCs	Quiescent HSCs
cHSCs	Culture-activated HSCs
iHSCs	Induction-activated HSCs
HCC	Hepatocellular carcinoma

Y. Xia and R. Chen contributed equally to this study.

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## Introduction

Hepatocellular carcinoma (HCC) is one of the major causes of cancer-related death throughout the world. Several studies have shown that the stroma of HCC is markedly infiltrated with myofibroblasts, which are identified through their positive staining with an antibody directed against  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Schmitt-Gräff et al. 1991; Enzan et al. 1994; Terada et al. 1996; Aishima et al. 2008). Myofibroblasts are a key player in the control of tumor cell

**Table 1** RT-PCR primers

Gene symbol	Primer sequence	Product size
Il7r-5'	5'-ACAGAGAAGGTGACGGAAATAGGT-3'	99
Il7r-3'	5'-ACTGGAGAGGAAATGGTTGAGG-3'	
Cystatin F-5'	5'-GCCTTGAAGCGGACATTACAC-3'	93
Cystatin F-3'	5'-AAGTCAGTGGCAGCGGAGA-3'	
Rac2-5'	5'-CTGGACCTTCGAGATGACAAGGA-3'	141
Rac2-3'	5'-TCGCTGGGTGAGTGCAGAAC-3'	
Raf1-5'	5'-AACAGTGAAGTCGCGCTGGA-3'	145
Raf1-3'	5'-CAGCACAATGCCATAGGAGTAGACA-3'	
Vcam1-5'	5'-GATCTTCGGAGCCTCAACGGTA-3'	131
Vcam1-3'	5'-CTGTGGTGTGCAAGTCAGGA-3'	
Cd44-5'	5'-GCTTTCGCAAGCCAGTTGGTA-3'	190
Cd44-3'	5'-GGCATCCTGGCAGATGAACA-3'	
Sdc2-5'	5'-AATCTGTTCAAGCGGACGGAAG-3'	167
Sdc2-3'	5'-TTCTGGTAAGCTGCGCTGGAC-3'	
Ctsl-5'	5'-GGCAATCAGGGCTGTAATGGA-3'	145
Ctsl-3'	5'-CGTTAGCCACAGCATACTCAGCTC-3'	
Il1a-5'	5'-GGAGGCCATAGCCCATGATTTAG-3'	68
Il1a-3'	5'-TATTCTGGAAGCTGTGAGGTGCTG-3'	
Adam17-5'	5'-CCTGAACAACGACACCTGCTG-3'	112
Adam17-3'	5'-CTTCTGGGCCGTCTCAAACCTG-3'	
Ccr1-5'	5'-GGTTGGGACCTTGAACCTTGAAT-3'	143
Ccr1-3'	5'-AGGAACTGGTCAGGAACAATAGCTT-3'	
Jun-5'	5'-CTGCCACCGAGACCGTAAAGA-3'	88
Jun-3'	5'-GCTAGCACTCGCCCAACTTCA-3'	
Tgfb2-5'	5'-CCCTGCTGTACCTTCATACCGTCTA-3'	102
Tgfb2-3'	5'-GGCATATGTGGAGGTGCCATC-3'	
Wnt4-5'	5'-AGGTGTGGCCTTTGCAGTGAC-3'	138
Wnt4-3'	5'-GCTACGCCATAGGCGATGTTG-3'	
Ifngr1-5'	5'-GAAACCATGTTTGGTGACGGAAATA-3'	174
Ifngr1-3'	5'-ACAGTAATTGGAATTCAGCGTGGAC-3'	
Il13ra2-5'	5'-AGATACGTACGCACTTGTGAGAGCA-3'	167
Il13ra2-3'	5'-AGGCTTCCAAGAGCAGACCAAATA-3'	
Colla15'	5'-TGAGCCAGCAGATTGAGAAC-3'	150
Colla13'	5'-TGATGGCATCCAGGTTGCAG-3'	
CCL24-5'	5'-CTGTGACCATCCCCTCATCT-3'	204
CCL24-3'	5'-TTCTCTTGGCATCCAGGTTTC-3'	
MMP9-5'	5'-TTCGACGCTGACAAGAAGTG-3'	156
MMP9-3'	5'-AGGGAGTCCTCGTGGTAGT-3'	
$\alpha$ -SMA-5'	5'-CGTGCCTGACATTAAGAGAAGC-3'	205
$\alpha$ -SMA-3'	5'-TGGATGCCACAGGATTCATACC-3'	
MT-2-5'	5'-GATCTTGAGATGTGCGCTAGAGC-3'	183
MT-2-3'	5'-TTGAACTGAACTAATTGGGTCGAA-3'	
PRAP-5'	5'-CGTTCGCACTTACTCGCCTTCAT-3'	125
PRAP-3'	5'-AAGTGACCTTAATTTGAACATCGT-3'	
ET-1-5'	5'-GCGCTGCGAAGCGAGGCCACTTC-3'	89
ET-1-3'	5'-GTACTTATACTCAGATGGGTGGA-3'	
PIP4-5'	5'-TACCCGATGCTGAACATGTC-3'	112
PIP4-3'	5'-TCTTCACTTTCTCACACTAAC-3'	

**Table 1** continued

Gene symbol	Primer sequence	Product size
c-Myc-5'	5'-TGGGCCGACGCGACGAGGCGACGGC-3'	205
c-Myc-3'	5'-GGTTGAGAACGTTGAAGAATGTTGA-3'	
Tlr4-5'	5'-AAGATTGAAGGGAGGACTCTACCGA-3'	193
Tlr4-3'	5'-ATCACATGAAATAATAATCACATGA-3'	
OSP-5'	5'-CTCGGTGGTCGTCGTCCTGACTCC-3'	125
OSP-3'	5'-ACTTATTTAGAACATAGAACTTACA-3'	
LI-1R $\alpha$ -5'	5'-TTGTCTCATTGTGCTCTGCTGTC-3'	137
LI-1R $\alpha$ -3'	5'-GCTGATGAATCCTGGAGTCCTTGTC-3'	
Robo-1-5'	5'-TACTTTACCTTTGTAGAAGGAGAG-3'	213
Robo-1-3'	5'-TTCTTAATCAGGTTTGACTTTGAC-3'	
CD72-5'	5'-CCTCTCCTCGGTCCCTCCACACCA-3'	98
CD72-3'	5'-CTCGGTCCGTCAAATAAAATT-3'	
Il7r-5'	5'-TCTCGACCCAAACCAGAGGGAGGA-3'	164
Il7r-3'	5'-ATTTCTTACCCGTTTAAAGAACCT-3'	

All primers were designed to have an optimum annealing temperature of 60°C

behavior and are very rare in normal liver tissues (Desmoulière et al. 2004). They are, however, abundant in diseases such as liver fibrosis and cirrhosis (Svegliati-Baroni et al. 2008). In these cases, although some data show that they could be derived in part from portal fibroblasts (Tuchweber et al. 1996), most studies suggest that they originate through the phenotypic modulation of hepatic stellate cells (HSCs) (Svegliati-Baroni et al. 2008; Musso et al. 1997). HSCs constitute approximately 8–14% of cells in the normal liver and are the primary site for retinoid storage in the body (Bataller and Brenner 2005). Liver fibrosis is characterized by progressive accumulation of extracellular matrix (ECM) proteins, synthesized by activated HSCs. When HSCs are isolated and grown in dishes in DMEM/10% fetal bovine serum (FBS), they spontaneously undergo similar activation (Rockey et al. 1992). Moreover, rat and human tumoral hepatocyte-conditioned medium (CM) were able to directly activate cultured HSCs, which could lead to specific features that would distinguish it from fibrosis-type activation (Nhieu et al. 1998; Faouzi et al. 1999a, b). To discover genes involved in activation of HSCs, several studies used DNA microarrays to compare gene expression between normal and cirrhotic livers (Honda et al. 2005; Lau et al. 2005; De Minicis et al. 2007). The differentially expressed genes identified include ECM proteins, genes involved in liver regeneration, proinflammatory genes and genes encoding serum proteins. However, it is not known whether culture activation of HSCs can reproduce the underlying changes in gene expression of HSC activation in HCC, and whether it represents a suitable model to study in vivo HSC activation. Moreover, it is not known whether HSC activation in HCC results in a stimulus-specific activation of HSCs with certain gene expression patterns. To answer these questions, we prepared rat C5F cell line CM and induced HSC activation in vitro. Subsequently, we performed DNA

microarray studies and compared gene expression profiles between culture-activated HSCs (cHSCs) and induction-activated HSCs (iHSCs), aiming to provide theoretical foundations for investigating the biology of HSCs in HCC.

## Materials and methods

### Animal and cell line

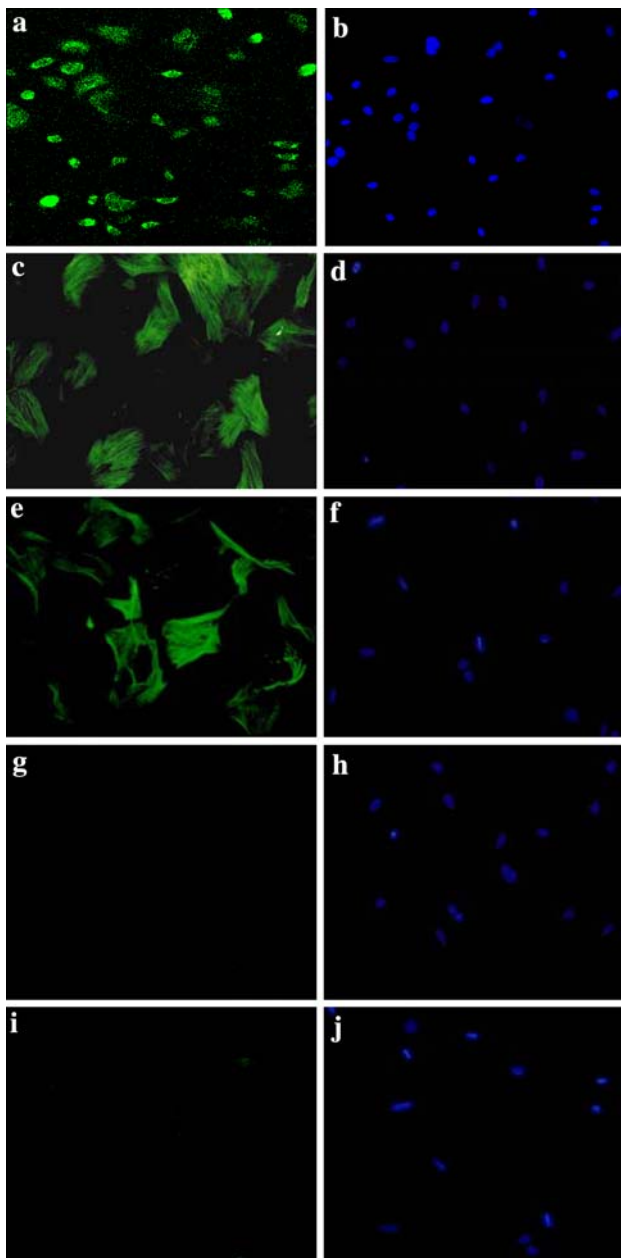
F344 rats (200–250 g) were obtained from Charles River Laboratory (USA). The rat C5F cell line was a gift from Dr. Kumiko Ogawa (First Department of Pathology, Nagoya City University, Japan). All animals received humane care according to the criteria outlined by Zhongshan Hospital, Fudan University, China.

### Preparation of CM

Conditioned medium was prepared as described previously (Faouzi et al. 1999a, b), with some modifications. Briefly, C5F cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. When subconfluent, cells were washed and incubated with serum-free DMEM for 2 h. This medium was then discarded and the cells were incubated with serum-free DMEM again. After 24 h, this CM was harvested and centrifuged at 400g for 8 min to remove debris, filtered through a 0.22  $\mu$ m filter, and stored at  $-20^{\circ}\text{C}$  until use.

### Isolation and culture of rat HSCs

Rat HSCs were isolated from rat livers by perfusion of collagenase and pronase, followed by centrifugation over a Nycodenz gradient, as described previously (Siegmund



**Fig. 1** Characterization of HSCs isolated from rat liver. After isolation, the cells were cultured for 2 days (quiescent HSCs, qHSCs), or for 7 days (culture-activated HSCs, cHSCs), or activated using C5F-CM for 7 days (induction-activated HSCs, iHSCs). **a** Immunofluorescence analysis of desmin expression in qHSCs. **c**  $\alpha$ -SMA expression in cHSCs. **e**  $\alpha$ -SMA expression-positive iHSCs. **g** Negative controls without primary antibody were performed in activated HSCs. **i**  $\alpha$ -SMA expression-negative qHSCs. DAPI nuclear staining for all conditions is shown in **b, d, f, h** and **j** ( $\times 100$ )

et al. 2006). After isolation, the cells were cultured in DMEM supplemented with 10% FBS for 2 days, when they were harvested as quiescent HSCs (qHSCs) (Jiang et al. 2006). To prepare cHSCs, HSCs were cultured in uncoated plastic dishes in DMEM supplemented with 10% FBS for a total of 7 days (Jiang et al. 2006). To prepare in vitro

iHSCs, HSCs were cultured in DMEM supplemented with 10% FBS for 24 h. Cells were then washed three times with serum-free DMEM and incubated with rat HCC-CM (CM diluted 1/2 with serum-free DMEM) for 6 additional days (Faouzi et al. 1999a, b). Cell purity was determined by desmin immunofluorescence staining and the typical light microscopic appearance of the lipid droplets as described previously (Liu et al. 2003). HSC activation was assessed by  $\alpha$ -SMA immunofluorescence staining. Immunofluorescence staining for desmin and  $\alpha$ -SMA were performed using anti-desmin and anti- $\alpha$ -SMA antibody (Boster, Wuhan, China). Cells were counterstained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR). DAPI (Sigma, Germany) stain was used to stain the nuclei. Negative controls were performed without primary antibody.

#### DNA microarray analysis

Total RNA extracted from quiescent, culture-activated and induction-activated rat HSCs was used for DNA microarray analysis. Two independent isolations and microarray analysis (experiment 1 and experiment 2) were performed using 27K Rat Genome Array chips (SmartArray<sup>TM</sup>, CapitalBio Corp., Beijing, China), according to the manufacturer's instructions. Data analysis was performed using Tomodel and CB-MAS V4.0.

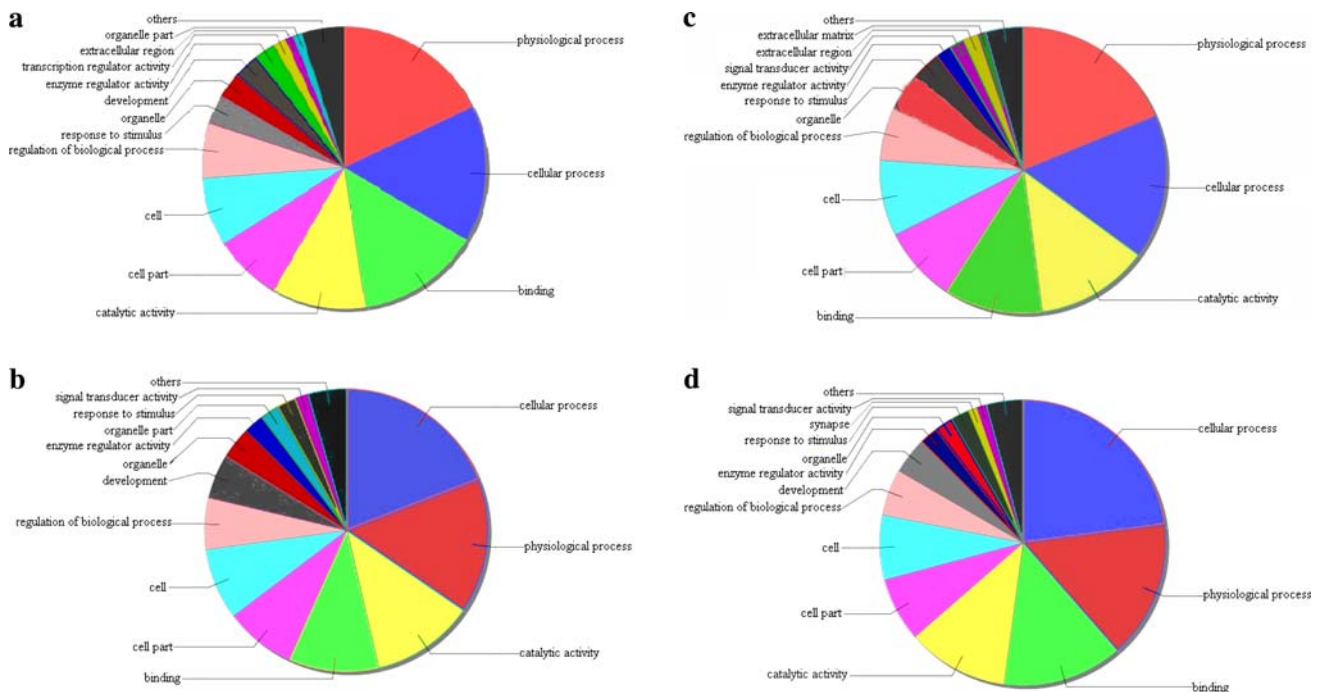
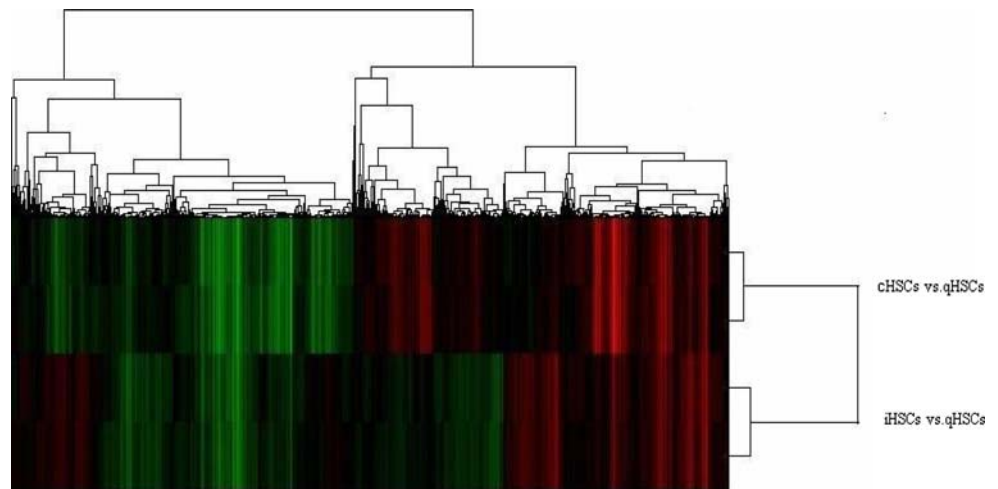
#### Real time RT-PCR analysis of gene expression

To quantify selected gene expression, HSCs were lysed with TRIzol reagent (Invitrogen Life Technologies) at a concentration of  $0.1 \text{ ml}/1 \times 10^6$  cells, and total RNA was extracted. mRNA was reverse transcribed using oligo(dT) 18–25 primer and Omniscript Reverse Transcriptase (TaKaRa, Danian, China). Selected gene transcripts were quantified by real-time RT-PCR using gene-specific primers and TaqMan Master Mix (TaKaRa). Expression of  $\beta$ -actin was used as an internal control. PCR amplification conditions were as follows:  $95^\circ\text{C}$  for 10 s, then 40 cycles of  $95^\circ\text{C}$  for 5 s and  $60^\circ\text{C}$  for 30 s. Samples were run in triplicate using a real-time PCR thermocycler (ABI PRISM 7000 Sequence Detection System; Applied Biosystems), and the results were analyzed by matched software. Relative expression of genes was determined by normalizing to  $\beta$ -actin expression in each set of samples according to the manufacturer's instructions. The gene-specific primers and sizes of the expected PCR products are listed in Table 1.

#### Western blot analysis

Total proteins were prepared by standard procedures. The total protein concentration was estimated by the Bradford

**Fig. 2** The gene expression pattern of cHSCs is different from that of iHSCs. HSCs were isolated from normal rat. HSCs were considered culture activated after 7 days of culture in 10% fetal bovine serum and induced after 7 days of culture in rat HCC conditioned medium. After isolation of mRNA and generation of cDNA, microarray was performed. Shown is a graph containing all genes that were more than 1.5-fold up-regulated (shown in red) or 0.5-fold down-regulated (shown in green) in comparison with qHSCs (*t* test, *P* < 0.05)



**Fig. 3** One thousand nine hundred sixty-seven genes were differentially expressed in cHSCs and iHSCs. The up-regulated genes in cHSCs, but not in iHSCs, comprised 755 genes, the expression of which was increased more than 1.5-fold compared to qHSCs. The down-regulated genes in cHSCs, but not in iHSCs, included 501 genes, the expression of which was decreased more than 0.5-fold compared to qHSCs. The up-regulated genes in iHSCs but not in cHSCs comprised 163 genes, the expression of which was increased more than 1.5-fold

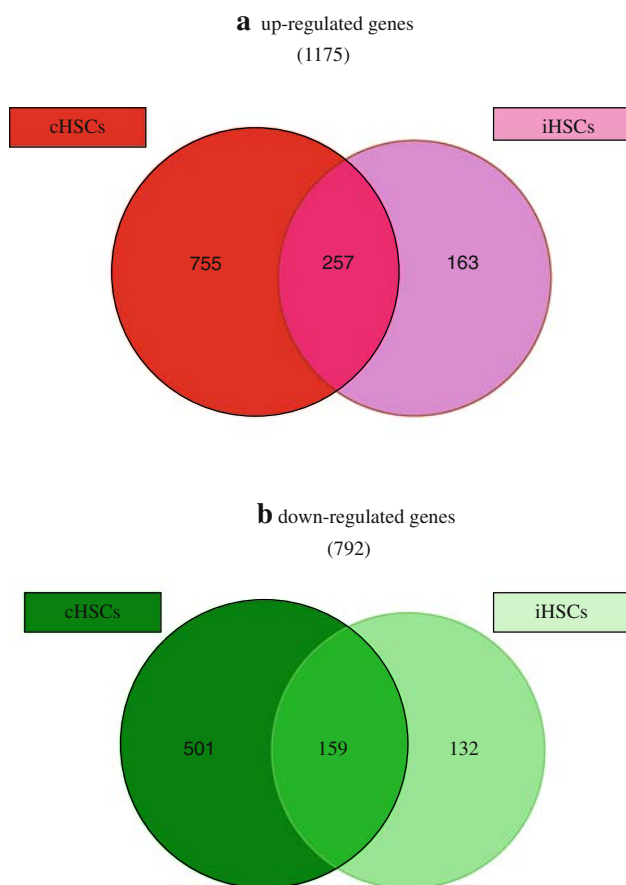
compared to qHSCs. The down-regulated genes in iHSCs, but not in cHSCs, included 132 genes, the expression of which was decreased more than 0.5-fold compared to qHSCs. Relative fractions of up-regulated and down-regulated genes in HSCs, as classified into 14 or 15 functional groups, are shown. **a** Up-regulated genes in cHSCs but not in iHSCs, **b** down-regulated genes in cHSCs but not in iHSCs, **c** up-regulated genes in iHSCs but not in cHSCs, **d** down-regulated genes in iHSCs but not in cHSCs

assay, with BSA as the standard. The proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 h at room temperature in 5% nonfat milk in 0.1% Tween, washed, and incubated overnight at 4°C with one of the following anti-rat antibodies: VCAM-1, MMP-9, Ccr1 and TGF-β2 (all from BD Pharmingen™, Biosciences, New Jersey, USA). Immunodetection was

performed using the ECL blotting detection system (Pierce, Rockford, IL).

Statistical analysis

All groups were compared with qHSCs. Data are given as mean ± SE. Statistical significance was determined using the Student’s *t* test. A value of *P* < 0.05 was



**Fig. 4** One thousand nine hundred sixty-seven genes were more than 1.5-fold up-regulated or 0.5-fold down-regulated in cHSCs and iHSCs. The Venn diagram shows overlapping genes that were significantly ( $P < 0.01$ ) and at least 1.5-fold up-regulated or 0.5-fold down-regulated in cHSC and iHSC. **a** 257 genes were concordantly up-regulated in cHSCs and iHSCs. 755 genes were up-regulated in cHSCs but not in iHSCs. 163 genes were up-regulated in iHSCs but not in cHSCs. **b** 159 genes were concordantly down-regulated in cHSCs and iHSCs. 501 genes were down-regulated in cHSCs but not in iHSCs. 132 genes were down-regulated in iHSCs but not in cHSCs

considered significant. SPSS15.0 software for Windows was used.

## Results

### Identification of HSC

A pure population of viable HSCs with the quiescent phenotype was obtained using our protocol 2 days after isolation. More than 95% of the qHSCs showed positive desmin staining, indicating pure HSCs (Fig. 1a). To obtain cHSCs, the cells were cultured for 7 days and stained for  $\alpha$ -SMA (Fig. 1c). To obtain iHSCs, the cells were cultured using C5F-CM for 7 days and stained for  $\alpha$ -SMA (Fig. 1e). More

than 95% of HSCs were  $\alpha$ -SMA positive, indicating a pure population. qHSCs were determined by staining  $\alpha$ -SMA negative (Fig. 1i).

Gene expression in iHSCs was different from that in cHSCs

The isolation and activation were repeated twice and expression of 27,100 genes was compared between qHSCs, cHSCs and iHSCs using cDNA microarrays in two independent experiments. One thousand nine hundred sixty-seven genes were more than 1.5-fold up-regulated or 0.5-fold down-regulated in cHSCs and iHSCs (Fig. 2). According to GO mapping analysis, these genes were classified into 14 or 15 groups and the fraction of genes in each group is shown in Fig. 3. Some genes encoding physiological process, cellular process and binding protein synthesis were preferentially up-regulated in cHSCs and iHSCs. 17.96% of up-regulated genes encoded physiological process proteins in cHSCs and 18.8% in iHSCs. The same was for cellular process proteins, where up-regulated genes represented 15.53% in cHSCs and 16.24% in iHSCs, and for genes involved in binding protein, with 14.08% of up-regulated genes in cHSCs and 11.11% of up-regulated genes in iHSCs. A less pronounced bias was observed for the genes related to enzyme regulator activity and the extracellular region. 2.43% in cHSCs and 1.71% in iHSCs of up-regulated genes were molecules involved in cell response to stimulus; 0.97% of up-regulated genes in cHSCs and 1.71% of up-regulated genes in iHSCs were extracellular region. Some genes encoding cellular process, physiological process and catalytic activity protein synthesis were preferentially down-regulated in cHSCs and iHSCs. 19.12% of down-regulated genes in cHSCs and 22.92% of down-regulated genes in iHSCs encoded cellular process proteins. The same was observed for physiological process proteins where down-regulated genes represented 15.44% in cHSCs and 15.63% in iHSCs and for genes involved in catalytic activity proteins, with 11.76% of down-regulated genes in cHSCs and 11.46% of down-regulated genes in iHSCs. A less pronounced bias was observed for the genes related to response to stimulus and signal transducer activity. 2.21% of down-regulated genes in cHSCs and 2.08% of down-regulated genes in iHSCs were cell response to stimulus molecules; 1.47% of down-regulated genes in cHSCs and 1.04% of down-regulated genes in iHSCs were signal transducer activity. Specifically up-regulated genes were related to organelles part, development and transcription regulator activity in cHSCs, but not in iHSCs. The up-regulated genes were involved in signal transducer activity and ECM in iHSCs but not in cHSCs. Specifically down-regulated genes were related to organelle part in cHSCs but not in iHSCs, and to synapses in iHSCs but not in cHSCs. Genes specifically up- and down-regulated in iHSCs were

**Table 2** Concordantly up-regulated genes in cHSCs and iHSCs

Gene	Symbol	cHSC↑		iHSC↑	
		exp 1	exp 2	exp 1	exp 2
Collagen alpha-1(I)	Col1α1	102.72	96.12	8.83	6.35
Collagen alpha-2(I)	Col1α2	83.39	74.98	43.73	38.51
Procollagen C-endopeptidase enhancer 1	Pcolce	56.41	79.49	8.51	23.43
Collagen alpha-1(III)	Col3α1	38.30	41.39	33.60	35.69
Alpha smooth muscle actin	α-SMA	15.56	16.33	15.53	12.44
Wingless-related MMTV integration site 4	Wnt4	23.41	22.85	32.75	32.86
Lysyl hydroxylase 2	LH2	20.84	32.77	4.44	6.06
Procollagen C-proteinase enhancer 1	PCPE-1	31.91	15.66	11.42	12.28
Small proline rich-like 10	Sprrl10	19.14	22.50	33.06	28.35
Decorin	Dcn	18.23	19.48	7.48	6.48
Chemokine receptor 1	Ccr1	17.89	18.04	2.44	2.99
Transgelin	Tagln	18.32	15.92	6.89	6.45
Neural-cadherin	N-cadherin	17.23	15.44	2.42	1.71
Glia derived nexin	GDN	19.41	12.06	5.40	4.90
Actin-associated protein paladin	paladin	14.80	14.94	3.51	3.39
Syndecan 2	Sdc2	14.32	29.35	3.82	4.73
Extracellular peptidase inhibitor	Expi	14.19	15.55	9.40	9.77
Vascular cell adhesion molecule-1	Vcam1	18.53	17.65	10.42	11.72
Complement C4	C4	13.49	15.09	4.93	4.20
Plasma glutamate carboxypeptidase	Pgcp	11.71	12.52	5.10	6.52
Adipocyte-specific adhesion molecule	Asam	10.79	10.30	2.09	1.95
Transforming growth factor, beta 2	Tgfb2	21.37	23.73	11.21	12.43
FK506-binding protein	Fkbp	10.22	8.81	3.48	2.18
Stem cell adaptor protein 1	STAP-1	9.21	7.36	12.82	13.55
Ectonucleotide pyrophosphatase 2	Enpp2	7.69	7.73	9.43	10.88
Collectin sub-family member 12	Colec12	6.99	6.98	12.57	13.07
Interleukin 13 receptor, alpha 2	Il13ra2	5.58	8.47	15.96	22.62
Calcium-regulated heat stable protein 1	CRHSP-1	6.46	5.37	3.01	2.60
Glb11 protein	Glb11	5.02	3.95	3.67	3.08
Lrrk2 protein	Lrrk2	3.96	3.48	2.66	2.53

Some genes were concordantly up-regulated in cHSCs and iHSCs. The fold increase in cHSCs or iHSCs compared to qHSCs obtained in two independent experiments is shown. When more than one hybridization sequence per gene was present on the chip, the fold increase is presented as the average cHSCs culture-activated HSCs, iHSCs induction-activated HSCs, ↑: up-regulated, ↓: down-regulated, exp 1 experiment 1, exp 2 experiment 2. The P values for cHSCs or iHSCs versus qHSCs for each gene are <0.01

involved in chemokines, chemokine receptors, signal transduction, inflammation, proliferation, development and metabolism.

iHSC gene expression was partially similar to that in cHSCs and also partially different. We compared up- and down-regulated genes between cHSCs and iHSCs. Gene expression in cHSCs overlapped only partially with iHSCs. Accordingly, the Venn diagram of all 1967 differentially regulated genes (supplementary Tables 1–6) showed that 257 up-regulated and 159 down-regulated genes from cHSCs were concordantly up- and down-regulated in iHSCs (Fig. 4). Some genes, such as α-SMA, Wnt4, Ccr1, Tagln, Vcam1, Tgfb2, STAP-1, Enpp2, Il13ra2, CRHSP-1 and Lrrk2, were concordantly up-regulated in cHSCs and iHSCs. Many genes including Mnda, Illa, Scin, Adam17, osteopontin, HIF-1α, LPTN, RT1-B, Crem and Ccl24 were concordantly down-regulated in cHSCs and iHSCs. Many

genes, however, were up- or down-regulated in iHSCs but not in cHSCs, and vice versa (Fig. 4). Some genes, such as MT-2, ET-1, Adora2b, Prrg4, Col5α1, Ccnd1, Foxp1, Mki67 and Pdlim1, were up-regulated in cHSCs, but were down-regulated in iHSCs. The other genes, such as cystatin F, MMP-9, Jun, IgG-2a, Il7r, IAPI and Igf1, were up-regulated in iHSCs, but were down-regulated in cHSCs (Tables 2, 3, 4, 5).

Verification of novel genes associated with HSC activation

To assess the validity of the obtained microarray data, we analyzed whether typical HSC activation markers were up-regulated both by microarray and real-time RT-PCR, and confirmed the strong up-regulation of α-SMA mRNA levels. In addition, we assessed the expression of the other selected genes whose expression has been associated with

**Table 3** Concordantly down-regulated genes in cHSCs and iHSCs

Gene	Symbol	cHSC↓		iHSC↓	
		exp 1	exp 2	exp 1	exp 2
Sorting nexin 12	Snx12	0.50	0.46	0.47	0.52
Toll-like receptor 4	Tlr4	0.48	0.48	0.49	0.41
Myeloid cell nuclear differentiation antigen	Mnda	0.45	0.51	0.47	0.39
Interleukin 1 alpha	Il1a	0.12	0.11	0.22	0.21
PHD finger protein 11	Phf11	0.45	0.45	0.44	0.43
Scinderin	Scin	0.47	0.41	0.20	0.17
Transcription factor MafB	Mafb	0.41	0.46	0.40	0.49
Small inducible cytokine A12	Scya12	0.37	0.49	0.43	0.48
Scotin	Scotin	0.41	0.42	0.45	0.49
A disintegrin and metalloproteinase 17	Adam17	0.31	0.35	0.32	0.41
RAB20, member RAS oncogene family	Rab20	0.46	0.36	0.51	0.47
Membrane-spanning 4-domains, subfamily A	Ms4a2	0.25	0.28	0.25	0.27
Osteopontin	OSP	0.28	0.25	0.22	0.25
Complement C1q subcomponent subunit C	C1QC	0.25	0.27	0.48	0.45
Hypoxia-inducible factor 1 alpha	HIF-1 $\alpha$	0.23	0.28	0.29	0.33
Tumor endothelial marker 7-related precursor	LOC30714	0.19	0.21	0.45	0.39
Lymphotactin	LPTN	0.20	0.20	0.21	0.24
RT1 class II, locus Ba	RT1-Ba	0.19	0.18	0.16	0.1
RAS guanyl releasing protein 1	Rasgrp1	0.18	0.18	0.37	0.39
Onzin	Onzin	0.19	0.16	0.31	0.33
cAMP-responsive element modulator	Crem	0.12	0.14	0.17	0.21
Ly6-C antigen	Ly6c	0.10	0.12	0.11	0.19
Dendritic cell-specific transmembrane protein	DCSTAMP	0.07	0.15	0.15	0.18
Interleukin-1 receptor antagonist	IL-1ra	0.10	0.09	0.30	0.30
Ribosomal protein L30	Rpl30	0.09	0.10	0.15	0.21
Pro-neuregulin-1	Pro-NRG1	0.08	0.10	0.03	0.05
Extracellular link domain-containing 1	Xlkd1	0.07	0.06	0.09	0.08
Chemokine (C–C motif) ligand 9	Ccl9	0.03	0.03	0.17	0.20
Chemokine (C–C motif) ligand 24	Ccl24	0.01	0.03	0.03	0.04

Some genes were concordantly down-regulated in cHSCs and iHSCs. The fold decrease in cHSCs or iHSCs compared to qHSCs for two independent experiments is shown. When more than one hybridization sequence per gene was present on the chip, the fold increase is presented as the average cHSCs culture-activated HSCs, iHSCs induction-activated HSCs,  $\uparrow$ : up-regulated,  $\downarrow$ : down-regulated, exp 1 experiment 1, exp 2 experiment 2. The *P* values for cHSCs or iHSCs versus qHSCs for each gene are  $< 0.01$ .

HSC activation (Fig. 4). We identified genes involved in proliferation (*cystatin F*, *c-Myc*, *ET-1*), inflammation (*Ccl24*, *Il-1a*), ECM organization and degradation (*Colla1*, *osteopontin*, *MMP9*), cell surface receptors (*Ccr1*, *Il-1Ra*, *Robo-1*, *PIP4*), cell adhesion molecules (*Vcam1*), signal transduction factors (*Wnt4*, *Jun*, *Raf1*, *Rac2*), and immunity factors (*CD44*, *CD72*, *Tgfb2*, *Ifngr1*, *Tlr4*, *Tlr7*). *MMP9* was previously reported to be down-regulated in activated HSCs in vivo and in vitro (Jiang et al. 2006; Tsuneyama et al. 2002). We confirmed that the expression of *MMP9* was down-regulated in cHSCs, and up-regulated in iHSCs. *Ccr1*, *Colla1*, *Tgfb2*, *Vcam1* and *Rac2* were concordantly increased in cHSCs and iHSCs. *Ccl24*, *Il-1a*, *Il-1Ra* and *Tlr4* were down-regulated in both cHSCs and iHSCs. *Robo-1*, *Raf1* and *CD72* were up-regulated in iHSCs, but down-regulated in cHSCs. *ET-1*, *c-Myc* and *PIP4* were up-regulated in cHSCs, but down-regulated in iHSCs (Fig. 5). These results suggest that the data obtained by cDNA microarrays are reliable.

Expression patterns of several genes by Western blot analysis

Expression patterns of four genes were confirmed by Western blot analysis. Expression patterns of all investigated proteins were similar to our PCR and microarray analysis data, with *MMP-9* being up-regulated in iHSC but not in cHSCs, *Ccr1* being significantly up-regulated in cHSCs but not in iHSCs, and *TGF- $\beta$ 2* and *Vcam1* being up-regulated in both cHSC and iHSCs (Fig. 6).

## Discussion

Myofibroblasts are major components of the stroma of many tumors, such as those of colon, pancreas, oral cavity and breast (Adegboyega et al. 2004; Masamune et al. 2008; Kellermann et al. 2007; Surowiak et al. 2007). Although their precise functions remain to be determined, several



**Table 4** Genes up-regulated in iHSCs but not in cHSCs

Gene	Symbol	cHSC↓		iHSC↑	
		exp 1	exp 2	exp 1	exp 2
Complement factor D	Cfd	0.41	0.38	2.18	2.09
Immunoglobulin kappa-chain	RGD1563231	0.39	0.37	1.96	1.68
Rho GTPase binding protein 2	Rhpn2	0.47	0.46	1.58	1.49
Roundabout homolog 1	Robo-1	0.43	0.46	2.90	2.53
Sialic acid binding Ig-like lectin G	Siglecg	0.39	0.46	2.20	2.27
Inhibitor of apoptosis protein 1	IAP1	0.42	0.45	2.09	2.04
Plakophilin 4	Pkp4	0.42	0.37	2.43	1.80
Cartilage glycoprotein 39	GP-39	0.11	0.14	1.52	1.48
RT1 class I, A3	RT1-A3	0.49	0.43	1.89	2.11
Interleukin-1 beta	IL-1 beta	0.11	0.09	1.68	1.48
Immunoresponsive gene 1	Irg1	0.10	0.11	1.47	1.57
Mucolipin 2	Mcoln2	0.44	0.46	1.54	1.66
Matrix metalloproteinase 9	Mmp-9	0.21	0.19	8.61	7.41
Oncogen jun	Jun	0.35	0.38	8.21	7.86
CaM-kinase II inhibitor alpha	Camk2n1	0.42	0.54	1.55	1.61
Toll-like receptor 7	Tlr7	0.29	0.26	1.47	1.55
Tumor necrosis factor receptor	Tnfrsf1a	0.20	0.18	1.53	1.70
Insulin-like growth factor 1	Igf1	0.45	0.41	1.47	1.57
P2X purinoceptor 4	P2X4	0.38	0.53	1.91	1.81
CD72 antigen	Cd72	0.27	0.29	1.49	1.69
Integrin beta 2	Itgb2	0.50	0.45	1.59	1.86
Glia maturation factor gamma	GMF-gamma	0.45	0.49	2.41	1.80
Platelet factor 4	Pf4	0.22	0.30	1.65	1.59
Plexin D1	Plxnd1	0.36	0.34	2.47	2.55
Cystatin F	Cst7	0.13	0.22	2.05	1.95
Hephaestin	Heph	0.27	0.33	1.93	2.17
Ig gamma-2B chain C region	LOC299353	0.38	0.51	2.26	2.27
Growth/differentiation factor 15	GDF-15	0.43	0.22	3.03	3.37
Gamma-2a immunoglobulin heavy chain	LOC299353	0.38	0.51	2.26	2.27
Interleukin 7 receptor	Il7r	0.28	0.31	3.68	4.15
Sclerostin	Sost	0.35	0.45	5.93	4.78

Some genes were up-regulated in iHSCs but not in cHSCs. The fold increase in iHSCs or fold decrease in cHSCs compared to qHSCs for two independent experiments is shown. When more than one hybridization sequence per gene was present on the chip, the fold increase is presented as the average cHSCs culture-activated HSCs, iHSCs induction-activated HSCs, ↑: up-regulated, ↓: down-regulated, exp 1 experiment 1, exp 2 experiment 2. The P values for iHSCs or cHSCs versus qHSCs for each gene are <0.001

arguments point to their role in favoring tumor progression (Dimanche-Boitrel et al. 1994; De Wever et al. 2008). Studies have already shown that human hepatic myofibroblasts are able to greatly increase the invasiveness of HCC cell lines through the secretion of hepatocyte growth factor and that myofibroblasts are also involved in the synthesis of many ECM components of the HCC stroma (Neaud et al. 1997; Guirouilh et al. 2000; Faouzi et al. 1999a, b). By activating qHSC into myofibroblasts, HCC cells themselves contribute to tumor progression (Faouzi et al. 1999a, b; Guirouilh et al. 2000). However, the precise mechanism of HSC activation in HCC is not well understood at present. Accordingly, we induced HSC activation by the rat HCC cell line C5F, and used cDNA microarray analysis to investigate gene expression levels of HSCs, to understand the role of iHSCs in this process. Our results showed that HCC cells

were able to induce the in vitro activation of HSCs. HSC activation was characterized by typical morphologic modifications, proliferation, and increased expression of α-SMA.

Our microarray study detected a large number of previously known and unknown genes that were up- or down-regulated during HSC activation, thus confirming the ECM-producing and ECM-degrading, proliferative, and inflammatory phenotype of activated HSCs. Although cHSCs up-regulated typical HSC activation markers such as α-SMA, they did have the same changes of gene expression patterns seen in iHSCs. The finding that induced activation of HSCs up-regulates a different set of genes than cultured activation of HSCs is not completely surprising, in view of the fact that the microenvironment of HSCs in the CM is complex and exposes HSCs to a number of cytokines secreted by HCC cells, whereas culture activation occurs in

**Table 5** Genes up-regulated in cHSCs but not in iHSCs

Gene	Symbol	cHSC↑		iHSC↓	
		exp 1	exp 2	exp 1	exp 1
Metallothionein-2	MT-2	6.04	5.36	0.48	0.43
Collagen alpha-1(V)	Col5α1	5.69	5.82	0.48	0.45
Adenine nucleotide translocator 1	ANT 1	3.12	3.50	0.45	0.43
Cyclin-D1	Ccnd1	4.15	5.05	0.41	0.47
Forkhead box P1	Foxp1	4.02	4.88	0.48	0.41
UDP-Glc dehydrogenase	UDPGDH	3.73	2.72	0.44	0.49
Heat-shock protein beta-1	HspB1	4.29	8.11	0.40	0.43
PRL receptor associated protein	PRAP	2.75	2.35	0.44	0.43
PPAR-interacting protein 4	PRAP	2.75	2.35	0.44	0.43
Polymerase, delta 2	PIP4	4.87	4.32	0.43	0.46
Ubiquitin-conjugating enzyme	UbcM2	2.86	2.61	0.48	0.44
Nucleolar protein 5A	Nol5a	2.23	1.98	0.39	0.38
Endothelin-1	ET-1	8.56	7.35	0.22	0.23
Chemokine (C–C motif) ligand 6	Ccl6	2.63	2.34	0.39	0.37
Activin beta-C chain	INHBC	5.36	5.80	0.33	0.44
CDC28 protein kinase 1b	Cks1b	3.05	2.68	0.45	0.50
RS21-C6 protein	Rs21c6	2.38	2.02	0.37	0.35
Matrix Gla-protein	MGP	4.31	4.33	0.43	0.40
Thyroid receptor-interacting protein 13	Trip13	1.95	2.64	0.36	0.49
Adenosine A2b	Adora2b	2.58	2.20	0.48	0.37
A-kinase anchoring protein 18	Akap18	2.11	2.24	0.30	0.33
Dithiolethione-inducible gene 1	DIG-1	2.84	2.95	0.42	0.43
Calcyclin binding protein	Cacybp	2.60	2.96	0.41	0.46
Adrenomedullin	AM	2.23	2.25	0.12	0.16
Glycoprotein 38	GP38	2.65	2.41	0.31	0.33
Myelocytomatosis oncogene	c-Myc	2.00	1.95	0.35	0.35
Helix-destabilizing protein	HDP	2.10	1.99	0.19	0.29
Ephrin-A1	Efna1	1.76	1.87	0.22	0.34
Proline-rich Gla 4	Prrg4	1.75	1.82	0.17	0.18
Fos-related antigen 1	FRA-1	1.61	1.63	0.16	0.19

Some genes were up-regulated in cHSCs but not in iHSCs. The fold increase in cHSCs or fold decrease in iHSCs compared to qHSCs for two independent experiments is shown. When more than one hybridization sequence per gene was present on the chip, the fold increase is presented as the average

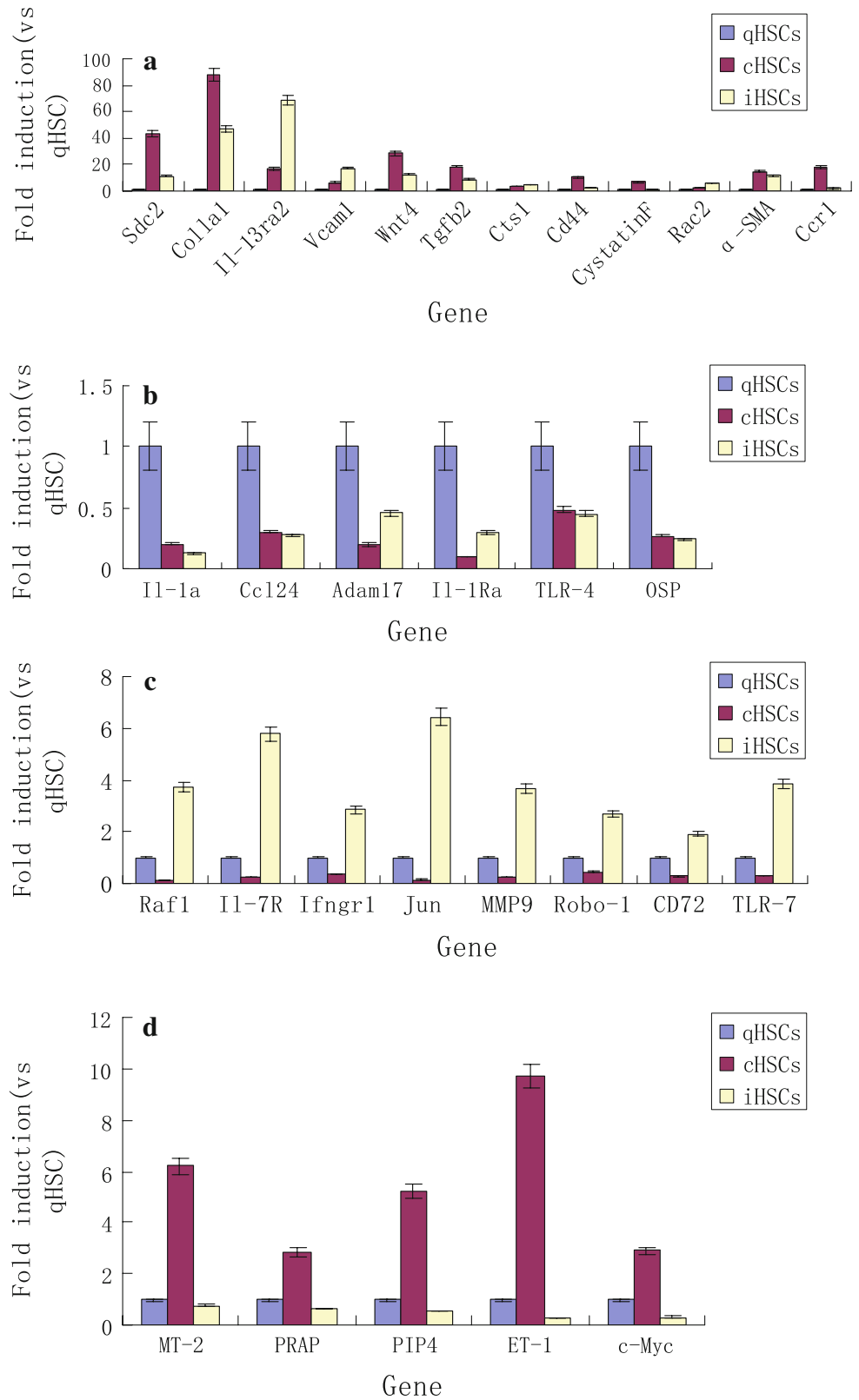
cHSCs culture-activated HSCs, iHSCs induction-activated HSCs, ↑: up-regulated, ↓: down-regulated, exp 1 experiment 1, exp 2 experiment 2

The *P* values for cHSCs or iHSCs versus qHSCs for each gene are <0.01

an artificial environment and does not include interactions with cytokines secreted by HCC cells. Culture activation with FBS is by far the most common model of HSC activation. Our data suggest that the common practice of evaluating genetic manipulations during HSC culture activation may produce results that do not reflect effects on HSCs and HCC in vivo. Our study compared gene expression patterns of cHSCs and iHSCs to that of qHSCs, and thus had a higher capacity to detect differentially regulated HSC-specific genes. Specific gene expression patterns of iHSCs indicated their distinct role in HCC. In addition to detecting classical HSC activation genes, such as  $\alpha$ -SMA, collagen  $\alpha 1(I)$ , cyclin D2 and fibronectin1, our study identified a large number of genes that have not been previously described to be associated with HSC-induced activation. Among novel differentially regulated genes identified in

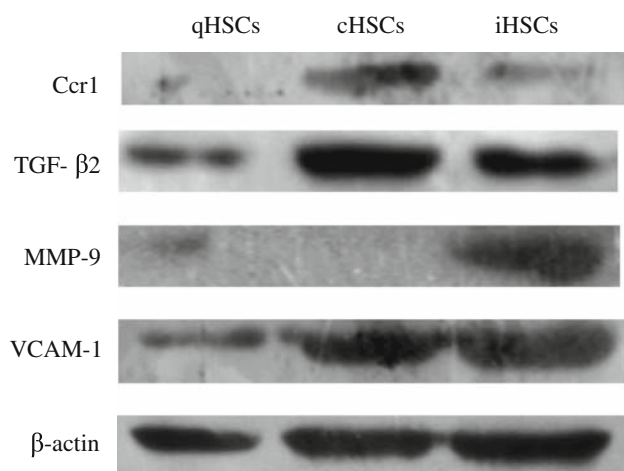
iHSCs are genes involved in (i) ECM formation and degradation, e.g. Col5α1 (down-regulated) and MMP-9 (up-regulated); (ii) apoptosis including IAP1 (up-regulated); (iii) proliferation, such as cystatin F (up-regulated); (iv) inflammation, e.g. Il-1a (up-regulated) and osteopontin (down-regulated); (v) adhesion, such as Vcam1 (up-regulated); (vi) signal transduction, including Wnt6, Jun, Raf1 (all up-regulated), and (vii) immune factors, such as CD44, Tgfb2 and Ifngr1 (all up-regulated). In addition, we identified differential expression of several receptors such as Ccl24, PIP4 (both down-regulated), Robo1, Il7r, CD72, Tlr7 and Ccr1 (all up-regulated). Increased expression of VCAM1 can facilitate the adherence of HCC cells to the vessel wall (Futakuchi et al. 2002). MMP-9 expression can degrade ECM and promote invasion and metastasis of HCC (Hong et al. 2005). Up-regulated TGF-β2 expression can induce

**Fig. 5** Identification of previously known and novel differentially regulated genes during HSC activation in CM and in culture. Expression of differentially regulated genes was confirmed in qHSCs, cHSC, iHSCs by real-time RT-PCR. Results were normalized to  $\beta$ -actin expression and are expressed as fold induction  $\pm$  standard error of the mean in comparison with qHSCs. Each group of HSC samples consisted of at least three different isolations (paired *t* test, *P* < .05). **a** Concordantly up-regulated genes, **b** concordantly down-regulated genes, **c** genes up-regulated in iHSCs but not in cHSCs, **d** genes up-regulated in cHSCs but not in iHSCs



HCC immune inhibition (Maggard et al. 2001), suggesting that iHSCs may indirectly facilitate recurrence and metastasis of HCC by inhibiting organism immune function. The

*IAP* family member *IAP1* was up-regulated in iHSCs, suggesting a potential role for *IAP1* in protecting HSCs from apoptosis during HCC progression.



**Fig. 6** Expression of selected genes was confirmed by Western blot analysis. Each Western blot is based on HSCs isolated from three normal rats, and is representative of at least two independent experiments

The fact that cHSCs and iHSCs display a significantly different gene expression pattern suggests that iHSCs might play an important role in HCC different from that of cHSCs. Moreover, cHSCs dramatically up-regulated genes such as *Ccr1*, *Sdc2*, *CD44* and *TGF-β2*, which were slightly up-regulated or down-regulated in iHSCs, suggesting that in vitro culture artificially drives the expression of some genes, and thus contributes to aberrant gene expression during culture activation. Our data indicate that, to some extent, iHSCs can reflect interactions with HCC in vivo, but cHSCs cannot truly reflect interactions with HCC. Therefore, this study has provided theoretical foundations for investigating the biology of HSCs in HCC. Nevertheless, the microenvironment of HSC in CM is not completely identical to the microenvironment in the liver. The in vivo activation of HSCs should be considered as the standard for the study of HSC biology in HCC.

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**Conflict of interest statement** We declare that we have no conflict of interest.

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