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Research Paper

In-vitro promoted differentiation of mesenchymal stem cells towards hepatocytes induced by salidroside

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Abstract

Objectives The present study aimed to investigate whether salidroside can induce differentiation of rat mesenchymal stem cells (rMSCs) towards hepatocytes *in vitro* and the mechanism of hepatic differentiation of rMSCs.

Methods rMSCs were subject to hepatic differentiation. One, two and three weeks later, the expression of alpha fetoprotein (AFP) and albumin (ALB), cytochrome P450 (CYP450)-dependent activity and inducibility, cellular uptake of low density lipoprotein (LDL) and urea synthesis were assessed and the hepatic differentiation of rMSCs was evaluated. In order to unravel the mechanism of hepatic differentiation of rMSCs *in vitro*, inhibitors of extracellular regulated kinase1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K) and p38 were applied. When the process of hepatic differentiation was completed, special proteins of hepatic differentiation were detected and blocking of inhibitors was evaluated.

Key findings Salidroside significantly induce differentiation of rMSCs towards hepatocytes. Differentiated rMSCs have typical functional hepatic characteristics. The results also showed that the ERK1/2 and PI3K signalling pathways play important roles in the regulatory effects of salidroside on hepatic differentiation of rMSCs and are involved in cell fate determinations, while the p38 signalling pathway does not.

Conclusions Salidroside can induce differentiation of rMSCs towards hepatocytes *in vivo*, and the ERK1/2 or PI3K signalling pathway underlie the process of hepatic differentiation.

Keywords differentiation; hepatocytes; mesenchymal stem cells; salidroside; signalling pathway

Introduction

Mesenchymal stem cells (MSCs) from bone marrow retain a dual capacity of renewal and versatile differentiation. In the past few years, these potentialities have been rigorously demonstrated, not only for the various cells of mesodermal origin,[1] but also for neuroectodermal[2] and endodermal cells.[3] This differentiation potential of MSCs, along with their powerful ex-vivo expansion capability, gives them excellent potential for tissue repair and organ regeneration,[4] and MSCs could also be used as tools for preclinical pharmacotoxicological research and regulatory testing.[5]

Since MSCs are easy to obtain from bone marrow (BM), the prospect of using these cells to correct liver diseases is promising.[6] Recently, many researchers have successfully induced MSCs to differentiate into hepatocytes *in vitro*[7–13] or *in vivo*.[14–16] However, some problems remain to be solved.

While the hepatic differentiation mechanism of MSCs remains unknown, a high concentration of hepatocyte growth factor (HGF) in media (or sera) and a continuous administration of liver-toxic substances, such as trichostatin A, play a vital role. It is necessary to find novel drugs to replace the current methods of induction.

In addition, results of hepatic differentiation *in vitro* seem to be similar, while they are widely divergent when differentiation is induced *in vivo*. The discrepancy between positive in-vitro experimental results and negative in-vivo experimental outcomes, and the unsatisfactory methods used in the induction of hepatocytes have led us to propose searching for drugs that may have specific functions, such as inducing differentiation of MSCs towards hepatocytes directly, reducing liver injury and ameliorating liver fibrosis.

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Salidroside, also known as rhodioside, is an extract from *Rhodiola rosea* L., which has been known for its pleiotropic characteristics and has been used in a wide range of applications for about 2000 years. Salidroside, an extensive hepatoprotective drug with low toxicity, has been reported to have various pharmacological properties, including adaptogenic, antifatigue, antioxidant, antihypoxia, antiapoptosis and anticancer activity, etc.[17–18] Salidroside has been found to play an extensive hepatoprotective role in liver diseases, such as inhibiting apoptosis of hepatocytes and proliferation of hepatic stellate cells, decreasing serum aminotransferase, reversing hepatic fibrosis and improving liver function.[19–21]

We have hypothesised that salidroside may induce differentiation of MSCs into hepatocytes, with minimal toxicity, by means of a direct improvement in the body's internal environment. If salidroside is found to induce differentiation of MSCs towards hepatocytes, it may provide a basis for clinical applications that will bridge in-vitro and in-vivo applications. In the present study we examined whether salidroside can facilitate the induction of rMSCs to hepatic differentiation *in vitro* and determined the hepatic differentiation mechanism of MSCs.

Materials and Methods

Experimental animals

Sprague Dawley (SD) rats obtained from the Laboratory Animal Unit of Zhejiang Academy of Medical Sciences (Hangzhou, China) were used in the experiments. All animal experiments were carried out in accordance with a legal regulation that includes approval by a local ethical committee.

Isolation and culture of rMSCs

Rat MSCs (rMSCs) were obtained from femurs of 6-week-old female SD rats according to Friedenstein's protocol.[22] Cold nutrient Dulbecco's minimum essential medium (DMEM, low glucose; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) was flushed into the bone shaft, the pellet was resuspended, washed and then added into erythrocyte lysis buffer (NH_4Cl 0.155 mol, $KHCO_3$ 10 mM, ethylenediaminetetraacetic acid (EDTA; Amresco, Canada), 1 mM) for 5 min to lyse the erythrocytes at room temperature. After centrifugation for 3 min at 1000 rpm, the supernatant was removed and culture commenced with the complete DMEM (low glucose) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cell concentration was adjusted to a density of 0.5×10^5 cells/cm^2 in a humidified atmosphere of 5% CO_2, at 37°C. When the culture reached 80% confluence, 6 days later, differentiated MSCs were trypsinised and a serial subcultivation begun at a density of 1×10^4 cells/cm^2.

Phenotype of the cells purified from rat bone marrow

To determine the antigen expression profiles of the adherent cells from bone marrow, cells in culture were detached using 0.25% trypsin and 1 mM EDTA (Gibco). Cells were stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies against: cluster designation (CD) 29, CD44 (both from Santa Cruz Biotechnology, Tokyo, Japan), CD45 (Biolegend, San Diego, CA, USA) and CD90 (Caltag Lab., Burlingame, CA, USA), washed and analysed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). An isotype control (FITC- or PE-labelled) was included in each experiment. Analyses were performed by Cell Quest Software (FACSCalibur, Becton-Dickinson, USA).

rMSC multi-differentiation potential assays

To verify the multipotential mesenchymal characteristics, rMSCs of passage 3 were analysed for adipogenic, chondrogenic and osteogenic differentiation as previously described.[23] Briefly, to assess the osteoblastic phenotype, alkaline phosphatase (ALP) activity was measured after 2 weeks of induction; Von Kossa staining was used to assess matrix mineralisation after 3 weeks. For chondrogenic differentiation, MSCs were stained with Alcian Blue and expression of the cartilage-specific collagen type II isoform was also determined after 2 weeks. The acquisition of the adipogenic phenotype was determined by staining the monolayers with 2% Oil Red-O solution after 2 weeks.

Inducing hepatic differentiation

rMSCs of passage 3 were incubated in basic differentiation DMEM (high glycose), supplemented with 2% FBS (Gibco), 0.1% dimethyl sulfoxide (DMSO) (Amresco), 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 serum deprived for 2 days in DMEM supplemented with 10 ng/ml fibroblast growth factor 4 (FGF4; PeproTech Inc, New Jersey, USA), prior to induction. Differentiation was induced by treating rMSCs with differentiation medium. rMSCs were divided into three groups according to an earlier report.[8] Groups A, B and C were cultured in basic differentiation DMEM, supplemented with 10 ng/ml FGF4, 10 ng/ml FGF4 and 20 ng/ml hepatocyte growth factor (HGF; PeproTech), and 10 ng/ml FGF4 and 2 μM salidroside (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), respectively. Cultures were maintained by medium exchange every 3 days. Cell morphology was observed under an Olympus phase contrast microscope.

Western blot analysis

rMSCs, induced or not, were lysed in Nonidet P 40 (NP40) lysis buffer, and lysates were recovered by centrifugation. Protein from each sample was used in Western blots.[24] Equal amounts of protein (30 μg) were separated by 10% SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were incubated with the relevant primary antibodies against α-fetoprotein (AFP) and albumin (ALB) (Santa Cruz Biotechnology) or β-actin. The membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The protein bands were visualised by enhanced chemiluminescence (Amersham, London, UK) and the images were captured on medical X-ray film (Kodak, USA). Rat β-actin was used as an internal control for production of protein

while extracts from HepG 2 and hepatocytes were used as positive control for AFP and ALB, respectively.

Alkoxyresorufin *O*-dealkylase assay

The activities of ethoxyresorufin *O*-deethylase (EROD) and 7-pentoxyresorufin-*O*-dealkylase (PROD) were assessed as previously described,[25] with some minor modifications: rMSCs were incubated with 20 μM 7-ethoxyresorufin and 18 μM 7-pentoxyresorufin (all from Sigma, Germany) for 3 h and subsequently for 30 min with β-glucuronidase/arylsulfatase.

To evaluate the inducibility of cytochrome P450 (CYP)IIB1 and CYPIA1, rMSCs were, after 2 weeks of differentiation, exposed to phenobarbital (PB, final concentration 1 mM) and 3-methylcholanthrene (MC, final concentration 2 μM; all from Sigma), respectively. Media, supplemented with either PB or MC, were renewed daily. Fresh culture media and adult hepatocytes (cultured for 6 h) were used as negative and positive controls, respectively.

Cellular uptake analysis of low density lipoprotein

rMSCs were incubated in culture media containing 20 μg/ml 1,10-dioctadecyl-1–3,3,30,30-tetramethyl-indo-carbocyanine perchlorate conjugated to acetylated low density lipoprotein (DiI-Ac-LDL; Invitrogen, USA) after 2 weeks of differentiation. Fluorescence of supernatants was measured using a fluorospectrophotometer (Infinite F200, Tecan, Austria). Fresh differentiation media and adult rat hepatocytes cultured for 6 h were used as negative and positive controls, respectively.

Evaluation of urea synthesis

After 2 weeks of differentiation, rMSCs were incubated in culture media containing 6 mM NH_4Cl (Sigma) for 24 h at 37°C. Urea concentrations in the supernatant were measured. Fresh differentiation media supplemented with 6 mM NH_4Cl and adult rat hepatocytes cultured for 6 h were used as negative and positive controls, respectively.

Signalling pathways on salidroside-induced hepatic differentiation in MSCs

To test whether ERK1/2, p38, and PI3K signalling pathways were involved in salidroside-induced hepatic differentiation of MSCs, MSCs were treated with 30 μM SB 203580, a p38 inhibitor, 30 μM LY294002, a PI3K inhibitor, or 10 μM U0126, an ERK1/2 inhibitor, respectively, for 2 weeks. Because it takes a long time for MSCs to be differentiated into hepatocytes and transient molecular events have nothing to do with it, the level of molecular production, such as AFP and ALB, rather than ERK1/2, phosphorylated ERK1/2, p38, phosphorylated p38, Akt (a downstream signalling molecule of PI3K) and phosphorylated Akt would be detected under these conditions, with or without salidroside. All inhibitors were purchased from Promega Corporation (Madison, WI, USA).

Western analysis was carried out using the primary antibodies AFP and ALB. After extensive washing, immunocomplexes were detected with appropriate horseradish peroxidase conjugated secondary antibodies followed by enhanced chemiluminescence (ECL).

Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis test. $P < 0.05$ was considered to indicate statistical significance and $P < 0.01$ to show high statistical significance.

Results

Separation and identification of rMSCs

Without interference from erythrocytes, the adherent spindle-shaped cells were observed on day 2. Cells that rapidly grew colonies and exhibited homogeneous morphology (Figure 1a) were selected for culture expansion. After being induced for 2 weeks, the differentiated rMSCs became round (Figure 1b), while rMSCs not induced to differentiate showed the post-midsized state, which was difficult to observe clearly (Figure 1c). There was no obvious difference between growth factor (FGF4 and HGF)-induced and salidroside-treated rMSCs (data not shown).

Immunophenotypic analyses were performed to rule out the contamination of macrophages and a few of the hematopoietic (stem) cells. The majority of the adherent fibroblastic cells of passage 3 were negative for CD45 while they were positive for CD29, CD44 and CD90, known to be cell surface antigens of rMSCs (Figure 2).[26]

To study the multilineage capacity of MSCs, MSCs were induced to differentiate toward the osteogenic, chondrogenic and adipogenic lineages using lineage-specific induction factors. MSCs have been shown to differentiate toward the osteogenic, chondrogenic and adipogenic lineages with appropriate medium supplementation (see Materials and Methods section). Differentiation of MSCs into osteoblasts is induced by culturing MSCs in an osteoblastic medium. The expression of ALP was examined (Figure 3a). To further confirm the osteoblastic differentiation, some mineralised cells were found using von Kossa staining after culturing for 3 weeks (Figure 3c). Chondrogenic differentiation can be induced *in vitro* using a micromass culture technique. After culturing with chondrogenic induction for 2 weeks, pellet cultures of the cells exhibited collagen type II, a marker of chondrogenic differentiation (Figure 3e). A more even staining profile was seen for Alcian Blue staining of sulfated proteoglycans in the pellets after chondrogenic induction for 2 weeks (Figure 3g). MSCs were treated with adipogenic induction medium for 2 weeks. The cells containing lipid vesicles exhibited an expanded morphology with the majority of intracellular space occupied by droplets and lipid vesicles, which is consistent with the phenotype of mature adipocytes. As early as 1 week after inducement, lipid droplets were detectable (data not shown), and lipid accumulation thereafter increased along with the inductive periods. Intracellular fat droplets were chemically stained by Oil Red-O (Figure 3i). Undifferentiated rMSCs maintained in control medium (Figures 3b, d, f, h and j, respectively) were examined as a negative control.

Hepatocyte specific protein expression

Western blot analyses were performed to examine expressions of liver-specific proteins. Results showed that rMSCs of group A, B or C all expressed ALB or AFP protein,

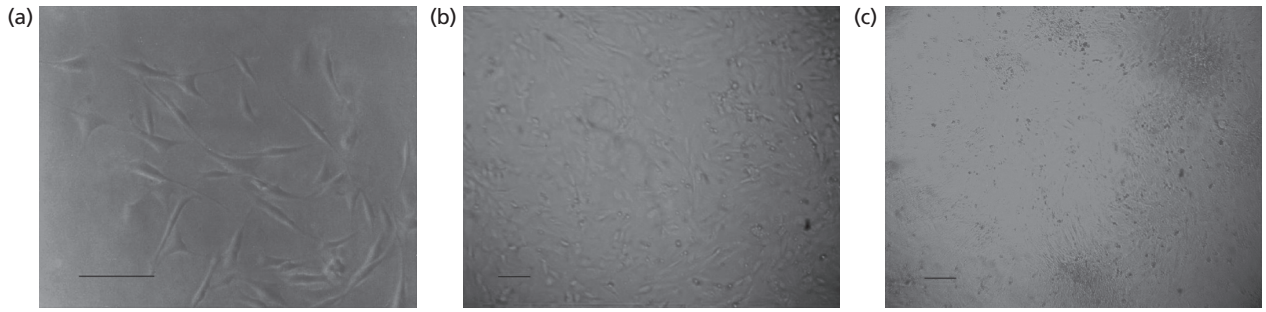

Figure 1 Photomicrograph of rat mesenchymal stem cells (rMSCs). When erythrocytes lysed, rMSCs were expanded in DMEM supplemented with 10% FBS. Cell colonies exhibited homogeneous morphology and were fibroblast-like (Figure 1a). When cultured for 2 weeks, morphologies were changed, and rMSCs showed the post-midsized state (Figure 1c) while induced MSCs became round (Figure 1b). (scale bar = 50 μm)

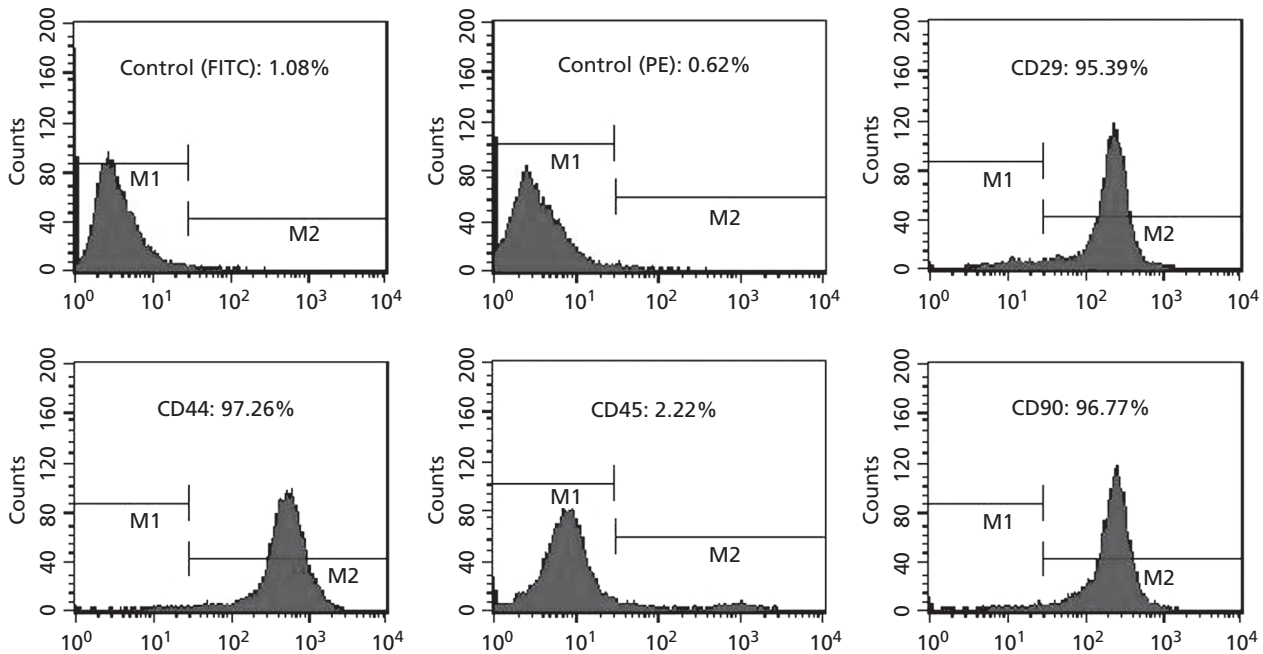

Figure 2 Phenotype of the cells. Analysis by flow cytometry shows that bone-marrow-derived rat mesenchymal stem cells are negative for the expression of CD45, but positive for the expression of CD29, CD44 and CD90

while undifferentiated rMSCs did not. A marker of hepatic differentiation during the early stage, AFP, was observed on day 4, peaked on day 7, and disappeared on day 14. ALB, a marker of hepatic differentiation during the mid/late stage, was detected on day 7, and then continued being expressed in a time-dependent manner. Expression of AFP and ALB of group B or C was significantly enhanced when compared to medium A (Figure 4).

Cytochrome P450-dependent activity and inducibility

rMSC of group A, B or C expressed functionally active CYPIA1 and CYPIIB1. Salidroside, as well as FGF4 and HGF, significantly promoted the metabolising capacity of CYPIA1 and CYPIIB1 from days 18 onwards (data not shown). By the end of 3 weeks, EROD and PROD even increased towards levels measured in primary hepatocytes cultivated for 6 h.

The potential to induce CYP dependent monooxygenases is considered as one of the most representative functional parameters for evaluating the adult hepatic phenotype.[27] The responsiveness of both CYPIA1 and CYPIIB1 to their respective prototype inducers, methylcholantrene (MC) and phenobarbital (PB), was therefore studied in parallel.

We performed an alkoxyresorufin-*O*-dealkylase assay to investigate P450-inducing activities of differentiated rMSCs. After 2 weeks postinduction, rMSCs of each group exhibited inducible CYP activities. More importantly, EROD and PROD activities of groups B and C were significantly induced after 3 days exposure to MC and PB, respectively. The inducibility persisted throughout the culture time. rMSCs of group A also showed inducible activities of EROD and PROD, but to a significantly lesser extent (Table 1).

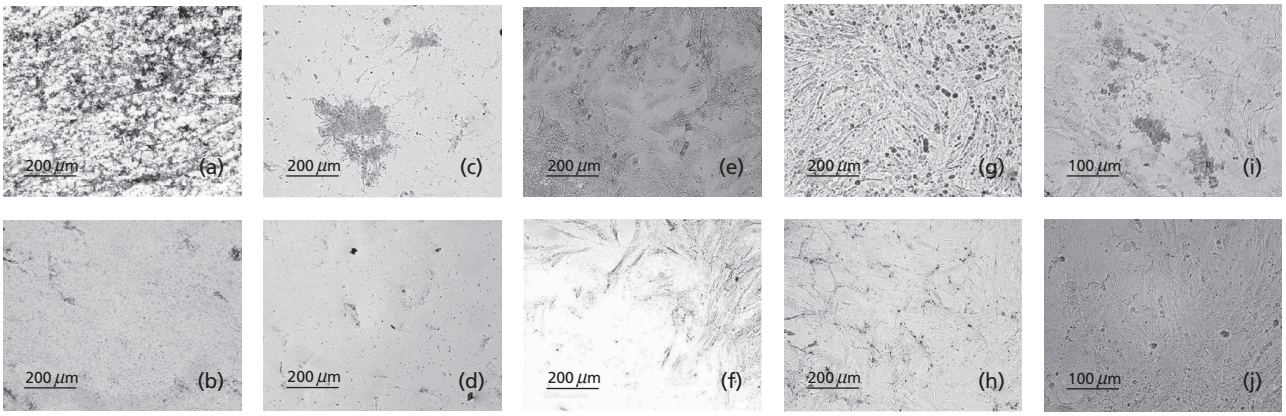

Figure 3 Rat mesenchymal stem cell (rSMC) multi-differentiation potential assays. rMSCs induced with osteogenic medium express alkaline phosphatase (ALP) and are associated with a calcified extracellular matrix (ECM). MSCs were cultured in osteogenic medium to induce osteogenesis. Cells were stained at 2 weeks for ALP activity (a). The presence of a calcified ECM was examined at 4 weeks (von Kossa; c). Undifferentiated MSCs maintained in control medium were examined for ALP expression (b) and matrix calcification (d) as a negative control. rMSCs treated with chondrogenic medium are associated with a proteoglycan-rich matrix and express collagen type II. MSCs were cultured for 2 weeks in chondrogenic medium to induce chondrogenesis. The cells were fixed and the expression of collagen type II (collagen II) was examined (e). MSCs cultured in control medium were stained (immunohistochemistry) for collagen II expression as a negative control (f). In addition, the cells were fixed and processed for the presence of sulfated proteoglycans with Alcian Blue under acidic conditions (g) while undifferentiated MSCs maintained in control medium were processed as a negative control (h). rMSCs accumulate lipid-filled droplets on treatment with adipogenic medium. MSCs were cultured for 2 weeks in adipogenic medium and stained with Oil Red-O to identify lipid-filled intracellular vacuoles (i). Undifferentiated MSCs maintained in control medium were stained as a negative control (j)

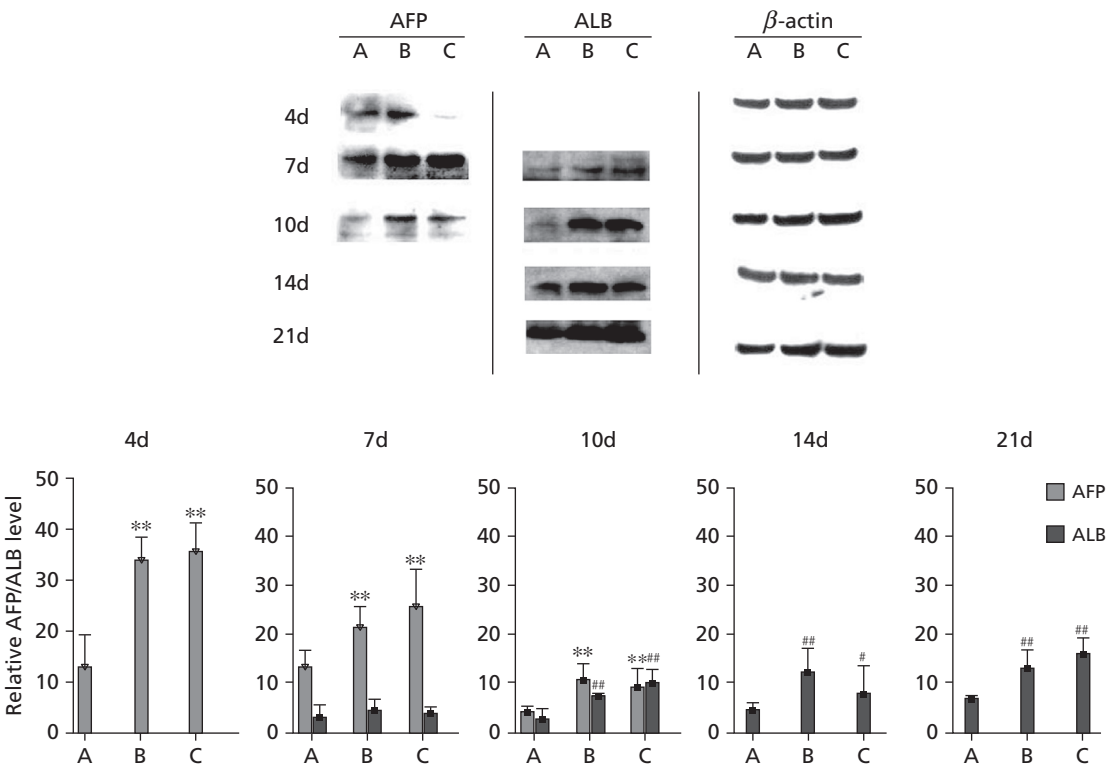

Figure 4 Western blot analyses of the expression of liver special proteins in rat mesenchymal stem cells (rMSCs) before and after differentiation. Under the hepatogenic conditions, rMSCs differentiated into hepatocyte-like cells expressing liver-specific protein α-fetoprotein (AFP) (left), albumin (ALB) (right). rMSCs of passage 3 were incubated in basic differentiation DMEM (Materials and Methods); group A was supplemented with 10 ng/ml FGF4, group B was supplemented with 10 ng/ml FGF4 and 20 ng/ml HGF, group C was supplemented with 10 ng/ml FGF4 plus 2 μM salidroside. β-actin was used as an internal control. PC, positive control (liver cells); A, B or C represents group A, B or C, respectively. The experiments were repeated at least three times and similar findings were observed. * (or #) $P < 0.05$; ** (or ##) $P < 0.01$, vs A (group A)

Table 1 Activities or characteristics of hepatic differentiation of rat mesenchymal stem cells

	Group		Time			Hepatocyte
			2 weeks	3 weeks	4 weeks	
Activities of EROD (pmol/well × min)	A	FGF4	1.75 ± 0.10	6.77 ± 0.74	10.04 ± 1.53	
	B	FGF4+HGF	2.45 ± 0.09	14.24 ± 0.73**	19.85 ± 1.66**	
	C	FGF4+salidroside	2.44 ± 0.13	15.09 ± 0.74**	23.00 ± 2.45**	
		Hepatocytes				6.18 ± 0.16
Inducibility of EROD (pmol/well × min)	A	FGF4+MC	2.85 ± 0.06	12.85 ± 1.17	18.49 ± 1.55	
	B	FGF4+HGF+MC	3.69 ± 0.03	36.96 ± 1.15**	46.21 ± 1.90**	
	C	FGF4+salidroside+MC	3.67 ± 0.10	39.24 ± 3.41**	49.89 ± 2.13**	
		Hepatocytes				8.44 ± 0.11
Activity of PROD (pmol/well × min)	A	FGF4	0.79 ± 0.01	2.07 ± 0.54	2.82 ± 0.36	
	B	FGF4+HGF	0.94 ± 0.06	3.78 ± 0.78**	4.47 ± 0.15**	
	C	FGF4+salidroside	0.91 ± 0.11	3.78 ± 0.27*	4.66 ± 0.32**	
		Hepatocytes				2.02 ± 0.11
Inducibility of PROD (pmol/well × min)	A	FGF4+PB	0.86 ± 0.08	3.15 ± 0.17	4.73 ± 0.25	
	B	FGF4+HGF+PB	1.06 ± 0.10	5.60 ± 0.29**	6.86 ± 0.12**	
	C	FGF4+salidroside+PB	1.13 ± 0.05	6.32 ± 0.33**	7.59 ± 0.50**	
		Hepatocytes				2.88 ± 0.04
Urea synthesis (pg/cel per h)	A	FGF4	3.38 ± 0.80	5.64 ± 0.49	5.34 ± 0.66	
	B	FGF4+HGF	5.53 ± 0.38	9.16 ± 0.51**	10.20 ± 0.47**	
	C	FGF4+salidroside	4.79 ± 0.41	9.21 ± 0.63**	10.38 ± 0.38**	
		Hepatocytes				10.32 ± 0.49
Uptake of LDL (ng/well)	A	FGF4	47.64 ± 2.23	167.09 ± 21.02	177.66 ± 20.50	
	B	FGF4+HGF	68.84 ± 7.95	308.45 ± 27.35**	381.32 ± 50.62**	
	C	FGF4+salidroside	76.65 ± 6.32	333.36 ± 29.02**	410.83 ± 73.58**	
		Hepatocytes				217.43 ± 18.40

Under the hepatogenic conditions, rMSCs differentiated into hepatocyte-like cells. Differentiated cells possess cytochrome P450 activity and inducibility, enable the utility of cellular uptake of low density lipoprotein (LDL) and secrete urea in a time-dependent manner. The experiments were repeated at least three times and similar findings were observed. A, B or C represents group A, B or C, respectively. Values are means ± SD, $^{*}P < 0.05$ vs A, $^{**}P < 0.01$ vs A. EROD, ethoxyresurofin-*O*-deethylase; PROD, 7-pentoxyresorufin-*O*-dealkylation; PB, phenobarbital; MC, 3-methylcholanthrene.

Cellular uptake analysis of low density lipoprotein

LDL is a lipoprotein that carries cholesterol around the body, for use by various cells. Most LDL is metabolised in the liver.[28]

We analysed the cellular uptake of LDL to investigate hepatic differentiation of rMSCs. After 3 weeks of differentiation, we observed LDL uptake by the differentiated rMSCs in each group. The uptake of LDL increased in a time-dependent manner, while undifferentiated rMSCs did not show increased uptake. Uptake of LDL significantly increased in rMSCs of group B or C. The rMSC of group A also had LDL uptake ability. However, levels remained stable and were significantly lower than those of groups B or C (Table 1).

Evaluation of urea synthesis

Urea synthesis, one of the hepatic metabolic functions, is an indicator of nitrogen metabolism. To investigate the hepatic differentiation of groups A, B and C, we evaluated the urea synthesis of rMSCs. Results showed that differentiated rMSCs enabled the synthesis of urea after 2 weeks, and they synthesised urea in a time-dependent manner, while undifferentiated rMSCs did not. Urea production of rMSCs of groups B or C was significantly higher than that of group A (Table 1).

Effects of ERK1/2, p38 and PI3K signalling pathways on salidroside-induced hepatic differentiation in MSCs

ERK1/2, p38, and PI3K all play an important role in mediating cellular effects in MSCs.[29] HGF is a pleiotropic cytokine, displaying mitogenic, motogenic, morphogenetic and antiapoptotic activities in a cell type-specific manner.[30] We determined whether these pathways were involved in salidroside's induction effect of hepatic differentiation, exposing MSCs to salidroside for 1 week (after which AFP was detected) or 2 weeks (after which ALB was detected).

Results showed that each inhibitor clearly inhibited the growth of MSCs; moreover, U0126 inhibited the salidroside-induced hepatocyte-specific protein (AFP and ALB) expression of differentiation MSCs, LY294002 did partially and SB 203580 did not. This means that salidroside induces differentiation of MSCs towards hepatocytes by the ERK1/2 and PI3K signal pathways (Figure 5).

Discussion

Cell-based therapies are emerging as an alternative to whole-organ transplantation. Recently many researchers have reported the generation of hepatocyte-like cells from MSCs. MSC-based therapies for liver diseases open new

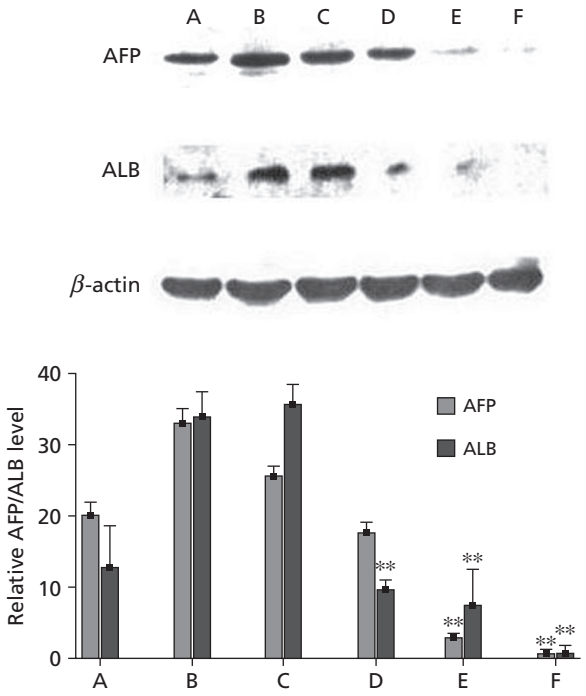

Figure 5 Western blot analyses of the expression of liver-specific proteins in the rat mesenchymal stem cells (rMSCs) when inhibitors were applied or not. Under the hepatogenic conditions, rMSCs were induced to differentiate into hepatocyte-like cells. All inhibitors (30 μM SB 203580, 30 μM LY294002 or 10 μM U0126) were added to the medium in the absence or presence of salidroside. Inhibitors were dissolved in DMSO (0.1%, v/v final concentration); controls contained 0.1%, v/v DMSO. The proteins α-fetoprotein (AFP) and albumin (ALB) in MSCs were detected by Western blot. The blots shown were representative of three independent experiments with similar results. Groups A, B, C, D, E and F were divided with different supplements: group A (10 ng/ml FGF4), group B (FGF4 plus 2 μM salidroside), group C (10 μM salidroside), group D (10 μM salidroside plus 30 μM SB 203580), group E (10 μM salidroside plus 30 μM LY294002) and group F (10 μM salidroside plus 10 μM U0126). $^{*}P < 0.05$; $^{**}P < 0.01$, vs C (group C)

perspectives. Many categories of liver disease can be distinguished and principally addressed by stem cell therapy.[31] However, some researchers doubt that rMSCs differentiate towards hepatocytes *in vivo* or improve liver function and prolong survival when transplanted.[32,33] The contrast between efficient differentiation effects *in vitro* and invalid treatments for liver diseases *in vivo* has represented an experimental bottleneck for researchers. The clinical application of MSCs remains promising but, so far, ambiguous.

This concern could lead to the failure of rMSCs-based therapies for liver disease. There is a contrast between differentiation of rMSCs towards hepatocytes *in vitro* and *in vivo*.[34] Growth factors (high concentration) play an important role in the process of differentiation *in vitro*, while the microenvironment (hepatic fibrosis, liver injury, etc.) affects hepatic differentiation of MSCs to a great extent.

HGF is a pleiotropic cytokine.[30] When high concentrations (or repeated doses) of HGF, required by hepatic differentiation of MSCs,[35] is systemically administered, adverse effects are caused in extra-hepatic organs.[36,37] It is very uncertain whether the microenvironment of liver disease is suitable for the survival of MSCs, not to mention their proliferation and differentiation. It has therefore become even more necessary to search for drugs that can not only improve the microenvironment, but also induce hepatic differentiation directly. Experimental studies have shown that salidroside has extensive hepatoprotective effects, which make it a good candidate in line with this requirement and it is this that has aroused our attention.

As is known, FGF4 acts as the priming factor of differentiation.[38,39] It has been reported that FGF4, a differentiation initiator, alone may weakly induce differentiation of rMSCs towards hepatocytes *in vitro*, while HGF plus FGF4 may strongly induce differentiation of rMSCs towards hepatocytes.[35] The same results were obtained in our laboratory. Moreover, FGF4 plus 2 μM salidroside or 10 μM salidroside may strongly induce differentiation of rMSCs towards hepatocytes.

In this study, whether exposed to FGF4 (10 ng/ml) plus salidroside (2 μM) or salidroside (10 μM) alone, rMSCs differentiated towards hepatocytes (Figure 5), and differentiation effects were similar to those of HGF plus FGF4, which has been reported by Schwartz.[8] Salidroside has been shown to be a differentiation agent with characteristics of positive differentiation agents, for example HGF. Our results have also shown that in the concentration range used (0–20 μM for salidroside), rMSCs differentiated towards hepatocytes in a time-dependent manner, whereas at salidroside concentration ranges greater than 20 μM, the differentiation rate of rMSCs plateaus (data not shown). The discovery has some significance in the application of rMSCs.

In the presence of salidroside, hepatocyte-like cells differentiated from MSCs showed typical functional hepatic features, such as the expression of AFP (an early marker protein of hepatic differentiation) and ALB, inducible CYP-dependent activity, cellular uptake of LDL and urea synthesis. Hepatocytes have many other characteristics, such as cytokeratin 18, cytokeratin 19, a1-antitrypsin, glucose-6-phosphatase, tyrosine aminotransferase (TAT), phosphoenolpyruvate carboxykinase (PEPCK) and transthyretin (TTR), etc. As a systematic study, it is difficult to identify the true differences between hepatocytes and hepatocyte-like cells differentiated from MSCs.

All experimental data indicated that the hepatogenic potential of MSCs is progressively engaged under differentiation conditions. In addition, the cells were gradually shown to gain genuine functions of mature hepatocytes. Functions were gained in a time-dependent manner.

It has been reported that both Wnt/β-catenin signals and hepatocyte nuclear factor 3 beta play an important role in the hepatic fate specification of MSCs.[40,41] HGF is a pleiotropic cytokine, displaying mitogenic, motogenic, morphogenetic and antiapoptotic activities in a cell type-specific manner.[30] Recently, some studies have shown that HGF can also induce biological responses in stem cells.[42] Binding of HGF to its receptor induces multiple biological responses by the downstream effectors p38MAPK, PI3K and ERK1/2. We found that the ERK1/2 signalling pathway plays an important role in the regulatory effects of salidroside on hepatic

differentiation of rMSCs, the PI3K signalling pathway did so partially while the p38 signalling pathway did not.

It has been shown that salidroside can improve the microenvironment *in vivo*, and in this study we have demonstrated that salidroside induces hepatic differentiation *in vitro*. Both of these properties of salidroside will make significant contributions to hepatic differentiation of MSCs *in vivo*, which makes salidroside a promising drug. Results did not show the functional advantage of salidroside over HGF, but indicated that salidroside could at least not cause adverse effects in extra-hepatic organs.

In summary, we have provided in-vitro experimental data to show that salidroside induces hepatic differentiation. Work to induce hepatic differentiation (in the presence of salidroside) *in vivo* and uncover the molecular mechanism of hepatic differentiation of rMSCs is ongoing.

Conclusions

Salidroside in combination with FGF4 significantly induce differentiation of rMSCs towards hepatocytes. Differentiated rMSCs have typical functional hepatic characteristics, such as expression of AFP and ALB, CYP-dependent activity and inducibility, cellular uptake of LDL and urea synthesis. The ERK1/2 and PI3K signalling pathways play important roles in the regulatory effects on hepatic differentiation of rMSCs and are involved in cell fate determinations, while the p38 signalling pathway does not.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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