Studying the Functions of TGF-β Signaling in the Ovary

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Abstract

In mammals, ovulation is a multistep physiological process that includes preovulatory follicle growth, oocyte meiotic maturation, cumulus-oocyte complex (COC) expansion, follicle rupture, and luteinization. TGF-β signaling pathway has multiple functions in mammalian ovary, as its complexity in ovarian function has been demonstrated by mouse models with knockouts of TGF-β receptors and SMADs. We describe the protocol that we use to study functions of TGF-β signaling pathway in follicle development and ovulation. Because total knockout of TGF-β pathway components often causes embryonic lethality, which prevents further investigation of these genes in ovarian functions, people have generated ovarian cell type-specific knockout mouse strains for TGF-β signaling pathway genes. These mouse models are also described.

Key words Ovary, Follicle, Oocyte, Ovulation, Female fertility

1 Introduction

In mammals, ovulation is a multistep physiological process that includes preovulatory follicle growth, oocyte meiotic maturation, cumulus-oocyte complex (COC) expansion, follicle rupture, and luteinization. The pituitary-secreted gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH) are major regulators of these events. During each estrus cycle, FSH facilitates the rapid growth of preantral and early antral follicles to enter the preovulatory stage. Then, a mid-estrous LH surge triggers the initiation of ovulation and the terminal differentiation of GCs into luteal cells [1].

In addition to gonadotropins, local ovarian signaling factors also play crucial roles during specific steps of ovulation. For example, EGF-like factors are intrafollicular mediators of LH effects, including triggering oocyte germinal vesicle breakdown (GVBD) and COC expansion [2, 3]. Granulosa and cumulus cell-produced prostaglandin E2 (PGE2) is important for organizing the COC matrix and in positive feedback regulation of EGF-like factors [4].

It is now well accepted that the oocyte is not only a passenger carried by the follicle, but is also an active regulator of follicle growth and ovulation. Oocyte-derived factors, including growth and differentiation factor 9 (GDF9) and bone morphogenic protein 15 (BMP15), promote COC expansion and the expressions of several key target genes involved in ovulation regulation by LH (*Has2*, *Ptgs2*, *Tnfaip6*, and *Ptx3*) [5]. GDF9 and BMP15 are both signaling molecules of the TGF-β super family. They bind to their respective membrane receptors and trigger the serine/threonine protein kinase activity of these receptors [6]. As a result, several members of the SMAD family of transcription factors become phosphorylated; SMAD1/5/8 in response to BMPs and SMAD2/3 in response to other TGF-β family ligands. Subsequently, phosphorylated SMAD1/5/8 and SMAD2/3 form heterodimers with SMAD4 and enter the nucleus, which regulates the expression of their target genes. This is the canonical TGF-β signaling pathway [7].

The TGF-β signaling pathway has multiple functions in the mammalian ovary. For example, in the ovaries of new born Gdf9 KO mice, oocytes cannot recruit the surrounding ovarian somatic cells to form follicles, which indicate that this paracrine factor is essential for follicle formation [8]. Knockout of inhibin and activin, TGF-β family ligands that are secreted by GCs, causes multiple ovarian defects by disrupting the feedback regulation between the pituitary and ovary [9]. In both murine and human ovaries, the TGF-β family ligand AMH is an intraovarian growth factor that regulates primordial follicle recruitment and the FSH sensitivity of growing follicles in an inhibitory manner [10].

The complexity of the TGF-β signaling pathway in ovarian functions has been further demonstrated by mouse models with knockouts of TGF-β receptors and SMADs. Conditional knockout of BMP receptor 1A/1B (BMPR1A/B) [11] or SMAD1/5/8 [12] in the GCs of developing follicles results in the oncogenic transformation of these cells. Deletion of both SMAD2 and 3 dramatically reduces female fertility, which is associated with disrupted multiple ovarian processes, including follicular development, ovulation, and COC expansion [13]. Most importantly, GC-specific depletion of SMAD4, the central component of the canonical TGF-β signaling pathway, causes premature luteinization of GCs followed by ovulation failure [14].

2   Materials

2.1   Cell Culture Reagents

Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), calcium and magnesium free phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin/streptomycin sulfate, and 0.25 % Trypsin/EDTA were purchased from Invitrogen. Bovine serum albumin (BSA), mineral oil, milrinone, and M2 and M16 media are purchased from Sigma.

2.2 Chemicals   Recombinant amphiregulin, GDF9, BMP15, and activin are purchased from R&D systems. TGF-β receptor I inhibitor SB431542 and pregnant mare serum gonadotropin (PMSG) are purchased from Calbiochem. EGF, prostaglandin E2, forskolin, and PMA are purchased from Sigma. Human chorionic gonadotropin (hCG) is purchased from American Pharmaceutical Partners (Schaumburg, IL).

2.3 Solutions   Acidic M2 medium: M2 medium containing 1% saturated hydrochloric acid.

Immunofluorescent staining wash buffer: PBS with 0.1 % Tween and 0.01 % Triton X-100.

Immunofluorescent staining blocking buffer: wash buffer with 1% BSA.

Tail buffer: 5 mL of 1 M Tris–HCl, pH 8.0, 7.5 mL of 5 M NaCl, 25 mL of 0.5 M EDTA, 10 % SDS, and adjusted to 500 mL with dH$_2$O.

2.4 Cre-expressing Mouse Strains   *Amhr2-Cre:* The expression of CRE recombinase in this mouse strain is under the control of the anti-Müllerian hormone type II receptor (*Amhr2*) promoter. CRE activity is found in the ovary as early as embryonic day 17.5. Throughout the postnatal ovary, CRE activity is found in GCs of all secondary and small antral follicles. However, low CRE activity is also found in some theca cells [14]. In addition, as expected, the muscular layer of the uterus is CRE positive, as Amhr2 is expressed in the mesenchyme of the Müllerian duct that gives rise to the uterine musculature.

Cyp19-Cre: The expression of Cre DNA recombinase in Cyp19-Cre mice is under the control of GC-specific Cyp19a1 promoter II. CRE activity is primarily detected during the preantral/early antral stage of follicle development.

Gdf9-Cre: Oocyte-specific deletion of ovarian genes using Cre/loxP technology provides an excellent tool for determining their physiological roles in the TGF-β signaling pathway during folliculogenesis, oogenesis, and pre-implantation embryonic development. A transgenic mouse line that expressed improved Cre recombinase (*iCre*) driven by the mouse growth differentiation factor-9 (GDF-9) promoter was used. In this mouse strain, Cre recombinase is only expressed in postnatal ovaries. Within the ovary, Cre recombinase is exclusively expressed in the oocytes of primordial follicles and follicles at later developmental stages. The expression of iCre in GDF-9-iCre mice is earlier than Cre expression in Zp3-Cre mice (see below).

Zp3-Cre: The expression of Cre recombinase in this mouse strain is under the control of the *Zp3* promoter. The *Zp3* gene encodes the mouse zona pellucida protein ZP3. In female *Zp3-Cre* transgenic mice, Cre activity is first detected in activated oocytes of primary follicles, but not in dormant oocytes of primordial follicles.

Therefore, *Zp3-Cre* is used to efficiently delete target genes in activated oocytes without affecting the ovarian oocyte storage pool (primordial follicles). Both *Gdf9-Cre* and *Zp3-Cre* are not expressed in male germ cells.

3   Methods

3.1   Mouse Granulosa Cell Harvesting and Culture

1. Intraperitoneally inject pubertal female mice (21-days-old) with 4 IU of PMSG to stimulate granulosa cell proliferation and follicle maturation. Humanly sacrifice mice 24 h after PMSG injection (*see* **Notes 1** and **2**).

2. Humanely sacrifice mice and isolate their ovaries. Place ovaries on a piece of clean wet paper tower, and carefully remove the fat and connective tissues and the oviduct adjacent to the ovaries. Then, place the ovaries in a sterile 3.5 mm cell culture dish (5–6 ovaries per dish) containing 2 mL of DMEM/F12 medium.

3. Release undifferentiated GCs from antral follicles by repeatedly puncturing the ovary with a 26.5 gauge needle. Remove the ovarian tissue debris from the dish after thorough puncturing.

4. Place the cell suspension in a sterile 15 mL centrifuge tube, and centrifuge at $700 \times g$ for 5 min.

5. Carefully remove the supernatant and resuspend the cells in 10 mL of DMEM/F12. Repeat **step 4** twice.

6. After a final wash, resuspend at a density of 1×10^6 cells/mL in DMEM/F12 medium (Invitrogen) supplemented with 5 % fetal bovine serum (FBS; Invitrogen), 100 U/mL of penicillin, and 100 µg/mL of streptomycin, and culture in 24-well culture dishes. After overnight incubation, cells are washed and cultured in serum-free DMEM/F12 medium before any further treatments.

3.2   In Vitro Hormone Treatment

3.2.1   Evaluating the Role of the TGF-β Signaling Pathway in FSH Actions

1. Pre-treat cultured granulosa cells with the ALK inhibitor SB431542 (10 µM) to block the TGF-β pathway, or with recombinant TGF-β ligands (GDF9, BMP15, activin, or AMH) to activate the TGF-β pathway.

2. Treat the cells with FSH (100 ng/mL) by directly adding this hormone to the medium.

3. FSH-induced phosphorylation of the cAMP/PKA pathway, the RAS/MAPK pathway, and the PI3K/AKT pathway can be determined by Western blot analysis. Harvest cells 30–60 min after FSH treatment by adding SDS protein loading buffer directly to the wells of a cell culture dish (*see* **Note 3**).

4. To determine transcriptional regulation of ovarian genes by FSH and TGF-β, harvest cells at 4, 12, and 24 h after FSH treatment by adding Trizol Reagent (300 µL) directly to each

well of a cell culture dish. The following genes are usually analyzed by real-time RT-PCR: *Cyp19a1*, which encodes aromatase, the key enzyme of estradiol biosynthesis that is upregulated by FSH and TGF-β signals; *Fshr*, which encodes the FSH receptor that is upregulated by FSH in a positive feedback manner; *Lhcgr*, which encodes the LH receptor that is upregulated by FSH, but is downregulated by TGF-β ligands.

3.2.2 Evaluating the Role of the TGF-β Signaling Pathway in LH Actions

1. Pre-treat cultured granulosa cells with the ALK inhibitor SB431542, or with recombinant TGF-β ligands, as described in **step 1** of Subheading 3.2.1.

2. To induce in vitro luteinization, treat the cells with LH (500 ng/mL) by directly adding this hormone to the medium. Alternatively, treat cells with forskolin (10 μM) plus PMA (20 nM) to mimic LH actions and induce more robust luteinization in cultured granulosa cells.

3. To determine the transcriptional regulation of ovulation-related genes by FSH and TGF-β, harvest the cells 2–4 h after LH treatment. The following genes are usually analyzed by real-time RT-PCR: *Areg, Ereg,* and *Btc*, which encode the EGF-like factors amphiregulin, epiregulin, and beta-cellulin, respectively, which are intrafollicular mediators of LH actions and whose expressions are known to be downregulated by TGF-β signals; *Has2, Ptgs2, Ptx3*, and *Tnfaip6*, which are essential for cumulus expansion and ovulation and are known to be significantly upregulated by LH in granulosa cells and cumulus cells in a TGF-β-dependent manner; *Lhcgr*, which encodes the LH receptor that is upregulated by FSH, but is downregulated by TGF-β ligands.

4. To determine the transcriptional regulation of luteinization-related genes by FSH and TGF-β, harvest the cells 24 h after LH treatment. The following genes are usually analyzed by real-time RT-PCR: *Star* and *Cyp11a1*, which encode steroidogenic acute regulatory protein and cholesterol side-chain cleavage enzyme, respectively, which are essential for progesterone biosynthesis; *Lhcgr*, which encodes the LH receptor; and *Sfrp4*, which encodes secreted frizzled-related protein 4. The expressions of these genes are known to be strongly induced by LH, but are repressed by TGF-β ligands. Primer sequences are listed in Table 1.

RNA Sample Preparation and Reverse Transcription

1. Intraperitoneally inject female mice with 5 IU of pregnant mare serum gonadotropin (PMSG) to stimulate preovulatory follicle development.

2. After 44–48 h, humanely sacrifice mice by cervical dislocation, dissect out the ovaries, and remove fat and connective tissues.

Table 1
Primers used for RT-PCR

Gene name	Primer sequence
Cyp19a1	Forward: 5′-TGTTGTGGACTTGGTCATGC-3′ Reverse: 5′-GGCTGAAAGTACCTGTAGGGAA-3′
Fshr	Forward: 5′-ACCATGGCTTAGAAAATCTGAAG-3′ Reverse: 5′-GATCCCCAGGCTGAGTCATA-3′
Lhcgr	Forward: 5′-TGCCCTCCAAAGAAAAATTC-3′ Reverse: 5′-AATCGTAATCCCAGCCACTG-3′
Areg	Forward: 5′-TTTATCTTCACACATCTCTTTATGTACAG-3′ Reverse: 5′-GAGGCTTCGACAAGAAAACG-3′
Ereg	Forward: 5′-GTCCCCTGAGGTCACTCTCTC-3′ Reverse: 5′-ACACTGGTCTGCGATGTGAG-3′
Btc	Forward: 5′-CTCCCTCCTGCATCTGTGAG-3′ Reverse: 5′-CTCTTGAATATCTTCACTTATGGGAG-3′
Has2	Forward: 5′-GTTGGAGGTGTTGGAGGAGA-3′ Reverse: 5′-ATTCCCAGAGGACCGCTTAT-3′
Ptgs2	Forward: 5′-TGTACAAGCAGTGGCAAAGG-3′ Reverse: 5′-CCCCAAAGATAGCATCTGGA-3′
Ptx3	Forward: 5′-GTGGGTGGAAAGGAGAACAA-3′ Reverse: 5′-GGCCAATCTGTAGGAGTCCA-3′
Tnfaip6	Forward: 5′-TTCCATGTCTGTGCTGCTGGATGG-3′ Reverse: 5′-AGCCTGGATCATGTTCAAGGTCAAA-3′
Star	Forward: 5′-CACACATTTTGGGGAGATGC-3′ Reverse: 5′-GAACTCTATCTGGGTCTGCGATA-3′
Cyp11a1	Forward: 5′-GTCTTACACAGACGCATCAAGC-3′ Reverse: 5′-ACACTGGTGTGGAACATCTGG-3′
Sfrp4	Forward: 5′-ATGCTCCGCTCCATCCTGGTG-3′ Reverse: 5′-TGGCCAGGATGGCGTTCTCC-3′

3. Place the ovaries in M2 medium and prick follicles using sharp needles under a stereoscope.

4. Collect fully grown germinal vesicle (GV)-stage oocytes under a stereoscope with a mouth-controlled micropipette.

5. Extract total RNA from 100 to 150 GV-stage oocytes using an RNeasy Micro kit (Qiagen) according to the manufacturer's instructions.

6. Add 1× first-strand buffer, 10 mM DDT, 1 mM dNTP, 25 nM oligo-dT, 20 U of RNaseOUT, and 25 U of SuperScriptII.

7. Reverse transcribe total RNA for 60 min at 42 °C and then 10 min at 95 °C.

Identify samples by PCR.

3.3   Mouse Cumulus-Oocyte Complex (COC) Culture and In Vitro COC Expansion Assay

The TGF-β family members GDF9 and BMP15 are oocyte-secreted factors that regulate cumulus cell differentiation and ovulation. Therefore, in vitro COC expansion experiments are typically used to study the role of TGF-β signaling in ovulation.

1. Intraperitoneally inject mice (3-weeks-old) with 5 IU of pregnant mare serum gonadotropin (PMSG) to stimulate preovulatory follicle development.

2. After 44–48 h, humanely sacrifice mice by cervical dislocation, dissect out the ovaries, and remove fat and connective tissues.

3. Cumulus-oocyte complexes (COCs) are released from antral follicles by puncturing with a 26.5 gauge needle and placed in COC medium (MEM w/ES, 0.25 mM sodium pyruvate, 3 mM L-GLUTAMINE, 1 mg/mL of BSA, 100 U/mL of penicillin, 100 μg/mL of streptomycin) with 1 % FBS.

4. Collect nonexpanded cumulus-oocyte complexes (COCs) with a micropipette of a suitable diameter, transfer the cumulus-oocyte complexes (COCs) in 50 μL of COC medium with 1 % FBS, and cover with mineral oil in a nunclon plate.

5. COC expansion can be induced by adding FSH (100 ng/mL), amphiregulin (250 ng/mL), epidermal growth factor (EGF, 10 ng/mL), forskolin (10 μM), or prostaglandin E2 (PGE2, 500 nM) to the medium. Culture COCs overnight in a 5 % CO_2 incubator at 37 °C.

6. Check the expansion status of COCs with a microscope the next day.

3.4   Mouse Denuded Oocyte Harvesting and Culture

1. Intraperitoneally inject female mice with 5 IU of pregnant mare serum gonadotropin (PMSG) to stimulate preovulatory follicle development.

2. After 44–48 h, humanely sacrifice mice by cervical dislocation, dissect out the ovaries, and remove fat and connective tissues.

3. Place the ovaries in M2 medium with 2.5 mM milrinone and prick follicles using sharp needles under a stereoscope.

4. Frequently agitate and pipette the tissues.

5. Collect fully grown germinal vesicle (GV) oocytes under a stereoscope with a mouth-controlled micropipette of a suitable diameter.

6. Wash GV oocytes three times by transferring the oocytes into fresh M2 medium without milrinone.

7. Transfer fully grown GV oocytes to a mini-drop of M16 medium covered with mineral oil by micropipette.

8. Culture germinal vesicle (GV)-stage oocytes in a 5 % CO_2 incubator at 37 °C.

3.5 Mouse Superovulation Procedures

1. Intraperitoneally inject female mice with 5 IU of pregnant mare serum gonadotropin (PMSG).

2. After 48 h, inject female mice with 5 IU of human chorionic gonadotrophin (hCG).

3. After 16 h, humanely sacrifice mice by cervical dislocation, dissect out the oviducts, and remove fat and connective tissues.

4. Place oviducts in M2 medium, blow out oocytes from oviducts using a mouth-controlled micropipette under a stereoscope.

5. Record the numbers of oocytes harvested from oviducts.

3.6 Immuno-fluorescent and Confocal Microscopy Imaging

1. Humanely sacrifice mice by cervical dislocation, dissect out the ovaries, and remove fat and connective tissues.

2. Place ovaries in M2 medium and prick follicles using sharp needles under a stereoscope.

3. Agitate and pipette the tissues, and collect oocytes under the stereoscope with a mouth-controlled micropipette of an appropriate diameter.

4. Remove the Zona Pellucida of oocytes with acidic M2 medium, 2–3 s each time.

5. Wash oocytes in fresh M2 medium.

6. Transfer oocytes to 4 % paraformaldehyde in PBS, and fix oocytes for at least 30 min at room temperature.

7. Permeabilize oocytes with a membrane-permeabilizing solution (PBS plus 0.3 % Triton X-100) for 30 min at room temperature in a humid chamber.

8. Incubate oocytes with a blocking buffer (1 % BSA) for 1 h at room temperature.

9. Incubate oocytes with a primary antibody (diluted in blocking buffer) overnight at 4 °C or for 1 h at room temperature.

10. Wash oocytes three times (5 min each) in wash solution (PBS containing 0.1 % Tween 20).

11. Label oocytes with a secondary antibody (diluted 1:200 in wash solution) for 1 h at room temperature.

12. Counterstain oocytes with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg/mL in wash solution) for 5–10 min.

13. Wash oocytes three times (5 min each) in wash solution.

14. Drop approximately 2–3 μL of 80 % glycerol with a pipette tip on a glass slide and carefully transfer oocytes to the slide with a minimal amount of wash solution.

15. Place four small columns of Vaseline around the glycerol drop with a pipette tip, and mount a cover slip on the slide.

16. Examine the pattern and localization of the fluorescent signals in oocytes using a confocal microscope.

17. Take oocyte images for each 0.5–1 μm thickness using the Z-stack function of the confocal microscope.

3.7 Genotyping

1. Cut approximately 0.5 cm from the end of a mouse tail, place in a centrifuge tube, and label it.

2. Add 400 μL of tail buffer (contains protease K; 1:200) into the tube, and digest the tail overnight at 65 °C.

3. Add 400 μL of a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1), vortex, and let stand for 5 min at room temperature.

4. Centrifuge at $15,000 \times g$ for 10 min using a microfuge.

5. Transfer 350 μL of the supernatant to a clean centrifuge tube.

6. Add 1 mL of 100 % ethanol into the tube, and gently tip the tube upside down a few times.

7. Centrifuge at 12,000 rpm for 5 min using a microfuge.

8. Pour out the supernatant, add 1 mL of 70 % ethanol into the tube, and shake.

9. Centrifuge at 12,000 rpm for 5 min using a microfuge.

10. Pour out the 70 % ethanol, and completely remove any remaining ethanol with a pipette.

11. Dry DNA at room temperature, and then add 300–400 μL of TE buffer to dissolve the DNA (65 °C, 30 min).

12. PCR reaction.

13. Apply the PCR products to agarose gel electrophoresis using an appropriate gel concentration to determine mouse genotypes.

PCR reaction mixtures for genotyping include the following:

Template: 1 μL.

10× PCR buffer: 2 μL.

dNTP (10 μM): 0.5 μL.

Forward primer: 0.5 μL.

Reverse primer: 0.5 μL.

DNA polymerase: 0.2 μL.

Add ddH_2O up to 20 μL.

4   Notes

1. To harvest undifferentiated granulosa cells and study ovarian responses to exogenous gonadotropins, PD21 female mice should be used to avoid the complexity of ovarian functions associated with estrous cycles and endogenous gonadotropin surges. In addition, the ovaries of adult mice contain the corpus lutea of different developmental stages, which will affect the purity of harvested undifferentiated granulosa cells.

2. Based on our experience, 24 h after PMSG injection is the best time point for harvesting undifferentiated granulosa cells. If the mouse granulosa cells are isolated at later time points, such as 44–48 h after PMSG injection, they usually spontaneously luteinize in culture, which precludes the analyses of TGF-β functions in undifferentiated granulosa cells.

3. It is not necessary to measure the protein concentrations in each sample, as only small amounts of protein are present in this experiment. Typically, 200 μL of SDS-loading buffer is added to each well of a 24-well plate to lyse cells, and 25–30 μL of the lysate is loaded into each lane for Western blotting. These lysates are sticky and difficult to load on SDS-PAGE gels. Repeated freezing and thawing can reduce the lysates' stickiness.

Acknowledgements

References

1. Fan HY, Richards JS (2010) Minireview: physiological and pathological actions of RAS in the ovary. Mol Endocrinol 24(2):286–298, PMCID: 2817603

2. Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM et al (2009) MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. Science 324(5929):938–941

3. Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303(5658):682–684

4. Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS (2006) Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. Mol Endocrinol 20(6):1352–1365

5. Diaz FJ, Wigglesworth K, Eppig JJ (2007) Oocytes determine cumulus cell lineage in mouse ovarian follicles. J Cell Sci 120(Pt 8): 1330–1340

6. Pangas SA, Matzuk MM (2005) The art and artifact of GDF9 activity: cumulus expansion

and the cumulus expansion-enabling factor. Biol Reprod 73(4):582–585

7. Pangas SA (2012) Bone morphogenetic protein signaling transcription factor (SMAD) function in granulosa cells. Mol Cell Endocrinol 356(1-2):40–47, PMCID: 3203253

8. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383(6600):531–535

9. Pangas SA, Woodruff TK (2000) Activin signal transduction pathways. Trends Endocrinol Metab 11(8):309–314

10. van Houten EL, Themmen AP, Visser JA (2010) Anti-Mullerian hormone (AMH): regulator and marker of ovarian function. Ann Endocrinol (Paris) 71(3):191–197

11. Edson MA, Nalam RL, Clementi C, Franco HL, Demayo FJ, Lyons KM et al (2010) Granulosa cell-expressed BMPR1A and BMPR1B have unique functions in regulating fertility but act redundantly to suppress ovarian tumor development. Mol Endocrinol 24(6):1251–1266, PMCID: 2875809

12. Pangas SA, Li X, Umans L, Zwijsen A, Huylebroeck D, Gutierrez C et al (2008) Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. Mol Cell Biol 28(1):248–257, PMCID: 2223289

13. Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M, Matzuk MM (2008) Redundant roles of SMAD2 and SMAD3 in ovarian granulosa cells in vivo. Mol Cell Biol 28(23):7001–7011, PMCID: 2593383

14. Pangas SA, Li X, Robertson EJ, Matzuk MM (2006) Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. Mol Endocrinol 20(6):1406–1422