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ORIGINAL ARTICLE

Wnt5a participates in hepatic stellate cell activation observed by gene expression profile and functional assays

Wu-Jun Xiong, Li-Juan Hu, Yi-Cheng Jian, Li-Jing Wang, Ming Jiang, Wei Li, Yi He

Wu-Jun Xiong, Li-Juan Hu, Yi-Cheng Jian, Li-Jing Wang, Ming Jiang, Wei Li, Yi He, Department of Gastroenterology, Shanghai East Hospital Affiliated to Tongji University, Shanghai 200120, China
Author contributions: Xiong WJ designed the research; Hu LJ, Jian YC, Wang LJ, Jiang M, Li W, He Y performed the research; Hu LJ wrote the paper.
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Correspondence to: Wu-Jun Xiong, Associated Professor, Department of Gastroenterology, Shanghai East Hospital Affiliated to Tongji University, 150 Jimo Road, Shanghai 200120, China. xiongwujun@hotmail.com
Telephone: +86-21-38804518-2063 Fax: +86-21-58798999
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Abstract

AIM: To identify differentially expressed genes in quiescent and activated hepatic stellate cells (HSCs) and explore their functions.

METHODS: HSCs were isolated from the normal Sprague Dawley rats by *in suit* perfusion of collagenase and pronase and density Nycodenz gradient centrifugation. Total RNA and mRNA of quiescent HSCs, and culture-activated HSCs were extracted, quantified and reversely transcripted into cDNA. The global gene expression profile was analyzed by microarray with Affymetrix rat genechip. Differentially expressed genes were annotated with Gene Ontology (GO) and analyzed with Kyoto encyclopedia of genes and genomes (KEGG) pathway using the Database for Annotation, Visualization and Integrated Discovery. Microarray data were validated by quantitative real-time polymerase chain reaction (qRT-PCR). The function of Wnt5a on human HSCs line LX-2 was assessed with lentivirus-mediated Wnt5a RNAi. The expression of Wnt5a in fibrotic liver of a carbon tetrachloride (CCl_4)-induced fibrosis rat model was also analyzed with Western blotting.

RESULTS: Of the 28 700 genes represented on this chip, 2566 genes displayed at least a 2-fold increase or decrease in expression at a $P < 0.01$ level with a false discovery rate. Of these, 1396 genes were upregulated, while 1170 genes were downregulated in culture-activated HSCs. These differentially expressed transcripts were grouped into 545 GO based on biological process GO terms. The most enriched GO terms included response to wounding, wound healing, regulation of cell growth, vasculature development and actin cytoskeleton organization. KEGG pathway analysis revealed that Wnt5a signaling pathway participated in the activation of HSCs. Wnt5a was significantly increased in culture-activated HSCs as compared with quiescent HSCs. qRT-PCR validated the microarray data. Lentivirus-mediated suppression of Wnt5a expression in activated LX-2 resulted in significantly impaired proliferation, downregulated expressions of type Ⅰ collagen and transforming growth factor-β1. Wnt5a was upregulated in the fibrotic liver of a CCl_4-induced fibrosis rat model.

CONCLUSION: Wnt5a is involved in the activation of HSCs, and it may serve as a novel therapeutic target in the treatment of liver fibrosis.

Key words: Hepatic stellate cells; Wnt5a; Microarray; Bioinformatics analyses; Liver fibrosis

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INTRODUCTION

Liver fibrosis is a common pathological change characterized by excessive deposition of extracellular matrix (ECM) that occurs in most types of chronic liver diseases. Hepatic stellate cells (HSCs) are a major source of ECM and activation of HSCs is one of the key steps in the development of liver fibrosis[1,2]. However, the molecular mechanisms underlying the activation of HSCs are not fully understood.

Previous studies have reported the differentially expressed gene profiles in quiescent and activated HSCs with gene chip. De Minicis *et al*[3] determined gene expression changes in three different models of HSC activation: culture-activated HSCs, HSCs isolated from CCl_4-treated mice and mice underwent bile duct ligation (BDL). They found that the gene expression pattern of culture-activated HSCs was different from that of BDL- and CCl_4-activated HSCs. Woo *et al*[4] performed a time-based study to investigate the differences in gene expression patterns during the activation of HSCs with microarrays and revealed a number of newly discovered genes involved in fibrogenesis during the activation of HSCs. Sancho-Bru *et al*[5] investigated the differences in phenotypic, genomic and functional characteristics between HSCs from human cirrhotic livers and HSCs from normal livers after prolonged culture. However, these three studies are lack of intense bioinformatics analyses.

In this study, we performed gene expression profile analyses using oligonucleotide microarrays to identify the genes involved in HSCs activation. These analyses revealed the altered expression of 2566 genes. On this basis, bioinformatics analyses were done to classify the function and explore the involved signaling pathway of these differentially expressed genes. Bioinformatics analyses revealed that these differentially expressed transcripts were grouped into 545 GO based on biological process GO terms. The most enriched GO terms included response to wounding, wound healing, regulation of cell growth, vasculature development, and actin cytoskeleton organization. It is reported that Wnt5a signaling takes part in wound healing, regulation of cell proliferation, vasculature development and the formation of cell polarity [6-9]. After intensive analyses of Kyoto encyclopedia of genes and genomes (KEGG) pathways, we found that Wnt5a signaling pathway participated in the HSCs activation, an observation verified by quantitative real-time polymerase chain reaction (qRT-PCR).

Because the role of Wnt5a in HSCs activation is unknown and the direct evidence has not been reported, we treated human HSCs line LX-2 with lentivirus-mediated Wnt5a ShRNA and found that knockdown of *Wnt5a* gene expression in LX-2 led to significantly impaired proliferation, downregulated expressions of type I collagen and transforming growth factor-β1 (TGF-β1). Wnt5a was upregulated in fibrotic liver of a carbon tetrachloride (CCl_4)-induced fibrosis rat model. Collectively, our data suggest that Wnt5a may play a role in HSCs activation and liver fibrogenesis. We expect that our results will help illustrate the molecular mechanisms involved in liver fibrogenesis and provide preliminary experimental evidence for the feasibility of targeting Wnt5a in gene therapy of liver fibrogenesis.

MATERIALS AND METHODS

Cell isolation, identification and culture

HSCs were isolated from normal Sprague Dawley (SD) rats by collagenase-pronase perfusion and subsequent density centrifugation on Nycodenz gradients as described previously[10]. Purity was tested by retinoid autofluorescence and exceeded 95% in all isolations. HSCs were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and antibiotics. After 1 (quiescent HSCs) and 7 (activated HSCs) d of culture, total RNA was extracted using an RNeasy mini-kit (Qiagen, Valencia, CA, United States). Human HSCs line LX-2 was cultured in DMEM (Invitrogen) supplemented with 10% FBS.

Microarray experiments and bioinformatics analysis

We used rat genome 430 2.0 array gene chips (Affymetrix, Santa Clara, CA). The arrays were hybridized, washed and scanned according to the standard Affymetrix protocol. The gene chip tests were performed by professional staffs of Shanghai Biochip Company. Chips were scanned with a Genechip Scanner 3000 7G (Affymetrix). Data analysis was performed using GCOS1.2 software. After background correction, we performed normalization for each array and gene. Data were filtered based on both signal intensity and detection call. Gene activity was considered to differ between quiescent HSCs and culture-activated HSCs ($P < 0.01$) when compared by the unpaired Student's t test using multiple testing correction (Benjamini and Hochberg False Discovery Rate). Molecular function and classification of differentially expressed genes were analyzed with GO and KEGG pathway using the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7[11] (http://david.abcc.ncifcrf.gov).

Quantitative real-time polymerase chain reaction

Five differentially expressed genes were selected for qRT-PCR analysis, with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as a control, to verify the reliability of the array data. The primer sequences of analyzed genes are shown in Table 1. Super Script Ⅲ reverse transcriptase (Invitrogen) was used for reverse transcription reactions. PCR amplification was performed using TaqMan universal Master Mix (Applied Biosystems, Foster City, CA, United States). Amplified reactions were quantified using an ABI PRISM 7900 Sequence detection system (Applied Biosystems, Foster City, CA, United States). Experiments were performed in triplicate. Relative gene quantities were obtained using the comparative Ct method after normalization to GAPDH control genes.

Table 1 Primer sequences of genes validating the microarray analysis by quantitative real-time polymerase chain reaction

Gene name	Sense primer	Antisense primer
Wnt5a	AGGGCTCCTACGAGAGTGCT	GACACCCCATGGCACTTG
Frizzled2	TCTCTGAGGACGGTTATCGCA	CAGAATCACCCACCAGATGGA
Calcium calmodulin mediated kinase Ⅱ delta	TCGGCTCACACAGTACATGGA	CCCCGAACGATGAAAGTGAA
α-1 type Ⅰ collagen	ACAGACTGGCAACCTCAAGAAG	AAGCGTGCTGTAGGTGAATCG
Connective tissue growth factor	GTTGGCGAACAAATGGCCTT	TGCCTCCCA AACCAGTCATAG
Glyceraldehydes-3-phosphate dehydrogenase	TCCTGCACCACCAACTGCTTAG	AGTGGCAGTGATGGCATGGACT

Construction of lentivirus-mediated Wnt5a RNAi

Lentiviral vector pll3.7 expressing human Wnt5a ShRNA targeting 5'-GGACUUUCUCAAGGACAGA-3' (538-556) was constructed by Shanghai Biyang Biotechology. Plasmids from correct clones were amplified by transforming into DH5α *Escherichia coli* cells. We used tronolab lentiviral vector system (pRsv-REV, pMDlg-pRRE and pMD2G) to perform lentivirus-mediated Wnt5a RNAi. An additional scrambled sequence was produced as a negative siRNA control. The scrambled sequence was not homologous with any known gene in mammalian cells as determined by a Basic Local Alignment Search Tool. 293T cells were transfected with polybrene. The crude virus lysates were amplified in 293T cells to generate higher titre viral stocks. Virus particles were purified and concentrated. The lentivirus titer was determined by a limited dilution plaque assay. LX-2 transfected with lentivirus-mediated pll3.7 Wnt5a ShRNA at concentrations of MOI 10 and MOI 20 was used as study cells; cells transfected with negative siRNA were negative controls, and cells without transfection were blank controls. The effect of Wnt5a knockdown was validated with Western blotting. Blots were developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology Inc., CA, United States), exposed on Kodak Xdmat blue XB-1 film and quantified by Bandscan 5.0 software using β-actin as internal control.

Effect of Wnt5a RNAi on cell proliferation

Cells were spread onto 96-well flat-bottom plates at a density of 5×10^4/mL, 100 μL per well, and cultured in DMEM including 5% fetal calf serum. Subgroups included: control group, negative siRNA control group and Wnt5a ShRNA group. Each group was repeated for 6 wells. The plates were incubated in a 5% CO_2 humidified incubator at 37 ℃ for 24 h and 200 μL of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) was added into each well. The plates were then incubated for 4 h and the supernatants were removed. After 150 μL DMSO was added into each well, the plates were agitated for 20 min to dissolve the crystals. Finally, the A values were measured using the enzyme-linked immunoassay at a wave-length of 450 nm.

Effect of Wnt5a RNAi on the expression of collagen Ⅰ and transforming growth factor-β1

Effects of Wnt5a RNAi on the expression of collagen Ⅰ and TGF-β1 in LX-2 were analyzed with RT-PCR *in vitro*. Experiments were performed in triplicate. The primer of α-1 type Ⅰ collagen (COL1A1) : sense primer: 5'-TGCCAAGGGAGATGCTGGTC-3', antisense primer: 5'-TTCACCCTTAGCACCAACAGCA-3', product size 238 bp; TGFβ1: sense primer: 5'-TGCAAGACTATCGACATGGAG-3', antisense primer: 5'-ACTTGAGCCTCAGCAGACGCA-3', product size 389 bp. The amplified products were applied to electrophoresis on 1% agarose gel analysis. The relative expression levels of COL1A1 and TGFβ1 were assessed based on the β-actin control by calculating the average ratios of light density using symmetry computerized gel imaging system.

Experimental fibrotic model

Liver fibrosis was induced by subcutaneous injection at hind leg of 40% CCl_4-tea oil , 0.5 mL/100 g body weight for the first time and then 0.3 mL/100 g body weight twice a week for 8 wk. Control rats received only tea oil. The amount of CCl_4 administered was adjusted for body weight each week. Liver tissue samples obtained from CCl_4-treated and non-treated control rats were dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin and Masson staining. The expression of Wnt5a was analyzed with Western blot with antibodies to Wnt5a (sc-30224, Santa Cruz, United States). The bands were semi-quantitatively evaluated by densitometric analysis. Protein expression levels of Wnt5a were thereby normalized to those of the housekeeping gene β-actin.

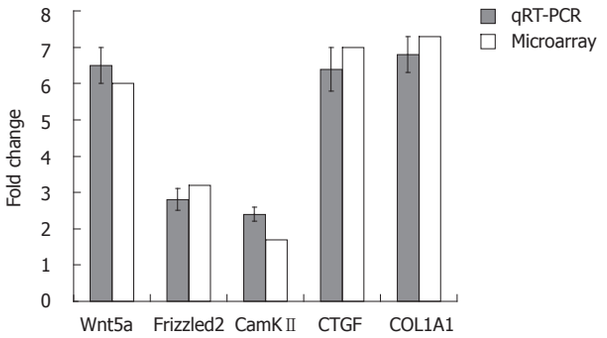
Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. Two-way analysis of variance was performed to detect the effects of Wnt5a knockdown on cell proliferation and Student *t* test was used to detect the difference between any two groups. The results were considered statistically significant at $P < 0.05$.

RESULTS

Microarray analysis and validation of microarray data

Culture-activated HSCs exhibited myofibroblast-like appearance. cRNAs, prepared from quiescent HSCs and culture-activated HSCs, were hybridized to a rat genome 430 2.0 array gene chips. Of the 28 700 genes represented therein, 2566 genes displayed at least a 2-fold increase or decrease in expression at the $P < 0.01$ level with a false discovery rate. Of these, 1396 genes were upregulated, while 1170 genes were downregulated in culture-activated



	Wnt5a	Frizzled2	CamKⅡ	CTGF	COL1A1
qRT-PCR	6.5	2.8	2.4	6.4	6.8
Microarray	6	3.2	1.7	7	7.3
SD	0.5	0.3	0.2	0.6	0.5

Figure 1 Validation of microarray data by quantitative real-time polymerase chain reaction. Comparison between expression values of five selected differently expressed genes estimated by quantitative real-time polymerase chain reaction (qRT-PCR) and gene expression data. The detected changes measured by qRT-PCR reflected different changes in gene expression between quiescent and activated hepatic stellate cells obtained by microarray analysis. SD: Sprague Dawley; CTGF: Connective tissue growth factor; CamKⅡ: Calcium calmodulin mediated kinase Ⅱ.

HSCs. As expected, genes such as COL1A1 and connective tissue growth factor (CTGF) were significantly upregulated. We were particularly interested in Wnt proteins in view of their potential role in HSCs activation. Wnt5a was upregulated by approximately 6-fold in activated HSCs in comparison with quiescent HSCs , its downstream factors Frizzled2 was upregulated by 3.2-fold and calcium calmodulin mediated kinase Ⅱ (CamKⅡ) was upregulated by 1.7-fold. Changes in gene expression profiles observed in the microarrays were validated by qRT-PCR analysis using the same total RNAs analyzed in the microarrays. We verified the upregulation of Wnt5a, Frizzled2 and CamKⅡ as well as COL1A1, CTGF (Figure 1). This verification confirmed that all the qRT-PCR results were in agreement with the microarray data.

Gene ontology analysis

To elucidate the relationship between gene differential expression patterns of quiescent HSCs and culture-activated HSCs, we examined the functional bias of 2566 differentially expressed transcripts according to Gene Ontology (GO) classifications. These differentially expressed transcripts were grouped into 545 GO based on biological process GO terms. The most enriched GO terms included response to wounding, wound healing and regulation of cell growth (Table 2). Analyses of GO also indicated that there were 102 GO terms identified by cellular component classification, and 90 GO terms identified by molecular function classification.

Kyoto encyclopedia of genes and genomes pathway analysis

Analysis of KEGG pathways revealed many enrichment-related pathways including focal adhesion, ECM-receptor interaction, regulation of actin cytoskeleton, pathways in cancer, dilated cardiomyopathy, hypertrophic cardiomyopathy, TGF-β signaling pathway, chemokine signaling pathway, p53 signaling pathway, renal cell carcinoma, Wnt signaling pathway, leukocyte transendothelial migration, MAPK signaling pathway in HSCs activation. The top 20 significantly perturbed pathways are listed in Table 3. Of these, Wnt signaling pathway is shown in Figure 2. It revealed that Wnt5a/Frizzled2/PLC/CamKⅡ pathway participated in the activation of HSCs.

Effect of Wnt5a knockdown on LX-2 cells

LX-2 cells were infected with lentiviral vector encoding GFP at a multiplicity of infection (MOI) of 10, resulting in GFP expression in the majority of cultured cells. Western blot analysis showed that the protein expression levels of Wnt5a were lower in the Wnt5a ShRNA group than in the control and negative siRNA group ($P < 0.01$) (Figure 3). MTT assay indicated that the proliferation of LX-2 cells was inhibited significantly after transfection with Wnt5a ShRNA as compared with the control and negative siRNA group ($P < 0.05$) (Figure 4). RT-PCR showed that *Wnt5a* gene silencing remarkably decreased the levels of COL1A1 and TGF-β1 mRNA ($P < 0.05$, respectively) as shown in Figure 5.

Upregulation of Wnt5a in a CCl_4-induced fibrosis model

Next, we investigated whether Wnt5a was induced during liver fibrosis in *vivo*. Compared with the normal group, 8 wk after CCl_4 treatment, collagen deposition was increased, pseudolobules formed, and numerous inflammatory cells infiltrated the portal area. Western blotting revealed that Wnt5a was upregulated as significantly as αSMA after 8 wk of CCl_4 treatment ($P < 0.01$) (Figure 6).

DISCUSSION

To discover novel genes involved in activation of HSCs, we compared gene expression profile between quiescent and culture activated HSCs. We showed that 2566 genes displayed at least a 2-fold increase or decrease in expression. Of these, 1396 genes were upregulated, while 1170 genes were downregulated in culture-activated HSCs. GO classification showed the most enriched GO terms included response to wounding, wound healing and regulation of cell growth. Analysis of KEGG pathways revealed that Wnt signaling pathway participates in HSCs activation. We were particularly interested in Wnt proteins in view of their potential role in HSCs activation.

The Wnt signaling pathway is a network of proteins best known for their roles in embryogenesis and cancer, but also involved in normal physiological processes in adult animals[12]. There are at least three different Wnt pathways: the canonical pathway, the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway. In the canonical Wnt pathway, Wnt ligands bind to the receptor of frizzled family, which transduce signal to β-catenin causing β-catenin to enter the nucleus and activate the target

Table 2 Top 20 enriched Gene Ontology terms based on Gene Ontology classifications

Biological process	Count	Percent	Molecular function	Count	Percent	Cellular component	Count	Percent
Response to wounding	78	7.24	Cytoskeletal protein binding	73	6.78	Extracellular region part	125	11.61
Wound healing	46	4.27	Actin binding	52	4.83	Extracellular region	180	16.71
Regulation of cell growth	43	3.99	Growth factor binding	24	2.23	Extracellular matrix	69	6.41
Vasculature development	50	4.64	Glycosaminoglycan binding	24	2.23	Proteinaceous extracellular matrix	61	5.66
Actin cytoskeleton organization	42	3.90	Calcium ion binding	76	7.06	Extracellular matrix part	34	3.16
Actin filament-based process	43	3.99	Extracellular matrix structural constituent	14	1.30	Cytoskeleton	125	11.61
Blood vessel development	48	4.46	Heparin binding	19	1.76	Basement membrane	25	2.32
Cell migration	51	4.73	Polysaccharide binding	24	2.23	Extracellular space	71	6.59
Cell adhesion	72	6.69	Pattern binding	24	2.23	Cell projection	92	8.54
Biological adhesion	72	6.69	Insulin-like growth factor binding	11	1.02	Cytoskeletal part	88	8.17
Regulation of growth	55	5.11	Integrin binding	12	1.11	Cell surface	53	4.92
Regulation of cellular component size	46	4.27	Carbohydrate binding	39	3.62	Actin cytoskeleton	39	3.62
Extracellular matrix organization	27	2.51	Chemokine activity	10	0.93	Actin filament bundle	14	1.30
Regulation of cell proliferation	87	8.08	Chemokine receptor binding	10	0.93	Plasma membrane	216	20.06
Cytoskeleton organization	54	5.01	Protein complex binding	29	2.69	Collagen	12	1.11
Response to organic substance	109	10.1	Identical protein binding	57	5.29	Actomyosin	14	1.30
Response to hormone stimulus	72	6.69	Collagen binding	8	0.74	Stress fiber	13	1.21
Response to endogenous stimulus	77	7.15	Growth factor activity	20	1.86	Microtubule cytoskeleton	53	4.92
Cell motion	62	5.76	Platelet-derived growth factor binding	5	0.46	Cell leading edge	26	2.41
Blood vessel morphogenesis	38	3.52	Phospholipid binding	20	1.86	Vesicle lumen	15	1.39

Table 3 Signaling pathway related to activation of hepatic stellate cells

Terms	Count	Percent	*P* value	Fold enrichment	Benjamini
rno04510: Focal adhesion	51	4.73	6.34E-19	4.16	9.90E-17
rno04512: Extracellular matrix-receptor interaction	27	2.50	9.93E-13	5.30	7.75E-11
rno04810: Regulation of actin cytoskeleton	36	3.34	5.18E-08	2.74	2.69E-06
rno05200: Pathways in cancer	40	3.71	2.74E-05	2.00	0.001066
rno05414: Dilated cardiomyopathy	18	1.67	3.27E-05	3.18	0.00102
rno05410: Hypertrophic cardiomyopathy	17	1.57	5.01E-05	3.22	0.001302
rno04350: Transforming growth factor-beta signaling pathway	17	1.57	6.76E-05	3.148	0.001505
rno04540: Gap junction	15	1.39	4.27E-04	2.94	0.008302
rno04110: Cell cycle	19	1.76	7.69E-04	2.40	0.013254
rno04666: Fc gamma R-mediated phagocytosis	15	1.39	0.001011	2.71	0.015653
rno05222: Small cell lung cancer	14	1.29	0.001758	2.68	0.024645
rno04062: Chemokine signaling pathway	22	2.04	0.002121	2.04	0.027229
rno04115: p53 signaling pathway	12	1.11	0.002359	2.89	0.027945
rno05211: Renal cell carcinoma	12	1.11	0.003385	2.76	0.03708
rno04310: Wnt signaling pathway	19	1.76	0.003846	2.08	0.039281
rno04670: Leukocyte transendothelial migration	16	1.48	0.005063	2.21	0.048281
rno04010: MAPK signaling pathway	28	2.59	0.008006	1.67	0.071107
rno05219: Bladder cancer	7	0.64	0.022754	3.09	0.172201
rno04270: Vascular smooth muscle contraction	14	1.29	0.0235	1.97	0.169299
rno05212: Pancreatic cancer	10	0.92	0.027028	2.30	0.184168

genes. Wnt-Ca^{2+} signaling is mediated through G proteins and phospholipases and leads to transient increases in cytoplasmic free calcium that subsequently activate the kinase protein kinase C and CamKⅡ and the phosphatase calcineurin[13-15].

With KEGG pathway analyses, we found that the canonical pathway, the PCP pathway and the Wnt/Ca^{2+} pathway participated in the activation of HSCs. In recent years, researches have shown that activation of Wnt signaling pathway was closely related with the development and progress of fibroproliferative diseases, such as pulmonary fibrosis[16-19], renal fibrosis[20-22], post-infarct cardiac remodeling[23] and hypertrophic scars[24,25], which makes it easy to think that Wnt pathway may have a definite effect on liver fibrosis. It is reported that Wnt/β-catenin signaling contributes to "antiadipogenic" activation of HSCs and may be used as a new target for liver fibrogenesis[26].

The study about the role of Wnt5a in HSCs activation is still in its initial stage. Jiang *et al*[27] reported that expressions of Wnt5a and its receptor Frizzled 2 were highly upregulated in culture-activated HSCs in *vitro*.

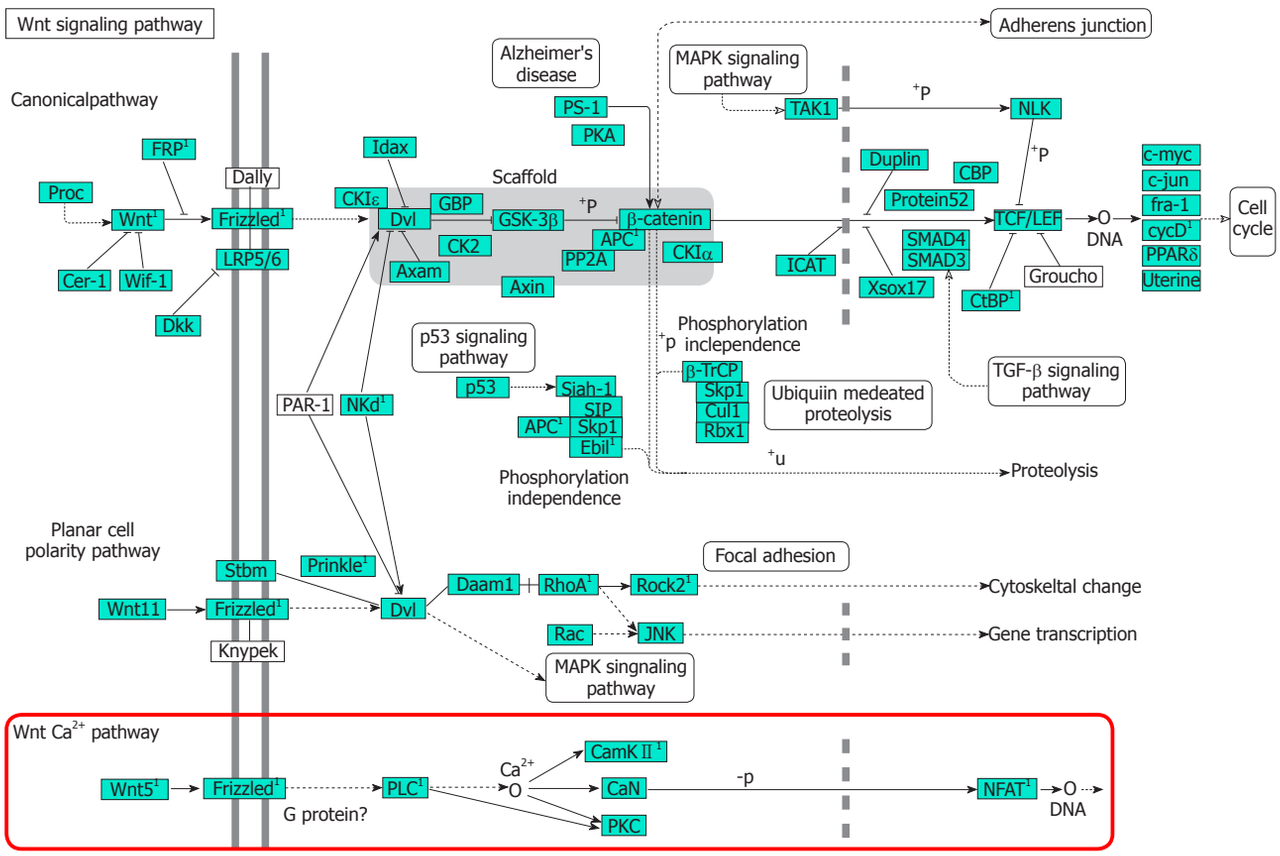


Figure 2 Wnt signaling pathway participated in the activation of hepatic stellate cells. [1]Differentially expressed genes revealed by microarray. TGF-β1: Transforming growth factor-β1; PKC: Protein kinase; CamKⅡ: Calcium calmodulin mediated kinase Ⅱ; LRP: Low density lipoprotein receptor-related protein; GBP: Paternally expressed 12; APC: Adenomatous polyposis coli; CBP: CREB binding protein; PKC: Protein kinase C; NFAT: Nuclear factor of activated T cells; PLC: Phospholipase c.

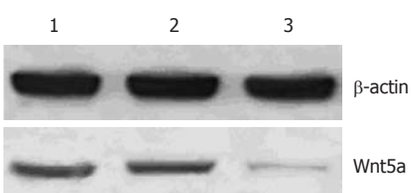


Figure 3 Expression levels of Wnt5a proteins in three groups detected by Western blotting. 1: Control; 2: Negative siRNA; 3: Wnt5a ShRNA.

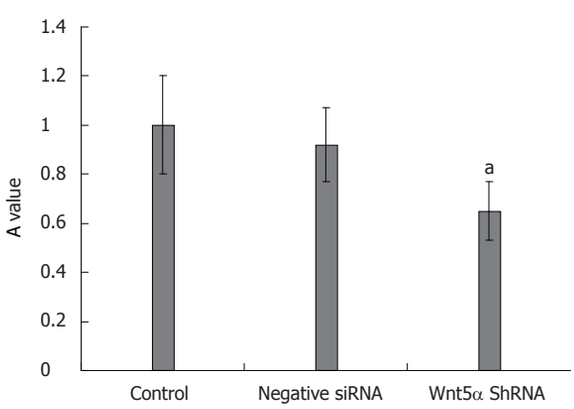


Figure 4 Knock down of Wnt5a decreases the cell proliferation. The role of Wnt5a in regulating LX-2 proliferation was determined by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Compared with the negative siRNA and control group, Wnt5a ShRNA could inhibit the proliferation of LX-2, [a]$P < 0.05$.

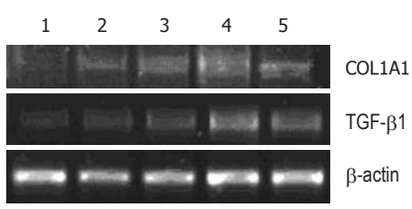


Figure 5 Knockdown of Wnt5a decreases expressions of α-1 type Ⅰ collagen and transforming growth factor-β1. Experiments were repeated three times and representative results are shown. Compared with the negative siRNA, vector and control groups, the expressions of α-1 type Ⅰ collagen (COL1A1) and transforming growth factor-β1 (TGF-β1) mRNA were downregulated remarkably in the Wnt5a shRNA group ($P < 0.05$). 1: Wnt5a ShRNA multiplicity of infection (MOI) 20; 2: Wnt5a ShRNA MOI 10; 3: Negative siRNA; 4:Vector; 5: Control.

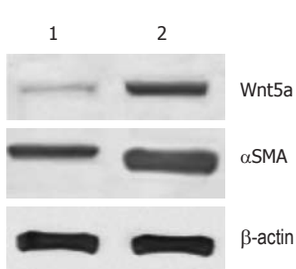


Figure 6 Expression of Wnt5a in fibrotic livers determined with Western blotting. The bands were semi-quantitatively evaluated by densitometric analysis. Wnt5a protein was upregulated as significantly as αSMA in the CCl_4-induced liver fibrosis model when compared with the normal rats ($P < 0.01$). 1: Normal group; 2: Model group.

In addition, the expressions of Wnt5a and Frizzled2 in HSCs isolated from the mouse liver fibrosis model induced by bile duct ligation were higher as compared with HSCs isolated from normal mice. It suggests that Wnt5a signaling pathway may be involved in differentiation of quiescent HSCs into myofibroblasts. Shackel *et al*[28] reported the highly expression of Wnt5a in primary biliary cirrhosis. We found that Wnt5a was upregulated in activated HSCs in comparison with quiescent HSCs as well as its downstream factors such as Frizzled2 and CamK Ⅱ. Changes in gene expression profiles observed in the microarrays were validated by qRT-PCR analysis. The direct evidence of Wnt5a on the activation of HSCs has not been reported. Because of this, we treated human HSCs line LX-2 with Wnt5a ShRNA, and found that knockdown of *Wnt5a* gene expression in LX-2 led to significantly impaired proliferation, downregulated expressions of type I collagen and TGF-β1. Wnt5a was upregulated in fibrotic liver of a CCl_4 -induced fibrosis rat model.

In summary, our data suggest that Wnt5a may play a role in HSCs activation and liver fibrogenesis, thus revealing the mechanism of HSCs activation from a new perspective, which may provide a novel means and therapeutic target for the prevention and treatment of liver fibrosis.

COMMENTS

Background

Hepatic stellate cells (HSCs) activation is one of the key steps in the development of liver fibrosis. However, the molecular mechanisms underlying the activation of HSCs are not fully understood. It is reported that the expression levels of Wnt5a and its downstream signaling molecules were significantly increased in activated HSCs and experimental liver fibrosis model, indicating that Wnt5a signaling pathways may be involved in liver fibrosis. But the direct evidence of Wnt5a on the activation of HSCs has not been reported. In this study, the authors aimed to identify differentially expressed genes in quiescent and activated HSCs and explore the role of Wnt5a in the activation of HSCs.

Research frontiers

Chip technology is rapid and concise in detecting mRNA expression. Previous studies have reported the differentially expressed gene profiles between quiescent and activated HSCs with gene chip. But, intense bioinformatics analyses and functional assays are deficient. No study has provided the direct evidence of Wnt5a in the mechanism of HSCs activation.

Innovations and breakthroughs

Bioinformatic analysis of the differentially expressed gene profiles between quiescent and activated HSCs reveals the role of Wnt5a signaling pathway in HSCs activation. This is the first study addressing the role of Wnt5a in the mechanism of HSCs activation.

Applications

This study suggests that Wnt5a participates in the HSCs activation and liver fibrogenesis, which may provide a novel means and therapeutic target for the prevention and treatment of liver fibrosis.

Terminology

Wnt signaling molecules are involved in diverse developmental and pathological processes. Based on the different intracellular events activated after Wnt-Frizzled binding, Wnt signaling is divided into two subclasses: the canonical pathway and the non-canonical pathway. Wnt5a is a non-canonical member of the Wnt family. In the Wnt5a signaling pathway, Wnt5a and Frizzled binding triggers the intra-cytoplasmic release of calcium. Increased intracellular calcium concentration activates calcineurin and calcium calmodulin mediated kinase Ⅱ. Wnt5a signaling takes part in wound healing, regulation of cell proliferation, vasculature development and the formation of cell polarity.

Peer review

The research is the *in vitro* experiments showing that Wnt5a ShRNA decreases proliferation, downregulates expression of collagen type Ⅰ and transforming growth factor beta, that probes into a direct Wnt5a participation in the mechanism of HSC activation.

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