

# A novel therapeutic regimen for hepatic fibrosis using the combination of mesenchymal stem cells and baicalin

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Baicalin, isolated from the root of *Scutellaria baicalensis* Georgi, has shown anti-inflammatory and antioxidant activities, while mesenchymal stem cells (MSCs) have the capability of self-renewal and multilineage differentiation. In the present study, we found that baicalin could promote differentiation of bone marrow-derived MSCs into hepatocytes *in vitro*. We then compared the therapeutic effects of five therapeutic regimens for hepatic fibrosis induced by carbon tetrachloride *in vivo* by analysis of serum enzymes, morphological characteristics, cytokines and cell engraftment. Transplantation of MSCs alone was able to promote partial recovery of liver function and suppression of liver inflammation, but showed little effect on reducing the fibrotic area; transplantation with baicalin-treated MSCs gave an improved therapeutic effect; MSC transplantation and baicalin administration showed a synergistic effect; transplantation with baicalin-treated MSCs in combination with baicalin administration had the best therapeutic effect for hepatic fibrosis. Therefore, we conclude that transplantation of pre-differentiated MSC together with baicalin administration may serve as an effective therapeutic regimen for severe liver diseases.

## 1. Introduction

Liver transplantation has become remarkably effective and has been the treatment of choice for several end-stage liver diseases (Neuberger 2004). However, shortage of donors and immunological rejection have limited the clinical application of liver transplantation. MSCs are adherent fibroblast-like cells with nonhematopoietic multipotent differentiation potential (Gregory et al. 2005; Tocci and Forte 2003), which can differentiate not only into most mesodermal cell types, but also into functional neuroectodermal and endodermal cell types (Schwartz et al. 2002), including hepatocytes. Therefore, transplantation of MSCs may be a promising therapeutic strategy in the treatment of end-stage liver diseases in the future.

Hepatocyte growth factor (HGF) has been associated with endodermal specification, and plays an essential role in the development and regeneration of the liver (Michalopoulos 1997). Growing evidence (Hong et al. 2005; Lee et al. 2004; Oyagi et al. 2006; Schwartz et al. 2002; Sgodda et al. 2007) has indicated that HGF at the proper concentration is a key factor in the hepatic differentiation of MSCs (Oh et al. 2000). However, endogenous HGF, at levels below 0.5 ng/mL (Hillan et al. 1996), is not sufficient to induce MSC differentiation into hepatocytes *in vivo*. Therefore, sustained stimulus by exogenous differentiated reagents is distinctly necessary in hepatic differentiation *in vivo*. Our current study aimed to seek non-toxic drugs from traditional Chinese medicine with the potential to promote MSC differentiation into hepatocytes and serve as alternatives to HGF. Baicalin (7-D-glucuronic acid,5,6-dihydroxyflavone), isolated from the root of *Scutellaria baicalensis* Georgi, has shown anti-inflammatory and antioxidant activities (Chen et al. 2001). Further evidence (Liu et al. 2007; Park et al. 2008; Taira et al. 2004; Wan et al. 2008; Zhao et al. 2005) has confirmed its hepato-

protective effects. To date, the effects of baicalin on hepatic differentiation of MSCs have not been investigated. In preliminary experiments, we found that baicalin failed to induce differentiation of MSCs into functional hepatocytes alone, but significantly up-regulated protein expression of alpha-fetoprotein after 7 days of differentiation in the presence of FGF-4 (data not shown). Thus, the aim of the present study was to elucidate the effects of baicalin on promoting differentiation of MSCs into functional hepatocytes *in vitro* and to find an effective therapeutic strategy for hepatic fibrosis induced by CCl<sub>4</sub> *in vivo*.

## 2. Investigations and results

### 2.1. Effects of baicalin on functional characterization of hepatocytes differentiated from MSCs *in vitro*

Expression of AFP, an early-stage marker of hepatic differentiation, started on day 4, peaked on day 7, and disappeared on day 14. ALB expression started on day 7 and increased gradually. Baicalin significantly enhanced the expression of the two proteins, which MSCs did not (Fig. 1).

Urea was synthesized only in hepatocytes rather than in MSCs (Schwartz et al. 2002; Lee et al. 2004). The urea production of differentiated MSCs increased in a time-dependent manner but not in a linear fashion. Moreover, in the baicalin group, urea production increased significantly compared with the FGF-4 group (Fig. 2A), and reached a level similar to that of 6-hour-cultured adult hepatocytes after differentiation for 4 weeks.

Hepatocytes have the ability for uptake of LDL; MSCs, on the contrary, do not. In our study, cells showed low levels of LDL uptake after 2 weeks' differentiation, and a significant increase at 3 and 4 weeks of differentiation. Furthermore, baicalin significantly increased the ability of cells for uptake of

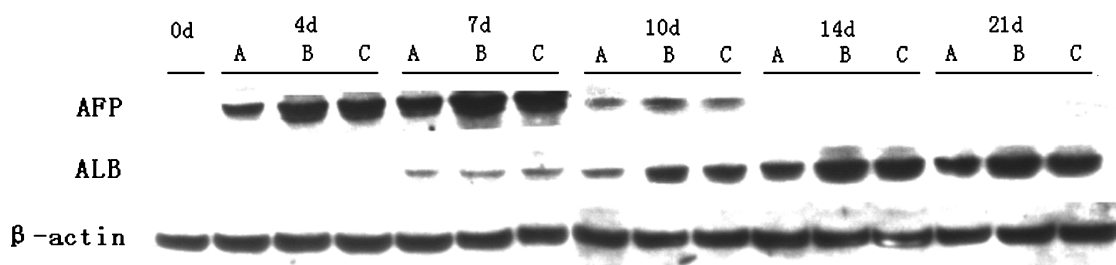


Fig. 1: Western blot analyses of the temporal expression pattern of hepatocyte-specific markers during hepatic differentiation of MSCs. A: FGF-4 group; B: HGF group; C: baicalin group.  $\beta$ -actin was used as reference protein in our study

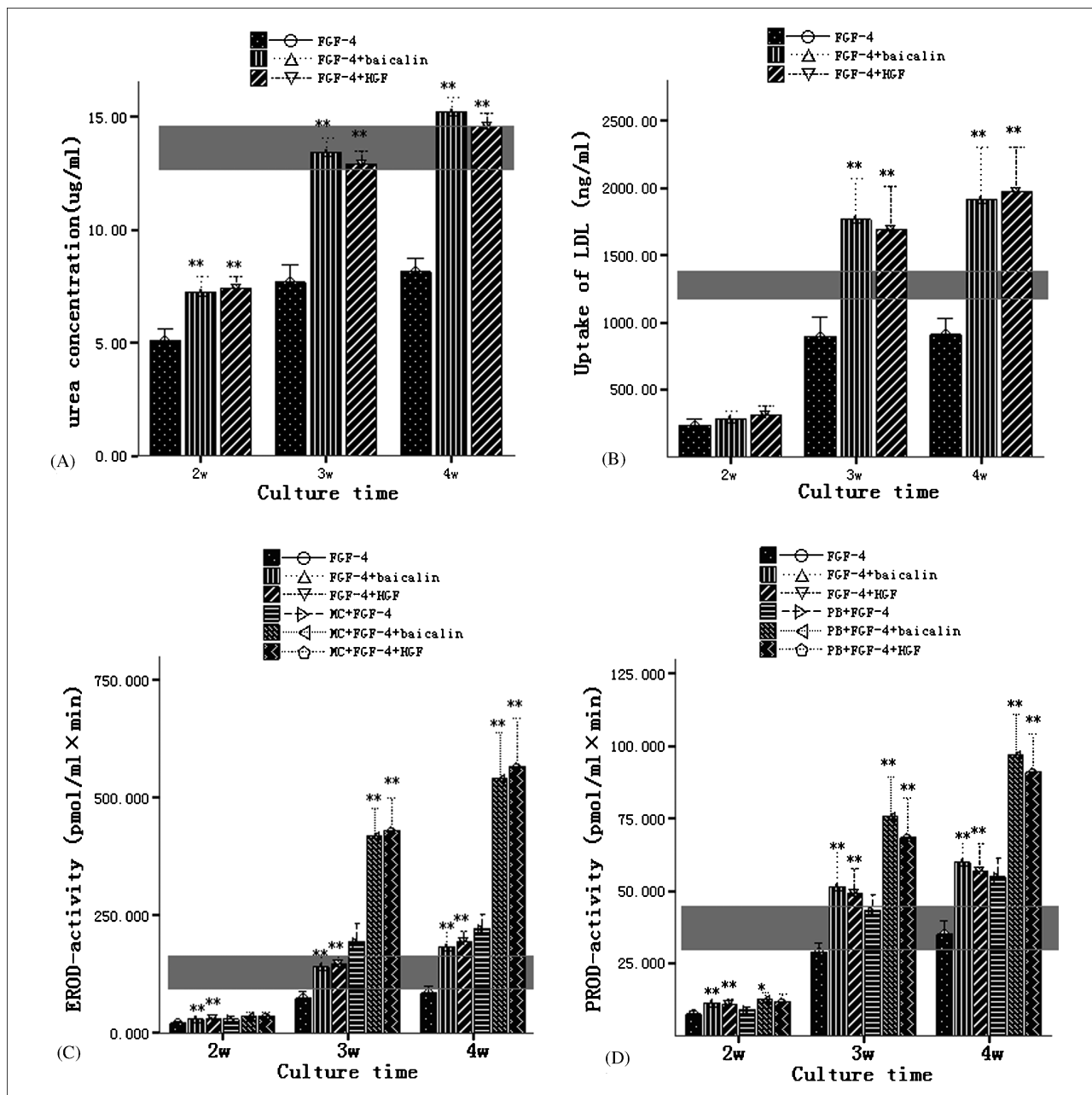


Fig. 2: Facilitative effects of baicalin on functional characterization of hepatocytes differentiated from MSCs *in vitro*. (A) urea production, (B) uptake of LDL, (C) EROD-activities and (D) PROD-activities. Gray area represents functional level of adult rat hepatocytes during primary culture. Values are expressed as mean  $\pm$  S.D. (n=6). Level of significance established at \* $P < 0.05$  or \*\* $P < 0.01$ , compared with FGF-4 group

LDL, compared with the FGF-4 group ( $P < 0.01$ ) (Fig. 2B), and its level was even higher than that measured in 6-hour-cultured adult hepatocytes.

Alkoxyresorufin-O-dealkylase assay is a sensitive method to quantify CYP-dependent monooxygenases in microwells (Donato et al. 1993). Ethoxyresorufin and pentoxyresorufin are

nonfluorescent compounds, metabolically activated by CYP1A1 or CYP2B1 into resorufin which emits red fluorescence. Therefore, the responsiveness of both CYP1A1 and CYP2B1 to their respective prototype inducers MC and PB were studied in parallel. Two weeks after induction, differentiated cells yielded low levels of fluorescence products in the absence of the prototype

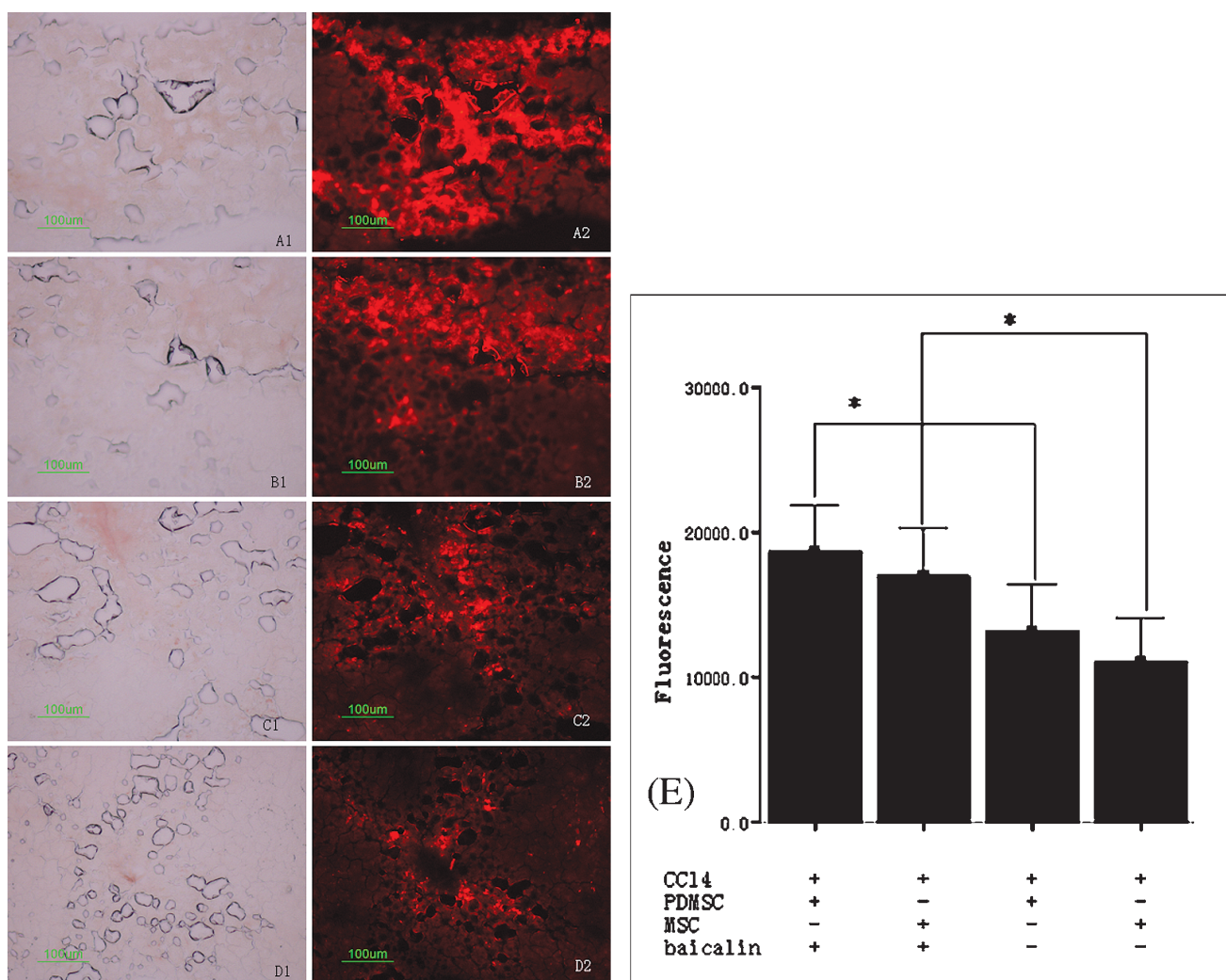


Fig. 3: Engraftment of Dil-stained cells in CCl<sub>4</sub>-injured rat liver. Liver frozen-sections were observed under fluorescence microscope, and fluorescence intensity was digitalized (E). A: baicalin plus PDMSC treatment group; B: baicalin plus MSC treatment group; C: PDMSC treatment group; D: MSC treatment group. Left-hand (A1-D1) and right-hand (A2-D2) pictures are bright-field and fluorescence images, respectively. Values expressed as mean  $\pm$  S.D. (n = 6). Level of significance was established at \* $P < 0.05$  or \*\* $P < 0.01$

inducers, and a small increase was observed after induction by the prototype inducers (Fig. 2C, D), suggesting the presence of endogenous P450 enzymes in differentiated cells. The differentiated cells were also demonstrated to have the ability to metabolize ethoxyresorufin or pentoxyresorufin to resorufin in the absence of the prototype inducers; and a significant increase could be observed after 3 weeks' differentiation in all groups when cells were treated with the prototype inducers. In addition, compared the with FGF-4 group, EROD- (Fig. 2C) and PROD-activities (Fig. 2D) of the baicalin group were significantly increased, regardless of stimulus by the prototype inducers.

## 2.2. MSCs engraftment in CCl<sub>4</sub>-injured liver

Dil-stained cells, both pre-differentiated and untreated, were able to settle in CCl<sub>4</sub>-injured liver (Fig. 3). Compared with groups F and G, the fluorescence intensity in livers was significantly increased in groups D and E, respectively, indicating that a sustained stimulus with baicalin was effective for improving the engraftment of MSCs to the injured recipient liver. However, there was no significant difference in fluorescence intensity between PDMSCs and MSCs (Fig. 3E).

## 2.3. Comparison of effects of five therapeutic regimens on hepatic injury and hepatic fibrosis in CCl<sub>4</sub> rat model

CCl<sub>4</sub> causes severe hepatic injury and fibrogenesis, resulting in a significant increase of serum ALT, AST (Table 1). After

cell transplantation, the levels of serum ALT and AST were significantly reduced. The group treated with baicalin accompanied with PDMSCs showed the best effects. In addition, the serum ALB level was significantly reduced in rats injected with CCl<sub>4</sub> ( $P < 0.01$ ), and cell transplantation attenuated the role of CCl<sub>4</sub>.

Hydroxyproline is an amino acid, existing almost exclusively in collagens. Measurement of the hydroxyproline content of the fibrotic tissue is regarded as a good method to quantify fibrosis (Fu et al. 2008). As shown in Table 1, the content of hepatic hydroxyproline was significantly increased in rats injected with CCl<sub>4</sub> ( $P < 0.01$ ), and significantly reduced in rats treated with cell transplantation ( $P < 0.05$ ,  $P < 0.01$ ). Among the five therapeutic groups, that using baicalin accompanied with PDMSCs obtained the best effects.

Various kinds of cytokines, such as proinflammatory cytokines TNF- $\alpha$ , profibrogenic cytokines TGF- $\beta$ 1, are released during hepatic fibrogenesis and inflammatory reaction (Bahcecioglu et al. 2008; Stalnikowitz and Weissbrod 2003). As shown in Fig. 4, compared with the vehicle group, the levels of TNF- $\alpha$  and TGF- $\beta$ 1 in the model group were significantly elevated in both liver and serum. Cell transplantation reduced the levels of the two cytokines, and the effects of suppression on inflammation were better in the groups using baicalin plus cell treatment (Group D and E) than in the cell transplantation groups (Group F and G).

**Table 1: Determination of the content of hepatic hydroxyproline and the levels of serum ALB, ALT and AST in the rat model with CCl<sub>4</sub>-induced liver injury**

Group	Vehicle	Model	Baicalin	Baicalin + PDMSCs	Baicalin + MSCs	PDMSCs	MSCs
ALT (U/L)	34.71 ± 3.07	84.31 ± 21.16##	74.15 ± 16.16	46.88 ± 11.83***\$\$	50.33 ± 5.44***\$\$	55.40 ± 13.36**	61.95 ± 11.24*
AST (U/L)	74.35 ± 11.64	214.00 ± 38.90##	185.24 ± 23.49	106.89 ± 18.31***\$\$	129.10 ± 30.24***\$\$	148.58 ± 32.14**	159.28 ± 23.07***
ALB (g/L)	33.22 ± 0.92	28.33 ± 1.31##	32.38 ± 1.89**	32.27 ± 1.77**	32.13 ± 1.42**	32.26 ± 1.61**	33.02 ± 1.54**
Hydroxyproline (μmol/g)	2.18 ± 0.25	4.60 ± 0.73##	3.73 ± 0.74	2.97 ± 0.53**	3.19 ± 0.66**	3.34 ± 0.38**	3.49 ± 0.74*

Values are expressed as mean ± S.D. (n = 6).

#P < 0.05; ##P < 0.01 as compared with vehicle group;

P < 0.05; \*\*P < 0.01 as compared with model control group;

\$P < 0.05; \$\$P < 0.01 as compared with baicalin treatment group

Histological analysis with Azan staining revealed that CCl<sub>4</sub> caused prominent hepatic steatosis, necrosis, and fibrotic septa (Fig. 5B). Cell transplantation attenuated the degree of pathological changes induced by CCl<sub>4</sub> (Fig. 5D–G). The effect of the five therapeutic regimens on ameliorating fibrosis was evaluated by digitalization of the blue-stained area (Fig. 5H). The results illustrated that cell transplantation reduced the fibrotic area, while better effects were achieved in the treatment groups using baicalin plus cells (Groups D and E).

All the results above suggested that the baicalin accompanied with PDMSCs treatment group (Group D) showed the best effects on hepatic injury and hepatic fibrosis in the CCl<sub>4</sub> rat model.

### 3. Discussion

MSCs are resident in various tissues and organs and possess certain characteristics including self-renewal and pluripotency. Upon appropriate induction, they can differentiate *in vitro* into multiple lineages, such as osteoblasts, adipocytes, chondrocytes, neural cells, and cardiomyocytes (Lee et al. 2004; Makino et al. 1999; Sanchez-Ramos et al. 2000). Therefore, MSCs represent a promising cell source for cell-based therapeutic strategy (Baksh et al. 2004). Moreover, there is growing evidence (Hong et al. 2005; Lee et al. 2004; Oh et al. 2000; Schwartz et al. 2002) indicating that MSCs are able to differentiate into functional hepatocyte-like cells under induction by various cytokines such as FGF-4 and HGF, which is also confirmed in our study.

Transplantation of MSCs is an easy, efficient, and safe way to cure severe liver diseases (Cho et al. 2009; Seo et al. 2005; Sgodda et al. 2007; Tsai et al. 2009), free from untoward immunological rejection. However, there is still controversy surrounding the effectiveness of MSCs in the treatment of prolonged hepatic injury (Popp et al. 2007). In the present study, we demonstrated that transplantation of MSCs alone restored partial liver function and suppressed liver inflammation, but had little effect on reducing the fibrotic area. Furthermore, Oyagi et al. (2006) found that the therapeutic effect of transplanting HGF-treated MSCs was better than that of untreated MSCs for CCl<sub>4</sub>-induced liver fibrosis. Wu et al. (2005) demonstrated that drugs could synergize with MSCs to improve recovery from liver fibrosis. Therefore, in order to find a better therapeutic strategy for hepatic fibrosis, we designed five different therapeutic regimens using different combinations of baicalin and MSCs (Groups C–G). Our study showed that low-dose baicalin or transplanted MSCs alone had a limited effect on amelioration of liver fibrosis. However, the therapeutic effect was greatly boosted after MSC transplantation in conjunction with baicalin administration. When treated with baicalin *in vitro* for two weeks prior to transplantation, PDMSCs possessed partial hepatocyte functions. The curative effects of transplantation of PDMSCs for CCl<sub>4</sub>-induced liver fibrosis were better than those of untreated MSCs, in agreement with a study by Oyagi et al. (2006). The combination of PDMSC transplantation with baicalin administration achieved the best therapeutic effects in our study.

The fluorescence intensity of MSC engraftment represents the quantity of cells settling in CCl<sub>4</sub>-injured livers. Oyagi et al. (2006) showed that significantly higher fluorescence was observed in liver receiving HGF-treated MSCs as compared with untreated MSCs. MSCs pre-differentiated *in vitro* facilitate long-term functional hepatic integration *in vivo* (Aurich et al. 2009). In our study, there was significant difference of fluorescence intensity between group D and group F, and between group E and group G, suggesting that baicalin improved the engraftment of MSCs into the injured recipient livers and that a better therapeutic effect was achieved. However, there was no significant difference between PDMSCs and MSCs. This may be attributed

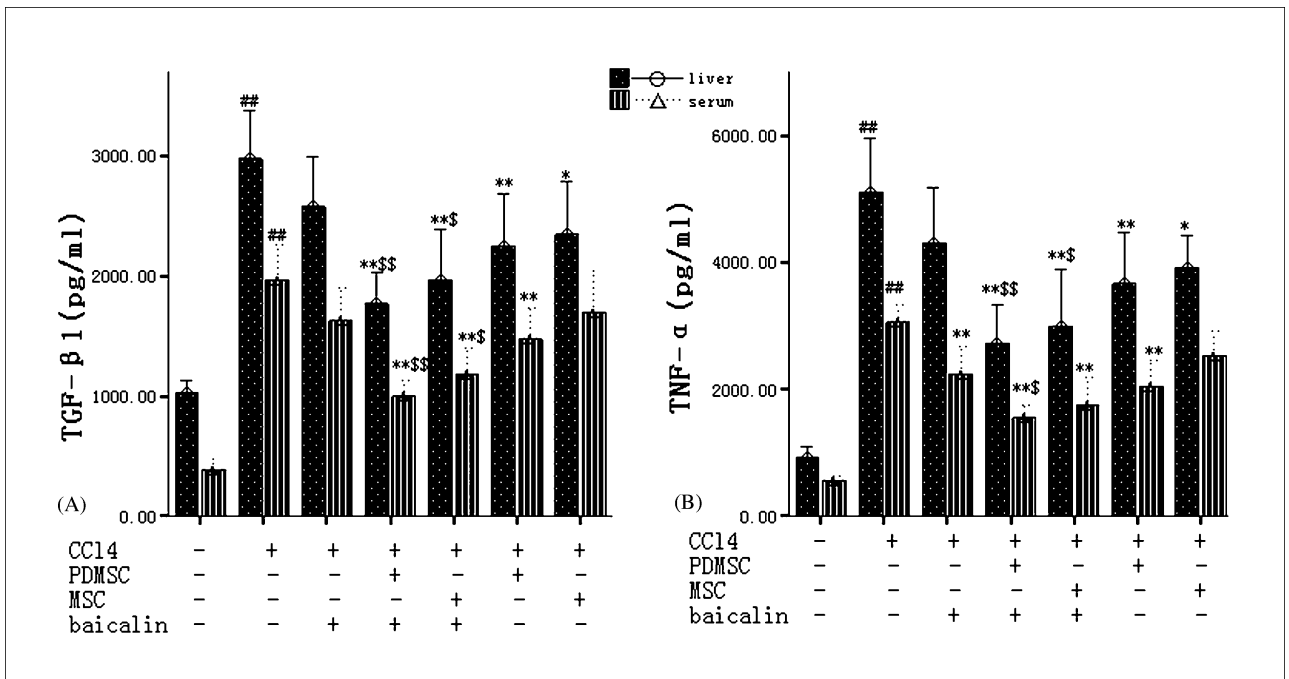


Fig. 4: Levels of TGF-β1 (A) and TNF-α (B) in liver and serum determined by ELISA. Values are expressed as mean ± S.D. (n=6). Level of significance was established at \* $P < 0.05$  or \*\* $P < 0.01$ , compared with model control group; # $P < 0.05$  or ## $P < 0.01$ , compared with vehicle control group; \$ $P < 0.05$  or \$\$ $P < 0.01$ , compared with baicalin treatment group

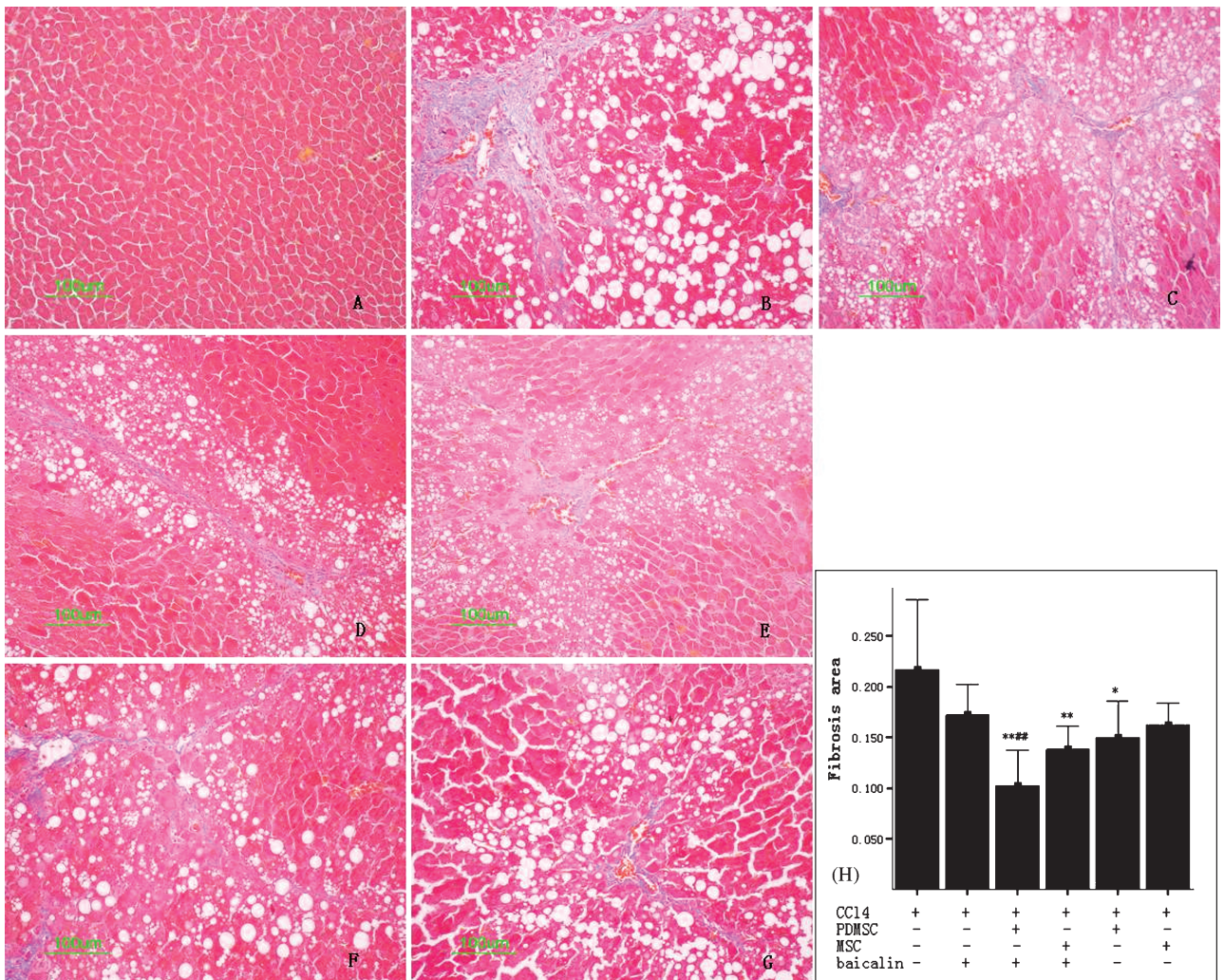


Fig. 5: Effect of MSC transplantation shown by Azan-staining (A-G) after CCl<sub>4</sub>-induced liver fibrosis in rats (original magnification, ×200). A: Vehicle group; B: model group; C: baicalin treatment group; D: baicalin plus PDMSC treatment group; E: baicalin plus MSC treatment group; F: PDMSC treatment group; G: MSC treatment group. Quantitative analysis of the degree of liver fibrosis in CCl<sub>4</sub>-injured rat liver using Azan-staining (H). Values expressed as mean ± S.D. (n=6). Level of significance established at \* $p < 0.05$  or \*\* $P < 0.01$ , compared with model control group; # $P < 0.05$  or ## $P < 0.01$ , compared with baicalin treatment group

to different internal environment of the liver in different models of liver injury and different time points of interventional therapy. Nevertheless, the exact causes remain to be elucidated in further studies. The way in which PDMSCs and MSCs exert their hepatoprotective effects *in vivo*, and the exact role of baicalin in improving therapeutic effects for hepatic fibrosis, will also be the focus of our study in future.

Taken together, our study shows that baicalin promotes differentiation of MSCs into functional hepatocytes *in vitro*, yet the underlying mechanisms remain to be determined. The order of therapeutic effects on CCl<sub>4</sub>-induced hepatic injury and hepatic fibrosis is as follows: PDMSC transplantation plus baicalin group > MSC transplantation plus baicalin group > PDMSC transplantation group > MSC transplantation group > baicalin treatment group. Therefore, transplantation of PDMSCs in combination with baicalin administration offers an effective therapeutic strategy for severe liver diseases.

## 4. Experimental

### 4.1. Cell isolation and culture

Bone marrow cells (MSCs) were harvested by flushing out the femurs and tibias of four-week-old Sprague-Dawley (SD) male rats with low-glucose Dulbecco's modified Eagle medium (L-DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin G (Amerisco), 100 µg/mL streptomycin (Amerisco), 2 mM/L glutamine (Sigma), and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (HEPES, Amerisco), using a 26-gauge needle. After centrifugation and resuspension, isolated cells were seeded on to 100-mm plastic culture plates, and non-adherent cells were washed out with medium changes after 24 h. Medium changes were performed every 3 d thereafter.

Hepatocytes were isolated from 4-week-old male SD rats by two-step collagenase perfusion as described previously (Berry and Friend 1969), and the viability of the hepatocyte preparations (>85%) was determined by trypan blue dye exclusion assay. Hepatocytes were cultured with high-glucose Dulbecco's modified Eagle medium (H-DMEM, Gibco), including 10% FBS, 2 mM glutamine, 100 U/mL penicillin G, 100 µg/mL streptomycin, 20 ng/mL HGF (PeproTech), 1 × ITS (Sigma) and 200 µg/L dexamethasone (Sigma).

### 4.2. *In vitro* differentiation

The MSCs of the fifth passage were divided into three groups, the FGF-4, HGF and baicalin groups. The FGF-4 group was treated with 10 ng/mL FGF-4 (PeproTech), the HGF group was treated with 10 ng/mL FGF-4 and 20 ng/mL HGF together, and the baicalin group was treated with 10 ng/mL FGF-4 and 2 µM of baicalin (National Institute for the Control of Pharmaceutical and Biological Products). At 100% confluence, cells were exposed to basic differentiation medium consisting of the following: H-DMEM, 2% FBS (Gibco), 0.1% DMSO (Amerisco), 2 mM of glutamine, 100 U/mL penicillin G, 100 µg/mL streptomycin and 10 mM of HEPES. From day 4 onwards, 1 × ITS (Sigma) and 200 µg/L dexamethasone (Sigma) were added.

### 4.3. Western blot analysis for hepatocyte-specific proteins

The cells were lysed by a lysis buffer. The protein content was determined by the Bradford assay (Applygen Technologies Inc. China). The samples were fractionated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Whatman). After washing, the membranes were blocked with 10% skim milk at room temperature for 1 h and incubated with polyclonal goat antibody to alpha-fetoprotein (AFP) (1:200 dilution; Santa Cruz), polyclonal goat antibody to albumin (ALB) (1:200 dilution; Santa Cruz), and polyclonal goat antibody to β-actin (1:1000 dilution; Santa Cruz), respectively, at 4 °C overnight. They were then washed three times and incubated with secondary antibody (1:10000 dilution; Santa Cruz) at room temperature for 1 h. The protein band was visualized by ECL (Amersham Bioscience) western blotting system.

### 4.4. Urea assay

After exposure to 6 mM of NH<sub>4</sub>Cl (Sigma) for 24 h, urea production was measured by an Automated Chemistry Analyzer (AU400, Olympus) in the culture medium using a Quantichrom Urea assay kit (Olympus). Fresh differentiation medium and adult rat hepatocytes cultured for 6 h served as the negative and positive controls, respectively.

**Table 2: Experimental process and animal groups**

Subcutaneously injected with CCl<sub>4</sub> twice a week for 8 weeks

Group	Baicalin administration everyday for 4 weeks						
	Cell transplantation			Sacrifice all rats			
	↓	↓	↓	↓	↓	↓	↓
0w	4w	4w	4w	8w	8w	8w	8w
Group	Vehicle Group A	Model Group B	Baicalin Group C	Baicalin+PDMSCs Group D	Baicalin+MSCs Group E	PDMSCs Group F	MSCs Group G
CCl <sub>4</sub>	-	+	+	+	+	+	+
Baicalin	-	-	+	+	+	-	-
PDMSC	-	-	-	+	-	+	-
MSC	-	-	-	-	+	-	+

### 4.5. Uptake of low-density lipoprotein (LDL)

Cells were incubated in differentiation media containing 10 µg/mL Dil-Ac-LDL (Invitrogen) for 24 h at 37 °C. Then, the supernatant was withdrawn from each well and transferred to another 96-well plate. Fluorescence of the supernatants was measured using a fluorospectrophotometer (Infinite F200, TECAN) with 549-nm excitation and 565-nm emission filters. A standard curve of Dil-Ac-LDL was prepared in differentiation medium. Fluorescence was linear within the range 0-10 µg/mL. Fresh differentiation medium and adult rat hepatocytes cultured for 6 h were used as negative and positive controls, respectively.

### 4.6. Alkoxyresorufin-O-dealkylase assay

Ethoxyresorufin (EROD)- and pentoxyresorufin (PROD)-activities were measured as previously described (Donato et al. 1993) with some minor modifications: in our setup, cells were exposed to phenobarbital (PB) (final concentration 1 mM) and 3-methylcholantrene (MC) (final concentration 2 µM, Sigma), respectively, then incubated with 20 µM 7-ethoxyresorufin and 18 µM 7-pentoxyresorufin (all from Sigma) for 1 h and subsequently for 1 h with β-glucuronidase/arylsulfatase (Roche Applied Science).

### 4.7. Preparation of cells for transplantation

There were two kinds of cell used for transplantation. One was MSCs without any treatment; the other was pre-differentiated MSCs (PDMSCs) treated with FGF-4 and baicalin for 14 days, as described in the section on "*In vitro* differentiation (4.2)". All cells were stained using Dil-dye (Invitrogen) before transplantation.

### 4.8. CCl<sub>4</sub>-induced liver fibrosis model and MSC transplantation

Male SD rats weighing 180-200 g were randomized into seven groups (Table 2). Group A served as a vehicle control, in which rats were not administrated CCl<sub>4</sub>; instead, they were subcutaneously injected with edible oil (1 ml/kg body weight). Rats in group B to G were subcutaneously injected with a mixture of CCl<sub>4</sub> and edible oil [1:1 (v/v)] (1 ml/kg body weight) twice a week for 8 weeks. Group B (model control) was orally administered distilled water daily (1 ml/ 100 g body weight) from the 5th week; Group C (baicalin treatment group) was given baicalin 25 mg/kg orally daily (1 ml/ 100 g body weight) from the 5th week. Group D (baicalin plus PDMSC treatment group) was administrated baicalin 25 mg/kg daily from the 5th week, and PDMSC transplantation was performed at the 5th week in the caudal vein (5 × 10<sup>6</sup> cells/rat); Group E (baicalin plus MSC treatment group) was administrated baicalin 25 mg/kg, and MSC transplantation was performed at the 5th week (5 × 10<sup>6</sup> cells/rat). Group F was the PDMSC treatment group, and PDMSC transplantation was performed at the 5th week (5 × 10<sup>6</sup> cells/rat). Group G was the MSC treatment group, and MSC transplantation was performed at the 5th week (5 × 10<sup>6</sup> cells/rat). 48 hours after the last CCl<sub>4</sub> injection, rats were sacrificed after being anesthetized by intraperitoneal injection of urethane (1 g/kg). Blood and livers were immediately harvested for analysis.

### 4.9. Analyses of the biochemical indicators for hepatic injury

The serum albumin (ALB), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured with diagnostic kits (Olympus) using an Olympus AU400 Automatic Biochemistry Analyzer (Olympus). The hepatic hydroxyproline level was monitored with a hydroxyproline test kit (Jiancheng Bioengineering Institute).

#### 4.10. Enzyme-linked immunosorbent assays

Levels of hepatic or serum TNF $\alpha$  and TGF- $\beta$ 1 were quantified using a corresponding ELISA kit purchased from Boster (Boster) according to the manufacturer's instructions.

#### 4.11. Histological analysis

A portion of the liver specimens were fixed with 10% formalin solution immediately and embedded in paraffin. The sections were mounted on slides and Azan staining was performed to analyze the extent of fibrosis. Another portion of the liver specimens were fixed with embedding medium to make frozen sections, which were used to observe Dil-labeled cells in the liver.

#### 4.12. Statistical analysis

Results are expressed as mean  $\pm$  SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Dunnett t-test.  $P < 0.05$  was considered statistically significant.

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