Lentiviral-mediated RNAi targeting p38MAPK ameliorates high glucose-induced apoptosis in osteoblast MC3T3-E1 cell line*

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The p38 mitogen activated protein kinase (p38MAPK) pathway is an important signaling cascade involved in cell growth, differentiation and apoptosis. High glucose activates p38MAPK pathway in different cells, including osteoblasts. In the present study, role of p38MAPK in high glucose induced osteoblast apoptosis and potential of RNA interference (RNAi) targeting p38MAPK as a therapy strategy have been reported. Lentiviral-mediated RNAi effectively reduced p38MAPK and p-p38MAPK expressions in osteoblastic cell line (MC3T3-E1) following high glucose (22 mM) induction. Inhibition of p38MAPK activity significantly suppressed high glucose induced apoptosis of MC3T3-E1 cell and was confirmed by flow cytometry and ultra-structural examination by transmission electronic microscope. Inhibition of p38MAPK also significantly attenuates caspase-3 and bax protein expressions, but increased significantly bcl-2 expression as determined by Western blot analysis. The results suggested that p38MAPK mediates high glucose induced osteoblast apoptosis, partly through modulating the expressions of caspase-3, bax and bcl-2. Inhibition of p38MAPK with lentiviral-mediated RNAi or its specific inhibitor provides a new strategy to treat high glucose induced osteoblast apoptosis.

Keywords: Apoptosis, High glucose, Lentivirus, p38MAPK, MC3T3-E1 cell line, RNA interference

Osteoporosis, a public health problem, is characterized by reduction of bone mass and abnormal bone micro-structure, leading to bone fragility and an increased risk of fractures of hip, spine and wrist¹. The pathologic process of osteoporosis includes increased bone resorption by osteoclasts and deficient new bone formation by osteoblasts².

Diabetes is one of the most prevalent diseases worldwide, and continues to increase in numbers and significance³,⁴. Long term hyperglycemia leads to chronic diabetic complications, including cardiovascular disease, neuropathy, nephropathy and retinopathy. Osteoporosis is also an understated complication of diabetes⁵,⁸. Diabetic patients are at an increased risk of osteoporotic fractures⁸,¹¹. Diabetes induced osteoporosis is a growing concern. Emerging evidences suggest the role of increased osteoblast apoptosis due to elevated glucose concentrations. It has been indicated that db/db mice had more apoptotic osteoblasts and reduced formation of new bone compared to normoglycemic mice¹². Advanced glycation end product (AGEs), which increased in response to hyperglycemia, stimulated osteoblast apoptosis by activating p38MAPK thereby contributing to deficient bone formation¹³,¹⁴. Moreover, high glucose also exerts a pro-apoptotic effect on mesenchymal stem cell, which may reduce the functionality and regenerative capacity of their osteogenic offspring, the developing osteoblasts¹⁵.

Higher concentration of glucose (22 and 44 mM), induced apoptosis of rat primary osteoblasts in a dose-dependent manner, and this deleterious effect had been reversed by metformine, a widely used hypoglycemic medication¹⁶. However, the underlying mechanisms by which high glucose induces osteoblast apoptosis have not been fully understood. p38MAPK is a 38 kD tyrosine phosphatase protein kinase. The p38MAPK signaling pathways mediate variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis. Recent studies have shown that p38MAPK signal transduction pathway plays an important role in regulation of osteoblast
apoptosis. p38MAPK mediates hypoxia induced osteoblast apoptosis \cite{17,18}, but prevents dexamethasone-mediated apoptosis of osteoblast \cite{19}. Both anandamide and paroxetine induce apoptosis through activating p38MAPK and caspase-3 in human osteosarcoma cells \cite{20,21}.

Hyperglycemia has been shown to activate p38MAPK pathway in many tissues, including vascular smooth muscle, aortic smooth muscle, glomeruli, nerve tissue, and retina of patients with type 1 and type 2 diabetes, leading to development of late complications of diabetes \cite{22,23}. High glucose induces cell death through phosphorylation of p38MAPK in human endothelial cell \cite{24}, and accelerates endothelial progenitor cell senescence via activation of p38MAPK \cite{25}. High glucose activates osteoclast p38MAPK in a time and concentration-dependent manner \cite{26}.

It has been reported that hyperglycemia increases p38MAPK activity in osteoblast \cite{27}, contributing to osmotic response to high glucose \cite{28}. Considering the pivotal role of p38MAPK in various stimuli-induced osteoblast apoptosis, p38MAPK may also be implicated in high glucose induced apoptosis in osteoblast.

In the present study, role of p38MAPK in high glucose induced osteoblast apoptosis has been investigated. Inhibition of p38MAPK by lentiviral-mediated RNAi in osteoblastic cell line (MC3T3-E1) has been employed to understand the possible mechanisms involved in high glucose-induced apoptosis.

**Materials and Methods**

**Reagents**—Mouse osteoblastic MC3T3-E1 cell line and 293T cell line were obtained from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Short hairpin RNA (shRNA) plasmid vector P113.7 and competent cell preparation kit were purchased from Chongqing Western Technology Co. Ltd. Lipofectamine TM2000 was purchased from Invitrogen Co. Ltd. QiPrep Spin Miniprep Kit was purchased from OMEGA Co. Ltd. DNA ligation Kit, TRNZol and Time saver cDNA synthesis Kit were obtained from TaKaRa Co. Ltd. Trizol, M-MuLV (200 U/µl) and RNAasin (40 U/µl) were purchased from Invitrogen Co. Ltd. Rabbit anti-mouse antibody p38, p-p38, Caspase-3, bax and bcl-2 were all from Santa Cruz Co. Ltd. Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Zhongshan Goldenbridge Biotechnology Co. Ltd.

**Establishment and clone of shRNA vectors targeting p38MAPK**—Based on known sequence of p38 gene in GenBank, Reynolds’ the design principles \cite{28} and online design software, three disturbance sequences were selected for their common sequence, i.e.,

- siRNA1: 5’-CTCAGAGTCTGCAAGAAACTA-3’
- siRNA2: 5’-CCAACAATTCTGCTCTGGTTA-3’ and
- siRNA3: 5’- AGAGTCTGCAAGAAACTACAT-3’.

A non-specific target sequence was also designed: 5’-GACTTCATAAGGCGCATGC-3’.

The sequences of 3 shRNA vector sequences were shRNA1: 5’-

TCTCAGAGTCTGCAAGAAACTATTTCAAGAGA TAGTTTCTTTCAGACACTCTGAGTTTTTC3’;

3’- AGAGTCTCAGACGTCTTTGATAAGTCTCTA TCAGAAGACGTCTGAGCTAAAAAGAGCT -5’; shRNA2: 5’- TCCAACAATTCTGCTCTGTTA TCCAAGAGA TAACCAGAGAATTGTTTGG TTTTTTC3’; 3’-A GGTTGTTAAGAGCAGACAAT AAGTTCTCT ATTGCTCGTCTTAAACC AAAAG AGCT-5’; shRNA3: 5’-T AGAGTCTGCAAGAAACTACAT TCCAAGAGA ATGTAGTTTCTTGACTCTCCT TTTTTTC3’; 3’-A TCTCAGACGTCTTTGAGTGA AAGTTCTCT TACATCAAGAGCAGTCAGA AAAAG AGCT-5’.

The terminator in the end was TTTTT, and in the end HpaI and XhoI were designed as the restriction enzyme cutting sites. The DNA sequence was synthesized by Chongqing Western Technology Co. Ltd. Chimaera synthesized sense chain and antisense chain were annealed with the formation of shRNA expression templates which were connected with linearized P113.7 vector cut by HpaI/XhoI enzyme. Sequence identification was carried out after screening and identification of enzyme cut. The plasmids obtained were named si-p38-1, si-p38-2 and si-p38-3.

**Packaging and purification of lentiviral vectors**—Lentiviral shuttle plasmids and vector plasmids form secondary package were prepared and subjected to high-purity endotoxin-free extraction. Co-transfection of 293T cells and was followed by replacement of complete medium after 8 h of transfection. After 48 h of incubation, cell supernatant rich in lentiviral particles were collected the virus titer was detected through flow cytometry after HeLa cell transfection.
**Cell culture and transfection of MC3T3-E1 cell**—Mouse osteoclast cell line (MC3T3-E1) was cultured in RPMI 1640 culture solution containing 10% fetal calf serum; 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO₂. The cells were harvested and seeded in 6-well plates and transfected MC3T3-E1 cell line at a cell density of 70-90% by lentiviral supernatant. After 48 h of transfection, enhanced green fluorescent protein expression in cells was observed under an inverted fluorescence microscope.

**Cell treatment**—Transfected cells were treated as per the following schedule: normal culture with no stimulating factor (normal control); culture supplemented with D-glucose (22.2 mM) (high glucose treatment); pre-culture with p38MAPK inhibitor (SB203580; 20.0 uM) for 2 h, then supplemented with D-glucose (22.2 mM) (signal transduction inhibitor treatment); transfection with shRNA-p38MAPK for 24 h, supplemented with D-glucose (22.2 mM) (shRNA transfection); transfection with negative control shRNA and liposome complexes for 24 h and then supplemented with D-glucose (22.2 mM) (negative control). All the transfected cell cultures were incubated for 7 days after each treatment as mentioned above.

**Determination of p38MAPK mRNA expression levels in MC3T3-E1 cells by RT-PCR**

**RNA extraction**—Cells were collected directly into 10 ml centrifuge tubes with cell number of (1-5)x10⁶/mL for each sample followed by centrifugation (500-1000 r/min, 10 min). The cell culture medium was washed with incubation buffer followed by centrifugation (500-1000 r/min for 5 min). Lable solutions (100 µl) were used to re-suspend the cells followed by incubation at room temperature away from light for 10-15 min and centrifuged at 500-1000 r/min for 5 min. Cell pellet was washed with incubation buffer, and add fluorescence (SA-FLOUS) solution was added and incubated at 4°C for 20 min away from light with constant stirring. The excitation wavelength of flow cytometry was 488 nm, and a pass-band filter with the wavelength of 515 nm was used to detect FITC fluorescence and another pass-band filter with the wavelength of over 560 nm was used to detect PI staining, while the cells with intact membrane showed no fluorescence.

Expression levels of p38, p-p38, Caspase-3, bax and bcl-2 in MC3T3-E1 cells of lentiviral mediated...
transfection after high glucose induction using Western blot and flow cytometry—Cells (100 mg) were homogenized in 150 µl of pre-cooled single-detergent lysis buffer for 15 sec and kept in ice bath for 10 min. Cells were disrupted by ultrasound thrice for 5 sec at 80 W followed by centrifugation (12,000 g, 4°C for 10 min. Protein quantification was made according to the instructions of PIERCE protein determination kit. The samples were packaged in refrigerator at -80°C and to adjust the protein concentration of the extract to consistent. Proteins (50 µg) were subjected to 10% SDS-PAGE followed by Western blot onto PVDF membrane.

The membrane was sealed with skim milk powder 5% for 6 h and washed thrice for 5 min/time using TBS. Then rabbit anti-mouse p38 (38 KD) (1:300), p-p38 (38 KD) (1:300), Caspase-3 (32 KD) (1:300), bcl-2 (26 KD) (1: 300), bax (23 KD) polyclonal antibody and GAPDH monoclonal antibody (37 KD) (1:300) were added followed by incubation for 12 h at 4°C. The membrane was washed twice (5 min/time) using TBS. Equivalently mixed reagents of A and B was applied to PVDF membrane and incubated for 2 min and ECL coloration. Gel image processing system was used to analyse the bands and ratio of gray values of each protein and GAPDH indicated the relative expression levels of proteins.

Observation of ultra-structures of cells—Inverted microscope was used to view and compare morphological changes in cells. Trypsin (0.25%) was used to digest and the cells and cell sediment was collected. Glutaraldehyde (3%) was added slowly to fix cells and embedded in Epon 812 for observation using JEM2000EX TEM after uranium-lead double staining of ultrathin sections.

Statistical analysis—Statistical analysis were performed using SPSS11.0 software. Values are expressed as mean ± SD. Single factor variance analysis was used for group difference and values at P<0.05 were considered statistically significant.

Results

Sequences of shRNA vectors are represented in Fig 1.

Information and graphs of plasmids of lentiviral vector system—Lentiviral packaging systems was a four-plasmid system including pRsv-REV, pMDlg-pRRE, pMD2G and the target shuttle plasmid. The shuttle plasmid can express the green fluorescent protein (GFP). pRsv-REV, pMDlg-pRRE and pMD2G contain the necessary components for viral packaging (Fig. 2).

Transfection effect of lentiviral mediated shRNA targeting P38MAPK on MC3T3-E1—After 48 h of the MC3T3-E1 transfection of lentiviral mediated shRNA targeting p38MAPK, we observed the enhanced green fluorescent protein (EGFP) expression under inverted fluorescence microscope. The results showed that EGFP expression was significantly increased. The background of si-p38-2 transfection group was

![Fig. 1—Sequence of shRNA vectors. ((A) P38_S1; (B) P38_S2; and (C) P38_S3).](image-url)
Fig. 2—Plasmids of lentiviral vector. [(A) target shuttle plasmid, (B) pRsv-REV; (C) pMDl-g-pRRE; and (D) pMD2G].
stronger than those of si-p38-1 and si-p38-3 and EGFP expression was more than the other two groups. Therefore si-p38-2 was used as a lentivirus production interfering target (Fig. 3).

**Decrease of p38MAPK mRNA expression level after the MC3T3-E1 transfection of lentiviral mediated shRNA targeting p38MAPK**—After PCR amplification, p38MAPK PCR products fully complied with the expected design and appeared in the corresponding position. As an effective stimulus, high glucose significantly induced and increased p38MAPK mRNA expression levels in MC3T3-E1 cells (Fig. 4), while the lentiviral mediated shRNA targeting p38MAPK effectively decreased p38MAPK mRNA expression levels. There was a significant difference between the normal control group and high glucose inducing group (Fig. 4).

**Effect of lentiviral mediated shRNA targeting p38MAPK on MC3T3-E1 apoptosis**—Compared with normal control group, high glucose significantly increased the apoptosis of MC3T3-E1 cells, while lentiviral mediated shRNA targeting p38MAPK and p38MAPK inhibitor significantly reduced the apoptosis of MC3T3-E1 cells induced by high glucose. Inhibitory effect on MC3T3-E1 apoptosis of lentiviral mediated shRNA targeting p38MAPK was significantly higher than that of p38MAPK inhibitor (Figs 5 & 6).
Effect of lentiviral mediated shRNA targeting p38MAPK on MAPK signaling pathway and apoptosis-related protein expression—Under the stimulation of high glucose, the protein levels of p38, p-p38, Caspase-3 as well as pro-apoptotic gene bax in MC3T3-E1 cells showed significant increase compared to normal control group. The inhibitor of apoptosis gene bcl-2 was significantly inhibited with the expression level reduced (Fig. 7). After the transfection of lentiviral mediated shRNA targeting p38MAPK, the protein expression levels of p38, p-p38, caspase-3 as well as bax in MC3T3-E1 cells were significantly decreased, while bcl-2 protein expression was restored (Fig. 7).

Effect of lentiviral mediated shRNA targeting p38MAPK on the ultra-structure of MC3T3-E1 cells—Under the transmission electron microscope, MC3T3-E1 cells in the high glucose-induced group had typical apoptotic features: condensed cytoplasm, cell membrane disintegration, swelling of endoplasmic reticulum, nucleolus disappeared, chromatin into several clumps and increased electron density. The lentiviral mediated si-p38-2 targeting p38MAPK transfection group did not show obvious morphological changes of apoptosis. No apoptotic bodies appeared and other groups showed no apparent morphological changes of apoptosis. (Fig. 8)

Discussion
Osteoporosis is one of the most common metabolic bone diseases in developed countries and growing evidence suggests that osteoporosis is also an understated complication of diabetes. In the present study the role of high glucose on osteoblast dysfunction in osteoblastic MC3T3-E1 cells has been reported. Recent studies suggest that p38MAPK, a class of mitogen-activated protein kinase, plays a crucial role in the pathophysiology of diabetes complications.

Fig. 5—Lentiviral mediated shRNA targeting p38MAPK on MC3T3-E1 apoptosis. [(A) Normal control; (B) D-glucose; (C) shRNA; (D) SB203580; and (E) Negative control].

Fig. 6—MC3T3-E1 apoptosis rate of each group compared with normal control group. [(A) Normal control; (B) D-glucose; (C) shRNA; (D) SB203580; and (E) Negative control]. Data represents over 3 independent experiments. Significant at *P<0.05, **P<0.01, ***P<0.001.
induced osteoblast apoptosis, the role of p38MAPK in hyperglycemia-induced osteoblast apoptosis has been investigated.

Glucose at the level of 22.2 mM was a level typical of poorly controlled diabetes, and was frequently used in *in vitro* study. It has been shown that this glucose level effectively mimics hyperglycemia state and activates p38MAPK in osteoblasts. This was also observed in this study, where 22.2 mM glucose markedly up-regulated p38MAPK, as demonstrated by enhanced p38MAPK mRNA level and increased p38MAPK, p-p38MAPK protein expressions. p-p38MAPK was the active form of p38MAPK, when p38MAPK was phosphorylated and the p38MAPK pathway was activated.

To explore the potential role of p38MAPK in the regulation of osteoblast apoptosis induced by hyperglycemia, p38MAPK was inhibited by targeting it with a lentiviral-mediated RNAi, a useful tool for functional analysis of genes and developing a potential therapeutic strategy for various diseases. p38MAPK was knocked down as proved by significantly decreased mRNA expression and decrease p38MAPK, p-p38MAPK protein expressions when co-incubated with high glucose (22.2 mM), compared to the 22.2 mM glucose group. SB 203580, a specific p38MAPK inhibitor, has been shown to inhibit p38MAPK activity in a body of studies, while the inhibitory effect was relatively weaker than by RNAi in this study. Effect of inhibiting p38MAPK
on high glucose-induced apoptosis by flow cytometry assay has been investigated. Corresponding to the activation of p38MAPK, apoptosis of MC3T3-E1 incubated with 22 mM glucose was also significantly potentiated relative to normal control, and this was consistent with a previous study\textsuperscript{16}. However, the pro-apoptotic effect of high glucose was potently abrogated in p38MAPK targeted group, and to a less extent in SB203580 treated group, in line with the profile of p38MAPK activities in the groups. Protective effects of RNAi and SB203580 on high glucose-induced apoptosis in MC3T3-E1 cells were further supported by ultrastructural observation with electronic microscope. Characteristic changes of apoptosis were apparently present in high glucose group, while no significant apoptosis alterations were observed in RNAi group. However, there was no observed markedly improved ultrastructures in SB203580 treated cells, which probably caused by the relatively weaker anti-apoptosis effect due to its lower inhibitory efficacy on p38MAPK.

There are evidences suggesting that caspase-3 functions as an important downstream target of p38MAPK\textsuperscript{17,20,24}, and there exist the expressions of bcl-2 and bax in osteoblast, which ratio are thought to determinate the susceptibility of osteoblast to apoptosis induced by a variety of stimuli\textsuperscript{33-36}. The results of this study showed that RNAi targeting p38MAPK and p38MAPK specific inhibitor might reverse high glucose induced up-regulation of caspase-3 and bax, and down-regulation of bcl-2, markedly alleviating osteoblast apoptosis.

The results also suggest a potential role of p38MAPK pathway in regulating of apoptosis induced by high glucose. High glucose activates p38MAPK signal cascade by modulating expressions of caspase-3, bax and bcl-2 resulting in apoptosis of osteoblast. Inhibition of p38MAPK may partly suppress the pro-apoptotic effect of high glucose on osteoblast.

Qi et al. have reported that peroxynitrite (ONOO\textsuperscript{-}), a strong oxidant, may mediate high glucose–induced osteoblast apoptosis\textsuperscript{37}. However, the mechanism suggested was different from the present study, indicating existence of diverse pathways in which high glucose affects osteoblast function.

In conclusion, the present study identified a novel pathway for high glucose induced osteoblast apoptosis and suggested inhibition of p38MAPK by
RNAi or specific inhibitors as a new strategy to protect osteoblast from high-glucose induced apoptosis.

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