Int J Clin Exp Med 2016;9(7):13965-13971
www.ijcem.com /ISSN:1940-5901/IJCEM0024230

Original Article

Effect of nuclear receptor corepressor on bone marrow mesenchymal stem cells proliferation

Hongwei Gao[1*], Deguo Xing[1*], Liang Wang[2], Lan Liu[3], Zhonghao Liu[1], Mingzhi Gong[1]

Departments of [1]Trauma and Orthopaedics, [2]Ultrasonography, [3]Gastroenterology, The Second Hospital of Shandong University, Jinan, Shandong Province, Peoples Republic of China. [*]Equal contributors.

Received January 17, 2016; Accepted May 20, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: Objective: Bone marrow mesenchymal stem cells (BMSCs) are considered a promising cell source for tissue engineering. Methods to advance the proliferation and anti-apoptotic capacity of mesenchymal stem cells (MSCs) are required to improve the efficiency of MSC-based therapy. In this study, we aimed to observe the effect of nuclear receptor corepressor (NCoR) on BMSC proliferation, as well as the relationship between NCoR and insulin. Method: Cells from Wistar rats were isolated, cultured, and transfected with three NCoR small interfering RNA (siRNA), and the most successful sequence was used. After cells were transfected with NCoR siRNA, we estimated cell growth and proliferation using methyl thiazolyl tetrazolium (MTT) and cell growth curve assays. Finally, BMSCs were treated with insulin at various concentrations (0, 5, 15, and 45 mmol/L) before the rate of cell proliferation was examined. Result: The third sequence was the most successful. After MTT assay and cell growth curve assay, NCoR siRNA was shown to inhibit rat BMSC proliferation after 3 days. Insulin at 15 mmol/L remarkably enhanced BMSC proliferation. When the gene NCoR was knocked down from BMSCs, the effect of various insulin concentrations on cell proliferation was inhibited. Conclusion: NCoR siRNA inhibited BMSC growth and proliferation. Insulin could promote BMSC growth and proliferation. After NCoR was knocked out, the effect of insulin on cell proliferation was inhibited. Thus, NCoR inhibited the function of insulin in cell proliferation, and PI3K signaling may play an important role in this event. Future cell studies may seek to investigate the potential underlying mechanism.

Keywords: NCoR, BMSC, proliferation, insulin

Introduction

Adult or bone marrow mesenchymal stem cells (BMSCs) are plastic adherent stromal cells found in special tissues and organs of human adults. With the capacity for self-renewal and multi-lineage differentiation, BMSCs are considered a promising cell source for tissue engineering as they are easily accessible and not associated with ethical issues in relation to their use [1, 2]. Mesenchymal stem cell (MSC)-based therapy has shown significant improvement of tissue regeneration in pre-clinical models and clinical trials. However, many challenges limit the use of MSC-based therapy [3, 4]. Thus, methods to advance the proliferation and anti-apoptotic capacity of MSCs are required to improve the efficiency of MSC-based therapy [5].

Nuclear receptor corepressor (NCoR) was initially identified based on its ability to bind unliganded retinoic acid and thyroid hormone receptors and to mediate active repression of its respective target genes through the recruitment of additional corepressor molecules [6, 7]. In addition to the nuclear receptor role, NCoR has been implicated in the pathogenesis of resistance to thyroid hormone, hypothyroidism, and certain types of leukemia [8]. In vitro studies have suggested that NCoR can exert various repressing functions via interactions with numerous other transcription factors, including NF-êB [9] and AKT [10].

NF-êB controls the expression of genes involved in a number of physiological responses, including immune inflammatory responses, acute-phase inflammatory responses, oxidative stress responses, cell adhesion, differentiation, and apoptosis [11]. The PI3K/AKT signaling pathway has been reported to play an important role in MSC growth [12], and survival [13]. PI3K plays a critical role in insulin signaling for cell

Table 1. Four pairs of siRNA squences designed based on target genes

Name	Sequence 5' to 3'
siNCoR-1-F	GGCGGAGCAGAATTACTGCTATGCCACTGA
siNCoR-1-R	TCGAGAATAAGCCGACGAGAATAACTGCA
siNCoR-2-F	TGCATTGATGGCTATTATCACCTTAACTGGA
siNCoR-2-R	CCGAGTTAAACGATAGATGGCTATATTCAGC
siNCoR-3-F	GATCCAGGAAGAGTGTTCCTGATTTTCAAGA
siNCoR-3-R	GAAATCAGGAACACTCTTCCTTTTTTTGGAAA
Negative-F	TGCCCGCTTTGATGGATTGCCATGGGCGAGCG
Negative-R	TGGAGTTAACGGCGCTTTGTACGAATCGGTCG

proliferation [14]. The activity of PI3K is necessary to elicit many of the effects of insulin on glucose and lipid metabolism, indicating that it is an essential downstream effector of insulin signaling.

In our previous study, we reported that NCoR can negatively regulate adipogenic differentiation in rat MSCs. However, little information is available regarding the effects of NCoR on the biological functions of MSCs. In this study, we observed the beneficial effects of NCoR on MSC proliferation and survival, and the role of NCoR in insulin-enhanced BMSC proliferation.

Materials and methods

Cell isolation and culture

A total of 40 four-week-old female Wistar rats were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, and maintained under pathogen-free conditions for a week. All animals received humane care in compliance with the Guide for the Care and Use of Experimental Animals. BMSCs were isolated from bilateral femora and tibias of rats and incubated in medium containing á-minimal essential medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO_2. The medium was changed after 2 days for the first time and then every 3 days thereafter. When cells were grown to 90% confluence, the cells were expanded into dishes as the first generation. All cells used for this experiment were the MSCs of the fourth generation.

Plasmids and small interfering RNA (siRNA)

According to the NCoR sequence from Gen Bank (EU006039.1), we designed three sequences of NCoR siRNA and a negative Non-target control siRNA (**Table 1**). For transient transfection, cells were cultured with serum-free medium for 2 days and then transfected with 6 ìg of NCoR siRNA construct for 2 days using HiPerFect (Qiagen) according to the manufacturer's protocols. The efficiency of NCoR siRNA was determined by Western blot analysis, and NCoR expression was detected using Western blot assay. Gene expression of NCoR was analyzed by real-time PCR.

Western blot analysis

For Western blot analysis, cells were collected and incubated in RIPA buffer containing a protease inhibitor cocktail on ice for 30 min. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to PVDF membranes. The membranes were blocked in 5% non-fat milk for 2 h at room temperature and incubated at 4°C overnight with polyclonal anti-NCoR (1:1000 dilution; Sigma). After overnight incubation, the membranes were washed and immunoblotted with HRP-conjugated anti-rabbit IgG antibody (1:1000 dilution; Amersham Biosciences, Tokyo, Japan) at 37°C for 1 h. The membranes were then developed with enhanced chemiluminescence substrate (Beyotime, Shanghai, China) and exposed to X-ray film. â-Actin (monoclonal anti-â-actin, 1/1000, Beyotime) was used to ensure adequate sample loading for all Western blots. Band density was quantitated using Image J software.

Real-time RT-PCR

The expression of NCoR in MSC was determined at indicated times by RNA preparation and quantitative reverse transcription polymerase chain reaction (RT-PCR). In brief, total cellular RNA was isolated from cells on six-well plates using TRIZOL reagent following the manufacturer's instructions (Invitrogen). RNA quality was assessed by agarose gel electrophoresis, and complementary DNA was synthesized with a random hexamer (TaKaRa, Osaka, Japan). Real-time PCR analysis was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) under the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. The reaction ran at one

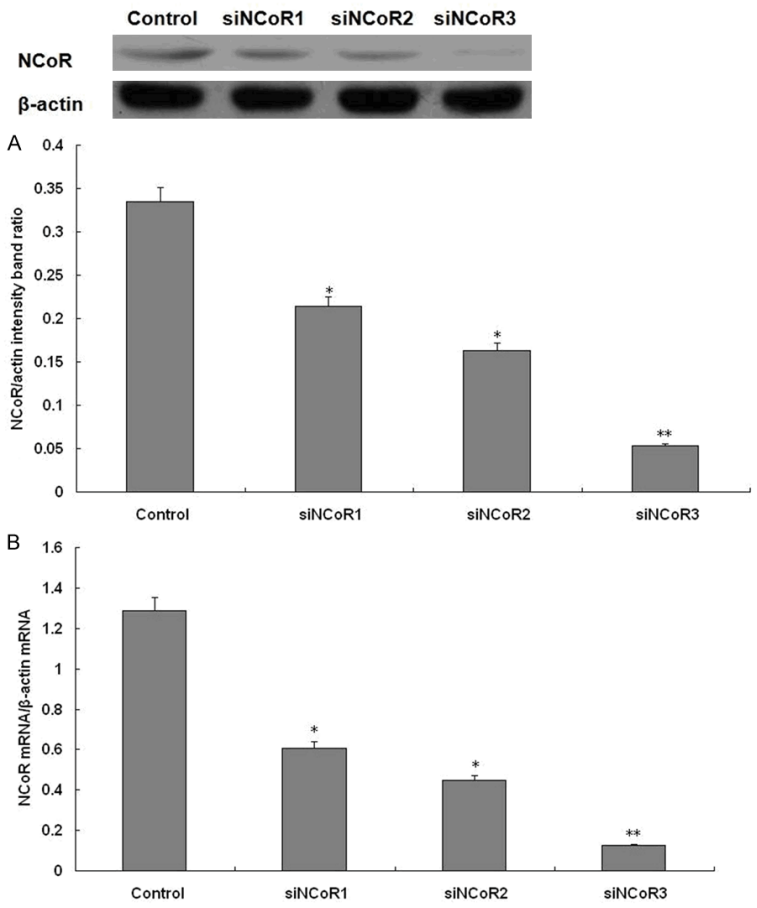

Figure 1. The interference effect of NCoR siRNA on rat BMSCs. Cells were seeded in 6-well plates and then transfected with 6 ìg non-target (negative control) or NCoR siRNA in medium for 2 d. Western blot (A) and RT-PCR (B) were performed to determine the efficiency of the siRNA knockdown. Data are expressed as mean ± SD. *P<0.05; **P<0.01; or P>0.05 vs control.

cycle of 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. We used â-actin expression as an internal control. Specific primer sequences were synthesized in BIOSUNE Biological Technology Corp. (Shanghai, China). The sequences of the primers of NCoR were TAGTCATACTTCCTGGTGAC (sense) CGATCGTGGAGTCAACACTG (anti-sense).

Cell proliferation assay

The endogenous effects of NCoR on cell viability were evaluated by methyl thiazolyl tetrazolium (MTT) assay. In brief, isolated and identified rat MSCs were seeded in 96-well plates at a density of 1.0×10^6/mL in medium. When cells attained 65% confluence, cells were transfected with NCoR siRNA and non-target NCoR siRNA. After incubation for 1, 2, 3, 4, and 5 days in medium, cell viability was evaluated by adding 15 ìL of 5 mg/mL MTT (Sigma-Aldrich Corp., St. Louis, MO, USA) solution to each well of one cell culture plate and further incubating for 4 h. The medium was removed, and 150 ìL of dimethyl sulfoxide was added to each well. Subsequently, the plate was agitated for 10 min on a shaker to dissolve formazan. Absorbance at 490 nm was determined using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell growth curve assays

BMSCs were seeded in 60 mm plates at a density of 1.0×10^4 cells/plate for cell growth curve assay. Cells were counted at 0, 1, 2, 3, 4, and 5 days after seeding. Cells were digested with 0.25% trypsin (Invitrogen), resuspended in 1 mL of PBS, and counted with an automated cell counter (TC10™, Bio-Rad Laboratories, Hercules, CA, USA). An equivalent volume of trypan blue was added to the cell suspension to exclude nonviable cells.

Insulin interference

We added 4 different concentrations of insulin (0, 5, 15, and 45 mmol/L) to the medium for interfering with MSC growth. We added insulin to the medium for every group, and cell viability was evaluated by MTT and the cell counter after incubation for 2 days.

Statistical analysis

Statistical analysis was carried out with one-way ANOVA using SPSS17.0 software. Values are expressed as the means ± standard deviation (SD). The mean values and SDs were calculated from three independent experiments. Differences were considered statistically significant at P<0.05.

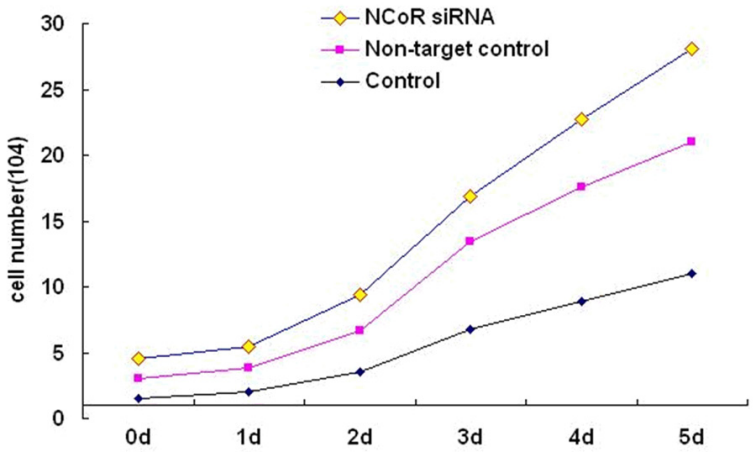

Figure 2. Effect of NCoR siRNA on the growth of rat BMSCs. Cells attaining 65% confluency in medium were transfected with NCoR siRNA, and cell growth was determined by cell growth curve assay at 0, 1, 2, 3, 4 and 5 d. NCOR siRNA was shown to decrease BMSC number after 2 d. Data are expressed as mean ± SD. *P<0.05; **P<0.01; or P>0.05 vs control.

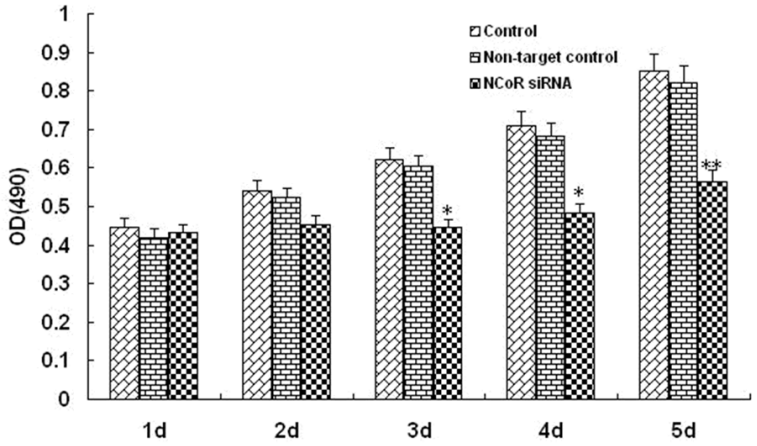

Figure 3. Effect of NCoR siRNA on the proliferation of rat BMSCs. Cells attaining 65% confluency in medium were transfected with NCoR siRNA, and cell proliferation was determined by MTT method at 1, 2, 3, 4 and 5 d. NCOR siRNA was shown to inhibit rat BMSC proliferation after 3 d. Data are expressed as mean ± SD. *P<0.05; **P<0.01; or P>0.05 vs control.

Result

Efficiency of NCoR siRNA

To investigate the effects of NCoR siRNAs, cells cultured in medium of 65% confluence were transfected with 3 NCoR siRNAs or control sequence. The expression of NCoR in MSCs cultured in medium was determined by Western blot assays and real-time PCR. Following NCoR transfection of three different sequences of siRNA for 2 days, the expression levels of primary NCoR was significantly decreased compared with the cells that were transfected with the control sequences. The third sequence, siNCoR3, has the most significant effect of silence NCoR expression than the first and second sequence, so this sequence was used in following experiment (**Figure 1**).

Effect of NCoR on BMSC proliferation

BMSCs were transfected with NCoR siRNA before cells were counted with the cell counter. The rate of cell proliferation was examined. NCoR siRNA was shown to decrease BMSC proliferation afterward 2 days by MTT assay (**Figure 2**) and afterward 3 days by growth curve assay (**Figure 3**).

NCoR siRNA inhibits the effect of insulin-induced BMSC proliferation

BMSCs were treated with insulin at 4 different concentrations (0, 5, 15, and 45 mmol/L) before the rate of cell proliferation was examined using MTT assay. Insulin at 15 mmol/L was shown to remarkably enhance BMSC proliferation, where as insulin at 45 mmol/L had no effect on BMSC proliferation. However, when the gene NCoR was knocked down from BMSC, the effect of various concentrations of insulin on cell proliferation was inhibited. This result was also confirmed by the cell growth curve assay (**Figure 4**).

Discussion

BMSCs can readily be isolated and expanded from bone marrow aspirates. Their abilities to immunosuppress and promote tissue repair have led to clinical trials for exploring their broad therapeutic potential. MSCs can differentiate into multiple cell lineages [15], secrete

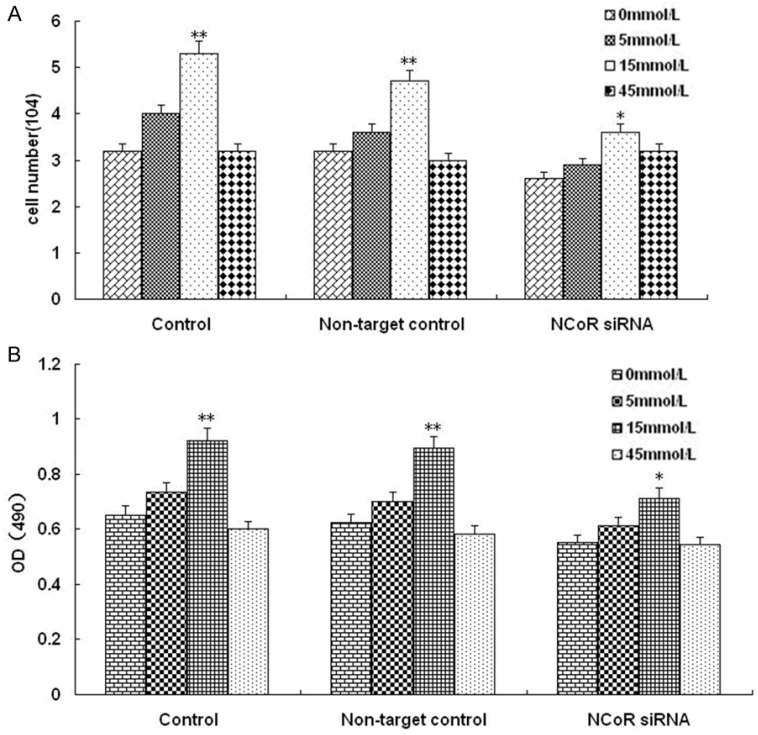

Figure 4. Effect of NCoR siRNA on insulin-induced cell proliferation of rat BMSCs. BMSC were treated with insulin at several concentrations (0, 5, 15, and 45 mmol/L), the rate of cell proliferation was examined with cell growth curve assay (A) and the MTT assay (B). Data are expressed as mean ± SD. *P<0.05; **P<0.01; or P>0.05 vs control.

several factors (growth factors and cytokines) with important functions in tissue regeneration [16], are immune privileged [17], and secrete immunomodulatory factors [18, 19]. Therefore, the proliferation of BMSC is critical for MSC-based therapy or cell trials.

NCoR forms corepressor complexes with histone deacetylase 3 to induce changes in the local chromatin structure and cause transcriptional repression. In vitro studies have suggested that NCoR can exert various repressing functions via interactions with numerous other transcription factors, including NF-êB [9], AKT [10], and PPARã [20]. PPARã is a member of the nuclear hormone receptor family and is highly enriched in adipose tissue, where it plays a critical role in adipocyte differentiation, insulin sensitivity, and adipokine/cytokine secretion [21]. AKT plays an important role in osteogenic differentiation of MSCs [10]. In our previous study, we reported that NCoR can negatively regulate adipogenic differentiation in rat MSCs. Thus, NCoR is related to cell differentiation of BMSCs, but the effect of NCoR on cell proliferation is the basic problem for subsequent cell trials and MSC-based therapy in a clinical setting.

The transcriptional activity of NCoR and its complex is involved in regulating important pathways that set the criteria for tumor characteristics; the NCoR signaling cascade promotes prostate tumorigenesis [22], and NCoR controls glioblastoma tumor cell characteristics [23]. Our results demonstrated that NCoR siRNA could inhibit BMSC growth and proliferation, and cell morphology did not change. Therefore, NCoR played an important role in cell growth and proliferation, which was consistent with the findings of other studies.

Several studies have shown that insulin therapy may increase the cancer risk [24]. Hyperinsulinemia in a non-obese mouse model of type 2 diabetes has been reported to lead to increased mammary tumor growth [25]. Blocking the insulin receptor (IR) tyrosine kinase via the inhibitor BMS-536924 reduces tumor growth in these mice [25]. Moreover, reduction of circulating insulin levels using a beta 3-adrenergic receptor agonist (CL-316243) or downregulation of IR in cancer cells and xenografts decreases tumor growth [26] and reduces cell proliferation and metastasis. Our results revealed that insulin could promote BMSC growth and proliferation, and insulin at 15 mmol/L produced the best effect.

The PI3K signaling pathway critically regulates cell growth and survival, and it is necessary for insulin action. PI3K activity is necessary to elicit many of the effects of insulin on glucose and lipid metabolism, indicating that it is an essential downstream effector of insulin signaling. Hence, insulin promotes cell growth and proliferation by the PI3K signaling pathway [27]. One study reported that NCoR, via protein-protein

interaction, is a novel regulator of PI3K signaling and can modulate thyroid tumor progression [28]. In our study, cell proliferation was inhibited when various concentrations of insulin were added to the medium for incubating BMSCs with knocked out NCoR. This finding indicated that NCoR inhibited the function of insulin in cell proliferation, and PI3K signaling may be the important link between NCoR and insulin.

Conclusion

BMSC proliferation is critical for MSC-based therapy or cell trials. Our results showed that NCoR siRNA could inhibit BMSC growth and proliferation, and cell morphology did not change. Insulin could promote BMSC growth and proliferation, and insulin at 15 mmol/L produced the best effect. When various concentrations of insulin were added to the medium for incubating BMSCs with knocked out NCoR, cell proliferation was inhibited. Thus, NCoR inhibited the function of insulin in cell proliferation, and PI3K signaling may play an important role in the relationship between NCoR and insulin. Future cell studies may seek to investigate the potential underlying mechanism.

Acknowledgements

This work was supported by the grant from the National Natural Science Foundation of Shandong Province, China (No. 2013ZRE27251).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Mingzhi Gong, Department of Trauma and Orthopaedics, The Second Hospital of Shandong University, Jinan, Shandong Province, Peoples Republic of China. E-mail: gongmingzhi2014@163.com

References

[1] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.

[2] Bianco P, Riminucci M, Gronthos S and Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001; 19: 180-192.

[3] Potier E, Ferreira E, Meunier A, Sedel L, Logeart-Avramoglou D and Petite H. Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. Tissue Eng 2007; 13: 1325-1331.

[4] Chavakis E and Dimmeler S. Homing of progenitor cells to ischemic tissues. Antioxid Redox Signal 2011; 15: 967-980.

[5] Xu J, Qian J, Xie X, Lin L, Zou Y, Fu M, Huang Z, Zhang G, Su Y and Ge J. High density lipoprotein protects mesenchymal stem cells from oxidative stress-induced apoptosis via activation of the PI3K/Akt pathway and suppression of reactive oxygen species. Int J Mol Sci 2012; 13: 17104-17120.

[6] Chen JD and Evans RM. A transcriptional corepressor that interacts with nuclear hormone receptors. Nature 1995; 377: 454-457.

[7] Guenther MG, Lane WS, Fischle W, Verdin E, Lazar MA and Shiekhattar R. A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. Genes Dev 2000; 14: 1048-1057.

[8] Jepsen K and Rosenfeld MG. Biological roles and mechanistic actions of co-repressor complexes. J Cell Sci 2002; 115: 689-698.

[9] Perissi V, Aggarwal A, Glass CK, Rose DW and Rosenfeld MG. A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. Cell 2004; 116: 511-526.

[10] Payne KA, Meszaros LB, Phillippi JA and Huard J. Effect of phosphatidyl inositol 3-kinase, extracellular signal-regulated kinases 1/2, and p38 mitogen-activated protein kinase inhibition on osteogenic differentiation of muscle-derived stem cells. Tissue Eng Part A 2010; 16: 3647-3655.

[11] Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999; 18: 6853-6866.

[12] Zhao Y, Zhang S, Zeng D, Xia L, Lamichhane A, Jiang X and Zhang F. rhPDGF-BB promotes proliferation and osteogenic differentiation of bone marrow stromal cells from streptozotocin-induced diabetic rats through ERK pathway. Biomed Res Int 2014; 2014: 637415.

[13] Wang J, Li Z, Zhang Y, Liu X, Chen L and Chen Y. CX43 change in LPS preconditioning against apoptosis of mesenchymal stem cells induced by hypoxia and serum deprivation is associated with ERK signaling pathway. Mol Cell Biochem 2013; 380: 267-275.

[14] Luo J and Cantley LC. The negative regulation of phosphoinositide 3-kinase signaling by p85 and it's implication in cancer. Cell Cycle 2005; 4: 1309-1312.

[15] Kode JA, Mukherjee S, Joglekar MV and Hardikar AA. Mesenchymal stem cells: immu-

nobiology and role in immunomodulation and tissue regeneration. Cytotherapy 2009; 11: 377-391.
[16] Meirelles Lda S, Fontes AM, Covas DT and Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine Growth Factor Rev 2009; 20: 419-427.
[17] Le Blanc K, Tammik C, Rosendahl K, Zetterberg E and Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol 2003; 31: 890-896.
[18] Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A and Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002; 30: 42-48.
[19] Tse WT, Pendleton JD, Beyer WM, Egalka MC and Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003; 75: 389-397.
[20] Li P, Fan W, Xu J, Lu M, Yamamoto H, Auwerx J, Sears DD, Talukdar S, Oh D, Chen A, Bandyopadhyay G, Scadeng M, Ofrecio JM, Nalbandian S and Olefsky JM. Adipocyte NCoR knockout decreases PPARgamma phosphorylation and enhances PPARgamma activity and insulin sensitivity. Cell 2011; 147: 815-826.
[21] Tontonoz P and Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem 2008; 77: 289-312.
[22] Yoo JY, Lim BJ, Choi HK, Hong SW, Jang HS, Kim C, Chun KH, Choi KC and Yoon HG. CK2-NCoR signaling cascade promotes prostate tumorigenesis. Oncotarget 2013; 4: 972-983.
[23] Heldring N, Nyman U, Lonnerberg P, Onnestam S, Herland A, Holmberg J and Hermanson O. NCoR controls glioblastoma tumor cell characteristics. Neuro Oncol 2014; 16: 241-249.
[24] Hemkens LG, Grouven U, Bender R, Gunster C, Gutschmidt S, Selke GW and Sawicki PT. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. Diabetologia 2009; 52: 1732-1744.
[25] Novosyadlyy R, Lann DE, Vijayakumar A, Rowzee A, Lazzarino DA, Fierz Y, Carboni JM, Gottardis MM, Pennisi PA, Molinolo AA, Kurshan N, Mejia W, Santopietro S, Yakar S, Wood TL and LeRoith D. Insulin-mediated acceleration of breast cancer development and progression in a nonobese model of type 2 diabetes. Cancer Res 2010; 70: 741-751.
[26] Fierz Y, Novosyadlyy R, Vijayakumar A, Yakar S and LeRoith D. Insulin-sensitizing therapy attenuates type 2 diabetes-mediated mammary tumor progression. Diabetes 2010; 59: 686-693.
[27] Di Zazzo E, Feola A, Zuchegna C, Romano A, Donini CF, Bartollino S, Costagliola C, Frunzio R, Laccetti P, Di Domenico M and Porcellini A. The p85 regulatory subunit of PI3K mediates cAMP-PKA and insulin biological effects on MCF-7 cell growth and motility. ScientificWorldJournal 2014; 2014: 565839.
[28] Furuya F, Guigon CJ, Zhao L, Lu C, Hanover JA and Cheng SY. Nuclear receptor corepressor is a novel regulator of phosphatidylinositol 3-kinase signaling. Mol Cell Biol 2007; 27: 6116-6126.