Human Umbilical Cord Mesenchymal Stem Cells Alleviate Myocardial Endothelial-Mesenchymal Transition in a Rat Dilated Cardiomyopathy Model

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ABSTRACT

Background. Human umbilical cord–derived mesenchymal stem cells (HuMSCs) have been shown to suppress cardiac fibrosis; however, the underlying mechanisms are not fully understood. Recent studies have shown that endothelial-mesenchymal transition (EndMT) plays a crucial part in myocardial fibrosis. In the present study, we investigated the suppressive role of HuMSCs in cardiac fibrosis and related mechanisms in a rat dilated cardiomyopathy (DCM) model.

Methods. Male Lewis rats were randomly divided into 3 groups. Rats without any treatment served as a negative control group, while the DCM rats, which were generated by immunization with porcine myosin, were divided into 2 groups: a HuMSC group, in which HuMSCs (1×10^6 cells/rat) were injected intravenously, and a vehicle group, in which rats were injected with volume-matched solution containing no HuMSCs. Histologic and immunofluorescent measurements were used to evaluate the effects of HuMSCs on cardiac fibrosis and EndMT.

Results. We observed a significant increase in myocardial fibrosis, and elevated EndMT in rats of the vehicle group were observed compared with those in the negative control group along with the increased activity of transforming growth factor (TGF)-β1/extracellular signal-regulated kinase (ERK) 1/2 signaling. Treatment with HuMSCs repressed the increase in myocardial fibrosis and EndMT observed in DCM rats, which correlated with decreased activity of TGF-β1/ERK1/2 signaling.

Conclusion. The HuMSCs attenuated cardiac fibrosis at least partly through the inhibition of TGF-β/ERK-induced EndMT.

CARDIAC fibrosis, a pathologic change present in a variety of cardiac diseases including hypertrophic cardiomyopathy, myocardial infarction, and postviral dilated cardiomyopathy [1], is caused by the excessive deposition of extracellular matrix proteins, typically collagen, in the myocardium, leading to impaired cardiac function and eventually heart failure [2]. Currently, there are no effective therapies for cardiac fibrosis, although the mechanisms leading to cardiac fibrosis have been studied extensively.

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While it is widely accepted that multifactorial mechanisms underpin the pathogenesis of cardiac fibrosis, there is a general belief that myofibroblasts significantly contribute to tissue fibrosis [3,4] and that the activation of resident fibroblasts [5], infiltration of circulating bone marrow-derived fibrocytes [6], and epithelial to mesenchymal transition [7] are all involved in the generation of tissue fibroblasts. Another potential source of fibroblasts in the local tissues is from an endothelial-mesenchymal transition (EndMT) [8,9]. During EndMT, endothelial cells lose their characteristic markers, such as vascular endothelial cadherin and CD31, and acquire fibroblast markers such as α–smooth muscle actin, fibroblast-specific protein (FSP) 1, and vimentin, indicating that these endothelial cells are converted into activated fibroblasts, which break down basement membranes and migrate into interstitial tissues [10]. Therefore, inhibition of EndMT is a potential target to treat cardiac fibrosis and is currently under intensive investigation.

Transplantation of mesenchymal stem cells (MSCs) is a new and promising treatment for cardiac fibrosis [11]. The MSCs are broadly distributed throughout the body, such as in adipose tissue, peripheral blood, bone marrow, placenta, and umbilical cord [12]. The MSCs most commonly used in clinical trials to date originated from umbilical cord, adipose tissue, and bone marrow [13]. Among these, human umbilical cord MSCs (HuMSCs) are more appropriate for clinical applications because there are fewer ethical issues involved, and HuMSCs possess high self-renewal ability and low immunogenicity [14,15]. Our research team and others previously reported the reparative potential of HuMSCs for fibrosis in different organs including the heart, liver, pancreas, and kidney [16–19]. However, whether HuMSCs have any effect on EndMT has not been explored. In the present study, we measured the effect of HuMSCs on EndMT in a rat DCM model and probed molecular signaling pathways associated with EndMT.

MATERIALS AND METHODS

Animals and Experimental Protocols

Animals. Male Lewis rats (8 weeks old; weight, 180–200 g, n = 24) were purchased from Vital River Laboratories (Beijing, China) and housed in the animal facility at Shantou University Medical College (Shantou, China) under conditions of humidity (70%) and constant temperature (25°C) with a 12-hour light-dark shift, and the animal protocol used in this study was reviewed and approved by the Animal Care and Use Committee of the Shantou University Medical College.

Human Umbilical Cord–derived Mesenchymal Stem Cells Culture. The culture of HuMSCs was approved by the Institutional Review Board of Second Affiliated Hospital of Shantou University Medical College and was performed as detailed previously [20]. In short, human umbilical cords were obtained from full-term pregnant women who had a cesarean delivery, washed with phosphate-buffered saline (PBS), and cut into small fragments. Afterward, the veins and arteries were removed, and the Wharton's jelly was cut into small pieces and transferred to 75-cm^2 flasks that contained Dulbecco's Modified Eagle's Medium/F-12 media (Sigma-Aldrich; EMD Millipore, Billerica, Mass, United States) supplemented with 5 ng/mL basic fibroblast growth factor (Sigma-Aldrich; EMD Millipore), 1 g/mL amphotericin B (Gilead Sciences Inc, San Dimas, Calif, United States), 5 ng/mL epidermal growth factor (Invitrogen; Thermo Fisher Scientific Inc, Waltham, Mass, United States), 10% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc), and 100 μg/mL penicillin/streptomycin (Shanghai Bioscience, Shanghai, China). The HuMSCs were incubated at 37°C in 5% CO_2 for 5 to 7 days for cells to migrate from the explants. We used HuMSCs at third passage for all experiments performed in this study.

Generation of Rat DCM Model and HuMSCs Transplant. The DCM was generated by injection of Lewis rats with an antigen-adjuvant emulsion in the footpads according to a previously detailed procedure [18]. Briefly, porcine cardiac myosin (Sigma-Aldrich; EMD Millipore) was dissolved in PBS and emulsified with complete Freund's adjuvant with 10 mg/mL *Mycobacterium tuberculosis* (Sigma-Aldrich; EMD Millipore). Rats were subcutaneously administered 0.2 mL emulsion per rat on the rear footpads on day 0. On day 28, DCM rats (n = 16) were randomly separated into 2 groups: a vehicle group, in which DCM rats (n = 8) received 0.2 mL PBS only, and an HuMSC group, in which DCM rats (n = 8) received 0.2 mL HuMSCs (1×10^6 cells/rat), respectively. The PBS or HuMSCs were injected intravenously via the tail vein. A negative control group contained age-matched Lewis rats without immunization (n = 8).

Morphologic Study. Standard histologic protocols were used for humane animal killing, heart collection, paraffin embedding, and sectioning followed by Masson's trichrome staining. The degree of fibrosis of each sample was scored under a high-power light microscope. The area of myocardial fibrosis (blue) in the left ventricle was calculated using a color image analyzer (Mac Scope; Mitani Co, Fukui, Japan). Collagen volume fraction (CVF) represents the level of cardiac fibrosis and was calculated using the following formula: CVF = (area of the collagen/area of field of vision) × 100. The average CVF was calculated from 10 randomly selected fields per sample.

Immunofluorescence Staining. Heart tissues were fixed in 4% polyoxymethylene at room temperature and sectioned. After 10 minutes × 3 washes in PBS, slides were incubated with the primary antibodies, rabbit anti-FSP1 (Abcam, Cambridge, United Kingdom) and rabbit anti-CD31 (Abcam), at 4°C overnight. Slides were then incubated with the secondary antibodies for 1 hour at room temperature. The secondary antibodies are goat antirabbit, Alexa Fluor 488 (Beyotime, Shanghai, China), and goat antirabbit, Cy3 (Beyotime). Nuclei were counterstained with DAPI (Beyotime). Two investigators analyzed the staining using Olympus BX51 confocal microscopy (Olympus, Tokyo, Japan) independently. Ten visual fields per heart were randomly selected and analyzed for coexpression of CD31 and FSP1.

Western Blot. The protein levels of CD31, collagen III, FSP1, transforming growth factor (TGF)-β1, and total and phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2 and p-ERK1/2) were assessed by Western blot, according to the previously described protocol [18]. In brief, total protein (30 μg) was resolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were incubated with the following primary antibodies prepared in 5% nonfat dry milk in Tris-buffered saline at 4°C overnight: antibodies against glyceraldehyde 3-phosphate dehydrogenase (catalog no. KC-5G5, 1:10,000) and CD31 (catalog no. AF3628, 1:1000) were purchased from R&D Systems, Inc (Minn, United States). Antibodies against FSP1 (catalog no. ab197896, 1:1,000) and collagen III (catalog no.

ab7778, 1:5,000) were obtained from Abcam (Cambridge, United Kingdom). Antibodies against ERK1/2 (catalog no. 9258, 1:1,000) and p-ERK1/2 (catalog no. 4668, 1:2,000) were obtained from Cell Signaling Technology, Inc (Danvers, Mass, United States). Anti-TGF-β1 antibody (catalog no. 146, 1:1000) was obtained from Santa Cruz Biotechnology, Inc (Dallas, Tex, Untied States). Then the membranes were washed and reprobed with the appropriate second antibody. The specific protein bands were visualized with chemiluminescence (EMD Millipore).

Statistical Analysis

Data are presented as mean (SD). One-way analysis of variance and the Tukey's multiple comparison test were used to assess the statistical difference between groups. A P value less than .05 was considered statistically significant.

RESULTS

Human Umbilical Cord–Derived Mesenchymal Stem Cells Treatment Significantly Reduced Cardiac Fibrosis in DCM Rats

We first examined whether HuMSCs could reduce cardiac fibrosis in DCM rats. As shown in Figure 1A and B, the area of cardiac fibrosis of the vehicle group was significantly increased compared with the negative control group, and this increase was greatly attenuated by HuMSCs treatment. ($^{*}P < .01$ vs negative control; $^{\dagger}P < .01$ vs vehicle).

Human Umbilical Cord–derived Mesenchymal Stem Cells Treatment Significantly Reduced EndMT in the Hearts of DCM Rats

We next studied endothelial cells undergoing phenotypic transition into mesenchymal cells in the myocardium of rats of these 3 groups. We used immunofluorescence staining to detect the expression of CD31, the marker of endothelial cell (green), and FSP1, the marker of fibroblast (red). Cells that coexpress both of these 2 markers are indicative of cells that have undergone EndMT. As shown in Figure 2, we observed an increase in the number of CD31+/FSP+ cells in the vehicle group compared with the negative control group, but this increase was suppressed in the HuMSCs treatment group, suggesting that DCM rat hearts exhibited elevated EndMT and that HuMSCs were able to repress it.

Human Umbilical Cord–Derived Mesenchymal Stem Cells Treatment Altered the Expression of Genes Related to Cardiac Fibrosis and EndMT

Next, we evaluated the expression of FSP1, collagen III, CD31, TGF-β1, ERK1/2, and p-ERK1/2 protein by Western blot, among which collagen III was one of the major components of ECM [21], and TGF-β1, ERK1/2 and pERK1/2 were activators of EndMT. As shown in Figure 3, levels of cardiac FSP1, collagen III, TGF-β1, and p-ERK1/2 protein were significantly upregulated in DCM rats of the vehicle group compared with the negative control group, and their expression was mitigated by HuMSCs treatment. On the contrary, the expression level of cardiac CD31 was significantly downregulated in the vehicle group compared with the negative control group but was upregulated by HuMSCs treatment ($^{*}P < .01$ vs negative control; $^{\dagger}P < .05$ vs vehicle; $^{\ddagger}P < .01$ vs vehicle).

DISCUSSION

Cardiac fibrosis promotes stiffness and undermines compliance of the heart tissue, thus impairing cardiac contraction and relaxation and leading to heart failure [2,22]. Though the etiology of cardiac fibrosis is multifactorial, one of the common characteristics of cardiac fibrosis is a large amount of activated fibroblasts or myofibroblasts in the affected tissues [10]. Nevertheless, the origin of those activated fibroblasts is not completely known. A recent study suggested that EndMT, which was activated in cardiac injury, was the key process that transforms endothelial cells to fibroblasts in heart tissues [23]. Hence, targeting EndMT represents a potentially novel therapeutic approach to suppress activation of fibrosis and treat cardiac fibrosis.

During EndMT, endothelial cells lose their markers including CD31 and vascular endothelial cadherin, acquire the mesenchymal markers such as vimentin, FSP1, and α–smooth muscle actin, and assume the function and morphology of activated fibroblasts [10,24]. The EndMT plays a critical role in cardiac structural formation during embryogenesis [25] and significantly contributes to cardiac fibrosis, as supported by recent findings that up to 35% of fibroblasts are of endothelial origin [26]. Recent studies support the notion that endothelial cells are able to undergo

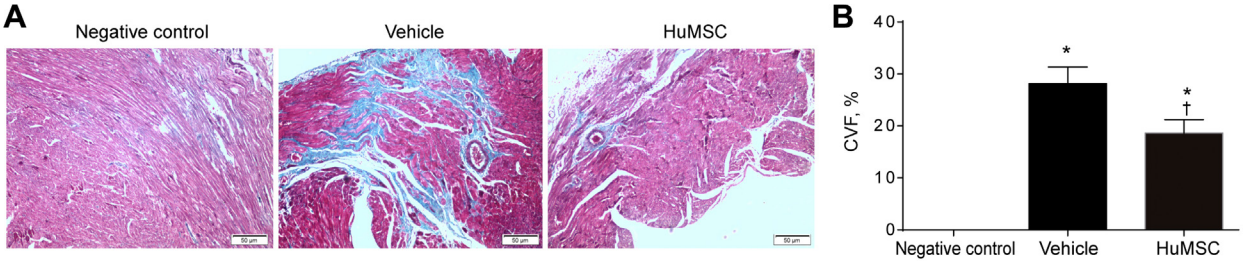

Fig 1. HuMSC treatment significantly reduced cardiac fibrosis in DCM rats. **(A)** Representative images of Masson's trichrome staining showing cardia fibrosis of rats from negative control group, vehicle group, and HuMSC group (magnification: 100 ×) **(B)** Statistical analysis of (A). $^{*}P < .01$ vs negative control group, $^{\dagger}P < .01$ vs vehicle group. n = 8/group. CVF, collagen volume fraction; DCM, dilated cardiomyopathy; HuMSC, human umbilical cord–derived mesenchymal stem cells.

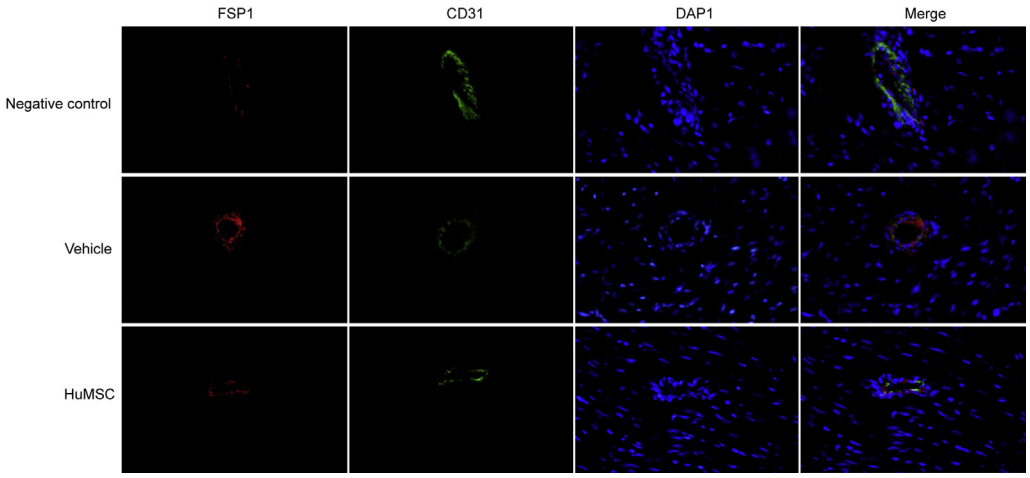

Fig 2. HuMSC treatment significantly reduced EndMT in the hearts of DCM rats. Representative images of confocal microscopy showing immunofluorescence staining for CD31 (green), FSP1 (red), and DAPI (blue) in the left ventricles of rats from negative control group, vehicle group, and HuMSC group (magnification: 40 ×). DCM, dilated cardiomyopathy; EndMT, endothelial-mesenchymal transition; FSP, fibroblast-specific protein; HuMSC, human umbilical cord–derived mesenchymal stem cells.

EndMT, which we hypothesize makes up an essential source of the mesenchymal cells involved in the cardiac fibrosis process [19,27]. In the present study, we found that the vast majority of CD31 and FSP1 protein was expressed in separate cardiac cells in rats of the negative control group, indicating low EndMT. As we had hypothesized, coexpression of CD31 and FSP1 was observed in the left ventricles of DCM rats from the vehicle group, indicating increased EndMT in DCM rat hearts. The HuMSC treatment significantly reduced the number of cells double positive for CD31 and FSP1. These data suggest that treatment with HuMSCs may inhibit EndMT in the rat DCM. Given that HuMSC also reduced cardiac fibrosis and that EndMT significantly contributed to the pathogenesis of cardiac fibrosis, we reasoned that HuMSCs suppressed cardiac fibrosis at least in part through repressing EndMT. Further

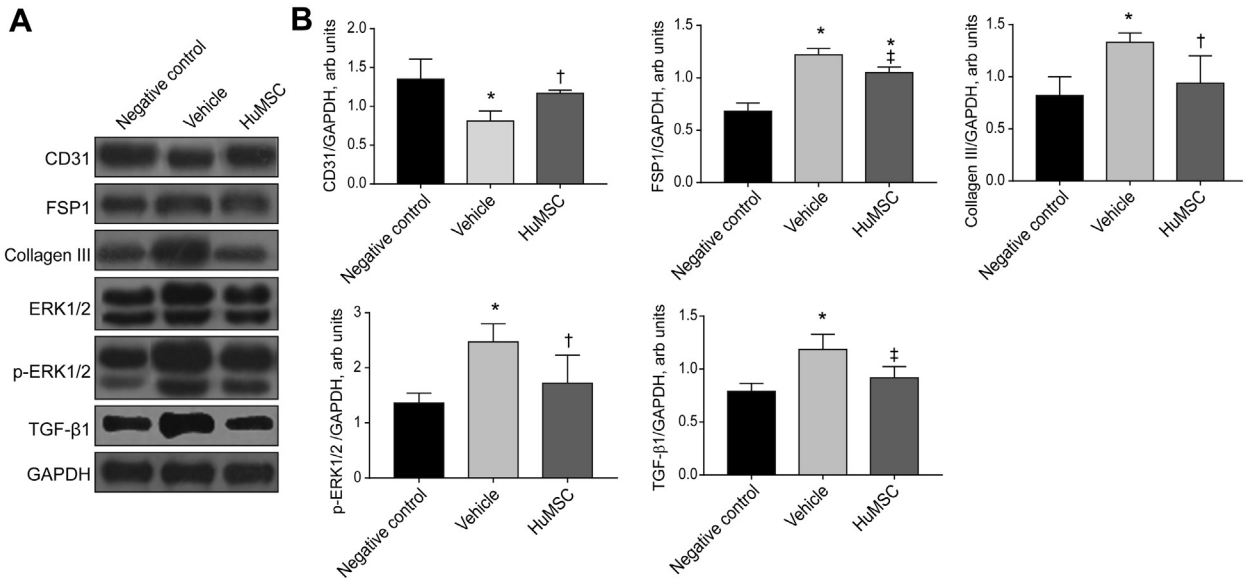

Fig 3. HuMSC treatment altered the expression of genes related to cardiac fibrosis and EndMT. **(A)** The expression of FSP1, collagen III, CD31, TGF-β1, ERK1/2, and p-ERK1/2 in the left ventricles of rats from negative control group, vehicle group, and HuMSC group was evaluated by Western blot. GAPDH serves as an internal control. **(B)** Statistical analysis of (A). $^{*}P < .01$ vs negative control group; $^{\dagger}P < .05$ vs vehicle group; $^{\ddagger}P < .01$ vs vehicle. n = 8/group. ERK, extracellular-signal regulated kinase; p-ERK, phosphorylated-ERK; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HuMSC, human umbilical cord–derived mesenchymal stem cells; TGF, transforming growth factor.

experiments are necessary, however, to determine if inhibition of EndMT is required for the protective actions of HuMSCs on cardiac fibrosis.

Diverse bioactive molecules and/or signaling, including TGF-β, mediate the EndMT process. Goumans et al [28] showed that a significant fraction of interstitial fibroblasts in cardiac fibrosis were derived from the endothelium through a TGF-β–dependent EndMT, indicating that TGF-β–dependent EndMT was the major contributor to the production of fibrotic tissue. This group proposed that EndMT served as a profibrotic switch in fibrotic disorders including cardiac fibrosis. Zhang et al [29] showed that menstrual blood–derived mesenchymal cells ameliorated myocardial fibrosis in myocardial infarction rats at least partially through the inhibition of TGF-β/Smad–induced EndMT. Additionally, Jiang et al [30] found that the inhibition of the ERK and Akt signaling–induced EndMT partly mediated the protective effect of evodiamine, a bioactive compound purified from the *Tetradium* genus of plants [31], on cardiac fibrosis induced by isoproterenol. Consistent with the above observations, we found that HuMSCs treatment mitigated the increase in TGF-β1/ERK1/2 signaling pathways in DCM rats, which was coincident with the decrease in the area of cardiac fibrosis and EndMT. Thus, we speculate that the protective effect of HuMSCs on DCM rats is, at least partially, through the suppression of TGF-β/ERK–induced EndMT. Whether beneficial effects of HuMSCs involve any other molecules and/or signaling merits further investigation.

In conclusion, we found that HuMSCs attenuate cardiac fibrosis in the DCM rats induced by immunization with porcine myosin, and these changes co-occur with markers of EndMT and TGFβ/ERK signaling.

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