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The Function Research of RUNX2 in Osteoblasts Differentiation from Rat Adipose-derived Stem Cells

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Abstract

RUNX2 was an essential for osteoblast differentiation and bone development. In our study, rat adipose-derived stem cells (ADSCs) were isolated from fat pads of abdomen and inguinal, and then purified and expanded *in vitro*. RUNX2 gene was over-expressed using *lentivirus* tools to induce the rat ADSCs differentiated into osteoblasts. The result shown that the transcription factors, RUNX2 could stable express in rat ADSCs and promote the directly osteoblasts differentiation of rat ADSCs. Western blotting assays revealed the up-regulation of osteoblast-related genes after osteoblastic differentiation of rat ADSCs. This study provides a theoretical basis and experimental evidence for the application of ADSCs into treatment of bone injury.

Keywords: Rat; ADSCs; RUNX2; Osteoblast

Introduction

The transcription factors, RUNX2 (Cbfa1/AML3/Pebp-2 A), a member of the Runt domain family, binds specific factors to regulate transcription of numerous genes and thereby control osteoblast differentiation from bone-marry mesenchymal stem cells [23]. Runx2 is also required for mesenchymal condensation, chondrocyte hypertrophy and vascular invasion of developing skeletons [12,18]. RUNX2 can directly stimulate transcription of osteoblast-related genes such as osteocalcin (OCN), collagen I, osteopontin (OPN) and collagen Ⅲ [5,9,24]. White adipose tissue has the ability to dynamically expand and shrink, like bone-marrow, adipose tissue is a mesodermally-derived organ that contains a stromal population containing endothelial progenitor cells, smooth muscle cells and mesenchymal stem cells [29]. So, adipose tissue have MSC-like cells, which can differentiated into bone, fat, muscle, and cartilage cells [2,16,20]. It has been reported that MSC derived from adipose tissues have similar characteristics of BMSCs [13]. Adipose-derived stem cells can differentiate along several lineages, including the osteogenic lineage, in response to stimulation by multiple environmental factors [3,7,27] and they involve complex pathways regulated at transcriptional levels. However, the regulation of these cellular pathways is not fully understood. Although Runx2 is expressed exclusively in mineralized tissues and their precursors, in many cases there is a poor correlation between actual Runx2 mRNA or protein levels and the expression of osteoblast-related genes. In this study, we focus on the RUNX2 gene regulation and the relationship of RUNX2 and other osteoblast-related genes in osteoblasts differentiation of rat ADSC.

Materials and Methods

Isolation and culture of ADSCs

Adipose tissues were separated from fat pads of rat abdomen and inguinal. All operations were finished under sterility condition. The adipose tissues were washed 3 times with phosphate buffer saline (PBS) containing 10^4 IU/mL penicillin/streptomycin to remove connective tissue membrane and capillaries. The tissues were chopped into small pieces (about 1 mm^3) and digested with 0.1% (m/v) type I collagenase (Sigma, USA) at 37°C for 1 h. Enzymatic digestion was then neutralized with fetal bovine serum (FBS, Gibco, USA). The suspension was filtered with an 80-μm Cell Strainer and centrifuged at 300 g for 5 min at room temperature. Then the supernatant was discarded and the cells were re-suspended with a complete medium containing L-DMEM+10% FBS. The cell suspension was plated and incubated at 37°C with 5% CO_2. The culture medium was changed every three days and the non-adherent cells were removed. When the cells reached 70-80% confluence, then 0.25% trypsin and 0.02% EDTA (Gibco, USA) were added to dissociate the cells from plates, and trypsinization was terminated with the complete medium. The cells were subcultured in new plates and incubated at 37°C with 5% CO_2.

Immunofluorescence for ADSCs surface antigen

Surface antigen of rat ADSCs from different passages were detected by immunofluorescence (IF) staining. The ADSCs were fixed in 4% paraformaldehyde for 20 min, then blocked for 15 min with methanol containing 0.1% Triton X-100, and then incubated in a goat serum working solution for 30 min to block nonspecific binding. The ADSCs were then incubated at room temperature for 1 h with primary antibodies including CD29, CD44, CD71, CD73, CD90, CD105, CD31 and STRO-1. The ADSCs were incubated with secondary antibodies conjugated with FITC (goat anti-mouse IgG and goat anti-rat IgG, Boster, China). For negative control, PBS was used to replace the primary antibodies. Finally, nuclei were labeled by incubation with 4, 6

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diamidino-2-phenylindole (DAPI) (Sigma, USA). The cells were examined by a phase contrast fluorescence microscope (Olympus, Japan).

Expression of RUNX2 by recombinant *lentivirus*

Recombinant lentiviruses were generated by using *Lentivirus*-delivered technology [22].The coding regions of RUNX2 was PCR-amplified using primer "F: 5' CCAGATGGGACTGTGGTTACC3'" "R:5'ACTTGGTGCAGAGTTCAGGG3'" and cloned into a *lentivirus* vector and then used to recombine *lentivirus* in HEK293T cells. The resulting lentiviruses designated as pLV/RUNX2/eGFP also express GFP as a marker for monitoring infection efficiency. Analogous *lentivirus* only expressing monomeric GFP was used as a control.

Osteogenic differentiation and transfection

The rat ADSCs were washed with PBS for 3 times. Then, DMEM medium containing 0.5nmol/L FGF-2 and 100 μg/L BMP-2 (Peprotech, US) was added to the factor-induced group (group A) [15,21,26]. The ADSCs at exponential growth were transfected by pLV/RUNX2/eGFP or eGFP (group B). 72 hours after transfection, the cells were selected and tested by western blotting. Or ADSCs were treated with 0.5nmol/L FGF-2 and 100 μg/L BMP-2 after RUNX2 transfection (group C). One week later, the cells were detected for the formation of calcium node using Alizarin Red staining, and the expression of osteoblasts specific genes via Western blot assay. ALP activity was assessed by colorimetric assay (using p-nitrophenyl phosphate as a substrate) and/or histochemical staining assay (using a mixture of 0.1 mg/ml napthol AS-MX phosphate and 0.6 mg/ml Fast BlueBB salt) [4,8].

Results

Morphological observation and surface antigen characteristics of rat ADSCs

Rat ADSCs were large, lucent and with strong refraction. The non-adherent cells were removed on the third day and ADSCs were fusiform and showed cell-like clone with growth being slower. Three days later, ADSCs proliferated rapidly. We observed a delayed outgrowth of exponentially growing populations of cells with spindle-shaped morphology. Immunoflourescence staining results showed that different passages of rat ADSCs expressed antigens CD13, CD44, CD29, CD71, CD73, CD90 and CD105, but did not express antigens CD31. CD31 antigen is special marker of endothelial cells. There was no significant difference in the positive rates of different passages (P>0.05) (Figure 1).

Alizarin Red staining and induce alkaline phosphatase (ALP) activity assay

The rat ADSCs were plated to 24-well plates for osteogenic differentiation. After the transfection of RUNX2, rat ADSCs had expression of GFP and significant changes in appearance (Figure 2A). Alizarin red staining of rat ADSCs was positive at one week after inducing. The positive region was brightly red, showing clear calcium nodules (Figure 2B), while the control was negative. Although the exact mechanisms remain to be fully delineated, RUNX2 is shown to regulate the osteogenic differentiation during skeletal development. As shown in Figure 2C, overexpression of RUNX2 was shown to effectively induce ALP activity in rat ADSCs.

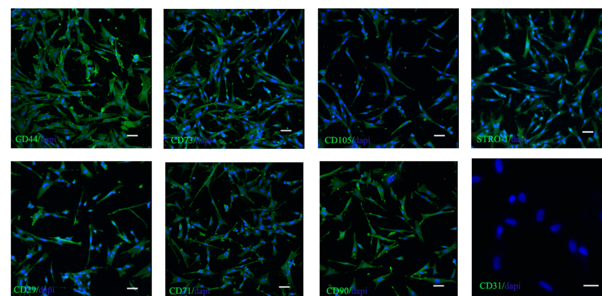

Figure 1: Surface antigen characteristics of rat ADSCs at different passages. Immunoflourescence staining results showed that rat ADSCs at different passages expressed antigens CD29, CD44, CD71, CD73, CD90, CD105 and STRO-1, but not antigens CD31.

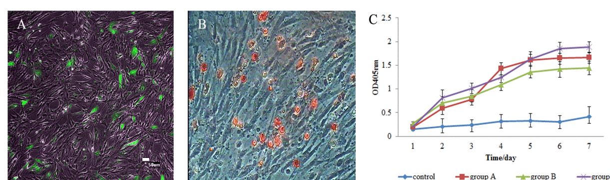

Figure 2: RUNX2 overexpression and Osteogenic differentiation of rat ADSCs. A: Transfection of RUNX2 after 48 h for Osteogenic differentiation. B: After incubation in osteogenic differentiation for one week, the cells metamorphosed from fusiform to tridimensional shapes and the nodules increased in number and size with prolonged inducing time. The nodules were obviously observed following Alizarin Red staining. (bar=50 μm). C: Detection of ALP concentration after Osteogenic differentiation. The ALP concentration assay of medium showed that ALP concentration increased with induced time extension. Group C is significantly different (P<0.05) then other groups.

Western blotting of osteoblast-related genes

Cellular total protein of three groups was extracted after one week of osteogenic induction respectively, and western blot were performed osteoblast-related genes, such as OCN, collagen I, OPN and collagen Ⅲ. Western blotting assay indicated that the osteoblast-related genes, OCN, collagen I, OPN and collagen Ⅲ increased protein expression level after osteogenic induction. However, the protein level of osteoblast-related genes had significant difference in three groups (Figure 3); the protein level of group C was highest in our research.

Discussion

Cell therapy has emerged as a strategy for the treatment of diseases, especially bone injury and repair. Adult stem cells were currently tentatively expanded and orientationally induced *in vitro* to seed cells that are needed, which are then implanted into patients to repair damage, to replace regressive tissue and improve the function of

hereditarily defective tissue. It was reported that ADSCs could be used to repair and reconstruct some tissues such as bone cartilage, lung, brain, liver, etc. [6,10,17]. At present, the number of ADSCs is not enough for tissue engineering. Accordingly, it is necessary to expand and purify ADSCs *in vitro*. The rat ADSCs were successfully isolated and expanded *in vitro* in our research, and the surface antigen characteristics of rat ADSCs were expressed CD13, CD44, CD29, CD71, CD73, CD90 and CD105 using immunoflourescence staining. It was reported that BMMSCs could be used to repair and reconstruct some tissues such as bone cartilage, lung, brain, liver, etc. [14,19,25,28]. RUNX2 is a transcription factor that belongs to the RUNX family [12]. RUNX2-deficient ($RUNX2^{-/-}$) mice completely lack bone formation owing to the absence of osteoblasts [11,18]. RUNX2 determines the osteoblast lineage from mesenchymal stem cells, induces osteoblastic differentiation at the early stage. Further, RUNX2 has been shown to ALP activity, expression of bone matrix protein genes, and mineralization in immature mesenchymal cells and osteoblastic cells *in vitro* [1]. RUNX2 regulates the expression of several osteoblastic genes including collagen I, OPN, bone sialoprotein and the skeletal-specific osteocalcin gene. The binding of nuclear RUNX2 to osteoblast-specific elements up-regulates skeletal genes and consequently the osteoblast phenotype. In our research, the RUNX2 were overexpressed using *lentivirus* tools in rat ADSCs, the osteoblast-related genes were expressed after RUNX2 transfection. However, the osteoblast-related genes expression level of group B (only RUNX2 expression) was lowest then other groups. The group C (RUNX2 expression added factors) was an effective method for osteoblastic differentiation from rat ADSCs.

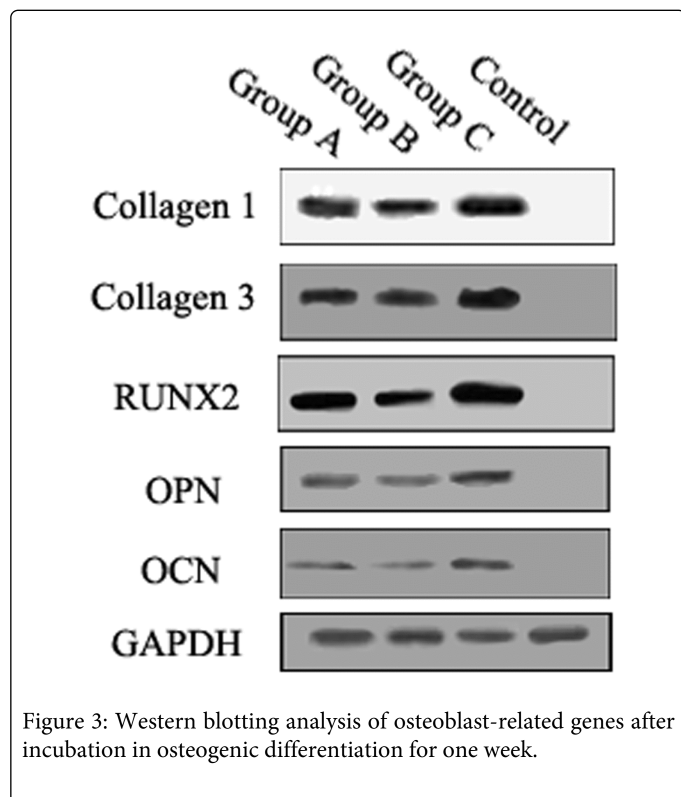

Figure 3: Western blotting analysis of osteoblast-related genes after incubation in osteogenic differentiation for one week.

Conclusion

In this study, rat ADSCs were isolated from adipose tissues, and investigated their morphology and antigen expression. We also induced the rat ADSCs to differentiate into osteoblasts, which proved that RUNX2 control osteoblasts differentiation. The present study has important bearing on the potential application of ADSCs as an adult stem cell source for regenerative therapies.

Reference

1. Banerjee C., McCabe L.R., Choi J.Y., Hiebert S.W., Stein J.L., Stein G.S. & Lian J.B.
Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex.
J. Cell. Biochem. 1997, 66: 1-8.

2. Brzoska M., Geiger H., Gauer S. & Baer P.
Epithelial differentiation of human adipose tissue-derived adult stem cells.
Biochem. Biophys.Res. Commun. 2005, 330: 142-150.

3. Chen G., Deng C. & Li Y.P.
TGF-beta and BMP signaling in osteoblast differentiation and bone formation.
Int. J. Biol. Sci. 2012, 8: 272-288.

4. Cheng H., Jiang W., Phillips F.M., Haydon R.C., Peng Y., Zhou L., Luu H.H., An N., Breyer B., Vanichakarn P., Szatkowski J.P., Park J.Y. & He T.C.
Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs).
J. Bone. Joint. Surg. Am. 2003, 85-A: 1544-1552.

5. Ducy P., Zhang R., Geoffroy V., Ridall A.L. & Karsenty G.
Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation.
Cell. 1997, 89: 747-754.

6. Gimble J. M., Katz A. J. & Bunnell B. A.
Adipose-derived stem cells for regenerative medicine.
Circ. Res. 2007, 100: 1249-1260.

7. Kang Q., Song W.X., Luo Q., Tang N., Luo J., Luo X., Chen J., Bi Y., He B.C., Park J.K., Jiang W., Tang Y., Huang J., Su Y., Zhu G.H., He Y., Yin H., Hu Z., Wang Y., Chen L., Zuo G.W., Pan X., Shen J., Vokes T., Reid R.R., Haydon R.C., Luu H.H. & He T.C.
A comprehensive analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells.
Stem Cells Dev. 2009, 18: 545-559.

8. Kang Q., Sun M. H., Cheng H., Peng Y., Montag A.G., Deyrup A.T., Jiang W., Luu H.H., Luo J., Szatkowski J.P., Vanichakarn P., Park J.Y., Li Y., Haydon R.C. & He T.C.
Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery.
Gene Ther. 2004, 11: 1312-1320.

9. Kern B., Shen J., Starbuck M. & Karsenty G.
Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes.
J. Biol. Chem. 2001, 276: 7101-7107.

10. Kingham P. J., Kalbermatten D. F., Mahay D., Armstrong S. J., Wiberg M. & Terenghi G.
Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro.
Exp. Neurol. 2007, 207: 267-274.

11. Komori T.
Runx2, a multifunctional transcription factor in skeletal development.
J. Cell. Biochem. 2002, 87: 1-8.

12. Komori T., Yagi H., Nomura S., Yamaguchi A., Sasaki K., Deguchi K., Shimizu Y., Bronson R. T., Gao Y. H., Inada M., Sato M., Okamoto R., Kitamura Y., Yoshiki S. & Kishimoto T.
Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts.
Cell. 1997, 89: 755-764.

13. Lee R.H., Kim B., Choi I., Kim H., Choi H.S., Suh K., Bae Y.C. & Jung J.S.
Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue.
Cell. Physiol. Biochem. 2004, 14: 311-324.

14. Liang Z.X., Sun J.P., Wang P., Tian Q., Yang Z. & Chen L.A.
Bone marrow-derived mesenchymal stem cells protect rats from endotoxin-induced acute lung injury.
Chin. Med. J. 2011, 124: 2715-2722.

15. Maegawa N., Kawamura K., Hirose M., Yajima H., Takakura Y. & Ohgushi H.
Enhancement of osteoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2).
J. Tissue. Eng. Regen. Med. 2007, 1: 306-313.

16. Miranville A., Heeschen C., Sengenes C., Curat C.A., Busse R. & Bouloumie A.
Improvement of postnatal neovascularization by human adipose tissue-derived stem cells.
Circulation. 2004, 110: 349-355.

17. Moon M.H., Kim S.Y., Kim Y.J., Kim S.J., Lee J.B., Bae Y.C., Sung S.M. & Jung J.S.
Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia.
Cell Physiol. Biochem. 2006, 17: 279-290.

18. Otto F., Thornell A.P., Crompton T., Denzel A., Gilmour K.C., Rosewell I. R., Stamp G.W., Beddington R.S., Mundlos S., Olsen B.R., Selby P.B. & Owen M.J.
Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development.
Cell. 1997, 89: 765-771.

19. Pereira R.F., O'Hara M.D., Laptev A.V., Halford K.W., Pollard M.D., Class R., Simon D., Livezey K. & Prockop D.J.
Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta.
Proc Natl Acad Sci U S A. 1998, 95: 1142-1147.

20. Planat-Benard V., Silvestre J.S., Cousin B., Andre M., Nibbelink M., Tamarat R., Clergue M., Manneville C., Saillan-Barreau C., Duriez M., Tedgui A., Levy B., Penicaud L. & Casteilla L.
Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives.
Circulation. 2004, 109: 656-663.

21 Rickard D.J., Sullivan T.A., Shenker B.J., Leboy P.S. & Kazhdan I.
Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2.
Dev. Biol. 1994, 161: 218-228.

22. Rubinson D.A., Dillon C.P., Kwiatkowski A.V., Sievers C., Yang L., Kopinja J., Rooney D.L., Zhang M., Ihrig M.M., McManus M.T., Gertler F.B., Scott M.L. & Van Parijs L.
A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference.
Nat. Genet. 2003, 33: 401-406.

23. Schroeder T.M., Jensen E.D. & Westendorf J.J.
Runx2: a master organizer of gene transcription in developing and maturing osteoblasts.
Birth. Defects. Res. C. Embryo Today. 2005, 75: 213-225.

24. Selvamurugan N., Chou W.Y., Pearman A.T., Pulumati M.R. & Partridge N.C.
Parathyroid hormone regulates the rat collagenase-3 promoter in osteoblastic cells through the cooperative interaction of the activator protein-1 site and the runt domain binding sequence.
J. Biol. Chem. 1998, 273: 10647-10657.

25. Thanabalasundaram G., Arumalla N., Tailor H.D. & Khan W.S.
Regulation of differentiation of mesenchymal stem cells into musculoskeletal cells.
Curr. Stem Cell Res. Ther. 2012, 7: 95-102.

26. Varkey M., Kucharski C., Haque T., Sebald W. & Uludag H.
In vitro osteogenic response of rat bone marrow cells to bFGF and BMP-2 treatments.
Clin. Orthop. Relat. Res. 2006, 443: 113-123.

27. Zhang W., Deng Z.L., Chen L., Zuo G.W., Luo Q., Shi Q., Zhang B.Q., Wagner E.R., Rastegar F., Kim S.H., Jiang W., Shen J., Huang E., Gao Y., Gao J.L., Zhou J.Z., Luo J., Huang J., Luo X., Bi Y., Su Y., Yang K., Liu H., Luu H.H., Haydon R.C., He T.C. & He B.C.
Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells.
PLoS One. 2010, 5: e11917.

28. Zhao D.C., Lei J.X., Chen R., Yu W.H., Zhang X.M., Li S.N. & Xiang P.
Bone marrow-derived mesenchymal stem cells protect against experimental liver fibrosis in rats.
World J. Gastroenterol. 2005, 11: 3431-3440.

29. Zuk P.A., Zhu M., Mizuno H., Huang J., Futrell J.W., Katz A.J., Benhaim P., Lorenz H.P. & Hedrick M.H.
Multilineage cells from human adipose tissue: implications for cell-based therapies.
Tissue Eng. 2001, 7: 211-228.