Differentiation of Human Umbilical Cord Derived Mesenchymal Stem Cells Into Neural Stem Cells Induced by hPRDX5

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Research Article

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Abstract

Background: The objectives of this study were to investigate the characteristics and capacity of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) differentiation into neural stem cells (NSCs) and whether this event enhanced by hPRDX5.

Methods and Results: The adherent cells were obtained from umbilical cord of normal full-term newborn by caesarean section under aseptic condition, and cultivated by tissue block culture method. The surface antigen expression profiles of hUC-MSCs were monitored and the multi-directional differentiation potential was identified. Following amplification, the cells of the 4th passage were divided into 5 groups (groups A-E). The morphology was observed under inverted microscope, and the positive expression rate of markers of neural stem cell was detected by immunocytochemical and western blot. Flow cytometry revealed that the hUC-MSCs expressed CD29, CD73, CD90 and CD105, but not CD19, CD34, CD45 or HLA-DR. Treatment with hPRDX5 led to the surface markers of neural stem cells which were positive for Nestin, but negative for NSE and GFAP expression.

Conclusions: Thus, the findings of the present study demonstrate that hPRDX5 effectively promotes hUC-MSCs to differentiate into neural stem cells possibly through TLR4 signaling pathway.

Background

Neurodegenerative disease is a kind of disease which is difficult to cure by conventional treatment. The degeneration of specific subpopulations of neurons in patients will lead to specific neurological disorders. For example, the degeneration of dopaminergic neurons can lead to the impairment of striatum function, which leads to Parkinson's disease [1]. In terms of current pharmacological and neurosurgical treatments, it can only relieve symptoms for a short time, but not prevent the progress of neurodegenerative diseases [2]. In recent years, studies have shown that neurogenesis not only exists in the embryonic stage, but also in the central nervous system of adult mammals. Adult neurogenesis is done by the proliferation and differentiation of neural stem cells into new neurons [3]. Adult NSCs are mesoderm derived stem cells with the potential of self replication and multi differentiation [4]. Neurogenesis in adults persists throughout life and is influenced by many factors. Under normal physiological conditions, the nervous system produces thousands of new nerve cells every day, but only a small part of them integrate into the neural network; When pathological changes occur in the central nervous system, NSCs can form new neurons and migrate to specific sites, integrate into the existing neural network, thus play the role of repairing the injury [5]. Recent studies have shown that transplanting neural stem cells (NSCs) with regenerative ability is an accurate and effective treatment method in the treatment of central nervous system diseases [6-8]. The transplanted NSCs play the role of cell substitution and paracrine in the patient's central nervous system. Many clinical studies on the use of NSCs in the treatment of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and spinal cord injury are also being carried out [9-11]. However, how to intervene in adult neurogenesis and promote neural cell regeneration is a difficult problem to be solved urgently [12].

Peroxiredoxins are a family of thiol-dependent peroxidases that are able to reduce peroxides and are constitutively expressed by virtually all cell types in mammals [13]. It has been reported that, PRDXs may act as cytoprotective antioxidant enzymes, modulators of peroxide signaling, protein chaperones, and redox relays, intracellularly [14]. PRDXs are also well studied in neural progenitor cell differentiation. Such as, PRDX1 and PRDX2 actively participate in the maintenance of embryonic stem cell stemness by opposing ROS/JNK activation during neurogenesis [15]. PRDX4 ablation causes premature neuronal differentiation and progenitor depletion [16]. Moreover, Shichita et al. [17] have shown that post-ischemic brain inflammation can be mediated through PRDXs and is dependent on TLR2 and TLR4. Unlike other PRDXs, the role of PRDX5 in neurogenesis has not been reported yet. In our previous studies, we found that PRDX5 can activate TLR4 signaling pathway and play the role of anti-pancreatic cancer. Thus, we could speculate that PRDX5 may intervene in neurogenesis through TLR4 signaling pathway. In this study, we will investigate the potential of hPRDX5 which was biosynthesis and preparation in *Escherichia coli* [18] to induce umbilical cord mesenchymal stem cells (UC-MSCs) into NSCs.

Methods

Materials

Human umbilical cords obtained from women with full-term pregnancies whose babies were delivered by cesarean section were provided by the Hospital of Changguo (Shangdong, China). This study was approved by the Ethics Committee of the Hospital of Changguo. The following reagents were obtained commercially: L-DMEM/F-12 was purchased from Lonza Group Ltd. Fetal bovine serum (FBS), trypsin, N-2 Supplement (100X), B-27™ Supplement (50X), epidermal growth factor (EGF) and human FGF-basic (bFGF) were obtained from Gibco. APC-CD19, FITC-CD34, APC-CD29, FITC-CD73, FITC-CD90, PE-CD45, PE-CD105 and PerCP-HLA-DR antibodies were obtained from Beijing DAKWE Biotechnology Co., Ltd. Anti-Nestin antibody, Anti-GAPDH antibody, Goat Anti-Rabbit IgG H&L (HRP) and Goat Anti-Mouse IgG H&L (HRP) was purchased from Abcam.

Isolation and culture of hUC-MSCs

The umbilical cord of healthy newborn baby with full-term pregnancy and caesarean section was selected under sterile conditions and placed in a special cord storage bottle. Cell extraction should be carried out within 12 hours. After removing the blood sample, umbilical artery and umbilical vein, the umbilical cord mesenchymal tissue was cut into 1-2 mm³ sections and placed in a 75cm² culture flask containing 5-10ml DMEM/F-12 culture medium. The flask was placed into an incubator at 37°C with 5% CO₂ and saturated humidity. The fibroblast-like adherent cells that migrated from the tissue fragments were primary umbilical cord mesenchymal stem cells (UC-MSC). When the cells achieved 80-90% confluency, the medium was removed, and the cells were rinsed two times with PBS and digested with trypsin (0.125%)-EDTA into single cells, then the fragments removed by filtering with a 100-µm cell strainer. These cells were defined as passage 1 (P1) cells for passaging until passage 4 (P4).

Identification of immunophenotype of hUC-MSC

Single cell suspensions of hUC-MSCs were placed into 12 tubes at 1×10^6 /tube in phosphate-buffered saline (PBS) and stained with PerCP anti-human HLA-DR, FITC anti-human CD34, APC anti-human CD19, FITC anti-human CD73, FITC anti-human CD90, PE anti-human CD105, APC anti-human CD29 and PE anti-human CD45 antibodies (BioLegend, USA), each were added 5 μ l separately. Anti-mouse IgG1-PE, anti-mouse IgG1-APC, anti-mouse IgG1- PerCP and anti-mouse IgG1-FITC antibody were added to the other 4 tubes as isotype controls. The cells and antibodies in all tubes were mixed thoroughly and incubated at room temperature for 30 min. The cells were then rinsed in PBS and resuspended in 200 μ l of PBS prior to detection by flow cytometry.

Induction of hUC-MSC into neural stem cells

The hUC-MSCs of the fourth passage in the logarithmic growth phase were obtained, rinsed twice with PBS and digested with trypsin. After centrifuging, resuspending in culture medium, the cells were seeded at 2×10^5 /ml into 6-well cell culture plates or 25cm² culture flasks. The plates or flasks were divided randomly into 5 groups (groups A-E). Each group was set with three parallel controls. Group A was treated with DMEM/F12 with 10% FBS (complete medium) only and served as blank. hPRDX5 was added to group A at the final concentrations of 1 μ g/ml and 10 μ g/ml and served as groups B-C. Group D was treated with DMEM/F12 + N2 + B27 + EGF (20 ng/ml) and bFGF (20 ng/ml) (neural stem cells induction medium) and served as control. hPRDX5 was added to group D at the final concentrations of 1 μ g/ml and served as group E. All the plates or flasks were cultured in an incubator at 37°C with 5% CO₂ and saturation humidity for 3 days. After 3 days of induction and differentiation, the above five groups of cells were digested with trypsin, and the cells were resuspended with maintenance medium (DMEM/F12 with 2% FBS). After 3 days of continuous culture, the cells were collected for subsequent detection.

Immunocytochemistry

The expression of neural protein markers was analyzed by means of immunocytochemistry. Briefly, after induction for 3 days and cultured for another 3 days with maintenance medium on the cell climbing films, the cells were rinsed gently once with PBS prior to fixing with 4% paraformaldehyde for 20 min at room temperature. The cell membranes were disrupted with 3% H₂O₂ firstly and then 0.3% Triton X-100 at room temperature. The goat serum was used for blocking for 15 min and then removed without washing, followed by overnight incubation with primary antibodies at 4°C. The primary antibodies included rabbit anti-human Nestin (1:200 dilution, BioLegend). The cells were then incubated with the appropriate biotinylated secondary antibody for 15 min, followed by horseradish peroxidase-conjugated streptavidin for 15 min. At the last, the cells were stained with freshly prepared diaminobenzidine (DAB) for 1 min and counterstained with hematoxylin before finally rinsed repeatedly with water. After dehydration and sealing piece, the images were acquired by use of a fluorescence microscope. The positive cells were counted in 20 randomly selected fields at a magnification of $\times 400$, and the proportion of positive cells was calculated as number of positive cells/total number of cells $\times 100\%$.

Western blot analysis

Cell lysates were obtained by lysing cells with cell lysis buffer and phenylmethylsulfonyl fluoride (Beyotime Biotechnology Co., Ltd.) followed by centrifugation at 12,000 g for 15 min. Protein samples were mixed with loading buffer, boiled for 10 min, electrophoresed in a 6% SDS gel and transferred to a polyvinylidene fluoride membrane (Beyotime Biotechnology Co., Ltd.). The membranes were blocked with QuickBlock™ Blocking Buffer (Beyotime Biotechnology Co., Ltd.) for 15 min and incubated with the Nestin, TLR4 and GAPDH primary antibodies at 4°C overnight followed by five washes for 5min in TBSTw and incubation with secondary antibodies (horseradish peroxidase–conjugated anti-rabbit IgG, horseradish peroxidase–conjugated anti-mouse IgG) for 1 h at room temperature. PVDF membranes were visualized by BeyoECL Plus using the ChemiDoc Touch Imaging System (BIO-RAD, USA).

Statistical analysis

Statistical analysis was performed by using SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA). Values are expressed as mean $\pm$ standard deviation (SD) from three different experiments. Single variable data were analyzed using a one-way ANOVA with Tukey multiple comparisons or two-tailed paired t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Culture and morphological observation of hUC-MSC

The primary cells were obtained at about the 12th day of culture of Wharton jelly and passaged at the ratio of 1:1 to produce the hUC-MSCs at passage 1. In the initial cultivation, the majority of the cells became adherent, but were not outstretched and were triangular or diamond-shaped. At about the 5th-7th day, the adherent cells proliferated rapidly and became significantly larger, appearing in a radial or spiral pattern. This situation is generally referred to as confluency. The passage cultivation was needed when the cells achieved 80-90% confluency.

Cellular phenotype of hUC-MSCs

The examination of the cellular phenotype of the hUC-MSCs at passage 4 was conducted by flow cytometry. The results revealed that the cells co-expressed CD105, CD90, CD29 and CD73, but not CD34, CD19, CD45 or HLA-DR (Figure 1).

Differentiation potential of hUC-MSCs

The P4 cells were induced into adipocytes, chondrocytes and osteoblasts to confirm the differentiation potential of hUC-MSCs. We first confirmed the capacity of osteogenic differentiation for hUC-MSCs, as demonstrated by alizarin red staining (Figure 2A). In adipocyte differentiation medium, an accumulation of oil red O–stained lipid drops were observed (Figure 2B). Furthermore, in histological sections obtained

of cells grown in chondrogenic induction medium, toluidine blue staining revealed the presence of extracellular matrix formation (Figure 2C).

Neural stem cells differentiation of hUC-MSCs detected by immunocytochemical

The morphology of hUC-MSCs plated in complete medium was almost the same as that of the blank group during 6 days of cultivation and presented a vortex-like confluency. The growth of cells in the experimental group (control + 1ug/ml hPRDX5) with the neural stem cells induction medium was almost the same as that of the control group. At the beginning of induction, cell aggregation was formed in most cells of adherent growth. A small number of cells scattered, showing a diamond or triangle-shaped. At the end of induction, the adherent cells gradually grew into cells clusters defined as neurospheres. This kind of neurosphere was difficult to be transformed into a single cell suspension by digestion or mechanical pipetting and carry out immunohistochemical detection, so it continued to culture in the maintenance medium with low concentration of FBS. At the initial stage of maintenance culture (12 hours), a few cells would crawl out from the center of the neurosphere. After maintaining culture for 48 hours, the neurospheres would gradually shrink, and a large number of cells separate from the neurospheres and grow with adherence in the culture flask. At the end of the culture (72 h), the contour profile of the neurospheres disappeared, and a large number of adherent cells could be observed under the microscope. The immunocytochemical staining of Nestin in the cells of the 5 groups was positive to different degrees. As shown in figure 3, differences in the expression levels of Nestin among the blank group and experimental group (control + 1ug/ml hPRDX5) were significant ($P<0.01$), and there were more Nestin-positive cells in the experimental group (control + 1ug/ml hPRDX5) compared with the control group (Figure 3).

(A-C) Immunocytochemical detection of positive rate of Nestin in treatment groups A, C and D, respectively (scale bar=20 μ m). (D) Relative expression levels of Nestin in every group. Data shown are representative of three independent experiments, and results are represented as mean values of three different samples $\pm$ SD.* $P<0.05$, ** $P<0.01$.

Neural stem cells differentiation of hUC-MSCs detected by western blot

We also examined their protein expression using western blotting, and their expression was consistent with the results of immunocytochemistry. The results showed that the experimental group (control + 1ug/ml hPRDX5) displayed the highest proportion of positive cells in Nestin staining. Although there was no significant difference between the hPRDX5 induction groups (group B and group C) and the blank group, the expression levels of Nestin in the induction groups were higher than that in the blank group, and there was a dose-dependent relationship between the two induction groups with different concentrations of hPRDX5 (Figure 4).

hPRDX5 induced differentiation of hUC-MSCs through TLR4 signaling pathway

We explored the potential mechanism of hPRDX5 inducing hUC-MSC into neural stem cells. Consequently, we found that hPRDX5 was associated with TLR4. In protein expression analysis by western blot, TLR4 exhibited significantly higher expression in the hPRDX5 induction groups (group B, group C and group E) than their counterparts in the same medium (Figure 5), and the differences in the expression levels of TLR4 among the blank group and hPRDX5 induction groups (group C), the control group and experimental group (control + 1ug/ml hPRDX5) showed statistical differences ($P < 0.05$).

Discussion

Human neural stem cells (hNSCs) are mainly derived from aborted embryos or fetal brain tissue, having the ability of self-renewal and differentiation into almost all nerve cells and great potential in the treatment of brain injury caused by various causes such as trauma, neuronal degeneration and stroke [19-21]. However, there are two problems limit the clinical application of hNSCs. One is that hNSCs obtained from embryo or fetal brain tissue have immunogenicity, and transplantation into patients will cause immune rejection, so the patients receiving transplantation need to take immunosuppressants for life. Second, the sources of hNSCs are limited, and there are ethical disputes. If there is a kind of cell which has a wide range of sources and does not cause graft rejection and can produce hNSCs through appropriate methods, the above problems will be solved. MSCs are adult stem cells derived from the mesoderm and ectoderm. MSCs are initially found in bone marrow and can differentiate into osteoblasts, adipocytes and nerve like cells expressing a variety of nerve cell surface markers under suitable conditions which opens a new way for stem cell transplantation in the treatment of nervous system diseases [22, 23]. If MSCs are induced to form NSCs in vitro, and then transplanted into animals or human, the enhanced neurogenic ability of MSCs may lead to better therapeutic effect. There have been animal experiments [24].

In the 1990s, scholars isolated "fibroblast like cells" from Wharton's jelly of human umbilical cord, which found a new source for mesenchymal stem cells. Umbilical cord, which used to be regarded as useless medical waste, has been paid more and more attention. In recent years, human umbilical cord mesenchymal stem cells (hUC-MSCs) have become a research hotspot because they have more abundant sources than bone marrow, more convenient access and can differentiate into neural cells. In this study, hUC-MSCs were cultured and passaged in vitro. It was found that hUC-MSCs had strong proliferation ability, the morphology of the cells was consistent with that of the primary cultured cells and did not change with time. We used flow cytometry to detect the molecular markers on the cell surface of the P4 generation hUC-MSCs. The results showed that hUC-MSCs expressed mesenchymal stem cell surface markers CD73, CD90, CD29 and CD105, but did not express CD19, CD34, CD45 and histocompatible antigen HLA-DR (MHC-II). This is consistent with the detection results of surface markers of mesenchymal stem cells from other sources. In addition, the P4 cells were induced into adipocytes, chondrocytes and osteoblasts to confirm the multi-directional differentiation potential. At present, there is no definite conclusion about the specific antigen of MSCs, which can only be judged by cell surface markers, cell morphology and the multi-directional differentiation ability of such cells.

There are many methods to induce MSCs to differentiate into neural cells, it can be generally divided into three groups: (1) chemical inducer; (2) neurotrophic factor; (3) effective components and preparations of traditional Chinese Medicine. All kinds of inducers have been proved to induce MSCs into neuron like cells and express various kinds of neuron surface antigens. The induction mechanism of different inducers is different. PRDX5 is widely expressed in various tissues constitutively and upregulated during inflammatory processes [25]. Frank Plaisant, et al. demonstrates that recombinant PRDX5 is protective against excitotoxic murine brain lesions that are similar to damage seen in human cerebral Palsy [26]. Therefore, we speculate that PRDX5 has the neuroprotective effects. In this experiment, we compared the effect of hPRDX5 on the induction of neural stem cells on the basis of neural stem cell standard medium and umbilical cord mesenchymal stem cell complete medium. We found that: in the complete medium, the neural induction effect of hPRDX5 was not very significant, there was the increased trend of protein expression levels for Nestin, but not statistically significant. By contrast, the protein expression levels for Nestin in the neural stem cell standard medium in the experimental group induced by hPRDX5 increased significantly. Based on these results, hPRDX5 can promote the differentiation of MSCs into neural cells, and it needs to work together with other factors in the neural stem cell standard medium to achieve more significant effect.

We further explored the mechanism of MSCs induced into NSCs. The results of Western blot showed that, compared with the blank and control group, the protein expression levels for TLR4 in the experimental groups induced by hPRDX5 increased in varying degrees. This suggests that hPRDX5 promotes the differentiation of hUC-MSCs into NSCs by activating TLR4. This result is similar to that of Zhang et al. [27]. They added LPS (TLR4 activator) under the condition of osteogenic culture, and found that TLR4 expression was increased after being activated, and promoted the differentiation of hUC-MSCs into osteoblasts. However, Grasselli et al. found that the expression of TLR4 in NSCs did not affect by LPS stimulation. Therefore, we analyzed that the increased expression of TLR4 was in the early stage of MSCs differentiation into NSCs [28].

Conclusion

In conclusion, we have obtained a macromolecular active substance which can induce hUC-MSCs to differentiate into NSCs in vitro, and revealed its potential mechanism of action. However, whether hPRDX5 can induce hUC-MSCs trans-planted in vivo to differentiate into neural cells effectively will require further study. All in all, it will greatly promote the application of NSCs in the treatment of neurodegenerative diseases.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Yuanyuan Jin: Methodology and Writing - original draft, Beichen Shi: Formal analysis and Investigation, Qiang Han: Validation, Kun Liu: Data curation, Shuai Fan: Writing - review & editing and Funding acquisition, Zaoyong Yang: Resources and Funding acquisition.

Competing Interest

The authors declare that they have no competing interests

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Not applicable

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Figures

Figure 1

Surface marker expression of CD34, HLA-DR, CD19, CD45, CD105, CD73, CD29 and CD90 in human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) detected by flow cytometry.

Figure 2

Differentiation into (A) osteocytes, (B) adipocyte and (C) chondrocytes of hUC-MSCs were identified by alizarin red staining, oil red O staining and alcian blue staining, respectively.

Figure 3

Staining for neural stem cells markers in human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) induced to differentiate by hPRDX5. (A-C) Immunocytochemical detection of positive rate of Nestin in treatment groups A, C and D, respectively (scale bar=20 μ m). (D) Relative expression levels of Nestin in every group. Data shown are representative of three independent experiments, and results are represented as mean values of three different samples $\pm$ SD.*P<0.05, **P<0.01.

Figure 4

(A) Protein expression of Nestin in every group was confirmed by Western blot. (B) Protein expression levels for Nestin in every group. Data shown are representative of three independent experiments, and results are represented as mean values of three different samples $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

Figure 5

(A) Protein expression of TLR4 in every group was confirmed by Western blot. (B) Protein expression levels for TLR4 in every group. Data shown are representative of three independent experiments, and results are represented as mean values of three different samples $\pm$ SD.* $P < 0.05$.