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Adiponectin AS lncRNA inhibits adipogenesis by transferring from nucleus to cytoplasm and attenuating *Adiponectin* mRNA translation

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ABSTRACT

Adiponectin (AdipoQ) is an adipocyte-derived hormone with positive function on systemic glucose and lipid metabolism. Long noncoding RNA (lncRNA) is emerging as a vital regulator of adipogenesis. However, *AdipoQ*-related lncRNAs in lipid metabolism have not been explored. Here, *AdipoQ* antisense (AS) lncRNA was first identified, and we further found that it inhibited adipogenesis. The half-life of *AdipoQ* AS lncRNA was 10 h, whereas that of *AdipoQ* mRNA was 4 h. During adipogenic differentiation, *AdipoQ* AS lncRNA translocated from nucleus to cytoplasm. *AdipoQ* AS lncRNA and *AdipoQ* mRNA formed an RNA duplex. Moreover, *AdipoQ* AS lncRNA delivered *via* injection of adenovirus expressing *AdipoQ* AS lncRNA decreases white adipose tissue (WAT), brown adipose tissue (BAT) and liver triglycerides (TG) in mice consuming a high fat diet (HFD). Interestingly, the non-overlapping region of *AdipoQ* AS lncRNA improved serum glucose tolerance and insulin sensitivity in HFD mice, but not *AdipoQ* AS lncRNA. In conclusion, *AdipoQ* AS lncRNA transfer from nucleus to cytoplasm inhibits adipogenesis through formation of an *AdipoQ* AS lncRNA/*AdipoQ* mRNA duplex to suppress the translation of *AdipoQ* mRNA. Taken together, we suggest that *AdipoQ* AS lncRNA is a novel therapeutic target for obesity-related metabolic diseases.

1. Introduction

Long noncoding RNA (lncRNA) is a class of ncRNA transcribed from the genome that does not encode proteins and is longer than 200 nt [1]. Interestingly, emerging evidence indicates that some lncRNAs can be translated into small peptides [2–4]. LncRNAs have been proven to participate in gene regulation and are involved in many biological processes that may be associated with some diseases [5–8]. They recruit complexes that transform chromatin structure to inhibit or promote transcription [9–11], form RNA duplexes to inhibit translation or enhance the stability of RNA [12–14], and absorb miRNAs like a “sponge” [15,16]. Antisense lncRNAs (AS lncRNAs) are transcribed from the strands that are opposite to the previously annotated transcripts [17]. Studies have revealed that AS lncRNAs positively or negatively regulate expression of neighboring genes through a complicated molecular mechanism [18–20].

Excess triglyceride (TG) accumulation and an increasing number of adipocytes are contributing factors to obesity and related diseases, including type 2 diabetes, inflammation and cardiovascular disease [21–23]. Deep RNA sequencing (RNA-Seq) revealed that lncRNAs were implicated in adipogenesis through the control of master adipogenic transcriptional factors, including PPARγ and C/EBPα [24,25]. LncRNA *ADINR* has been proven to transcriptionally activate the principal adipogenic factor C/EBPα by recruiting histone methyl-transferase complexes to change histone modifications in the C/EBPα locus, inducing human mesenchymal stem cells to differentiate into adipocytes [26]. Blnc1, an inducible lncRNA during brown adipogenesis, drives thermogenic gene expression by interacting with transcription factor early B cell factor 2 (EBF2) to form a ribonucleoprotein complex [27]. LncRNA ADNCR, a competing endogenous RNA, sequesters miR-204

Abbreviations: AdipoQ, Adiponectin; AS lncRNA, antisense long noncoding RNA; FISH, fluorescence *in situ* hybridization; RPA, ribonuclease protection assay; iWAT, inguinal white adipose tissue; eWAT, epididymal white adipose tissue; BAT, brown adipose tissue; TG, triglyceride; HFD, high fat diet; RNA-Seq, RNA sequencing; FFAs, free fatty acids; GTT, glucose tolerance test; ITT, insulin tolerance test; CPC, Coding Potential Calculator; CPAT, Coding Potential Assessment Tool; NOR, non-overlapping region of *AdipoQ* AS lncRNA; Ad-AS, adenovirus expressing *AdipoQ* AS lncRNA; Ad-NOR, adenovirus expressing NOR; *IR*, insulin receptor; *IRS*, insulin receptor substrate; *PPARγ*, peroxisome proliferator activated receptor γ; *C/EBPα*, CCAAT/enhancer binding protein α; *aP2*, fatty acid binding protein 4; *CPT1α*, carnitine palmitoyl transferase 1 α; *HSL*, hormone sensitive lipase; *ATGL*, adipose triglyceride lipase; *SREBP1c*, sterol responsible element binding protein 1c; *TNFα*, tumor necrosis factor α; *UCP1*, uncoupling protein 1; *PGC1-α*, peroxisome proliferator activated receptor gamma coactivator-1 alpha; *PRDM16*, PRD1-BF1-RIZ1 homologous domain-containing protein 16

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from *Sirt1* mRNA and inhibits adipocyte differentiation [28]. Although the above studies demonstrated the regulatory mechanism of lncRNAs on adipogenesis, the functions of novel vital antisense (AS) lncRNAs on lipid metabolism need to be further explored.

AdipoQ, a selectively expressed hormone in adipocytes, modulates glucose and lipid metabolism to balance whole-body energy homeostasis [29,30]. Studies have uncovered that *AdipoQ* overexpression promotes differentiation, insulin sensitivity, and lipid content in 3T3-L1 cells [31]. ob/ob mice that overexpressed *AdipoQ* were prone to fat deposition but were metabolically healthy, with lowering of glucose, triglycerides (TG) and free fatty acids (FFAs) [32]. The insulin sensitivity of tissues was enhanced by adiponectin through promotion of glucose utilization and fatty acid oxidation by activating AMP protein kinase [33,34]. The serum level of AdipoQ is negatively correlated with obesity, type 2 diabetes and cardiovascular disease [35]. Studies have demonstrated that SNPs within *ADIPOQ*, hypermethylation of the *AdipoQ* promoter, transcription factors including *PPARγ*, *FoxO1* and *C/EBPα*, inflammatory cytokines and miRNA modulate *AdipoQ* expression and secretion [36–39]. However, there is still no report on *AdipoQ*-related lncRNAs.

In this study, *AdipoQ* AS lncRNA, which was more stable than *AdipoQ* mRNA, was first identified and was shown to inhibit adipogenesis through forming an *AdipoQ* AS lncRNA/*AdipoQ* mRNA duplex to suppress translation of *AdipoQ* mRNA. During adipogenic differentiation, *AdipoQ* AS lncRNA translocated from nucleus to cytoplasm. Moreover, *AdipoQ* AS lncRNA decreases the weights of WAT, BAT and TG of the liver in HFD mice. Interestingly, the non-overlapping region of *AdipoQ* AS lncRNA improved the serum glucose tolerance and insulin sensitivity of HFD mice but not *AdipoQ* AS lncRNA. Therefore, we suggested that *AdipoQ* AS lncRNA is a novel therapeutic target for obesity-related diseases.

2. Materials and methods

2.1. Animal experiment

Eight-week-old C57BL/6 J male mice were purchased from the Experimental Animal Center of Xi'an Jiao-Tong University, and all animal experiments were approved by the Northwest A&F University Animal Care Committee. Mice were provided water and a standard laboratory chow diet and HFD (fat provided 40% of total energy) purchased from Trophic Animal Feed High-tech Co. Ltd.*ad libitum*.

Here, mice (n = 54) were divided into two cohorts: one cohort fed with chow diet (D12450B:RodentDiet with 10 kcal% Fat, Trophic Animal Feed High-tech Co., Ltd., China), and another cohort fed with HFD (D12451:RodentDiet with 45 kcal% Fat, Trophic Animal Feed High-tech Co., Ltd., China). For the adenovirus injection study, each cohort was divided into three groups. Mice were fed with a chow diet or HFD for 8 weeks. Purified adenovirus (1×10^9 pfu per mouse) was intraperitoneally injected into the mice every 7 days for one month. The body weight and food intake of the mice were recorded weekly. Mice were euthanized using ethyl ether, and the serum, liver, inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT) and brown adipose tissue (BAT) were collected for RT-qPCR, western blot, HE and ELISA.

2.2. RNA extraction, reverse transcription and qPCR

Total RNA was extracted from various mouse tissue samples and cells using TRIzol reagent (Takara, Kyoto, Japan) according to the manufacturer's instructions, treated with deoxyribonuclease (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using the RT Kit (Takara, Kyoto, Japan). Real-time qPCR was conducted using the SYBR-Green kit (Takara, Kyoto, Japan) and detected by an IQ5 real-time qPCR detection system (Bio-Rad, Hercules, CA). β-actin was applied as an internal control. Primers used in this study are shown in Table S1.

2.3. Protein extraction and western blot analysis

The cells and tissues were lysed in RIPA lysis buffer (Appligen, Beijing, China) with a protease inhibitor cocktail (Cwbiotech, Beijing, China). The protein concentration was detected by the BCA Protein Assay kit (Cwbiotech, Beijing, China). Proteins were separated by SDS-PAGE, transferred to a PVDF nitrocellulose membrane (Millipore, Boston, MA, USA), blocked with 5% non-fat milk and then incubated with primary antibodies. Antibodies against AdipoQ (C45B10, Cell signaling), aP2 (BA3262-2, BOSTER), PPARγ (ab191407, abcam) and β-tubulin (BM1453, Boster) were used. HRP-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies were added and incubated. The bands were visualized using a chemiluminescent peroxidase substrate (Millipore).

2.4. Separation and culture of primary adipocytes

Inguinal adipose tissues were washed with PBS buffer with penicillin–streptomycin (200 U/ml), minced and digested as in a previous publication [19]. Preadipocytes were seeded onto 35-mm culture dishes at a density of 8×10^4 cells per dish, and incubated at 37 °C under a humidified atmosphere of 5% CO_2. When cells reached 100% confluence (day 0), DMEM/F12 medium with 10% FBS (Gibco), 1 μM dexamethasone (Sigma), 10 μg/ml insulin (Sigma), and 0.5 mM IBMX (Sigma) were added to the dishes. After two days, the medium was changed every two days with induction medium containing 10% FBS and 10 μg/ml insulin.

2.5. Adenoviral vector construction and purification

Adenoviral vectors encoding *AdipoQ* AS lncRNA or the non-overlapping region of *AdipoQ* AS lncRNA (NOR) were created by the AdEasy™ Adenoviral Expression system. Adenovirus was produced in HEK 293A cells and purified by the Add-N-Pure Adenovirus Purification Kit (abm) according to the manufacturer's instructions.

2.6. Metabolic phenotyping

The glucose tolerance test (GTT) was performed by intraperitoneal injection of D-glucose (1.5 g/kg body weight) after a 16-h overnight fast. Blood glucose levels were measured at 0, 15, 30, 60, 90 and 120 min after injection with adenovirus expressing *AdipoQ* AS lncRNA (Ad-AS) or NOR (Ad-NOR). The insulin tolerance test (ITT) in mice was carried out after a 6-h fast, and then mice were injected intraperitoneally with human insulin at 0.75 U/kg body weight. Blood glucose levels were measured at 0, 30, 60 and 90 min post-injection. Serum AdipoQ and leptin levels were determined using ELISA kits (Njjcbio, Nanjing, China). Serum TG levels were measured using the triglyceride assay kit (Njjcbio, Nanjing, China) according to the manufacturer's instructions.

2.7. Ribonuclease protection assay

The sense/antisense RNA duplex was detected by an endogenous ribonuclease protection assay (RPA) and RT-qPCR. The total RNAs from primary preadipocytes were assessed according to previous studies with a few changes [14,40]. DNase I (Fermentas) and RPA-grade RNase A (Applied Biosystems) were added to remove all of the genomic DNA contamination and single-strand RNAs. Then, the residual endogenous double-strand RNAs (dsRNAs) were applied in the RT reaction catalyzed by the reverse transcriptase Superscript III (Invitrogen). The reaction system was incubated at 55 °C for 60 min and terminated at 75 °C for 10 min. Finally, the double strand cDNA was amplified in a 25-μl PCR system. After a 35-cycle amplification, the products were detected