Journal of Integrative Agriculture 2017, 16(9): 2019–2028

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RESEARCH ARTICLE

MicroRNA-34c regulates porcine granulosa cell function by targeting forkhead box O3a

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Abstract

Granulosa cells (GCs) are somatic cells of ovary, the behaviors of GCs are important for ovarian function. MicroRNAs (miRNAs) are a class of endogenous 18–24 nucleotide (nt) non-coding RNAs, some of which have been shown to be important regulators of GCs function. miR-34c involved in the regulation of various biological processes and was identified to be a pro-apoptotic and anti-proliferative factor in many cell types. However, the roles of miR-34c in GCs function remain unknown. In this study, we used Annexin V-FITC and EdU assays to demonstrate that miR-34c exerted pro-apoptotic and anti-proliferative effects in porcine GCs. Dual-luciferase reporter assays, quantitative real-time PCR (qRT-PCR) and Western blotting identified *Forkhead box O3a* (*FoxO3a*) as a direct target gene of miR-34c. The overexpression of FoxO3a rescued the phenotypic change caused by miR-34c in porcine GCs. In conclusion, miR-34c regulate the function of porcine GCs by targeting *FoxO3a*.

Keywords: porcine, microRNA-34c, FoxO3a, granulosa cell function

1. Introduction

Follicles are the functional units of the ovary, and each follicle consists of an oocyte surrounded by one or more layers of somatic granulosa cells (GCs). During follicular development, oocytes increase in size and progress to maturation, accompanied by the proliferation and apoptosis of surrounding GCs (McGee and Hsueh 2000). This GC behavior together with their release of paracrine signals are shown to be essential for ovarian function (Hillier 2001; Matzuk *et al*. 2002; Richards *et al*. 2002; Binelli and Murphy 2010). While GCs function requires tightly regulated expression and interaction of a multitude of genes, new regulators of GCs continue to be uncovered. With the discovery of small RNAs that exert additional layers of control on gene expression, studies have begun focusing on the roles of these molecules in regulating cellular events in GCs.

MicroRNAs (miRNAs) are a class of endogenous 18–24 nucleotide (nt) non-coding RNAs that regulate gene expression *via* the translational repression or degradation of mRNA by binding to the 3′ untranslated region (UTR) of target mRNA in a sequence-specific manner (Ambros 2004; Bartel 2004; Huntzinger and Izaurralde 2011). They have been identified and shown to participate in the regulation of various biolog-

Received 25 October, 2016 Accepted 7 February, 2017
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doi: 10.1016/S2095-3119(16)61582-4

ical processes, such as cell proliferation, differentiation and apoptosis (Ambros 2004; Lee *et al*. 2005). Its role in manipulating GCs function was not, however, recognized until the GCs dysfunction and infertility in Dicer-knockout mice were confirmed, indicating that miRNAs are critical for the function of GCs (Nagaraja *et al*. 2008). Furthermore, many recent studies have provided strong evidences for the regulatory functions of miRNAs in GCs, these miRNAs mainly mediated the apoptosis and proliferation process of GCs in many species (Carletti *et al*. 2010; Yao *et al*. 2010; Yan *et al*. 2012; Yin *et al*. 2012; Zhang *et al*. 2013; Liu *et al*. 2014).

miR-34c, a member of miR-34 family, involved in the regulation of various biological processes, such as spermatogenesis (Bouhallier *et al*. 2010), male infertility (Wang *et al*. 2011; Yu *et al*. 2014), and oncogene/tumour suppressor networks (Cannell and Bushell 2010). It was identified to be a pro-apoptotic and anti-proliferative factor in many cell types, including vascular smooth muscle cell (Choe *et al*. 2015) and various cancer cells (Corney *et al*. 2007; Cai *et al*. 2010; Hagman *et al*. 2010; Li *et al*. 2015). However, the roles of miR-34c in GCs function remain unknown. Therefore, this study aimed to investigate the effects of miR-34c on porcine GCs apoptosis and proliferation. In addition, many lines of evidence have shown that FoxO3a plays an important role in the activation and development of ovarian follicles (Reddy *et al*. 2005; Liu *et al*. 2007). However, the FoxO3a-mediated regulatory mechanism of miR-34c involvement of GCs function has not been well documented. Then, we identified *FoxO3a* as a miR-34c target gene that may be mediated in the process of regulating porcine GCs function.

2. Materials and methods

2.1. Cell culture

GCs were isolated as described (Xu *et al*. 2016). Briefly, porcine ovaries were collected from a local abattoir, transported to the laboratory within 2 h while maintained in sterile PBS (HyClone, Logan, UT) at 37°C. GCs were aspirated from follicles using a 1-mL syringe fitted to No. 5 fine needle. Cells and follicular fluid were centrifuged at 1 000×g for 6 min and cell pellet were dispersed by pipetting and washed twice using DMEM (HyClone). Finally, GCs were seeded in DMEM containing 10% fetal bovine serum (HyClone), 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (Gibco, Grand Island, NY) and incubated at 37°C with 5% CO_2. Medium was removed after 48 h, cells were washed with PBS, and fresh medium mentioned-above was added to continue the cell culture. Immunofluorescence for the granulosa cell-specific marker follicle-stimulating hormone receptor (FSHR) confirmed that the purity of the culture was above 97% (Gao *et al*. 2014).

2.2. miRNA target prediction

Prediction of miR-34c targets was performed using the MicroInspector (http://bioonfo.uni--plovdiv.bg/microinspector/), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html/), and RNAHybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html/) software packages.

2.3. Plasmid construction

miR-34c mimic, inhibitor, and negative control plasmids were synthesized by RiboBio (Guangzhou, China) (Appendix A). The 3′ UTR of *FoxO3a* (GenBank: DQ243691.2) containing putative miR-34c binding sites was amplified from the genomic DNA of porcine ovary tissue extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions using primers shown in Appendix A. *FoxO3a* 3′ UTR mutants were generated by nested PCR using the FoxO3a-Mutant-3′UTR primer pair (Appendix A). Both the wild-type (FoxO3a-3′UTR-WT) and mutated 3′ UTR of FoxO3a (FoxO3a-3′UTR-MUT) were cloned into the pmirGLO dual-luciferase reporter vector using *Xho*I and *Xba*I restriction sites. The FoxO3a expression vector was constructed by cloning the complete coding sequence of *FoxO3a* (NM_001135959.1) into the pcDNA3.1(–) vector (FoxO3a-pcDNA3.1(–)) at *Xho*I and *Bam*HI restriction sites using primers shown in Appendix A. All vectors were confirmed by sequencing.

2.4. Transient transfection and luciferase reporter assay

GCs were grown to 50–80% confluency in 24-well plates and transfected with miR-34c mimics (50 nmol L^{-1})/inhibitor (100 nmol L^{-1}) or FoxO3a plasmids (500 ng) using Lipofectamine® 3000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA and protein were extracted 48 h after transfection as described below and used for qRT-PCR or Western blotting. For luciferase reporter assays, GCs were transfected with miRNA/reporter constructs and harvested 48 h post-transfection using Glo Lysis Buffer (Promega, Madison, WI). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with a Synergy™ Multi-Detection Microplate Reader (BioTek, Winooski, VT). Firefly luciferase activity was normalized with the corresponding Renilla luciferase activity. All transfection experiments were performed in triplicate.

2.5. RNA isolation and qRT-PCR

Total RNA was isolated from porcine ovary tissue and GCs using Trizol® reagent (Invitrogen). cDNA was synthesized