Biochemical and Biophysical Research Communications xxx (2017) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

NRF2 overexpression in mesenchymal stem cells induces stem-cell marker expression and enhances osteoblastic differentiation

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ARTICLE INFO

Article history:
Received 13 July 2017
Accepted 14 July 2017
Available online xxx

Keywords:
NRF2
$CoCl_2$
Hypoxia
MSC
Stemness
Osteoblastic differentiation

ABSTRACT

Hypoxic environment has been suggested in stem cell culturing as their physiologic niche requires oxygen tension to maintain stemness. The administration of cobalt chloride ($CoCl_2$) was widely applied in mimicking hypoxia for its economic advantages and convenience. We confirmed that $CoCl_2$ could maintain stemness and promote the osteogenesis capacity of MSCs. However, $CoCl_2$ could induce the apoptosis and hinder the proliferation of MSCs. To find out a potential method maintaining their stemness without threatening their survival, we analyzed the database of Gene Expression Omnibus (GEO) and proposed that NRF2 (nuclear factor erythroid-derived 2-like 2) might be the potential target. We found that knocking down NRF2 expression in MSCs impaired the expression of stem cell markers and the osteogenesis process even under hypoxic environment, but with NRF2 overexpression, the proliferation of MSCs was increased with significantly reduced rate of apoptosis. Therefore, our findings suggested that overexpressing NRF2 could be a potential method for maintaining stemness and preventing apoptosis in MSCs under oxidative stress.

1. Introduction

Mesenchymal stem cells (MSCs) have been exploited extensively from animal models to clinical trials. Owing to the specific characteristics such as low immunogenicity, immunomodulation and tissue regeneration, MSCs have afforded promise in the treatment of numerous diseases ranging from myocardial infarction to hepatic failure [1–3]. Although MSCs for cell therapy have been shown to be safe and effective, challenges still exist in maintaining their original bioactivity, given the responsiveness of MSC characteristics to microenvironmental cues [4,5]. On the other hand, MSCs are inclined to loss their original stemness characteristics once derived from the niche, which would adversely affect the transplantation therapy, such as low rates of cell survival, abnormal differentiation and undesirable paracrine effects [6,7]. Therefore, the in vitro culture system need to be tackled in maintaining MSCs stemness before their wide application in the clinic.

MSCs are typically cultured under normoxic conditions, while tissues from which MSCs are isolated are associated with variable oxygen levels (1–7% in bone marrow, 10–15% in adipose tissue, and 2–8% at the MSC niche), therefore, oxygen tension has been suggested to be more beneficial as its similar to that present at the physiologic niche for MSCs [8,9]. As hypoxia was also found to maintain the stemness of bone marrow stromal cells and human dental pulp cells by suppressing their differentiation, some studies have administrated CoCl2 to stem cell cultures as an in vitro hypoxic system [10-12]. CoCl2 treatment have been proven to maintain the expression of stemness marker in human periodontal ligament

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http://dx.doi.org/10.1016/j.bbrc.2017.07.083
0006-291X/

(HPDL) cells [10], but hypoxia induced significant apoptosis as well. The question becomes whether we could maintain their stemness without threatens to their survival.

Under oxidative stress, the transcription factor NRF2 has been recognized as an important modifier during the cellular responses [13]. NRF2 is the master regulator of the antioxidant response element (ARE) driven genes, which coordinately regulated the promoters of many antioxidant genes including stress-response proteins heme oxygenase-1 (HO-1) and direct ROS-removing enzymes [14–16]. Extensive studies have supported that NRF2 defective cells or organisms are more susceptible to an array of oxidative insults, while NRF2 overexpression reduced oxidative stress-induced apoptosis and cytotoxicity [17,18]. Recent studies proposed NRF2 as a key pluripotency gene in human embryonic stem cells (hESCs) and NRF2 played an important role in the maintenance of MSC stemness via p53−SIRT1 regulation [19].

In this regard, we investigated whether $CoCl_2$ treatment with NRF2 overexpression in MSCs could reduce apoptosis while maintaining stemness. Notably, we found that under oxidative stress, stemness of MSCs was maintained with intact osteogenesis capacity, but MSCs expansion was limited as significant apoptosis was induced. As NRF2 deficiency impaired the beneficial effect of hypoxia on stemness maintenance and osteogenesis capacity, NRF2 overexpression promoted the maintenance of stem cell characteristics and prevented MSCs apoptosis [20]. Our results suggested that oxidative stress combined with NRF2 overexpression are essential for the stability of MSCs stemness maintenance and stable expansion, which provide important clues for efficient in vitro MSCs culture system in future therapy.

2. Materials and methods

2.1. Cell isolation and culture

The study was approved by the Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University. Human umbilical cord tissue was collected from Caesarean section deliveries from healthy donors with the approval of the relevant ethics committee and with anonymous consent from the donor, and transferred in phosphate-buffered saline (PBS) containing 100 UI/mL penicillin and streptomycin (Life Technologies) at 4°C [21]. The human umbilical cord-derived MSCs (UC-MSCs) were isolated according to previous method. In short, umbilical cord tissue was washed twice with PBS containing penicillin and streptomycin and the vessels were removed during this process. The whole tissue was manually dissociated into 0.5 cm pieces and floated in Dulbecco's modified Eagle's medium with low glucose (Life Technologies) containing 10% FBS (Life Technologies), 5% HS, penicillin, and streptomycin at 37 °C in a humidified atmosphere with 5% CO2. After 7 days nonadherent cells were removed and after 10 days, well-developed colonies of fibroblast-like cells appeared, the cultures were trypsinized and transferred into a new flask for further expansion. The medium was changed every 2 days.

2.2. Osteogenic and adipogenic differentiation

For osteogenic and adipogenic differentiation, cells were stimulated for 21 days and 14 days in differentiation media (Life Technologies) according to manufacturer's instructions. The medium was changed every 2 days. Cells cultured in original growth medium were used as the control. In the experiments intended for quantification of the differentiation potential, MSCs were cultured in differentiation media for 10 days. Osteogenic and adipogenic differentiation were assessed by staining for minerals (Alizarin red, Sigma) and for the presence of lipid droplets (Oil red O, Sigma) [22,23].

2.3. Flow cytometry

Cells were harvested with trypsin-EDTA and resuspended in the wash buffer. The Abs used for flow cytometry were FITC-, PE-, or APC- conjugated mouse anti-human CD73, CD90, CD105, CD45, CD34, CD166, CD44, CD29 (eBioscience). The isotype antibody was utilized as the negative controls.

2.4. Cell proliferation assay

Forty-eight hours after transfection, MSCs in logarithmic phrase of growth were obtained and adjusted to a density of 5×10^5 cells/mL. Cells were then inoculated in a 96-well plate and further left untreated or treated with $CoCl_2$ (Sigma) for 12, 24, and 72 h 100 μL of fresh culture medium containing 10 μl of CCK-8 reagent (Dojindo Molecular Technologies, China) was then added into each well for cell incubation. After 2 h, the medium was discarded, and the optical density (OD) value was measured at a wavelength of 450 nm using the Bio-Rad 680 micro-plate reader (Bio-Rad, USA).

2.5. Western blotting

Standard protocols for western blotting were used as described previously. Equal amounts of protein (30–50 μg) from each sample were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore). The proteins were then incubated overnight at 4 °C with primary antibodies against human HIF1α, Nanog, Bmi-1, BCl2, Cleaved-caspase3, HSP90, Histone H3, P-NRF2, NRF2, (Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology). The membrane was then washed 3 times (5min each time) and incubated with secondary antibody for 1 h at room temperature. After being washed for 3 times (5 min each time), the membranes were visualized by enhanced chemiluminescence (ECL; Millipore) and recorded on Tonon5200.

2.6. Nuclear and cytosolic fractionation

MSCs were collected by trypsinization and washed with phosphate-buffered saline (PBS) two times before nuclear and cytosolic fractionation. Nuclear and cytoplasmic fractionation were conducted using the Nuclear and Cytoplasmic Extraction Reagents Kit (Beyotime, China) according to the manufacturer's instructions. Each separated protein was analyzed by western blotting analysis.

2.7. Confocal assay

MSCs were plated on covered glass-bottom dishes (NEST), washed twice with phosphate-buffered saline, fixed in 4% paraformaldehyde for 15 min, and then washed 3 times. The cells on the dishes (0.5- mm thick) were prepared and stained with the primary antibodies overnight at 4 °C. The sections were washed 3 times with phosphate-buffered saline, incubated with fluorochrome-labeled secondary antibodies (1: 1000, Invitrogen) for 30 min, and then washed 3 times. The nuclei were stained with 4, 6-diamidino-2-phenyindole (DAPI, Sigma) and then examined using a Zeiss LSM700 scanning laser confocal microscope and analyzed with Zeiss confocal software.

To measure the number of fluorescent foci, a total of 10 cells were recorded with the Zeiss LSM700 confocal microscope and analyzed using ImageJ analysis software.

Please cite this article in press as: Z. Yuan, et al., NRF2 overexpression in mesenchymal stem cells induces stem-cell marker expression and enhances osteoblastic differentiation, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.07.083