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Determination of the Granulosa Cell-Specific Endothelin Receptor A Deletion on Ovarian Function

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ABSTRACTS

Endothelin 2 (EDN2) induces follicular rupture by constricting periovulatory follicles. In this study, it was investigated the mechanisms of EDN2 action on follicular rupture with respect of receptor using the conditionally granulosa cell specific EDN2 receptor type A (ETa) KO mice (gcETaKO; $ETa^{flox/-}$ · $Amhr2^{Cre}$). It was generated the gcETaKO mice by breeding with $ETa^{flox/-}$ mice after mono-alleic ETa knockout by $ZP3^{Cre}$ and $Amhr2^{Cre}$ mice. Fertility, ovulation and maturation rates of ovulated oocytes after super ovulation were investigated in the gcETaKO mice compared with wild-type mice ($ETa^{flox/flox}$ and $ETa^{flox/-}$) as a control group. In the gcETaKO mice, normal fertility after breeding with male mice was shown compared with wild-type mice. And, there was no significant differences in ovulation rates after super ovulation, however its maturation rates was lower than that of wild type mice. These findings show that EDN2 in follicular rupture for ovulation is related with an other ETa not in granulosa cells. Further studies are needed to investigate how EDN2 is acted in ovarian follicular rupture for ovulation.

(Key words : endothelin2, endothelin receptor type A, ovary, ovulation, granulosa cell)

INTRODUCTION

It was reported that endothelin 2 (EDN2) is essential in the ovarian contraction for follicular rupture (Ko *et al.*, 2006). In this study, EDN2 was produced in the granulosa cells but diffuses through the weakened follicular wall, reaching the theca externa where smooth muscle cells are located, and inducing muscle contraction for ovulation (Ko *et al.*, 2006). After report of EDN2 roles in follicular rupture for ovulation, several studies were reported to investigate how EDN2 is acted via which receptors out of receptor type A (ETa) and B (ETb) in the ovarian contraction by pharmaceutical approaches using antagonists of EDN2 receptors (Al-Alem *et al.*, 2007; Bridges *et al.*, 2010; Palanisamy *et al.*, 2006). However, limitations of these pharmaceutical approaches derived from different results among these studies were shown.

To overcome these limitations and make a definite conclusion, animal model systems were needed. For using animal models for ETa deletion, conditional ETa knockout (KO) mice are needed because total KO mice cannot be survived by severe craniofacial and cardiovascular defects (Clouthier *et al.*, 1998). For conditional ETa KO in the ovary, Amhr2Cre system was applied into $ETa^{flox/flox}$ model that ETa was delete donlyin the granulos a cells (Arango *et al.*, 2008; Hernandez Gifford *et al.*, 2009; Jorgez *et al.*, 2004). Using these animal models, roles of ETa in the granulosa cells (gcETa) in follicular rupture for ovulation by EDN2 could be elucidated more definitely than pharmaceutical approaches.

Here, it was investigated the reproductive abilities in the gcETa- KO mice including fertility, ovulation and maturation rates after superovulation compared with wild type (WT) mice to investi- gate the EDN2 mechanisms in ovarian follicular rupture for ovulation especially what type of EDN receptor is acted with EDN2.

MATERIALS AND METHODS

1. Materials

Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Sigma (St. Louis, MO). Cell strainers were supplied by Becton Dickinson Falcon (Billerica, MA). Media for experiments were obtained

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from Life Technologies, Inc. (Grand Island, NY). Molecular reagents were purchased from Invitrogen (Invitrogen, Carlsbad, CA).

2. Animals

To generate the granulosa cell-specific ETa KO mice with genotype of $ETa^{flox/-} \cdot Amhr2^{Cre}$, 3 mutant mouse lines were used as founders. Cre recombination by $Amhr2^{Cre}$ was too weak to delete the 2 loxP sites simultaneously, mono-alleic loxP was already deleted by $ZP3^{Cre}$ during the meiosis of oocytes. The floxed ETa ($ETa^{flox/-}$) mouse line, which carries two loxP sites in the introns flanking exon 6, 7 and 8 of the ETa gene (Kedzierski *et al.*, 2003), was used as a target of ETa gene excision. The transgenic mouse line was used to selectively delete ETa in the granulosa cells as was previously described (Jorgez *et al.*, 2004). Briefly, a female $ETa^{flox/flox}$ mouse was first bred with a male $Amhr2^{Cre}$ mouse, which resulted in the production of F1 heterozygotes, $ETa^{flox/wt} \cdot Amhr2^{Cre}$. Then $ETa^{flox/-} \cdot Amhr2^{Cre}$ female mice can be generated by breeding of male F1 $ETa^{flox/wt} \cdot Amhr2^{Cre}$ mice and female $ETa^{flox/-}$ mice (Bagchi & Ghosh, 2006). Mice with genotype of $ETa^{flox/flox}$ and $ETa^{flox/-}$ did not have $Amhr2^{Cre}$ gene after geno- typing as wild type mice for control group were used.

3. Fertility

In the procedure to generate the gcETaKO mice, many types of genotype by Mendelian inheritance were shown in the pups after genotyping. Out of these mice with different genotypes, it was used 2 types of genotype with Amhr2Cre gene ($ETa^{flox/flox} \cdot Amhr2^{Cre}$ or $ETa^{flox/-} \cdot Amhr2^{Cre}$) as gcETaKO mice and 2 genotypes of floxed ETa mice ($ETa^{flox/flox}$ or $ETa^{flox/-}$) without $Amhr2^{Cre}$ were used as WT mice for control group. These 4 genotypes of mice were bred with male mice to generated gcETaKO mice, fertility by litter size were compared among 4 genotypes.

4. Semiquantitative and Real Time RT-PCR

Semiquantitative RT-PCR to compare the relative deletion of ETa by $Amhr2^{Cre}$ in the granulosa cells was performed using primer sets that bind Exon 5 (5'-TCTACTTCTGCATGCCCT-TGGTGT-3') and Exon 7 (5'-TTCATGGTTGCCAGGTTAAT-GCCG-3'). Granulosa cells were isolated by follicular puncture after superovulation, as described previously (Ko *et al.*, 1999). Briefly, ovaries of gcETaKO and WT mice were collected from gonadotropin-primed mice and granulosa cell were collected in cold serum-free 4F medium consisting of 15 mmol/L HEPES (pH 7.4), 50% Dulbecco's modified Eagle's medium (DMEM; Invitrogen) and 50% Ham's F12 with bovine transferrin (5 μg/ml), human insulin (2 mg/ml), hydrocortisone (40 ng/ml), and antibiotics. Total RNA was isolated from 1×10^6 granulosa cells from each group using Trizol (Invitrogen) and RN easy kit (QIAGEN Inc., Valencia, CA). A DNase1 reaction buffer (Invitrogen), DNase1 (Invitrogen) and 25 mM EDTA (Invitrogen) were added to total RNA (1 μg) in 15.5 μl before heating at 65℃ for 15 min, then heated at 72℃ for 10 min after mixing the 12.5 μl of total RNA mixture and 1 μl oligo dT (Invitrogen). Then a cocktail of 5×1^{st} strand buffer, 10 mM dNTP, 40 IU RNase out, and 200 IU Moloney leukemia virus reverse transcriptase were added to a total volume of 20 μl. There action mixture was incubated at 42℃ for 60 min then heated at 94℃ for 5 min to in activate RNase H. And then 100 μl of DEPC H_2O was added to the mixture. Real-time PCR was performed using this cDNA by the Master cycler® ep realplex (Eppendorf, Hamburg, Germany). Expression levels of mRNA for ETa were determined relative to L19 as endogenous control. cDNA for ETa and L19 was amplified using iQTM SYBR® Green Supermix (Bio-rad) and the analysis was replicated 2～3 times on different days. DEPC-treated water was used to replace cDNA and act as a negative control for each analysis. The relative amount of transcript was calculated by the ΔΔCT method (Livak & Schmittgen, 2001) and normalized to L19.

5. Superovulation Assay

To investigate the ovulation activity in the gcETaKO mice, ovulation rate was examined in 1 gcETaKO mice ($ETa^{flox/-} \cdot Amhr2^{Cre}$) and 2 genotypes of WT mice after superovulation. These 3 genotypes of mice were treated with 5 IU PMSG to induce follicular development and 48 h later with 5 IU hCG to

Table 1. Fertility of 2 gcETaKO and 2 wild type mice in the procedure to generate and increase the gcETaKO mice

	Geno types	No. of mice	Total litters	Average litter size (±S.D)
gcETaKO	$ETa^{flox/-} \cdot Amhr2^{cre}$	6	21	3.5±2.81
	$ETa^{flox/flox} \cdot Amhr2^{cre}$	2	7	3.5±0.71
Wild type	$ETa^{flox/-}$	9	32	3.6±1.88
	$ETa^{flox/flox}$	15	55	3.7±1.84

induce the ovulatory cascade. At 18 hrs after hCG injection, cumulus-oocyte-complexes (COCs) in the oviduct ampullae were collected and placed into M2 medium with 0.1% (W/V) hyaluronidase. After 5 minutes of placement, cumulus cell were removed by gentle pipetting. And then, ovulated oocytes of each group were counted and its maturation was examined by extrusion of first polar body (Fig. 1).

6. Statistical Analysis

Oneway ANOVA using SigmaStat 3.5 (Systat Software Inc., Point Richmond, CA, USA) was used to determine differences in the real-time PCR analysis and the ovulation assays. If differences were detected, Tukey's test was used to determine which means differed.

RESULTS

1. Fertility

Fertility of 2 gcETaKO and 2 WT female mice that was derived from procedure to generate and increase the gcETaKO mice was summarized (Fig. 2). gcETaKO mice were fertile and had no significant difference in fertility among 2 gcETaKO ($ETa^{flox/-} \cdot Amhr2^{Cre}$ [3.5±2.81], $ETa^{flox/flox} \cdot Amhr2^{Cre}$ [3.5±0.71]) and 2 WT female mice ($ETa^{flox/-}$ [3.56±0.63], $ETa^{flox/flox}$ [3.67±1.84]) ($P<0.05$). However, litter sizes of 4 groups were below than 4 and these sizes were very low compared with normal litter size of mice.

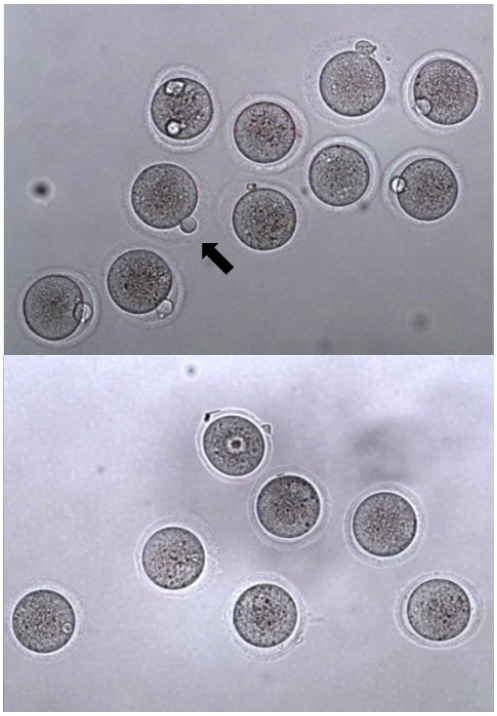

Fig. 1. Matured and immatured oocytes after ovulation by superovulation by PMSG and hCG. First polar body (black arrow) is shown in the matured oocytes.

2. Deletion of ETa in the Granulosa Cells

ETa deletion in the granulosa cells of gcETaKO mice was semi-quantified to measure the ETa mRNA expression by real time RT-PCR before experiment. ETa mRNA expression of granulosa cells of gcETaKO mice was below than that of WT mice (Fig. 3).

3. Ovulation and Maturation Rates

To evaluate the ovulation rates after ETa deletion in the granulosa cells, no. of ovulated oocytes was counted in the 1 gcETaKO ($ETa^{flox/-} \cdot Amhr2^{Cre}$) and 2 WT ($ETa^{flox/-}$, $ETa^{flox/flox}$) mice. There was no significant deficiency in the ovulation in the gcETaKO mice (Fig. 4). However, its maturation rates were significantly lower in the gcETaKO group than that of WT mice (Fig. 5).

DISCUSSION

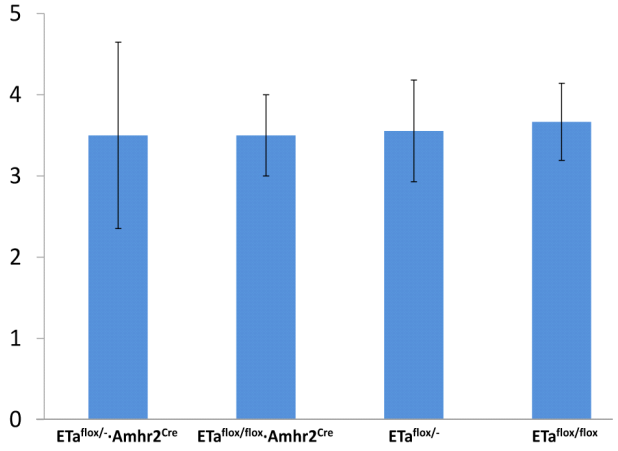

Fig. 2. Fertility of gcETaKO ($ETa^{flox/-} \cdot mhr2^{Cre}$, $ETa^{flox/flox} \cdot mhr2^{Cre}$) and wild-type ($ETa^{flox/-}$, $ETa^{flox/flox}$) mice. Female mice from the 4 different genotypes at the ages of 3～6 months were housed with males for the period of 2～5 months. gcETaKO mice were fertile and had no significant differences with WT mice. However, average litter size is very low compared with averages litter size of mice.

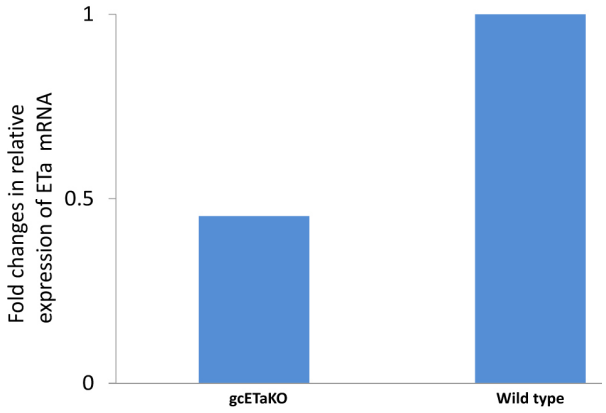

Fig. 3. Relative values of ETa deletion in the granulosa cells of gcETaKO mice compared with wild type mice. ETa was not deleted completely in the gcETaKO mice but it was deleted below than half compared with that of wild type.

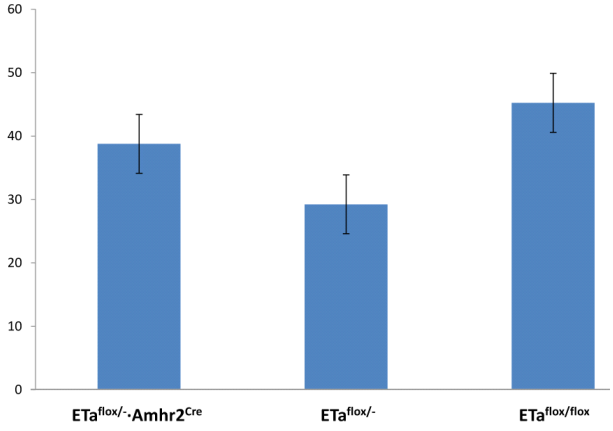

Fig. 4. No. of ovulated oocytes after superovulation by PMSG and hCG in the gcETaKO ($ETa^{flox/-} \cdot mhr2^{Cre}$) and 2 wild type mice ($ETa^{flox/-}$, $ETa^{flox/flox}$). No. of ovulated oocytes was not decreased significantly in the gcETaKO mice.

To investigate the EDN2 mechanisms in follicular rupture for ovulation with respect of its receptors, reproductive abilities were evaluated in this study using the conditional KO mice that ETa was deleted only in granulosa cells. It was confirmed the ovulation in the gcETaKO mice by their reproduction in the normal breeding, however it has decreased fertility. And, no. of ovulated oocytes was not decreased in the gcETaKO mice compared with WT mice, however its maturation rates were decreased.

Many of gcETaKO mice with $Amhr2^{Cre}$ were used in this experiment to overcome the limitations of pharmaceutical app-

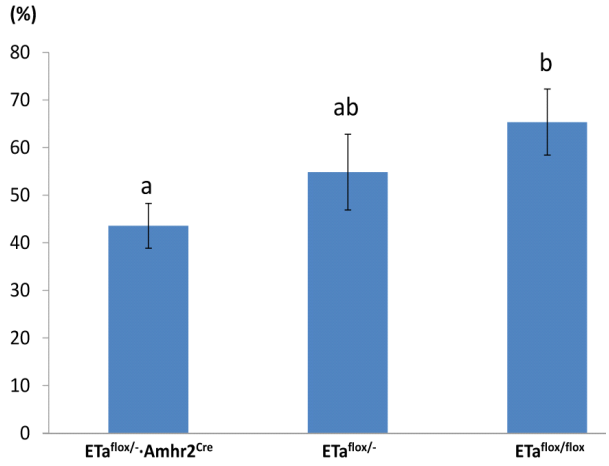

Fig. 5. Maturation rates of ovulated oocytes after superovulation by PMSG and hCG in the gcETaKO ($ETa^{flox/-} \cdot mhr2^{Cre}$) and 2 wild type mice ($ETa^{flox/-}$, $ETa^{flox/flox}$). No. of ovulated oocytes was not decreased significantly in the gcETaKO mice.

roaches. To generate and increase the gcETaKO mice, many of breeding was performed using ETa floxed mice and mice with $Amhr2^{Cre}$ gene. Additionally, ZP3Cre recombinase was applied in this breeding program for mono-alleic ETa deletion. Two different genotypes of gcETaKO mice ($ETa^{flox/-} \cdot Amhr2^{Cre}$, $ETa^{flox/flox} \cdot Amhr2^{Cre}$) could reproduce their offspring in this study, that means ovulation occurrence in the normal estrous cycles. And, their fertility by litter size is not decreased compared with WT mice. However, litter size of gcETaKO and WT female mice was below than 4 and it was very low compared with WT mice breeding. ETa floxed mice were also used in the breeding with mice of another Cre system, and they showed similar fertility with WT mice (unpublished data). It might be thought that these low fertility was caused by male mice because mice with genotype of $ETa^{flox/-} \cdot Amhr2^{Cre}$, $ETa^{flox/flox} \cdot Amhr2^{Cre}$ is used in this breeding program. Sertoli cells of these male mice were negatively affected by $Amhr2^{Cre}$ because ETa gene was deleted in the semale mice. Sertoli and germ cell interactions are essential for spermatogenesis and, thus, male fertility (Griswold, 1988; Jegou, 1993). Infertility or subfertility in the male mice with conditional KO of another gene as like CTNNB1, WNT4, GATA4 by $Amrh2^{Cre}$ recombinase was reported in previous studies (Boyer *et al.*, 2012; Kyronlahti *et al.*, 2011; Tanwar *et al.*, 2010).

ETa deletion must be quantified to use the mice with these genotypes as gcETaKO mice. Western blotting is appropriate method for this quantitation, however ETa protein did not ex-

pressed well in the granulosa cells after immunohistochemistry and this expressed only in oviduct (Ko *et al.*, 2006). Semi-quantitative real-time PCR was performed after making the cDNA of ETa in the isolated granulosa cells, and we could confirm the lower ETa mRNA expression in this mouse. However, it could be considered to use another cre recombinase system that is expressed in the granulosa cells because ETa mRNA was not deleted enough in the gcETaKO mice.

No. of ovulated oocytes were counted and examined their maturation rates by first polar body extrusion after superovulation. No significant differences in the ovulated oocytes were shown between gcETaKO and WT mice. It has suggested that EDN2 do not have direct relation with ETa in the granulosa cells in follicular rupture. ETb also could be thought be another candidate of EDN2 induced follicular rupture, however it can be rule out by our previous study (Cho *et al.*, 2012). In conclusion of this and our previous studies, EDN2 acted with ETa not ETb in follicular rupture for ovulation and this ETa is not that of granulosa cells. Ovarian follicle is also composed with theca interna, theca externa and smooth muscle cells enclose the granulosa cells. Therefore, EDN2 might be act with ETa in these cells in follicular rupture for ovulation. Maturation rates of ovulated oocytes after superovulation is deceased in the granulosa cells shows that ETa is important in the germinal vesicle break down (GVBD) after LH surge. Kawamura *et al.*, (2009) reported that EDN1 regulate the meiosis resumption via ETa in the granulosa cells, so GVBD was impaired in the gcETaKO mice after LH surge by superovulation.

We performed this study as first step to elucidate the EDN2 mechanisms in follicular rupture for ovulation using animal system not pharmaceutical approach. And then, we could make the conclusions that EDN2 act on ETa in other cells not in granulosa cells for this mechanism. Further studies are needed after ETa conditional deletion in other follicular cells to elucidate the EDN2 mechanisms for follicular rupture.

REFERENCES

Al-Alem L, Bridges PJ, Su W, Gong MC, Iglarz M and Ko C. 2007. Endothelin-2 induces oviductal contraction via endothelin receptor subtype A in rats. The Journal of Endocrinology 193: 383-391.

Arango NA, Kobayashi A, Wang Y, Jamin SP, Lee HH, et al. 2008. A mesenchymal perspective of Mullerian duct differentiation and regression in Amhr2-lacZ mice. Molecular Reproduction and Development 75: 1154-1162.

Bagchi A and Ghosh TC. 2006. Structural insight into the interactions of SoxV, SoxW and SoxS in the process of transport of reductants during sulfur oxidation by the novel global sulfur oxidation reaction cycle. Biophys. Chem. 119: 7-13.

Boyer A, Yeh JR, Zhang X, Paquet M, Gaudin A, et al. 2012. CTNNB1 signaling in sertoli cells downregulates spermatogonial stem cell activity via WNT4. PloS one 7: e29764.

Bridges PJ, Jo M, Al Alem L, Na G, Su W, et al. 2010. Production and binding of endothelin-2 (EDN2) in the rat ovary: endothelin receptor subtype A (EDNRA)-mediated contraction. Reproduction, Fertility, and Development 22: 780-787.

Cho J, Kim H, Kang DW, Yanagisawa M and Ko C. 2012. Endothelin B receptor is not required but necessary for finite regulation of ovulation. Life Sciences.

Clouthier DE, Hosoda K, Richardson JA, Williams SC, Yanagisawa H, et al. 1998. Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. Development 125: 813-824.

Griswold MD. 1988. Protein secretions of Sertoli cells. International Review of Cytology 110: 133-156.

Hernandez Gifford JA, Hunzicker-Dunn ME and Nilson JH. 2009. Conditional deletion of beta-catenin mediated by Amhr2cre in mice causes female infertility. Biology of Reproduction 80: 1282-1292.

Jegou B. 1993. The Sertoli-germ cell communication network in mammals. International Review of Cytology 147: 25-96.

Jorgez CJ, Klysik M, Jamin SP, Behringer RR and Matzuk MM. 2004. Granulosa cell-specific inactivation of follistatin causes female fertility defects. Mol. Endocrinol. 18: 953-967.

Kawamura K, Ye Y, Liang CG, Kawamura N, Gelpke MS, et al. 2009. Paracrine regulation of the resumption of oocyte meiosis by endothelin-1. Developmental Biology 327: 62-70.

Kedzierski RM, Grayburn PA, Kisanuki YY, Williams CS, Hammer RE, et al. 2003. Cardiomyocyte-specific endothelin a receptor knockout mice have normal cardiac function and an unaltered hypertrophic response to angiotensin II and isoproterenol. Molecular and Cellular Biology 23: 8226-8232.

Ko C, Gieske MC, Al-Alem L, Hahn Y, Su W, et al. 2006. Endothelin-2 in ovarian follicle rupture. Endocrinology 147: 1770-1779.

Ko C, In YH and Park-Sarge OK. 1999. Role of progesterone

receptor activation in pituitary adenylate cyclase activating polypeptide gene expression in rat ovary. Endocrinology 140: 5185-5194.

Kyronlahti A, Euler R, Bielinska M, Schoeller EL, Moley KH, et al. 2011. GATA4 regulates Sertoli cell function and fertility in adult male mice. Molecular and Cellular Endocrinology 333: 85-95.

Livak KJ and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. Methods 25: 402-408.

Palanisamy GS, Cheon YP, Kim J, Kannan A, Li Q, et al. 2006. A novel pathway involving progesterone receptor, endothelin-2, and endothelin receptor B controls ovulation in mice. Mol. Endocrinol. 20: 2784-2795.

Tanwar PS, Kaneko-Tarui T, Zhang L, Rani P, Taketo MM, Teixeira J. 2010. Constitutive WNT/beta-catenin signaling in murine Sertoli cells disrupts their differentiation and ability to support spermatogenesis. Biology of Reproduction 82: 422-432.

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