Delta-like 1 Serves as a New Target and Contributor to Liver Fibrosis Down-regulated by Mesenchymal Stem Cell Transplantation

Ruo-Lang Pan<sup>a,b</sup>, Ping Wang<sup>c</sup>, Li-Xin Xiang<sup>d</sup>,<sup>e</sup> and Jian-Zhong Shao<sup>d</sup>,<sup>e</sup>

From the <sup>a</sup>College of Life Sciences, Zhejiang University, Hangzhou 310058 and the <sup>b</sup>Key Laboratory for Cell and Gene Engineering of Zhejiang Province, China

Chronic liver injury always progresses to fibrosis and eventually to cirrhosis, a massive health care burden worldwide. Delta-like 1 (Dlk1) is well known as an inhibitor of adipocyte differentiation. However, whether it is involved in liver fibrosis remains unclear. Here, we provide the first evidence that Dlk1 is a critical contributor to liver fibrosis through promoting activation of hepatic stellate cells (HSCs) during chronic liver injury. We found that upon liver injury, Dlk1 was dramatically induced and initially expressed in hepatocytes and then into the HSCs by a paracrine manner. It leads to the activation of HSCs, which is considered to be a pivotal event in liver fibrogenesis. Two forms (~50 and ~25 kDa) of the Dlk1 protein were detected by Western blot analysis. In vitro administration of Dlk1 significantly promoted HSC activation, whereas in vivo knockdown of Dlk1 dramatically inhibited HSC activation and the subsequent fibrosis. The large soluble form (~50 kDa) of Dlk1 was shown to contribute to HSC activation. We were encouraged to find the Dlk1-promoted HSC activation and liver fibrosis can be depressed by transplantation of bone marrow-mesenchymal stem cells (BM-MSCs). Furthermore, we demonstrated that FGF2 was up-regulated in BM-MSCs under injury stimulation, and it probably participated in the inhibition of Dlk1 by BM-MSCs. Our findings provide a novel role of Dlk1 in liver fibrosis leading to a better understanding of the molecular basis in fibrosis and cirrhosis and also give insights into the cellular and molecular mechanisms of MSC biology in liver repair.

Fibrosis occurs in almost all patients with chronic liver injury, and its end stage, cirrhosis, represents a massive health care burden worldwide (1). It has long been a challenging problem because limited therapy exists, and the underlying detailed cellular and molecular mechanisms are still elusive (2). Recently, increasing evidence has suggested the therapeutic potential of MSCs<sup>i</sup> in the treatment of liver diseases (3–7), and the transplantation of exogenous MSCs can strongly alleviate acute or chronic liver injury and improve the fibrosis (8–10). However, the exact fate and role of MSCs played in these processes is still undetermined, and the cellular and molecular mechanisms of MSC involved liver repair remain unclear. Here, we provide the first evidence that MSCs exert an anti-fibrotic effect by suppression of hepatic stellate cell (HSCs) activation by inhibiting Delta-like 1 (Dlk1), a previously unknown modulator in liver fibrosis.

Liver fibrosis resulting from chronic liver damage is closely related to excessive extracellular matrix accumulation, in which hepatic stellate cells (HSCs) are the main contributor (11–13). During liver injury, quiescent HSCs become activated to a contractile myofibroblast-like phenotype, expressing cytoskeletal protein α-smooth muscle actin (α-SMA) and secreting fibrillar collagens (14). Therefore, the prevention of HSC activation is a promising strategy for anti-fibrotic therapies. Several cytokines, such as TGF-β, platelet-derived growth factor, and connective tissue growth factor, are involved in fibrosis by stimulating HSC proliferation, activation, and migration; however, the molecular and cellular responses involved in fibrogenesis seem more complex (15, 16). Therefore, our present finding that Dlk1 serves as a critical modulator in liver fibrosis provides a new target for anti-fibrotic therapy and also new insight into molecular basis in fibrosis and cirrhosis.

Dlk1, previously known as an inhibitor of adipocyte differentiation (17), is made as an epidermal growth factor repeat-containing transmembrane protein that can be proteolytically cleaved into large (50 kDa) and small (25 kDa) soluble forms. However, only the large soluble form is essential to the inhibition of adipocyte differentiation (18). In addition, Dlk1 possesses several other biological activities, including cellular interaction and development (19–21). Whether this factor is involved in liver fibrosis remains unclear. Previously, the expression of Dlk1 was found up-regulated during the progression of liver fibrosis in human biliary atresia (22), suggesting the implication of Dlk1 in fibrogenesis associated with biliary atresia. Nevertheless, the exact relationship among Dlk1 expression, HSC activation, and liver fibrosis remains elusive. In this study, we provide the first evidence that Dlk1 is a critical contributor to liver fibrosis through promoting HSC activation. We also found that this Dlk1-promoted HSC activation and fibrosis could be down-regulated by transplantation of MSCs in molecular weight; HMW, higher molecular weight; TRITC, tetramethylrhodamine isothiocyanate.
which FGF2 is involved, adding new insight into MSC biology in liver fibrosis.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals and Liver Injury Models**—Eight-week-old male ICR mice were obtained and housed, as described previously (5, 6). All animal experiments were performed in accordance with legal regulations, including approval by a local ethics committee. For acute or chronic liver injury models, mice were subjected to carbon tetrachloride (CCl4) intoxication according to previously described protocols (7, 23).

**Microarray and Western Blot Analyses**—A genome-wide microarray (Illumina Mouse WG-6 version 2.0 BeadChip) was used to evaluate the cytokines potentially involved in liver injury, as described previously (7). Western blotting was performed to verify the up-regulation of Dlk1 detected in the microarray analysis. Protein extracts from the liver tissues of the injured mice from 2 days and 1–4 weeks were electrophoresed and blotted. The blots were incubated with rat anti-Dlk1 antibodies (Enzo Life Sciences AG, Switzerland) and HRP-conjugated rabbit anti-rat IgG (Santa Cruz Biotechnology) and then visualized using enhanced chemiluminescence.

**In Situ Hybridization**—A 207-bp sequence amplified from Dlk1 cDNA was used to prepare riboprobes by in vitro transcription using the DIG RNA labeling kit (Boster, China) according to the manufacturer’s instructions. The primers are shown in supplemental Table 1. Sections of paraffin-embedded liver tissues (CCl4 injury for 4 weeks) were deparaffinized and digested with proteinase K (25 μg/ml) at 37 °C for 10 min, followed by hybridization with Dlk1 probes in hybridization solution at 48 °C for 15 h. The positive signals were developed with DAB color reaction, and tissue sections were counterstained with hematoxylin.

**Immunofluorescence Staining**—Tissue sections were deparaffinized and incubated with mouse anti-α-SMA (Boster, China) and goat anti-Dlk1 antibodies (Santa Cruz Biotechnology) for dual immunofluorescence staining as described previously (23), with some modifications. The secondary antibodies, including TRITC-conjugated rabbit anti-goat IgG and FITC-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology), were used according to the manufacturer’s instructions. Samples were counterstained with DAPI and photomicrographed under a fluorescent microscope (Axioskop Plus, Carl Zeiss Inc., Germany).

**Preparation of Dlk1 Proteins**—The large (Dlk1-L) and small (Dlk1-S) soluble forms of Dlk1 proteins were prepared as described previously (18), with some modifications. Briefly, the cDNA sequences encoding large soluble form of Dlk1 (Dlk1-L), which contains six EGF-like domains, and a small soluble form of Dlk1 (Dlk1-S), which contains EGF repeats 1–3, were inserted into pCDNA6 digested with HindIII and BamHI. The two constructs were verified by sequencing and transforming into HEK293 cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Serum-free culture media were collected 72 h after transfection, centrifuged at 500 × g for 10 min, and the supernatants stored at −20 °C for further use. The recombinant Dlk1-L and Dlk1-S proteins were identified by Western blot, and the concentrations were determined by ELISA.

**Activation of HSCs by Dlk1**—Mouse HSCs were isolated by collagenase-Pronase perfusion and density centrifugation on Nycodenz gradients as described previously (24), with some modifications. Briefly, the livers were perfused via portal veins with Pronase and collagenase (Invitrogen). After digestion, cell suspension was filtered through nylon mesh and purified via 8.2% Nycodenz (Axis-shield, Norway) gradient centrifugation. The isolated HSCs were cultured in uncoated plastic dishes with Iscove’s modified Dulbecco’s medium (Sigma) supplemented with 10% FBS (HyClone, MD) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in 5% CO2. The purity of HSCs was determined by their typical star-like shape and abundant lipid droplets. The cells were induced with cultural medium containing Dlk1-L and Dlk1-S (40 ng/ml). After 7–14 days, the activation of the HSCs was examined by morphological observation, real time PCR, and immunofluorescence staining of biomarkers.

**Real Time PCR**—The expression levels of Dlk1, α-SMA, and α1(I) collagen (coll1α1(I)) were quantified by real time PCR using Mastercycler ep realplex (Eppendorf, Germany) and detection system software using the primers shown in supplemental Table 1. The total amount of transcripts was normalized to endogenous β-actin mRNA.

**siRNA Treatment**—siRNA with 5′-phosphate modification for targeting Dlk1 was synthesized by GenePharma (China) and delivered in vivo as described previously (25). The sense and antisense strands of siRNA were 5′-P-CCCCUUAUGCAUGAUAAAdTdT-3′ (sense) and 5′-P-UUAAUCAGUAAUAGGGAG-3′ (antisense). Synthetic siRNAs were delivered in vivo using a modified “hydrodynamic transfection method,” by which 50 μg of siRNA dissolved in 1 ml of PBS was rapidly injected into the tail vein of mice with 2-week liver injury by CCl4. The injection was repeated 8 and 24 h later. Control mice were injected with an equal volume of PBS. Then the mice were injected for another week, and liver tissues were collected for real time PCR and Picrosirius Red staining to evaluate the levels of HSC activation and liver fibrosis.

**Picrosirius Red Staining**—For fibrosis examination, liver tissues were fixed with 4% parafomaldehyde and embedded in paraffin. Then 5-μm tissue sections were stained with Picrosirius Red (Sigma), and the extent of fibrosis was assessed under light microscope (Carl Zeiss, Germany) and by a software program.

**BM-MSCs Transplantation**—Mouse BM-MSCs were prepared and transplanted into liver-injured recipients through the caudal vein as described previously (7, 23). Briefly, bone marrow-derived cells were flushed from the femurs and tibias of ICR and enhanced GFP transgenic mice and cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO2. After 3 days, nonadherent cells and debris were removed, and the adherent cells were cultured continuously. At near confluence, the cells were re-plated at 100 cells/cm2. The mesenchymal lineage differentiation (osteogenic, adipogenic, and chondrogenic differentiation) was examined for functional identification. Then the BM-MSCs were transplanted into liver-injured...
mice through the caudal vein. Persistent liver damage was maintained after transplantation. The animals were sacrificed 2 weeks post-transplantation, and the livers were collected for analysis of Dlk1 expression, HSC activation, and liver fibrosis. Meanwhile, a “rescue” experiment was performed. Mice that received BM-MSC transplantation were managed with Dlk1 protein at different concentrations (2, 4, and 8 μg/mouse). Then the mice were injured for another 2 weeks, and their livers were collected for analysis of α-SMA and collagen I (α1) expression for HSC activation.

**Cellular Interaction Assay**—Hepatocytes from chronic liver-injured mice were isolated by a two-step collagenase perfusion as described previously (5). A co-culture system of hepatocytes with the BM-MSCs was assembled by transwell membranes (24 mm diameter, 0.4 μm pore size; Millipore). Approximately 1.0 × 10^6 hepatocytes were placed in the lower chamber, and an equal amount of the BM-MSCs was added to the upper chamber. After 72 h, both the hepatocytes and BM-MSCs were collected for gene expression analysis. The expression of Dlk1 in the hepatocytes and cytokines (FGF4, FGF2, hepatocyte growth factor, oncostatin M, and IL6) in the BM-MSCs was quantified by real-time PCR with the primers shown in supplemental Table 1. Normal cultured hepatocytes and BM-MSCs were used as controls. In a parallel experiment, after being co-cultured with the hepatocytes for 48 h, the BM-MSCs were placed in serum-free medium for another 24 h. Then the culture media were collected and passed through a 0.25-μm filter. The secreted cytokine (FGF2) in the filtrate was examined by ELISA using specific antibodies (Beijing Biosynthesis Biotechnology Co., China).

**Functional Evaluation of FGF2**—An in vitro FGF2 depletion or administration assay was performed to investigate the role of FGF2 in BM-MSC-inhibited Dlk1 expression. For the depletion of FGF2, the culture medium of co-cultured BM-MSCs was incubated with anti-FGF2 antibody (Beijing Biosynthesis Biotechnology Co., Ltd., China) overnight 4°C under agitation. Protein A-coupled Sepharose beads were prepared according to the manufacturer’s instruction (AbCAM, UK), and added into the antibody-pretreated culture medium. After being incubated at 4°C for 4 h, the samples were centrifuged at 10,000 × g for 5 min, and supernatants were collected and defined as media (dep) for the treatment on hepatocytes from injured liver. In parallel, a nonspecific antibody (IgG isotype) was used in control groups and the supernatants were defined as media (co). Then the expression of Dlk1 in the hepatocytes was determined after exposing the cells to a co-cultural medium of FGF2 depletion (media (dep)) or FGF2 (R & D Systems, UK) protein (20 ng/ml) for 72 h. For an in vivo functional assay, FGF2 was injected into liver-injured recipients through the caudal vein (50 μg/kg body weight). Persistent liver damage was maintained for another 2 weeks. Then the mice were sacrificed, and their livers were collected for Dlk1 expression, HSC activation, and liver fibrosis analyses.

**Statistical Analysis**—All experiments were replicated a minimum of three times. Data were expressed as mean ± S.D. The differences between the values were determined by the independent samples test. A p value of less than 0.05 was considered statistically significant.

## RESULTS

**Dlk1 Expression Was Up-regulated in Injured Liver**—Genome-wide microarray analysis unexpectedly found that the mRNA level of Dlk1, a known crucial gene involved in adipogenesis, was dramatically elevated (>1400-fold) in the CCl_{4}-injured livers. This finding encouraged us to speculate that Dlk1 might be associated with liver injury and HSC activation. Therefore, kinetic expression of Dlk1 during CCl_{4}-induced chronic liver injury was further examined at the protein level. The results show that two forms (~50 and 25 kDa) of Dlk1 proteins were constantly expressed in injured livers at different time points but were nearly undetectable in healthy mice (Fig. 1A). In situ hybridization and immunofluorescence staining demonstrated that Dlk1 mRNAs were expressed mainly in hepatocytes (Fig. 1B). However, the Dlk1 proteins were localized to most α-SMA-positive cells (indicative of the activated HSCs) but rarely in the parenchymal cells (Fig. 1C). These results mirror previous findings in human biliary atresia (22) and suggest that Dlk1 may be synthesized in hepatocytes, released, and transported into HSCs. To preclude the possibility that Dlk1 mRNA is also expressed in HSCs, cells were isolated from injured livers for RT-PCR analysis. As expected, little Dlk1 expression could be detected in HSCs, whereas injured hepatocytes demonstrated significant Dlk1 expression (Fig. 1D). These findings suggest that a paracrine signaling mechanism between hepatocytes and HSCs might be involved in HSC activation during liver injury.

**In Vitro Determination of Dlk1-promoted HSCs Activation**—Based on the above results, both soluble forms of Dlk1 (50-kDa large and 25-kDa small forms) appeared to be induced by CCl_{4} treatment. To evaluate the potential contribution of Dlk1 in HSC activation, we prepared two forms of Dlk1 (Dlk1-L and Dlk1-S) by HEK293 cell transfection (supplemental Fig. 1), and the culture media were collected for HSC activation assay. The results show that after incubation of the HSCs with Dlk1-L for 7 days, the expression of PPARy, a hallmark of adipocytes, significantly decreased, whereas the expression of α-SMA and collagen I (α1), two markers of activated HSCs, considerably increased. Compared with the Dlk1-L treatment, the expression of these genes changed to a lesser extent either in the control group or in the Dlk1-S-treated group, although they were also up-regulated or down-regulated because of the auto-activation of HSCs (Fig. 2, A and B). Similarly, morphological observations show that many lipid droplets deposited in control and Dlk1-S-treated cells, whereas hardly any lipid droplets could be detected in Dlk1-L-treated cells. Accordingly, immunofluorescence staining combined with cellular fluorescence intensity analysis demonstrate that more abundant α-SMA expression could be detected after Dlk1-L induction for 14 days (Fig. 2, C and D). All these results demonstrate that Dlk1-L, rather than Dlk1-S, effectively promoted the activation of HSCs in vitro.

**In Vivo Determination of Dlk1-promoted HSC Activation and Liver Fibrosis**—To further confirm the role of Dlk1 in HSC activation during liver injury, we performed in vivo RNAi against Dlk1 or additional administration of Dlk1-L. The up-regulated Dlk1 transcripts were significantly (p < 0.01) inhib-
ited after synthetic siRNA injection, accompanied by reduced levels of α-SMA and col I (α1) mRNAs (Fig. 3A). This result demonstrates that the activation of HSCs could be dramatically inhibited by the “knockdown” of Dlk1. By contrast, additional administration of Dlk1-L markedly (p < 0.05) promoted HSC activation, as determined by the expression of α-SMA and col I (α1) (Fig. 3, B and C). Accordingly, the extent of liver fibrosis changed in concordance with the alteration of HSC activation (Fig. 3D). These in vivo observations provide further support to the role of Dlk1 (Dlk1-L) in HSC activation and liver fibrogenesis.
**BM-MSCs Attenuated Fibrosis through Dlk1 Inhibition**—We further investigated whether the BM-MSCs could reduce fibrosis through inhibition of Dlk1 expression. BM-MSCs were identified by their mesenchymal lineage differentiation potency (supplemental Fig. 2). Then we found that, in accordance with our previous report (7), BM-MSCs efficiently migrated to the injured livers after 2 weeks of transplantation (supplemental Fig. 3). Quantification of Sirius Red-stained collagen fibers in images of liver tissues clearly shows that the transplantation of 1 × 10^6 BM-MSCs obviously attenuated liver fibrosis in CCl₄-injured mice (Fig. 4, A). Afterward, we investigated whether the inhibitory effect of the BM-MSCs on HSCs was caused by the down-regulation of Dlk1. Real time PCR and in situ hybridization show that BM-MSC transplantation markedly attenuated the up-regulation of Dlk1 transcripts in the hepatocytes during liver injury (Fig. 5, A). Using Dlk1 and α-SMA dual immunofluorescence staining, we found that the proportion of α-SMA-positive cells in transplanted mice decreased with the depression of Dlk1 (Fig. 5C). This suggests the participation of Dlk1 in BM-MSC-modulated HSC activation. For further evidence, a rescue experiment was performed by administering the recombinant Dlk1 (Dlk1-L) into the BM-MSC-transplanted mice. The down-regulated activation of HSCs in transplanted mice was...
significantly rescued by the increased administration of Dlk1, as determined by the changes in α-SMA and col I(α1) expression (Fig. 5D). These findings suggest that the BM-MSCs might down-regulate the expression of Dlk1 in hepatocytes and impair the subsequent paracrine of Dlk1 protein into HSCs, resulting in depression of HSC activation.

**BM-MSCs Inhibited Dlk1 Expression by Secreting FGF2**—An *in vitro* cellular interaction assay was performed to investigate the possible mechanisms underlying BM-MSC-inhibited Dlk1 expression. Hepatocytes from injured livers were isolated and co-cultured with the BM-MSCs using a transwell device. In agreement with the *in vivo* observations, the Dlk1 transcripts in the hepatocytes were significantly reduced after a 72-h co-culture, providing direct evidence that the BM-MSCs contributed to the suppression of Dlk1 in the hepatocytes upon liver injury (Fig. 6A). This result also suggests that cytokines may be released from BM-MSCs for participation in this process because no cell-cell interactions existed in the experimental system. Then a set of cytokines, including FGF2, FGF4, IL6, hepatocyte growth factor, and oncostatin M, well known for their participation in BM-MSC biology (including anti-scarring, support, angiogenesis, and liver development) (26, 27), were selectively examined. FGF2 was dramatically up-regulated (>4-fold) in BM-MSCs after co-culture (Fig. 6B). A similar result was also obtained in the co-cultural medium detected by ELISA at the protein level (Fig. 6C). Therefore, FGF2 might be a key cytokine in BM-MSC-modulated Dlk1 inhibition. When FGF2 was removed through a “depletion” experiment, the inhibitory effect of the cultural medium on Dlk1 expression was dramatically suppressed (Fig. 6D). Thus, FGF2 was cogently demonstrated to participate in the BM-MSC-induced inhibition of Dlk1 in the injured hepatocytes.

**FGF2 Influenced Dlk1-regulated HSC Activation and Fibrosis**—To directly observe if FGF2 is involved in BM-MSC-suppressed Dlk1 expression and to evaluate whether FGF2 alone has an inhibitory effect on Dlk1-regulated HSC activation and fibrosis, we performed an FGF2 assay both *in vitro* and *in vivo*. The results show that *in vitro* treatment of the hepatocytes with FGF2 at 20 ng/ml for 72 h caused a 45% decrease in Dlk1 expression compared with the controls (Fig. 7A). Similarly, *in vivo* administration of FGF2 to chronically liver-injured mice largely mimicked the BM-MSC transplantation, in which Dlk1 expression decreased, accompanied by the significant inhibition of α-SMA and col I(α1) expression and Sirius Red-stained collagen deposition (Fig. 7, B–D). These results reveal that FGF2 participated in BM-MSC-suppressed Dlk1 expression.
**HSC activation, and fibrosis, which may provide new insight into the possible use of FGF2 in anti-fibrotic therapy.**

**DISCUSSION**

Liver fibrosis resulting from the excessive deposition of extracellular matrix represents the final common pathway for most chronic liver diseases. Activated HSCs are the major source of extracellular matrix during liver injury. Although the relationship between HSC activation and liver fibrosis has been studied extensively, strategies or drugs for preventing HSC activation and treating liver fibrosis are only partially effective (28). Therefore, improving knowledge on cellular and molecular bases of HSC activation as well as hepatic fibrogenesis will increase the possibilities for therapeutic treatments (16). Our study provides both in vivo and in vitro evidence showing Dlk1 as a new contributor to liver fibrosis through promoting HSC activation during chronic injury. It is anticipated to provide a new target for anti-fibrotic therapy and lead to a better understanding of the functional characterization of Dlk1 in hepatic fibrosis and molecular basis in liver disease.

Dlk1 was shown as an adipocyte-differentiation inhibitor. It can prevent lipid accumulation and expression of various adipocyte transcription factors, such as peroxisome proliferator-activated receptor γ (17). Quiescent HSCs are lipocyte-like cells of retinoid storage. Following liver injury, they are activated by losing adipocyte characteristics and trans-differentiated into myofibroblast-like cells (29). The present study showed that the Dlk1 mRNA was mainly detected in the hepatocytes, whereas...
its proteins were localized in α-SMA-positive HSCs, indicating the paracrine of Dlk1 from hepatocytes to HSCs. Therefore, it seems reasonable to hypothesize that the paracrine of Dlk1 facilitates the loss of adipocyte characteristics in HSCs, which might accelerate HSC activation and cirrhosis. In addition, the Dlk1 has been recognized to proteolytically cleave into two soluble forms. In this study, we also demonstrated that two soluble forms of Dlk1 could be detected during liver injury. However, only the large soluble form could promote HSC activation and fibrogenesis. The exact biological function of the small form of Dlk1 remains unexplored. So we propose that hepatocytes may express Dlk1 in response to injury signals and generate two soluble forms of Dlk1. The large soluble form was then parasitized from the hepatocytes into HSCs, facilitating the activation of HSCs and subsequent fibrogenesis.

Accumulating studies have shown the beneficial effects of MSC-based therapy on liver structural and functional repair (8, 9, 30, 31). However, the exact cellular and molecular mechanisms underlying these processes are still undetermined. Previously, we have demonstrated that either exogenous or endogenous BM-MSCs could be recruited into injured livers with a low rate of hepatic specification but with clear therapeutic effects (7). The results from that study indicate the limited contribution of MSCs to hepatocyte differentiation, and other mechanisms may exist. In this work, we demonstrated that BM-MSCs could significantly inhibit the expression of Dlk1, followed by the reduced paracrine of Dlk1 into HSCs, as well as impaired HSC activation and liver fibrosis. Our results confirmed the anti-fibrotic effect of MSCs in liver-injured mice and uncovered the involved mechanism, which was probably uncovered through the inhibition of HSC activation by down-regulation of Dlk1 expression. In addition, an in vitro co-culture experiment demonstrates that the BM-MSC-suppressed Dlk1 expression in the hepatocytes was independent of cell-cell contact, indicating the involvement of cytokine secretion. From various determined candidate cytokines, we found that FGF2 was dramatically up-regulated in BM-MSCs after simulating by the hepatocyte from injured liver. Depletion of FGF2 from the co-cultural medium by immunoprecipitation significantly repressed the BM-MSC-induced inhibition of Dlk1. Similarly, both in vitro and in vivo administration of FGF2 could inhibit Dlk1 expression, HSC activation, and fibrogenesis. According to present observations, we suggest that under liver injury signals, BM-MSCs might secrete FGF2 to modulate Dlk1 expression and participate in liver repair, which is called the paracrine mechanism currently being exposed (32).

FGF2 is a pleiotropic protein expressed in various isoforms, including the 18-kDa low molecular weight (LMW) form and the 22–34-kDa high molecular weight (HMW) forms (33). Overexpression of FGF2 has been associated with several human liver diseases, including hepatocellular carcinoma (34). However, the role of FGF2 in liver fibrosis remains elusive. Previous animal studies suggested that LMW and HMW isoforms might play different roles in liver fibrosis. For example, FGF2-deficient mice were protected from CCl4-induced liver fibrosis (35), whereas the absence of FGFR4 resulted in profound liver fibrosis (36). In a rat model, the 22-kDa HMW FGF2 was the main isoform up-regulated during liver fibrogenesis (37). However, in our mouse model, we demonstrated that the LMW isoform had a therapeutic effect on liver fibrosis. Therefore, we propose that LMW and HMW FGF2 play opposing roles in liver fibrosis, but further studies are required to fully elucidate the role of FGF2. Furthermore, the optimal amount of LMW FGF2 for liver fibrosis therapy must be determined, and the susceptibility should be addressed in different animal models, as different concentrations of FGF2 have shown distinct effects in different animal lines (38). The unique pathological features of liver diseases and the multiple isoforms of FGF2 suggest a complex role for FGF2 in liver pathology.

In conclusion, we proposed a novel biological function for Dlk1 in liver fibrosis through promoting HSC activation. We also demonstrated that MSC can attenuate liver fibrosis by specifically down-regulating Dlk1 expression through FGF2 secretion. These findings largely improved the understanding of the molecular basis in liver fibrosis and provide not only a new target for liver fibrosis therapy but also insight into the cellular and molecular mechanisms underlying MSC-based tissue repair.

REFERENCES
1. Friedman, S. L. (2003) J. Hepatol. 38, S38–S53
2. Henderson, N. C., and Iredale, J. P. (2007) Clin. Sci. 112, 265–280
3. Luk, J. M., Wang, P. P., Lee, C. K., Wang, J. H., and Fan, S. T. (2005) J. Immunol. Methods 305, 39–47
4. Lee, K. D., Kuo, T. K., Whang-Peng, J., Chung, Y. F., Lin, C. T., Chou, S. H., Chen, J. R., Chen, Y. P., and Lee, O. K. (2004) Hepatology 40, 1275–1284
5. Chen, Y., Dong, X. J., Zhang, G. R., Shao, J. Z., and Xiang, L. X. (2007) J. Cell. Biochem. 102, 52–63
6. Pan, R. L., Chen, Y., Xiang, L. X., Shao, J. Z., Dong, X. J., and Zhang, G. R. (2008) Cytotherapy 10, 668–675
7. Chen, Y., Xiang, L. X., Shao, J. Z., Pan, R. L., Wang, Y. X., Dong, X. J., and Zhang, G. R. (2010) J. Cell. Mol. Med. 14, 1494–1508
8. Oyagi, S., Hirose, M., Kojima, M., Okuyama, M., Kawase, M., Nakamura, T., Ohgushi, H., and Yagi, K. (2006) J. Hepatol. 44, 742–748
9. Hirai, Y., Kato, H., Kato, T., and Ikeda, M. (2007) J. Cell. Biochem. 102, 52–63
10. Chen, Y., Xiang, L. X., Shao, J. Z., Pan, R. L., Wang, Y. X., Dong, X. J., and Zhang, G. R. (2010) J. Cell. Mol. Med. 14, 1494–1508
11. Ouyagi, S., Hirose, M., Kojima, M., Okuyama, M., Kawase, M., Nakamura, T., Ohgushi, H., and Yagi, K. (2006) J. Hepatol. 44, 742–748
12. Sul, H. S. (2009) Mol. Endocrinol. 23, 1717–1725
13. Mei, B., Zhao, L., Chen, L., and Sul, H. S. (2008) Biochem. J. 411, 1–18
14. Mei, B., Zhao, L., Chen, L., and Sul, H. S. (2008) Mol. Endocrinol. 23, 1717–1725
15. Sul, H. S. (2009) Mol. Endocrinol. 23, 1717–1725
16. Mei, B., Zhao, L., Chen, L., and Sul, H. S. (2008) Biochem. J. 411, 1–18
17. Wang, Y., and Sul, H. S. (2009) Cell Metab. 9, 287–302
18. Raghunandan, R., Ruiz-Hidalgo, M., Jia, Y., Ettenger, R., Rudikoff, E., Riggins, P., Farnsworth, R., Tesfaye, A., Laborda, J., and Bauer, S. R. (2008) Stem Cells Dev. 17, 495–507
19. Andersen, D. C., Petersson, S. J., Jørgensen, L. H., Bollen, P., Jensen, P. B., Teisner, B., Schroeder, H. D., and Jensen, C. H. (2009) Stem Cells 27, 898–908
20. Huang, C. C., Chuang, J. H., Huang, L. L., Chou, M. H., Wu, C. L., Chen, C. M., Hsieh, C. S., Lee, S. Y., and Chen, C. L. (2004) J. Pathol. 202, 172–179
21. Chen, Y., Pan, R. L., Zhang, X. L., Shao, J. Z., Xiang, L. X., Dong, X. J., and Zhang, G. R. (2009) J. Cell. Mol. Med. 13, 2582–2592
22. Uchinami, H., Seki, E., Brenner, D. A., and D’Armiento, J. (2006) Hepatol. 44, 420–429
23. Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shan-
Dlk1, a Novel Modulator in MSC-attenuated Liver Fibrosis

26. Meirelles Lda, S., Fontes, A. M., Covas, D. T., and Caplan, A. I. (2009) Cytokine Growth Factor Rev. 20, 419–427
27. Kinoshita, T., and Miyajima, A. (2002) Biochim. Biophys. Acta 1592, 303–312
28. Muddu, A. K., Guha, I. N., Elsharkawy, A. M., and Mann, D. A. (2007) Int. J. Biochem. Cell Biol. 39, 695–714
29. Friedman, S. L. (2008) Toxicology 254, 120–129
30. Sato, Y., Araki, H., Kato, J., Nakamura, K., Kawano, Y., Kobune, M., Sato, T., Miyaniishi, K., Takayama, T., Takahashi, M., Takimoto, R., Iyama, S., Matsunaga, T., Ohtani, S., Matsuura, A., Hamada, H., and Niitsu, Y. (2005) Blood 106, 756–763
31. Zhao, Q., Ren, H., Zhu, D., and Han, Z. (2009) Biol. Cell 101, 557–571
32. Psaltis, P. J., Zannettino, A. C., Worthley, S. G., and Gronthos, S. (2008) Stem Cells 26, 2201–2210
33. Chlebova, K., Bryja, V., Dvorak, P., Kozubik, A., Wilcox, W. R., and Krejci, P. (2009) Cell. Mol. Life Sci. 66, 225–235
34. Jin-no, K., Taninizu, M., Hyodo, I., Kurimoto, F., and Yamashita, T. (1997) J. Gastroenterol. 32, 119–121
35. Yu, C., Wang, F., Jin, C., Huang, X., Miller, D. L., Basilico, C., and McKeehan, W. L. (2003) Am. J. Pathol. 163, 1653–1662
36. Yu, C., Wang, F., Jin, C., Wu, X., Chan, W. K., and McKeehan, W. L. (2002) Am. J. Pathol. 161, 2003–2010
37. Peng, X., Wang, B., Wang, T., and Zhao, Q. (2005) J. Huazhong. Univ. Sci. Technol. Med. Sci. 25, 166–169, 222
38. Ishikawa, T., Terai, S., Urata, Y., Marumoto, Y., Aoyama, K., Murata, T., Mizunaga, Y., Yamamoto, N., Nishina, H., Shinoda, K., and Sakaida, I. (2007) Cell Tissue Res. 327, 463–470