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# Synergistic effects of rMSCs and salidroside on the experimental hepatic fibrosis

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Rat mesenchymal stem cells (rMSCs) and salidroside have been applied in the treatment of hepatic fibrosis. The present study aimed to investigate the mechanism of hepatic differentiation of rMSCs *in vitro* and synergistic effects of rMSCs and salidroside on the experimental hepatic fibrosis in rats. rMSCs treated with 10 μg/mL, 20 μg/mL and 50 μg/mL salidroside were taken at 14 days and the proteins were subjected to western blot analysis. Hepatic fibrosis was induced in rats by administration of porcine serum for 8 weeks. Then, rats were randomly divided into 6 groups: control group, hepatic fibrosis group (model), salidroside group, rMSCs group and rMSCs plus salidroside group. Four weeks later, the localization and differentiation of rMSCs were determined. To evaluate the improvement of liver injury, the pathology of hepatocytes (or liver) and serum transforming growth factor-β1 (TGF-β1) were assessed. Induced rMSCs expressed alpha-fetoprotein (AFP) and albumin (ALB), which suggested rMSCs differentiated towards hepatocytes; moreover, E-adherin and beta-catenin were involved in the hepatic differentiation of rMSCs. In experiments of rMSCs transplantation, the amount of collagen in the liver of rMSCs plus salidroside treated rats was significantly lowered accompanied by reduced expression of TGF-β1, when compared to the control group and rMSCs group. These findings suggested the synergistic effects of rMSCs transplantation and salidroside on hepatic fibrosis. Salidroside could differentiate rMSCs towards hepatocytes and E-adherin and beta-catenin were involved in the hepatic differentiation of rMSCs. Treatment with rMSCs transplantation and salidroside exerted synergistic effects on the experimental hepatic fibrosis via suppressing the expression of TGF-β1.

## 1. Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) have been an attractive cell source in regenerative medicine because they are easy to obtain. rMSCs are multipotent stem cells that can differentiate into a variety of cell types including mesodermal lineages, adipocytes, osteoblasts, and other mesodermal cells (Owen 1988; Bianco and Robey 2000). Under several conditions, MSCs could differentiate towards hepatocyte-like cells *in vitro* (Lee et al. 2004; Oh et al. 2000; Okumoto et al. 2005; Schwartz et al. 2002) and *in vivo* (Daan van Poll et al. 2008; Oyagi et al. 2006). As reported, hepatic differentiation might underlie some signaling pathway or molecules, such as Wnt-β-catenin (Shimomura et al. 2007; Yoshida et al. 2007b), Ras-mitogen-activated protein kinase (MAPK) (Okumoto et al. 2003), hepatocyte nuclear factor 3β (HNF3β) (Ishii et al. 2008), etc. Wnts are powerful regulators of cell proliferation and differentiation, and their signaling pathway involves proteins that directly participate in both gene transcription and cell adhesion (Bowen et al. 2004). In the absence of Wnt signaling, the level of β-catenin is kept low through degradation of β-catenin. As is known (Bowen et al. 2004), cadherins inlayed in the plasma membrane, for example, E-cadherin, and β-catenins in excess of E-cadherin would be degraded, so expression of E-cadherin should affect degradation of β-catenin, signal transduction and hepatic differentiation of MSCs, which should be examined.

Moreover, the results related to therapeutic effects of MSCs on hepatic fibrosis were not consistent in a rat model of severe liver injury (Carvalho et al. 2008; Donckier et al. 2004; Terai et al. 2003). Liver diseases are frequently caused by infectious agents, autoimmune attack and inborn genetic deficiencies etc, and a majority of liver diseases usually lead to hepatocyte dysfunction with the possibility of eventual organ failure (Seo et al. 2005). The progress made in the field of liver transplantation has revolutionized the treatment of a wide range of liver diseases, but the lack of donor livers and immune rejection are troubling the patients. Therefore, it is imperative to find novel strategies to substitute liver transplantation.

Salidroside has been reported to have various pharmacological properties (Song et al. 2004; Wang et al. 2004; Zhang and Liu 2006). We have found that salidroside, combined with fibroblast growth factor (FGF) could act to differentiate MSCs into hepatecytes with minimal toxicity *in vitro* (data shown elsewhere). In the present study, we examined whether salidroside could differentiate MSCs into hepatocytes and what is the mechanism of differentiation. Moreover, we examined whether MSCs can be induced to undergo morphologic and phenotypic changes consistent with hepatogenic differentiation, and reduce

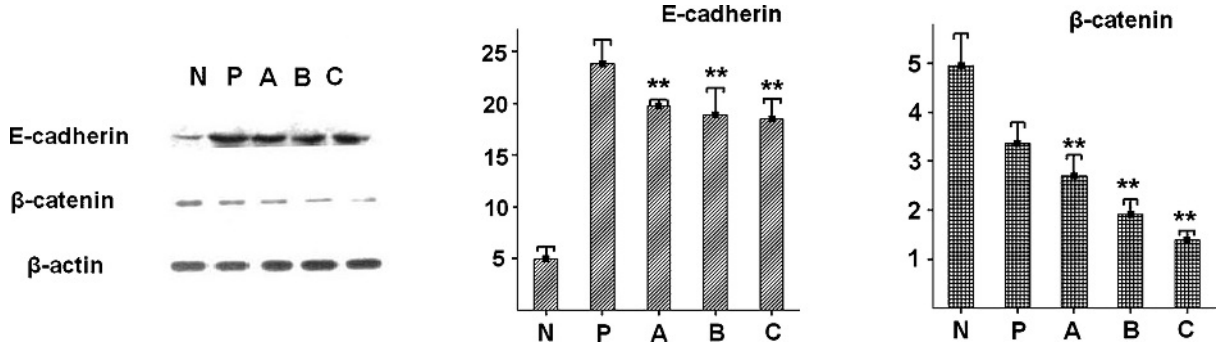


Fig. 1: E-cadherin and beta-catenin expression in hepatic differentiation of rMSCs. Data are expressed as the mean ± standard error of the mean of three experiments.*$P < 0.05$, **$P < 0.01$ compared with the protein level of Group N (native control)

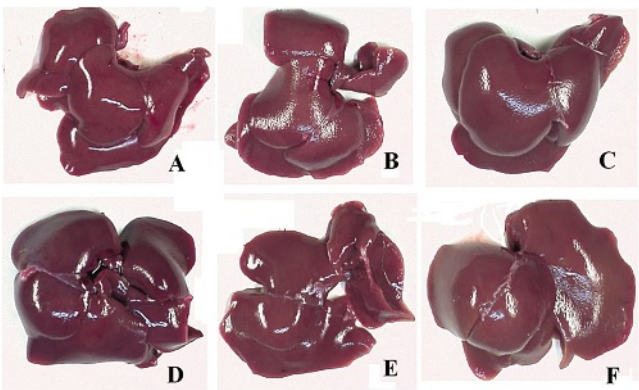


Fig. 2: The morphological changes of livers in each group. rMSCs transplantation improved the morphological changes. Normal livers in Group A were brown and bright, smooth and soft; those of Group B were dark and swelling, hard and rough accompanied by numerous fibrous nodules on the surface of the livers; rMSCs transplantation could significantly decrease the hepatic fibrosis in a time-dependent manner, and salidroside had the synergistic effects. A and B represent normal rats (control) and hepatic fibrosis rats without treatment, respectively; C and D independently represent rats receiving porcine serum and $5 \times 10^6$ or $1 \times 10^6$ rMSCs; E represents rats receiving porcine serum, $1 \times 10^6$ rMSCs and salidroside; F represents rats receiving porcine serum and salidroside

hepatic fibrosis or improve liver function, and whether there are synergistic effects of rMSCs transplantation and salidroside on hepatic fibrosis.

## 2. Investigations and results

### *2.1. MSCs differentiated towards hepatocytes and expressions of E-cadherin or β-catenin were changed*

Other groups showed that down-regulation of Wnt/β-catenin signaling plays a role in hepatic differentiation of MSCs (Shimomura et al. 2007; Yoshida et al. 2007a). We found that enhanced expression of E-adherin or the suppression of β-catenin signals both play an important role in the hepatic fate specification of MSCs induced by HGF or salidroside (Fig. 1).

### *2.2. Transplantation of rMSCs improved the morphology and histopathology of livers*

To evaluate the synergistic effects of rMSCs and salidroside, the morphology and histopathology of livers were examined. Normal livers in Group A were brown and bright, smooth and soft; those of Group B were dark and swelling, hard and rough accompanied by a large number of fibrous nodules on the surface; both rMSCs transplantation and salidroside administration could decrease hepatic fibrosis, and salidroside even acted synergistically (Fig. 2).

**Table 1: Plasma levels of AST and ALP in each group**

| Group | AST (U/L) | ALP (U/L) |
|---|---|---|
| A | 100.21 ± 15.62* | 123.13 ± 15.06** |
| B | 128.46 ± 11.77 | 242.30 ± 10.88 |
| C | 82.94 ± 6.94** | 113.77 ± 27.98** |
| D | 100.46 ± 16.38* | 144.77 ± 20.06** |
| E | 84.97 ± 5.56** | 128.01 ± 14.62** |
| F | 87.56 ± 17.58** | 131.64 ± 24.10** |

Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$, *vs* Group B. A and B represent normal rats and rats receiving porcine serum alone, respectively; C and D independenly represent rats receiving porcine serum and $5 \times 10^6$ or $1 \times 10^6$ rMSCs; E represents rats receiving porcine serum, $1 \times 10^6$ rMSCs and salidroside; F represents rats receiving porcine serum and salidroside

HE staining is often used for the pathological examination and azan staining is a complex method for general histology and suitable for color photographs (Shunji 2001). HE staining (Fig. 3 A1∼F1) and azan staining (Fig. 3 A2∼F2) revealed massive fibrosis and hepatocyte denaturation in the group B when compared with group A. The amelioration of liver pathology was found in the groups treated with rMSCs or salidroside, and the improvement of hepatic fibrosis and hepatocyte denaturation in Group E were more evident than in Group D and F. These results suggested synergistic effects of rMSCs and salidroside.

### *2.3. Distribution, proliferation or hepatic differentiation of transplanted rMSCs*

To localize rMSCs in the liver and investigate their status, MSCs were labeled with CM-Dil 24 h before being injected into the portal vein. At the end of the experiment, rats were sacrificed and the number of CM-Dil positive cells was detected. The presence of transplanted MSCs was determined by immunohistochemistry. Some CM-Dil positive cells were found in the groups treated with rMSCs 28 days after transplantation, part of which had differentiated towards hepatocytes. But it is difficult to prove the basal rate of MSCs proliferation and differentiation because differentiation of transplanted rMSCs into hepatocytes was a small probability event when compared to the hepatocytes in the liver (Fig. 4).

### *2.4. rMSCs Transplantation decreased transaminase activity*

Transaminase activity was measured to examine liver function. When massive hepatocyte death overwhelms the regenerative capacity of the liver during hepatic damage or failure, the release of liver enzymes increased accompanied by enhanced activities of peripheral liver enzymes.

As shown in Table 1, the levels of serum AST and ALP in the rats of Group B were significantly increased, which were

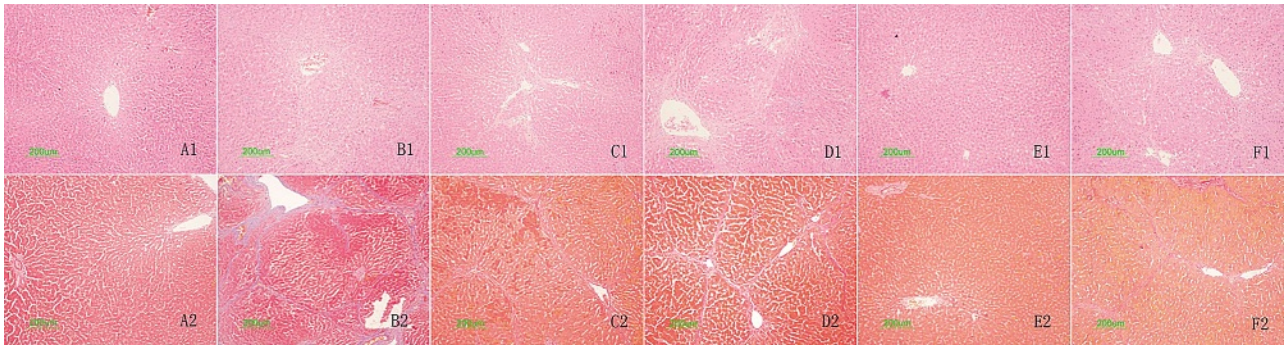


Fig. 3: HE-staining of livers in each group (A1∼F1, 200×). After 8 weeks of intraperitoneal administration of porcine serum, hepatic fibrosis was observed indicated by HE-staining. A large fibrotic area was seen in Group B. The therapeutic effects of rMSCs, salidroside and the synergistic effects of salidroside and rMSCs on hepatic fibrosis were proved. Azan-staining of livers in each group (A2∼F2, 200×). Sections stained with azan showed an extensive accumulation of collagens. The amount of collagens in Group B was higher than in other groups, and the therapeutic effects in Group E were more evident than in other groups suggesting salidroside had the synergistic effects. A and B represent normal rats (control) and hepatic fibrosis rats without treatment, respectively; C and D independently represent rats receiving porcine serum and $5 \times 10^6$ or $1 \times 10^6$ rMSCs; E represents rats receiving porcine serum, $1 \times 10^6$ rMSCs and salidroside; F represents rats receiving porcine serum and salidroside

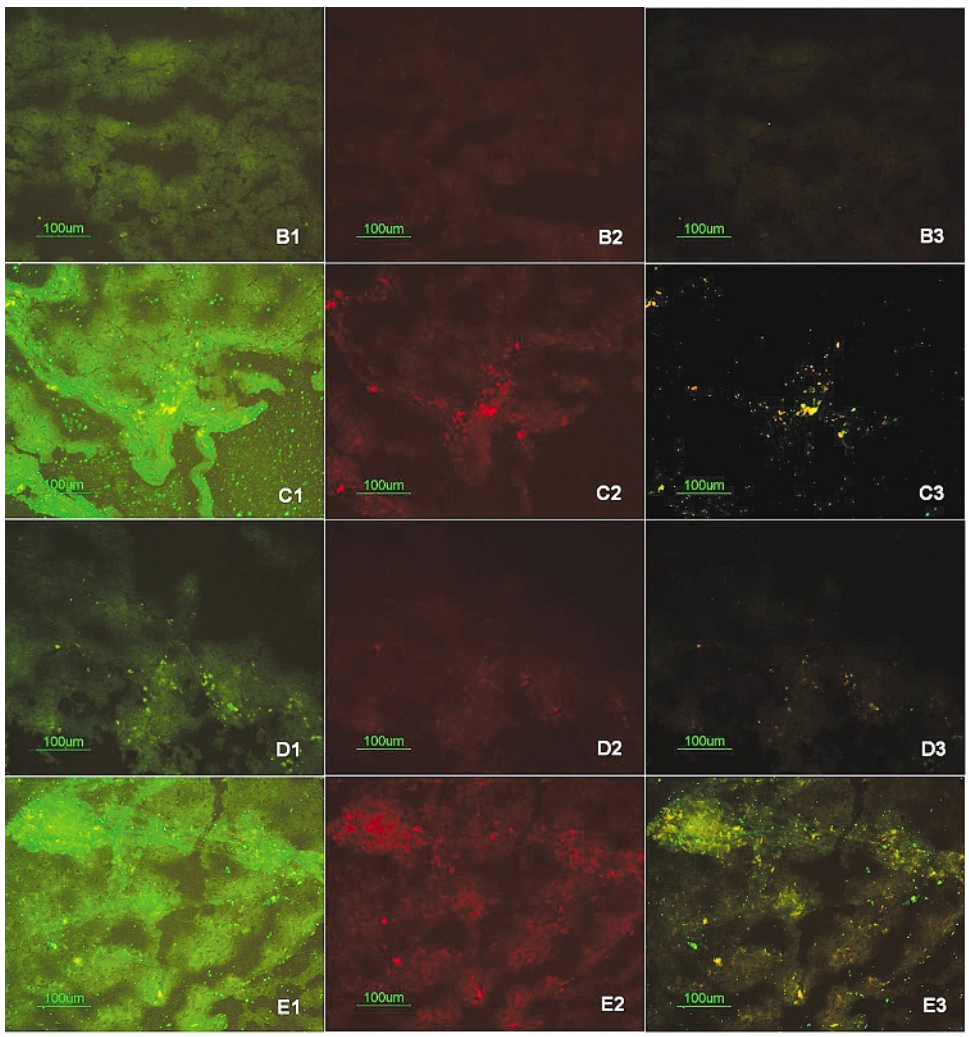


Fig. 4: Fluorescence (or immunofluorescence) analysis of liver sections from porcine serum -treated rats (100×). Double fluorescent images (red, CM-Dil; green, FITC; yellow, CM-Dil and FITC) on day 28. Analysis showed some rMSCs (CM-Dil) in the liver and positive for FITC and CM-Dil. B1, C1, D1 and E1 show green fluorescence (FITC); B2, C2, D2 and E2 show red fluorescence (CM-Dil); B3, C3, D3 and E3 show double-labeling with FITC and CM-Dil. No fluorescence was indicated in the negative controls. The experiments were performed in quintuplicates. B represents rats receiving porcine serum alone, C and D independently represent rats receiving porcine serum and $5 \times 10^6$ or $1 \times 10^6$ rMSCs; E represents rats receiving porcine serum, $1 \times 10^6$ rMSCs and salidroside

dramatically decreased by the transplantation of rMSCs and/or salidroside. In addition, the serum AST and ALP activities were decreased to normal levels when administration of rMSCs and salidroside was performed, which suggested salidroside and rMSCs transplantation had synergistic effects. However, no differences in TP, ALB and ALT levels were noted among these five groups (data not shown).

### *2.5. rMSCs transplantation suppressed collagen deposition*

Hydroxyproline (HyPro) is a product of nonenzymatic hydroxylation reaction of proline and a biochemical marker of collagen damage (Ozturk et al. 2008). To evaluate the inhibitory effects of rMSCs transplantation (with or without salidroside) on the progression of fibrosis in a quantitative manner, the hydroxyproline

**Table 2: Content of hydroxyproline and glutathione in the serum and liver tissue**

| Group | Serum glutathione (μmol/L) | Serum hydroxyproline (μg/ml) | Liver tissue hydroxyproline (μg/g) | Liver tissue glutathione (mgGSH/gprot) |
|---|---|---|---|---|
| A | $27.31 \pm 2.61$** | $50.70 \pm 6.87$** | $316.44 \pm 26.26$** | $1.73 \pm 0.37$** |
| B | $4.71 \pm 1.74$ | $111.71 \pm 6.62$ | $780.12 \pm 73.14$ | $0.44 \pm 0.06$ |
| C | $23.11 \pm 6.10$** | $67.94 \pm 5.41$** | $383.60 \pm 18.30$** | $1.06 \pm 0.16$**# |
| D | $12.89 \pm 2.04$*## | $74.58 \pm 11.40$** | $431.59 \pm 53.93$**## | $1.08 \pm 0.22$**# |
| E | $26.47 \pm 4.41$** | $66.63 \pm 7.21$** | $353.12 \pm 9.44$** | $1.38 \pm 0.23$** |
| F | $14.71 \pm 2.61$**## | $82.59 \pm 6.23$**# | $429.63 \pm 13.17$**## | $0.84 \pm 0.08$**## |

Data are presented as mean ± SD. *$P < 0.05$; ** $P < 0.01$, *vs* Group B; # $P < 0.05$; ## $P < 0.01$, *vs* group F. A and B represent normal rats and rats receiving porcine serum alone, respectively; C and D independenly represent rats receiving porcine serum and $5 \times 10^6$ or $1 \times 10^6$ rMSCs; E represents rats receiving porcine serum, $1 \times 10^6$ rMSCs and salidroside; F represents rats receiving porcine serum and salidroside

**Table 3: Content of TGF-β1 in the serum of each group**

| Group | Treatment | TGF-β1 |
|---|---|---|
| A | Group saline | $244.07 \pm 30.18$** |
| B | Group porcine serum | $524.80 \pm 45.57$ |
| C | Group porcine serum/5*$10^6$rMSCs | $333.02 \pm 38.05$**# |
| D | Group porcine serum/1*$10^6$rMSCs | $371.11 \pm 50.87$**## |
| E | Group porcine serum/salidroside/ 5*$10^6$ rMSCs | $241.62 \pm 22.45$** |
| F | Group salidroside | $340.54 \pm 69.54$**# |

Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$, *vs* Group B; #$P < 0.05$; ##$P < 0.01$, *vs* Group F. A and B represent normal rats and rats receiving porcine serum alone, respectively; C and D independenly represent rats receiving porcine serum and $5 \times 10^6$ or $1 \times 10^6$ rMSCs; E represents rats receiving porcine serum, $1 \times 10^6$ rMSCs and salidroside; F represents rats receiving porcine serum and salidroside

content in the liver was measured. As shown in Table 2, the hepatic hydroxyproline contents in Group B were 2 times higher than in the control group (Group A), indicating successful establishment of the hepatic fibrosis model. The hepatic hydroxyproline contents were decreased by the transplantation of rMSCs or salidroside, and hydroxyproline contents were decreased obviously when rMSCs and salidroside were administered, which suggested salidroside and rMSCs transplantation had synergistic effects.

### *2.6. TGF-β1 level in the liver after treatment with salidroside and rMSCs*

TGF-β1, a pro-fibrogenic factor in chronic liver disease, is a potent fibrogenic cytokine produced by Kupffer cells and HSCs (Hellerbrand et al. 1999; Jeong et al. 2005). There is a sustained increase of TGF-β1 expression during the progress of hepatic fibrosis (Nakatsukasa et al. 1990).

As shown in Table 3, the serum level of TGF-β1 was significantly increased after intraperitoneal administration of porcine serum for 8 weeks. Transplantation of rMSCs could decrease the level of serum TGF-β1, which was more evident in rats treated with rMSCs and salidroside.

## 3. Discussion

MSCs, a novel source of cells, are promising candidates for new cell-based approaches for the treatment of liver diseases (Mitaka 2001). MSCs can be easily harvested, tremendously expanded *in vitro* and evaded ethical issues. MSCs also have multiple proliferative potentials and the capacity to differentiate into various cell types. Experimental evidence suggested that MSCs could differentiate into hepatocytes (or hepatocyte-like cells) under appropriate conditions (*in vitro* or *in vivo*) (Lee et al. 2004; Okumoto et al. 2005; Oyagi et al. 2006; Schwartz et al. 2002; Wang et al. 2003; Yan et al. 2009), but the specific mechanisms underlying the differentiation of MSCs in the liver remain unclear.

It has been reported that Wnt/β-catenin signals play an important role in the hepatic fate specification of MSCs *in vitro* (Ke et al. 2008; Yoshida et al. 2007a), but E-adherin, binding site and a regulatory factor of β-catenin, could not be taken into account. β-Catenin usually exists in a cadherin-bound form while only a few β-catenin exist in the free state; under some conditions, enhanced expression of β-catenin in excess of E-cadherin, β-catenin could be phosphorylated and targeted for degradation; or only if it is transported into the nucleus combined with TCF/LEF transcription factors. Thus, it is necessary to consider the stabilization and accumulation of β-catenin in the cytoplasm and the role of E-adherin. Results showed that expression of β-catenin was enhanced while E-cadherin was depressed in the hepatic differentiation process of MSCs.

Hepatic fibrosis, which is a common disease caused by viral hepatitis, alcohol or drugs, is characterized by an increased deposition of extracellular collagen resulting in chronic hepatocyte injury and progressive deposition of fibrous tissue in the liver (Spee et al. 2005). Hepatic stellate cells are known to play an important role in this process. The activation of hepatic stellate cells is associated with liver injury or fibrosis. Hepatic stellate cells, by secreting TGF-β1 which stimulate production of large amounts of extracellular matrix, act as one of the major components of hepatic fibrosis (Hellerbrand et al. 1999). Studies reported hepatic fibrosis could be ameliorated, but few effective strategies are available against chronic hepatic fibrosis. Recently, the development of MSCs based therapeutic strategies for chronic hepatic injury or fibrosis are under ongoing experimental evaluation (Fiegel et al. 2006), but the potential mechanisms involved in the therapeutic effects of MSCs are still poorly understood.

There is convincing evidence that the two main counteracting factors, HGF and TGF-β1, determine the outcome of liver disease. HGF is the principal factor which stimulates the liver to grow or regenerate and suppresses fibrosis and apoptosis through blocking the nuclear translocation and accumulation of activated Smad-2/3 protein triggered by TGF-β1 in Smad pathway (Yang et al. 2003). TGF-β1 is a powerful stimulus for collagen formation *in vitro* (Kanzler et al. 1999) and induces formation of excessive fibrous tissue as well as a reduction of regenerative capacity. The findings with respect to the crucial counteracting effects of HGF and TGF-β1 in liver growth, regeneration and fibrosis, have prompted researchers to investigate the potential of administering HGF to experimental animals as a hepatotoxic and fibrogenic drug (Spee et al. 2005, 2006).

Although it was reported that MSCs could secrete HGF and suppress inflammation when transplanted into $CCl_4$ -injured rats, it is unclear that whether HGF could promote to differentiate MSCs towards hepatocytes and exert therapeutic effects on liver injury or fibrosis (Oyagi et al. 2006).

Moreover, there is an approach to avoid expansion of the MSCs before implantation in regenerative medicine (Kasten et al. 2008), but it is more ordinary to expand MSCs before implantation, and the latter approach is suspected (Popp et al. 2007).

It was reported that MSCs pre-induced to hepatocytes before implantation effectively treats liver injury in rats (Oyagi et al. 2006), which is a promising technique indeed, but pre-induced MSCs, condition of post-mitosis and assembled, result in population of MSCs and some pathological effects, such as blocking, so it is necessary to search for a promising technique for transplantation with liver injury.

Salidroside has been found to play a hepatoprotective role in liver diseases through inhibiting apoptosis of hepatocytes as well as proliferation of hepatic stellate cells, decreasing serum aminotransferase, reversing hepatic fibrosis and improving liver function (Song et al. 2004; Wang et al. 2004; Zhang and Liu 2006). Our previous study has shown that salidroside could directly induce the differentiation of rMSCs towards hepatocytes-like cells *in vitro* (Ouyang et al. 2009), but it is not known whether salidroside differentiates rMSCs towards hepatocytes *in vivo* and exerts synergistic effects in experimental hepatic fibrosis.

To investigate the effects of salidroside (see above) in the treatment of chronic hepatic fibrosis, porcine serum-induced hepatic fibrosis was introduced. Then, rMSCs were transplanted along with intraperitoneal administration of salidroside. Histopathologic staining, immunohistochemistry assay and biochemical analyses were performed to examine the hepatic differentiation of rMSCs and the reversion of hepatic fibrosis. The results showed that hepatic fibrosis was profoundly reversed and liver function was improved to a certain extent after rMSCs transplantation plus intraperitoneal administration of salidroside, which suggested a synergistic effects of rMSCs and salidroside. Immunohistochemistry indicated that albumin was expressed by transplanted rMSCs. It was suggested that salidroside could reverse the hepatic fibrosis and create a suitable internal environment for rMSCs, therefore, growth and hepatic differentiation of rMSCs were followed.

Our results suggested that transplanted rMSCs survived form the injured liver, and some of them had differentiated towards hepatocytes. According to morphological and histologic analysis, transaminases activity and collagen deposition in the rats with rMSCs transplantation and intraperitoneal administration of salidroside were significantly reduced when compared with those in other groups. It is well known that transplanted rMSCs could differentiate towards hepatocytes and secrete some growth factors to reverse hepatic fibrosis, but the results were conflicting. The survival, proliferation and differentiation of rMSCs under the poor conditions of fibrotic liver were unclear. In the present study, we confirmed that rMSCs could survive, proliferate and differentiate towards hepatocytes in the fibrotic liver, and salidroside, which could improve the liver microenvironment, exerted synergistic effects with rMSCs transplatation.

In conclusion, our findings demonstrated that rMSCs could ameliorate rat hepatic fibrosis and provide a potential new therapy for liver disorders or liver injury. Salidroside could contribute to the reverse of hepatic fibrosis and improvement of liver function and confer synergistic effects with rMSCs transplantation on the hepatic fibrosis. Notwithstanding, we did not prove the basal rate of MSCs proliferation and differentiation because the differentiation of rMSCs is a small probability event when compared with hepatocytes in the liver. Further studies are needed to clarify these problems.

## 4. Experimental

### *4.1. Preparation of rat bone marrow-derived MSCs*

rMSCs were prepared from rat bone marrow. In brief, the tibia and femur were flushed and bone marrow was obtained. The erythrocytes were lysed by erythrocyte lysis buffer. rMSCs were preferentially attached to the polystyrene surface and were further purified by passaging. rMSCs expressing cluster differentiation (CD)44 and CD90, but not CD45 were identified through detection of these receptors with a flow cytometer. The multiple differentiation potentials of rMSCs into adipocytes, osteoblasts and chondrocytes were also confirmed *in vitro* (Data shown elsewhere).

### *4.2. In vitro hepatic differentiation protocol*

Hepatic differentiation was performed as follows. Briefly, $1 \times 10^4$ cells at passage 3 were seeded per well in 24-well plates and hepatic differentiation was processed in high-glycose DMEM (differentiation medium), supplemented with 2% FBS (Gibco), 0.1% dimethyl sulfoxide (DMSO) (Amerisco), 2 mM glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid (pH 7.2), $10^{-8}$ M dexamethasone, 1 × insulin/transferrin/selenium, 100 U/mL penicillin G and 100 μg/mL streptomycin. rMSCs were divided into 5 groups by different media. Group C was high-glycose DMEM, negative control; Group P, acted as positive control, was differentiation medium, supplemented with 20 ng/mL hepatocyte growth factor (HGF; PeproTech); Group A, B and C were differentiation medium, supplemented with 10 μg/mL, 20 μg/mL and 50 μg/mL salidroside (National Institute for the Control of Pharmaceutical and Biological Products, China), respectively. The culture media was changed twice weekly and different assays were used to evaluate hepatic differentiation.

### *4.3. Western blot analysis*

rMSCs inoculated in differentiation media were taken at 14 days. Thirty micrograms of the proteins were subjected to western blot analysis. The rabbit polyclonal antibodies including anti- beta-catenin (Santa Cruz), anti-E-cadherin (Santa Cruz) and anti-Actin (BD) were used.

### *4.4. Experimental regimen in vivo*

One-month-old Sprague Dawley (SD) rats were supplied by the Laboratory Animal Unit of Zhejiang Academy of Medical Sciences (Hangzhou, China). All experiments were approved by the ethics committee of Zhejiang University.

A total of 48 rats weighing 140∼160 g were randomly divided into 6 groups. Hepatic fibrosis was induced by intraperitoneal administration of porcine serum twice a week for consecutive 8 weeks, as described by Bhunchet et al. (1992). rMSCs labeled with CM-Dil, as described by Weir et al. (2008), were administrated by tail vein injection on day 35 and salidroside was intraperitoneally injected once daily from day 35 to day 56.

Six groups were as follows: Group A, rats received normal saline (n = 8); Group B (hepatic fibrosis or model group): rats were intraperitoneally injected with porcine serum only (n = 8); Group C ($5 \times 10^6$ rMSCs group): rats received porcine serum and treated with $5 \times 10^6$ rMSCs (n = 8); Group D ($1 \times 10^6$ rMSCs group): rats received porcine serum and treated with $1 \times 10^6$ rMSCs (n = 8); Group E (rMSCs plus salidroside group): rats received porcine serum, treated with $1 \times 10^6$ rMSCs in combination with salidroside (intraperitoneal injection; 7 mg/kg) (n = 8); and Group F (salidroside group): rats received porcine serum and treated with salidroside (7 mg/kg) (n = 8).

### *4.5. Morphologic analysis and histopathologic staining*

All rats were sacrificed after determination of body weight on day 56 and the livers were removed. Morphologic analysis was performed. Part of liver was fixed in 10% paraformaldehyde, dehydrated in graded ethylic alcohol, and embedded in paraffin. Then, tissues were cut into 8 μm sections followed by staining with hematoxylin & eosin (HE) and azan to detect the severity of fibrosis, as described by Oyagi et al. (2006). The remaining livers (portal area) were processed for cryosection followed by immunofluorescence staining.

### *4.6. Immunofluorescence staining*

For immunofluorescence staining, tissues were cut into 8 μm cryosections and mounted on glass slides for immunocytochemistry with indirect immunofluorescence technique. Blocking was performed by incubation with 1% bovine serum albumin (BSA) in 0.10 M phosphate buffered saline (PBS; pH 7.4) for 60 min. Then, cryosections were incubated with primary antibody (1:50, goat anti albumin; Abcam) for 60 min followed by incubation with secondary antibody (1:100, fluorescein isothiocyanate-conjugated mouse anti goat IgG; Santa Cruz) for 60 min. Incubations were performed at room temperature. Each incubation was followed by washing with PBS thrice (3 × 5 min). Fluorescences of CM-Dil and fluorescein isothiocyanate were examined under fluorescence microscope (Nikon).

### 4.7. Hydroxyproline assay

To detect the amount of collagen in the liver, the content of hydroxyproline was determined with colorimetric assay as described by Nakamura et al. (2000). Briefly, lyophylized hepatic tissue was digested overnight by hydrolysis in 6 M hydrochloric acid, and hydrolysates were diluted to a constant volume with further acid. Aliquots of 200 µl were dried at 100 °C and were subsequently made to back up to volume with distilled water. Oxidation of the samples with chloramine-T results in the formation of a pyrrole that in turn forms a colored compound when reacted with Ehrlich's reagent. Hydroxyproline content of the samples was expressed as micrograms of hydroxyproline per gram of dry tissue, with the sample assayed in triplicate.

### 4.8. Biochemical analysis

Peripheral blood was collected for determination of plasma TP (total protein), albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and TGF-β1. The plasma levels of TP, ALB, ALP, AST and ALT were assessed by an automatic biochemical analyser (Olympus), and that of TGF-β1 detected by enzyme-linked immunosorbent assay (ELISA assay).

### 4.9. Statistical analysis

Data are expressed as mean ± SD and analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. A value of $P < 0.05$ was considered statistically significant.

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