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Downregulated microRNA-32 expression induced by high glucose inhibits cell cycle progression via PTEN upregulation and Akt inactivation in bone marrow-derived mesenchymal stem cells

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ABSTRACT

MicroRNAs regulate a host of physiological and pathological processes in mesenchymal stem cells (MSCs), although no published studies describe changes in microRNA expression or function in MSCs under *in vitro* hyperglycemic conditions. By using a microRNA microarray approach, we have identified that miRNA-32-5p expression is significantly reduced under hyperglycemic conditions in rat bone marrow-derived MSCs. Expression of miRNA-32-5p targets the 3′-untranslated region of the mRNA encoding phosphatase and tensin homologs deleted on chromosome 10 (PTEN), a negative regulator of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Exposure to high glucose levels reduced miR-32-5p expression, induced PTEN expression, and inhibited activation of the PI3K/Akt signaling pathway of MSCs. Conversely, overexpression of miR-32-5p inhibited the expression of PTEN, ameliorated the inhibitory effect of high glucose levels on the PI3K/Akt signaling pathway, and promoted cell cycle progression from G0/G1 to G2/M and S phases. Our study indicates that exposure of MSCs to hyperglycemic conditions reduces miR-32-5p expression and disturbs cell cycle progression through a PTEN-mediated inhibitory effect on the PI3K/Akt signaling pathway. In summary, MiR-32-5p is a potentially important therapeutic agent for preventing MSC dysfunction under hyperglycemic conditions.

1. Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent stromal cells capable of self-renewal with multiple differentiation potentials. MSCs are mobilized from the bone marrow niche, subsequently traveling to the injured tissue target and participating in tissue repair and regeneration. Based on these capabilities, MSCs have garnered increasing attention in the field of regenerative medicine [1]. Under diabetic or stress-induced hyperglycemic conditions, the high glucose levels affect MSC mobilization [2], promoting the production of reactive oxygen species (ROS) [3], accelerating cellular senescence [4], and eventually leading to an apparent decline of the body's self-repairing capabilities [5]. The studies of MSCs subject to long-term *in vitro* culturing under hyperglycemic conditions are greatly important in the application of fundamental research knowledge and quality control systems for application in stem cell therapy.

It has been reported previously that many types of abnormal genes induced by hyperglycemic conditions result in MSC dysfunction, although the precise molecular mechanism remains unclear. By investigating the stability of microRNAs during the post-transcriptional processes and the translation state of the abnormal gene induced by high glucose levels, the molecular mechanism of MSC dysfunction may be uncovered.

MicroRNAs (miRNAs) are small, non-coding RNAs that interact with the 3′-untranslated region (UTR) of their target mRNA transcripts, causing RNA degradation and inhibited translation, thereby suppressing target gene expression. MicroRNAs regulate cellular senescence, cancer development, proliferation, differentiation, apoptosis, and additional biological processes [6,7]. No published reports exist to date describing changes in microRNA expression or function in bone marrow-derived MSCs under *in vitro* hyperglycemic conditions.

We observed changes in microRNA expression in rat bone marrow-derived MSCs exposed to chronically high glucose conditions by using an miRNA microarray platform. High glucose levels reduced the expression levels of the microRNAs miR-1843-3p, miR-196c-5p, and miR-32-5p, where the decrease of miR-32-5p was most pronounced. One of the predicted targets of miR-32 lies in the 3′-UTR of phosphatase and tensin homologs deleted on chromosome (PTEN), which negatively regulates the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. The PTEN/PI3K signal pathway is adversely impacted by the pathological process of

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several diseases, such as cancer [8], inflammation [9], and diabetes [10]. We further validated whether miR-32 regulates PTEN expression, affects the activation of the PI3K/Akt signaling pathway, and inhibits cell cycle progression of MSCs.

2. Materials and methods

2.1. Materials

Rat MSCs, Dulbecco's modified Eagle media (DMEM) with 4.5 mM and 25 mM D-glucose, MSC-Qualified fetal bovine serum (FBS), Trizol reagent, and Lipofectamine 2000 were all obtained from Invitrogen (Life Technologies, Beijing, China). TIANScript Reverse Transcription Kit was obtained from Tiangen Biotech Co. (Beijing, China). SYBR FAST qPCR Kit Master Mix (2×) Universal was obtained from Kapa Biosystems (Woburn, MA, USA). Protein assay kit and peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse) were obtained from Santa Cruz Biotechnologies, Inc. (Beijing Zhongshan, China). PTEN, pan-Akt, phospho-Akt (p-Akt), GSK-3β, phospho-GSK-3β (p-GSK-3β), cyclin D1, and GAPDH antibodies were obtained from Cell Signaling Technology (Beijing Kexing, China). Rno-miR-32-5p mimics and negative oligonucleotide were synthesized (GenePharma, Shanghai, China).

2.2. Cell cultures

MSCs were cultured in 4.5 mM D-glucose DMEM with 4.5 mM D-glucose (NG group) or DMEM with 25 mM D-glucose (HG group) media supplemented with 10% MSC-Qualified FBS in a humidified atmosphere containing 5% CO_2 at 37 °C. MSCs were passaged 2–3 times a week during the exponential growth phase. After 7 days, cultured cells were collected for microRNA analysis.

2.3. Synthesis and transfection of miR-32-5p mimics oligonucleotide

The sequences for rno-miR-32-5p mimic oligonucleotides were 5′-UAU UGC ACA UUA CUA AGU UGC A-3′ for the sense strand and 5′-CAA CUU AGU AAU GUG CAA UAU U-3′ for the anti-sense strand. The negative control sequences were 5′-UUC UCC GAA CGU GUC ACG UTT-3′ for the sense strand and 5′-ACG UGA CAC GUU CGG AGA ATT-3′ for the anti-sense strand. After 24 h in serum-free medium, MSCs were transfected with 50 nM miR-32 mimic or negative control oligonucleotide using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, the medium was replaced with 4.5 mM or 25 mM D-glucose DMEM (LG and HG, respectively). After 48 h, MSCs were collected for quantitative real-time polymerase chain reaction (qRT-PCR), Western blotting, and cell cycle analysis.

2.4. Quantitative real-time PCR (qRT-PCR) analysis for miR-32 and PTEN mRNA expression

Total RNA was extracted from cells by using Trizol reagent according to manufacturer's protocol and reverse transcribed for quantification with the TIANScript RT Kit. Mature microRNAs and mRNA were quantitated using the SYBR FAST qPCR Kit according to manufacturer's protocol. The highly conserved snRNA U6 was used as an internal normalizing control for microRNA.

The primer sets for PCR (forward and reverse, respectively) were designed as follows: 5′-GCGG CGTA TTGC ACAT TACT-3′ and 5′-TCGT ATCC AGTG CAGG GTC-3′ for miR-32; 5′-CTCG CTTC GGCA GCAC ATAT ACT-3′ and 5′-ACGC TTCA CGAA TTTG CGTG TC-3′ for U6; 5′-TTCC ACAA ACAG AACA AGAT GC-3′ and 5′-GTCA TTAT CCGC ACGC TCTA T-3′ for PTEN; and 3′-GCAC CATG AAGA TCAA GATC ATT-3′ and 5′-TAAC AGTC CGCC TAGA AGCA TT-3′ for beta-actin. The quality of PCR products were assessed by performing a melting curve analysis. The relative expression ratio of miR-32 and PTEN were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blotting

MSCs were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer. Cellular protein was prepared. Equal amounts of protein (30 μg) were subjected to 6–15% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and transferred to nitrocellulose membranes. The membranes were then blocked with 5% (w/v) milk in PBS-Tween 20 for 1 h before overnight incubation with rabbit polyclonal antibody against PTEN, Akt, p-Akt, GSK-3β, p-GSK-3β, or cyclin D1, and then blotted with a horseradish peroxidase (HRP)-linked secondary antibody for 1 h. Enhanced chemiluminescence was used to analyze the proteins. The protein band intensity on each blot from three repeated experiments was quantified by scan densitometry. The fold increase was calculated by dividing the band intensity by the control sample.

2.6. Cell cycle analysis

About 1×10^6 cells were harvested and washed twice with PBS, then fixed overnight in 75% ethanol at −20 °C. Fixed cells were then washed and incubated in 100 μg/mL propidium iodide (PI) and 20 ng/mL RNase in PBS for 30 min at 37 °C. The cell cycles were assessed by flow cytometry (FACSCalibur, BD Bioscience), and FCS Express V3 software (BD Bioscience; San Jose, CA, USA) was used to perform the analysis.

2.7. Statistical analysis

All values are expressed as the mean ± SEM. For analysis of the difference among three or more groups, we used either Independent Samples *t*-Test or ANOVA with Tukey's Test. All statistical analyses were performed using SPSS v16.0 (IBM Corp., Endicott, NY, USA). The statistical significance level was set to $p < 0.05$.

3. Results

3.1. Reduced expression of miR-32-5p in high glucose-treated MSCs

Quantitative real-time PCR assays validated the observation that the expression level of miR-32-5p was significantly reduced in MSCs treated under hyperglycemic conditions. The decrease was statistically significant in MSCs treated with HG media compared with MSCs treated with NG media ($P < 0.01$; Fig. 1).

3.2. PTEN is a potential target of miR-32 in MSCs

Through the use of bioinformatics tools and the application of web-based miRNA target gene prediction portals (TargetScan 6.0, miRbase), we searched the potential target gene of miR-32 action. We found that the 3′-UTR area of the PTEN gene area contained 12 base sites matching with the miR-32 sequence (schematic representation). PTEN is considered a negative regulatory factor of the PI3K/Akt signaling pathway.

The results of qRT-PCR and Western blotting showed that PTEN mRNA (Fig. 2A) and protein levels (Fig. 2B) of MSCs exposed to hyperglycemic conditions for 7 days were significantly increased compared to mRNA and protein levels of MSCs exposed to normal glucose levels ($P < 0.05$ for both).

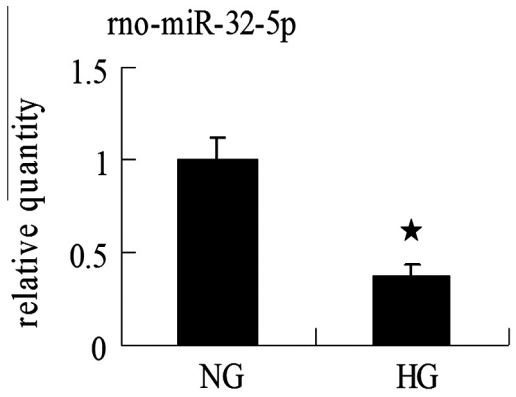

Fig. 1. MiR-32-5p is downregulated in MSCs by high glucose levels. MSCs were exposed to 4.5 mM D-glucose (NG) or 25 mM D-glucose (HG) in DMEM for 7 days. The expression of miR-32 in MSCs was verified using qRT-PCR assays. ★ Stars depict statistically significant differences in the HG group compared to the NG group, $P < 0.05$. Bars represent mean values ± SEM, $n = 6$.

3′-UTR of PTEN has a potential miR-32 binding site

2812 5′————AGUUUUAGAAAAUUUGUGCAAUA
————3′ PTEN 3′-UTR
3′–ACGUUGAAUCAUUA————CACGUUAU
————5′ rno-miR-32-5p
Position 2812–2819 of PTEN 3′ UTR

TargetScan 6.0 was used to examine all predicted miR-32 target sites in the 3′-UTR of the rat PTEN gene. The schematic representation above displays the putative miR-32-5p – binding sites within the 3′-UTR of the rat PTEN gene.

3.3. Expression of PTEN is regulated by miR-32 mimics

To further investigate the effect of miR-32 on the expression of PTEN in MSCs, the cells were transfected with 50 nM miR-32 mimic or negative control oligonucleotide using Lipofectamine 2000 for 24 h, followed by continuous culturing in NG or HG media for another 48 h. MiR-32 mimic oligonucleotide can apparently improve the production inadequacy of miR-32 induced by high glucose levels (Fig. 3A), while simultaneously reducing PTEN mRNA (Fig. 3B) and protein expression (Fig. 3C). There were no differences in the expression of miR-32 and PTEN in the miR-32–negative oligonucleotide transfected group compared with the HG-treated group.

3.4. Overexpression of miR-32 alleviates the inhibitory effect of high glucose levels on the PI3K/Akt signaling pathway

Reduced expression of miR-32 induced by high glucose levels decreased the inhibitory effects on PTEN transcription, leading to higher expression levels of PTEN. PTEN is a negative regulatory factor of the PI3K/Akt signaling pathway. Increased levels of PTEN promote GSK-3β phosphorylation, inhibit Akt phosphorylation and cyclin D1 expression, and lead to restrained cellular proliferation. MiR-32 mimic oligonucleotides could reduce the inhibitory effects of high glucose levels on the PI3K/Akt signaling pathway, promoting Akt phosphorylation and the expression of cyclin D1. MiR-32-negative control oligonucleotides did not mediate any change in the inhibited Akt signaling pathway induced by high glucose levels (Fig. 4).

3.5. MiR-32 mimic oligonucleotides alleviate the inhibitory effect on cell cycle progression induced by high glucose levels

MSCs were transfected with miR-32 mimic or negative oligonucleotide for 24 h, and then cultured in NG or HG media for another 48 h. Using flow cytometry to examine cell cycle distribution, we observed that most MSCs in the HG-treated group were arrested in the G0/G1 phase. Addition of MiR-32 mimic oligonucleotide

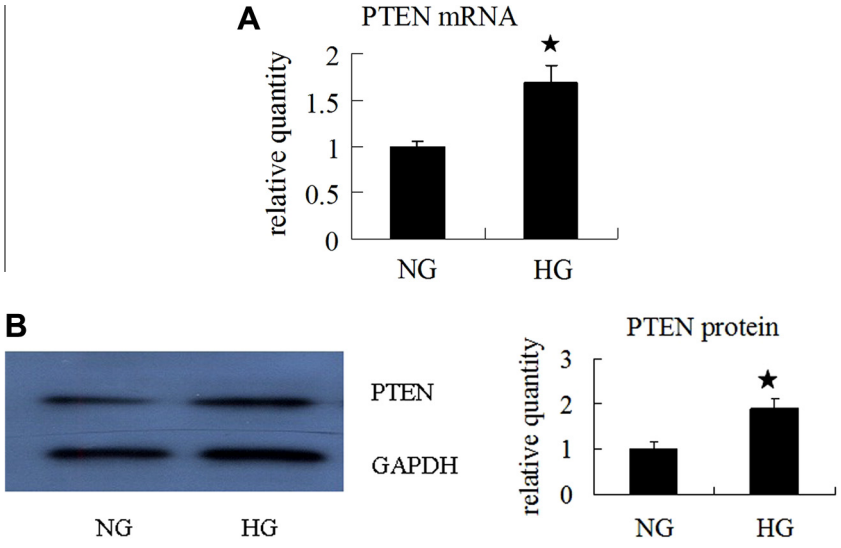

Fig. 2. Changes in the cellular level of PTEN in MSCs exposed to high glucose. PTEN has a potential miR-32-5p-binding site and is upregulated by DMEM with 25 mM D (HG) in MSCs. The expression of PTEN mRNA and protein increased in MSCs exposed to high glucose levels for 7 days compared with MSCs treated with normal glucose levels. (A) The level of PTEN mRNA was determined by qRT-PCR. Beta-actin was used as an internal control. (B) The levels of PTEN protein were determined by Western blotting, with representative blots of 4 experiments displayed alongside signal densities indicated in a bar chart. GAPDH was used as an internal loading control. ★ Stars depict statistically significant differences in the HG group compared to the corresponding controls (NG). $P < 0.05$.

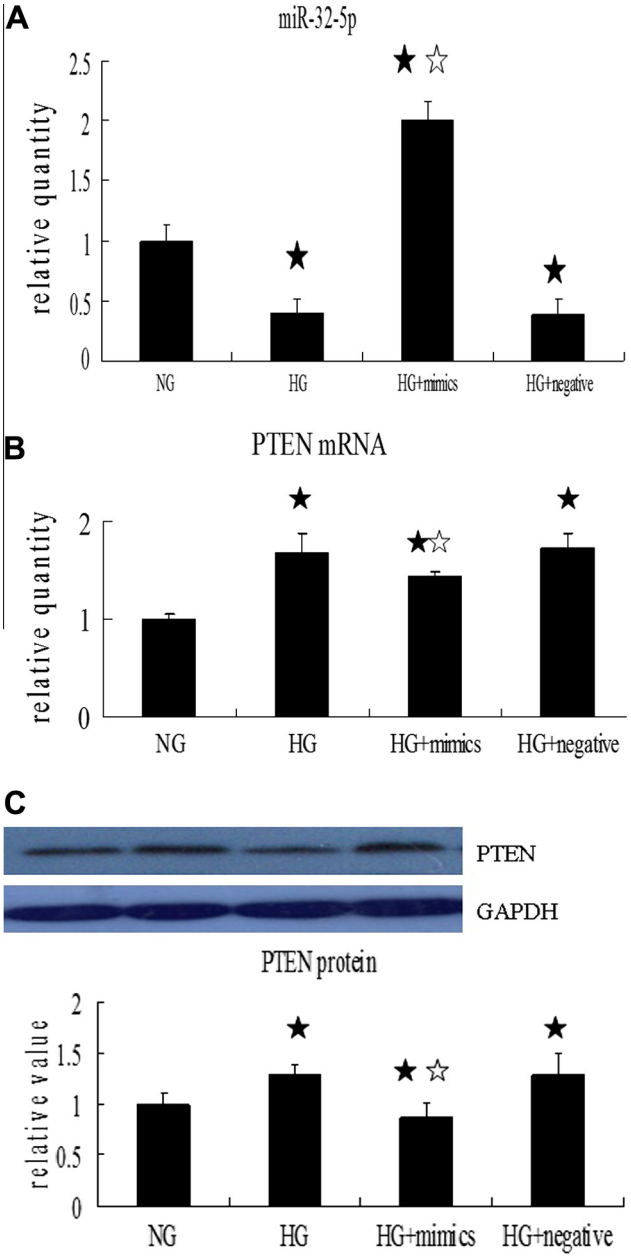

Fig. 3. The expression of PTEN protein is regulated by miR-32. Transfection of miR-32 mimic oligonucleotide resulted in a reversal of the high glucose-induced reduction in miR-32 expression and increased PTEN protein expression in MSCs. The expression of miR-32 was determined by qRT-PCR analysis (A). U6 was used as an internal control. The expression levels of PTEN mRNA (B) and protein (C) were determined by qRT-PCR and Western blotting. Beta-actin and GAPDH were used as internal controls, respectively. Relative expression levels were calculated as relative change from normal glucose-treated MSCs group. ★ Black stars depict statistically significant differences from the NG group; ☆ White stars depict statistically significant differences from the HG group. $P < 0.05$; bars represent mean values ± SEM, $n = 4$.

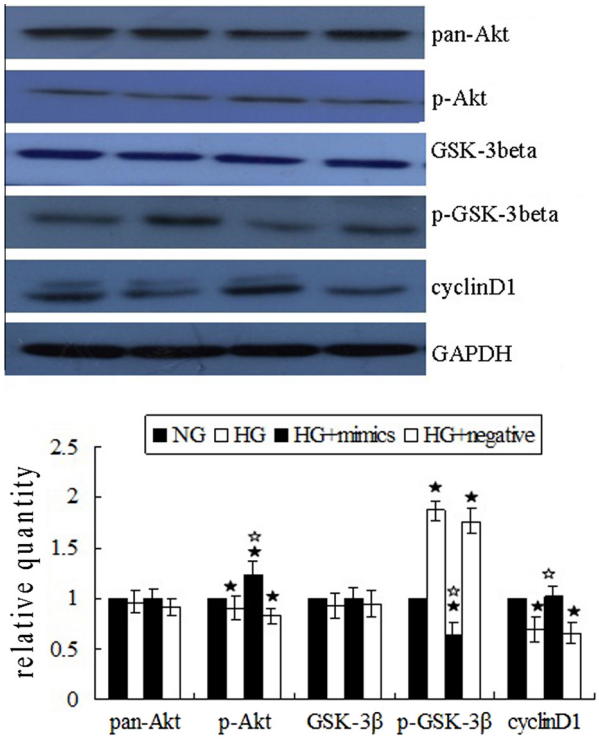

Fig. 4. High glucose levels negatively regulate PI3K/Akt signaling pathway and miR-32 mimics ameliorate the inhibitory effect. High glucose levels inhibit the Akt signaling pathway activation in MSCs, experimentally manifested in the reduced phosphorylation levels of Akt and cyclin D1, and increased phosphorylation of GSK-3β. MiR-32 mimics ameliorates the inhibitory effect of high glucose levels on the Akt signaling pathway. By contrast, miR-32–negative control oligonucleotides demonstrate no effect on the Akt signaling pathway. ★ Black stars depict statistically significant differences from the NG group; ☆ White stars depict statistically significant differences from the HG group. $P < 0.05$; bars represent mean values ± SEM, $n = 4$.

Table 1
Inhibition of cell cycle progression by high glucose is alleviated by miR-32 mimic oligonucleotides.

MSCs treatment	G0/G1	G2/M	S
	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)
NG	32.94 ± 2.93	22.01 ± 1.26	45.05 ± 4.28
HG	42.52 ± 5.00[a]	21.15 ± 2.05	36.33 ± 3.76
HG + miR32 mimics	35.25 ± 3.18[a, b]	21.30 ± 2.43	43.45 ± 4.32
HG + miR32 negative	43.56 ± 4.37[a]	19.91 ± 1.87	36.53 ± 2.92

[a] The statistically significant differences from the normal glucose treated MSCs (NG).
[b] The statistically significant differences from high glucose treated MSCs (HG). $P < 0.05$.

could alleviate the cell cycle arrest of high glucose levels, reducing the proportion of MSCs in the G0/G1 phase. By contrast, MiR-32-negative control oligonucleotides displayed no positive effect on cell cycle progression induced by high glucose levels (Table 1).

4. Discussion

Bone marrow-derived stem cells are multipotent stromal cells that have been widely used in tissue repair and regeneration [11]. The ability of MSCs to participate in tissue repair is closely related to the functional state of MSCs. Persistent hyperglycemia is observed in several diseases, including diabetes, sepsis, system inflammatory response syndrome, trauma, and serious burn injury, resulting in MSCs dysfunction [12].

There are conflicting reports about the effect of high-glucose culture conditions *in vitro* and hyperglycemia *in vivo* on MSCs. Several *in vitro* studies have previously demonstrated that high glucose levels are toxic to MSCs, reducing cellular proliferation, increasing cellular senescence and apoptosis [13,14], and enhancing adipogenic potential [15,16]. These variables may account for the high percentage of failures of many MSC transplantation treatments for various diseases associated with hyperglycemia [17], as

well as the poor wound healing capabilities of patients with diabetes and hyperglycemia [17]. By contrast, several reports suggest that MSCs under conditions of short-term exposure to high glucose levels will not affect cellular proliferation and secretion of growth factors [12,14,18]. Several studies even suggest that high glucose levels actually promote MSC proliferation [18].

An increasing number of studies suggest that microRNAs participate in the development and progression of several diseases that are accompanied by hyperglycemia. Kim and colleagues (2012) reported that high glucose levels can lead to cellular senescence by improving the miR-486-5p expression of MSCs of human fat (hAT-MSCs) [19]. High glucose levels induced the expression of miR-221/miR-222 or miR-503 in human umbilical vein endothelial cells, vascular endothelial cells, and endothelial progenitor cells, leading to the inhibition of cellular proliferation, migration, and the dysfunction of endothelial cells [20–22]. High glucose levels also induced podocyte and cardiocyte apoptosis by increasing miR-29c or miR-195 expression in mouse renal podocytes [23], and increasing miR-1 or miR-206 expression in cardiomyocytes [24,25], respectively.

The results of our analysis of the microRNA expression profile indicate that the mRNA expression levels of miR-1843-3p, miR-196c-5p, and miR-32-5p are more than threefold lower in rat MSCs treated with high glucose levels compared with normal glucose levels. The most prominent difference was observed in microRNA-32-5p. Several studies have shown that miR-32, in combination with its downstream target SLC45A3 (solute carrier family 45, member 3), plays an important role in myelin protein expression in oligodendrocytes and myelin maintenance, through the modulation of glucose and lipid metabolism. MiR-32 may also promote cell growth by inhibiting the pro-apoptosis gene *BIM*, thereby reducing the extent of cellular apoptosis [26].

Using bioinformatics technology and the application of a web-based miRNA target gene prediction portal (TargetScan 6.0, miRbase), we searched the potential target gene of miR-32-5p action, and found that the 3′-UTR area of the PTEN gene contained 12 base sites whose sequences matched those with miR-32-5p. PTEN catalyzes the dephosphorylation of PtdIns(3,4,5)-triphosphate, resulting in the inactivation of Akt kinase. PTEN is a negative regulator of the PI3K/Akt signaling pathway, disturbing cellular proliferation and survival. PTEN has also been implicated in the development and progression of several disease and pathological processes that are dependent upon the PI3K signal pathway as a primary control channel, such as cancer, inflammation, and diabetes [27]. PTEN also plays an important role in the modulation of glucose metabolism [28].

The role of PTEN varies based on the cell type, especially regarding cellular proliferation. Increased PTEN activation and inhibited Akt phosphorylation induced by hyperglycemia leads to cellular apoptosis in renal tubular cells [29] and insulin resistance in atrial biopsies [30]. By contrast, hyperglycemia promotes increased expression of miRNA-21 and inhibits the expression of PTEN in renal mesangial cells, increasing Akt phosphorylation levels and leading to cellular hypertrophy [31].

Our study found that the miR-32 expression levels were significantly decreased in MSCs under hyperglycemic conditions and negatively correlated with PTEN expression. Low expression of miR-32 increases the expression of PTEN mRNA and protein, thereby inhibiting Akt phosphorylation and promoting GSK-3β phosphorylation, and resulting in reduced cyclin D1 expression. Ultimately, this process results in the arrest of most MSCs at the G0/G1 phase, negatively regulating cell cycle progression. This observation is consistent with previous reports that high glucose levels can arrest cells at the G0/G1 phase [32,33]. By contrast, alternate suggestions have been provided stating that high glucose levels increase the expression of cell cycle-associated regulatory proteins of mouse embryonic stem cells via the PI3K/Akt and MAPK signaling pathways and promote cellular proliferation [34]. We conclude that high glucose levels have varying effects on cell cycle processes depending on the cell type [35].

MicroRNA-32-5p mimic oligonucleotides alleviate the inhibitory effects of high glucose levels on the function of MSCs. Additionally, microRNA-32-5p mimic oligonucleotides inhibit PTEN transcription and translation, increase Akt phosphorylation, promote the expression of cyclin D1, and promote MSC progression from the G0/G1 phase into the G2/M and S phases. Our experimental results support the hypothesis that high glucose levels have inhibitory effects on MSC cell cycle progression through miR-32-mediated PTEN upregulation, Akt inactivation, and inhibition of the PI3K/Akt signaling pathway.

MicroRNAs have been implicated in numerous developmental processes affected in various disease states. Our studies have revealed that miR-32 plays a critical role in the influence of high glucose levels on MSC cell cycle progression. MicroRNA-32-5p may function as a putative novel therapy that is effective in preventing MSC dysfunction under hyperglycemic conditions.

Acknowledgments

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