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Differentiation of human umbilical cord-derived mesenchymal stem cells into cardiomyocytes and effect of it in myocardial infarction

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ABSTRACT

To test the myocardiogenic potential of human umbilical cord -derived mesenchymal stem cells (MSCs), MSCs were incubated for 24 hours in serum-free Dulbecco's Modified Eagle's Medium (DMEM)containing 10μmol/L 5-azacytidine(5-aza). The medium was then changed to DMEM/ 10% FBS for 4 weeks. Also, MSCs were incubated for 24 hours in serum-free DMEM containing 0.8% dimethyl sulfoxide (DMSO). The medium was then changed to DMEM/10% FBS for 3 weeks. The expression of cardiac desmin, troponin I and troponin T was evaluated by immunohistochemistry in the differentiated myogenic cells. RT-PCR was used to test the expression of Nkx2 .5 and troponin I cDNA in the cells induced. Myofilament and change of cell shape were examined by Transmission Electron Microscope and Scanning Electron Microscopy. Our result showed that MSCs derived from human umbilical cord retained myocardiogenic potential and could be induced into cardiomyocytes by both 5-azacytidine and DMSO, suggesting a suitable approach for cell therapy and transplantation.

KEYWORDS

Umbilical cord;
Mesenchymal stem cells;
Cardiomyocytes;
Differentiation.

INTRODUCTION

Cardiomyocytes are short of regeneration capacity, so the death of cardiomyocytes can lead to cardiac failure. After myocardium infarction and cardiac cells massive necrosis, fiber hyperplasia and scar formation can not repair the damage. Method of traditional treatments only delays the progress of the disease and cannot solve the prime problem. Transplantation of stem cell will be the most important method of treatment for ischemic heart disease[1]. However, up to now, we still cannot decide which is the best type of cells for transplantation, because of issues such as the source of donor cells and immunological rejection.

Cell transplantation to enhance the cardiac function has therefore been actively explored. But the source of cells is limited. Currently the most important sources of MSCs are bone marrow and human cord blood. It is

also controversial whether human cord blood contain MSCs[2-4]. So it is important to find an easy source of MSCs. There were reports that MSCs from bone marrow[1, 5]and human cord blood[6, 7]could be induced into cardiomyocytes. MSCs from human umbilical cord Wharton's jelly and the marrow had the same homoioplastic property[8]. Our previous results revealed that MSCs from the human umbilical cord showed positive expressions of surface-marker CD29, CD44, CD59, but express neither hematopoietic lineage markers CD14, CD33, CD34, CD45, CD38 and CD117, nor GVHD-related markers CD80,CD86,CD40 and CD40L. The MSCs from the human umbilical cord have the potential of multi-directional differentiation. They could differentiate into nerve-like cells[9, 10].

Therefore, we suggested that human umbilical cord Wharton's Jelly contains multipotent progenitor cells that could proliferate and differentiate into cardiomyocytes.

MATERIALS AND METHODS

Cell culture and main reagents

Human umbilical cord Wharton's Jelly for this study was aseptically collected from infants delivered by full-term normal labor at Second Affiliated Hospital of Shantou University Medical College (SUMC). This was approved by the Hospital's ethics committee. 5-azacytidine was purchased from Sigma Co,USA. Dimethyl sulfoxide was bought from Shanghai DNA BioTechnologies Co.,Ltd. Desmin,troponin I, troponin T were donated by Lab Vision Corporation, USA. Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and 1% glutamine were purchased from Gibco Co, USA. Streptavidin-biotin-avidin complex (SABC) was from Boster Co, China. Trizol was from Invitrogen, France, and materials for reverse transcriptase-polymerase chain reaction (RT-PCR) were supplied by TaKaRa, Japan.

MSCs culture

The method of cell culture was previously described[9, 10].

Induction of Wharton's Jelly cells

When the cells reached the 3rd and 8th passages, induction of Wharton's Jelly primary culture was performed, with the cells at 60%-70% confluence. The MSCs were incubated for 24 hours in serum-free DMEM containing 10μmol/L 5-azacytidine. The medium was then changed to DMEM/10% FBS for 4 weeks, followed by 24 hours in serum-free DMEM containing 0.8%DMSO. The medium was then changed to DMEM/10% FBS for 3weeks[11]. The whole induction process was carefully monitored using the phase contrast microscope.

Morphology

Morphology of the cells before and after induction were compared using scanning electron microscope and transmission electron microscope. Any changes after induction were documented.

Immunohistochemistry

The cells were washed with PBS, fixed for 20 minutes at 37°C in 0.05% methyl alcohol, followed by 0.1%Triton-X for 10 minutes and 3%H_2O_2 20 minutes,incubated for 25 minutes at room temperature with a 1:9 dilution of normal goat serum in PBS to block nonspecific binding of the primary antibody. The slides were then incubated for 16 hours at 4°C with various nonlabeled mouse anti-human antibodies(Desmin, Troponin I and Troponin T), followed by biotin goat anti-mouse antibodies for 20 minutes, and then dyed and took pictures. The experiments were repeated at least three times.

Semi-quantitative RT-PCR

Total RNA of the cells was extracted by Trizol for semi-quantitative RT-PCR, which was performed under the following conditions: Nkx2.5, forward primer: :5'- GGA GAA GAC AGA GGC GGA CA -3' and reverse primer: 5'- ACG CCG AAG TTC ACG AAG TT -3' ▪ i525bp ▪ j, denaturation at 61 ▪ Ž. For Desmin, forward primer :5'- ACC GCT TCG CCA ACT ACA TC -3' and reverse primer 5'- TCA CTG GCA AAT CGG TCC TC -3 ▪ i727 bp ▪ j were used followed by denaturation at 61 ▪ Ž. β-actin was used as an internal reference in each reaction, with forward primer: 5'- CACACTGTGCCCATCTACGA -3' and reverse primer: 5'- TACAGGTCTTTGCGGATGTC -3' ▪ i400bp).

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RESULTS

MSCs culture

The MSCs resembled fibroblasts, similar to these in our previous studies. The duration of primary culture was 10-14 days. After passaging, the cells could proliferate 4 to 5 times in 3 to 5 days, but this proliferation somewhat decreased after 9 times of passaging.

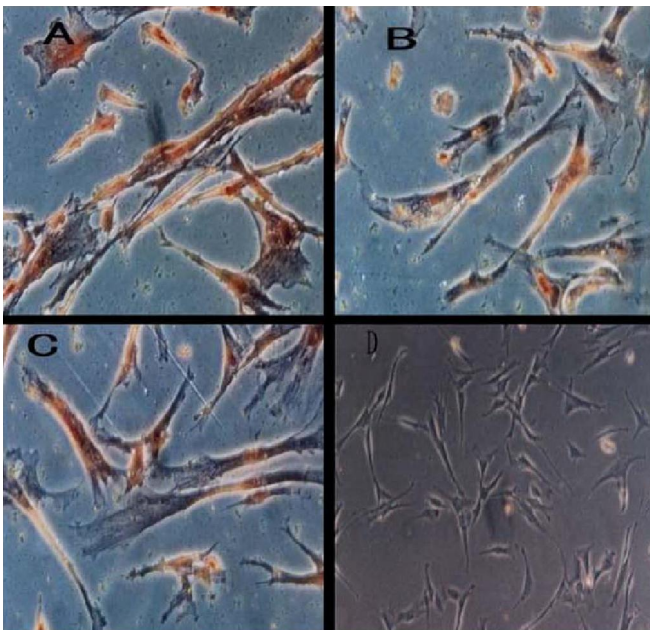

Figure 1 ▪ FThe expressions of cardiac Desmin(A), cardiac Troponin I(B) and cardiac Troponin T(C) were strong positive in MSC, after treatment with 5aza. Cells of human umbilical cord have a fibroblast-like morphology(D). ▪ i×200 ▪ j

Morphological change

The MSCs of the 3rd and 8th passages were cultured in DMEM and induction was performed with 5aza or DMSO. The morphology of these cells changed after 1 to 2 weeks: the cells became bigger, like rhabdocytes, and a few cells appeared irregular. Moreover, some streaky structure appeared inside the cytoplasm.

Immunohistochemistry

MSCs before induction grew as a flat single layer, like fibroblasts, and were negative for Desmin, Troponin I and Troponin T expressions. After incubating for 24 hours in serum-free DMEM containing 10μmol/L 5-azacytidine and then with DMEM/10% FBS for 4 weeks, the MSCs were strong expression for Desmin, Troponin I and Troponin T.(Figure 1)

Also, after incubating for 24 hours in serum-free DMEM containing 0.8%DMSO and then with DMEM/10% FBS for 3 weeks, the MSCs were strong expression for Desmin, Troponin I and Troponin T (Figure 2).

Results of RT-PCR

Figure 3 and 4 demonstrated that there were no desmin and Nkx2.5 expression before the induction.

However, the desmin and Nkx2.5 bands appeared after induction by 5aza.

Also, there was no desmin and Nkx2.5 band before induction, but the desmin and Nkx2.5 bands appeared after induction by DMSO(Figure 5 and 6).

Scanning electron microscope and transmission electron microscope

The MSCs of the 3rd and 8th passages were cultured in DMEM, and induction was performed by 5aza or DMSO. The morphology of these cells changed after 1 to 2 weeks. The cells became bigger, like rhabdocytes, and a few cells appeared irregular. (Figure 7)

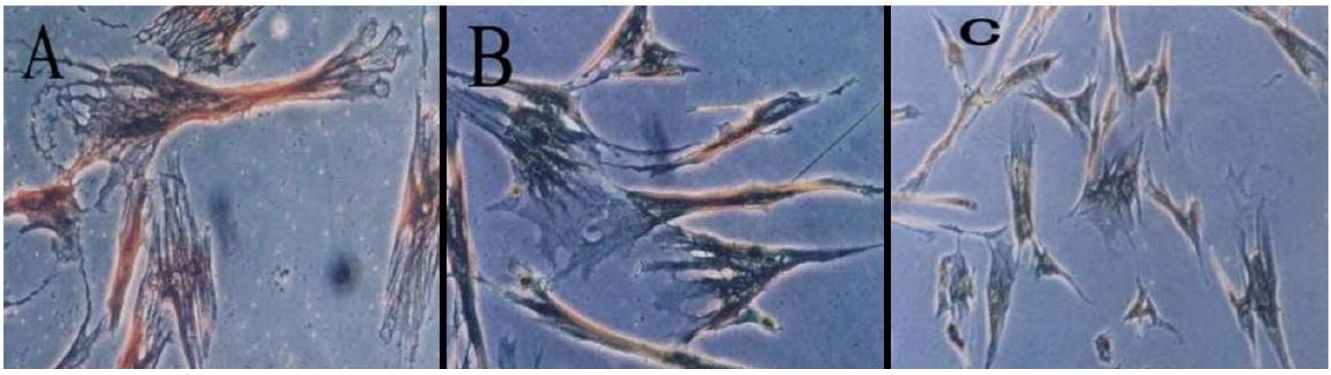

Figure 2 ▪ FThe expressions of cardiac Desmin(A), cardiac Troponin I(B) and cardiac Troponin T(C) were strong positive after treatment with DMSO. ▪ i×200 ▪ j

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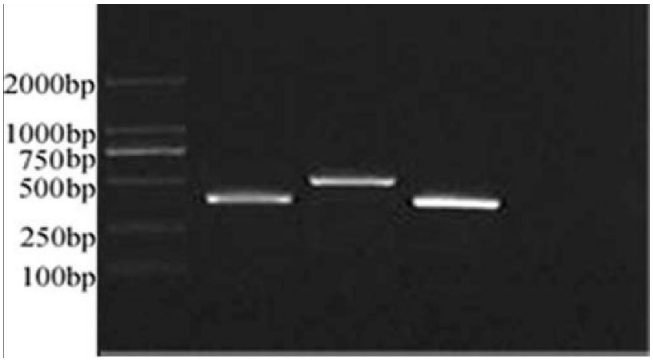

Figure 3: RT-PCR analysis of the Nkx2.5 in cells treated with 5aza. M, DL2,000 Marker ;1, β-actin ▪ i400bp ▪ j, after induction ;2, Nkx2.5 ▪ i525 bp ▪ j, after induction ;3, β-actin ▪ i400bp ▪ j,before induction ; 4, Nkx2.5 ▪ i525 bp ▪ j, before induction.

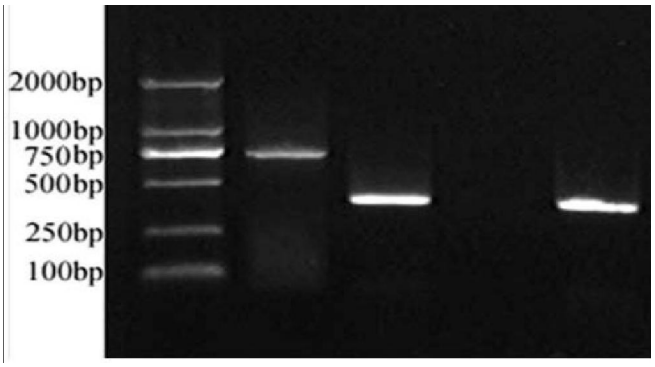

Figure 4: RT-PCR analysis of the desmin in cells treated with 5aza. M, DL2,000 Marker ;1, desmin ▪ i727 bp ▪ j, after induction ;2, β-actin ▪ i400bp ▪ j, after induction ;3, desmin ▪ i727 bp ▪ jbefore induction ; 4, β-actin ▪ i400bp ▪ j, before induction

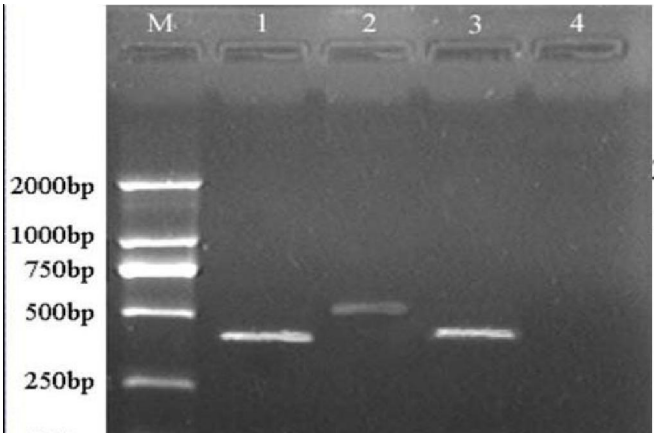

Figure 5:RT-PCR analysis of Nkx2.5 in cells treated with DMSO. M, DL2,000 Marker ;1, β-actin ▪ i400bp ▪ j, after induction ;2, Nkx2.5 ▪ i525 bp ▪ j, after induction ;3, β-actin ▪ i400bp ▪ j,before induction ; 4, Nkx2.5 ▪ i525 bp ▪ j, before induction.

Ultramicrostructural changes

Electron microscope revealed that the cells were full of ribosomes with some myofilament-like structures in the endochylema after induction by 5aza or DMSO. (Figure 8)

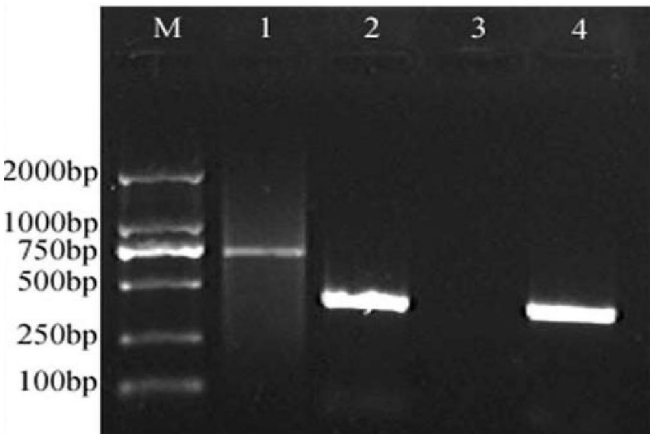

Figure 6: RT-PCR analysis of desmin in cells treated with DMSO. M, DL2,000 Marker ;1, desmin ▪ i727 bp ▪ j, after induction ;2, β-actin ▪ i400bp ▪ j, after induction ;3, desmin ▪ i727 bp ▪ jbefore induction ; 4, β-actin ▪ i400bp ▪ j, before induction

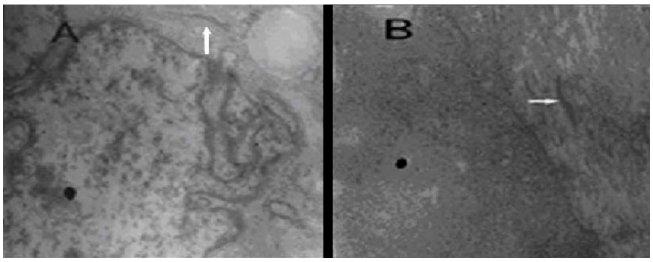

Figure 8 ▪ FThe nucleus was positioned in the center of the cell ▪ Cand myofilaments were founded in the cytoplasm after treatment with 5aza(A) or DMSO(B)

DISCUSSION

MSCs have the potential of multi-directional differentiation. The most important source of MSCs is bone marrow. But 10^4-10^5 bone marrow cells only has one MSC. It is now also controverial whether human cord blood contain MSCs[2-4]. In the previous study, we found that human cord blood contains MSCs, but it is difficult for serial subcultivation[12].

Human umbilical cord matrix cells have homoplastic property similar to the MSCs of bone marrow[13-15]. Mitchell et al[8] reported that umbilical cord matrix cells had the potentiality of multipotent stem cells, could proliferate 80 times in vitro, and also expressed the markers of stem cells, such as c-kit and telomerase. Umbilical cord matrix cells expressed markers of nerve cells

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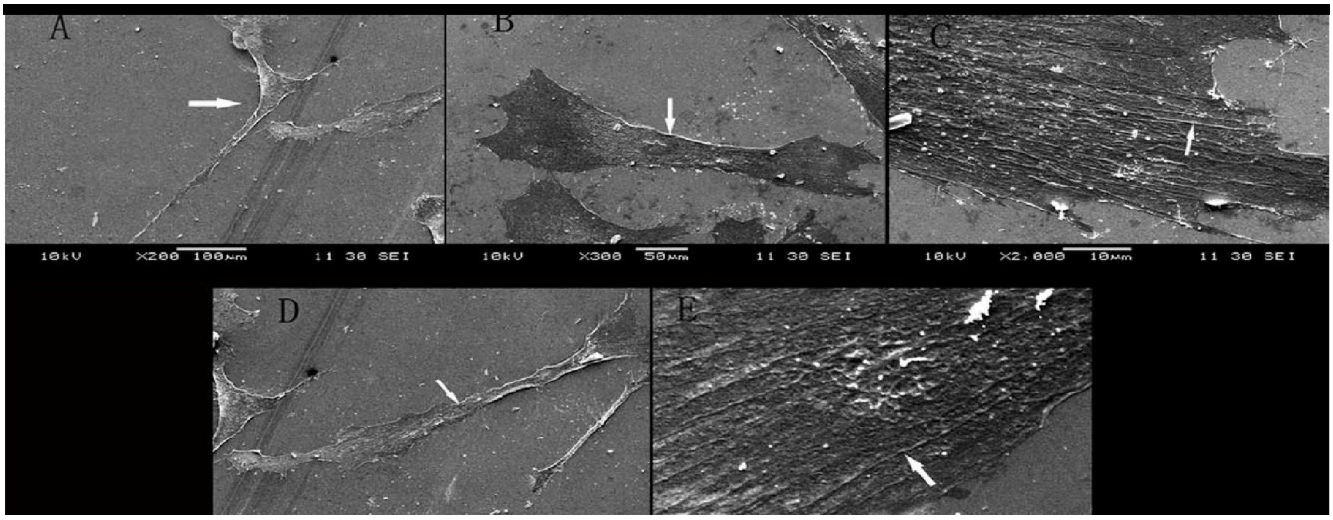

Figure 7 ▪ FCells from human umbilical cord have a fibroblast-like morphology(A). The MSCs became bigger, like rhabdocytes after treatment with 5aza (B) and DMSO (D), and myofilament-like structures appeared in the cytoplasm after induction with 5aza(C) and DMSO(E). (A×200 ▪ CB×300 ▪ CC×2000 ▪ CD×250 ▪ CE×2000)

after treatment with bFGF ▪ Aserum and DMSO. Weiss et al[16] injected pig umbilical cord matrix cells into the brain of mouse. The injected cells could live and migrate in the case of no immune depressant. The injected cells expressed the markers of porcine nerve cells. This provided evidences that umbilical cord matrix cells contained MSCs and could differentiate into nerve cells.

MSCs could be induced into cardiomyocytes. There were reports that MSCs from bone marrow[1, 5] and human cord blood[6, 7] could be induced into cardiomyocytes. Hwai-Shi Wang et al[17] found that MSCs from human umbilical cord Wharton's jelly and human cord blood had similar phenotype. MSCs from human umbilical cord Wharton's jelly could express N-cadherin and cardiac troponin I after treated with 5-aza or co-cultured with cardiomyocytes. All these findings suggested that human umbilical cord—derived mesenchymal stem cells could be another important source of cells for treatment of myocardial infarction.

The present study found that Wharton's jelly MSCs could proliferate rapidly in DMEM with FBS, EGF and bFGF added, and could proliferate 4 to 5 times after passage within 3 to 5 days. Moreover, cell proliferation could be maintained for 10 passages. These proliferated cells were mainly elongated fibroblast-like cells with or without branching, very similar to the MSCs from the bone marrow. Our results were consistent with previous reports showing that Wharton's Jelly contained some stem cells that could expand, self-replicate, and differentiate into multiple cell types under appropriate induction conditions. FACScan cytometry showed that there were no markers of hematopoietic stem cells and cells responsible for graft rejection in cultured MSCs. We concurred with recent studies that Wharton's Jelly could be more convenient and feasible for cell transplantation, and also could represent a more economical source of MSCs, compared with bone marrow[9, 10].

5aza was a analogue of cytidine and it could activete some genes with specific phenotype by provoking hypomethylation of some cytosine residues. The gene is normally in the state of transcription inactivation, and contains an important position that determine differentiation of the cells into cardiomyocytes. These cells could differentiate into cardiomyocytes after treatment with 5aza which provoked hypomethylation of the cytosine[18, 19]. Constantinides PG et al[20] firstly used 5aza to induce embryonic cells differentiating into cardiomyocytes. Makino S et al[21] reported that about thirty percent of the fibroblast-like cells had some morphological changes. 5-Aza treated cells could interconnect after one week, and some formation of myotube appeared in the cells two weeks later. These cells could beat synchronously three weeks later.

Paquin J and Draper Jos[22, 23] reported that DMSO could induce mouse embryonic stem cells and dermoid tumor cells to differentiate into cardiomyocytes. Shi et al[11] found that DMSO could induced human bone marrow MSCs to differentiate into cardiomyocytes. DMSO up-regulated the expression of prodynorphin and ynorphin B genes to switch on the expression of GATA4 and Nkx2 .5 and synthesis of α-MHC and cardiac ven-

tricle idio-myosin light chain-2[22]. Our study found that the shape of the MSCs changed after treatment with 5aza or DMSO. Most of them became rhabdocytes,some were irregular. The body of cells became bigger and there were some streaks formed in the cells.

The induced cells expressed Nkx2.5 and troponin I cDNA,and also showed positive staining for desmin, Troponin I and Troponin T and developed myofilament inside the cells. These results demonstrated that MSCs derived from human umbilical cord could be induced into cardiomyocytes by 5aza and DMSO. The induction effects showed no significant variation among different MSCs passages.

Nkx2.5 is an early transcription factor that was closely linked with the differentiation of cardiomyocytes. RT-PCR showed that Nkx2.5 and desmin were positive after, but not brfore induction with 5aza or DMSO. This finding suggested one of the mechanisms by which 5aza and DMSO exert its induction effects on MSCs. The myofilaments in the intracytoplasm indicated that the cells induced have contractile properties. So both 5aza and DMSO could induce human umbilical cord—derived mesenchymal stem cells to differentiate into cardiomyocytes.

We did not see differentiated cells beating. This phenomenon is rare due to the varied experiment conditions, because reference observed only a few beating cells in 192 clones.

Our results demonstrated that there were MSCs in human umbilical cord . Human umbilical cord—derived MSCs could differentiate into cardiomyocytes with changes in gene and protein expressions when induced by 5aza and DMSO. Compared with stem cells from other sources, human umbilical cord—derived MSCs have some advantages: (1) the human umbilical cord is collected very easily and readily awailable; (2) MSCs were easy to collect, culture and store;(3) MSCs were relative immunologic immaturity. However ▪ Cwhether the differentiated cells can live and connect with the cells around and form a functional syncytium after transplantation remains to be explored.

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