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ARTICLE

Sohlh2 inhibits the apoptosis of mouse primordial follicle oocytes via C-kit/PI3K/Akt/Foxo3a signalling pathway

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Abstract We previously reported that bone morphogenetic protein 4/drosophila mothers against decapentaplegic protein (BMP4/Smad) signalling pathway initiated primordial follicle growth and prevented oocyte apoptosis via up-regulation of Sohlh2 and receptor for kit ligand (c-kit). The mechanism underlying this process was not fully elucidated. In the present study, primary oocyte cultures were established from ovaries of 3-day-old female mouse pups by two-step enzyme digestion. Cultures were divided into Sohlh2 small interference RNA (SiRNA) group, negative SiRNA group, Sohlh2 overexpression plasmid group and pCAG-puro group. TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling assay was carried out to detect the oocyte apoptosis; immunocytochemical staining and quantitative real time-polymerase chain reaction detected the expression of c-kit and Forkhead box O3a (Foxo3a); Western blot was performed to detect the expression of Sohlh2, C-kit, saerine/threonine kinases (Akt1) and Foxo3a. The results showed that Sohlh2 inhibited oocyte apoptosis and upregulated c-kit expression; Sohlh2 decreased the endonuclear Foxo3a via the upregulation of phosphorylated Akt1 (P-Akt1) and phosphorylated Foxo3a (P-Foxo3a) but not total Akt1 (T-Akt1) or total Foxo3a (T-Foxo3a); Sohlh2 increased P-Akt1 but not T-Akt1; the PI3K (phosphotidylinsitol-3-kinase) inhibitor LY294002 ameliorated the role of Sohlh2 on phosphorylation of Akt1 and Foxo3a. Sohlh2 may inhibit oocyte apoptosis via c-kit/PI3K/Akt/Foxo3a signalling pathway.

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Introduction

The reproductive capacity of female mammals depends on the size of primordial follicle pool at late stage of embryogenesis or before birth and the ratio of apoptotic oocytes in primordial follicles (Sui et al., 2007). Excessive follicle atresia or exhaustion of primordial follicles leads to premature ovarian failure (POF) and endocrine disorders or infertility (Ding et al., 2013). The early follicular atresia is usually initiated by apoptosis of oocyte and subsequently granular cells (Sui et al., 2007). The mechanism underlying oocyte apoptosis, however, is not fully understood.

Our previous study (Ding et al., 2013) has shown that bone morphogenetic protein 4/drosophila mothers against decapentaplegic protein (BMP4/Smad) signalling pathway initiates primordial follicle growth and prevents oocyte apoptosis via up-regulation of Sohlh2 and c-kit. A recent study indicates that stem cell factor (SCF) initiates anti-apoptotic signalling from its membrane receptor for kit ligand (c-kit) to B-cell lymphoma-2 (Bcl-2) family members by phosphotidylinsitol-3 kinase/serine/threonine kinases (PI3K/Akt) cascades in the oocytes of primordial follicles (Jin et al., 2004). In the present study, the role of spermatogenesis- and oogenesis-specific basic helix-loop-helix (bHLH) transcription factor 2 (*Sohlh2*) on oocyte apoptosis was studied, along with the mechanism underlying it. It is hoped that the results of this study will facilitate an understanding of the molecular mechanism during the process of oogenesis and also provide theoretical and experimental evidence for diagnosis and treatment of clinical infertility and POF.

Materials and methods

Animals

Three-day-old female neonatal Kunming mice were used to prepare oocyte cultures. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the protocols were approved by the Animal Care and Use Committee of Shandong University (approved 28 February 2013, reference number 201002243).

Oocyte culture

Ovaries of 3-day-old Kunming mice were dissociated by a two-step enzymatic digestion. Briefly, the tunica albuginea was removed and the ovaries were incubated in 1 mg/ml collagenase IV with gentle agitation at 37°C for 30 min. After washing and pipetting in Dulbecco's phosphate buffered saline (DPBS) (Gibco, CA, USA), the dispersed ovary tissue was incubated at 37°C in 0.05% trypsin/ethylenediaminetetraacetic acid (Gibco, CA, USA) for 5 min. After digestion, the cell suspension was filtered through a mesh to remove large clumps of cells, and then centrifuged at 500 × g for 10 min. The cell pellets were resuspended in 5% fetal bovine serum (FBS, Gibco, CA, USA) at a concentration of about $3 \times l0^6$ cells per ml. The cells were then plated in a 24-well plate and cultured in an incubator with 5% CO_2 and saturated humidity at 37°C. Twenty-four hours later, the attached cells were eliminated and cells remained in the media were centrifuged at 500 × g. The cell pellet was then resuspended and maintained in alpha minimum essentiali medium (Gibco, CA, USA), supplemented with 3 g/L bovine serum albumin, 10^5 U/L penicillin and streptomycin, 5 mg/L transferrin, 1 × L- glutamine, 5 mg/L insulin and 5 mg/L sodium selenide (oocyte culture system, Sigma–Aldrich, MO, USA). One-half of the media were replaced every other day.

Transient transfection

For overexpression of *Sohlh2*, the mouse cDNA of *Sohlh2* were cloned into the pCAG-puro. The CAG-puro was generated by replacing cytomegalovirus (promoter in Puro3; Clontech, CA, USA) with CAG promoter (Qi et al., 2004). For *Sohlh2* knockdown, small interference RNA (SiRNA) specific to mouse *Sohlh2* (GenBank accession number NM-002467) was synthesized by Invitrogen Corporation (Invitrogen, Carlsbad, CA). The siRNA oligonucleotide sequences were shown in **Table 1**.

Transient transfection of cultured oocytes was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Cells were divided into four groups: the pCAG-puro group (labelled as P-Con) and the pCAG-*Sohlh2* group (labelled as P-Sohlh2); the negative control siRNA group (labelled as SiCon) and the *Sohlh2* siRNA group (labelled as SiSohlh2). At 5 h after transfection, cells were

Table 1 Small interference RNA sequences.

Gene		*sequence (5′ → 3′)*
negative control	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT
sohlh2 siRNA(1)	Sense	UAUACAGAUCCUCAAUAGAGCUCUC
	Antisense	GAGAGCUCUAUUGAGGAUCUGUAUA
sohlh2 siRNA(2)	Sense	AAUCCACGAAAGAUGCUGGCUGAGG
	Antisense	CCUCAGCCAGCAUCUUUCGUGGAUU
sohlh2 siRNA(3)	Sense	AAACAUAGCCUUUAAGUCUUUCAGG
	Antisense	CCUGAAAGACUUAAAGGCUAUGUUU