전용필 교수 지도
석사학위 청구논문

Role of estrogen in folliculogenesis through estrogen receptor alpha

2020

성신여자대학교 대학원

생물학과

양희선

Role of estrogen in folliculogenesis through estrogen receptor alpha

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Submitted in partial fulfillment of the
requirements for the degree of master.

December, 2019

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ABSTRACT

Role of estrogen in folliculogenesis through estrogen receptor alpha

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Estrogens are known to play an important role in the growth, differentiation and function of the female reproductive system. In the development of follicle until ovulation, estrogen actions on the follicular development as autocrine or paracrine. In growing follicles, estrogen promotes the action of gonadotropins, the formation of antrum in granulosa cell, gap junction formation between granulosa cells, and inhibits follicular atresia. Estrogens regulate the synthesis of androgens in theca cells. Estrogen action via the nuclear receptors, estrogen receptor alpha (ERα, Esr1) and beta (ERβ, Esr2). In the different cell types of the ovary, two different ER proteins are differentially localized. ERα is localized in theca/interstitial cell and ERβ is localized in granulosa cell. ERα is known as required for ovulation and ERβ is known as required for antrum formation and preovulatory follicle. However, the function formation and differentiation of follicle cells in ERα mediated folliculogenesis is controversy and not well understood. The importance of estrogen-mediated action via estrogen receptors (ERs) is suggested using estrogen receptor gene knockout mice. In this study, we investigated the role of estrogen receptor on folliculogenesis, especially the differentiation of follicle cells and its effect on oocyte growth. In estrogen receptor alpha knockout mice (ERαKO), estrogen cannot action through estrogen receptor alpha in theca cell. Therefore, the effect of estrogen in folliculogenesis through theca cell can be blocked. In the 2D culture the diameter of follicles was increased in E_2 treated group of wild type (WT), and was significantly reduced at day 8 in PHTPP treated WT. In 3D culture system, ICI 182,780 administered WT shows decreased follicle size entire experimental days. however KO follicle no significantly change between two different system. In both culture systems, expression of *Insl3*, theca cell-specific gene, tended to decrease in PHTPP treated ERαKO, but increased in WT. In both culture systems, the expression of *Fshr* and *Cyp19a1* significantly increased in E_2 treated ERαKO but significantly decreased in PHTPP treated ERαKO. Expression of Ptx3, Ptgs2, and Tnfaip6 in both ERαKO and WT, showed different results according to the culture systems. ERαKO also showed differences in the levels of steroid hormone between the two culture systems. The concentrations of E_2 and P_4 in both culture systems increased in and PHTPP treated WT. On the other hand, ERαKO mice showed decreased levels of E_2 and P_4 in 2D culture system, but increased in 3D culture system. These results indicate that estrogens might effect on the growth of follicles, differentiation of granulosa and theca cells, cumulus expansion and production of steroid hormones. In addition, this might provide insight into the local actions of estrogen receptors in folliculogenesis.

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INTRODUCTION

The mammalian ovary contains at various stages of developing follicles, including primordial, primary, secondary, tertiary and preovulatory follicles (Shiomi et al., 2015). The primordial follicles consisted with oocytes which arrested in the diplotene stage of the first meiotic division and surrounded flat granulosa cell. Through primordial follicle activation, primordial follicle leaves out in their dormant state and the primary follicle has cuboidal granulosa cell. The secondary follicle has more than two-layered follicles, and inner and outer theca cell. The antral cavity initiate to form at tertiary follicle (Rimon et al., 2018). Estrogen which is primarily produced in granulosa cells under the influence of follicle-stimulating hormone (FSH). Estrogen has a critical role in the growth, development and maintenance of female reproductive organs (Chen et al., 2009). In porcine, estradiol improves the meiotic and developmental competence of oocytes, cumulus expansion, and cumulus cell attachment to the oocytes (Kubo et al,. 2015). In granulosa cell of growing follicles estrogen has been shown to inhibit follicular atresia and to facilitate the actions of gonadotropin, the formation of gap junction (Bley et al., 1997). In theca cell of growing follicles, estrogen modulates steroidogenesis.

It is known that estrogen can work at least two pathways. One is the classical pathway: estrogens function via their nuclear receptors estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Pettersson et al., 2001). The second is the membrane receptor mediated pathway. ERβ is the most abundant ovarian estrogen receptor (Lenie et al., 2008). In the different cell types of the ovary, two different ER proteins are differentially localized. While the expression of ERα is localized in theca cell and interstitial cell, the expression of ERβ is localized in granulosa cell (Pelletier et al., 2000). In addition to acting as nuclear hormone receptors, there is evidence that ERs can be translocated to and act at the plasma membrane (Pedram et al. 2006) and these ER signaling is called nonclassical pathway. Several studies are suggesting estrogen may also signal through membrane-bound receptors including a G-coupled membrane receptor called GPR30 (Revankar et al. 2005) and further studies have shown that GPER is expressed on mouse oocytes plasma membrane (Li et al., 2013).

In vitro cultured ovarian follicle, ERα was localized in oocyte, granulosa cell and theca cell of secondary follicle. A shift of expressed ERα from cytoplasmic to nuclear was observed following in vitro ovarian follicle growth. ERβ was localized in nuclei of granulosa cell in all stage of in vitro ovarian follicular growth (Lenie et al., 2008).

The importance of ER-mediated estrogen signaling is suggested using mice lacking ERs. Adult female mice lacking ERα (ERαKO) is infertile and possesses ovaries that exhibit elevated steroid synthesis and multiple hemorrhagic/cystic follicles (Lubahn et al., 1993). The ERαKO is also characterized high circulating LH that interacted with LHR of the theca and granulosa cells (couse et al., 1999). These features lead to a failure of ovulation and luteinization. Therefore these processes might be related to hypothalamic–pituitary–gonadal (HPO) axis dependent upon ERα action. Adult female mice lacking ERα and ERβ (ERαβKO) have granulosa cell deficient large antral follicles and similar ovarian phenotype of ERαKO(Dupont et al., 2000). In contrast, adult female mice lacking ERβ (ERβKO) is subfertile and have ovaries that show normal granulosa cell but reduced numbers of growing follicles and corpora lutea(CL) (Krege et al., 1998). Therefore the folliculogenesis might be impaired in ERβKO (Dupont et al., 2000). ERβ is also required for antrum formation and preovulatory follicle (Drummond et al., 2012).

In vitro culture of follicle can be expose the mechanisms or roles of various factors in folliculogenesis. *In vitro* ovarian follicle culture can maintain the similar environment of *in vivo* ovarian environment, therefore ovarian follicles can growth and maturation *in vitro* (Desai et al., 2010). The two-dimensional culture system is conventionally used for *in vitro* ovarian follicle culture. However, follicular flattening leads to disruption of gap junctions (Desai et al., 2010). The three-dimensional culture system can more mimic *in vivo* ovarian environment. These culture systems can preserve the spherical morphology of ovarian follicles and gap junctions (Desai et al., 2010).

Many studies suggest that estrogen plays an important role in the differentiation of granulosa cells and in the maturation of the oocytes, but it is not clear yet how the mechanism of estrogen specifically actions. In this study we investigated the mechanism by which estrogen plays a role in differentiation of granulosa cells and oocytes growth using mice lacking ERα and estrogen receptor antagonists. In this study, we used both two-dimensional culture system and three-dimensional culture system and then compared to the effects of culture system on folliculogenesis. In addition, two types of in vitro ovarian follicle culture system were compared through diameter of follicle, difference of expressed specific gene and production of steroids.

MATERIALS AND METHODS

Experimental animals

Animal care and use were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. All animal procedures were approved by the Institutional Animal Care and Use Committee(IACUC) of Sungshin University (SSWIACUC-2019-009). Involving experiment animals were maintained under standard condition at Sungshin University. Diurnal rhythm kept under the 14L : 10D schedule with light-on at 06:00 and clean room system. Estrogen receptor α knockout (ERαKO) mouse were on a C57BL/6 background and deleted the exon 3 of ERα gene. Genomic DNA was isolated from ear and the primer combination of ERα primer1 (5’-TT GCCCGATAACAATAACAT-3’) and ERα primer2 (5’-GGCATTACCACTTCTC CTGGGAGTCT-3’) was used to determine the genotype. 2-3 weeks female estrogen receptor α knockout (ERαKO) mice were used. 2-3 weeks female C57BL/6J mice was used as wild type.

Isolation of Secondary follicles

All female mice using in this study were sacrificed using cervical dislocation. Multi-layered secondary follicles (150 –180 ㎛) were mechanically isolated from 2-3 weeks ovaries of C57BL/6J and ERαKO by using a 28-gauge syringe needle in α-minimal essential medium (α-MEM) (Gibco, Cat#:41061-029, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS) (Welgene, Namcheon, Korea). Follicles with intact basal membrane and a high density of granulosa cells were selected for further culture under microscope.

In vitro growth of Follicles

The standard culture medium was α-MEM (Gibco, Cat#:41061-029, Carlsbad, CA, USA) supplemented with 60.4 mg/ml penicillin (Sigma, USA),10 mg/ml streptomycin (Sigma, USA), 5 μg/ml insulin (Sigma, USA), 10 μg/ml transferrin (Sigma, USA), 2.5 ng/ml sodium selenite (Sigma, USA), 25 μg/ml Ascorbic acid (Sigma, USA) , 100 mIU/mL human FSH (Sigma, USA) and 5% FBS. The isolated follicles were cultured for two different systems. The follicles cultured by 2D system were allocated at one follicle/well in 96-well plates (Corning, USA) containing 50 μl/well culture medium with 35 μl mineral oil overlay. The follicles cultured by 3D system were allocated at one follicle/well in 96-well plates (SPL, Cat#: 13485, Pocheon, Korea) containing 50 μl/well culture medium and then inverted. The follicles were cultured for 8 days in both systems. Every other day, 25 μl of the conditioned medium was collected and stored at - 80℃ for hormone assay, and 25 μl of pre-conditioned medium was added to each well. All follicles were grown in an incubator at 37℃ with 5% CO_2 atmosphere. The diameter of follicles were measured using ImageJ 1.51 and based on a calibrated ocular micrometer at 10X magnification every other day. To examine the role of estrogen in folliculogenesis through estrogen receptor alpha, 10^{-8} M 17β - estradiol (Sigma, USA) and/or 10^{-5} M ICI 182,780 (TOCRIS, USA), 10^{-5} M PHTPP (TOCRIS, USA) was added into the standard culture medium. In group of the 10^{-5} M ICI 182,780 treated standard medium and 10^{-5} M PHTPP treated standard medium, isolated follicles were incubated in the each 10^{-5} M ICI 182,780 treated or 10^{-5} M PHTPP treated standard medium for 2 hr and then transferred in to the 10^{-8} M E_2 added 10^{-5} M ICI 182,780 or 10^{-5} M PHTPP treated medium.

Oocyte maturation

After 8 days, the follicles were transferred to maturation media composed of α MEM supplemented 5% FBS, 1.5IU/ml human chorionic gonadotropin (hCG) (Sigma, USA) and 5ng/ml epidermal growth factor (EGF) (Sigma, USA) and incubated for 16 h at 37℃ in 5% CO_2. After 16 h, oocytes were denuded from surrounding cumulus cells by 0.1% hyaluronidase and oocyte meiotic maturation was assessed by the detection of germinal vesicle (GV), germinal vesicle breakdown (GVBD), and metaphase Ⅱ (MⅡ).

Total RNA extraction and first strand cDNA synthesis

Total RNA of follicles were extracted using RNeasy® Micro Kit (Qiagen, CA, USA) following to the manual of manufacturer. Total RNA were used to perform reverse transcription. First strand cDNA synthesized using Accuscrpit High Fidelity Reverse Transcriptase (Agilent Technologies, CA, USA) in accordance with the manufacturer’s instruction. Briefly, reaction reagents were 5 µg total RNA, 5.0µL MMLV buffer (10X), 1.0 µL oligo dT primer (0.5 µg/µL), 1.0 µL random primers (0.1 µg /µL), 2 µL 100mM dNTP mix, and RNase-free DEPC-treated water. Reaction mixture was incubated at 65℃ for 5min, cooled at RT to allow the primers to anneal to RNA for 10min. After then, 4.0 µL 100mM DTT, 2.0 µL RNase block ribonuclease inhibitor (Agilent Technologies, CA, USA), and 1.0 µL MMLV RT were added. The mixture was incubated at 42℃ for 1hr and at 70℃ for 10min to terminate cDNA synthesis. The cDNA was stored at -20℃.

Real-Time PCR

Real-time PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa, Japan) and Thermal Cycler Dice Real Time System TP800 (TaKaRa, Japan). Each reaction was run in triplicate. Dissociation curves were run on all reactions to confirm amplification of a single product with the appreciate melting temperature. The fold change in gene expression was calculated using the △△Ct method with the housekeeping gene, β-actin, as an internal control. Average β-actin cycle threshold (CT) values were subtracted from each follicle cell specific gene to obtain △Ct values and then △Ct was subtracted from the average of △Ct to obtain △△Ct values. Relative follicle specific gene expression to β-actin was calculated using $2^{-\triangle\triangle Ct}$.

Enzyme-Linked Immunosorbent Assay (ELISA) for steroid hormone

The concentration of 17β-estradiol and progesterone in culture medium were measured by Estradiol ELISA Kit (CUSABIO, Cat#: CSB-E05109m, Houston, USA) and Progensterone ELISA Kit (CUSABIO, Cat#: CSB-E05104m, Houston, USA) based on the manufacturer’s instructions. The sensitivity of 17β-estradiol and progesterone ELISA kit is 0.04 ng/ml and 0.2ng/ml, respectively. The intra-assay precision and inter-assay precision was under 15%.

Statistics

All experiments were conducted at least in triplicate. The Student's t-test was performed to evaluate the statistical significance between control and experimental group. Results were presented as mean ± SEM. Values of $P < 0.05$ were considered to be significantly different.

Table 1. Thermal cycler schedule

step		Temperature (℃)	Time
Hold	Hold	95	30 sec
3 step PCR (45 cycle)	Denaturation	95	1 min
	Annealing	59	30 sec
	Extension	72	1 min
Dissociation	Denaturation	95	15 sec
	Annealing	60	30 sec
	Extension	95	15 sec
Hold		4	Indefinitely

Table 2. Sequence - specific of primers

Gene	Symbol	NCBI gene reference		Primer sequence(5'-3')	Amplified length (bp)
Follicle-stimulating hormone receptor	*Fshr*	NM_007810.4	S AS	TCTCTCTCGTAGATGATGAACCCAG TGTATTGGCTTGTGGTCAGGAC	239
Cytochrome P450 Family 19 Subfamily A Member 1	*Cyp19a1*	NM_007810.4	S AS	GGAGAGTTCATGAGAGTCTGGATCA ATTGTTCTCCAAAGGCTCGGGT	187
Cytochrome P450 Family 17 Subfamily A Member 1	*Cyp17a1*	NM_007809.3	S AS	AGTGAATCTCTCTCCAGCCTGACA AAAGCCAGGATCCAGTTCAGCA	184
Insulin-like 3	*Insl3*	NM_013564.7	S AS	TGCAGTGGCTAGAGCAGAGACATCT AGCAATTAAGGACGCACCG	221
Pentraxin 3	*Ptx3*	NM_008987.3	S AS	TAGTGTTGGTGGTGGGTGGAAA TTCTTGGCCAATCTGTAGGAGTCC	205

Prostaglandin-endoperoxide synthase 2	*Ptgs2*	NM_011198.4	S AS	CCCACAGTCAAAGACACTCAGGT TAGTTGCTCATCACCCCACTCA	195
TNF alpha induced protein 6	*Tnfaip6*	NM_009398.2	S AS	TACTGGCACATTCGGCTCAAGT GACGGATGCATCACTCAGAAACTTC	221
Actin, beta	*Actb*	NM_007393.5	S AS	TCCGTAAAGACCTCTATGCCAACAC TCAGTAACAGTCCGCGAGAAGCA	263

Results

Selection of culture media

To remove exogenous estrogen in the standard culture medium, we tried to use charcoal-stripped fetal bovine serum (cFBS) in the in vitro ovarian follicle culture. In the group of 5% cFBS-treated standard medium, the survival rates of follicles were only 1.89% at day 4 (Table 3). In the group of 1% or 2.5% or 5% cFBS and 3mg/ml BSA-treated standard medium, the survival rates of follicles were increased 6.25% to 100.00% as the amount of the cFBS increases in the standard medium. However, the survival rates of follicles was zero at day 8 in all the group of cFBS treated standard medium. Therefore, FBS was selected in this study.

Table 3. Selection of culture media

	Survival rate (%)			
	Day2	**Day4**	**Day6**	**Day8**
cFBS 1% + 3mg/ml BSA	6.25%	0.00%	0.00%	0.00%
cFBS 2.5% + 3mg/ml BSA	79.49%	38.46%	12.82%	0.00%
cFBS 5% + 3mg/ml BSA	100.00%	85.71%	75.00%	0.00%
cFBS 5%	26.42%	1.89%	0.00%	0.00%
FBS 5%	100.00%	100.00%	100.00%	90.48%

The morphology of *in vitro* cultured follicles

The ovarian follicles were cultured for 8 days using two different *in vitro* ovarian follicle culture system. The follicles cultured by 2D culture system were allocated at one follicle/well in 96-well plates containing 50 μl/well culture medium with 35 μl mineral oil overlay (Fig. 1A). The flattened follicles were observed in 2D culture system. The follicles cultured by 3D culture system were allocated at one follicle/well in 96-well plates containing 50 μl/well culture medium and then inverted (Fig. 1B).

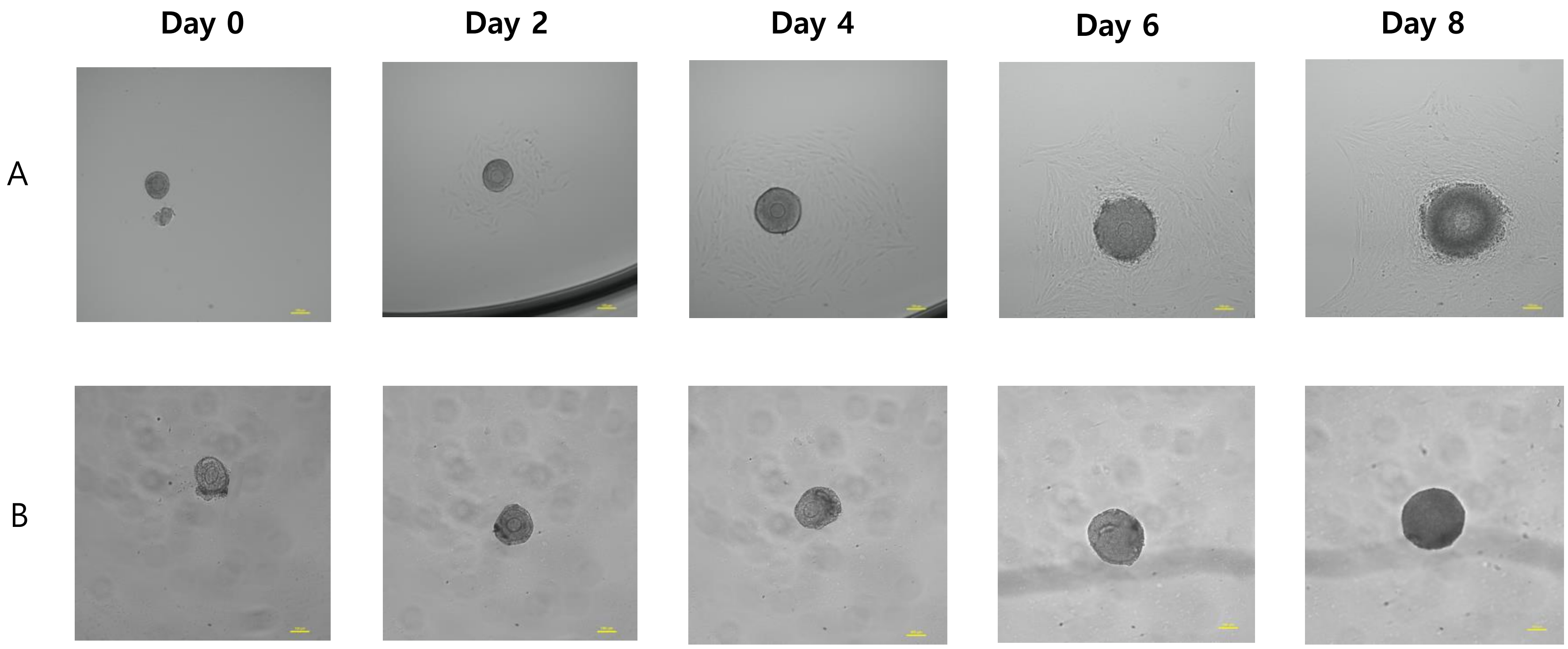


Figure 1. The morphology of in vitro cultured follicles

The ovarian follicles of Wild type (C57BL/6) were cultured for 8 days. A) Follicles were cultured in the way of 2-dimensional system. B) Follicles were inverted and cultured to maintain 3-dimensional environment. Scale bars, 100 ㎛

The growth of follicles from wild type (C57BL/6) and ERαKO in two different *in vitro* ovarian follicle culture system

All follicle cultures are treated or not with 10_{-8} M 17β – estradiol or their antagonists (10_{-5} M PHTPP or 10_{-5} M ICI 182,780) for 8 days. When the E_2 was treated standard medium, the diameter of ovarian follicles significantly increased at days 4, 6 and 8 of culture compared to control in wild type (C57BL/6) using 2D culture system (Fig. 2A). However, in the group of PHTPP treated standard medium, the diameters of follicles significantly decreased at day 8 (Fig. 2A). In the way of 3D culture system, the diameters of ovarian follicles cultured in ICI 182,780 treated standard medium significantly decreased at days 2, 4, 6 and 8 of culture (Fig. 2B). This shows estrogen might be influence on the growth of ovarian follicles. There was no significantly difference in all cultured group of ERαKO under both 2D culture system and 3D culture system (Fig. 3A,B).

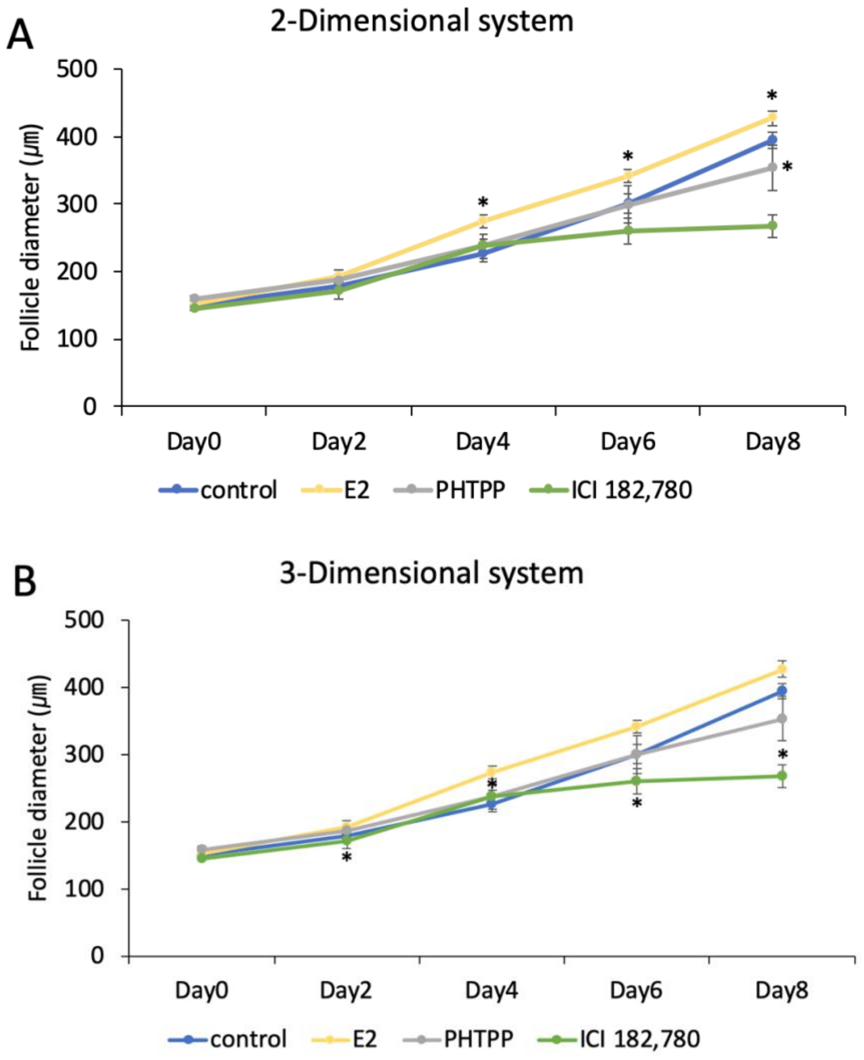


Figure 2. The growth of follicles from wild type (C57BL/6) in two different *in vitro* ovarian follicle culture system

A) Follicles of wild type were cultured for 8 days in the way of 2-dimensional culture system. B) Follicles of wild type were cultured for 8 days in the way of 3-dimensional culture system. Statistical analysis by Student’s t-test (*: p<0.05 vs control to group)

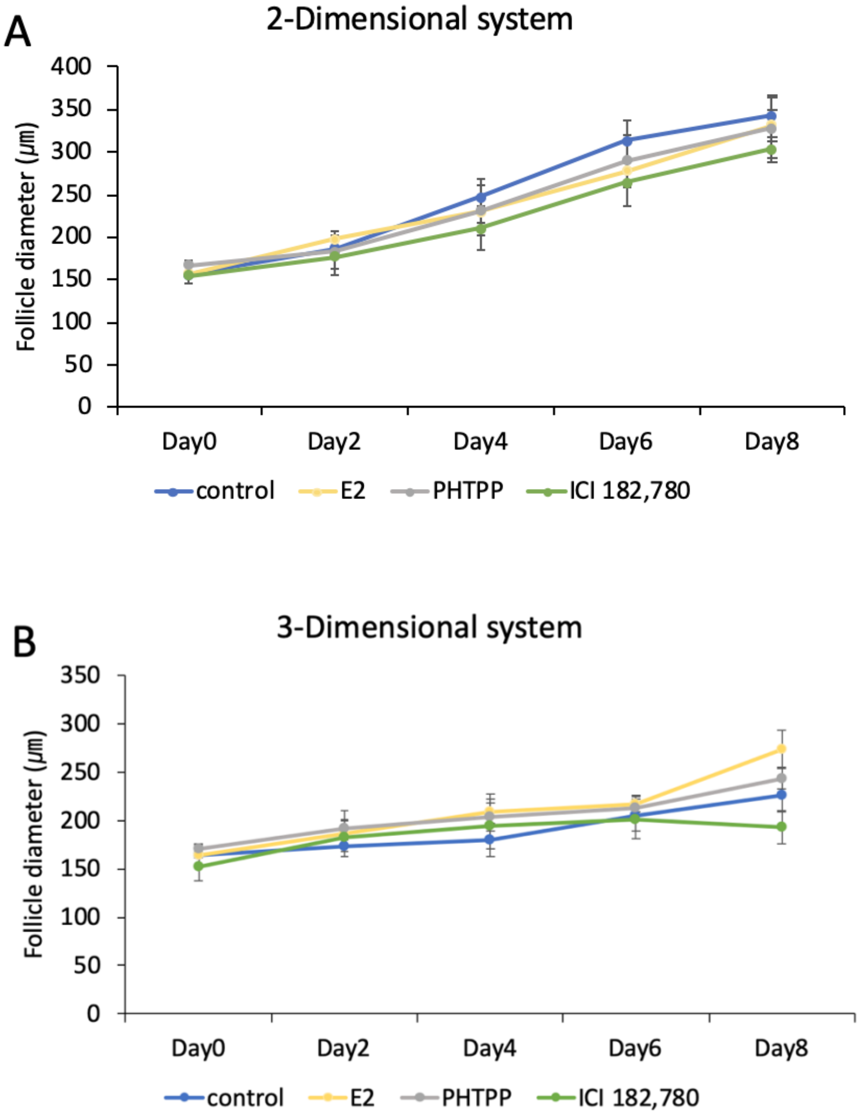


Figure 3. The growth of follicles from ERαKO in two different *in vitro* ovarian follicle culture system

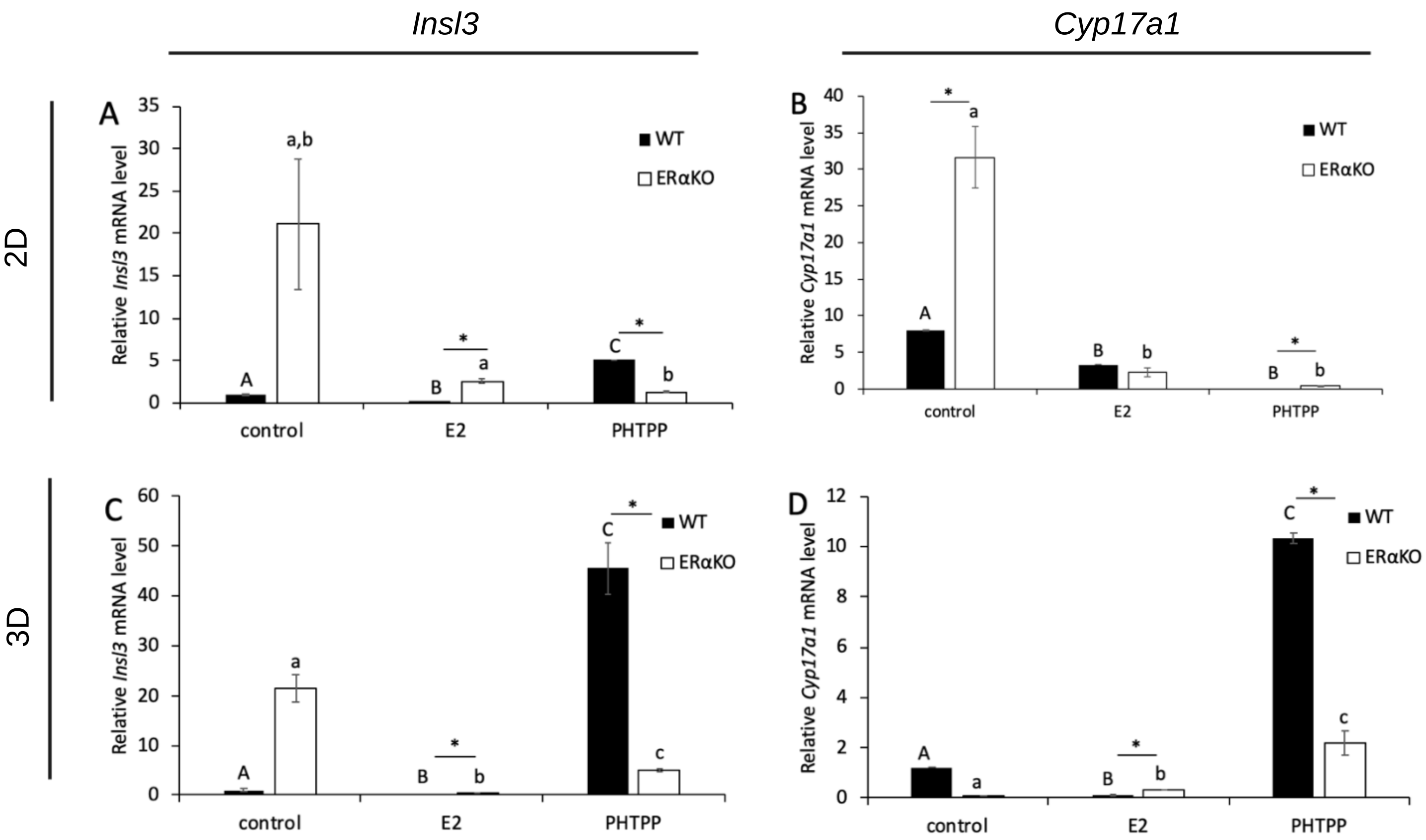
A) Follicles of wild type were cultured for 8 days in the way of 2-dimensional culture system. B) Follicles of wild type were cultured for 8 days in the way of 3-dimensional culture system. Statistical analysis by Student's t-test (*: $p<0.05$ vs control to each group)

The effect of ER β - specific antagonist (PHTPP) and ERαKO on expression of follicle cell specific gene

To evaluate the expression of the follicle cell specific genes in the cultured follicles at day 9, real-time PCR was performed. There was not observed germinal vesicle breakdown (GVBD) in ICI 182,780 treated group, so only standard, E_2 treated and PHTPP treated group were compared except for ICI 182,780 treated group. The stage of oocytes in all group was unified with GVBD. Under 2D culture system, the expression of *Insl3* significantly decreased compared to control in E_2 treated group in both WT and ERαKO (Fig. 4A). However, the expression of *Insl3* significantly increased compared to control in PHTPP treated group in both WT and ERαKO (Fig. 4A). The expression of *Insl3* in in ERαKO were up-regulated in E_2 treated group and down-regulated in PHTPP treated group compared to WT (Fig. 4A). In the case of cultured using 3D culture system, there was similar *Insl3* expression pattern like 2D culture system between the different cultured groups (Fig. 4C). The expression of *Cyp17a1* significantly decreased only in PHTPP treated group of ERαKO (Fig. 4B), but significantly decreased in both E_2 treated group and PHTPP-treated group in WT (Fig. 4B). The 3D culture system shows different *Cyp17a1* mRNA expression pattern compared to 2D culture system. The expression of *Cyp17a1* significantly decreased in E_2 treated group and significantly increased in PHTPP treated group in both WT and ERαKO (Fig. 4D).

In 2D culture system, the expression of *Fshr* was up-regulated in PHTPP treated group of WT, but down-regulated in E_2 treated group of WT. In ERαKO, the expression of *Fshr* significantly decreased in PHTPP treated group compared to control (Fig. 5A). Unlike 2D culture system, the expression of *Fshr* significantly increased in E_2 treated group of WT compared to control (Fig. 5C). In E_2 treated group, the expression of *Cyp19a1* significantly increased compared to control in both WT and ERαKO when follicles cultured in the way of 2D system (Fig. 5B). In PHTPP treated group, the expression of *Cyp19a1* was up-regulated in only WT (Fig. 5B). In 3D culture system, the expression of *Cyp19a1* was down-regulated in E_2 treated group of WT compared to control and up-regulated in PHTPP treated group of ERαKO compared to control unlike 2D culture system (Fig. 5D).

In 2D culture system, when the E_2 were treated in standard medium, the expression of *Ptx3* and *Ptgs2* significantly decreased compared to control in WT (Fig. 6AB). When the PHTTP were treated in standard medium, the expression of *Ptgs2* and *Tnfaip6* significantly decreased compared to control in WT (Fig.6BC). In ERαKO, the expression of *Ptx3* and *Tnfaip6* were down-regulated compared to control in both E_2 and PHTPP treated group (Fig. 6AC). In 3D culture system , the expression of *Ptx3* significantly increased and the expression of *Tnfaip6* significantly decreased in E_2 treated group of WT compared to control (Fig. 6DF). The expression of *Ptgs2* significantly decreased and the expression of *Tnfaip6* significantly increased in PHTPP treated group of WT compared to control (Fig. 6EF). In ERαKO, the expression of *Ptx3* was up-regulated and the expression of *Ptgs2* was down-regulated in E_2 treated group compared to control (Fig. 6DE), but the expression of *Ptgs2* was down regulated in PHTPP treated group compared to control (Fig. 6E).


Insl3
Cyp17a1
2D
3D
A
Relative Insl3 mRNA level
0
5
10
15
20
25
30
35
■ WT
☐ ERαKO
A
a,b
B
a
*
C
b
*
control
E2
PHTPP
B
Relative Cyp17a1 mRNA level
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5
10
15
20
25
30
35
40
*
A
a
B
b
*
B
b
control
E2
PHTPP
C
Relative Insl3 mRNA level
0
10
20
30
40
50
60
A
a
*
B
b
C
c
*
control
E2
PHTPP
D
Relative Cyp17a1 mRNA level
0
2
4
6
8
10
12
A
a
*
B
b
C
c
*
control
E2
PHTPP

Figure 4. Expression profile of theca cell specific mRNA level in cultured follicles at day 9.

Relative *Insl3* and *Cyp17a1* mRNA expression level in the cultured follicles from wild type and ERαKO treated or not with E_2 or PHTPP for 8 days. A,B) Follicles were cultured in the way of 2-dimensional system. C,D) Follicles were cultured in the way of 3-dimensional system. Different capital letters represent differences in the different cultured group of wild type. Different lower cases represent differences in the different cultured group of ERαKO. Statistical analysis by Student's t-test (*: $p<0.05$ WT vs ERαKO). Data are represented as the mean ± SEM.

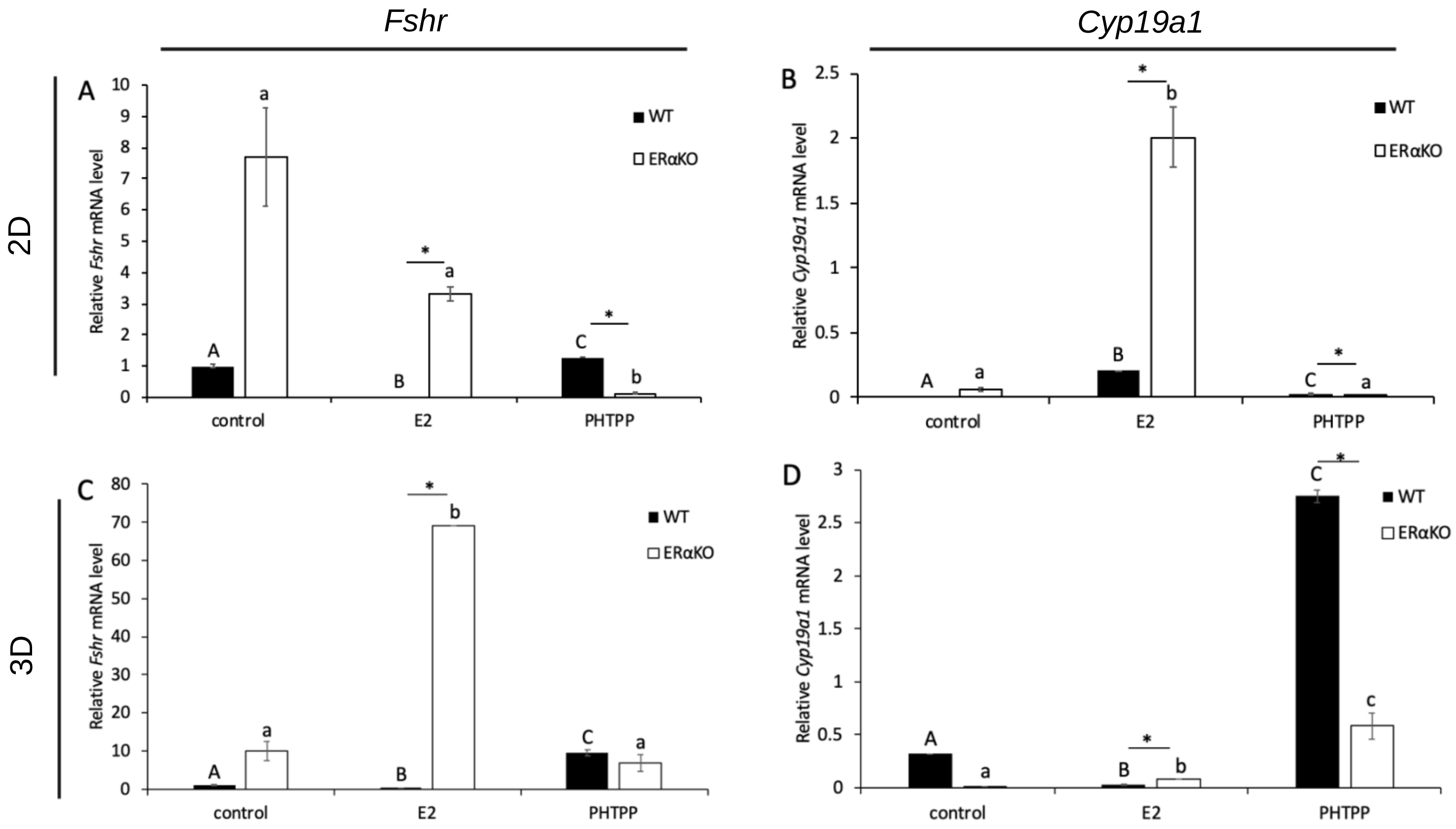
Fshr
Cyp19a1
2D
3D
A
Relative Fshr mRNA level
0
1
2
3
4
5
6
7
8
9
10
■ WT
□ ERαKO
a
*
a
*
A
B
C
b
control
E2
PHTPP
B
Relative Cyp19a1 mRNA level
0
0.5
1
1.5
2
2.5
■ WT
□ ERαKO
*
b
B
*
A
a
C
a
control
E2
PHTPP
C
Relative Fshr mRNA level
0
10
20
30
40
50
60
70
80
■ WT
□ ERαKO
*
b
a
A
B
C
a
control
E2
PHTPP
D
Relative Cyp19a1 mRNA level
0
0.5
1
1.5
2
2.5
3
*
C
■ WT
□ ERαKO
c
A
a
*
B
b
control
E2
PHTPP

Figure 5. Expression profile of granulosa cell specific mRNA level in cultured follicles at day 9.

Relative *Fshr* and *Cyp19a1* mRNA expression level in the cultured follicles from wild type and ERαKO treated or not with E_2 or PHTPP for 8 days. A,B) Follicles were cultured in the way of 2-dimensional system. C,D) Follicles were cultured in the way of 3-dimensional system. Different capital letters represent differences in the different cultured group of wild type. Different lower cases represent differences in the different cultured group of ERαKO. Statistical analysis by Student's t-test (*: $p<0.05$ WT vs ERαKO). Data are represented as the mean ± SEM.

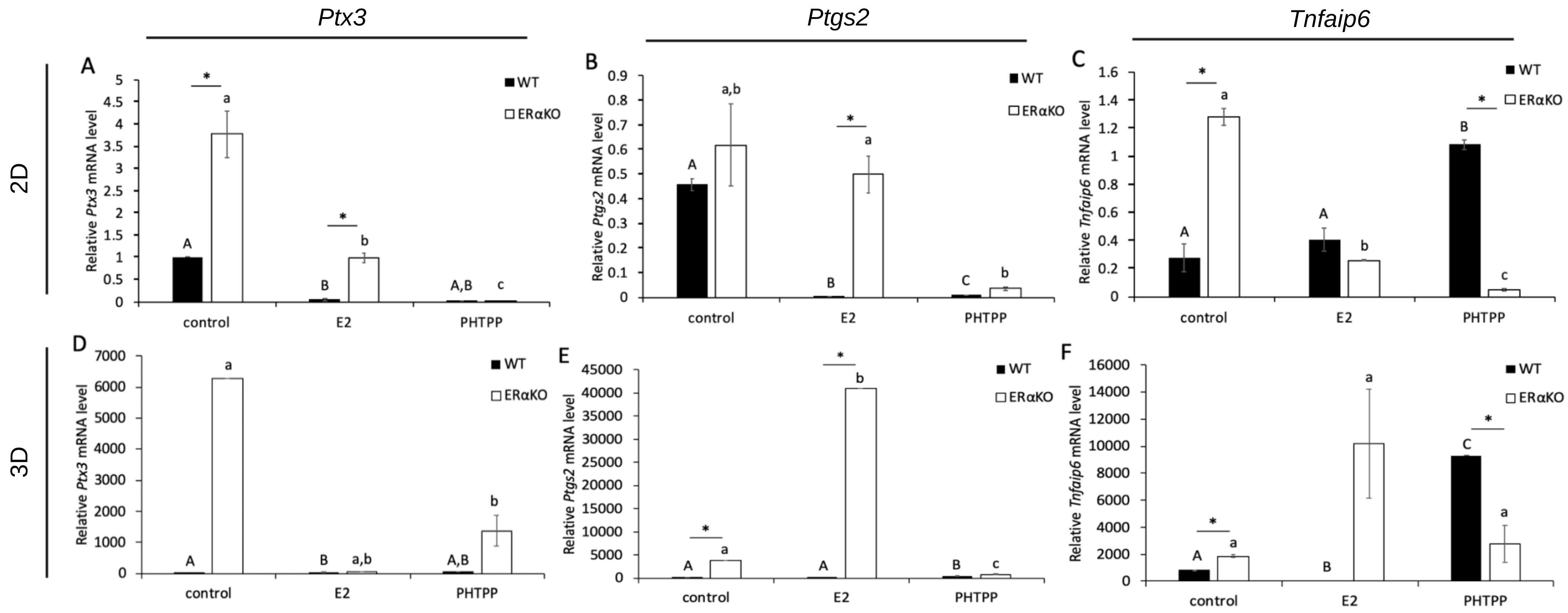
Ptx3
Ptgs2
Tnfaip6
2D
3D
A
Relative Ptx3 mRNA level
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
■ WT
□ ERαKO
*
a
A
*
b
B
A,B
c
control
E2
PHTPP
B
Relative Ptgs2 mRNA level
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
■ WT
□ ERαKO
a,b
A
*
a
B
C
b
control
E2
PHTPP
C
Relative Tnfaip6 mRNA level
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
■ WT
□ ERαKO
*
a
A
A
b
*
B
c
control
E2
PHTPP
D
Relative Ptx3 mRNA level
0
1000
2000
3000
4000
5000
6000
7000
■ WT
□ ERαKO
a
A
B
a,b
b
A,B
control
E2
PHTPP
E
Relative Ptgs2 mRNA level
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
■ WT
□ ERαKO
*
b
*
a
A
A
B
c
control
E2
PHTPP
F
Relative Tnfaip6 mRNA level
0
2000
4000
6000
8000
10000
12000
14000
16000
■ WT
□ ERαKO
a
*
C
a
*
A
a
B
control
E2
PHTPP

Figure 6. Expression profile of cumulus expansion specific mRNA level in cultured follicles at day 9.

Relative *Ptx3, Ptgs2* and *Tnfaip6* mRNA expression level in the cultured follicles from wild type and ERαKO treated or not with E_2 or PHTPP for 8 days. A,B) Follicles were cultured in the way of 2-dimensional system. C,D) Follicles were cultured in the way of 3-dimensional system. Different capital letters represent differences in the different cultured group of wild type. Different lower cases represent differences in the different cultured group of ERαKO. Statistical analysis by Student's t-test (*: $p<0.05$ WT vs ERαKO). Data are represented as the mean ± SEM.

The levels of 17β-estradiol and progesterone during *in vitro* culture

In 2D culture system, the concentrations of E_2 in control spent medium and E_2 treated spent medium were significantly higher in ERαKO compared to WT (not significant in E_2 treated group at days 9 of culture) (Fig.7 AB). However, the concentrations of E_2 in PHTPP treated spent medium were significantly higher in WT compared to ERαKO (Fig. 7C). In 3D system, the concentrations of E_2 in all group of ERαKO were significantly higher than WT at days 4 and 8 of culture (Fig. 7DEF). Fig 8 show the comparison of E_2 levels in all group of spent medium within WT or ERαKO. Under 2D culture system, E_2 levels were significantly higher in E_2 or PHTPP treated group compared to control in both WT and ERαKO (not significant in E_2 treated group of WT at days 9 of culture) (Fig. 8AB). In 3D culture system, the similar patterns observed in 2D culture system of WT (Fig. 8C). However, in ERαKO, E_2 levels in E_2 or PHTPP treated group were significantly higher than control medium at days 4, 8 and 9 of culture (Fig. 8D).

In 2D culture system, the concentrations of P_4 in control spent medium and E_2 treated spent medium were significantly higher in ERαKO compared to WT at days 8 of culture) (Fig.9 AB). In contrast, the concentrations of P_4 in PHTPP spent medium were significantly higher in WT than ERαKO at days 4,8 and 9 of culture (Fig. 9C). In 3D culture system, the concentrations of P_4 in all group of ERαKO were significantly higher than WT at days 4, 8 and 9 of culture (Fig. 9DEF). Fig 10 show the comparison of P_4 levels in all group of spent medium within WT or ERαKO. Under 2D culture system, P_4 levels were significantly higher in E_2 or PHTPP treated group compared to control in WT (not significant in PHTPP treated group at days 9 of culture) (Fig. 10A). In ERαKO, P_4 levels were significantly lower in E_2 or PHTPP treated group compared to control (Fig. 10B). In 3D culture system, the similar patterns observed in 2D culture system of WT (except for days 9 of culture in E_2 treated group) (Fig. 10C). In ERαKO, P_4 levels in E_2 or PHTPP treated group were significantly higher than control medium at days 4, 8 and 9 of culture (Fig. 10D).

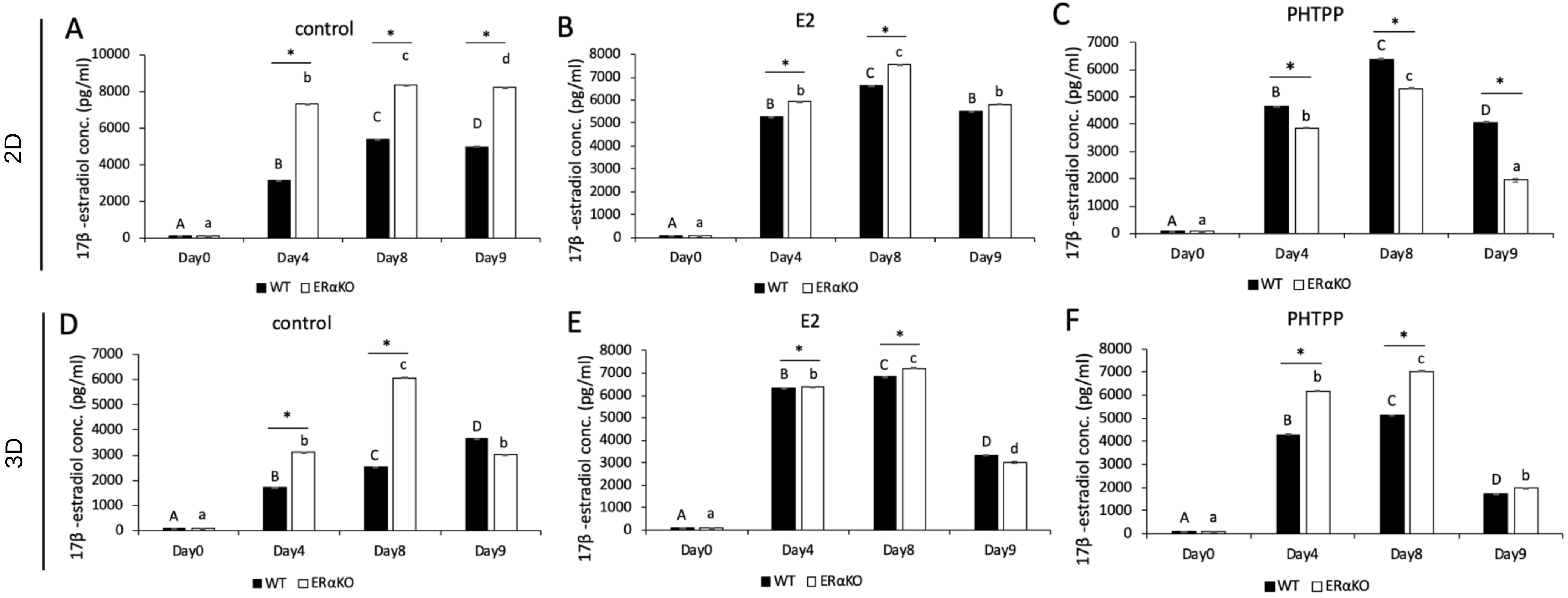
2D
3D
A
control
B
E2
C
PHTPP
D
control
E
E2
F
PHTPP
17β -estradiol conc. (pg/ml)
Day0
Day4
Day8
Day9
■WT
□ERαKO
A
a
B
b
C
c
D
d
*

Figure 7. The comparison of production of 17β-estradiol during *in vitro* culture between WT and ERαKO

The 17β-estradiol concentrations in spent culture medium from wild type and ERαKO treated or not with E_2 or PHTPP. A,B) Follicles were cultured in the way of 2D culture system. C,D) Follicles were cultured in the way of 3D culture system. Different capital letters represent differences in the different cultured group of wild type. Different lower cases represent differences in the different cultured group of ERαKO. Statistical analysis by Student's t-test (*: $p<0.05$ WT vs ERαKO). Data are represented as the mean ± SEM.

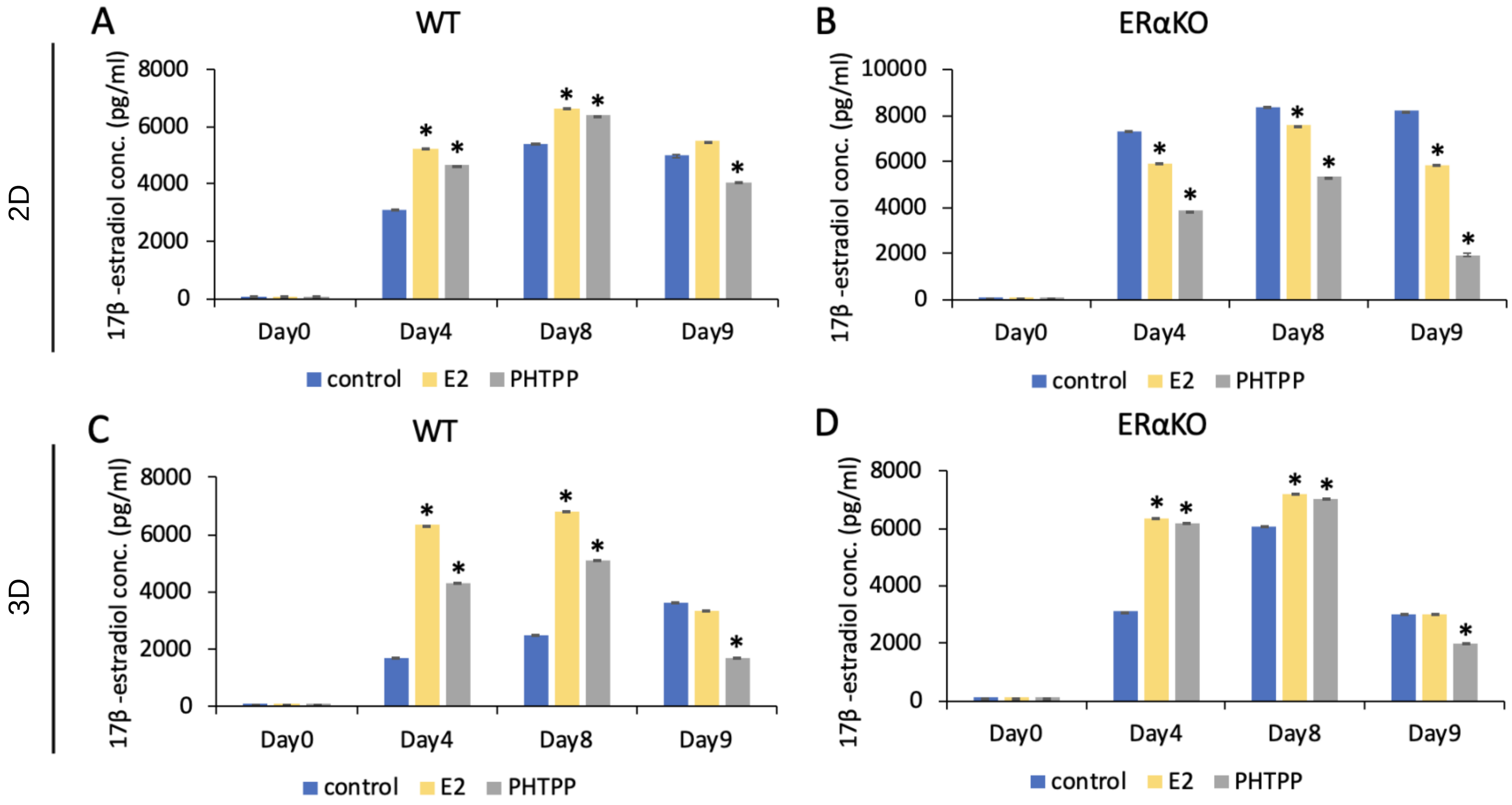
2D
3D
A
WT
B
ERαKO
C
WT
D
ERαKO
17β -estradiol conc. (pg/ml)
0
2000
4000
6000
8000
10000
Day0
Day4
Day8
Day9
*
control
E2
PHTPP

Figure 8. The levels of 17β-estradiol during *in vitro* culture

The 17β-estradiol concentrations in spent culture medium from wild type and ERαKO treated or not with E_2 or PHTPP. A,B) Follicles were cultured in the way of 2D culture system. C,D) Follicles were cultured in the way of 3D culture system. Different capital letters represent differences in the different cultured group of wild type. Different lower cases represent differences in the different cultured group of ERαKO. Statistical analysis by Student’s t-test (*: $p<0.05$ WT vs ERαKO). Data are represented as the mean ± SEM.

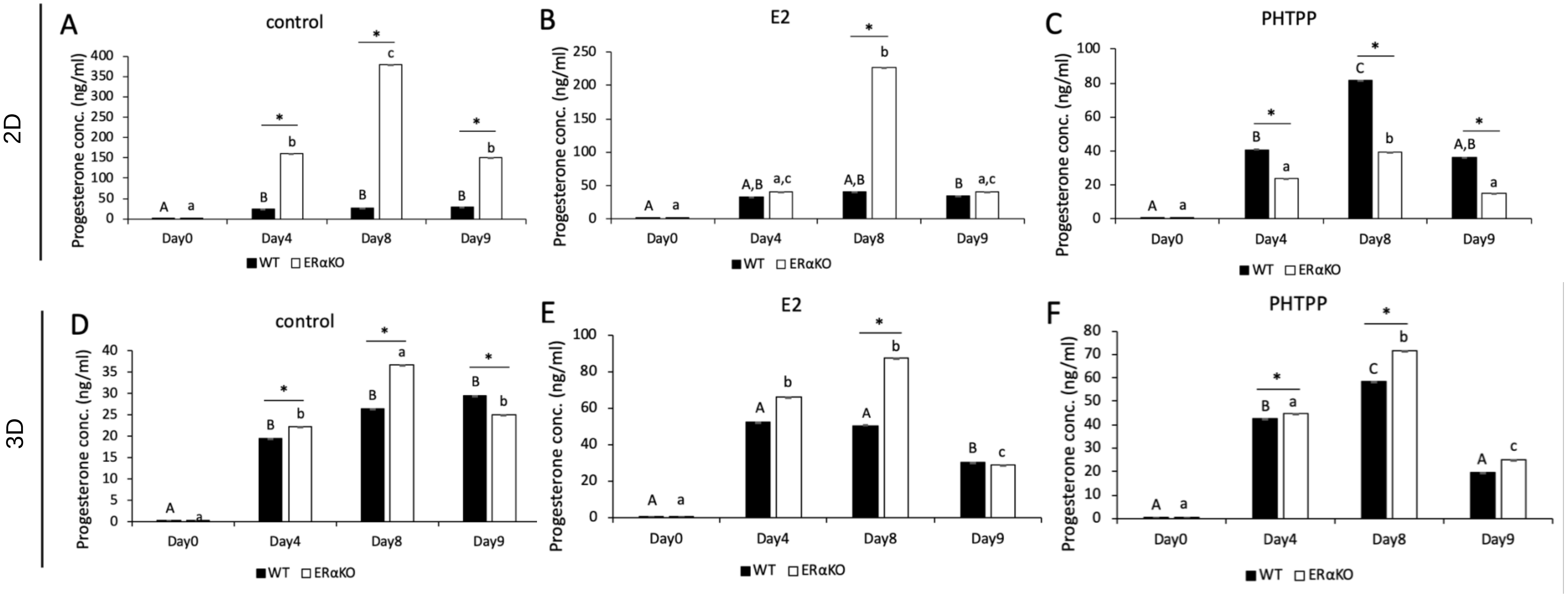
2D
3D
A
control
B
E2
C
PHTPP
D
control
E
E2
F
PHTPP
Progesterone conc. (ng/ml)
0
50
100
150
200
250
300
350
400
Day0
Day4
Day8
Day9
A
a
B
b
B
c
B
b
*
■ WT
□ ERαKO
0
50
100
150
200
250
A
a
A,B
a,c
A,B
b
B
a,c
0
20
40
60
80
100
A
a
B
a
C
b
A,B
a
0
5
10
15
20
25
30
35
40
A
a
B
b
B
a
B
b
A
b
A
b
B
c
0
10
20
30
40
50
60
70
80
A
a
B
a
C
b
A
c

Figure 9. The comparison of production of progesterone during *in vitro* culture between WT and ERαKO

The progesterone concentrations in spent culture medium from wild type and ERαKO mice treated or not with E_2 or PHTPP. A,B) Follicles were cultured in the way of 2D culture system. C,D) Follicles were cultured in the way of 3D culture system. Different capital letters represent differences in the different cultured group of wild type. Different lower cases represent differences in the different cultured group of ERαKO. Statistical analysis by Student's t-test (*: $p<0.05$ WT vs ERαKO). Data are represented as the mean ± SEM.

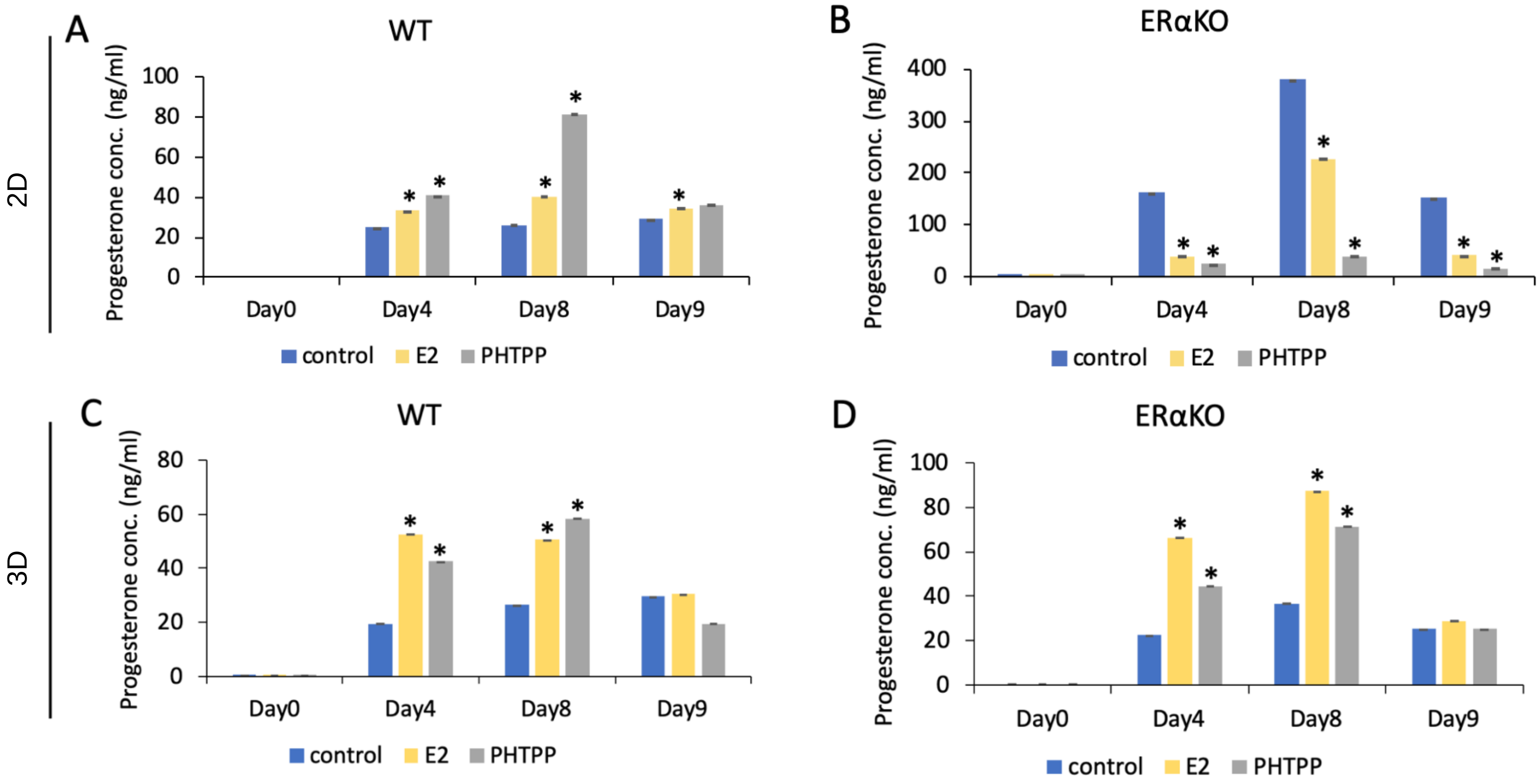
2D
3D
A
WT
B
ERαKO
C
WT
D
ERαKO
Progesterone conc. (ng/ml)
0
20
40
60
80
100
100
200
300
400
Day0
Day4
Day8
Day9
*
control
E2
PHTPP

Figure 10. The levels of progesterone during *in vitro* culture

The progesterone concentrations in spent culture medium from wild type and ERαKO mice treated or not with E_2 or PHTPP. A,B) Follicles were cultured in the way of 2D culture system. C,D) Follicles were cultured in the way of 3D culture system. Different capital letters represent differences in the different cultured group of wild type. Different lower cases represent differences in the different cultured group of ERαKO. Statistical analysis by Student’s t-test (*: $p<0.05$ WT vs ERαKO). Data are represented as the mean ± SEM.

Discussion

In this study, we investigated the effect of estrogen in folliculogenesis through ERα using two different in vitro ovarian follicle culture system. To observe the action of ERα in ovarian follicular growth we used ERαKO, ERβ-specific antagonist (PHTPP) and ERs antagonist (ICI 182,780). ERα is known to be localized in theca/interstitial cell in rodents (Sar M et al., 1999). Therefore, in this study demonstrates estrogen directly influence on folliculogenesis through ERα in theca cell.

When the 10^{-8} M 17β – estradiol was treated standard medium, the diameters of follicles from WT significantly increased at days 4, 6 and 8 of culture compared to control using 2D culture system (Fig. 2A). However, in the group of 10^{-5} M PHTPP treated standard medium, the diameters of follicles significantly decreased at day 8 (Fig. 2A). In the way of 3D culture system, the diameters of ovarian follicles cultured in 10^{-5} M ICI 182,780 treated standard medium significantly decreased at days 2, 4, 6 and 8 of culture (Fig. 2B). This shows estrogen might be influence on the growth of ovarian follicles through ERα and ER β. There was no significantly difference in all cultured group of ERαKO under both 2D culture system and 3D culture system (Fig. 3A,B).

Insulin-like 3 (Insl3) and Cytochrome P450 Family 17 Subfamily A Member 1 (Cyp17a1) are used for theca cell specific markers. Insl3 and its receptor (Relaxin family peptide 2, RXFP2) is expressed in theca cells and impacts on the functions of theca cell including modulation of LH-mediated androgen synthesis (Richard JS., 2018). Cyp17a1 involving the conversion of pregnenolone to androstenedione also expressed in theca cells (Fuentes N., 2019). The expression of *Insl3* mRNA significantly increased in E_2 treated group of ERαKO but significantly decreased in

PHTPP treated group of ERαKO in both two culture systems.

Follicle-stimulating hormone receptor (Fshr) and Cytochrome P450 Family 19 Subfamily A Member 1 (Cyp19a1) are used for mural granulosa cell specific markers. Cyp19a1 involving the conversion of androstenedione to estrone expressed in granulosa cells (Fuentes N., 2019). In both culture systems, the expression of *Fshr* and *Cyp19a1* significantly increased in E_2 treated group of ERαKO but significantly decreased in PHTPP treated group of ERαKO. In WT, the expression of Cyp19a1 was only up-regulated in E_2 treated group. These results show estrogen might effect on the differentiation of granulosa cell through ERβ.

Pentraxin 3 (Ptx3), Prostaglandin-endoperoxide synthase 2 (Ptgs2), and TNF alpha induced protein 6 (Tnfaip6) are used for cumulus expansion specific markers. Ptx3 and Tnfaip6 involved in stabilization of the cumulus complex (Fulop et al. 2003). Ptgs2 produced by cumulus cells is required for maximum cumulus expansion and ovulation (Lim et al., 1997). Expression of *Ptx3*, *Ptgs2*, and *Tnfaip6* in both ERαKO and WT, showed different results according to the two culture systems. ERαKO also showed differences in the levels of steroid hormone between the two culture systems. The concentrations of E_2 and P_4 in both cultures increased in E_2 and PHTPP treated group of WT. On the other hand, ERαKO mice showed decreased levels of E_2 and P_4 in 2D culture system, but increased in 3D culture system.

Based on these results, it is suggested that estrogens might effect on the growth of follicles, differentiation of granulosa and theca cells, cumulus expansion and production of steroid hormones. In addition, the two-dimensional and three-dimensional cultures may have different effects on the progress of the folliculogenesis.

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논문개요

에스트로겐은 여성의 생식 기관의 성장, 분화 그리고 기능에 중요한 역할을 하는 것으로 알려져 있다. 배란에 이르기까지 난포가 성장하는 과정에 있어서 에스트로겐은 자가 분비 또는 주변 분비를 통해 영향을 미친다. 성장하는 난포의 과립막세포에서 에스트로겐은 성샘자극호르몬의 작용, 난포 강 형성, 과립막세포 간 간극 연접 형성을 촉진하며 난포의 폐쇄를 억제한다. 성장하는 난포막세포에서 에스트로겐은 안드로겐의 합성을 조절한다. 스테로이드가 난포의 성장에 미치는 영향에 관한 연구는 활발하게 이루어졌음에도 불구하고 에스트로겐이 난포의 성장에 미치는 영향에 대해 모순된 결과를 보인다. 에스트로겐은 핵 수용체인 에스트로겐 수용체 알파와 베타를 통해 작용한다. 에스트로겐 수용체를 매개한 에스트로겐의 작용의 중요성은 에스트로겐 수용체 유전자 적중 생쥐를 이용하여 많은 연구가 이루어지고 있다. 이 연구에서는 난포의 체외 배양을 통해 에스트로겐 수용체 알파 유전자 적중 생쥐를 이용하여 난포막 세포를 통한 에스트로겐의 작용이 난포의 성장, 난자의 성숙에 미치는 영향을 알아보고자 하였다. 야생형의 생쥐에서는 이차원적인 배양 방식에서 에스트로겐 처리시 난포의 직경이 증가하였고, PHTPP 처리시 배양 8 일 째 유의하게 감소하였다. 야생형의 생쥐의 삼차원적인 배양 방식에서는 ICI 182,780 처리시 전체 배양 기간 동안 감소하는 경향을 보였다. 에스트로겐 수용체 알파 유전자 적중 생쥐에서는 두 배양 방식에서 모두 그룹 간의 유의한 난포 크기 변화를 나타내지 않았다. 두 배양 방식에서 모두 난포막 세포 특이적인 유전자인 Insl3 의 발현이 에스트로겐 수용체 알파 유전자 적중 생쥐에서 PHTPP 처리시 mRNA 수준에서 감소하는 경향을 보인 반면, 야생형의 생쥐에서는 증가하는 양상을 보였다. 두 배양 방식에서 모두 에스트로겐 처리시 과립막 세포 특이적인 유전자인 Fshr 와 Cyp19a1 은 에스트로겐 수용체 알파 유전자 적중 생쥐에서

유의한 증가를 보였고, 야생형의 생쥐에서는 Cyp19a1 만 증가하는 양상을 보였다. 그러나, PHTPP 처리시 에스트로겐 수용체 알파 유전자 적중 생쥐에서 Fshr 는 감소였다. 에스트로겐 수용체 알파 유전자 적중 생쥐와 야생형의 생쥐에서 모두 난구 세포의 분산 특이적인 유전자인 Ptx3, Ptgs2 그리고 Tnfaip6 의 발현 양상은 두 배양 방식에 따라 서로 다른 결과를 보였다. 에스트로겐 수용체 알파 유전자 적중 생쥐의 경우 스테로이드 호르몬 생산 수준에서도 두 배양 방식에 따라 차이를 보였다. 야생형의 생쥐는 두 배양 방식에서 모두 에스트로겐과 프로게스테론의 농도가 에스트로겐과 PHTPP 처리 시 증가하였다. 반면, 에스트로겐 수용체 알파 유전자 적중 생쥐에서는 이차원적인 배양 방식에서는 에스트로겐과 프로게스테론의 농도가 에스트로겐과 PHTPP 처리 시 감소하였지만, 삼차원적인 배양 방식에서는 증가하는 양상을 보였다. 이러한 결과를 통해 에스트로겐이 난포의 크기, 과립막세포와 난포막세포의 분화, 난구세포의 분산, 스테로이드 호르몬의 생산에 영향을 미칠 수 있음을 알 수 있었다. 또한 이는 난포의 성장 과정에서 에스트로겐 수용체의 국소 작용에 대한 통찰력을 제공할 수 있다.

감사의 글

석사 학위 과정 동안 도움을 주신 모든 분께 감사드립니다. 먼저, 2년 동안 좋은 방향으로 이끌어주시고 지도해주신 전용필 교수님께 감사드립니다. 항상 랩실을 묵묵히 이끌어주고 있는 순영언니 같이 고민해주고 조언해주셔서 감사합니다. 사소한 부분까지도 챙겨주고 알려주는 연정언니 항상 감사하게 생각하고 있어요. 실험 정말 많이 도와준 민영이 진짜 너무 고마워요... 같이 졸업하는 우리랑 주혜 덕분에 외롭지 않게 실험할 수 있었어요. 입학 초부터 느꼈지만 다들 모두 열심히 하는 동기들을 만나서 2년 동안 즐겁고 행복했습니다. 마지막 학기에 굉장히 일도 많고 실험 양도 많아서 혼자 많이 버거웠었는데 옆에서 정말 많은 분들이 도와주셔서 힘들었지만 정말 행복했어요. 제가 표현을 잘 못했지만 항상 감사하게 생각하고 있어요. 랩실에 있으면서 제 자신이 많이 성장한 것을 최근에 많이 느꼈는데 모두 옆에서 도와주신 분들 덕분입니다. 석사 하는 동안 힘들면 가족들에게 투정도 많이 부리고 했는데 항상 옆에서 응원해준 우리 엄마, 아빠, 희원이 모두 고맙고 사랑해요. 그리고 요즘 집에 없어서 많이 못 놀아준 별,달,아치...언니가 이제 많이 놀아 줄게요.

다시 한번 제가 성장할 수 있는 발판을 마련해주셔서 감사드리고 앞으로 더 열심히 배우고 발전하는 사람이 되겠습니다. 감사합니다 ☺