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N-CoR MODULATES OSTEOGENIC DIFFERENTIATION OF RAT MESENCHYMAL STEM CELLS THROUGH THE PI3K/AKT-CELL SIGNALING PATHWAY

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Abstract: The nuclear receptor corepressor (N-CoR) is involved in the regulation of diverse transcription factors. We previously found that N-CoR could regulate adipogenic differentiation of rat mesenchymal stem cells (MSCs), but whether it modulated osteogenic differentiation of this type of cells was uncertain. Therefore, in the present study, we investigated the effect and mechanism of N-CoR on osteogenic differentiation of rat MSCs. The results showed that MSCs cultured in osteogenic medium successfully differentiated into osteogenic cells. Overexpression of N-CoR decreased cell proliferation, alkaline phosphatase (ALP) activity, calcium accumulation, mRNA expression of genes including bone sialoprotein (BSP), osteocalcin (OCN), osteopontin (OPN), Osterix and Runx2, and protein expression of phosphor-Akt (pAkt). Conversely, knocking down cellular N-CoR by small interfering RNA (siRNA) promoted pAkt activity and cell differentiation. Overexpression or knockdown of N-CoR had no significant influences on the protein expression of pyruvate dehydrogenase kinase isozyme 1 (PDK1), phosphatidylinositol 3-kinase (PI3K) and total Akt, indicating that N-CoR regulated the changes in the PI3K/Akt signaling pathway by targeting pAkt. To further prove the function of the PI3K/Akt signaling in N-CoR-regulated osteogenic differentiation, we used the PI3K inhibitor (LY294002) to block the activation of the PI3K/Akt pathway and found that overexpression of N-CoR showed no effects on ALP activity, calcium level and mRNA expression of BSP, osteocalcin OCN, OPN, Osterix and Runx2 in rat MSCs following the inhibition of the PI3K/Akt pathway. These findings suggest that N-CoR regulates osteogenic differentiation of rat MSCs through suppression of the PI3K/Akt signaling pathway.

Key words: N-CoR; MSCs; osteogenic differentiation; PI3K; Akt

INTRODUCTION

Mesenchymal stem cells (MSCs) isolated from bone marrow with properties of low immunologic rejection and self-renewal potential [1,2] can differentiate into a variety of cell types, including fibroblasts, adipocytes, osteoblasts, chondrocytes and myoblasts [3]. Osteogenesis of MSCs is a complex biological process that involves the differentiation of mesenchymal cells into preosteoblastic cells and mature osteoblasts, and finally results in the synthesis of bone matrix proteins and calcium deposition. Furthermore, osteoblastic differentiation from MSCs plays a crucial role in bone formation [4]. In order to treat osteoporosis and other bone diseases, it is important to understand the roles of the factors that regulate differentiation of MSCs.

The nuclear receptor corepressor (N-CoR) was reported to play an important role in the regulation of diverse transcription factors. Recent studies have shown that N-CoR is localized in both the nucleus and the cytoplasm [5,6]. The redistribution of N-CoR to the cytoplasm reveals a novel molecular mechanism for controlling the differentiation of neural stem cells into astrocytes [5]. Since the discovery of N-CoR, numerous studies have mainly focused on its functions in transcription regulation [7]. A new study found that the motility and proliferation of tumor cells were

suppressed by cellular N-CoR siRNA knockdown [8], indicating a novel regulatory role of N-CoR.

PI3K/Akt signaling pathways exist in a large number of mammalian cells and exert multiple pro-survival functions, including cell proliferation, differentiation, survival, migration, invasion and metabolism. Studies proved that aberrant regulation of PI3K/Akt signaling led to diseases such as cancer and autoimmunity [9,10]. In the PI3K/Akt signaling pathway, the lipid kinase-PI3K generates phosphatidylinositol-3,4,5-phosphate (PIP3), which further activates PDK1 and Akt [11]. After being fully activated by PDK1, Akt becomes powerful and translocates into the cell cytosol and nucleus, where it causes the activation of a variety of other important signals [12]. Previous results revealed N-CoR as a novel regulator of PI3K signaling, which modulates thyroid tumor progression [8]. A more recent study suggested that N-CoR negatively regulated adipogenic differentiation of rat MSCs [13]. However, whether N-CoR and its activated signaling pathways were involved in the osteogenic differentiation of rat MSCs remained uncertain. Therefore, in the present study, we made a mechanistic study of the proliferation and osteogenic differentiation of rat MSCs induced by N-CoR, which might provide a base knowledge for our understanding the correlation between N-CoR and its meditated PI3K/AKT-cell signaling pathway in the regulation of osteogenic differentiation of rat MSCs.

MATERIALS AND METHODS

Ethics statement

The *in vivo* experiment was carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Shanghai Laboratory Animal Center (Permit Number: 15-632). All animals received humane care in compliance with the Guide for the Care and Use of Experimental Animals (Animal Care Committee, 2002).

Isolation, culture and osteogenic differentiation induction of rat MSCs

Sixty 6-week-old Wistar rats (200±10 g) were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Bone marrow cells were isolated from the bilateral femora and tibias of rats and incubated in medium containing an α-minimal essential medium (α-MEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2. When cells had grown to 90% confluency, they were cultivated as the first generation. All cells used for this experiment were MSCs of the third generation. Rat MSCs of passage 3 were cultured in the osteogenic medium, which was α-MEM supplemented with 10% FBS, 100 nM dexamethasone, 5 mM β-glycerophosphate (Sigma-Aldrich, St. Louis., MO, USA) and 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis., MO, USA), as previously described [14]. Rat MSCs and MSC-derived cells in culture were identified through visualizing the morphology under an inverted microscope (Olympus, Tokyo, Japan). After culture in osteogenic medium for 1, 7, 14, 21 and 28 days, ALP activity and calcium accumulation were tested and the expression levels of BSP, OCN, OPN, Osterix and Runx2 gene expression were analyzed by real-time PCR.

XTT assay

The endogenous effects of N-CoR on cell viability were evaluated using the 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) Cell Viability Assay Kit (Sigma, CA, USA), as described [15]. Briefly, isolated and identified rat MSCs were seeded in 96-well plates at a density of 1.0×10^6/mL in osteogenic medium. When cells attained 65% confluency, they were transfected with N-CoR siRNA or non-targeting N-CoR siRNA. Further, cells were transfected with pCMV-N-CoR plasmid constructed in our laboratory. On days 1, 2, 3 and 5, after discarding the medium, 100 μL fresh medium and 25 μL XTT solution were added to the cells in each well. The plate was then put into an incubator. After 5 h, optical density (OD) in each well was estimated at a wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Real time RT-PCR

Expressions of BSP, OCN, OPN, Osterix and Runx2 in MSC-derived cells were measured by RNA preparation and quantitative reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA isolation (Invitrogen, Carlsbad, CA, USA) and real-time RT-PCR analysis (Qiagen, Valencia, CA) were performed according to the manufacturers' instructions. The reaction run was 1 cycle of 56°C for 2 min and 94°C for 15 min, followed by 40 cycles of 94°C for 10 s, 58°C for 30 s and 72°C for 45 s. We used β-actin expression as an internal control. Specific primer sequences were synthesized in the BIOSUNE Biological Technology Corp (Shanghai, China), and sequences of the primers are shown in Table 1.

Western blot analysis

The Western blot method was used to analyze protein expression of N-CoR, PI3K, PDK1, total Akt and pAkt. Briefly, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Then the membranes were blocked in 5% non-fat milk for 2 h at room temperature and incubated at 4°C overnight with polyclonal anti-N-CoR (1:1000 diluted; Sigma, CA, USA), PI3K (1:1000 diluted; Cell Signaling Technology, MA, USA), PDK1 (1:1000 diluted; Cell Signaling Technology, MA, USA), total Akt (1:1000 diluted; Cell Signaling Technology, MA, USA) and pAkt (1:1000 diluted; Cell Signaling Technology, MA, USA). After overnight incubation, the membranes were immunoblotted with HRP-conjugated anti-rabbit IgG antibody (diluted 1:1000; Abcam, Cambridge, UK) at 37°C for 40 min, developed with enhanced chemiluminescence (ECL) substrate (Pierce, Wisconsin, USA) and exposed to X-ray film. β-actin (diluted 1/1000, Santa Cruz Biotechnology, CA, USA) was used to ensure adequate sample loading for all Western blots. Band density was quantitated using Image J software.

Table 1. Primers used in this study

Gene	Serial number	Primers
β-actin	NM_031144.3	Sense: 5'-GACATGCCGCCTGGAGAAAC-3' Antisense: 5'-AGCCCAGGATGCCCTTTAGT-3'
Runx2	NM_001278483.1	Sense: 5'-GGCTGTGGAGTTTGGTGTCTA-3' Antisense: 5'-TCTGCTAAATTCTGCTTGGGT-3'
BSP	X86100.1	Sense: 5'-CTGGCACAGGCTATACAGGGT-TAG-3' Antisense: 5'-ACTGGTCCGGTTTATGCCTTG-3'
OCN	M23637.1	Sense: 5'-AAGGTGGTGAATAGACTCCG-3' Antisense: 5'-AAACGGTGGTGCCATAGATG-3'
Osterix	Y177399.1	Sense: 5'-TAATGGGCTCCTTTCACCTG-3' Antisense: 5'-CACTGGGCAGACAGTCAGAA-3'
OPN	M99252.1	Sense: 5'-CACTCCAATCGTCCCTAC-3' Antisense: 5'-AGACTCACCGCTCTTCAT-3'

Small interference RNA (siRNA) assay

N-CoR-specific siRNA was as follows: GATCCAGGAAGAGTGTTCCTGATTTTCAAGA (sense) GAAATCAGGAACACTCTTCCTTTTTTTGGAAA (anti-sense). For transient transfections, cells cultured in osteogenic medium were grown to 60% confluence and then transfected with 6 μg of N-CoR-specific siRNA construct or the negative control siRNA using HiPerFect (Qiagen, Valencia, CA, USA), according to the manufacturer's protocols.

ALP activity

Measurement of cellular ALP activity was performed as previously described [16]. On days the experiments were performed, the medium was removed, and the cell layers were rinsed with PBS three times and lysed by lysis buffer containing 0.1% Triton X-100 (Sigma, CA, USA). Twenty μL of cell lysate was mixed with 100 μL of Tris-glycine buffer (PH 10.3) (BioRad, Hercules, CA, USA), 2 mM $MgCl_2$ (Sigma, CA, USA) and 100 Ml p-nitrophenyl phosphate (Sigma, CA, USA). The reaction mixture was incubated at 37°C for 30 min and the reaction was stop by adding 50 μL of NaOH. Absorbance was read at 405 nm using a microplate reader (Olympus, Tokyo, Japan).

Calcium assay

Calcium deposition was measured in 0.5 N HCl extracts according to the manufacturer's instructions contained in Diagnostic Kit 587 (Sigma, CA, USA). Total calcium was expressed as μg/mg cellular protein. Cell numbers were determined using the nuclear dye, crystal violet [17].

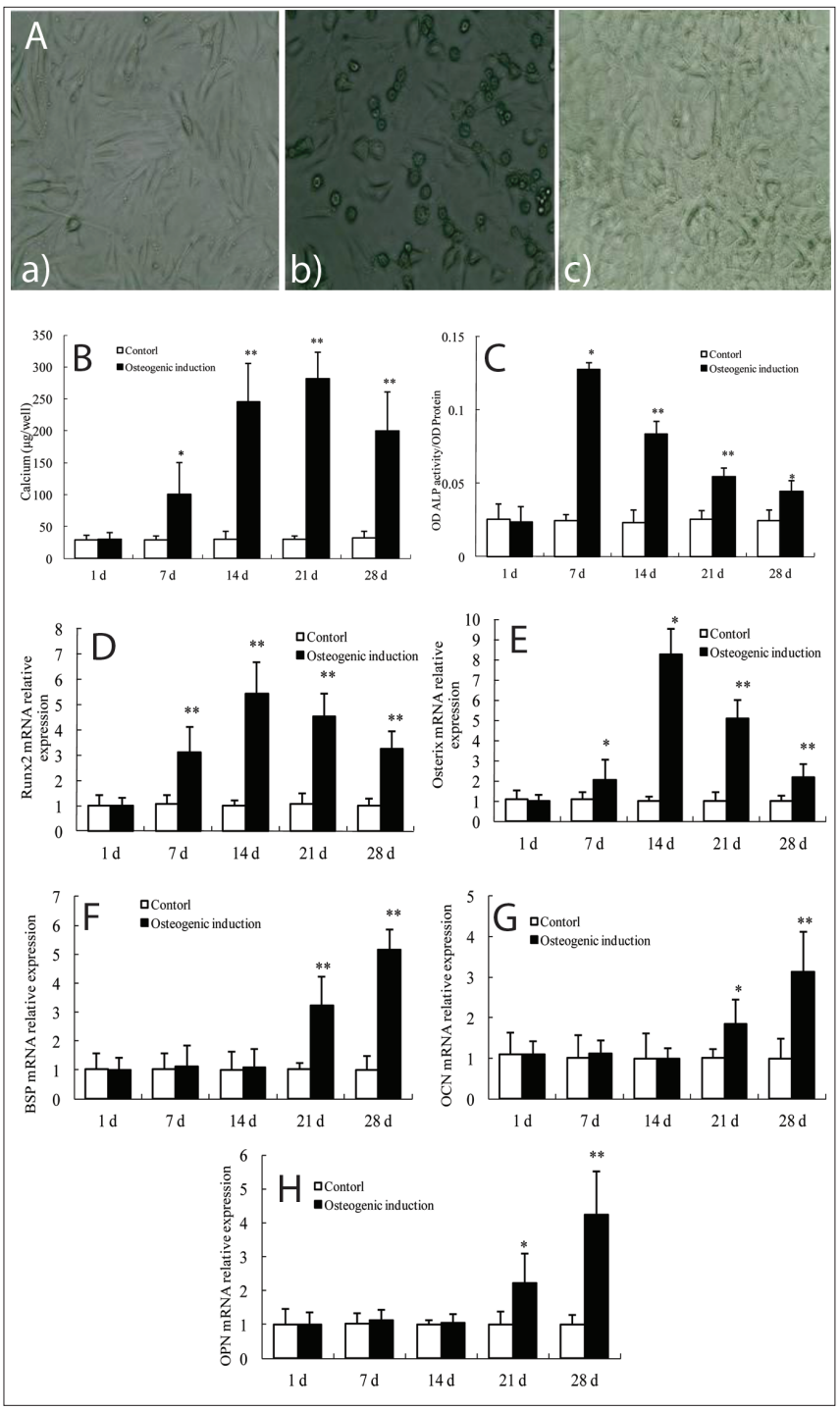

Fig. 1. Osteogenic differentiation induction of rat MSCs at different times of cultureing. MSCs (a) were induced to osteogenic differentiation (b,c) in osteogenic medium following 28 days of culture. (**A**). On days 1, 7, 14, 21 and 28, calcium deposition (**B**), ALP activity (**C**) and osteogenic differentiation-related marker genes Runx2 (**D**), Osterix (**E**), BSP (**F**), OCN (**G**) and OPN (**H**) were measured by different methods, as indicated. All measured items were induced at different times, indicating successful induction of osteogenic differentiation of rat MSCs in the osteogenic medium. Data are expressed as means±SD of three independent experiments in six replicates.

Statistical analysis

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

RESULTS

Induction of rat MSC osteogenic differentiation

It was found that, as shown in Fig. 1A, MSCs were derived from rat bone marrow, and MSCs of the third generation (a in Fig. 1A) were successfully induced to differentiate into osteogenic cells (b and c in Fig. 1A) by adding osteogenic medium. Under this condition, the calcium deposition level, ALP activity and marker genes related to rat MSC osteogenic differentiation were measured at 1, 7, 14, 21 and 28 days. Calcium deposition was detected at 7 days, and increased markedly at 21 days (Fig. 1B) ($P<0.05$). ALP activity was increased at 7 days, while decreasing the following days (Fig. 1C) ($P<0.05$). Moreover, real-time RT-PCR analyses showed that mRNA expression of Runx2 (Fig. 1D) and Osterix (Fig. 1E) was found at 7 days and reached a peak at 14 days ($P<0.05$), and BSP (Fig. 1F), OCN (Fig. 1G) and OPN (Fig. 1H) expression was found at 21 days of osteogenic differentiation induction ($P<0.05$). Therefore, under the osteogenic induction condition, the onset of osteogenic differentiation of rat MSCs began 7 days post induction initiation.

Effects of N-CoR on MSC-derived cell proliferation

To further determine the individual roles of N-CoR in osteogenic differentiation of rat MSCs, we established osteogenic differentiation cells constitutively expressing siRNA targeting N-CoR and pCMV-N-CoR at 7 days when rat MSCs were induced to differentiate. After 2 days, the efficiency of knockdown and overexpression of N-CoR in osteogenically differentiated rat MSCs was tested by Western blot. It was seen that, following N-CoR inhibition or overexpression, the protein expression of N-CoR was significantly decreased as compared with cells that were transfected with non-targeting siRNA (Fig. 2A), and increased heavily in pCMV-N-CoR-treated cells in comparison with the control (Fig. 2B). On days 1, 2, 3 and 5 after transfection, cell viability was detected by the XTT method. It was suggested that, compared to the control, XTT activity of cells in N-CoR siRNA was significantly promoted at the examined times, whilst it

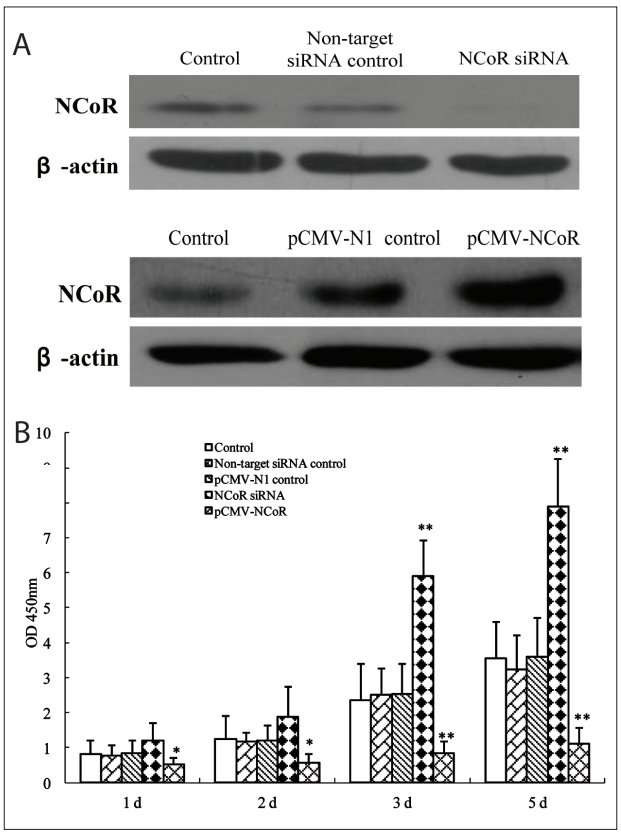

Fig. 2. Effects of siRNA knockdown or overexpression of cellular N-CoR on osteoblast-derived proliferation of rat MSCs. **A** – expression of N-CoR after siRNA knockdown. Cells were seeded in 6-well plates and then treated with 6 μg negative control and N-CoR siRNA for 48 h. Western blot was performed to determine the efficiency of the siRNA knockdown of N-CoR. **B** – expression of N-CoR after transfection of cells with pCMV-N-CoR. Cells were seeded in 6-well plates and then treated with 2 μg negative control and pCMV-N-CoR for 48 h. Western analysis was performed to determine the efficiency of overexpression of N-CoR. **C** – cell proliferation in osteoblast-derived rat MSCs was measured by XTT Cell Viability Assay Kit on days 1, 2, 3 and 5 after cells transfected with siRNA N-CoR or pCMV-N-CoR. Data are expressed as means±SD of three independent experiments in six replicates.

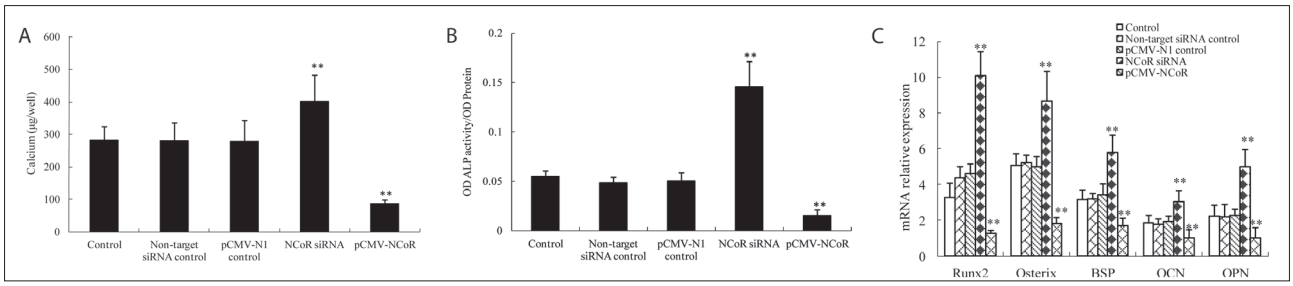

Fig. 3. Effects of siRNA knockdown or overexpression of cellular N-CoR on osteogenic differentiation of rat MSCs. On day 21, calcium deposition (**A**), ALP activity (**B**) and osteogenic differentiation-related marker genes Runx2, Osterix, BSP, OCN and OPN (**C**) significantly decreased in overexpressing N-CoR-treated cells, and increased in siRNA knockdown N-CoR-treated cells as compared with the control. Data are expressed as means±SD of three independent experiments in six replicates.

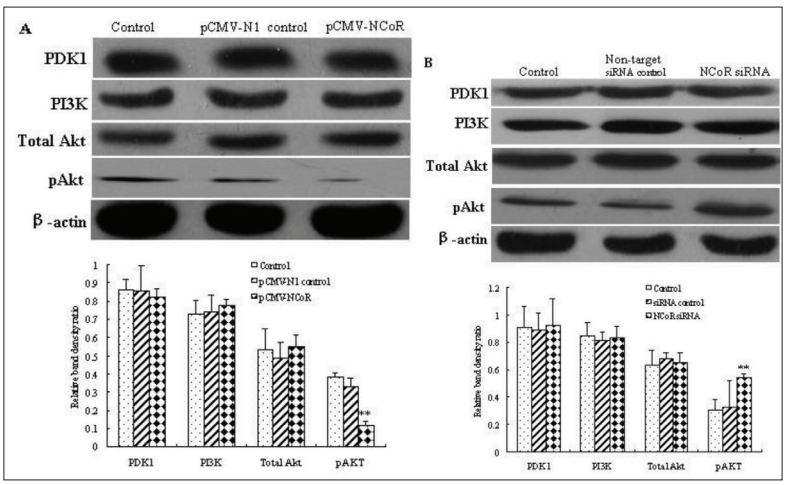

Fig. 4. Effects of siRNA knockdown or overexpression of cellular N-CoR on the PI3K/Akt signaling pathway. **A** – activities of PI3K, PDK1, total Akt and pAkt of osteogenic differentiation cells treated with N-CoR siRNA or negative control were measured by Western analysis. **B** – protein expression of PI3K, PDK1, total Akt and pAkt of osteogenic differentiation cells treated with pCMV-N-CoR or negative control were determined by Western blotting. Blots are representative of three independent experiments.

was inhibited in the N-CoR overexpressing cells (Fig. 2C) ($P<0.05$).

Effects of N-CoR on osteogenic differentiation of rat MSCs

Following 21 days of osteogenic differentiation induction, cells were transfected with N-CoR siRNA, non-targeting N-CoR siRNA, pCMV-N-CoR and pCMV-N1. About 3 days later, calcium deposition, ALP activity and osteogenic differentiation related genes were measured by different methods. As shown in Fig. 3, it demonstrated that, compared to the control, overexpression of N-CoR in cells significantly abrogated calcium deposition (Fig. 3A), ALP activity (Fig. 3B), and Runx2, Osterix, BSP, OCN and OPN expression (Fig. 3C), whereas N-CoR siRNA induced higher activity of these indexes ($P<0.05$).

Effects of N-CoR on the activation of the PI3K/Akt signaling pathway

The effects of N-CoR on the activation of the PI3K/Akt signaling pathway were determined by treatment

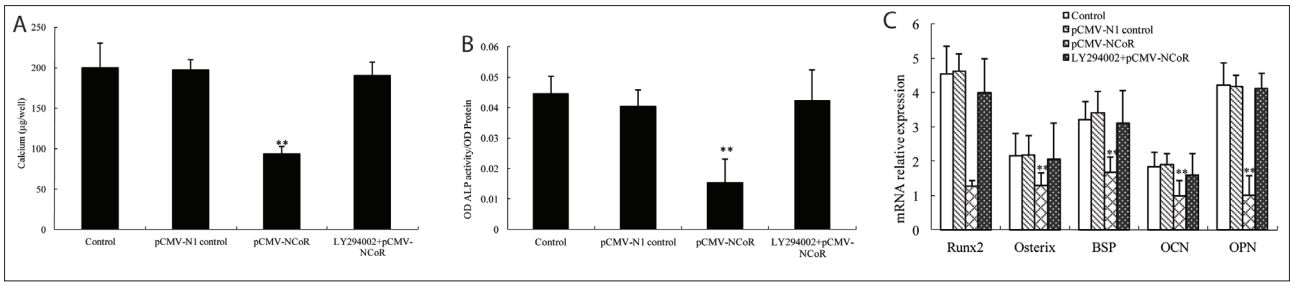

Fig. 5. The PI3K/Akt signaling was involved in N-CoR-induced osteogenic differentiation of MSCs. PI3K inhibitor Ly294002 was used to assess the inhibition of the PI3K/Akt pathway on N-CoR-induced osteogenic differentiation of MSCs. Overexpression of N-CoR showed no effects on the level of calcium deposition (**A**), ALP activity (**B**) and osteogenic differentiation genes Runx2, Osterix, BSP, OCN and OPN expression (**C**), after blocking the PI3K/Akt pathway. Data are expressed as means±SD of three independent experiments in six replicates.

of cells with pCMV-N-CoR and N-CoR siRNA. The results showed that overexpression of N-CoR decreased the expression of pAkt (Fig. 4A) ($P<0.05$), whereas N-CoR siRNA produced a promotion of pAkt as determined by Western blot (Fig. 4B) ($P<0.05$). Total Akt, PDK1 and PI3K in both pCMV-N-CoR cells and N-CoR siRNA cells showed no changes in comparison with that in the control ($P>0.05$). Taken together, these findings indicated that N-CoR influenced the activation of the PI3K/Akt signaling pathway.

The PI3K/Akt pathway signaling is involved in N-CoR-dependent functions

To elucidate whether PI3K/Akt signaling is necessary for N-CoR-dependent osteoblast differentiation, LY294002, a selective inhibitor of PI3K, at a dose of 50 mM, was used to prevent the activation of PI3K/Akt signaling. pCMV-N-CoR or N-CoR siRNA was stably transfected in cells, and osteogenic differentiation was quantified after 2 days of stimulation. The results suggested that, compared to the control, overexpression of N-CoR heavily prohibited calcium deposition (Fig. 5A), ALP activity (Fig. 5B), and Runx2, Osterix, BSP, OCN and OPN mRNA expression (Fig. 5C) induced by the osteogenic medium ($P<0.05$). However, when PI3K/Akt signaling was blocked by LY294002, N-CoR overexpression showed no influences on calcium deposition, ALP activity and osteogenic differentiation-related gene expression ($P>0.05$). These findings indicated that PI3K/Akt signaling was involved in N-CoR-dependent rat MSCs osteoblast differentiation.

DISCUSSION

Mesenchymal stem cells (MSCs) isolated from bone marrow and other adult tissues are proved to be multipotent progenitor cells, and they can be differentiated into multiple cell lineages, including fat, bone, muscles, ligament, cartilage and neurons [18,19]. Recent studies have reported that several factors, such as bone morphogenetic proteins (BMPs) [20], osteogenic growth peptide (OGP) [21] and 1, 25-dihydroxyvitamin D3 [22] play an important role in the differentiation of MSCs. N-CoR was also found to interact with the ligand-binding domains of PPARδ as well as PPARγ and PPARα, indicating that N-CoR was involved in cell differentiation. Therefore, in the present study, the role and mechanism of N-CoR on osteogenic differentiation of rat MSCs were investigated.

We found that rat MSCs began to differentiate after cells were cultured in osteogenic medium for 7 days. The XTT assay was used to determine cell viability in osteogenically differentiated rat MSCs. It was indicated that N-CoR was able to negatively regulate cell proliferation. To further elucidate the function and mechanism of N-CoR, six marker genes related to osteoblast differentiation were investigated. ALP secreted by osteoblasts is an early marker frequently used to demonstrate osteoblast differentiation [23,24]. Runx2, which belongs to the Runx family, contributes to osteoblastic differentiation of multipotent mesenchymal cells [25], and induces ALP activity and bone matrix protein expression in osteoblastic cells [26]. Furthermore, Runx2 regulates its target genes such as

BSP, OCN and OPN by binding the promoter region. OPN and Osterix act as direct downstream targets of Runx2 [27]. Osterix is also required for differentiation of preosteoblasts into mature osteoblasts [28]. OCN regulates osteoblast maturation and matrix mineralization at the late stage of bone formation [29]. This study suggested that N-CoR overexpression down-regulated ALP activity and Runx2, Osterix, BSP, OCN and OPN expression, while N-CoR knockdown exerted the contrary functions, indicating N-CoR negatively regulated osteogenic differentiation of rat MSCs.

The PI3K/Akt signaling has been implicated in differentiation of cells such as skeletal muscle cells, adipocytes, osteoblasts, chondrocytes and myoblasts [30-32]. Furthermore, Akt1/Akt2 is the key element in osteoclast differentiation [33], and is involved in bone development [34]. We assessed three members of the PI3K/Akt signaling pathway in order to confirm pathway activation. PDK1, a novel serine/threonine kinase, can activate a group of protein kinases in the AGC kinase subfamily in response to different stimulation [35]. Numerous studies suggest that PDK1 has pivotal roles in mediating cell proliferation, migration and energy metabolism [36,37]. We found that overexpression of N-CoR could inhibit protein expression of pAkt, while knockdown of N-CoR could promote the expression of this protein, indicating the regulation of N-CoR in the PI3/Akt signaling pathway. We further hypothesized that this inactivation of the PI3K/Akt pathway could be an important factor in N-CoR-exerted proliferation and differentiation of rat MSCs. To further verify our hypothesis, we used LY294002, the specific inhibitor of PI3K to block the activation of the PI3K/Akt pathway. We observed that N-CoR overexpression was capable of inhibiting osteogenic differentiation of rat MSCs, and that this effect was not significant following blockage of the PI3K/Akt pathway by LY294002, indicating that N-CoR could inhibit osteogenic differentiation of rat MSCs through suppression of the PI3K/Akt signaling pathway.

CONCLUSIONS

This study underscores the importance of N-CoR in regulating the differentiative responses of rat MSCs cultured in osteogenic medium. N-CoR could suppress osteogenic differentiation of rat MSCs through targeting the PI3K/Akt signaling pathway. These observations might provide a potential mechanism for modulating PI3K/Akt inactivation and the commitment of rat MSCs to osteogenic differentiation.

Authors' contributions: Yi Qin and Guoping Cao designed this study, carried out Western blot analysis and drafted the manuscript; Yi Qin and Guoping Cao contributed equally to this work; Jichao Ye and Peng Wang performed RT-PCR and participated in the interpretation of data; Liangbin Gao and Suwei Wang maintained the cell culture and performed data analysis; as the corresponding author of this study, Huiyong Shen participated in the design of the study and revised the manuscript. All authors read and approved this version to be published.

Conflict of interest disclosure: The authors declare that they have no competing interests.

REFERENCE

1. Ding DC, Shyu WC, Lin SZ. Mesenchymal stem cells. Cell Transplant. 2011;20:5-14.
2. Ma L, Feng XY, Cui BL, Law F, Jiang XW, Yang LY, Xie QD, Huang TH. Human umbilical cord Wharton's Jelly-derived mesenchymal stem cells differentiation into nerve-like cells. Chin Med J (Engl). 2005;118:1987-93.
3. Caplan AI. Mesenchymal stem cells. J Ortho Res. 1991;9:641-50.
4. Aubin J, Triffitt JT. Mesenchymal stem cell and osteoblast Differentiation. In: Bilezikian J, Raisz LG, Rodan GA, editors. Principles of bone biology. San Diego: Academic Press; 2002. p. 59-82.
5. Hermanson O, Jepsen K, Rosenfeld MG. N-CoR controls differentiation of neural stem cells into astrocytes. Nature. 2002;419:934-9.
6. Sardi SP, Murtie J, Koirala S, Patten BA, Corfas G. Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. Cell. 2006;127:185-97.
7. Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature. 1995;377:397-404.
8. Furuya F, Guigon CJ, Zhao L, Lu C, Hanover JA, Cheng SY. Nuclear receptor corepressor is a novel regulator of phosphatidylinositol 3-kinase signaling. Mol Cell Biol. 2007;27(17):6116-26.

9. Jiang BH, Liu LZ. PI3K/PTEN signaling in angiogenesis and tumorigenesis. Adv Cancer Res. 2009;102:19-65.
10. Oak JS, Fruman DA. Role of phosphoinositide 3-kinase signaling in autoimmunity. Autoimmunity. 2007;40:433-41.
11. Akanbi KA, Brodie AE, Suryawan A, Hu CY. Effect of age on the differentiation of porcine adipose stromal-vascular cells in culture. J Anim Sci. 1994;72(11):2828-35.
12. Kim EH, Suresh M. Role of PI3K/Akt signaling in memory CD8 T cell differentiation. Front Immunol. 2013;4:20.
13. Gao HW, Liu Lan, Xing DG, Liu ZH, Ren P, Li ZQ, Shan GQ, Gong MZ. NCoR negatively regulates adipogenic differentiation of mesenchymal stem cells. In Vitro Cell Dev Biol Anim. 2015;51(7):749-58
14. Li B, Dong Z, Gao L, He X, Liao L, Hu C, Wang Q, Jin Y. Lipopolysaccharide differentially affects the osteogenic differentiation of periodontal ligament stem cells and bone marrow mesenchymal stem cells through Toll-like receptor 4 mediated nuclear factor κB pathway. Stem Cell Res Ther. 2014;5(3):67.
15. Huyck L, Ampe C, Van Troys M. The XTT cell proliferation assay applied to cell layers embedded in three-dimensional matrix. Assay Drug Dev Technol. 2012;10(4):382-92.
16. Son KM, Park HC, Kim NR, Lee IS, Yang HC. Enhancement of the ALP activity of C3H10T1/2 cells by the combination of an oxysterol and apatite. Biomed Mater. 2010;5(4):044107.
17. Westergren-Thorsson G, Onnervik PO, Fransson LA, Malmström A. Proliferation of cultured fibroblasts is inhibited by L-iduronate-containing glycosaminoglycans. J Cell Physiol. 1991;147(3):523-30.
18. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JM, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-7.
19. Wang YK, Chen CS. Cell Adhesion and mechanical stimulation in the regulation of mesenchymal stem cell differentiation. Cell Mol Med. 2013;17(7):823-32.
20. Chen D, Ji X, Harris MA, Feng JQ, Karsenty G, Celeste AJ, Rosen V, Mundy GR, Harris SE. Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. J Cell Biol. 1998;142:295-305.
21. Tontonoz P, Hu E, Spiegelman B. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor gamma. Curr Opin Genet Dev. 1995;5:571-6.
22. Kveiborg M, Flyvbjerg A, Eriksen EF, Kassem M. 1,25-Dihydroxyvitamin D3 stimulates the production of insulin-like growth factor-binding proteins-2, -3 and -4 in human bone marrow stromal cells. Eur J Endocrinol. 2001;144(5):549-57.
23. Allen MJ. Biochemical markers of bone metabolism in animals: uses and limitations. Vet Clin Pathol. 2003;32:101-13.
24. Sila-Asna M, Bunyaratvej A, Maeda S, Kitaguchi H, Bunyaratavej N. Osteoblast differentiation and bone formation gene expression in strontiunm-inducing bone marrow mesenchymal stem cell. Kobe J Med Sci. 2007;53:25-35.
25. Komori T. Runx2, a multifunctional transcription factor in skeletal development. J Cell Biochem. 2002;87:1-8.
26. Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A. Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem. 1999;274:6972-8.
27. Westendorf JJ, Zaidi SK, Cascino JE, Kahler R, van Wijnen AJ. Runx2 (Cbfa 1, AML-3) interacts with histone deacetylase 6 and represses the p21 (CIP1/WAF1) promoter. Mol Cell Biol. 2002;22(22):7982-92.
28. Zhang C. Transcriptional regulation of bone formation by the osteoblast specific transcription factor Osx. J Orthop Surg Res. 2010;5:37.
29. Peng S, Zhou G, Luk KD, Cheung KM, Li Z. Strontium promotes osteogenic differentiation of mesenchymal stem cells through the Ras/MAPK signaling pathway. Cell Physiol Biochem. 2008;23:165-74.
30. Ghosh-Choudhury N, Abboud SL, Nishimura R, Celeste A, Mahimainathan L, Choudhury GG. Requirement of BMP-2-induced phosphatidylinositol 3-kinase and Akt serine/threonine kinase in osteoblast differentiation and Smad-dependent BMP-2 gene transcription. J Biol Chem. 2002;277:33361-8.
31. Kaliman P, Vinals F, Testar X, Palacin M, Zorzano A. Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. J Biol Chem. 1996;271:19146-51.
32. Sakaue H, Ogawa W, Matsumoto M, Kuroda S, Takata M, Sugimoto T, Spiegelman BM, Kasuga M. Posttranscriptional control of adipocyte differentiation through activation of phosphoinositide 3-kinase. J Biol Chem. 1998;273:28945-52.
33. Sugatani T, Hruska KA. Akt1/Akt2 and mammalian target of rapamycin/Bim play critical roles in osteoclast differentiation and survival, respectively, whereas Akt is dispensable for cell survival in isolated osteoclast precursors. J Biol Chem. 2005;280(5):3583-9.
34. Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev. 2003;17:1352-65.
35. Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol. 1997;7(10):776-89.
36. Mora A, Komander D, van Aalten DM, Alessi DR. PDK1, the master regulator of AGC kinase signal transduction. Semin Cell Dev Biol. 2004;15(2):161-70.
37. Toker A, Newton AC. Cellular signaling: pivoting around PDK-1. Cell. 2000;103(2):185-8.