Experimental Cell Research 349 (2016) 221–229

Contents lists available at ScienceDirect

Experimental Cell Research

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

Research Article

Downregulation of Nrf2 promotes autophagy-dependent osteoblastic differentiation of adipose-derived mesenchymal stem cells

Jiang Tao[a,1], Haining Wang[a,1], Yue Zhai[a], Hyun Park[a], Jian Wang[a], Fang Ji[b,*], Zhiyong Zhang[c,d,**]

[a] Department of General Dentistry, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai, China
[b] Department of Orthodontics, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, No. 639 Zhi Zao Ju Road, Shanghai 200011, China
[c] Department of Plastic and Reconstructive Surgery, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Tissue Engineering, Shanghai, China
[d] National Tissue Engineering Center of China, Shanghai, China

ARTICLE INFO

Keywords:
Nrf2
Oxidative stress
Adipose derived stem cells
Differentiation

ABSTRACT

Adipose derived stem cells (ADSCs) are an important source of stem cells for tissue repair and regeneration; therefore, understanding the mechanisms that regulate stem cell differentiation into a specific lineage is critical. The NF-E2-related factor 2 (Nrf2) pathway and autophagy promote cell survival in response to oxidative stress. However, the roles of Nrf2 and autophagy in bone metabolism under oxidative stress are controversial. Here, we explored the involvement of Nrf2 signaling and autophagy on the differentiation of ADSCs under conditions of oxidative stress. Exposure of ADSCs to H_2O_2 promoted reactive oxygen species (ROS) accumulation concomitant with the reduction of cell viability, upregulation of Nrf2, the induction of apoptosis and autophagy, and the promotion of osteogenesis. Suppression of autophagic activity at particular stages resulted in the activation of the Nrf2 pathway, whereas osteoblastic differentiation of ADSCs was inhibited upon ROS stimulation. Silencing of Nrf2 promoted autophagy and osteoblastic differentiation upon ROS stimulation *in vitro*, and this effect was confirmed *in vivo* in a mouse model, in which bone formation was enhanced in mice receiving Nrf2-knockdown ADSCs. Taken together, these findings indicate that a negative interaction between the Nrf2 pathway and autophagy may modulate oxidative stress-induced ADSC osteogenesis, and suggest that Nrf2 is a potential target to regulate the differentiation of ADSCs into a specific lineage.

1. Introduction

Stem cells, which are characterized by their self-renewal and multilineage differentiation capacity, have recently attracted attention for their potential medicinal applications [1]. Adipose-derived stem cells (ADSCs) are multipotent cells with the ability to differentiate into many lineages, including adipogenic, chondrogenic, myogenic, and osteogenic cells. In addition to their multipotent properties, ADSCs are good candidates for tissue engineering and regenerative medicine because they can be easily isolated from adipose tissues [2]. ADSCs are affected by oxidative stress, and low levels of intracellular reactive oxygen species (ROS) activate cellular processes associated with stem cell function [3,4]. ADSCs cultured under hypoxic conditions show enhanced proliferative and migratory capacities, as well as increased growth factor secretion [5].

Several signaling pathways exist in cells that trigger antioxidant responses upon sensing oxidative stress [6]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcriptional factor that promotes cell survival and protects cells against oxidative stress-induced damage [7]. Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap-1), which binds to Nrf2 in the cytoplasm and directs it for proteasomal degradation [8]. Exposure to oxidants disrupts the KEAP1-Nrf2 interaction, stabilizing Nrf2 and inducing its translocation to the nucleus, where it regulates the expression of antioxidant and anti-inflammatory genes through consensus cis-elements termed anti-oxidant response elements [9]. Alterations in Nrf2 signaling impair

** Correspondence to: Department of Plastic and Reconstructive Surgery, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine,Shanghai Key Laboratory of Tissue Engineering, National Tissue Engineering Center of China, No. 639 Zhi Zao Ju Road, Shanghai 200011, China.
* Corresponding author.
E-mail addresses: smilefang98@hotmail.com (F. Ji), mr.zhiyong@gmail.com (Z. Zhang).
[1] Jiang Tao and Haining Wang equally contributed to this work.

http://dx.doi.org/10.1016/j.yexcr.2016.09.013
Received 25 July 2016; Received in revised form 6 September 2016; Accepted 22 September 2016
Available online 23 September 2016

antioxidant gene expression and enhance oxidative stress-mediated inflammation.

Autophagy is a catabolic process in which organelles or macromolecules are sequestered and transported to the lysosome for degradation [10]. The p62/sequestosome 1 (SQSTM1) protein acts as a cargo receptor for autophagic degradation of ubiquitinated targets and its induction by oxidative stress is mediated by Nrf2. When autophagy is impaired, p62 accumulates, resulting in increased binding to the Nrf2 inhibitor KEAP1 and reduced proteasomal degradation of Nrf2 in a feed forward loop triggered by oxidative stress [11]. As an autophagic adapter, p62 interacts with the autophagosomal marker LC3, which in its cytosolic form (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II and is recruited to autophagosomal membranes and degraded after fusion with the lysosome [12]. The ratio of LC3-II to LC3-I is therefore used as a marker of autophagy.

In the present study, we examined the effect of oxidative stress on the osteoblastic differentiation of ADSCs and explored the involvement of the crosstalk between Nrf2 and autophagy in this process.

2. Materials and methods

2.1. Ethical approval

Sprague-Dawley rats and nude mice (6–7 weeks old) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals. All study protocols were approved by the Ethics Committee of Shanghai Ninth People's Hospital of China.

2.2. Reagents

Antibodies were obtained from the following companies: GAPDH, beclin-1, LC3-I, LC3-II, P62, Nrf2, BMP2 and Runx2 (Sigma, St. Louis, MO), L-asparagine and 3-methyladenine (Abcam, Cambridge, USA). DMEM (low-glucose/high glucose), osteogenic differentiation media, and fetal bovine serum were from Gibco (Gaithersburg, MD, USA). Cell lysis buffer (10×) was obtained from Cell Signaling Technology (Massachusetts, USA). The RT-PCR kit was purchased from Gibco (Gaithersburg, MD, USA). Other reagents included DAPI (Roche, Germany), hematoxylin and eosin (H & E, Toronto Chemicals, Toronto, Canada), and trypsin (Sigma). Ad-mRFP-GFP-LC3 was purchased from Biovector Science Lab, Inc. (Beijing, China). All pairs of real-time PCR primers were synthesized by Shenggong Biotechnology (Shanghai, China). Other chemicals and reagents were of analytical grade.

2.3. Isolation and culture of ADSCs

ADSCs were extracted from the subcutaneous fat at the hind leg and side abdominal region of two female Sprague-Dawley rats. The adipose tissues were minced and treated with 200 U/ml type I collagenase (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) for degradation at 37 °C with gentle agitation for 30 min. Following mechanical and enzymatic treatment, the tissues were homogenized in medium containing low-glucose DMEM/Ham's F12 (1:1) (Gibco) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). After homogenization, cells were cultured in an incubator (37 °C/5% CO_2, 95% relative humidity). The culture medium was refreshed two times every week. Cells of the 2nd to 5th passages were used in this study.

2.4. Osteoblastic differentiation and H_2O_2 exposure

After ADSCs reached 70–80% confluence in basal medium, the medium was changed to osteogenic differentiation media to allow the cells to differentiate into osteoblasts. To mimic the oxidative stress environment, ADSCs with/without siNrf2 were exposed to 0, 0.1, 0.25, 0.5, 1.0 and 1.5 mM of H_2O_2 for 12, 24 and 48 h. To examine the effect of ROS on osteoblastic differentiation, ADSCs were exposed to 0.1 mM H_2O_2 for 24 h, and then cultured in osteogenic differentiation medium for 21 days, with fresh culture medium changed every 3 days. To suppress autophagic activity, the inhibitor L-asparagine (Asn, 250 mM) or 3-methyladenine (3-MA, 10 mM) was added for 24 h pretreatment before H_2O_2 exposure.

2.5. ALP activity assay and Alizarin red staining

ADSCs were seeded into 96-well plates at a density of 2×10^3 cells/well and cultured in routine medium or mineralization-inducing medium (MM) containing α-MEM, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM ascorbic acid, 2 mM 2-glycerophosphate, and 10 nM dexamethasone. The ALP activity assay was performed using an ALP activity kit (Sigma) and the activity was normalized to total protein content in the cell. Alizarin red staining was performed on day 21 after treatment. The staining of calcified nodules was eluted by using 10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate (pH7.0). Calcium concentration was determined by measuring the absorbance at 526 nm with a universal microplate reader (BioTek Instruments, USA). This experiment was performed in triplicate and the results are presented as the mean ± SD.

2.6. Apoptosis assay

Apoptosis of ADSCs was measured by flow cytometry. ADSCs were harvested and resuspended in 500 μl binding buffer. Cells were then incubated with 5 μl annexin V-FITC and 5 μl propidium iodide (PI, Sigma) at room temperature in the dark for 15 min. Subsequently, the samples were analyzed by flow cytometry using a FACSCalibur flow cytometer and FlowJo software (Flowjo, Ashland, OR, USA).

2.7. Quantitative real-time PCR

Total RNA was extracted and purified using the Trizol reagent (Sigma) following the manufacturer's instructions. cDNA was prepared by PCR. Quantitative real-time PCR was performed using the SYBR Green Real-time PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI Step One Plus real-time PCR system. The specific primers used in the PCR reaction were as follows: GAPDH, 5′-ATGGGGAAGGTGAAGGTCG-3′ (sense) and 5′-GGGGTCATTGATGGCAACAATA-3′ (antisense); Nrf2, 5′-TGATTGACAT CCTTTGGAGG-3′ (sense) and 5′-CCGGCTGA-ATTGGGAGGAAT-3′ (antisense). The cycle threshold (Ct) values were standardized to the Ct values of GAPDH. Fold differences were determined by the $2^{-\Delta\Delta Ct}$ method.

2.8. Construction and infection

Small interfering RNAs (siRNA) specific for Nrf2 (siNrf2) and a scrambled siRNA used as control (SCR) were synthesized by GenScript (siNrf2, 5′-GCAGCCATGACTGATTTAATT-3′ SCR, 5′-CGCTGGCGAA-CAAGATTCATT-3′). ADSCs were transfected with siRNA using oligofectamine following the manufacturer's instructions (Sigma). The Ad-mRFP-GFP-LC3 vector was used to infect ADSCs cells to monitor puncta formation by immunofluorescence microscopy using a Fluoview 1000 System (Olympus, Irving, TX).

2.9. Western blot analysis

Cell cultures were washed with PBS and trypsinized (0.05% trypsin w/v with 0.02% EDTA). The pellets were lysed in buffer [Tris-Cl 50 mM, EDTA 10 mM, Triton X-100 1% v/v, phenylmethylsulfonyl fluoride (PMSF) 1%, pepstatin A 0.05 mM, and leupeptin 0.2 mM].