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Comparing the Biological Characteristics of Adipose Tissue-Derived Stem Cells of Different Persons

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ABSTRACT
Scientists have found that cell sex is a variable that considerably influences the regeneration abilities of muscle-derived stem cells' in mice. We try to find out whether the cell sex or cell age (the age of donor) will influence the biological characteristics of human adipose tissue-derived stem cells (H-ADSCs). The results indicate that cell sex influences the proliferation, differentiation, paracrine, and anti-apoptosis abilities of the H-ADSCs, and cell age may also affect the H-ADSCs' differentiation and anti-apoptosis abilities. J. Cell. Biochem. 113: 2020–2026, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: ADIPOSE TISSUE-DERIVED STEM CELLS; DIFFERENTIATION; PARACRINE; ANTI-APOPTOSIS

Stem cells have attracted more and more attention in treating ischemic heart disease in the past decade, due to their potential in repairing damaged cardiac tissue. Researchers have isolated a population of multilineage cells from human adipose tissue in 2001 [Zuk et al., 2001]. After that adipose tissue-derived stem cells (ADSCs) have been gradually accepted as a seed cell for cytological engineering, as they are free of ethical, oncological, and immunological concerns, which present in pluripotent cells (embryonic stem cells or induced pluripotent stem cells). Obtaining ADSCs lacks the painful and time-consuming process which is associated with cells from other sources such as skeletal muscle or bone marrow and evidence suggests that multiple mechanisms could be involved in the functions of stem cells in treating myocardial infarction (MI), including: differentiation, paracrine secretion, angiogenesis, cell fusion, passive mechanical effects, and influence of endogenous cardiac stem cells [Wei et al., 2009]. Scientists have found that cell sex is a variable that considerably influences the regeneration abilities of muscle-derived stem cells (MDSCs) and the female MDSCs regenerate skeletal muscle more efficiently in mice [Deasy et al., 2007]. In this study, we try to find out whether the cell sex or cell age will influence the human ADSCs (H-ADSCs)' biological characteristics, including proliferation, differentiation, paracrine, and anti-apoptosis abilities.

MATERIALS AND METHODS

ADIPOSE TISSUE ACQUIREMENT AND PATIENT SELETION

Human subcutaneous adipose tissue samples were obtained from abdominal operations. The Institutional Review Board of Chinese PLA General Hospital approved the study, and the patients gave written consent before the procedure. We acquired about 2.5 ± 1 g visceral adipose tissue from per patient, and examined H-ADSCs from 30 people (15 female and 15 male) in terms of two variables: sex, age.

Patients who were excluded were those with: chronic disease of respiratory system, digestive system, urinary system, or endocrine system; disease of hematological system, disease of connective tissue, rheumatism, tumor, or other genetic disease.

CELL ISOLATION AND CULTURE

The connective tissue and small blood vessels were rejected from the adipose tissue with ophthalmology scissors, and the adipose tissue was cut into small pieces of about 1 mm^2. The fragments were digested with 0.25% trypsogen (Amresco) at 37°C for 10 min, then the adipose tissue in the upper layer was left and the digestive juice in the lower layer was removed (to remove the erythrocyte). Then the adipose tissue was digested in 0.1% collagenase type I (Invitrogen) under gentle agitation at 37°C for 40 min. The liquid filled with

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TABLE I. Lineage-Specific Induction Factors and Identification Method

Direction	FBS (ml/L)	Other composition	Identification
To fat	100	10 μmol/L methylprednisolone; 10 umol/L insulin; 200 μmol/L antinfan; 0.5 mmol/L IBMX	Oil red O staining
To bone	100	0.1 μmol/L methylprednisolone; 50 μmol/L ascorbic acid-sodium phosphate; 10 μmol/L β-glycerophosphate	Alkaline phosphatase staining
To myocardium	100	10 μmol/L 5-azacytidine	TNT FCM

ADSCs was centrifuged at 1,200/rpm for 6 min, then the supernatant removed, and the precipitated cells were then inoculated into the culture flask with bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM, Gibco), which was supplemented with 10% fetal bovine serum (Gibco). The Cells were cultured at 37°C and 5% CO_2. The first medium exchange was carried out 24 h later to remove cells without adherence, and then the medium was exchanged every 2 days. When occupying about 80% of the culture flask, the cells were passaged.

IMMUNOHISTOCHEMISTRY STAIN

The third passage cells cultured on cover slips were fixed with 4% paraformaldehyde for 60 min, then followed by 30 min in 3% H_2O_2, after that the cover slips were covered with normal goat serum at 37°C for 20 min. Then, we added rabbit anti-human CD13, 34, 44, 45, HLA-DR, VWF multiclone antibodies (1:200, Sigma) on the cover slips and left the cover slips in a wet box 4°C overnight. After washing them with distilled water the next morning, the cover slip were stained with biotin at 37°C for 60 min, then we added HRP to mark the bio-epiderm antibodies at 37°C for 60 min, and then used the DAB stain for coloration at last.

MTT ASSAY

The cells' proliferation ability was assessed via the MTT assay, as the more viable cells the more metabolic dye MTT turned to a blue formazan product. H-ADSCs at passage 3 were seeded at a density of 3,000 cells per well in 96-well plates and the medium was exchanged every 2 days. For each 24 h, 20 μl of a stock (MTT solution 5 mg/ml in PBS) were added to related wells, and the incubation was then continued for 4 h. The medium was aspirated, and the cells were treated with DMSO (150 μl per well). Levels of MTT were determined by measuring differences in absorbance at 492 nm, and we measured each cell population for 11 days.

MULTILINEAGE DIFFERENTIATION ASSAY

The third passage cells were induced by lineage-specific induction factors to bone, fat, and myocardium [Zammit and Beauchamp, 2001] (Table I). The cells which were induced to bone or fat were determined with staining methods of alkaline phosphatase or Oil red O.

The third passage H-ADSCs, which had been adherent for 24 h after passage, were treated with 10 μmol/L 5-azacytidine and incubated for 24 h. Later, the medium was replaced back with normal medium [Planat-Bénard et al., 2004]. After culturing for 4 weeks, the cells' expression of TNT was detected by flow cytometry (FCM) and the data was transported to an Apple G3 computer, and analyzed by CellQuest software (Becton Dickinson company).

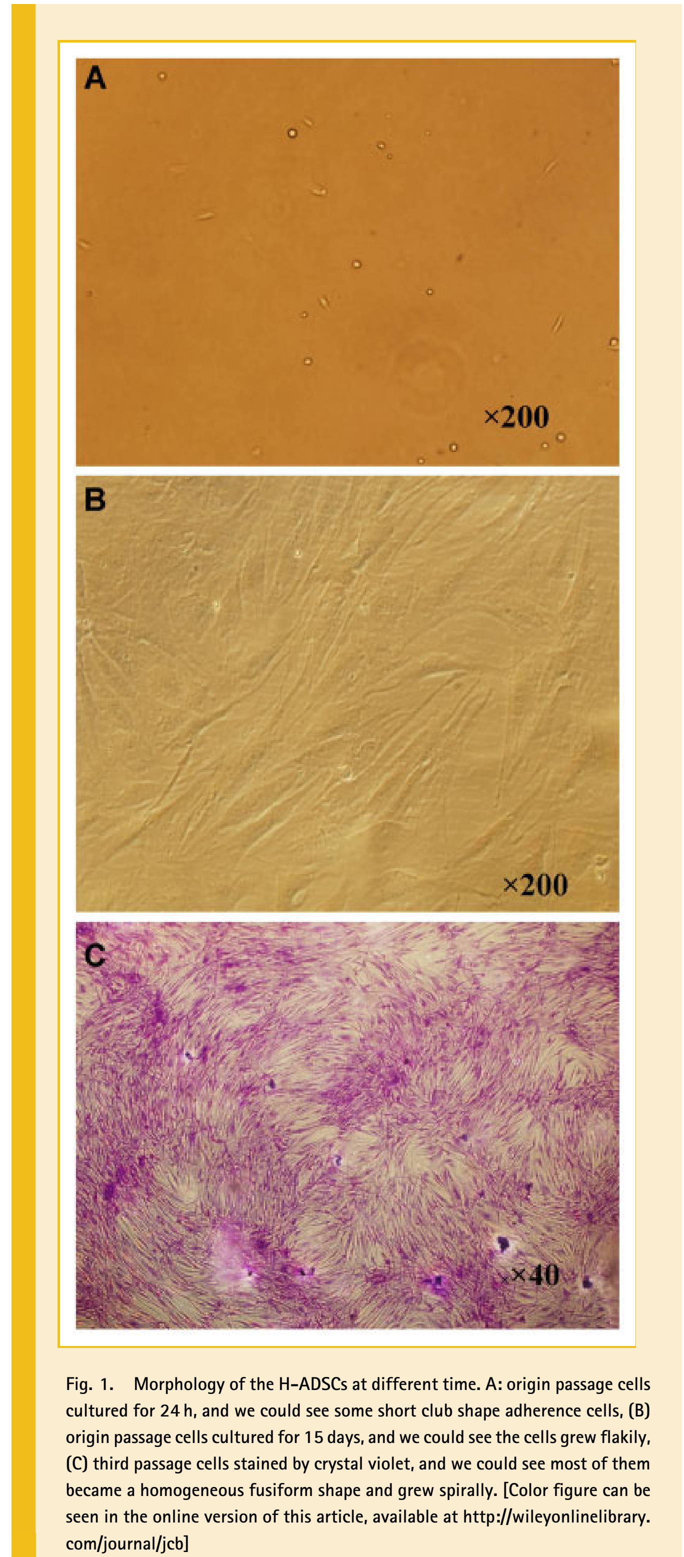

Fig. 1. Morphology of the H-ADSCs at different time. A: origin passage cells cultured for 24 h, and we could see some short club shape adherence cells, (B) origin passage cells cultured for 15 days, and we could see the cells grew flakily, (C) third passage cells stained by crystal violet, and we could see most of them became a homogeneous fusiform shape and grew spirally. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

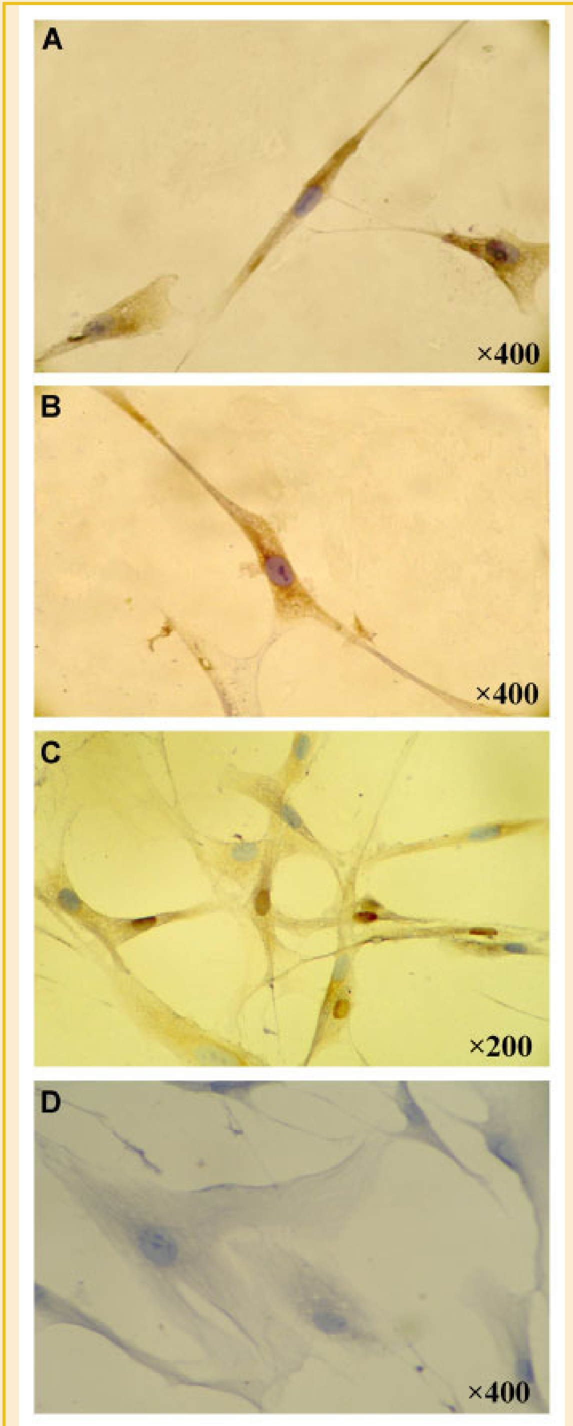

Fig. 2. The surface antigens of H-ADSCs were identified by Immunohistochemistry stain with different antibodies. A: The third passage cells were immunohistochemistry stained by CD 13 and the nucleus was contrast stained by hematine. The hyalomitome were positive, (B) The third passage cells were immunohistochemistry stained by CD 44 and the nucleus was contrast stained by hematine. The hyalomitome were positive, (C) The third passage cells were immunohistochemistry stained by CD 13 and the nucleus was contrast stained by hematine. The hyalomitome and part of the nucleus were positive, (D) The third passage cells of negative immunohistochemistry stain. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

DETECTION OF THE SECRETION OF HGF AND VEGF

The H-ADSCs at passage 4 were seeded at a density of 50,000 cells per liter medium in six-well plates. We collected supernatant of the cells after cultured for 72 h, then kept the supernatant in −80°C freezer, while the number of cells was determined by a hemacytometer. The secretion abilities of cells in HGF and VEGF were gauged by ELISA method.

INDUCTION OF THE H-ADSCs TO APOPTOSIS WITH H_2O_2

The H-ADSCs at passage 3 were plated at a density of 700 cells/cm^2. After culturing for 72 h, the cells were incubated with 500 μmol/L H_2O_2 for 30 min to stimulate oxidative stress injury. Then the adherent cells were collected, added with Annexin V-FITC and PI, and deposited for 15 min away from light. The apoptosis and necrosis degrees of the cells were tested by FCM of which were: Normal cells (An− PI−), cells in early apoptosis stage (An+ PI−), injured cells. An− PI+), cells in late apoptosis stage and necrosis (An+ PI+).

STATISTICAL ANALYSIS

Data was presented as mean ± SE and statistically significant difference was defined as P-value < 0.05. Categorical variables were compared by paired t-test or linear correlation analysis and statistical analysis was performed using SPSS, version 12.0.

RESULTS

MORPHOLOGY FEATURE OF H-ADSCs

The newly isolated H-ADSCs were round, and we could see some short club shape adherence cells after 24 h (Fig. 1A). The cells grew flakily after culturing for about 2 weeks and needed passage (Fig. 1B). When the cells came to the third generation, most of them became a homogeneous fusiform shape and grew spirally (Fig. 1C).

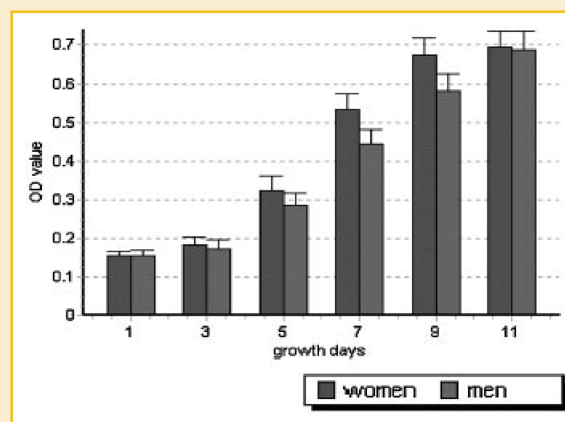

Fig. 3. We used the Mtt assay to compare the growth curve of different stem cells and the F-ADSCs had a higher OD values than the M-ADSCs in the 5 (0.325 ± 0.037 vs. 0.284 ± 0.031, P < 0.01), 7(0.532 ± 0.042 vs. 0.443 ± 0.038, P < 0.01), and 9 (0.674 ± 0.046 vs. 0.582 ± 0.043, P < 0.01) growth days, which indicated that the F-ADSCs might have better proliferation ability than M-ADSCs.

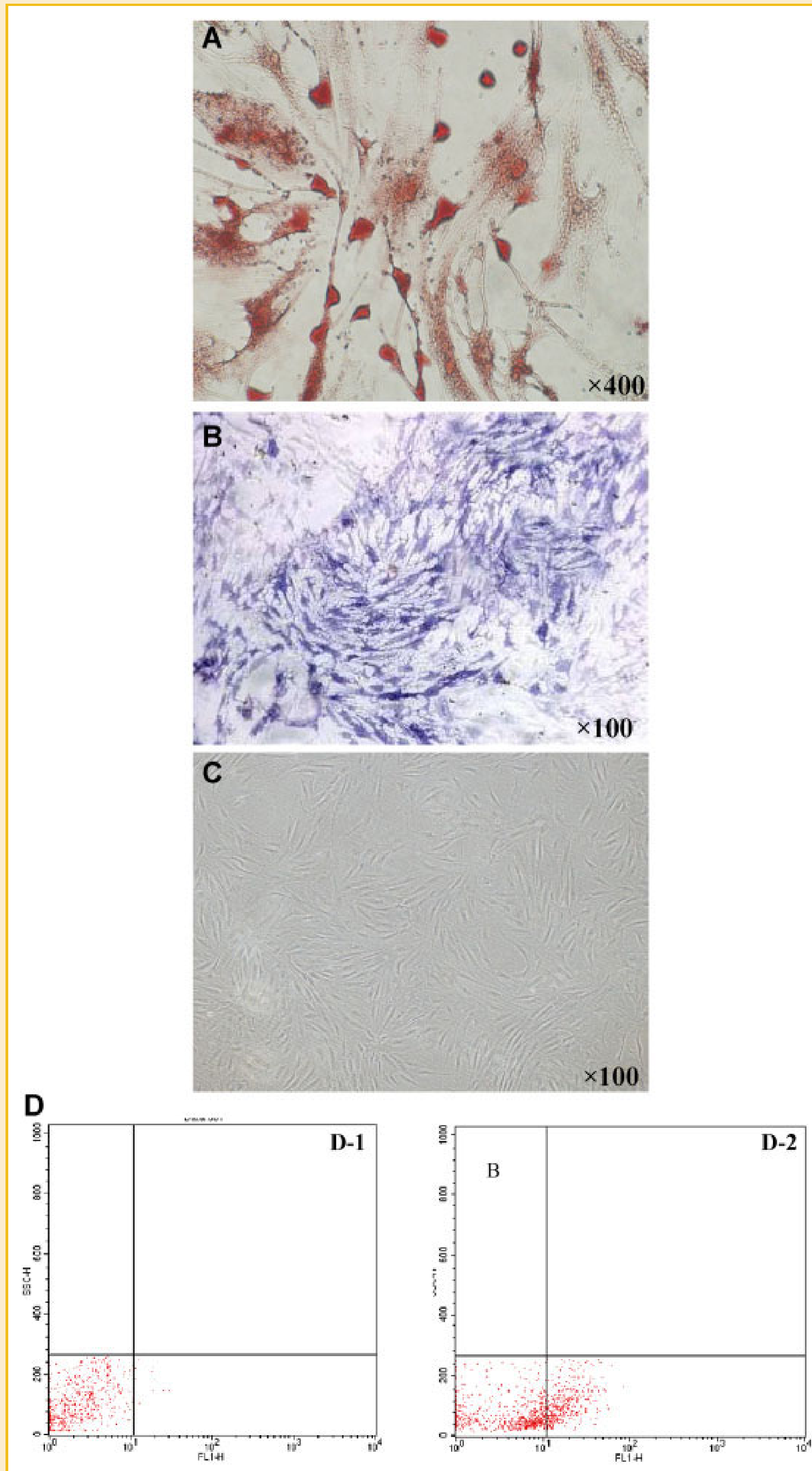

Fig. 4. The third passage cells were induced by lineage-specific induction factors to bone, fat, and myocardium. A: the cells were induced with 10 μmol/L methylprednisolone, 10 μmol/L insulin, 200 μmol/L antinfan, 0.5 mmol/L IBMX to fat and the fat was stained by Oil red O, (B) the third passage cells were induced with 0.1 μmol/L methylprednisolone, 50 μmol/L ascorbic acid-sodium phosphate, 10 μmol/L β-glycerophosphate and then the cells were stained by alkaline phosphatase, (C) the cells were not induced by any induction factor, (D) the myocardial cell differentiation scatter diagram by FCM. We used the normal cells (which had not been induced by 5-aza) as the contrast, and took the fluorescence intensity of coal maceral 10^1 as the limit of the cells, which could express TNT. In the contrast group Picture 1, there is almost none positive cells. At the same time, in the Picture 2, we could see that after induced by 5-aza, there are about 20% positive cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

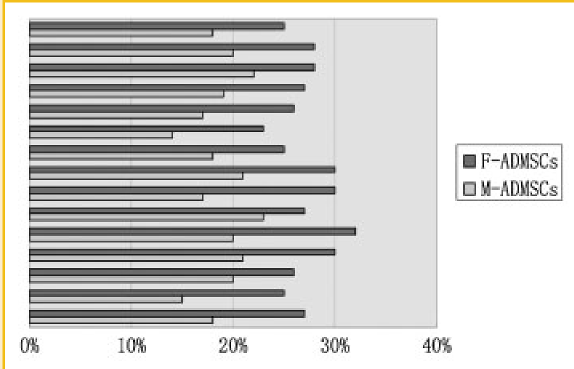

Fig. 5. Comparing the cells' expression rate of TNT by FCM. We took the cell for matching in age, and made the match cells (one men, one women) for a group. So we divided the 30 presons' samples into 15 groups. After induced by 5-azacytidine and detected by FCM, the F-ADMSCs showed a higher expression of TNT than M-ADMSCs (27.3% ± 2.4% vs. 18.9 ± 2.5%, $P < 0.01$).

SURFACE ANTIGEN IDENTIFICATION

The immunocytochemistry stain showed that most of the cells were positive for CD13, CD44, and negative for CD45, VIII factor, HLA-DR, VWF. And some cells had positive nucleus with CD 13 antibody (Fig. 2A–D).

COMPARING THE GROWTH CURVE BY USING MTT ASSAY

The stem cells had similar shape of growth curve. They had lag period for about 1–2 days after passage, then came into the logarithmic phase on the third day, and would reach platform phase on about the 11th day. The MTT assay showed that F-ADSCs had a significant higher OD value than M-ADSCs at the 5 days (0.325 ± 0.037 vs. 0.284 ± 0.031 $P < 0.01$), 7 days (0.532 ± 0.042 vs. 0.443 ± 0.038 $P < 0.01$), and 9 days (0.674 ± 0.046 vs. 0.582 ± 0.043 $P < 0.01$; Fig. 3).

MULTILINEAGE DIFFERENTIATIONS

Induced by lineage-specific induction factors for about 2 weeks, the H-ADSCs were positive for Oil red O staining, alkaline phosphatase staining and their expression of TNT was positive by FCM (Fig. 4A–D).

COMPARING THE DIFFERENTIATION RATE

The H-ADSCs could express TNT as showed above and we compared the expression rate of TNT to find out whether different H-ADSCs had different differentiation abilities. We found that F-ADSCs showed a higher expression of TNT than M-ADSCs (27.3% ± 2.4% vs. 18.9% ± 2.5%, $P < 0.01$; Fig. 5). With linear correlation analysis, the cell age had a negative correlation to the rate of TNT expression (correlation coefficient = −0.7714, $P < 0.01$). Thus the stem cells derived from younger donors might be induced to express more TNT.

COMPARING THE PARACRINE CAPACITY DIFFERENCE IN HGF AND VEGF

We found that all of the H-ADSCs could secrete HGF and VEGF. And F-ADSCs had a better secretion ability comparing to M-ADSCs in HGF and VEGF (Tables II and III).

COMPARING THE APOPTOSIS RATE OF THE CELLS WITH OXIDATIVE STRESS INJURY

Induced by 500 μmol/L H_2O_2 and detected by FCM, F-ADSCs showed a lower apoptosis rate than M-ADSCs (17.99% ± 3.15% vs. 25.31% ± 4.22%, $P < 0.01$; Fig. 6). With linear correlation analysis, the cell age had a negative correlation to the apoptosis rate (correlation coefficient = −0.5824, $P < 0.05$). Thus the stem cells derived from younger donors might be induced to apoptosis more easily.

DISCUSSION

We show here that the sex of H-ADSCs influences their abilities of proliferation, differentiation, paracrine, and anti-apoptosis. And the age of the donor may influence the cells' differentiation and anti-apoptosis abilities. This study may help to explain the broad heterogeneity that has been reported in other stem cell populations [Deasy et al., 2004; Collins et al., 2005; Wagers and Conboy, 2005].

TABLE II. Comparing the Secretion Capacity of HGF of Different Cells

F-ADMSCs			M-ADMSCs		
Cell mean	HGF density of supernatant (pg/ml)	The secretion capacity of HGF (pg/10^6)	Cell mean	HGF density of supernatant (pg/ml)	The secretion capacity of HGF (pg/10^6)
60,000	489.71	13,467	54,000	423.07	12,927
80,000	748.07	15,429	68,000	570.87	13,852
75,000	715.50	15,741	70,000	606.12	14,287
1,25,000	907.88	11,984	88,000	527.20	9,885
78,000	609.58	12,895	75,000	479.45	10,548
73,000	509.85	11,524	69,000	459.75	10,994
90,000	674.45	12,365	82,000	511.13	10,285
68,000	598.44	14,521	65,000	483.28	12,268
82,000	710.02	14,287	75,000	597.32	13,141
73,000	601.39	13,593	63,000	447.68	11,725
1,02,000	766.05	12,392	87,000	579.05	10,982
65,000	439.75	11,163	58,000	350.14	9,961
91,000	806.54	14,624	86,000	719.85	13,811
89,000	654.72	12,138	83,000	504.34	10,026
74,000	610.97	13,623	67,000	493.89	12,163

F-ADMSCs secreted more HGF than M-ADMSCs (13,316 ± 1,404 pg per 10^6cells vs. 11,790 ± 1,541 pg per 10^6cells, $P < 0.01$).

TABLE III. Comparing the Secretion Capacity of VEGF of Different Cells

F-ADMSCs			M-ADMSCs		
Cell mean	VEGF density of supernatant(pg/ml)	The secretion capacity of VEGF (pg/10^6)	Cell mean	VEGF density of supernatant(pg/ml)	The secretion capacity of VEGF (pg/10^6)
60,000	52.99	1,457	54,000	37.41	1,143
80,000	82.28	1,697	68,000	49.50	1,201
75,000	73.77	1,623	70,000	54.60	1,287
1,25,000	112.65	1,487	88,000	62.67	1,175
78,000	57.39	1,214	75,000	48.50	1,067
73,000	70.48	1,593	69,000	49.18	1,176
90,000	87.93	1,612	82,000	58.74	1,182
68,000	65.53	1,590	65,000	49.79	1,264
82,000	77.13	1,552	75,000	59.27	1,304
73,000	74.42	1,682	63,000	50.63	1,326
1,02,000	97.73	1,581	87,000	65.33	1,239
65,000	65.87	1,672	58,000	52.09	1,482
91,000	90.12	1,634	86,000	69.16	1,327
89,000	83.17	1,542	83,000	60.97	1,212
74,000	80.64	1,798	67,000	64.64	1,592

F-ADMSCs secreted more VEGF than M-ADMSCs (1,582 ± 132 pg per 10^6cells vs. 1265 ± 133 pg per 10^6cells, $P < 0.01$).

After MI, cardiac tissue remain chronically scarred and even in the case of an early reperfusion, myocardium is rarely completely rescued. So differentiation into the desired lineages (cardiac muscle) used to be the best option to definitively heal the scar and studies have pointed out that ADSCs have the potential to turn into the cardiac phenotypes [Planat-Bénard et al., 2004; Bai et al., 2007].

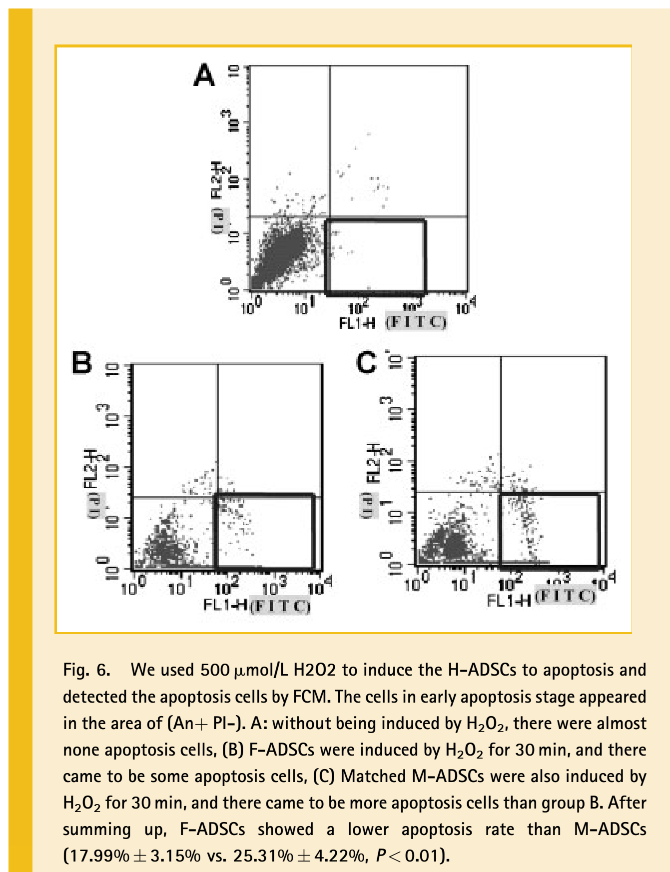

Fig. 6. We used 500 μmol/L H2O2 to induce the H-ADSCs to apoptosis and detected the apoptosis cells by FCM. The cells in early apoptosis stage appeared in the area of (An+ PI-). A: without being induced by H_2O_2, there were almost none apoptosis cells, (B) F-ADSCs were induced by H_2O_2 for 30 min, and there came to be some apoptosis cells, (C) Matched M-ADSCs were also induced by H_2O_2 for 30 min, and there came to be more apoptosis cells than group B. After summing up, F-ADSCs showed a lower apoptosis rate than M-ADSCs (17.99% ± 3.15% vs. 25.31% ± 4.22%, $P < 0.01$).

However, one of the major drawbacks of cell therapy is the low rate of engraftment of the transplanted cells [Haider and Ashraf, 2008], yet on the other hand, paracrine activity has demonstrated that even a few engrafted cells can exert a beneficial effect on cardiac tissue [Fedak, 2008]. So more people think that paracrine, except differentiation plays the main role in the cell transplantation therapy in MI. VEGF is a potent inductor of angiogenesis at the capillary level [Ferrara et al., 2003], and it has been reported to be expressed by ADSC [Song et al., 2007]. HGF has been demonstrated an ability to alleviate the adverse effects of MI through increased angiogenesis, cardiac hypertrophy and fibrosis, even to improve pump function [Nakamura et al., 2005]. In the present study, we have found that there are sex differences in the differentiation and paracrine abilities of the H-ADSCs and we think the sex differences in the cells' paracrine ability of VEGF and HGF may be an important point in the cell transplantation therapy.

Oxidative stress is expected to be suffered by the cells in the microenvironment after cell transplantation and in the present study F-ADSCs showed a lower apoptosis rate than M-ADSCs, when induced by H_2O_2. This finding indicates that F-ADSCs may have better anti-apoptosis ability in the exceptional environment than M-ADSCs. Deasy et al. [2007] have found that in the mice MDSDs, the RNA and protein levels of the anti-apoptotic gene Bcl2 is lower in M-MDSDs as compared with F-MDSDs. This differential gene expression may be the reason for the distinction of anti-apoptosis abilities, of which needs further study.

By linear correlation analysis, we observe that the cell age is related to the cells' differentiation and anti-apoptosis ability and there could be a hypothesis that H-ADSCs may have age related biological characteristics differences. It is known to all that stem cells are "multipotent", however, will the stem cell's power change as its host is getting older? Madonna et al. [2011] have found that aging may alter the H-ADSCs' angiogenic functional capacity and it will be interesting to determine whether other stem cell types also exhibit sex or age-related differences. Moreover, what we have done are all in vitro experiments and whether the cells will work in vivo to show similar results is still waiting for confirmation.

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