OPEN

Citation: Cell Death and Disease (2014) 5, e1308; doi:10.1038/cddis.2014.257

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Chronic restraint stress decreases the repair potential from mesenchymal stem cells on liver injury by inhibiting TGF-β1 generation

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Chronic psychological stress has been demonstrated to play an important role in several severe diseases, but whether it affects disease therapy or not remains unclear. Mesenchymal stem cells (MSCs) have been demonstrated to have therapeutic potentials in treating tissue injury based on their multidifferentiation potential toward various cell types. We investigated the effect of chronic restraint stress on therapeutic potential of MSCs on carbon tetrachloride (CCl_4)-induced liver injury in mice. CCl_4-induced mice were injected with enhanced green fluorescent protein–MSCs, which was followed by chronic restraint stress administration. Corticosterone and RU486, a glucocorticoid receptor (GR) antagonist, were employed *in vivo* and *in vitro*, too. In the present study, we illustrated that MSCs could repair liver injury by differentiating into myofibroblasts (MFs) which contribute to fibrosis, whereas stress repressed differentiation of MSCs into MFs displayed by reducing α-smooth muscle actin (α-SMA, a solid marker of MFs) expression. Whereas RU486 could maintain the liver injury reduction and liver fibrosis increases induced by MSCs in stressed mice and block the decrease of α-SMA expression induced by stress. Furthermore, chronic stress inhibited MFs differentiation from MSCs by inhibiting transforming growth factor-β1 (TGF-β1)/Smads signaling pathway which is essential for MFs differentiation. Chronic stress reduced autocrine TGF-β1 of MSCs, but not blunted activation of Smads. All these data suggested that corticosterone triggered by chronic stress impaired liver injury repair by MSCs through inhibiting TGF-β1 expression which results in reduced MFs differentiation of MSCs.
Cell Death and Disease (2014) **5,** e1308; doi:10.1038/cddis.2014.257; published online 26 June 2014

Liver fibrosis is a wound repairment event in response to chronic injuries induced by a series of causes, such as viral hepatitis infection, alcohol, drugs, autoimmune reaction and metabolic diseases,[1] which is characterized by excessive deposition of extracellular matrix proteins. If liver injury could not be repaired in time, the fibrosis would continue and come to a bad cycle that will alter the balance of matrix secretion and degradation. Cirrhosis, the end stage of fibrosis, mortality rate which increases speedily worldwide,[2] appears to be a large health burden in the world. There is still no effective and feasible treatment of cirrhosis apart from orthotopic liver transplantation.[3] Therefore, treating liver injury at early stage seems to be crucial to arrest cirrhosis progression. In general, some factors resulting in liver injury could not be removed; hence, alternative strategies to repair liver injury at early stage needs to be developed.

With the growing enthusiasm of stem cell therapy, the application of mesenchymal stem cells (MSCs) on liver injury repair attracts more and more attention. In the trend of stem cell therapy, there are still unresolved problems in clinical application, such as the risk of teratoma formation, ethical issue, heterogeneity rejection and normalized production. However, MSCs become the most promising candidates for treatment in recent years because they are free of ethical concerns, without the risk of teratoma formation, and with low immunogenicity. MSCs have been isolated from a wide array of tissues successfully[4–15] and can be cultured *in vitro*. Dependent on the nature of injurytropism and multipotent differentiation capacity, they have been shown to be highly effective to treat various tissue injury and degenerative diseases, such as myocardial infarction, liver cirrhosis, spinal cord injury, bone damage, cornea damage, burn-induced skin defects and other tissue injuries.[16] There have been reports demonstrating that exogenous MSCs can repair damaged liver, but the mechanisms are diverse.[17,18]

It has been reported that myofibroblasts (MFs) are activated and contribute to wound healing after tissue injury.[19] Hepatic stellate cells are not the only sources of MFs,[20] as MSCs can

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Abbreviations: α-SMA, α-smooth muscle actin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl_4, carbon tetrachloride; EGFP, enhanced green fluorescent protein; GC, glucocorticoid; GR, glucocorticoid receptor; MSCs, mesenchymal stem cells; MFs, myofibroblasts; TGF-β1, transforming growth factor-β1

Received 24.1.14; revised 19.4.14; accepted 29.4.14; Edited by M Agostini

differentiate into MFs too.[21,22] Hence, it is suggested that MSCs repair liver injury through differentiating into MFs which is consistent with our results to some extent. In our study, exogenerous MSCs in early stage of liver injury could differentiate into MFs which contribute to liver fibrosis, and repaired liver injury in carbon tetrachloride (CCl_4)-induced mouse model. Therefore, we illustrated that elevated fibrosis exerted by MSCs at early stage of liver injury could reduce liver damage, even though fibrosis at late stage of liver injury results in liver failure. Transforming growth factor-β1 (TGF-β1), as a known growth factor associated with liver fibrosis, was documented to be involved in MFs differentiation from stromal cell types by inducing the expression of α-smooth muscle actin (α-SMA), a reliable marker of differentiated MFs.[19,23–31] There has been reports demonstrating that MSCs express α-SMA after TGF-β1 treatment,[24,25,32,33] and autocrine of TGF-β1 from MSCs after TGF-β1 administration has been reported too.[33]

During stem cell treatment, there are various factors affecting the therapy efficiency. Despite the attention paid to their own properties of MSCs, there is little consideration on the mental status of patients. Chronic stress, as a negative emotion,[34] accompanies with patients and exists in the process of disease therapy. Chronic stress has an important role in the occurrence and development of various considerable diseases among cardiovascular system, digestive system, immune system and nervous system. However, the role of chronic stress in the efficiency of MSCs therapy continues to be unclear. In stress system, the hypothalamic–pituitary–adrenal and the sympathetic–adrenal–medullary axises are activated, and thereby provoke the releasing of glucocorticoid (GC) (corticosterone in rodents and cortisol in humans[35,36]) and adrenal hormones, which are the main stress hormones. We are eager to know whether response to psychological stress of central nervous system influences therapeutic effect of MSCs on liver injury. In our study, mice were subjected to restraint stress after MSCs injection in CCl_4-induced liver fibrosis model. Here, we demonstrated that stress repressed the function of MSCs in liver injury repair through directly affecting on MSCs.

Results

Increased liver fibrosis induced by MSCs contributed to liver injury repair, and chronic stress reversed this process. We established a CCl_4-induced liver injury model in 6–8-week-old mice. To determine the role of MSCs in liver injury repair, we isolated enhanced green fluorescent protein (EGFP)–MSCs from bone marrow of EGFP transgenic mice and transplanted them into the mice injected with CCl_4 intravenously. We found that when MSCs were injected at the fourth week after CCl_4 intragastric administration, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were reduced significantly. From the data above, we could conclude that exogenerous MSCs reduced liver injury significantly. On the other hand, liver fibrosis was tested by Sirius Red staining. As shown in Figures 1c and d, compared with CCl_4 group, exogenerous MSCs injection made the pseudolobules formation clear and they promoted liver fibrosis in a large extent. These data showed that injury repair is consistent with fibrosis promotion, which suggested that liver fibrosis induced by MSCs contributed to liver damage reduction.

Chronic stress, which always accompanies with patients through the process of disease, has drawn little attention in clinical therapy. In order to investigate what effect chronic stress has on MSCs repairing liver injury, restraint was used to simulate chronic stress. At the fourth day, when almost all of exogenerous MSCs have migrated into the injury sites after injection (Supplementary Figure 2A), mice were exposed to restraint stress. At the second week after the end of stress, mice were killed. As shown in Figure 1, MSCs repaired liver injury and promoted liver fibrosis, but compared with CCl_4 + MSCs group, stress increased ALT and AST level and reduced liver fibrosis. The hematoxylin eosin staining also showed the same result of liver injury indicated by ALT and AST level (Figure 1e), which suggested that stress reversed the function of exogenous MSCs on liver injury repair and fibrosis.

Chronic stress inhibited MFs differentiation of MSCs. Liver fibrosis happened as an injury repair event induced by the activation of MFs. As liver is injured, not only cells such as hepatic stellate cells and fibroblasts *in situ* contribute to injury repair but MSCs recruited from bone marrow have also been shown to be involved in injury repair process.[22] To explore how chronic stress attenuated the therapeutic effect of MSCs on liver injury, we tested the effect of chronic stress on differentiation of MSCs into MFs *in vivo* and *in vitro*. Double staining with anti-GFP and anti-α-SMA antibodies of immunofluorescence assay was done to determine the distribution of MSCs and expression of α-SMA. As shown in Figure 2A, MSCs recruiting to liver injury sites could differentiate into MFs determined by co-expression of EGFP and α-SMA. However, in the MSCs plus stress group, without affecting migration of MSCs into liver injury sites (Supplementary Figure 2B) from bone marrow, the efficiency of MFs differentiation from MSCs was reduced significantly (Figures 2A and B).

To verify this result further, we detected the effect of stress on MFs differentiation from MSCs *in vitro*. We plated MSCs into 24-well plates, and after cells reached 50% confluence, 2 ng/ml of TGF-β1 and 2% serum from mice with or without stress were added. TGF-β1 application was used as a positive control, as MSCs express α-SMA after TGF-β1 stimulation. Four days after treatment, the immunofluorescence assay showed that TGF-β1 could induce expression of α-SMA in MSCs. With the existence of TGF-β1, serum from control mice did not alter the expression of α-SMA compared with only TGF-β1-treated cells. However, serum from stressed mice decreased α-SMA expression strongly (Figure 2C), which indicated that stress repressed MFs differentiation from MSCs.

Chronic stress promoted corticosterone production. As the main stress hormone, GC has been demonstrated to be critical in neuroendocrine system, which is an important effector of hypothalamic–pituitary–adrenal axis activation. GC has also been reported to function on MSCs through bonding with GR in MSCs.[37] To detect the GC secretion in

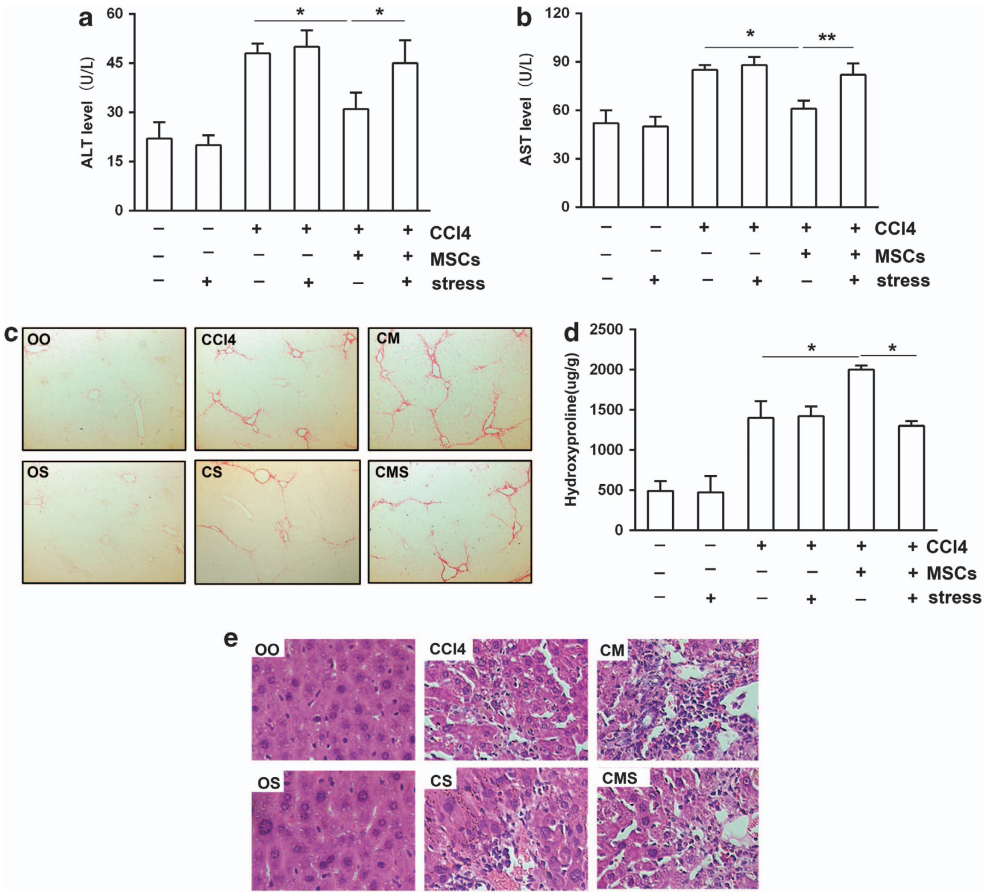

Figure 1 Chronic stress repressed the function of exogeneous MSCs in liver injury repair and hepatic fibrosis. Mice serum was analyzed for ALT (**a**) and AST (**b**). Sirius Red staining ($\times$ 100) (**c**) and liver hydroxyproline analysis (**d**) were performed to detect liver fibrosis. Hematoxylin eosin (HE) staining was done to detect liver injury ($\times$ 200) (**e**). OO, olive oil; OS, oil + stress; CS, CCl_4 + stress; CM, CCl_4 + MSCs; CMS, CCl_4 + MSCs + stress. $^*P<0.05$, $^{**}P<0.01$

mice after restraint stress, we sent the mice with or without CCl_4 injection for 3 weeks to stress. Corticosterone level in serum was determined from mice with or without stress by enzyme-linked immunosorbent assay (ELISA). In the serum of stressed mice, corticosterone concentration was upregulated approximately two to threefold compared with non-stressed mice (Figure 3), immaterial of whether the mice were administrated with CCl_4 or not.

Corticosterone impaired the liver injury repair by MSCs by inhibting MFs differentiation of MSCs. To verify the role of corticosterone in chronic stress on liver injury repair by MSCs, corticosterone was subcutaneously administrated into mice at the third day after MSCs injection in parallel with restraint stress in another group. We also used RU486 to block the function of stress *in vivo*. Results indicated that MSCs could reduce liver injury implied by decreased ALT and AST level, but corticosterone application attenuated the effect of MSCs which was consistent with stress group. Surprisingly, RU486 could downregulate ALT and AST level compared with the stress group, which indicated that RU486 could reverse the effect of stress (Figures 4a and b). The liver injury extent indicated by hematoxylin eosin staining was consistent with that the ALT and AST level suggested in (Figure 4e).

Liver fibrosis analysis was performed. Fibrosis level in MSCs plus corticosterone group was reduced compared with the MSCs group, which indicated that corticosterone could reverse the promoted liver fibrosis by MSCs, which could also be observed when mice was subjected to stress. Furthermore, RU486, as a GC receptor antagonist, could block the liver fibrosis reduced by stress (Figures 4c and d). Therefore, we concluded from all the above data that stress repressed therapeutic effect of MSCs through corticosterone production.

To assess the role of corticosterone in the MFs differentiation from MSCs, in the differentiation medium with 2 ng/ml of TGF-β1, we added 2% N (serum from normal mice), 2% S (serum from stressed mice), 2% S plus RU486 (100 nM) or corticosterone (200 nM), respectively. As shown in Figure 4f, corticosterone had the same effect with stress serum, which could inhibit α-SMA expression displayed by decreased fluorescence, and RU486 reversed the reduction of α-SMA expression induced by stress serum. Collectively, chronic stress inhibited MFs differentiation of MSCs by stimulating corticosterone secretion.

Corticosterone downregulated TGF-β1 production by MSCs. At the initial stage of MFs differentiation, TGF-β1 is the key-inducing factor. TGF-β1 induces expression of α-SMA by oligomerization of type I and type II TGFβ

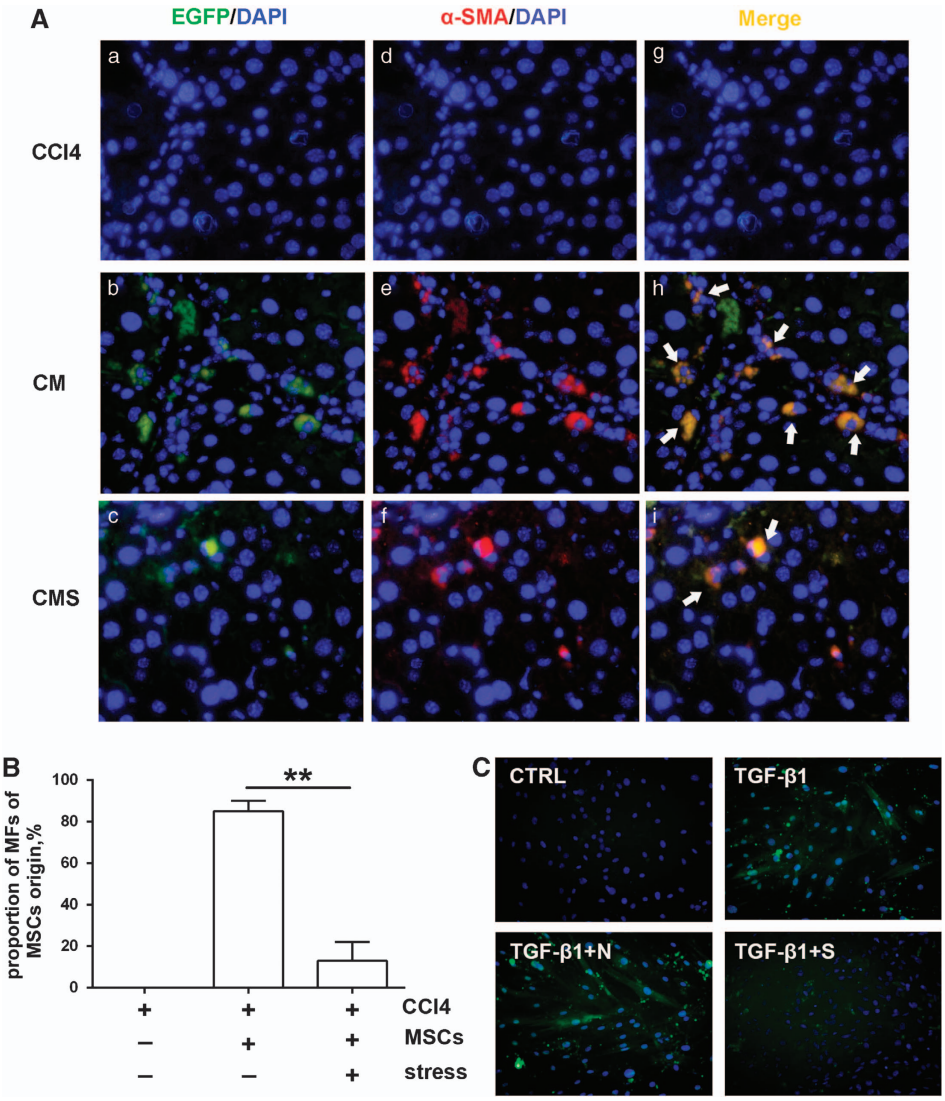

Figure 2 Chronic stress inhibited MFs differentiation of MSCs *in vivo* and *in vitro*. Liver sections were stained by immunofluorescence for MSCs (EGFP, green) and MFs (α-SMA, red) ($\times$ 400), co-positive stands for MFs differentiation of MSCs (yellow, white arrow) (**A**). Percentage of co-positive cells in recruited MSCs was calculated (**B**). MSCs were induced to differentiate into MFs *in vitro*. Cytoimmunofluorescence was done to detect α-SMA expression on MSCs (**C**). 4′,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei (blue). OO, olive oil; CM, CCl_4 + MSCs group; CMS, CCl_4 + MSCs + stress group. N, serum from normal mice; S, serum from stressed mice. $^{**}P<0.01$

receptors and then activates Smads by phosphorylation. In order to assess whether stress alters TGF-β1 expression, we extracted RNA from freezed liver specimens and tested mRNA expression of TGF-β1. As shown in Figure 5a, TGF-β1 expression was upregulated in injured liver induced by CCl_4, MSCs injection increased TGF-β1 expression to a higher level. However, stress played a negative role on TGF-β1 expression. MSCs injection with subsequent stress could not promote TGF-β1 expression compared with CCl_4 group. In other words, stress repressed MSCs-associated TGF-β1 expression increase. But stress together with RU486 could not repress the function of MSCs.

In addition, we tested TGF-β1 expression on protein level in liver by IHC assay. Tissue sections were stained with anti-TGF-β1 antibody. Data from immunohistochemistry assay were consistent with the real-time PCR results. Interestingly, TGF-β1 expressed at the position where MSCs were recruited (Figure 5b). To verify the effect of corticosterone on TGF-β1 expression of MSCs, we stimulated MSCs with 2 ng/ml TGF-β1, and we added 2% N, 2% S, 2% S plus 100 nM of RU486, or 200 nM of corticosterone in the culture; 12 h later, medium was replaced by serum-free medium. ELISA and real-time PCR were performed after 24 h. The data showed that MSCs could secret TGF-β1 after TGF-β1 stimulation and stress could inhibit TGF-β1 expression both in extracellular secretion and intracellular mRNA expression of MSCs, and RU486 could reverse the effect of stress (Figures 5c and d). α-SMA expression was consistent with TGF-β1 expression level (Figure 5e), which provided further evidence to support that TGF-β1 stimulates the MFs differentiation of MSCs. To reinforce the effect of GC on TGF-β1 production, we detected TGF-β1 production in human liver cell line Chang Liver after cortisol treatment. As shown in Supplementary Figure 3, cortisol could inhibit TGF-β1 production.

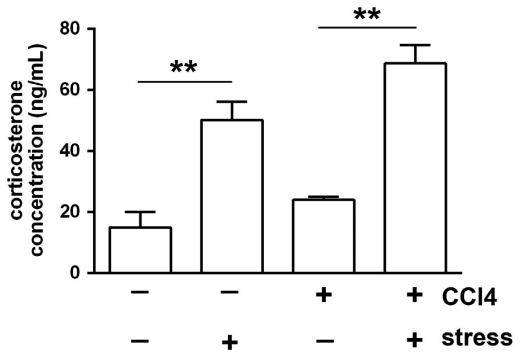

Figure 3 Corticosterone concentration in mice serum. Naive or CCl_4-treated mice were stressed or not, then killed and serum was collected and detected by ELISA for corticosterone. $^{**}P<0.01$

Activation of Smads in TGF-β1/Smads pathway was not affected by corticosterone directly. TGF-β1/Smads-dependent signal pathway is known to be critical for MFs differentiation and this pathway is activated by oligomerization of type I and type II TGFβ receptors and subsequent phosphorylation of Smad2/3. To examine whether chronic stress affects activation of Smads or not directly apart from downregulating TGF-β1 expression, we treated MSCs with 50 ng/ml TGF-β1, and with 2% N, 2% S, 2% S plus 100 nM of RU486, or 200 nM of corticosterone in addition for 4 days, then detected phosphorylation of Smads in MSCs by western blotting assay. As shown in Figure 6, with the same level of TGF-β1 stimulus, stress could not inhibit phosphorylation of Smad2 and Smad3. Therefore, chronic stress only inhibited production of TGF-β1 without affecting downstream of the TGF-β1 signaling pathway.

Discussion

MSCs, with the presence of cell surface marker CD29, CD44, CD90, Sca1 and absence of hematopoietic antigens CD34, CD45, are a population of heterogenerous tissue stem cells, which were isolated from the bone marrow originally. In addition, they express MHC class I^{low} and do not express MHC class II, which makes them having low immunogenicity. They are characterized by the property of self-renewal, plastic adhesion and fibroblast-like morphology. They have the capacity of differentiating into multiple cell lines, such as adipocytes, osteocytes and chondrocytes, even other cell types besides mesoderm lineages. They have been shown to be isolated from a wide array of tissues, such as bone marrow, fat, skin and can be cultured *in vitro*. Furthermore, MSCs exhibit a strong capacity of immunomodulation and inflammation tropism. Due to their properties above and free of teratoma formation and ethical issues, they have a strong potential in clinical application.

After tissue is injured, the repair process involves two stages: a regenerative phase, injured tissues are replaced by cells of the same type; and a fibrosis phase, connective tissue replaces the normal tissue. When normal cells cannot repair the injury completely, MFs will be activated to repair injury and come to the fibrosis phase.[19,38] Fibrosis contributes to damage reduction and is a result of injury repair. There is an increasing evidence showing that the hepatic fibrosis of early stage is beneficial and reversible if the stimuli are removed and injury is repaired successfully.[39] Activation of MFs will be terminated and MFs will disappear by apoptosis when the tissue is repaired.[40] MFs are generally considered to have key roles in physiological reconstruction of connective tissue and wound healing. α-SMA has been accepted as a solid marker of activation of MFs. From previous reviews, we can conclude that MFs originate from various cell populations, including local tissue fibroblasts, resident hepatic stellate cells, epithelial cells undergoing epithelial–mesenchymal transition and fibroblast-like cells from bone marrow.[21,41–48] However, contribution of MSCs to MFs activation is still unclear even if differentiation of MSCs into MFs has gained increasing attention. In CCl_4-induced mouse liver injury model, MSCs recruited into injury sites have been demonstrated to express α-SMA. However, whether this fate is beneficial or detrimental for liver injury repair remains to be an unresolved problem.[38,49,50]

While in clinical therapy, the efficiency of MSCs therapy is limited and varies among different patients. To explore the problem and optimize the treatment efficiency of MSCs, concerns have been focused on the quality of MSCs, including the cell viability, culture condition of cells, passage of cells and even origins where the cells come from. But there is little attention paid to the patients personally. Indeed, emotion of the patients who accepted MSCs as treatment is equally important. The fate of MSCs in recipients is responsible for the efficiency of treatment. In almost all the patients, chronic stress accompanies them through the disease progression, the negative emotion affects the development and treatment of disease. In our study, we found that chronic stress could repress the therapeutic effect of MSCs in CCl_4-induced liver injury model. While, from this point, self-healing by endogenous MSCs can also be impaired.

Chronic stress-associated corticosterone, which is one type of steroid hormone coming from adrenal gland, has been considered to affect almost every tissue and has an important role in stress-related diseases.[36] There were also evidences showing that glucocorticoid induced by stressors could repress immune responses by diminishing pro-inflammatory cytokines.[3] Interestingly, glucocorticoid has been demonstrated to have the capacity to slow wound healing.[3] They explain the phenomenon as the same cause with immunosuppression. In the present study, we analyzed this problem from another aspect which is a novel standpoint. We found that stress repressed differentiation of MSCs into MFs, which resulted in impaired liver injury repair. More recently, GC has been investigated to interfere in differentiation of MSCs into adipocytes, osteoblasts, myoblasts and chondrocytes. The underlying mechanism of GC participating in the differentiation process remains unknown, apart from bonding with GR. The effects of GC on mature fibroblasts have been usually investigated in skin. Surprisingly, previous results came as the negative effect of GC on fibroblasts proliferation and the ability of synthesizing mature collagen.[51] However, there has been no subtle investigation on the mechanisms of GC affecting MFs differentiation.

In the present study, corticosterone had the same effect with chronic stress on MSCs in reducing liver injury.

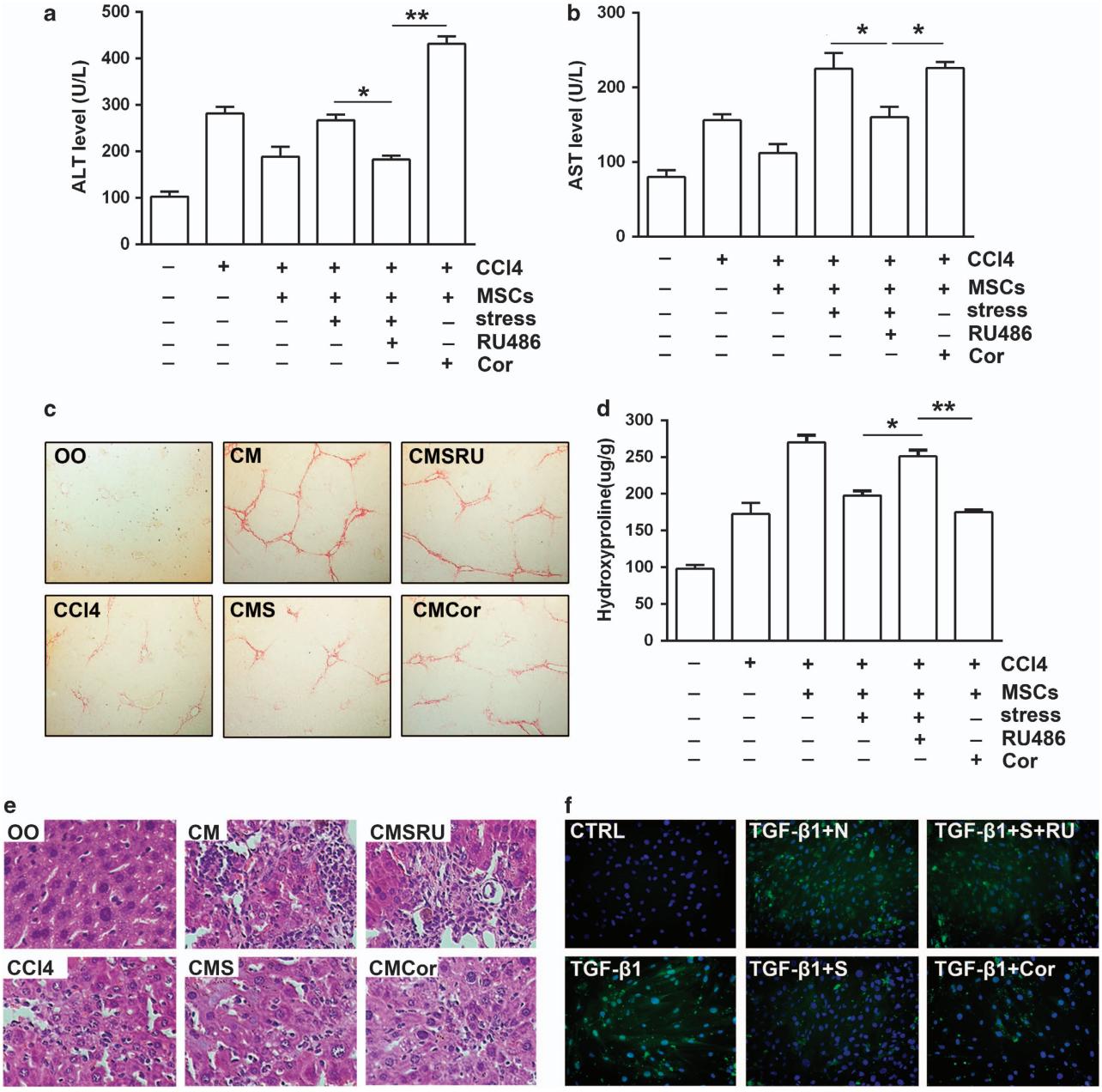

Figure 4 Corticosterone repressed MSCs differentiation into MFs. Mice serum was collected and analyzed for ALT (**a**) and AST (**b**). Sirius Red staining (× 100) (**c**) and liver hydroxyproline analysis (**d**) were done to detect liver fibrosis. Hematoxylin eosin (HE) staining was done to detect liver injury (× 200) (**e**). MSCs were induced to differentiate into MFs *in vitro*. MFs differentiation efficiency of MSCs was determined by cytoimmunofluorescence assay (**f**). 4′,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei (blue). OO, olive oil; CM, CCl_4 + MSCs group; CMS, CCl_4 + MSCs + stress group; CMSRU, CCl_4 + MSCs + stress + RU486 group; CMCor, CCl_4 + MSCs + corticosterone group; N, serum from normal mice; S, serum from stressed mice. RU, RU486; Cor, corticosterone. *$P<0.05$, **$P<0.01$

In addition, RU486 could reverse the effect of chronic stress. These results suggested that chronic stress repressed liver injury repair by MSCs by producing corticosterone. Then, we addressed the problem why chronic stress-associated corticosterone attenuates MFs differentiation of MSCs for the first time. *In vivo* and *in vitro* experiments both showed that corticosterone reduced the production of TGF-β1. Interestingly, immunohistochemistry assay showed that TGF-β1 was expressed in the injury sites where MSCs were recruited. Whereas, after corticosterone or chronic stress was administrated to mice, TGF-β1 expression was reduced. *In vitro* assay showed that corticosterone could block autocrine and intracellular expression of TGF-β1 in MSCs, which is compatible to the *in vivo* assay. Both the *in vivo* and *in vitro* results suggested that chronic stress-reduced expression of TGF-β1 was associated with the reduction of TGF-β1 generation from MSCs. To elucidate whether corticosterone has a negative role in TGF-β1 production through interacting with GR, we used RU486 *in vivo* and *in vitro*; data showed that RU486 could block the function of corticosterone, which

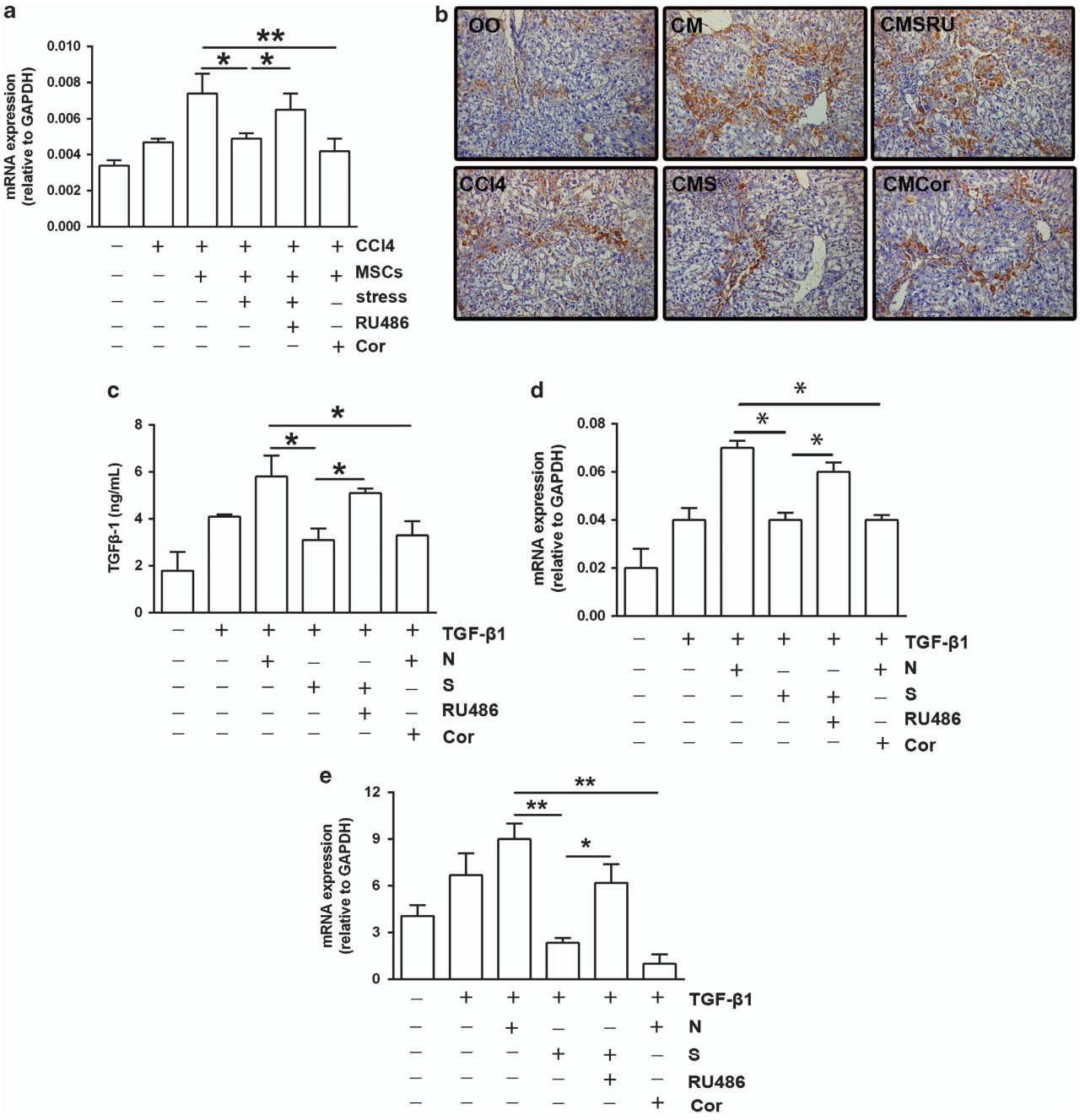

Figure 5 Corticosterone inhibited TGF-β1 expression in livers and MSCs. TGF-β1 expression level in livers was detected by real-time PCR (**a**) and immunohistochemistry (**b**). Conditioned medium from MSCs under different conditions was collected and tested by ELISA (**c**). mRNA expression of TGF-β1 (**d**) and corresponding α-SMA expression in MSCs were tested by real-time PCR (**e**). OO, olive oil; CM, CCl_4 + MSCs group; CMS, CCl_4 + MSCs + stress group; CMSRU, CCl_4 + MSCs + stress + RU486 group; CMCor, CCl_4 + MSCs + corticosterone group; N, serum from normal mice; S, serum from stressed mice. Cor, corticosterone. *$P<0.05$, **$P<0.01$

suggested that corticosterone functioned through the GC/GR pathway.

TGF-β1 is the crucial factor in MFs differentiation[3,33] and it induces α-SMA expression through the TGF-β1/Smads pathway involving phosphorylation of Smad2/3. With the same concentration of TGF-β1 inducing MFs differentiation of MSCs, additional stress serum or corticosterone in the culture medium had no effect on the phosphorylation of Smad2/3 and expression of α-SMA. With the same level of TGF-β1, the bonding of TGF-β1 with its receptors and activation of Smads could not be altered by corticosterone. These results suggested that corticosterone inhibited MFs differentiation by preventing TGF-β1 generation, without inhibiting the activation of downstream of the TGF-β1 pathway directly. And how corticosterone reduces production of TGF-β1 needs further study.

Taken together, our study demonstrated that chronic stress repressed liver injury repair by MSCs by increasing

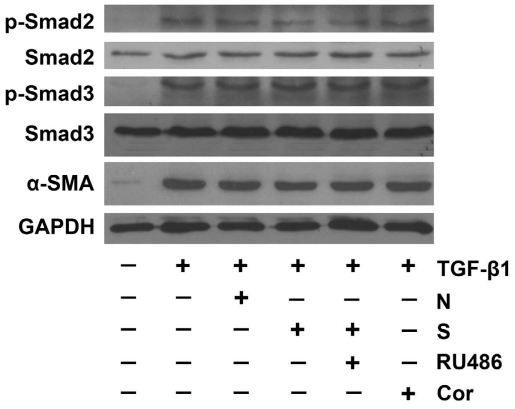

Figure 6 Corticosterone did not affect downstream of the TGF-β1/Smads pathway. Expression of Smad2, p-Smad2, Smad3, p-Smad3, α-SMA was tested in MSCs by western blotting assay. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. N, serum from normal mice; S, serum from stressed mice. Cor, corticosterone

corticosterone in mice, which resulted in inhibition of MFs differentiation from MSCs. TGF-β1 production is one target of corticosterone in inhibiting MFs differentiation without affecting downstream of the TGF-β1/Smads signaling pathway. On the other hand, RU486 may contribute to increasing efficiency of MSCs in liver injury repair in stressful patients. Further research should be done to discover a safe and efficient candidate to ensure the function of MSCs. Importantly, the mental state of patients under treatment needs more attention.

Materials and Methods

Materials. Dulbecco's modified eagle medium (low glucose), fetal bovine serum, glutamax (catalog number: 35050-061), penicillin–streptomycin (catalog number:15140-122), trypsin–EDTA (catalog number: 15400-054) and fibroblast growth factor basic (catalog number: PMG0035) were purchased from Gibco (Grand Island, NY, USA). Hydroxyproline detection kit (catalog number: A030-2) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Recombinant mouse TGF-β1 (catalog number: 7666-MB-005) was purchased from R&D system (Minneapolis, MN, USA). Mouse TGF-β1 ELISA kit was purchased from Shanghai Hengyuan Biotechnology (Shanghai, China). Corticosterone ELISA kit (catalog number: ADI-900-097) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Corticosterone (catalog number: 46148), cortisol (catalog number: C-113), RU486 (catalog number: M8046) were purchased from sigma (St. Louis, MO, USA). Anti-αSMA antibody (catalog number: ab5694) was purchased from Abcam (Cambridge, UK). GFP antibody (catalog number: NB100–1770) was purchased from Novus Biologicals (Littleton, CO, USA). Alexa Fluor 568 goat anti-rabbit IgG(H + L) (catalog: A-11011), Alexa Fluor488 donkey anti-goat IgG(H + L) (catalog number: A-11055) were purchased from Invitrogen (Carlsbad, CA, USA). Smad2/3 antibody (catalog number:5678S), phosphate-smad2/3 rabbit mAb (catalog number: 8828S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-TGF-β1 antibody (catalog number: ab92486) was purchased from Abcam.

Isolation and culture of mouse MSCs. Male EGFP transgenic mice aged 6–8 weeks were used to isolate bone marrow MSCs. MSCs were isolated and cultured as described by Heng Zhu *et al.*[52] Mice were killed by cervical dislocation and the limbs were removed. We flushed bone marrow cells from the medullary cavities from the tibias and femurs with physiological saline by using a 2-ml needle. Derived bone marrow cells were suspended in single-cell suspension and seeded in DMEM medium (low glucose) with 10% fetal bovine serum, 1 $\times$ glutamax, 1 $\times$ penicillin–streptomycin by 1 $\times$ 10^7 cells/ml. Three days later, suspended cells were removed and medium was replaced with fresh medium. Thereafter, medium was replaced every three days. At the seventh day after isolation, attached MSCs were removed by trypsin–EDTA, then resuspended in fresh medium. After cells were passaged for three times, they were taken as purified MSCs and identified by adipocytes and osteoblasts differentiation (see Supplementary Figure 1).[53] In the following culture of MSCs, fibroblast growth factor basic was added in a concentration of 0.5 ng/ml.

Chronic restraint stress procedure. Restraint stress model was based on previous studies.[54–56] Mice were assigned into home cage control or the restraint tubes (50 ml conical tubes containing many holes) to limit their actions for 12 h/day for three consecutive days. They were put into tubes at 2000–2100 h with five mice per group, and released at 0800–0900 h next day. In the tubes, they could not move freely, couldn't get water and food, but they were not pressed and could breath freely.

Mouse model of liver fibrosis and treatment. In order to induce chronic liver injury, male C57BL/6 mice aged 6–8 weeks were feeded with 20% (v/v, dissolved in olive oil) CCl_4 by intragastric method at a concentration of 5.0 ml/kg, twice a week. At the fourth week, EGFP-MSCs (1 $\times$ 10^6 per mouse) or saline was injected through the tail vein. Cell transplantation mice were divided into four groups: control group, without any treatment; stress group, mouse were administrated with stress at the fourth day after MSCs injection and in the following week; stress plus RU486 group, RU486 was administrated subcutaneously at a dosage of 25 mg/kg one day before the beginning of the stress sessions, thereafter RU486 was injected 2 h before stress period; and corticosterone group, corticosterone was injected subcutaneously at a dosage of 5 mg/kg once a day in parallel with the stress treatment. In addition, we had olive oil and stress group as control. Mice were killed at the sixth week after liver injury induction.

Liver injury analysis. Serum of mice was collected by centrifugation at 3000 $\times$ *g*, 4 °C for 10 min with anticoagulants. The ALT and AST were analyzed with a biochemical analyzer.

Hematoxylin and eosin staining. Mouse liver samples were obtained after killing and fixed in 4% paraformaldehyde, and then embedded in paraffin. In total, 5-μm-thick sections were prepared for the experiments. Mouse liver sections embedded in paraffin were stained with hematoxylin and eosin according to the manufacturer's protocol.

Liver fibrosis analysis. To detect liver fibrosis, the paraffin sections were stained with Sirius Red. To detect liver fibrosis quantitatively, fresh liver tissues were tested for hydroxyproline using hydroxyproline detection kit.

Immunohistochemistry and immunofluorescence staining. Immunohistochemistry assay was performed according to method described before,[33] anti-α-SMA antibody, anti-GFP antibody, anti-TGF-β1 antibody were used. All of these antibodies were used at a dilution of 1/100. Immunofluorescence and horseradish peroxidase-conjugated secondary antibodies were used correspondingly.

Real-time PCR. Total RNA from cultured cells or freezed liver tissues was extracted by trizol method. In total, 1 μg RNA in 10 μl for all reagents was transcripted to cDNA using bestar qPCR RT kit. The real-time PCR was performed using bestar real-time PCR master mix with an ABI Prism 7300 system (Grand Island, NY, USA). The primers of mouse genes for real-time PCR were as follows: α-SMA, sense, 5′-ATGCTCCCAGGGCTGTTTT-3′, and antisense, 5′-TTCCAACC ATTACTCCCTGATGT-3′; TGF-β1, sense, 5′-CTCCCGTGGCTTCTAGTGC-3′, and antisense, 5′-GCCTTAGTTTGGACAGGATCTG-3′; glyceraldehyde-3-phosphate dehydrogenase, sense, 5′-AGGTCGGTGTGAACGGATTTG-3′; and antisense, 5′-TGTAGACCATGTAGTTGAGGTCA-3′. The primers of human genes for real-time PCR were as follows: TGF-β1, sense, 5′-CGAGCCTGAGGCCGACTAC-3′; and antisense, 5′-AGATTTCGTTGTGGGTTTCCA-3′; glyceraldehyde-3-phosphate dehydrogenase, sense, 5′-CCAGGAAATGAGCTTGAGAAAGT-3′; and antisense, 5′-CCCACTCCTCCACCTTTGAC-3′.

ELISA. Serum from mice of control, restraint stress, CCl_4-treated and CCl_4 plus stress-treated mice was assayed for corticosterone with a corticosterone ELISA kit according to the manufacturer's recommendation. MSCs were treated by corresponding reagents for 12 h, then medium was replaced by fresh

serum-free culture medium and cells were cultured for another 24 h, then supernatant was collected as conditioned medium and tested for TGF-β1 by a TGF-β1 ELISA kit.

Western blotting assay. MSCs treated with appropriate conditions were lysed in lysis buffer. The lysates were qualified using BCA kit and boiled with loading buffer for 10 min. Thereafter, denatured protein was segregated by SDS-polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membrane. The membranes were blocked by 5% nonfat milk, and then immunoblotted by various primary monoclonal antibodies. The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and ECL were used to examine the expression of protein. Glyceraldehyde-3-phosphate dehydrogenase was used as internal conference. Expression of target proteins was normalized to glyceraldehyde-3-phosphate dehydrogenase before comparing among different groups.

Statistical analyses. Results were presented as means $\pm$ S.E.M. and the statistical differences were analyzed by Student's *t*-test, $n=3$ ($P<0.05$ were considered significant).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This project was supported by Key Basic Research Project of China (Grant No. 2012CBA01303, 2011CB966200, 2010CB945600, 2011CB965100); Key project of National Natural Science Foundation of China (Grant no 81030041); National Natural Science Foundation of China (Grant No. 31171321, 81101622, 81372330, 81101830); Special Funds for National key Sci-Tech Special Project of China (Grant No. 2012ZX10002-016, 2012ZX10002011-011); Shanghai Science and Technology Committee (Grant No. 11ZR1449500, 12ZR1439800); Shanghai Municipal Health Bureau (Grant No. 20114004) and Science Fund for Creative Research Groups, NSFC, China (Grant No. 81221061).

Author contributions

XY, Z-PH and S-SZ participated in the design and performance of the study. XY carried out cell culture, molecular studies and analyzed the data. XY and P-XZ did the mouse experiments. YY made the tissue sections. L-XW conceived this study and participated in its design and coordination. The manuscript was drafted by XY, revised by S-SZ and reviewed by all authors. All authors approved the final version of the manuscript to be published.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)