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# MiR-29 family members interact with SPARC to regulate glucose metabolism

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ABSTRACT

MicroRNA (miR)-29 family members have been reported to play important regulatory roles in metabolic disease. We used TargetScan to show that “secreted protein acidic rich in cysteine” (SPARC) is a target of the miR-29s. SPARC is a multifunctional secretory protein involved in a variety of biological activities, and SPARC dysregulation is associated with a wide range of obesity-related disorders, including type 2 diabetes mellitus (T2DM). We explored whether miR-29s played roles in glucose metabolism and whether miR-29s directly targeted SPARC. We also examined the effect of SPARC on glucose metabolism and how the association of miR-29s with SPARC affected glucose metabolism. We found that overexpression of miR-29s reduced glucose uptake and GLUT4 levels; that miR-29 directly targeted SPARC, resulting in degradation of SPARC-encoding mRNA and reduction in the SPARC protein level; that SPARC increased glucose uptake and GLUT4 levels; that shRNA-mediated knockdown of SPARC reduced GLUT4 protein levels in 3T3-L1 adipocytes; that miR-29s reduced glucose uptake and GLUT4 levels; and that miR-29s inhibited glucose uptake by suppressing SPARC synthesis. Thus, the miR-29 family negatively regulates glucose metabolism by inhibiting SPARC expression.



## 1. Introduction

MicroRNAs (miRNAs) are endogenous, small, noncoding RNA molecules 20–24 nucleotides in length that regulate the expression levels of protein-encoding genes. MicroRNAs are involved in many biological processes and diseases [1–4], and previous reports have suggested that microRNAs critically regulated the progression of type 2 diabetes mellitus (T2DM) [5–7]. Of the many miRNAs, the miR-29 family includes potential regulators of metabolism [8–10] and may serve as a useful, novel therapeutic target in patients with metabolic diseases and T2DM [11].

The miR-29 family contains three members—miR-29a, miR-29b, and miR-29c—all of which share a common seed sequence. The interactions between miR-29s and target genes are mediated by miRNA binding to the 3′-untranslated regions (UTRs) of the targets [12]. miR-29s regulate cell differentiation and pathological formation of the extracellular matrix; dysregulation of miR-29 expression contributes to the development of disease [13,14], often by inappropriately affecting extracellular matrix synthesis.

Here, we used TargetScan to show that “secreted protein acidic rich in cysteine” (SPARC) was a target of miR-29s. SPARC is a multifunctional protein secreted by adipose tissue (mostly adipocytes) and is involved in many biological activities, including angiogenesis, cell adhesion, and remodeling of the extracellular matrix [15–17]. SPARC reportedly plays roles in adipogenesis, adipocyte differentiation, and adipose tissue hyperplasia [18]. Aberrant SPARC expression is associated with a variety of obesity-related diseases, including insulin-resistance and T2DM [19]. SPARC secretion from adipose tissue is increased by insulin and leptin [20]. SPARC may represent a key link between diabetes and obesity. However, the molecular mechanisms in play remain largely unclear. Our objective was to explore whether miR-29s played roles in glucose metabolism and whether miR-29s directly targeted SPARC; we also examined the effect of SPARC on glucose metabolism and assessed whether miR-29s modulated the effects of SPARC in this context.

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## 2. Materials and methods

### 2.1. Antibodies

SPARC (sc-25574, Santa Cruz), GLUT4 (SC-7938, Santa Cruz), GAPDH (MAB374, Millipore).

### 2.2. Cell culture

Human embryonal kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin, and 1% (w/v) streptomycin, under 5% (v/v) $CO_2$ at 37 °C.

### 2.3. Preadipocyte differentiation

Preadipocytes of the 3T3-L1 line (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptozotocin to induce cell differentiation. Confluent cells were cultured in medium containing 10% (v/v) FBS and MDI (0.5 mM IBMX, 1 μM dexamethasone, and 10 μg/mL insulin). The medium was replaced at 2-day intervals until small lipid droplets appeared in the cytoplasm.

### 2.4. Effects of miR-29a/b/c on glucose uptake by 3T3-L1 adipocytes

3T3-L1 adipocytes were inoculated into six-well plates. Mmu-miR-29a/b/c and negative control RNAs were then transfected into the adipocytes; the amount of Lipofectamine RNAiMAX (from the Invitrogen glucose uptake assay kit) added was 7.5 μL/well and that of RNA was 75 pmol/well. After 24 h, the cells were counted, and groups 20,000 cells were inoculated into wells of a 96-well plate and cultured in 100 μL of medium overnight. Then, the medium was replaced with phosphate-buffered saline (PBS, 100 μL) without glucose, and the cells treated with 0, 50, 100, and 150 nM insulin for 15 min. Labeled glucose analogs were added to the glucose-free medium; the cells were treated with insulin overnight, washed five times in PBS, and fluorescent signals were measured using a microplate reader.

### 2.5. Effects of SPARC silencing and overexpression on glucose uptake by 3T3-L1 adipocytes

3T3-L1 adipocytes were inoculated into six-well culture plates, transfected to ensure SPARC silencing or overexpression (with a negative control) and, after 48 h, puromycin was added, and incubation was continued for another 72 h. The monolayers were suspended and the cells counted. Groups of 20,000 cells were inoculated into wells of a 96-well plate and cultured in 100 μL of medium overnight. Then, the medium was replaced with PBS (100 μL) without glucose, and the cells were treated with 0, 50, 100, or 150 nM insulin for 15 min. Labeled glucose analogs were added to the glucose-free medium, and the cells were treated with insulin overnight, washed five times in PBS, and fluorescent signals were measured using a microplate reader.

### 2.6. Effect of SPARC on GLUT4 expression by 3T3-L1 cells

Reagents: Invitrogen LipofectamineRNAiMAX kit; Generay Trizol RNA extraction reagent; Takara reverse transcription kit; qPCR kit; DMEM basic medium (per L): 13.7 g DMEM, 3.7 g $NaHCO_3$, adjust pH to 7.2; PBS: 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L $Na_2HPO_4$, 2 mmol/L $KH_2PO_4$, pH 7.4. 3T3-L1 adipocytes were inoculated into six-well culture plates, transfected to ensure SPARC silencing or overexpression (with a negative control) and, after 48 h, puromycin was added, and incubation was continued for another 72 h. The monolayers were suspended and the cells counted. Groups of 60,000 cells were inoculated into wells of a 96-well plate and cultured in 500 μL of medium overnight. Then, the medium was replaced with PBS (500 μL) without glucose, and the cells were treated with 0, 50, 100, or 150 nM insulin for 15 min. DMEM containing a high level of glucose was added; this was followed by incubation for 5 h, and RNA was then prepared for Real-Time RT-PCR.

### 2.7. miRNA target prediction

Targets of the miR-29 family (mmu-mir-29a-3p: 5′-UAGCACCAUCUGAAAUCGGUUA-3′, mmu-mir-29b-3p: 5′-UAGCACCAUUUGAAAUCAGUGUU-3′, and mmu-mir-29c-3p: 5′-UAGCACCAUUUGAAAUCGGUUA-3′) were identified using the miRGen Web tool TargetScan, microRNA.org, and miRanda.

### 2.8. Construction of a recombinant SPARC expression vector

Full-length SPARC cDNA was obtained with the aid of a Reverse cDNA Synthesis Kit (Thermo Scientific) from mouse cell total RNA and isolated and purified. The forward primer was 5′-CTAGTCTAGACATAGATTTAACTGAATACA-3′ and the reverse primer was 5′-CCGGAATTCGCACAGAGTCTGGGTGAGTGT-3'. The PCR products were digested by *Xba*I and *Eco*RI and subcloned into PGL3-3′ UTR (Invitrogen). The accuracy of all constructs was confirmed by DNA sequencing.

### 2.9. Luciferase reporter assay

The 3′-UTR sequence of the SPARC gene was amplified by PCR using specific primers: forward, 5′-CTAGTCTAGACATAGATTTAACTGAATACA-3'; reverse, 5′- CCGGAATTCGCACAGAGTCTGGGTGAGTGT-3'. The PCR product was cloned into the PGL3-3′-UTR vector (RiboBio) between the *Xba*I and *Eco*RI sites. To generate a mutant, seven nucleotides in the target site (GGUGCUA) of the miRNA-29a/b/c seed region were mutated (to CCACGAT). HEK293T cells were transfected with a SPARC 3′-UTR luciferase reporter (100 ng) or the mutant or the empty vector together with mmu-mir-29a-3p, mmu-mir-29b-3p, and mmu-mir-29c-3p mimics (100 ng of each) or mimic controls, with the aid of Lipofectamine 2000. Cells were collected 48 h after transfection, and firefly and *Renilla* luciferase activities were measured using a luciferase assay system (Promega).

### 2.10. Real-time RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time RT-PCR was performed with the aid of a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The primers were as follows: SPARC forward, 5′-CCCTGCCACTTGAAACCTTC-3′, reverse, 5′-AACGAAGCACCGGAGAGTAA-3′; GLUT4 forward, 5′-CTGTGCCATCTTGATGACCG-3′, reverse 5GAAACCCATGCCGACAATGA3; and GAPDH forward, 5′-CATGAGAAGTATGACAACAGCCT-3′, reverse, 5′-AGTCCTTCCACGATACCAAAGT-3′. GAPDH served as the endogenous control. The mature miRNA sequence for mouse mir-29a-3p (MIMAT0000535) was 5′-UAGCACCAUCUGAAAUCGGUUA-3′; for mouse mir-29b-3p (MIMAT0000127), it was 5′-UAGCACCAUUUGAAAUCAGUGUU-3′; and, for mouse mir-29c-3p (MIMAT0000536), it was 5′-UAGCACCAUUUGAAAUCGGUUA-3′.

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