Original Research

Initiation of oogenesis and meiosis in the fetal ovary depends on Dennd1a-mediated production of Wnt5a and retinoic acid from the somatic niches

Jingjing Shi[1,2,3,4], Qun Niu[1,2,3,4], Qing Gao[5], Jiang Fu[1,2,3,4,*], Jinlong Ma[1,2,3,4,*]

[1]*Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, 250012 Jinan, Shandong, China,* [2]*Key Laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, 250012 Jinan, Shandong, China,* [3]*Shandong Key Laboratory of Reproductive Medicine, Shandong University, 250012 Jinan, Shandong, China,* [4]*Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, 250012 Jinan, Shandong, China,* [5]*Department of Histology and Embryology, School of Basic Medicine, Cheeloo College of Medicine, Shandong University, 250012 Jinan, Shandong, China*

TABLE OF CONTENTS

1. Abstract

Background: The DENN (differentially expressed in neoplastic versus normal cells) domain containing 1A (Dennd1a), a guanine nucleotide exchange factor (GEF) for the small GTPase Rab35, is essential for mouse embryogenesis. Disruption of Dennd1a impairs the migration and differentiation of fetal germ cells. In the present study, we further elucidated the role of Dennd1a in oogenesis and meiosis in the fetal ovary. **Results**: Ablation of Dennd1a disrupted the mRNA expression of Sohlh2, Figla, Stra8, and Rec8 in the ovary of Dennd1a-/- mutants at E13.5. Using *ex vivo* culture of E12.5 female gonads and adenoviral Dennd1a shRNA infection, we demonstrated that transcription of Sohlh2, Figla, Stra8 and Rec8 were not activated in the fetal ovary lacking Dennd1a. Dennd1a in

Submitted: 1 September 2021 Revised: 1 November 2021 Accepted: 5 November 2021 Published: 30 December 2021

the somatic cells might stimulate Sohlh2 expression at early stage of oocyte differentiation via regulating Wnt5a synthesis. On the other hand, meiotic initiation of the fetal germ cells required Dennd1a-mediated RA production from the somatic cells, which induced the expression of Stra8 and Rec8. **Conclusions**: Dennd1a could be involved in multiple signal pathways in the somatic cells that are critical for various processes of oogenesis and meiosis in the fetal ovary.

2. Introduction

The DENN (differentially expressed in neoplastic versus normal cells) domain containing 1A (Dennd1a) functions as a guanine nucleotide exchange factor (GEF) for the small GTPase Rab35. It mediates the switch from GDP-bound Rab35 to GTP-bound form, to promote endocytic recycling of various cargoes on clathrin-coated vesicles (CCVs) after internalization [1–3]. Dennd1a has been identified as a susceptible gene in patients with polycystic ovary syndrome (PCOS), a complex endocrine disorder causing infertility in women of reproductive age [4, 5]. Dennd1a protein was found increased in the theca cells of PCOS patients, and knockdown of a Dennd1a isoform in isolated PCOS theca cell cultures reduced the androgen biosynthesis, indicating that Dennd1a plays a role in the hyperandrogenemia associated with PCOS [6]. However, the role of Dennd1a in the ovaries at either developmental or adult stage is largely unknown.

Previous study revealed that Dennd1a is essential for mouse embryogenesis, and homozygous deletion of Dennd1a causes embryonic mouse lethality. Moreover, disruption of Dennd1a impairs the migration and differentiation of fetal germ cells [7]. In mouse, the population of primordial germ cells (PGCs) arise during gastrulation in the extra-embryonic mesoderm of the posterior amniotic fold, distinguished by high alkaline phosphatase (AP) activity [8]. PGCs at embryonic day 7.5 (E7.5) migrate from the base of allantois, then through the hindgut endoderm and mesentery before colonizing the genital ridge by E11.5 [9]. Dennd1a deficiency affects the migration of PGCs and results in a significant reduction in gonadal germ cell numbers [7].

Following migration of PGCs to the genital ridge, the long and narrow gonadal primordium becomes thicker and undergoes fate determination into testis or ovary by the action of sex-determining gene *Sry* [10]. In female mice, expression of spermatogenesis and oogenesis bHLH transcription factor 2 (Sohlh2) begins in the germLine as early as E12.5, which is an essential regulator of oocyte differentiation, independent of meiosis [11]. Since Sohlh2 mRNA expression is significantly low in the fetal ovary of Dennd1a-null embryo at E13.5, it suggests that Dennd1a may be involved in the regulation of Sohlh2 during fetal ovary development [7].

Table 1. The sequences of primers for sexual identification and real-time PCR.

Gene	Primer sequence (5′ to 3′)
Sry-forward	TTGTCTAGAGAGCATGGAGGGCCATGTCAA
Sry-reverse	CCACTCCTCTGTGACACTTTAGCCCTCCGA
Aldh1a1-forward	CCTCCTGGCGTGGTAAACAT
Aldh1a1-reverse	TTGATCCAGTGAAGGCCACC
Aldh1a2-forward	AGCCACAGGAGAGCAAGTGT
Aldh1a2-reverse	GTCTGCAAGCTTGTCCAACA
Axin2-forward	CTTCCAGATCCCAGCAGCAG
Axin2-reverse	AACGGGCATAGGTTTGGTGG
Dennd1a-forward	ACATTGACGGCCTGCATCCA
Dennd1a-reverse	AGCACAGCAGTAGTCCAGCA
Figla-forward	ACAGAGCAGGAAGCCCAGTA
Figla-reverse	CAGCTGGTAGGTTGGGTAGC
Rec8-forward	CCCGCTTCTCCCTCTATCTC
Rec8-reverse	TGGGAAGAAGCAAGCTAGGT
Stra8-forward	GCTTTTGACGTGGCAAGTTT
Stra8-reverse	AACACAGCCAAGGCTTTTGA
Wnt5a-forward	AAGTATGATAGCGCGGCGGC
Wnt5a-reverse	TTGCGCACACAGTAGTCCGG
Sohlh2-forward	ATGGCCCAGGTTACAGAAGC
Sohlh2-reverse	CTCCTGCAGCAGTCATGGAA
Gapdh-forward	AACTTTGGCATTGTGGAAGG
Gapdh-reverse	ACACATTGGGGGTAGGAACA

Meiosis is another big event in the fetal ovary, which begins approximately at E13.5. Retinoic acid (RA) produced by the somatic cells of the gonad and mesonephros is required for female germ cells to initiate meiotic prophase [12]. RA is derived from retinol (vitamin A), and members of aldehyde dehydrogenase family 1 subfamily A (Aldh1a1-3) catalyze the last step of RA synthesis [13]. In the fetal ovary, Stra8 (Stimulated by Retinoic acid gene 8), a gene required for meiotic initiation, and Rec8, a gene required for meiotic progression, are induced by RA [14].

Wnt5a and its receptor, Ror2, have been implicated in migration and proliferation of PGCs. Wnt5a-null and Ror2-null embryos display similar phenotypes, resulting in a diminished number of PGC in the embryonic gonad due to abnormal PGC migration prior to gonadal colonization [15, 16]. In specific somatic niches, Wnt5a-Ror2 pathway suppresses canonical Wnt/β-catenin signaling to limit the proliferation of migratory PGCs and promote their movement [17]. Moreover, Wnt5a promotes oocyte maturation and meiotic resumption during postnatal oocyte development [18, 19].

In this study, we revealed that Dennd1a plays a role in Wnt5a and RA synthesis in the somatic cells of the fetal ovary and mesonephros. Dennd1a deficiency impairs the expression of genes associated with oocyte differentiation and initiation of meiosis in the fetal ovary, due to insufficient production of Wnt5a and RA respectively.

3. Materials and methods

3.1 Mouse strain and materials

The Dennd1a mutant strain used in this study was reported previously [7]. Wild-type Kunming mice were purchased from the Laboratory Animal Center of Shandong University. The sex was determined by PCR analysis using Sry primers (Table 1) [20]. Care and use of experimental animals described in this work comply with the guidelines and policies of the University Committee on Animal Resources at Shandong University.

3.2 Immunofluorescence and immunohistochemistry

Mouse tissues were dissected and fixed in 4% paraformaldehyde at 4 °C overnight. The fixed and paraffin-embedded tissues were sectioned in a thickness of 5 μm. For immunofluorescence double-labeling, the sections were incubated with mixture of primary antibodies overnight, and then incubated with fluorescently labeled secondary antibody. The sections were mounted in medium with DAPI (abcam, ab104139). Primary antibodies used include Rabbit anti-Aldh1a1 (cell signaling technology, 12035), Rabbit anti-Aldh1a2 (cell signaling technology, 83805S), Rabbit anti-Dennd1a (abcam, ab125347). Secondary Antibodies include Goat anti-Chicken IgY (H + L) Alexa Fluor 488 (Invitrogen, A-11039), Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Alexa Fluor 546 (Invitrogen, A-11010), Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Alexa Fluor 488 (Invitrogen, A11029). Images were obtained by confocal fluorescence microscope (Dragonfly, Andro, England) and analyzed by Imaris 9 software (Oxford Instruments, England).

For immunohistochemistry, the sections of E11.5 embryos were incubated with Dennd1a antibodies which were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (ZSGB-BIO, PV-9001), followed by enzymatic color reaction (Vector Laboratories, SK-4100). Images were taken using OLYMPUS BX53F microscope and analyzed by OLYMPUS cellSens Imaging 1.15 software (OLYMPUS, Japan).

3.3 Tissue and cell culture

The fetal gonads were obtained from E12.5 embryos, and divided into 2 pieces and cultured on 24-well plates (corning) with 1 mL of growth medium in each well at 37 °C with 5% CO_2 for 24–96 h. The growth medium was composed of α-minimal essential medium (α-MEM) (Gibco, 12571063), supplemented with 10% heat-inactivated fetal bovine serum (BI, 04-001-1ACS), 0.23 mM pyruvic acid (Gibco, 11360070), 10 mIU/mL FSH (Merckserono), 100 mIU/mL penicillin G, and 100 mg/mL streptomycin sulfate (Gibco, 10378016). The gonad tissues were treated with Retinoic acid (R&D, 0695/50) at a concentration of 1 μM, Recombinant human/mouse Wnt5a Protein (R&D, 645-WN-010) at a concentration of 350 ng/mL and combination respectively.

The somatic cells were obtained from E12.5 wild-type female mesonephros, which were cultured in each well of a 6-well plate with 2 mL of Dulbecco's modified Eagle's medium (Gibco, 11995065) containing 10% (v/v) fetal bovine serum (FBS) (BI, 04-001-1ACS) and 1% Penicillin/Streptomycin (Gibco, 10378016) in a humidified incubator at 37 °C with 5% CO_2 for 24–96 h. The somatic cells were treated with LY294002 (Sigma, 10 μM) for 24 h to inhibit PI3K activity, or treated with Wnt3a (R&D, 5036WN, 100 ng/mL) for 24 h to induce Wnt/β-catenin signaling.

3.4 Adenovirus transfection in gonads and mesonephros

The adenoviral Dennd1a shRNA (Vigene Biosciences) containing GFP labeling was added into medium of cultured tissues. The adenovirus containing no inserted gene was used as control. The gonad tissues were successfully transfected with adenovirus at the dose of 1×10^{11} PFU/mL, while the somatic cells were successfully transfected at the dose of 5×10^{7} PFU/mL. The efficiency of Dennd1a knockdown was measured using Western blot and real-time RT-PCR analysis.

3.5 Real-time RT-PCR analysis

Total RNA kit I (OMEGA, R6834-02) was used to extract total RNA from gonads and mesonephros. The total RNA was subject to the first strand cDNA synthesis using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan, RR047A). RNeasy Mini Kit (QIAGEN,74104) was used to extract RNA from the fetal germ cells and somatic cells of the gonads of E11.5 or E13.5 embryos. The first strand cDNA synthesis was performed using the Evo M-MLV RT for PCR Kit (Accurate Biology, AG11603). The cDNAs were then amplified by real-time PCR with TB Green Premix Ex Taq (Takara, RR420A) in Light Cycler real-time PCR instrument (Roche, Basel, Switzerland, LC480) according to the manufacture's specification. Gene expression changes were normalized to Gapdh and analyzed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 1.

3.6 Western blot

Proteins were extracted from the fetal gonads using Minute™ Total Protein Extraction Kit for Animal Cultured Cells/Tissues (Invent, SD-001/SN-002). Proteins from the tissue or cell cultures were extracted using T-PER™ Tissue Protein Extraction Reagent (Thermo, 78510). Complete™ Protease Inhibitor Cocktail (Roche, 04693116001) were used to inhibit the degradation of a broad spectrum proteases. The extracted samples were separated, then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, PFL00010). The membranes were probed with Rabbit anti-Dennd1a (abcam,

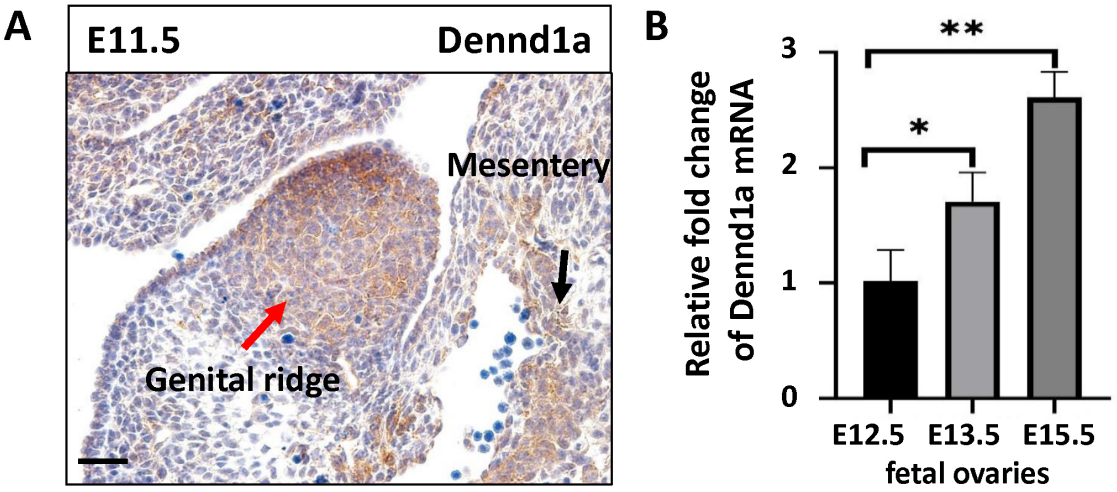

Fig. 1. Expression of Dennd1a in the fetal ovary. (A) Immunohistochemistry shows the localization of Dennd1a in the genital ridge and posterior mesentery of E11.5 mouse embryo. The red arrow indicates genital ridge. The black arrow indicates posterior mesentery. Scale bar = 20 μm (n = 3). (B) Quantitative analysis of *Dennd1a* mRNA expression levels in the wild-type fetal ovaries of E12.5, E13.5, and E15.5 embryos by real time RT-PCR (n = 5).

ab125347), Rabbit anti-ALDH1A1(cell signaling techonogy, 12035, Rabbit anti-ALDH1A2 (cell signaling technology, 83805S), Rabbit anti-Wnt5a (cell signaling technology, 2392S), Rabbit anti-non-phospho β-catenin (cell signaling technology, 8814), Rabbit anti-phospho-AKT (cell signaling technology, 4060), Rabbit anti-AKT (cell signaling technology, 9272), Rabbit-anti-phospho-Stat3 (cell signaling technology, 9145), Rabbit anti-Stat3(cell signaling technology, 9134), Rabbit anti-Axin2 (abcam, ab109307) primary antibodies at 4 °C overnight, followed by incubation with peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G antibodies (ZSGB-BIO, ZB-2301, ZB2305) for 1 h at room temperature. The interaction was monitored with Thermo Scientific SuperSignal West Pico PLUS (Thermo, 34577). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Proteintech, 60004-1-Ig) or Alpha Tubulin (proteintech, 11224-1-AP) antibodies were used to monitor the loading amount. BIO RAD software was used for analysis.

3.7 Enzyme-linked immunosorbent assay (ELISA)

Supernatant culture medium of gonads or somatic cells was collected to quantify concentrations of RA using Mouse Retinoic Acid ELISA kit (CUSBIO, CSB-EQ028019MO) according to the manufacture's specification, using a microplate reader (Molecular devices; SPECTRA MAX 384plus) set to 450 nm. The software used was SoftMax Pro7 (Molecular Devices, USA). Each standard and sample were in duplicates, calibrated with blank well without any solution, and a standard curve was set up by using software Curve Expert. The data was linearized by plotting the log of the Retinoic acid concentrations versus the log of the O.D., and the best fit line was determined by regression analysis.

3.8 Statistics

Graphpad PRISM8 analysis software was used for plotting, and SPSS (version 21.0, IBM Corp., Chicago, IL, USA) analysis software was used for statistical analysis. $p < 0.05$ was statistically significant.

4. Results

4.1 Expression of Dennd1a is gradually increased in the fetal ovary

At E11.5 during mouse embryo development, Dennd1a protein was found in both the hindgut mesentery and genital ridge of mouse embryos (Fig. 1A), suggesting Dennd1a may play a role when PGCs are migrating through the mesentery and colonized in the genital ridge. In fetal ovaries, the levels of Dennd1a mRNA were gradually increased on E12.5, E13.5, and E15.5, during early stages of the oocyte differentiation and meiosis (Fig. 1B). The findings suggest that Dennd1a-mediated signaling pathway may be involved in the regulation of fetal ovary development.

4.2 Dennd1a deficiency impairs initiation of oogenesis and meiosis in the fetal ovary

To assess the oocyte differentiation and initiation of meiosis during embryonic development, we examined mRNA expression of *Sohlh2*, *Figla* (Factor in the germLine α), *Stra8*, and *Rec8* in the wild-type fetal ovaries at E12.5, E13.5, and E15.5. As transcription factors in the germ

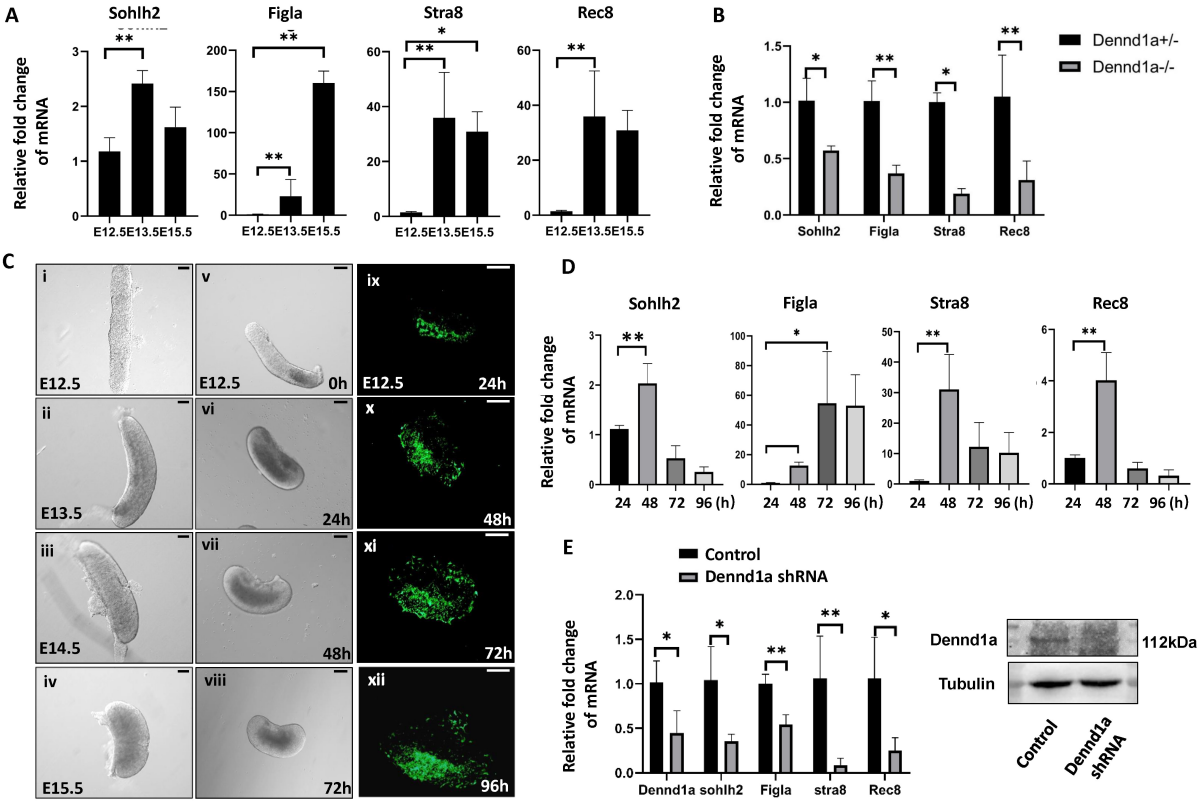

Fig. 2. Dennd1a deficiency impairs the initiation of oogenesis and meiosis of the fetal germ cells. (A) Real-time RT-PCR analysis shows the mRNA expression levels of *Sohlh2*, *Figla*, *Stra8* and *Rec8* in wild-type fetal ovaries of E12.5, E13.5 and E15.5 mouse embryos (n = 5). (B) Real-time RT-PCR analysis of the mRNA levels of *Sohlh2*, *Figla*, *Stra8*, and *Rec8* in the fetal ovaries of E13.5 Dennd1a+/- and Dennd1a-/- embryos (n = 3). (C) (i–iv) Morphological changes of E12.5, E13.5, E14.5, E15.5 wild-type fetal ovaries, scale bar = 50 μm (n = 3); (v–viii) Morphological changes of E12.5 wild-type fetal ovaries *ex vivo* cultured for 0, 24, 48, or 72 h, scale bar = 100 μm; (n = 3) (ix–xii). The *ex vivo* culture of E12.5 wild-type fetal ovaries were infected with adenoviral Dennd1a shRNA carrying a GFP fluorescent tag for 24, 48, 72, or 96 h; scale bar = 100 μm (n = 3). (D) The mRNA expression of *Sohlh2*, *Figla*, *Stra8*, and *Rec8* in E12.5 wild-type fetal ovaries cultured *ex vivo* for 24, 48, 72 and 96 h were examined by Real-time quantitative RT-PCR analysis. (E) The mRNA expression of *Sohlh2*, *Figla*, *Stra8*, and *Rec8* in the E12.5 gonad culture infected with Dennd1a shRNA adenovirus for 72 h was shown as representative (n = 3). The knockdown efficiency of Dennd1a was examined by real time PCR and Western Blot (n = 3).

cells or oocytes, *Sohlh2* mRNA level was elevated at E13.5 and then declined at E15.5, while mRNA expression of *Figla* was activated at E13.5 and dramatically promoted at E15.5 (Fig. 2A). The transcription of *Stra8* and *Rec8*, two genes essential for oocyte meiosis, were both activated circa E13.5 (Fig. 2A). However, using Dennd1a gene knockout mice, we discovered that in the ovaries of homozygous Dennd1a-/- mutants at E13.5, mRNA expression of these genes were insufficiently induced. Deletion of Dennd1a affected the transcription of not only *Sohlh2*, but *Figla*, *Stra8* and *Rec8* as well at this developmental stage (Fig. 2B).

Since homozygous Dennd1a-/- mutants die circa E14.5, they are not suitable to determine whether loss of Dennd1a eventually disrupts the expression of genes associated with oocyte differentiation and meiotic initiation. Moreover, due to limited availability of Dennd1a-/- gonads, we therefore established an *ex vivo* gonad culture by incubating genital ridges isolated from E12.5 wild-type female embryos. The long and narrow E12.5 gonads *ex vivo* became short and thick after being cultured for 24, 48, and 72 h (Fig. 2C, v–viii), recapitulating the morphological changes of developing gonads *in vivo* from E13.5 to E15.5 (Fig. 2C, i–iv). The mRNA expression of *Sohlh2*, *Figla*, *Stra8* and *Rec8* in wild-type gonads *ex vivo* were activated after being incubated for 48 h (Fig. 2D), showing a similar expression pattern as that in gonads *in vivo* at E13.5 (Fig. 2A). To knockdown the expression of Dennd1a, the *ex vivo* E12.5 gonads were infected with adenoviral Dennd1a shRNA carrying a GFP fluorescent tag for 24, 48, 72, or 96 h (Fig. 2C, ix–xii). After 48 h or longer of infection, the expression of Dennd1a mRNA and protein were decreased to approximately half the wild-type level (Fig. 2E). In gonads with Dennd1a knockdown, the mRNA expression of *Sohlh2*, *Figla*, *Stra8* and *Rec8* were not fully activated, as shown in representative samples treated for 72 h (Fig. 2E). These results suggest that Dennd1a deficiency disrupted the proper activation of genes associated with oocyte differentiation and initiation of meiosis in fetal ovaries.

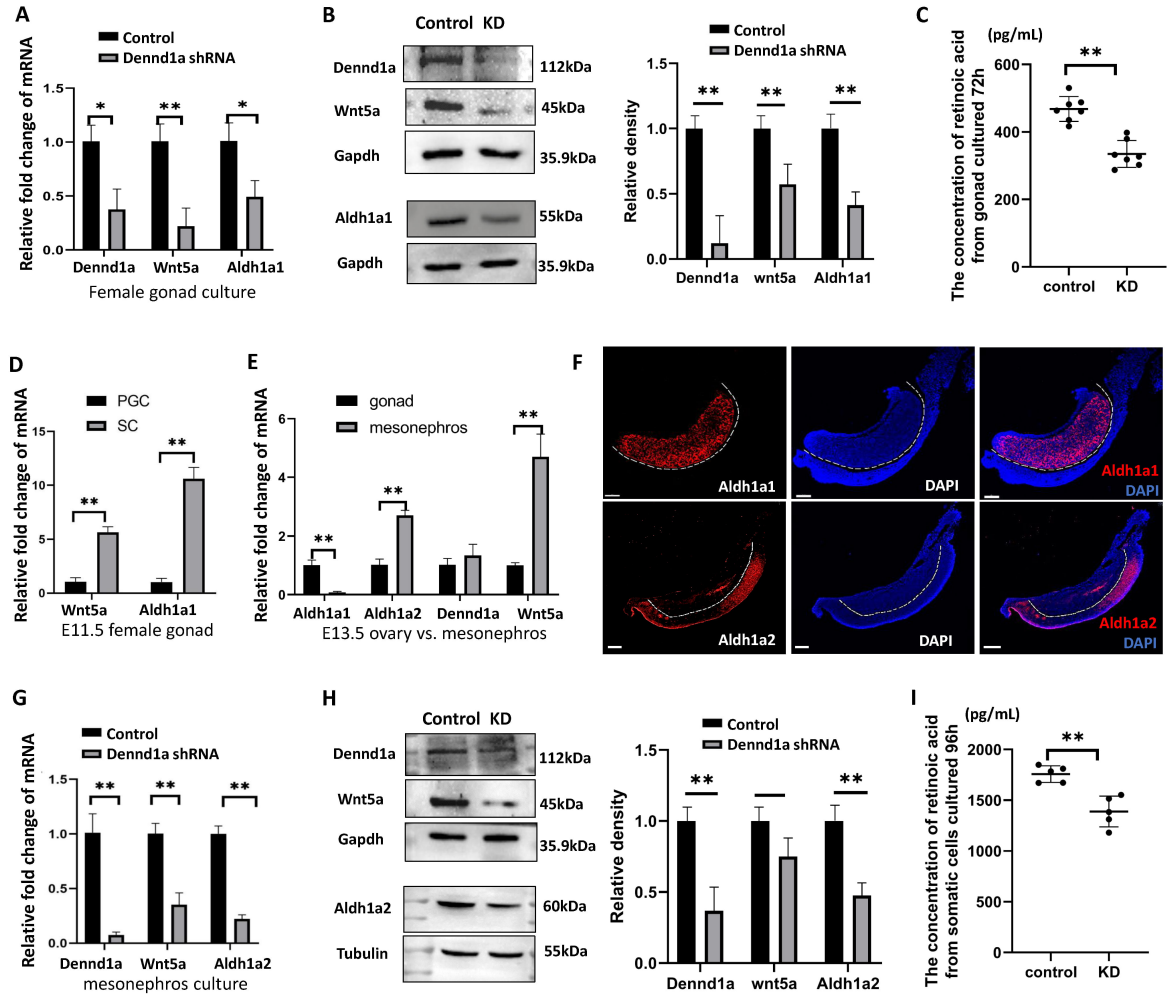

Fig. 3. Production of Wnt5a and RA is reduced in the somatic cells of the fetal ovary lacking Dennd1a. (A,B) Real time PCR and Western blot show the mRNA and protein levels of Dennd1a, Wnt5a, and Aldh1a1 in E12.5 wild-type fetal ovaries cultured *ex vivo* and infected with Dennd1a shRNA adenovirus (KD) or adenovirus containing no inserted gene as control for 72 h (n = 3). (C) ELISA shows the concentrations of RA in the culture medium of E12.5 wild-type fetal ovaries cultured *ex vivo* and infected with Dennd1a shRNA adenovirus (KD) or adenovirus containing no inserted gene as control for 72 h (n = 5). (D) The primordial germ cells (PGC) and the somatic cells (SC) were isolated from E11.5 female gonads. The mRNA expression of Aldh1a1 and Wnt5a in the PGC and SC isolated from E11.5 female gonads were examined by real time PCR (n = 5). (E) Total RNA was extracted from E13.5 wild-type fetal ovaries and mesonephros. The mRNA expression of *Dennd1a*, *Aldh1a1*, *Aldh1a2*, and *Wnt5a* was analyzed by real-time quantitative PCR (n = 5). (F) Immunofluorescence shows the localization of Aldh1a1 in the ovary and Aldh1a2 in the mesonephros of E13.5 embryos (Red). The section was counterstained with DAPI (blue). The Dashed line indicates the boundary between the fetal ovary and mesonephros (scale bar 40 μm, n = 3). (G,H) The mRNA and protein of Wnt5a and Aldh1a2 in E12.5 wild-type fetal mesonephros cultured *in vitro* and infected with Dennd1a shRNA adenovirus or adenovirus containing no inserted gene as control for 96 h were examined by real time PCR and Western blot (n = 3). (I) ELISA shows the concentrations of RA in the culture medium of E12.5 wild-type fetal mesonephros cultured *in vitro* and infected with Dennd1a shRNA adenovirus (KD) or adenovirus containing no inserted gene as control for 72 h (n = 5).

4.3 Production of Wnt5a and RA is reduced in the somatic cells of the fetal ovary and mesonephros lacking Dennd1a

We suspected that Dennd1a deficiency disturbed somatic components which are important for inducing fetal oocyte differentiation and meiosis. It has been reported that Wnt5a was predominantly expressed in the somatic niches and necessary for gonadal differentiation [15]. Furthermore, Wnt5a was involved in oocyte maturation and meiotic resumption during oocyte development [19]. Therefore, we examined whether loss of Dennd1a disrupts Wnt5a production in the somatic cells. In E12.5 female gonad culture infected with adenoviral Dennd1a shRNA for 48, 72 or 96 h, the expression of Dennd1a mRNA and protein was disrupted (Fig. 3A,B). Knockdown of Dennd1a decreased the mRNA and protein levels of Wnt5a in the gonad culture (Fig. 3A,B).

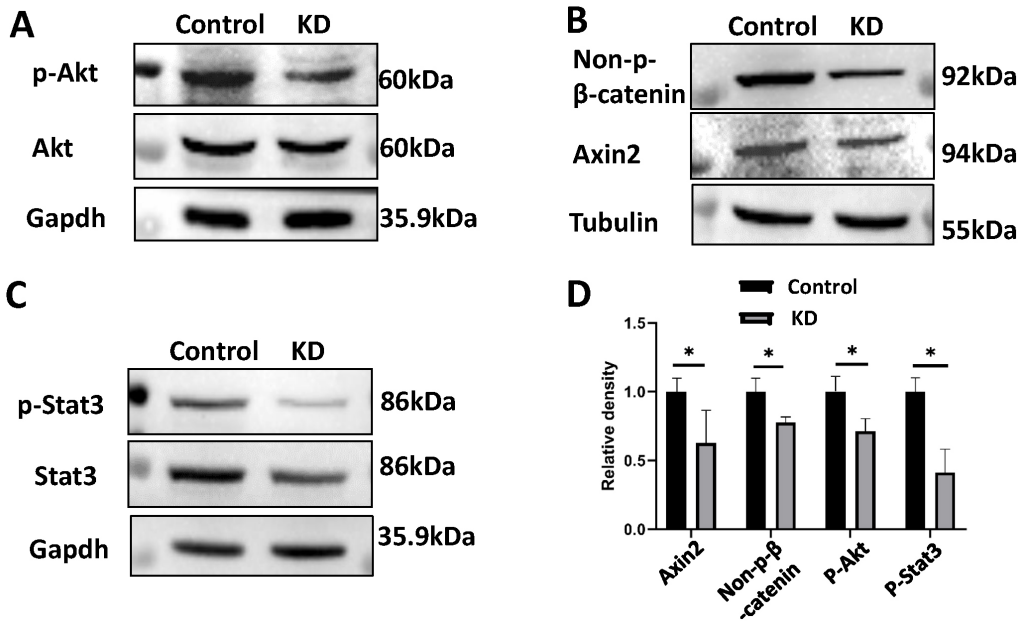

Fig. 4. Knockdown of Dennd1a decreases the phosphorylation level of Akt and accumulation of active β-catenin in the mesonephros. Proteins extracted from E12.5 wild-type fetal mesonephros, which was cultured *in vitro* and infected with Dennd1a shRNA adenovirus (KD) or adenovirus containing no inserted gene as control for 96 h, were examined by Western blot. The expression level of Tublin or Gapdh was analyzed as a loading control (n = 5). (A) Phosphorylation levels of Akt. (B) Protein levels of non-phosphorylated active β-catenin and Axin2. (C) Phosphorylation levels of Stat3 were analyzed by Western blot. (D) Quantitative analysis of protein levels.

Fetal germ cells in the ovary initiate meiosis in response to RA [12]. Deficiency of Dennd1a also reduced the mRNA and protein expression of Aldh1a1, which encodes RA-synthesizing enzyme in the fetal ovary (Fig. 3A,B), and reduced secretion of RA into the culture medium (Fig. 3C). The main source of Wnt5a and RA in the fetal ovary is the somatic cells as confirmed by quantitative analysis of the mRNA expression of Wnt5a and Aldh1a1 in isolated PGCs and somatic cells from E11.5 female gonads (Fig. 3D). It seemed that Dennd1a was involved in the production of Wnt5a and RA from the somatic cells of the fetal ovary.

An alternative source of RA and Wnt5a is the fetal mesonephros. At E13.5, unlike the ovary which expressed mainly Aldh1a1, the mesonephros expressed RA-synthesizing enzymes Aldh1a2 (Fig. 3E,F) and Aldh1a3 [12]. The mRNA expression of Dennd1a in the mesonephros was at the same level as in the ovary, while the mesonephros expressed more Wnt5a than the ovary (Fig. 3E). The mesonephros were then isolated from E12.5 wild-type female embryos, and dissociated cells were cultured *in vitro* and infected with adenoviral Dennd1a shRNA for 48, 72 or 96 h (**Supplementary Fig. 1**). Knockdown of Dennd1a resulted in decreased mRNA and protein levels of Wnt5a and Aldh1a2 in the culture (Fig. 3G,H), and decreased production of RA in the medium (Fig. 3I). Therefore, Dennd1a may play a role in regulating the production of Wnt5a and RA from the somatic cells of the fetal ovary and mesonephros.

4.4 Dennd1a mediates Wnt5a and RA synthesis in the somatic cells through Akt and β-catenin pathways

To explore the mechanism by which Dennd1a regulates RA and Wnt5a in the somatic cells, we used the culture of fetal mesonephros which produce RA and Wnt5a, and also contribute to the somatic niche for germ cells, in order to avoid the complexity associated with the mixture of germ cells and somatic cells in gonadal culture. As a major GEF for Rab35 to regulate endosomal membrane trafficking, Dennd1a may be involved in Rab35-mediated PI3K/Akt pathway [21]. To determine whether Dennd1a deficiency may compromise PI3K/Akt pathway, the E12.5 fetal mesonephros infected with or without adenoviral Dennd1a shRNA for 48 or 72 h were assessed. Dennd1a knockdown led to decreased phosphorylation of Akt in the somatic cells (Fig. 4A,D). Dennd1a deficiency also caused reduced accumulation of non-phosphorylated β-catenin and expression of Axin2, a downstream target of β-catenin-dependent transcription (Fig. 4B,D). In addition, Dennd1a knockdown resulted in decreased expression and phosphorylation of Stat3 (Fig. 4C,D).

To provide evidence of a direct causal link between PI3K/Akt axis and Wnt5a or RA production in the mesonephros culture, we treated the somatic cells with LY294002 to inhibit PI3K activity, the efficacy of which was tested by measuring the phosphorylation levels of the PI3K target protein Akt. Treatment with LY294002 (10 μM) inhibited the phosphorylation of Akt, which was ac-

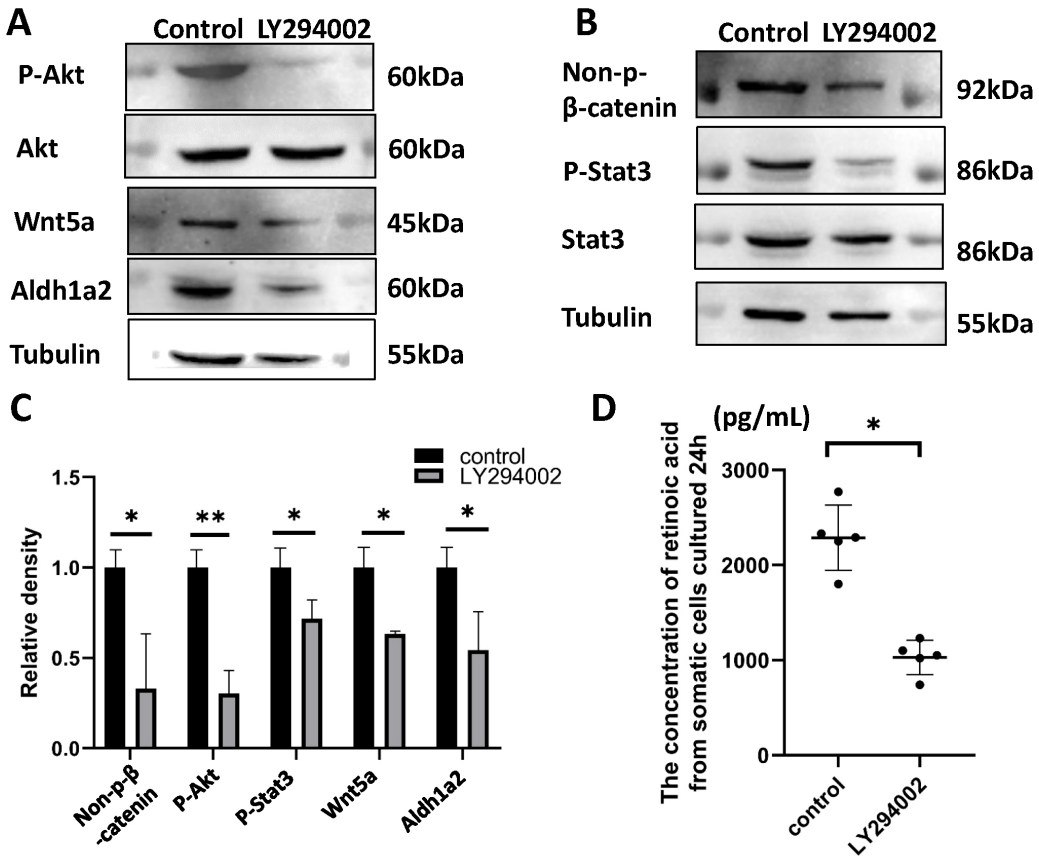

Fig. 5. Inhibition of PI3K/Akt pathway downregulates β-catenin signaling and reduces the production of Wnt5a and RA in the mesonephros. (A) The mesonephros culture was treated with LY294002 to inhibit PI3K activity, the efficacy of which was tested by measuring the phosphorylation levels of the PI3K target protein Akt. Western blots also show the protein levels of Wnt5a and Aldh1a2 (n = 5). (B) Immunoblot analysis shows the levels of non-phosphorylated β-catenin (active), phospho-Stat3, and Stat3 (n = 5). (C) Quantitative analysis of protein levels shown in A and B. (D) ELISA shows the concentrations of RA in the culture medium of E12.5 wild-type fetal mesonephros cultured *in vitro* and treated with LY294002 for 24 h (n = 5).

companied with reduced protein levels of both Wnt5a and Aldh1a2 in the somatic cells (Fig. 5A,C), as well as less RA production in the culture medium (Fig. 5D). Moreover, in the mesonephros cell culture treated with LY294002, accumulation of active non-phosphorylated β-catenin was reduced (Fig. 5B,C), suggesting that β-catenin signaling is downstream of PI3K/Akt pathway. Inhibition of PI3K/Akt pathway also reduced the phosphorylation level of Stat3 (Fig. 5B,C), which has been linked to the regulation of Wnt5a expression.

The role of Wnt/β-catenin signaling in regulating the production of Wnt5a or RA was then examined. Treatment with Wnt3a (100 ng/mL) induced accumulation of non-phosphorylated β-catenin in the mesonephros culture (Fig. 6A,C). Interestingly, the protein level of Wnt5a and phosphorylation level of Stat3 was increased by Wnt3a treatment (Fig. 6A–C), indicating that Wnt/β-catenin signaling may induce Wnt5a expression in the mesonephros. Treatment with Wnt3a also increased the levels of Aldh1a2 in the mesonephros and RA in the culture medium (Fig. 6A,C,D). The results suggest that Akt/β-catenin pathways may be involved in Dennd1a-mediated Wnt5a and RA production in the somatic cells during fetal germ cell development.

4.5 Exogenous Wnt5a and RA stimulate oocyte differentiation and meiotic initiation in the fetal ovary

To determine the effect of Wnt5a and RA on the regulation of genes associated with oocyte differentiation and meiotic initiation, we treated the fetal gonad culture from E12.5 female wild-type mice with recombinant Wnt5a (350 ng/mL) or RA (1 μM) or both for 48 h. Exogenous Wnt5a alone induced mRNA expression of Sohlh2 in the fetal ovary, but didn't affect gene expression of Figla, Stra8, or Rec8 (Fig. 7A). RA alone did not affect Sohlh2 expression while it decreased the expression of Figla, and as expected, RA induced the expression of Stra8 and Rec8 in the fetal ovary (Fig. 7A). Addition of both Wnt5a and RA did not significantly alter the expression of Sohlh2, Figla, and Rec8, although they increased the expression of Stra8 (Fig. 7A). These findings suggested that Wnt5a could induce oocyte differentiation, whereas it had no effect on meiotic initiation of the fetal germ cells. Furthermore, RA did

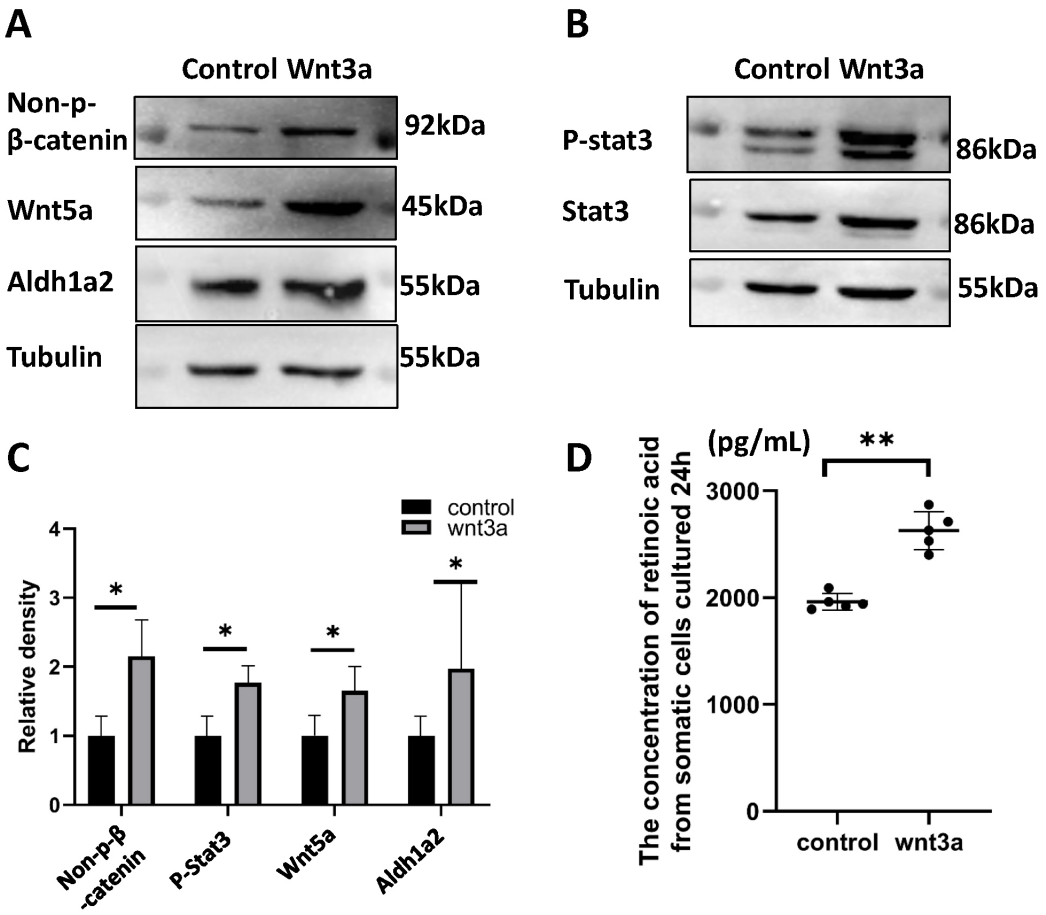

Fig. 6. Wnt3a/β-catenin signaling induces Wnt5a and RA synthesis in the mesonephros. (A) Immunoblot analysis shows the protein levels of non-phosphorylated β-catenin, Wnt5a, and Aldh1a2 in the mesonephros with or without treatment of Wnt3a (100 ng/mL) for 24 h (n = 5). (B) Phosphorylation levels of Stat3 in the mesonephros were examined by Western blot (n = 5). (C) Quantitative analysis of protein levels shown in A and B. (D) ELISA shows the concentrations of RA in the culture medium of E12.5 wild-type fetal mesonephros cultured *in vitro* and treated with or without Wnt3a (100 ng/mL) for 24 h (n = 5).

not work synergistically with Wnt5a to affect oocyte differentiation while it stimulated initiation of meiosis in the fetal ovary.

Next, we investigated whether insufficient Wnt5a and RA was responsible for dysregulation of genes associated with oocyte differentiation and meiosis initiation in the fetal ovary lacking Dennd1a. In the E12.5 fetal ovary culture infected with adenoviral Dennd1a shRNA for 48 h or 72 h, Dennd1a knockdown impaired the expression of Sohlh2. Treatment with exogenous Wnt5a (350 ng/mL) could stimulate the expression of Sohlh2 in Dennd1a knockdown fetal ovaries to the level comparable to that in wild-type control (Fig. 7B showing representative samples treated for 48 h). However, exogenous Wnt5a could not rescue the decreased mRNA expression of Figla, Stra8, or Rec8 in the fetal ovaries affected by Dennd1a knockdown (Fig. 7B). On the other hand, RA treatment (1 μM) stimulated the expression of Stra8 and Rec8 in both wild-type and adenoviral Dennd1a shRNA infected fetal ovaries, although it had no effects on the expression of Sohlh2 and Figla (Fig. 7B). The above results suggested that Dennd1a might play a role in regulating the somatic cells of fetal ovaries to produce Wnt5a and RA, which were required for the oocyte differentiation and meiotic initiation respectively (Fig. 7C).

5. Discussion

The importance of Dennd1a in human reproduction is highlighted by its involvement in PCOS, an endocrine disorder causing female infertility [4–6]. The well-studied function of Dennd1a is to mediate the switch from GDP-Rab35 to GTP-bound form and regulate endocytic recycling of cellular components on clathrin-coated vesicles [21, 22]. During embryogenesis, Dennd1a is essential for the development of fetal organs such as brain, liver, and ovary [7]. In the present study, we further elucidated the role of Dennd1a in oogenesis and meiosis in the fetal ovary.

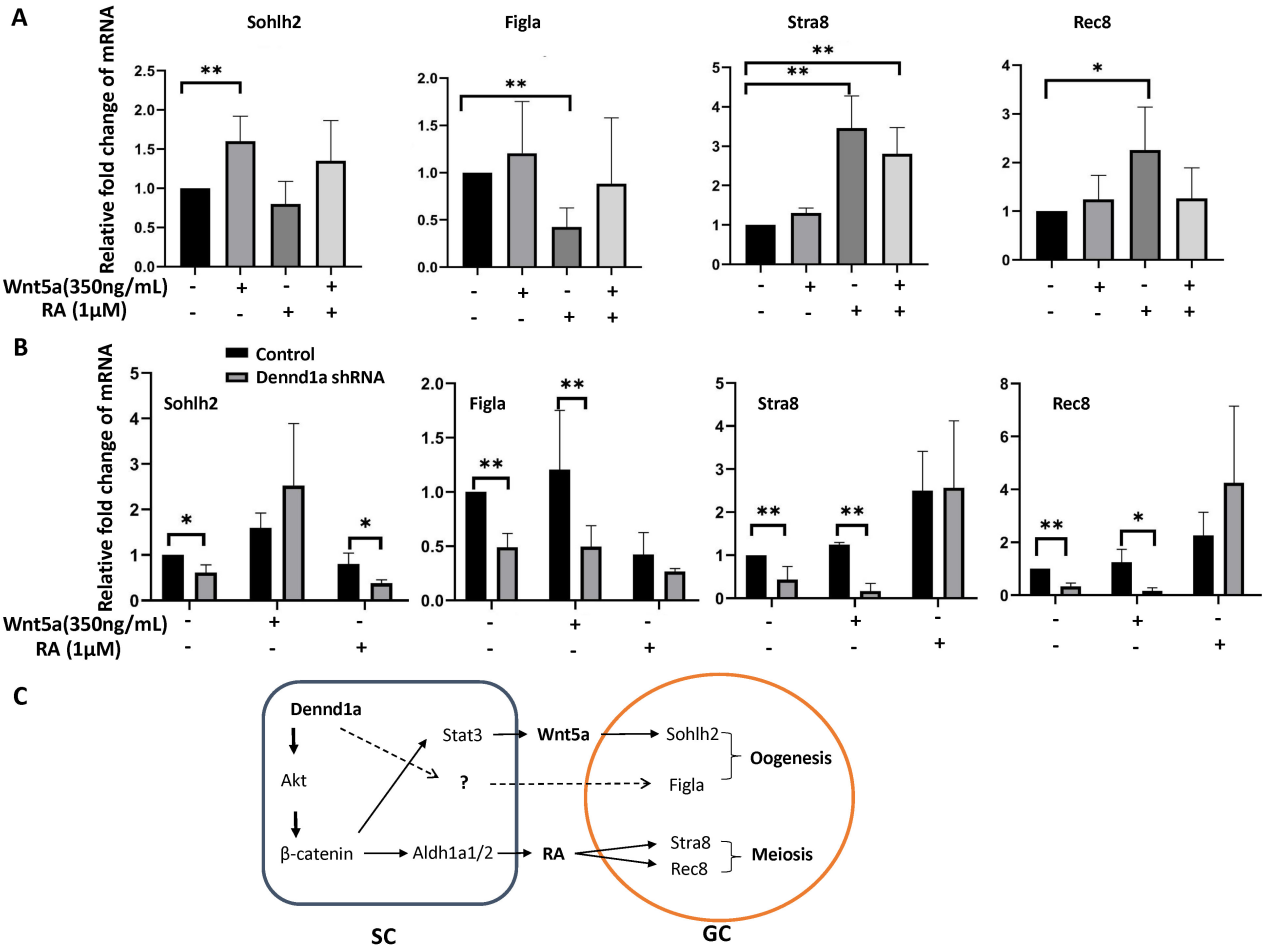

Fig. 7. Exogenous WNT5A and RA stimulate the expression of genes associated with initiation of oogenesis and meiosis in the fetal germ cells. (A) The fetal gonad culture of E12.5 female wild-type mouse embryos was treated with recombinant Wnt5a (350 ng/mL) or RA (1 μM) or both for 48 h. The mRNA expression of Sohlh2, Figla, Stra8, Rec8 were examined by real time PCR (n = 5). (B) The control and Dennd1a knockdown fetal ovaries were cultured *ex vivo* with or without Wnt5a or RA for 48 h. The mRNA expression of Sohlh2, Figla, Stra8, and Rec8 were examined by real time PCR (n = 5). (C) The schematic shows that Dennd1a could be involved in multiple signal pathways in the somatic cells that are critical for various processes and stages of oocyte differentiation and meiosis in the fetal ovary.

We revealed that Dennd1a is mainly expressed in the somatic cells of the fetal ovary. Ablation of Dennd1a disrupted the mRNA expression of Sohlh2, Figla, Stra8, and Rec8 in the ovary of Dennd1a-/- mutants at E13.5. Using *ex vivo* culture of E12.5 female gonads and adenoviral Dennd1a shRNA infection, we further demonstrated that the transcription of Sohlh2, Figla, Stra8 and Rec8 were not activated in deficiency of Dennd1a. In mouse embryo, oogenesis begins with the differentiation of PGCs into oogonia following sexual differentiation at approximately E12.5 [11]. Meanwhile, PGCs receives RA signals from the somatic cells of the ovary and mesonephros and gains the capacity for meiotic initiation [12]. Sohlh2 protein is expressed in the fetal germ cells as early as E12.5, while Sohlh1 protein expression occurs circa E15.5. Sohlh2 is among the early oocyte-specific transcription factors that regulates oocyte differentiation without affecting meiosis [11, 23]. Figla is another basic helix-loop-helix transcription factor that is primarily expressed in female germ cells and plays a critical role in oocyte differentiation. Moreover, Figla deficiency impairs oocyte meiotic progression [24, 25]. RA signal activates transcription of Stra8 and Rec8 in ovarian germ cells. Stra8 is required for meiotic initiation including meiotic DNA replication as well as the subsequent processes of meiotic prophase. Rec8 encodes a component of the cohesin complex that accumulates during meiotic S phase, and is essential for chromosome synapsis and segregation. These two factors are independently activated by RA and precede the expression of other meiotic markers [12, 14]. Thus, Dennd1a in the somatic cells seems necessary for activation of these genes in the fetal germ cells, which are critical for oocyte differentiation and meiotic initiation.

Dennd1a in the somatic cells may be involved in regulating the production of Wnt5a and RA because Dennd1a deficiency impaired the expression of both Wnt5a and genes that encoding RA-synthesizing enzymes in the fetal ovary and mesonephros. Wnt5a has been identified as an important cytokine for oocyte maturation and meiotic resumption [18]. We therefore focused our study on the effect of Wnt5a on fetal oocyte differentiation and meiosis. Wnt5a stimulated Sohlh2 during early stage of oocyte differentiation in fetal ovary, though it had no effect on the expression of Figla, Stra8 or Rec8. Moreover, the compromised activation of Sohlh2 in the fetal ovary in deficiency of Dennd1a could be rescued by exogenous Wnt5a. These findings suggest that Dennd1a in the somatic cells might stimulate Sohlh2 expression at early stage of oocyte differentiation via regulating Wnt5a synthesis. On the other hand, Dennd1a-mediated expression of Figla, Stra8 and Rec8 seems independent of Wnt5a. Although the concentrations of RA detected in the culture medium of both gonads and mesonephros by ELISA are lower than the average concentrations in the tissues of mouse embryos [26], our data is consistent with previous reports about the source of RA during germ cell development [12]. Knockdown of Dennd1a in gonad and mesonephros culture resulted in a reduced release of RA in the culture medium, suggesting Dennd1a is implicated in RA synthesis. Furthermore, RA treatment could rescue the compromised activation of Stra8 and Rec8 due to Dennd1a deficiency, indicating that meiotic initiation of the fetal germ cells depends on Dennd1a-mediated RA production. Taken together, Dennd1a could be involved in multiple signal pathways in the somatic cells that are critical for various processes and stages of oocyte differentiation and meiosis.

Dennd1a is a GEF for the small GTPase Rab35, which plays a regulatory role in PI3K/Akt pathway [21, 27]. In our study, Dennd1a deficiency could be responsible for reduced phosphorylation of Akt by compromising Rab35 activity. Reduced phosphorylation of Akt could result in downregulation of β-catenin and β-catenin-dependent target genes. In this study, Dennd1a may mediate PI3K/Akt and β-catenin pathways in the somatic cells of mesonephros, which is consistent with previous finding that loss of Dennd1a disrupted β-catenin signaling in the developing brain [7]. It has been reported that β-catenin signaling promotes the expression and phosphorylation of Stat3, which has been identified as an upstream regulator of Wnt5a [28]. β-catenin also induces the expression of Aldh1a1 [29]. Downregulated β-catenin signaling could be the altered upstream regulators for Wnt5a and RA synthesis, through decreased expression and phosphorylation of Stat3 and reduced expression of Aldh1a2. Therefore, β-catenin could be pivotal for regulating Wnt5a and RA synthesis in the somatic cells, which in turn mediate the initiation of oogenesis and meiosis of germ cells in the fetal ovary (Fig. 7C). However, the cross-talk among Akt, β-catenin, Wnt5a and Aldh1a1 signaling was complex and controversial [17–19, 29, 30]. Therefore, the signaling targets of Dennd1a in the somatic cells and the mechanism by which they control fetal oocyte differentiation and meiosis need further investigation.

6. Conclusions

We discovered that the ablation of Dennd1a in the somatic cells disrupts transcription of Sohlh2, Figla, Stra8, and Rec8 in the fetal germ cells of developing ovary. Dennd1a could be involved in multiple signal pathways in the somatic cells and mediate Wnt5a and RA synthesis, which are necessary for the initiation of oocyte differentiation and meiosis during embryonic ovary development.

7. Author contributions

JF and JM conceived and designed the experiments; JS and QN performed the experiments; JF and QG analyzed the data; JF, QG and JM contributed reagents and materials.

8. Ethics approval and consent to participate

All procedures performed in studies were in accordance with the ethical standards for the care and use of laboratory animals. Approval (#20160704) was obtained from the Institutional Review Board of Shandong University.

9. Acknowledgement

Thanks to all the peer reviewers for their opinions and suggestions

10. Funding

This work was supported by the Basic Science Center Program, China (Grant number: 31988101), the National Key Research and Development Program of China (Grant number: 2016YFC1000601), the Natural Science Foundation of Shandong Province, China (Grant numbers: ZR2016HM79 and ZR2014HM017), and Shandong Provincial Key Research and Development Program, China (Grant number: 2020ZLYS02).

11. Conflict of interest

The authors declare no conflict of interest.

12. References

[1] Allaire PD, Marat AL, Dall'Armi C, Di Paolo G, McPherson PS, Ritter B. The Connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes. Molecular Cell. 2010; 37: 370–382.

[2] Marat AL, McPherson PS. The Connecdenn Family, Rab35 Guanine Nucleotide Exchange Factors Interfacing with the Clathrin Machinery. Journal of Biological Chemistry. 2010; 285: 10627–10637.
[3] Chaineau M, Ioannou MS, McPherson PS. Rab35: GEFs, GAPs and effectors. Traffic. 2013;14: 1109–1117.
[4] Chen ZJ, Zhao H, He L, Shi Y, Qin Y, Shi Y, *et al*. Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. Nature Genetics. 2011; 43: 55–59.
[5] Shi Y, Zhao H, Shi Y, Cao Y, Yang D, Li Z, *et al*. Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome. Nature Genetics. 2012; 44: 1020–1025.
[6] McAllister JM, Modi B, Miller BA, Biegler J, Bruggeman R, Legro RS, *et al*. Overexpression of a DENND1a isoform produces a polycystic ovary syndrome theca phenotype. Proceedings of the National Academy of Sciences. 2014; 111: E1519–E1527.
[7] Shi J, Gao Q, Cao Y, Fu J. Dennd1a, a susceptibility gene for polycystic ovary syndrome, is essential for mouse embryogenesis. Developmental Dynamics. 2019; 248: 351–362.
[8] Ginsburg M, Snow MH, McLaren A. Primordial germ cells in the mouse embryo during gastrulation. Development. 1990; 110: 521–528.
[9] Molyneaux KA, Stallock J, Schaible K, Wylie C. Time-lapse analysis of living mouse germ cell migration. Developmental Biology. 2001; 240: 488–498.
[10] Wilhelm D, Palmer S, Koopman P. Sex Determination and Gonadal Development in Mammals. Physiological Reviews. 2007; 87: 1–28.
[11] Shin Y, Ren Y, Suzuki H, Golnoski KJ, Ahn HW, Mico V, *et al*. Transcription factors SOHLH1 and SOHLH2 coordinate oocyte differentiation without affecting meiosisi. Journal of Clinical Investigation. 2017; 127: 2106–2117.
[12] Endo T, Mikedis MM, Nicholls PK, Page DC, de Rooij DG. Retinoic Acid and Germ Cell Development in the Ovary and Testis. Biomolecules. 2019; 9: 775.
[13] Ghyselinck NB, Duester G. Retinoic acid signaling pathways. Development. 2019; 146: dev167502.
[14] Koubova J, Hu Y, Bhattacharyya T, Soh YQS, Gill ME, Goodheart ML, *et al*. Retinoic acid activates two pathways required for meiosis in mice. Public Library of Science Genetics. 2014; 10: e1004541.
[15] Chawengsaksophak K, Svingen T, Ng ET, Epp T, Spiller CM, Clark C, *et al*. Loss of Wnt5a disrupts primordial germ cell migration and male sexual development in mice. Biology of Reproduction. 2012; 86: 1–12.
[16] Laird DJ, Altshuler-Keylin S, Kissner MD, Zhou X, Anderson KV. Ror2 enhances polarity and directional migration of primordial germ cells. Public Library of Science Genetics. 2011; 7: e1002428.
[17] Cantú AV, Altshuler-Keylin S, Laird DJ. Discrete somatic niches coordinate proliferation and migration of primordial germ cells via Wnt signaling. Journal of Cell Biology. 2016; 214: 215–229.
[18] Liu X, Hao Y, Li Z, Zhou J, Zhu H, Bu G, *et al*. Maternal Cytokines CXCL12, VEGFA, and WNT5A Promote Porcine Oocyte Maturation via MAPK Activation and Canonical WNT Inhibition. Frontiers in Cell and Developmental Biology. 2020; 8: 578.
[19] Zhang NN, Zhang T, Gao WY, Wang X, Wang ZB, Cai JY, *et al*. Fam70A binds Wnt5a to regulate meiosis and quality of mouse oocytes. Cell Proliferation. 2020; 53: e12825.
[20] Wambach CM, Patel SN, Kahn DA. Maternal and fetal factors that contribute to the localization of T regulatory cells during pregnancy. American Journal of Reproductive Immunology. 2014; 71: 391–400.
[21] Wheeler DB, Zoncu R, Root DE, Sabatini DM, Sawyers CL. Identification of an oncogenic RAB protein. Science. 2015; 350: 211–217.
[22] Klinkert K, Echard A. Rab35 GTPase: a Central Regulator of Phosphoinositides and F-actin in Endocytic Recycling and beyond. Traffic. 2016; 17: 1063–1077.
[23] Choi Y, Yuan D, Rajkovic A. Germ cell-specific transcriptional regulator sohlh2 is essential for early mouse folliculogenesis and oocyte-specific gene expression. Biology of Reproduction. 2008; 79: 1176–1182.
[24] Soyal SM, AmLeh A, Dean J. FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. Development. 2000; 127: 4645–4654.
[25] Wang Z, Liu C, Zhao Y, Dean J. FIGLA, LHX8 and SOHLH1 transcription factor networks regulate mouse oocyte growth and differentiation. Nucleic Acids Research. 2020; 48: 3525–3541.
[26] Horton C, Maden M. Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. Developmental Dynamics. 1995; 202: 312–323.
[27] Allaire PD, Seyed Sadr M, Chaineau M, Seyed Sadr E, Konefal S, Fotouhi M, *et al*. Interplay between Rab35 and Arf6 controls cargo recycling to coordinate cell adhesion and migration. Journal of Cell Science. 2013; 126: 722–731.
[28] Katoh M, Katoh M. STAT3-induced WNT5a signaling loop in embryonic stem cells, adult normal tissues, chronic persistent inflammation, rheumatoid arthritis and cancer (Review). International Journal of Molecular Medicine. 2007; 19: 273–278.
[29] Condello S, Morgan CA, Nagdas S, Cao L, Turek J, Hurley TD, *et al*. B-Catenin-regulated ALDH1a1 is a target in ovarian cancer spheroids. Oncogene. 2015; 34: 2297–2308.
[30] Wang W, He S, Zhang R, Peng J, Guo D, Zhang J, *et al*. ALDH1A1 maintains the cancer stem-like cells properties of esophageal squamous cell carcinoma by activating the AKT signal pathway and interacting with β-catenin. Biomed Pharmacother. 2020; 125: 109940.

Supplementary material: Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/26/12/10.52586/5045.

Keywords: Dennd1a; Wnt5a; Retinoic acid; Fetal oogenesis; Meiosis

Send correspondence to:

Jiang Fu, Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, 250012 Jinan, Shandong, China, Key Laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, 250012 Jinan, Shandong, China, Shandong Key Laboratory of Reproductive Medicine, Shandong University, 250012 Jinan, Shandong, China, Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, 250012 Jinan, Shandong, China, E-mail: fujiang@sdu.edu.cn

Jinlong Ma, Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, 250012 Jinan, Shandong, China, Key Laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, 250012 Jinan, Shandong, China, Shandong Key Laboratory of Reproductive Medicine, Shandong University, 250012 Jinan, Shandong, China, Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, 250012 Jinan, Shandong, China, E-mail: majinlong@sduivf.com