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RESEARCH ARTICLE

EFFECTS OF NCoRsiRNA ON PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS IN HIGH GLUCOSE MICROENVIRONMENT

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ABSTRACT

Background: Chronic hyperglycemia enhances osteoporosis in diabetic patients mainly by inhibiting osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). Nuclear receptor co repressor (NCoR) has been reported to negatively modulate osteogenic differentiation of Rat BMSCs in a standard culture medium, conversely, its knockdown promoted osteogenic differentiation. In this study, we investigated the effects of NCoR knockdown on proliferation and osteogenic differentiation of MSCs under a high glucose microenvironment.

Methods: Cells from Wistar rats were isolated, transfected with NCoR small interfering RNA (NCoRsiRNA), cultured in various glucose concentrations (5.5, 16.5,25 and 35mmol/L) and then cell proliferation determined using methyl thiazolyltetrazolium (MTT). Osteogenic differentiation of cells cultured in normal (5.5mmol/L) and high glucose (25mmol/L) was determined through quantitative changes in mineralization (calcium accumulation), alkaline phosphatase (ALP) activity and expression of osteoblast marker genes including Runx2, osteocalcin (OCN), Osterix, bone sailoprotein (BSP) and osteopontin (OPN)using quantitative Real Time PCR.

Results: NCoR knockdown in MSCs cultured in high glucose (25mmol/L) inhibited cell proliferation but resulted in increased Calcium accumulation, alkaline phosphatase (ALP) activity and elevated mRNA expression of all osteoblast related genes.

Conclusion: NCoR knockdown inhibited proliferation but promoted osteogenic differentiation of BMSCs under a high glucose micro-environment.

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INTRODUCTION

Mesenchymal stem Cells (MSCs) are fibroblast multi-potent adult stromal cells found in several human tissues including bone marrow and fat (Kolf *et al*., 2007). With their regenerative and multi-lineage potential to differentiate into osteoblasts, chondrocytes or adipocytes (Holovská *et al*., 2010),coupled with lack of ethical and Medical legal issues concerning their use (Si *et al*., 2011), MSCs have become the center of attention in tissue regeneration therapy(Steinert *et al*. 2012). Promising results in the clinical application of musculoskeletal tissue regeneration therapyhas led to the recent outburst of research studies involving biological and physical factors affecting proliferation and osteogenic differentiation of mesenchymal stem cells (MSCs). Different methods of enhancing proliferation and successful induction of MSCs towards osteoblast differentiation have been devised in vitro (Glueck *et al*., 2015; Heino and Hentunen, 2008). Several factors have been reported to regulate osteoblast differentiation of MSCs including mechanical, physical and biological (Arnsdorf *et al*., 2009; Peng *et al*., 2009; Yourek *et al*., 2010). However, primary differentiation of MSCs into osteoblasts is mainly controlled by a transcription factor Runx2 (Ducy *et al*. 1997; Shakibaei *et al*., 2012). Runx2/Cbfa1 regulates osteogenic differentiation of MSCs by working as the targeted gene for many signaling pathways including but not limited to transforming growth factor 1(TGF-1), Bone morphogenetic protein (BMP), wingless type(Wnt) and (Nel)-like protein type 1(NELL) during preliminary stages of differentiation (Franceschi and Xiao 2003).

Chronic hyperglycemia is known to be the cause of derangements in many systemic metabolic processes diabetic patients, including bone metabolism (Moreira *et al*., 2015), leading to enhanced bone loss or osteoporosis; a metabolic bone disorder characterized by low bone mass and decreased bone quality (Chau *et al*., 2003). This is evidenced by the high incidence of fractures and fracture non-unions in diabetic

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patients compared to the general population (Moreira *et al*., 2015). Previous studies have reported that high glucose inhibited MSC proliferation(Zhang *et al*., 2016). Another in-vitro study demonstrated that high glucose lowers intracellular Bone Morphogenetic Protein-2(BMP-2), an important bone inducing growth factor, hence inhibiting osteoblast differentiation (Wang *et al*., 2013). However, the same study reported that high glucose promoted MSC proliferation in a dose dependent manner with the highest proliferation obtained at 25mmol/L.

Nuclear receptor co- repressor (NCoR), a known transcriptional co- regulatory protein, exert various repressing functions by interacting with numerous other transcription factors, including NF-Eb, AKT, and PPARa (Ghisletti *et al*., 2009). It has recently been shown that NCoR inhibited proliferation and osteogenic differentiation of MSCs via P13k/AKT pathway, conversely, its knockdown with small interfering RNA (siRNA) promoted their osteogenic differentiation in a standard osteogenic culture medium. However, the effects of NCoR knockdown on osteogenic differentiation of MSCs under a high glucose microenvironment has never been reported. This study was carried out to investigate this effect, testing the hypothesis that NCoR knockdown could still promote osteogenic differentiation of MSCs under high glucose. Combined with our previously reported results that NCoR knockdown promoted tissue insulin sensitivity in obese diabetic mice (Li *et al*., 2011), our results would provide an alternative preventive regeneration therapy for hyperglycemic induced osteoporosis in diabetic patients with poorly controlled blood glucose levels.

MATERIALS AND METHODS

Cell isolation and culture

A total of 40 female Wistar rats at age of 4weeks old 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. Cells were then isolated and cultured as we previously described (Hong-Wei *et al*., 2015). All cells used for this experiment were the MSCs of the fourth generation.

Plasmids and small interfering RNA (siRNA)

We designed expression plasmids for NCoRsiRNA and a negative non-target siRNA as previously done, according to the NCoR sequence from Gen Bank (EU006039.1) NCoR-specific siRNA was as follows: GATCCAGGAAGAGTGTTC CTGATTTTCAAGA (sense) GAAATCAGGAACA CT CTT CCTTTTTT TGGAAA (anti-sense). Non target siRNA: TGCCCGCTT TGA TGGATTGCCA TGG GCG AGCG (sense) TGGAGTTAACGGCGCTTTGTACGAATCGGTCG (antisense). For transient transfections, cells were cultured with serum-free osteogenic medium for 2 d and then cultured in osteogenic medium for 2 d, then transfected with 6 μg of NCoR-specific siRNAconstruct or non-target siRNA for 2 d using HiPerFect (Qiagen) according to the manufacturer's protocols. The efficiency of NCoRsiRNA was then determined by western blot analysis.

Western Blot analysis

For western blot analysis, cells were collected and incubated in RIPA buffer solution for 30 min, proteins separated by 10% sodium dedocylsulphatepolyacramide gel electrophoresis then transferred to PVDF membranes. The membranes were blocked in 5% no fat milk for 2 hours at room temperature and incubated at 4 degrees Celsius overnight with anti-NCoR (1:1000 dilution; sigma) thereafter washed and immunoblotted with HRP- conjugated anti-rabbit igG antibody 1:1000 dilution; Ameri-sham Biosciences, Tokyo, japan) at 37 degrees for 1 hour. The membranes were then developed with enhanced chemiluminescence (ECL) substrate (Beyotime, shanghai, China) and exposed to X-ray film. B-actin (Monoclonal anti B-actin, 1:1000, Beyotime) was used to ensure adequate sample loading on all western blots.

Cell proliferation assay

The endogenous effects of NCoR knock-down on BMSC cell proliferation under a high glucose micro-environment was evaluated by methyl thiazolyltetrazolium (MTT) essay. Isolated and identified rat MSCs were plated at a density of 5 x 10 3cells/well in 96 –well plates for 24h. After attaining a confluence of 65%, Cells samples were transfected with NCoR small interfering RNA (siRNA) or non-targetsiRNA then treated with Dulbecco's modified Eagles medium (DMEM; Invitrogen Gibco Rockville, MD, USA) containing normal (5.5 mmol/L) or high glucose (16,5 mmol/L; 25 mmol/L and 35mmol/L) concentrations. After incubation in the medium for nine days, MTT assay was carried out according cells proliferation kit protocol (Sigma, St. Luis, MO/USA0). Absorbance was determined at 490 nm with a micro-plate reader (Bi-Tek Instruments, Winooski, VT, USA). All the reported values are presented as the means of triplicate samples. This test was repeated three times.

Calcium assay

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

ALP activity analysis

Separate Rat BMSCs were transfected with NCoR siRNA or non-targetNCoR siRNA then cultured in osteogenic medium under normal and high glucose environment. Cells were then harvested from each culture medium using a cell scraper after seven days and placed in 1ml phosphate buffer solution. By repeated freezing and thawing, cells were lysed and centrifuged at 1500r/min for 10min, supernatant was then collected and measured for alkaline phosphate activity using ALP - ELISA kits according to the manufacturer's instructions. This test was repeated three times.

Real-time RT-PCR

Expression of osteogenic related genes Runx2, Osterix, BSP, OCN and OPN in MSC-derived osteoblast was determined at indicated times by RNA preparation and quantitative reverse transcription polymerase chain reaction (RT-PCR). Briefly, total cellular RNA was isolated from cells on 6-well plates using TRIZOL reagent following the manufacturer's

instructions (Invitrogen). RNA quality was assessed by agarose gel electrophoresis, and complementary DNA (cDNA) was synthesized with random hexamer (TaKaRa, Osaka, Japan). The RT-PCR analysis was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) under the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA), according to the manufacturers' instructions. The reaction run at 1 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 β-actin expression as an internal control.

Statistical analysis

Statistical analysis was carried out with one-way analysis of variance (ANOVA) using SPSS 17.0 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

Efficiency of NCoRsiRNA

In order to determine the effects of NCoR siRNA, MSCs cultured in medium were transfected with NCoR siRNA or no-target NCoR siRNA then using B-actin as a medium, western blot analysis was carried out to determine the expression of NCoR. Results showed significant decrease in the levels of NCoR expression after 2 days of culture in samples transfected with NCoR siRNA as compared to the non-target control and the control Fig.1.

NCoR-knock inhibited proliferation of BMSCs in high glucose

To prove the recently reported results that high glucose promoted BMSC proliferation and to observe the effects of NCoR knock down on this effect, we systematically cultured BMSC samples transfected with NCoR siRNA in four different glucose concentrations (5.5, 16.5, 25 and 35mmol/L). Results demonstrated that proliferation of BMSC cultured in DMEM containing moderately high glucose (25mmol/L) was better than that cultured in DMEM containing 5.5mmol/L glucose after 5 days. DMEM containing 16.5 and 25 mmol/L glucose both promoted BMSC proliferation, However, DMEM containing 25mmol/L glucose exerted the best proliferation effects in the four groups ($P<0.05$). Cells cultured in very high glucose concentration (35mmol/L) had the worst proliferation in the experiment. NCoR knockdown resulted in slight decrease cell proliferation in both normal (5.5mmol/L) and high glucose (16.5, 25 and 35mmol/L) media, denoting that it inhibited proliferation. However, increased proliferation was noted in both control and knockdown samples with increasing glucose concentrations, with the highest proliferation obtained at 25mmol/L as shown in Figure 2. We selected 5.5mmol/L glucose as the normal group (control) and 25mmol/L glucose as the high glucose group for further experiments.

NCoR knockdown promoted osteogenic differentiation of BMSC under high glucose micro environment

With the aim to investigate the effects of NCoR knockdown on BMSC osteogenic differentiation under high glucose conditions, cells transfected with NCoR siRNA or non-target NCoR siRNA were cultured in osteogenic media containing 25mmol/L(high glucose) and 5.5mmol/L glucose (control) respectively for 21 days. We then used previously described methods to observe quantitative changes in cellular ALP activity, calcium deposition and expression of related genes; Run2, Osterix, OCN, OPN and BSP to monitor osteogenic differentiation.

ALP Activity

After 7 days of culture in normal (5.5mmol/L) glucose, ALP activity analysis revealed a marked increased ALP activity in cell samples treated with NCoR siRNA compared to both the control and non-target siRNA controls, denoting that NCoR knockdown promoted osteogenic differentiation of BMSCs in a standard culture medium. There was a marked decrease in ALP activity noted in all cell samples (experimental and controls) after culturing in high (25mmol/L) glucose medium compared to those cultured in normal glucose (5.5mmol/L), showing that high glucose inhibited osteogenic differentiation. However, a preserved increase in ALP activity was noted in NCoR gene knockdown (NCoR siRNA treated) cells compared to both controls in samples cultured under the same high glucose concentration. This showed that NCoR knock down promoted osteogenic differentiation under a high glucose microenvironment. Obtained results were as shown in Fig 3a.

Calcium sedimentation

To determine effects of NCoR knock down on cell mineralization in high glucose, we measured quantitative amount of calcium deposition in cell samples after 21days of culture as described above. Under normal glucose (5.5mmol/L), NCoRsiRNA transfected cell samples showed a significantly higher calcium accumulation compared to both control and non-target NCoRsiRNA control, confirming that NCoR knockdown promoted osteogenic differentiation of MSCs in normal glucose. Cell culture in high a glucose (25mmol/L) medium resulted in global decrease in cell mineralization, demonstrated by the decreased absorbance, denoting inhibited osteogenic differentiation in both control and experimental samples. A persistent increase in calcium sedimentation in knocked down samples compared to both controls was observed (Fig. 3b). This persistent increase in cellar calcium sedimentation after culture in high glucose further confirmed that NCoR knockdown promoted osteogenic differentiation under high glucose.

Real Time –PCR

In order to further confirm the promoting effect of NCoR knockdown on osteogenic differentiation of BMCs in high glucose, we extracted mRNA from BMSC samples cultured in osteogenic media containing 5.5mmol/L (normal) and 25mmol/L (high) glucose for seven days and performed a quantitative real time PCR to determine changes in the expression levels of the five osteoblast marker genes; Runx2, osterix, OPN, BSP and OCN. As shown in Fig .3c, a marked increase in expression levels of all marker genes was noted in cell samples transfected with NCoR siRNA compared to both controls in culture medium containing 5.5mmol\L glucose, showing that NCoR knock down promoted osteogenic differentiation in standard conditions. Conversely, culture in high glucose resulted in decrease in the gene expression of all osteoblast maker genes in both the control and experimental cell samples. The most significant change was noted in Runx2 gene expression levels.

Gene	Accession No.	Primer sequence
β-actin	NM_031144.3	sense: 5'-GACATGCCGCCTGGAGAAAC-3' antisense: 5'-AGCCCAGGATGCCCTTTAGT-3'
NCoR	AF124821.1	sense: 5'-TAGTCATACTTCCTGGTGAC-3' antisense: 5'-CGATCGTGGAGTCAACACTG-3'
Runx2	NM_00127848 3.1	sense: 5'-GGCTGTGGAGTTTGGTGTCTA-3' antisense: 5'-TCTGCTAAATTCTGCTTGGGT-3'
BSP	X86100.1	sense: 5'-CTGGCACAGGCTATACAGGGTTAG-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'

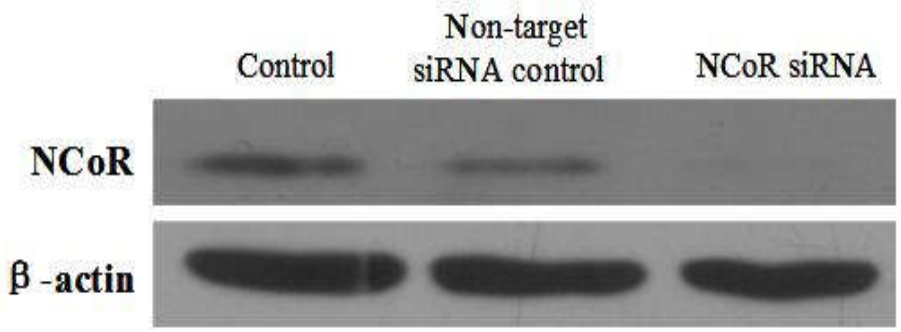

Figure 1. The interference effect of NCoRsiRNA on rat BMSCs. Cells were seeded in 6-well plates and then transfected with 6 ugNCoRsiRNA or non-target (negative control) in osteogenic medium for 2 d. Western blot analysis was performed to determine the efficiency of siRNA knockdown

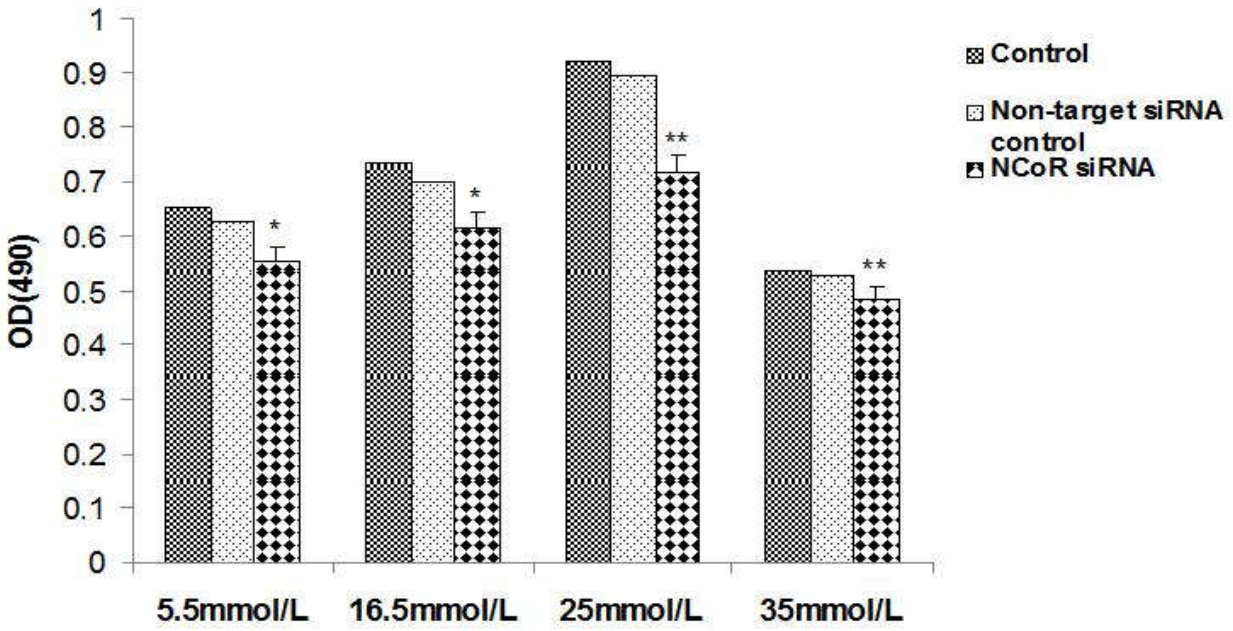

Figure 2. Effects of NCoRsiRNA on the proliferation of rat BMSCs in different glucose concentrations. Cells were transfected with NCoRsiRNA, cultured in 5.5, 16.5,25 and 55mmol/L glucose respectively for 7d and then cell proliferation determined by MTT method. NCoRsiRNA was shown to inhibit rat BMCs proliferation after 3days. Data are expressed a mean +/- SD.*P<0.05; **P<0.01; P>0.05 vs control

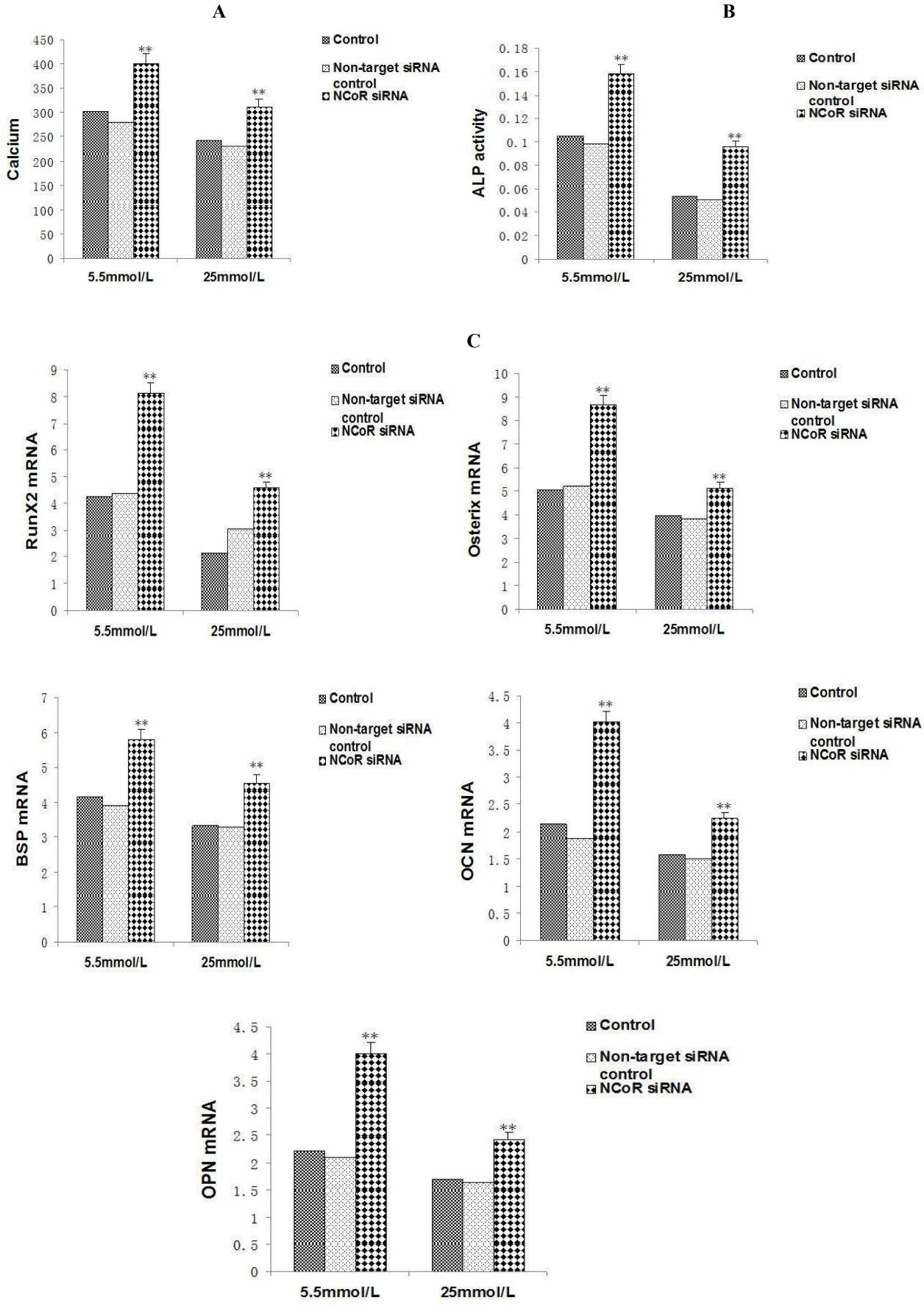

Figure 3. Effects of siRNA knockdown on osteogenic differentiation of rat MSCs in high glucose microenvironment. On day 21, calcium deposition (A), ALP activity (B) and osteogenic differentiation-related marker genes Runx2, Osterix, BSP, OCN and OPN (C) significantly increased in siRNA knockdown NCoR-treated cells as compared with the controls. Data are expressed as means±SD from three independent experiments performed in triplicates. *P<0.05 vs control

Under high glucose, NCoR gene knockdown in MSCs was accompanied by significant increase in the expression of all osteoblast maker genes compared to non-knockdown samples cultured under the same condition, confirming that it promoted osteoblast differentiation.

DISCUSSION

Hyperglycaemia, a characteristic feature a common metabolic disorder diabetes, is the cause of almost all systemic complications of the disease. Resulting from impaired insulin secretion or insufficient insulin action or both, chronic hyperglycemia has been implicated with disorders in bone metabolism leading to bone loss (Moreira *et al*., 2015). This is evidenced by the high incidence of osteoporosis in diabetic patients with poorly controlled blood sugar levels. One promising solution to combat this scourge is through the application of preventive regeneration therapy using mesechymal stem cells (MSCs) (Steinert *et al*., 2012). The ability of mesechymal stem cells to differentiate into multiple tissues like bone and fat makes them a suitable cell source for tissue regeneration therapy (Holovská *et al*., 2010). However, their fate to differentiate into a specific lineage is regulated by multiple factors yet to be fully understood. It has been established that osteoblast differentiation of MSCs is primarily controlled by Cbfa1/Runx2, however, multiple other factors regulate this process through the stages of proliferation, matrix maturation and matrix mineralization. The inhibitory effect of certain physical factors like a high glucose environment on osteogenic differentiation of MSCs has been demonstrated in vitro (Wang *et al*., 2013). Studies to devise methods of promoting osteogenic differentiation of MSC at molecular level in altered microenvironments are therefore needed to advance the application of MSC based regeneration therapy in patients with medical co-morbidities like diabetes. NCoR forms corepressor complexes with histone deacetylase 3 to induce to induce changes in the local chromatin structure and cause transcription repression. Its interaction with a number of important transcription factors (NF-Kb, AKT, PPARa) shows that it is involved in various metabolic and differentiation processes. In our previous study, we demonstrated that NCoR negatively regulated adipogenic differentiation in rat MSCs (Hong-Wei *et al*., 2015).We also demonstrated thatNCoR modulatedosteogenic differentiation of MSCs through P13K/AKT pathway in a standard osteogenic culture media. In this study we investigated the effects of NCoR knock down on proliferation and osteogenic differentiation of BMSC under a high glucose microenvironment.

We found that NCoR knockdown inhibited BMCs proliferation in high glucose medium. Similarly, this inhibition effect was observed in control samples too which was consistent with our earlier published results that NCoR siRNA could inhibit BMSCs proliferation in standard culture media. This inhibition effect was, however, insignificant ($p>0.05$) and did not seem to interfere with the enhanced proliferative effects of high glucose on MSCs. This was evidenced by the increase in cell proliferation even in knockdown samples as glucose concentration increased. The exact mechanism through which this occurs needs to be delineated, however, our results demonstrated that NCoR knockdown did not interfere with the MSCs proliferation promotion pathway of high glucose. To elucidate the effects of NCoR siRNA on osteogenic differentiation of BMSCs under a high glucose microenvironment, six marker genes related to osteoblast differentiation were investigated. ALP secreted by osteoblasts is an early marker frequently used to demonstrate osteoblast differentiation. Runx2 induces ALP activity and bone matrix protein expression in osteoblast cells (Franceschi and Xiao 2003; Shakibaei *et al*., 2012; Westendorf *et al*., 2002). OPN and osterix acts as direct downstream targets of Runx2. Osterix is also required for differentiation of preosteoblasts into mature osteoblasts (Zhang, 2010). OCN regulates osteoblast maturation and matrix mineralization at the end stage of bone formation. This study suggested that reduced expression of NCoR under high glucose up-regulated ALP activity and Runx2, Osterix, BSP, OCN and OPN expression indicating that NCoR knockdown promoted osteogenic differentiation of rat MSCs under high glucose microenvironment. In addition, we also found that transfection with NCoRsiRNA resulted in increased intracellular calcium sedimentation (mineralization), further confirming enhanced osteogenic differentiation. Future cell studies may seek to investigate the exact mechanism through which this occurs. All in all, our results demonstrated that high glucose did not affect NCoR modulation of rat MSCs osteogenic differentiation via P13K/AKT pathway, since NCoR knockdown promoted osteogenic differentiation of MSCs even in standard culture media. In our study, we demonstrated that NCoR knockdown slightly inhibited cell proliferation but greatly promoted osteogenic differentiation of rat BMSCs under a high glucose microenvironment, confirming our hypothesis.

Conclusion

Preventive regeneration therapy, through modulation of BMSC osteogenic differentiation, may just be thesolution to the hyperglycemic induced osteoporosis in diabetic patients. Our results showed that although NCoR knockdown could slightly inhibit proliferation, it significantly promoted osteogenic differentiation of BMSCs under a high glucose micro-environment. This could be used as an alternative to counteract the inhibitory effect high glucose on osteogenic differentiation of BMSCs in diabetic patients consequently help combating the scourge of osteoporosis in these patients. Combined with earlier reports that NCoR knock down promoted tissue insulin sensitivity, it would not only be used as a preventive regeneration therapy for osteoporosis, but would also optimize insulin therapy in diabetic patients by increasing tissue insulin sensitivity.

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Conflict of interest

The authors declare that they have no competing interests.

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