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MicroRNA-145 suppresses mouse granulosa cell proliferation by targeting activin receptor IB

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ABSTRACT

MicroRNAs (miRNAs) are a class of 21- to 25-nucleotide non-coding RNAs, some of which are important gene regulators involved in folliculogenesis. In this study, we used CCK-8, real-time PCR and Western blot assays to demonstrate that miR-145 inhibits mouse granulosa cell (mGC) proliferation. Combined with the results of luciferase reporter assays that studied the 3′-untranslated region of ACVRIB mRNA, these assays identified ACVRIB as a direct target of miR-145. The ectopic expression of miR-145 reduced the levels of both ACVRIB mRNA and protein and also interfered with activin-induced Smad2 phosphorylation. Altogether, this study revealed that miR-145 suppresses mGC proliferation by targeting ACVRIB.

1. Introduction

Previous studies have shown that follicles, the functional units of the ovary, consist of a central oocyte surrounded by one or more layers of somatic granulosa cells (GCs). Folliculogenesis is a complex process during which oocytes increase in size and develop into a mature form, accompanied by the proliferation and differentiation of the surrounding granulosa cells [1–3]. The pituitary gonadotropins FSH and LH play an important role in stimulating folliculogenesis during follicular development. This process is also regulated by various extra- and intraovarian factors such as activins, inhibins, BMPs and GDF-9 [4–6].

Activins belong to the transforming growth factor β (TGF-β) superfamily, are made up of two subunits (βA and βB) and are classified into three types: activin A (βAβA), activin B (βBβB) and activin AB (βAβB) [7]. Increasing evidence suggests that activins are involved in reproductive dysfunction and cancer, and that they apparently play important roles in the regulation of folliculogenesis and follicular function [8–11]. Activins have been shown to promote the maturation of oocytes and regulate the proliferation of GCs, and the different expression of TGF-β proteins (such as inhibins, activins and follistatin) imply a significant functional role for these peptides in the development of follicles within the ovary [12–14].

The two types of activin receptors that have intrinsic serine/threonine kinase activity are designated as type I (ACVRIA and ACVRIB) and type II (ACVRIIA and ACVRIIB). Activin binds directly to the type II activin receptor, leading to the phosphorylation and activation of the type I activin receptor (also known as ALK4). Activated ALK4 transiently interacts with and phosphorylates two intracellular signal transducers, Smad2 and Smad3 (R-Smads), which, upon phosphorylation, form complexes with the common partner Smad4 (Co-Smad). Subsequently, the Smad complexes translocate into the nucleus and bind to the promoters of target genes to regulate their expression [15–17].

MicroRNAs (miRNAs) are a class of 21- to 25-nucleotide non-coding RNAs that have been recognized as important gene regulators. miRNAs function as regulators of gene expression by targeting mRNAs for degradation or blocking mRNA translation by binding to the 3′-untranslated region (3′-UTR) [18,19]. Hundreds of miRNAs have been identified and shown to participate in the regulation of various biological processes, such as cell proliferation, differentiation and apoptosis [19–21]. For example, miR-145 is capable of

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; BMPs, bone morphogenetic proteins; GDF-9, growth differentiation factor-9; TGF-β, transforming growth factor-β; mGCs, mouse granulosa cells; 3′-UTR, 3′-untranslated region; ACVRIA, activin receptor typeIA; ACVRIB, activin receptor typeIB; ACVRIIA, activin receptor typeIIA; ACVRIIB, activin receptor typeIIB; ER-alpha, estrogen receptor-α; OCT4, octamer-binding transcription factor-4; SOX9, SRY-related high mobility group-box gene-9; ITGB8, integrin, beta-8; CCND2, cyclin D2; Smad, Sma and Mad related family; POF, premature ovarian failure

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inhibiting tumor cell growth and invasion by targeting genes such as c-Myc, mucin 1 and ER-alpha [22–24]. In addition, miR-145 targets pluripotency factors such as OCT4, SOX9 and ITGB8 and functions as a key regulator of human stem cells [25–27]. Evidence indicates that some miRNAs may play critical roles in controlling the expression of genes that are essential for ovarian folliculogenesis and endocrine function [28,29], which suggests that miRNAs may be important for follicular development. However, miRNAs involvement in the regulation of ovarian function has not been well documented.

Therefore, this study aimed to investigate the effects that miR-145 have on mouse granulosa cell proliferation. In addition, ACVRIB was identified as a miR-145 target that may be mediated in the process of regulating mouse granulosa cell proliferation.

2. Materials and methods

2.1. Animals

Three-week-old ICR mice were purchased from the Lab Animal Center of Yangzhou University (Yangzhou, China) and maintained in the Animal Laboratory Center of Drum Tower Hospital (Nanjing, China) on a 12:12 h light/dark cycle (lights off at 19:00) with food and water available ad libitum. All of the experiments involving animals were performed according to the guidelines of the Experimental Animal Management Committee (Jiangsu Province, China).

2.2. Cell lines

HEK293 cells and HEK293A cells were maintained in DMEM/High Glucose (Gibco BRL/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL/Invitrogen, Carlsbad, CA, USA) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin, Gibco BRL/Invitrogen, Carlsbad, CA, USA). The cells were kept at 37 °C in a humidified environment with 5% CO_2.

2.3. Mouse primary granulosa cell collection and culture

Mouse granulosa cells (mGCs) were collected from the ovaries of 21-day-old immature ICR mice using the follicle puncture method, as described previously [30]. The mGCs were cultured in DMEM/F12 (Gibco BRL/Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 1% sodium pyruvate (Gibco BRL/Invitrogen, Carlsbad, CA, USA), 1% glutamine (Gibco BRL/Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin in the presence or absence of activin A (R&D Systems, Minneapolis, MN, USA). The cells were incubated at 37 °C and supplemented with 5% CO_2 in a humidified chamber.

2.4. Construction of recombinant adenovirus

Adenoviruses harboring a 441-bp DNA fragment encompassing the has-miR-145 gene (Ad-miR-145), Flag-tagged mouse ACVRIB without 3′-UTR (Ad-flag-mACVRIB), Flag-tagged mouse ACVRIB with wild-type 3′-UTR (Ad-flag-mACVRIB-3′-UTR) and flag-tagged mouse ACVRIB with 3′-UTR-mutant (Ad-flag-mACVRIB -3′-UTR mu) were generated using the AdMax (Microbix) system according to the manufacturer's recommendations. The adenovirus bearing LacZ (Ad-LacZ) was obtained from Clontech. The viruses were packaged and amplified in HEK293A cells and purified using CsCl banding followed by dialysis against 10 mM Tris-buffered saline with 10% glycerol. The viral titer was determined using HEK293A cells and the Adeno-X Rapid Titer kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

2.5. Cell proliferation assays

The cell proliferation assays were conducted using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The cells were seeded in 96-well plates at approximately 0.5–1 × 10^4 cells per well and cultured in growth medium. The level of cellular proliferation was detected 48 h after treatment with Ad-miR-145 or the miR-145 inhibitor (Ribobio, Guangzhou, China). A 10-μl aliquot of CCK-8 reagent was added to each well, and the plates were then incubated for 1–3 h. The cell numbers were detected on a microplate reader by measuring the absorbance at 450 nm (OD450).

2.6. RNA isolation and quantitative real-time PCR

Total RNA was isolated from tissues and cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of purified RNA using a PrimeScript RT reagent kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The specific primers used for the quantitative polymerase chain reaction (PCR) analysis are listed in Table 1. Each sample was analyzed in triplicate, and the experiment was repeated three times. Each real-time PCR reaction was composed of 1 μl RT product, 10 μl SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA), and 500 nM forward and reverse primers. The real-time PCR was performed on a MyiQ Single Color Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) in 40 cycles (miRNAs: 95 °C for 15 s, 60 °C for 1 min after an initial 15 min incubation at 95 °C. Genes: 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s after an initial 3 min incubation at 95 °C). The data were analyzed with the 2-ΔΔCT method [31], and the fold changes in gene expression were normalized to 18S rRNA or U6 as endogenous controls.

2.7. Western blotting

Total protein was isolated from mGCs, which were harvested at 48 h after treatment. The cells were rinsed with ice-cold PBS (pH 7.4) and lysed with whole cell lysis buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1.0% NP-40, Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail (Sigma, St. Louis, MO, USA)). The protein concentrations were determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein (30 μg) were separated on a 10% SDS–polyacrylamide gel, transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), incubated with antibodies, and visualized using a chemiluminescence detection kit (Amersham Biosciences Corp., Piscataway, NJ, USA). The following antibodies were used for immunoblotting: anti-phospho-Smad2 (1:800, Cell Signaling Technology, Danvers, MA, USA), anti-Smad2 (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-cyclin D2 (1:500, Cell Signal-

Table 1
DNA sequences of primers used for real-time PCR.

Genes	Primers(5′–3)	Products size (bp)
miR-145	CGCGCTCGAGCCCAGAGCAATAAGCCACAT GGTGTCGTGGAGTCGGCAATTCAGTTGAG	69
U6	CTCGCTTCGGCAGCACA AACGCTTCACGAATTTGCGT	94
mCCND2	ACACCGACAACTCTGTGAAGC GCCAGGTTCCACTTCAGCTTA	79
mACVRIB	GCTTGCATGGTCTCCATCTT GGGACCCTGAGGTCAATCTT	176
m18S rRNA	ATGGCCGTTCTTAGTTGGTG CGGACATCTAAGGGCATCAC	183