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Original Article

Down-regulation of SENCR promotes smooth muscle cells proliferation and migration in *db/db* mice through up-regulation of FoxO1 and TRPC6

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ABSTRACT

Background: The inappropriate proliferation of vascular smooth muscle cells (VSMCs) plays a crucial role in the atherosclerotic process. SENCR was reported to be associated with cell migration in human smooth muscle cells. However, the regulation role of SENCR in SMCs is still not fully understood.
Methods: qRT-PCR and Western blotting were performed to detect the mRNA and protein levels of SENCR, FOXO1 and TRPC6 in SMCs of *db/db* mice and SMCs exposed to high glucose. The regulation of SENCR on the expression of FoxO1 and TRPC6 were examined with luciferase report assays. Furthermore, we investigated the effect of SENCR on VSMCs proliferation and migration using MTT assay and cell migration assay, respectively.
Results: Here, we found that SENCR was down-regulated in *db/db* mice and in SMCs exposed to high glucose. According to the result of luciferase assays, it was shown that SMCs knockdown enhanced the expression of FoxO1 and FoxO1 overexpression increased the expression of TRPC6. In addition, qRT-PCR revealed that SENCR overexpression reversed the effect of high glucose on mouse VSMCs proliferation and migration.
Conclusion: In this study, our data indicated that down-regulation of SENCR promoted smooth muscle cells proliferation and migration in *db/db* mice through up-regulation of FoxO1 and TRPC6.

1. Introduction

The increased risk for type 2 diabetes mellitus (T2DM) is the developing of several serious complications, among which cardiovascular disease, is the most common disease and mainly involves the accelerated development of atherosclerotic vascular changes [1]. The most common pathological change in atherosclerosis is the inappropriate proliferation of vascular smooth muscle cells (VSMCs), which occurs in response to arterial injury and plays a crucial role in the atherosclerotic process [2]. It is reported that hyperproliferation and migration of VSMCs have been shown to lead to lesion formation in restenosis, atherosclerosis, and hypertension [3]. However, the molecular mechanism behind abnormal VSMC proliferation in diabetes is still not fully understood.

Long noncoding RNAs (lncRNAs) have emerged as novel regulators of gene expression and played roles in diverse biological processes, such as proliferation, differentiation, and development through various modes of action [4,5]. In cardiovascular disease, few lncRNAs are reported to involved in cardiovascular development and pathophysiology, such as lncRNA Braveheart, CHRF, *MALAT1* and LIPCAR [6]. Bell et al. used RNA sequencing of human coronary artery smooth muscle cells and identified several unannotated lncRNAs. Among these lncRNAs, they pinpointed a vascular cell-enriched lncRNA that they termed smooth muscle and endothelial cell-enriched migration/differentiation-associated long noncoding RNA, or in short SENCR, which resides within the first intron of *Friend leukemia virus integration 1* (*FLI1*) in an antisense orientation [7]. Several studies demonstrated that SENCR is likely to be related with maintaining a normal, non-motile contractile phenotype in SMCs and its down-regulation in human coronary artery smooth muscle cells (HCASMCs) displayed increase in cell migration in scratch wound [8]. Therefore, elucidating the regulation role of SENCR in SMCs is helpful for further understanding its function.

Various growth factors and cytokines are also known to be involved in SMCs proliferation and migration. FoxO1 (FKHR) proteins is a member of the transcription factor Forkhead box O (FoxO) family, which have important roles in regulating cellular differentiation, proliferation, survival in various cell lines, including cancer cells, fibroblasts, myoblasts, endothelial cells, and

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SMCs. It has been shown that contraction of VSMs mostly relies on the elevation of the intracellular free Ca^{2+} concentration. TRPC6 is a member of the short transient receptor potential (TRP) channel gene subfamily, which has been implicated as molecular candidates for channels mediating receptor-stimulated Ca^{2+} influx. In this study, we investigated the dysregulation of SENCR, FoxO1 and TRP6 in SMCs of *db/db* mice or SMCs exposed to high glucose. And we found that SMCs knockdown might enhance the expression of FoxO1 and FoxO1 overexpression increased the expression of TRPC6. In addition, SENCR overexpression reversed the effect of high glucose on mouse VSMCs proliferation and migration.

2. Materials and methods

2.1. Experimental animals and collection of VSMCs

Animal experiments were approved by the Experimental Animal Ethics Committee of Changzhou No. 2 People's Hospital. Male *db/db* mice at 8-, 12- and 16-weeks-old were used. Age matched non-diabetic *db/m* mice were used as the controls. Mice were housed in micro-isolator cages in a pathogen-free facility. After 1-week acclimation, mice were euthanized with CO_2 and decapitated. The thoracic aorta was immediately dissected and enzymatically digested at 37 °C for ~3.5 h using a 0.25% trypsin solution. Following digestion, tissue fragments were explanted in a 35-mm culture dish. Contaminated fibroblasts were separated from the VSMCs due to their differing adhesion abilities. The VSMCs used for real-time polymerase chain reaction (PCR) and western blotting experiments were frozen on dry ice and stored at −80 °C.

2.2. Cell culture, transfection, and luciferase assays

Mouse vascular smooth muscle cells and human smooth muscle cell lines C-12511 were obtained from ATCC (Manassas, Virginia) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C. pcDNA-SENCR and were used to overexpress SENCR and FoxO1. The luciferase reporter constructs were cotransfected using Lipofectamine 2000 (Invitrogen) with pcDNA-SENCR, pcDNA-FoxO1, si-SENCR or si-FoxO1 into SMCs and luciferase activity was measured using the luciferase assay kit. Relative promoter activities were expressed as luminescence relative units normalized for co-transfected β-galactosidase expression in the cell extracts.

2.3. Chromatin-immunoprecipitation sequencing

Chromatin immunoprecipitation (ChIP) assays were carried out as described previously using the ChIP Assay Kit from Upstate Biotech following the manufacturer's protocol [9]. Primer sequence for FoxO1 is available upon request.

2.4. VSMC proliferation assay

MTT assay was performed to measure the VSMC proliferation. Briefly, VSMCs were harvested by trypsinization and plated in a 96-well plate at a density of 2×10^3 cells/mL. Then, VSMCs were grown in 100 μL of medium at 37 °C for 24 h, followed by incubation with 20 μL MTT (3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) for 4 h. Then, 150 μL dimethyl sulfoxide (DMSO) was added to each well and the absorbance was measured at 490 nm using a microplate reader.

2.5. VSMC migration assay

Cell migration assay was performed in a 24-well plate with 8 mm pore size chamber inserts (Corning). 5×10^4 cells were placed into the upper chamber per well with the non-coated membrane. Platelet-derived growth factor (PDGF) at 1 and 10 ng/mL dissolved in DMEM medium containing 0.1% FBS was added in the bottom chamber. VSMCs (5104 cells per well) suspended in 100 L of DMEM containing 0.1% BSA was added to the upper chamber. After incubation for 5 hours at 37 °C and 5% CO_2, cells on both side of the membrane were fixed and stained with Diff-Quick staining kit (Baxter Healthcare Corp). Cells on the upper side of the membrane were removed with a cotton swab. The average number of cells from 5 randomly chosen high power (200) fields on the lower side of the membrane was counted.

2.6. Real-time RT-PCR

Quantitative real-time reverse transcription (RT-PCR) was used to determine the mRNA expression of SENCR, FoxO1 and TRP6 in SMCs. Total RNA was extracted with Trizol and treated with RNase-free DNase I. First-strand cDNA was generated from total RNA by RT with the RevertAidHMinus First Strand cDNA synthesis kit. RT-PCR was performed using primers with either 5′-6-carboxyfluorescein (FAM) labeled probes (Integrated DNA Technologies, Inc.) or SybrGreen master mix according to the manufacturer's instructions. The expression level of each candidate gene was internally normalized against that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blot analysis

VSMCs were re-suspended in lysis buffer and 2 mg/mL protease inhibitor cocktail (Roche Diagnostics Corp). Protein concentrations were determined using the Bradford Protein assay kit (Beyotime Biotechnology, China). Equal amount of protein were analyzed by SDS-PAGE and immunoblotting. Primary antibodies used include the following: FoxO1, TRPC6 and GAPDH (BD Transduction Laboratories). Secondary antibodies were fluorescence-labeled antibodies. Bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

2.8. Statistics

All data are given as means ± SD. The statistical significance of differences between mean values was assessed with Student's *t*-test. Differences were regarded as statistically significant for $P < 0.05$.

3. Results

3.1. SENCR was down-regulated in db/db mice

The thickness of aortic smooth muscle layer in *db/db* mice was significantly enhanced comparing to that in control mice. Moreover, in *db/db* mice, aortic smooth muscle layer appeared to be more thick in a time-dependent manner (Fig. 1A). Then, SMCs were separated from *db/db* mice at different weeks and corresponding control mice. RT-PCR and western blot were performed to examine the expression of SENCR, FoxO1 and TRPC6 in separated SMCs. It has been shown that SENCR was down-regulated in *db/db* mice in a time-dependent manner (Fig. 1B). On the other hand, the expression of FoxO1 and TRPC6 were significantly higher in *db/db* mice than that in control at transcription and translation level (Fig. 1C).

3.2. SENCR was down-regulated in mouse VSMCs exposed to high glucose

To further verify the dysregulation of SENCR, FoxO1 and TRPC6 in *db/db* mice, we detected their expression in primary mouse