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REGULATORY MECHANISMS OF G PROTEIN-COUPLED RECEPTOR (GPCR) SIGNALING AT FOLLICLE SELECTION IN THE HEN OVARY

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ABSTRACT

In vertebrate species that ovulate a species-specific number of eggs each ovulation cycle, ovarian follicles undergo a process of selection for further development and final maturation prior to ovulation. Subsequent to follicle selection, ovarian follicles mature into preovulatory follicles and are eventually ovulated. In monovulatory vertebrate species, which maintain reproductive cycles, only one follicle from the limited growing follicle population is typically selected for fertilization. Ovarian follicle selection is largely dependent upon the transition of granulosa cells (GCs) from an undifferentiated to a fully differentiated state, which accompanies the morphological and functional changes that initiate follicle development. In both mammals and birds, it has been well established that GC differentiation during follicle development is dependent upon gonadotropin-responsiveness (*e.g.* initially follicle stimulating hormone (FSH)- and subsequently luteinizing hormone (LH)-responsiveness). In the hen ovary, the selection of a single follicle each day occurs from a small cohort of prehierarchal follicles measuring 6-8 mm in diameter. To date, considerable research has been focused on identifying the role of growth factors or the signaling pathways in granulosa cells to understand the principle of follicle selection. Nonetheless, the ultimate signals responsible for cellular events which result in a single follicle being selected have yet to be demonstrated in any vertebrate.

The present studies were conducted to investigate the regulatory mechanisms of G protein-coupled receptor (GPCR) signaling to better understand mechanisms that initiate the differentiation of GCs at follicle selection in the hen ovary. It is hypothesized that prior to follicle selection, FSH receptor (FSHR) and vasoactive intestinal peptide (VIP) receptors (VPAC-1 and -2) are desensitized by a βARRESTIN-mediated event in undifferentiated GCs. Additionally, it is proposed that receptor-desensitization is maintained and/or promoted by inhibitory mitogen-activated protein kinase (MAPK) signaling. Therefore, at follicle selection, actively

differentiating GCs are able to acquire receptor-responsiveness due to reduced MAPK signaling, that results in inducing cyclic 3,5-adenosine monophosphate (cAMP)-induced signaling. Finally, the present studies demonstrate that the acquisition of receptor-responsiveness initiates GC differentiation by promoting cAMP-induced steroidogenesis, clock gene expression, and angiogenesis.

Undifferentiated GCs from hen prehierarchal follicles failed to initiate signaling via cyclic 3,5-adenosine monophosphate (cAMP) following a 3-4 h challenge with recombinant human (rh)FSH (10 ng/ml) and chicken (ch)VIP (1 μM), despite the finding that undifferentiated GCs from prehierarchal follicles express *FSHR* mRNA and FSHR protein plus *VPAC1/2* mRNA during follicle development. In addition, these cells demonstrated an inability to induce steroidogenic acute regulatory (STAR*)* protein expression and progesterone production due to the absence of GPCR-mediated cAMP formation. Specifically, knockdown studies using small interfering RNA (siRNA) specific for *Gallus βARRESTIN1* revealed that reduced βARRESTIN expression in undifferentiated GCs results in increased cAMP formation, STAR protein expression, progesterone production following a rhFSH or chVIP treatment compared to cells transfected with non-targeting (scrambled) siRNA. Furthermore, co-transfection of bovine *βARRESTIN* and *GPCR kinase 2* (*GRK2)* constructs in actively differentiating GCs significantly decreased cAMP and progesterone production following a 3 h rhFSH treatment.

In chickens, VIP, unlike the unchanging concentrations of circulating of FSH, is rhythmically expressed within the hypothalamus during a photo-induced reproductive cycle [1, 2]. Accordingly, it was hypothesized that immediately subsequent to follicle selection in the hen, VIP derived from a neuronal and/or humoral origin can serve to regulate expression of clock genes within the GC layer. Significantly, it was determined that cAMP-induced clock gene expression was not initiated by a 4 h challenge with chVIP (1 μM) in undifferentiated GCs. Using *βARRESTIN*1-siRNA, it was confirmed that undifferentiated GCs with reduced levels of

βARRESTIN became competent to respond to chVIP, which resulted in increasing cAMP-dependent *BRAIN AND MUSCLE ARNT-LIKE PROTEIN 1 (BMAL1)* gene expression. Finally, *BMAL1* and *CIRCADIAN LOCOMOTOR OUTPUT CYCLES KAPUT (CLOCK)* gene expression in actively differentiating GCs varied in a time-in-culture-dependent fashion, compared to undifferentiated GCs in which clock gene expression demonstrated no rhythmicity.

The number and size of blood vessels surrounding and within the follicle theca layer increases dramatically beginning at the time of follicle selection when compared to prehierarchal follicles. However, processes regulating angiogenesis have not been studied in chicken ovarian follicles. There is elevated expression of *VASCULAR ENDOTHELIAL GROWTH FACTOR* (*VEGF)* mRNA in GCs from the selected (preovulatory) follicle, compared to GCs from unselected (prehierarchal) follicles. In addition, it was determined that *ANGIOPOIETIN* (*ANGPT)-2*, a natural antagonist for *ANGPT-1* with angiogenic functions, is highly expressed in undifferentiated GCs and its gene expression decreases after follicle selection. In hen GCs, it was determined that *VEGF* and *ANGPT-2* gene expression is regulated by gonadotropins and several transforming growth factor beta (TGFβ) superfamily members, including TGFβ1 and BMP4. Levels of *VEGF* and *VEGF RECEPTOR* (*VEGFR)* mRNA were determined to be dramatically lower in atretic follicles compared to healthy prehierarchal follicles, which suggests VEGF signaling may play a role in maintaining follicle survival.

Collectively, the present studies provide novel evidence that prior to follicle selection FSHR and VPACs in undifferentiated hen GCs are, at least in part, desensitized by a βARRESTIN-mediated event. This receptor desensitization contributes to the absence of cAMP-mediated signaling, that results in the inability to synthesize steroids, the arrhythmicity of peripheral clock genes, and a minimal extent of vasculature within prehierarchal follicles.

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ABBREVIATIONS LIST

AC : Adenylyl cyclase

ACTH : Adrenocorticotrophic hormone

ADP : Adenosine diphosphate

AMH : Anti-müllerian hormone

ANGPT : Angiopoietin

AP-2 : Adaptor protein-2

ARF : ADP ribosylation factor

BCL-2 : B-cell lymphoma 2

bHLH : basic Helix-loop-helix

BMAL : Brain and Muscle ARNT-like

BMP : Bone morphogenetic protein

CAM : Chorioallantoic membrane

cAMP : cyclic 3,5-adenosine monophosphate

CKε : casein kinase ε

CLOCK : Circadian locomotor output cycles kaput

CNS : Central nervous system

CREB : cAMP response-element binding protein

CREM : cAMP response-element modulator

CRY : Cryptochrome

DAG : Diacylglycerol

EC : Endothelial cell

EGFR : Epidermal growth factor receptor

EGFRL : EGFR ligand

EIA : Enzyme immunoassay

ER : Estrogen receptor

ERK1/2 : Extracellular signal-regulated kinase 1/2

FSH : Follicle stimulating hormone

FSHR : Follicle stimulating hormone receptor

GDP : Guanosine diphosphate

GnIH : Gonadotropin inhibitory hormone

GnRH : Gonadotropin releasing hormone

G protein : Guanine-nucleotide binding protein

GPCR : G protein-coupled receptor

GRK : GPCR kinase

ICER : Inducible cAMP early repressor

JNK3 : c-Jun N-terminal Kinase 3

ID : Inhibitor of differentiation

IP3 : Inositol triphosphate

LDL : Low density lipoprotein

LH : Luteinizing hormone

LHR : LH receptor

MAPK : Mitogen-activated protein kinase

MMP : Matrix metalloproteinase

NSF : N-ethylmaleimide-sensitive fusion protein

PER : Period

PI3K : Phosphainositide3-kinase

PKA : Protein kinase A

PKB : Protein kinase B (AKT)

PKC : Protein kinase C

PLC : Phospholipase C

PMA : Phorbol 12-myristate 13-acetate

P450scc : Cytochrome P450 side-chain cleavage (CYP11A)

PRL : Prolactin

qRT-PCR : quantitative real time-polymerase chain reaction

SCN : Suprachiasmatic nucleus

SMAD : portmanteau word; *Caenorhabditis elegans* protein SMA (from gene sma for small body size) and the *Drosophila* protein, mathers against decapentaplegic (MAD)

STAR : Steroidogenic acute regulatory

7-TM : Seven transmembrane

3βHSD : 3-beta-hydroxysteroid dehydrogenase

TGFα : Transforming growth factor alpha

TGFβ : Transforming growth factor beta

TIE-2 : Tyrosine kinase with immunoglobulin-like and EGF-like domain 2

TIMP : Tissue inhibitors of metalloproteinase

TSH : Thyroid-stimulating hormone

VEGF : Vascular endothelial growth factor

VEGFR : Vascular endothelial growth factor receptor

VLDL : Very low density lipoprotein

VTG : Vitellogenin

VIP : Vasoactive intestinal peptide

VPAC : Vasoactive intestinal peptide receptor

Chapter 1

INTRODUCTION

Infertility is a global issue for about 10% of couples worldwide [3]. In the United States, about 11 % of women (~6 million) have difficulty getting pregnant or maintaining pregnancy, according to the Centers for Disease Control and Prevention (CDC) [4]. The causes of female infertility include genetic abnormalities, aging, exposure to environmental risks (*e.g.*, smoking and toxins), certain acute or chronic diseases (*e.g.*, stress, obesity, and diabetes), and hormone imbalance (*e.g.*, polycystic ovarian syndrome) [5-8]. The above conditions cause irregular reproductive cycles and are mainly associated with ovarian dysfunction in approximately 25% of all infertility cases. Ovarian dysfunction is mainly caused by failure in the process of follicle/oocyte development [9, 10]. Currently, these issues are often overcome with *in vitro* fertilization (IVF), a revolutionary assisted treatment for infertility beginning with the first test tube baby born in 1978 [11]. From the genetic point of view, babies born by IVF may have potential defects, because of the genetic background of their parents who have reproductive problems. Human infertility issues may, in turn, continue to exist from generation to generation. Therefore, basic research to understand cellular mechanisms (*e.g.*, epigenetics), which can be a desirable method to solve the fundamental causes of infertility, is required in order to overcome current technological limitations.

Ovarian follicle selection in monovulatory species (*e.g.*, women, cattle, domestic hen) is the process that gives rise to the dominant or ovulatory follicle from a cohort of slow growing follicles. The proximal mechanism mediating follicle selection is currently unclear in vertebrates, but it is clear that follicle selection is controlled by a combination of factors. In the reproductively

active domestic hen, the single ovary experiences almost daily ovulations. The mature, reproductively active ovary is composed of resting primordial, slow growing prehierarchal, and rapidly growing preovulatory follicles, the latter of which is characterized by the large size and degree of yolk formation [12]. In addition, each follicle consists of an oocyte, granulosa layer and theca layer (**Figure 2.4**), similar to the structures found in mammals [13, 14]. It is well established that one of the prehierarchal follicles (6-8 mm) is selected each day for a characteristic number of days (clutch length) and grows with the accumulation of lipoprotein-rich yolk and the differentiation of theca into interna and externa layers. In prehierarchal follicles, the theca layer has steroidogenic activity, while the granulosa layer is inactive [15-19]. Following follicle selection, preovulatory follicles rapidly grow from ~9 mm to ~50 mm until ovulation due to the uptake of large amount of lipoprotein available from the abundant vasculature. GCs from the most recently selected follicle (9-12 mm) initially acquire FSHR-responsiveness and begin to produce progressively greater amounts of steroid hormones (in particular, progesterone) [18]. The largest preovulatory follicle eventually is ovulated along the avascular stigma region. Compared to the granulosa layer, considerably less work has been conducted in theca layers that are composed of multiple types of cells [20-22]. This layer provides both the structural support and the synthesis of steroid precursors required for successful follicle development and maturation [16, 23, 24]. Importantly, it is the GC layer that undergoes the greatest change during follicle development in that differentiation is initiated only after follicle selection. Nevertheless, to date most research on avian follicle development has focused on the regulation of steroidogenesis in differentiated GCs from preovulatory follicles [25, 26].

Prior to follicle selection in the hen ovary, GCs from prehierarchal follicles are mitotically active, but steroidogenically inactive due to negligible expression of steroidogenic factors, whereas GCs from preovulatory follicles are capable producing significant (μg) amounts of progesterone [18, 27, 28]. Since steroidogenesis is mediated primarily by cAMP signaling in

response to FSH and subsequently LH, it is predicted that FSHR-responsiveness initiates the process of final differentiation in the hen follicles. Considering the important roles of FSHR-responsiveness at ovarian follicle selection, it is essential to identify how undifferentiated GCs become responsive to FSH and how the absence of FSHR-responsiveness is maintained in undifferentiated GCs prior to follicle selection. Furthermore, it is also important to understand the proximal mechanisms by which follicle selection is accomplished on a daily basis and what additional factors are required to support follicle growth following selection. Therefore, it is necessary to establish the mechanisms that regulate receptor responsiveness and subsequently enable GC differentiation at the time of follicle selection. In this dissertation, the regulatory mechanisms of FSH and vasoactive intestine peptide (VIP) signaling through their respective GPCR receptors were investigated to understand the regulation of GPCR signaling prior and immediately subsequent to follicle selection. In addition, the mechanisms of receptor responsiveness were applied to studies regarding the circadian rhythmicity of peripheral (ovarian) clock genes and angiogenesis to identify additional mechanisms in GC differentiation following follicle selection in the hen ovary.

RATIONALE

In mono-ovulatory vertebrate species (*e.g.*, women, cattle, domestic hen), only a single ovarian follicle is normally selected per cycle for the final growth and ovulation. It has been determined that follicle selection in the hen ovary occurs from within a cohort of 8-12 follicles measuring 6-8 mm in diameter [19]. According to previous studies, critical processes in follicle selection are mediated by the FSH-FSHR interaction and are closely associated with the transition of GCs from an undifferentiated to differentiated state [19, 29, 30]. To date, considerable research

has been focused on identifying the role of growth factors and the signaling pathways within GCs in an effort to understand the mechanism of follicle selection in vertebrates [18, 27-33].

Previously, our lab has reported that undifferentiated GCs express negligible levels of steroidogenic enzyme genes and fail to produce steroids in response to FSH, although they express FSHR with similar level to actively differentiating GCs from the most recently selected (9-12 mm) follicle, and with even higher levels than that of more differentiated GCs [19]. These results indicate that there is the absence of FSH-induced signaling via FSHR in undifferentiated GCs. Moreover, it has been reported that mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) signaling maintains GCs in an undifferentiated state by inhibiting *FSHR* mRNA expression and cAMP formation [19, 34]. The FSHR and LH receptor (LHR) belong to the family of GPCRs, seven transmembrane proteins with intracellular and extracellular domains for signal transduction and ligand binding, respectively [35]. However, either continuous exposure to the ligands or high concentration of the ligands leads to decreased responsiveness and the down-regulation of intracellular signaling, a process called desensitization. In Sertoli cells from mouse testes, it was observed that FSH-induced adenylyl cyclase (AC) activity is attenuated in both a time- and concentration-dependent manner [36]. Since FSHR represents a member of the GPCR family which has regulatory mechanisms mediated by βARRESTIN and GPCR kinases (GRKs), it is predicted that FSH-responsiveness in undifferentiated GCs is inhibited by a βARRESTIN-mediated event and that this inhibition is maintained by active MAPK signaling prior to follicle selection.

The development of ovarian follicles in vertebrates is regulated not only by gonadotropins and ovarian-derived growth factors, but also by neuroendocrine factors. It has been reported that VIPergic nerve fibers extend throughout the theca interna of hen prehierarchal follicles, and that paracrine VIP signaling is involved in the regulation of steroidogenesis by stimulating cAMP-dependent factors, including STAR and cytochrome P450 side-chain cleavage

(P450scc/CYP11A) in cultured GCs [31, 37-39]. Additionally, recent studies have demonstrated that VIP promotes follicle development and maintains cell survival in mammalian GCs [40-42]. Significantly, transgenic mice lacking VIP or VPAC2 show weak or no circadian rhythms, suggesting that VIP is required for regulating circadian rhythms and clock-related genes [43-46]. In non-mammalian vertebrates such as domestic hens and Japanese quail, it has been previously shown that clock genes (*BMAL1*, *PER2*, *CLOCK*) are expressed in granulosa and theca layers and these ovarian clock proteins are involved in steroidogenesis. In addition, they are involved in regulating the rhythm of ovulation and oviposition that are entrained to photoperiod and regulated partially by the preovulatory LH surge [47, 48]. These studies were primarily focused on fully differentiated GCs within preovulatory follicles, but also demonstrated that there is no rhythmic expression of clock genes in GCs from quail prehierarchal follicles [47]. Since there is evidence that the expression of clock genes such as *BMAL* and *PER* is mediated by cAMP signaling [45, 49, 50], it is speculated that the rhythmicity of their expression in undifferentiated GCs is prevented by the absence of VPAC signaling. Several recent studies have reported significantly reduced fertility and fecundity in *PER1* and *PER2*-deleted mice [51] and prolonged estrous cycles in clock-mutated animals [52, 53]. In addition, *BMAL1*-knockout female mice display irregular estrous cycles, abnormal steroidogenesis, and reduced progesterone production [54-56]. Therefore, these results strongly support that clock genes play pivotal roles in regulating female reproductive systems. However, there is currently no evidence that VIP promotes ovarian follicle development by regulating peripheral circadian rhythms. To date, research has demonstrated that clock gene expression can be regulated by cAMP-mediated signaling [45, 49, 57]. In particular, *VIP* mRNA is shown to be expressed rhythmically in the SCN of female [1, 58, 59], and *VPAC2* null mice fail to induce circadian expression of clock genes in the SCN of mice. This suggests that VIP-induced signaling is essential for the maintenance of circadian function in the SCN [60]. Collectively, it is speculated that a rhythmic expression of VIP transmitted through VIPergic

nerves from the SCN to the ovary may influence the expression of clock genes to regulate female reproductive cycles.

Ovarian follicles are known to contain and produce angiogenic factors that facilitate follicle growth by transporting growth factors, hormones and nutrients [61, 62]. It has been well established that angiogenesis is essential for the tissue growth and morphogenesis during embryonic gonad development [63]. Vascular endothelial growth factor (VEGF) is highlighted, because it is known to be a potent and primary angiogenic factor secreted by GCs from mammalian ovaries [64-66]. The two most important angiogenic factors, VEGF and ANGPT, are expressed in the ovary and regulate follicular angiogenesis in normal follicle growth, ovulation, and the formation of the corpus luteum [65, 67-69]. Moreover, these studies support the role of VEGF supporting follicle development by promoting proliferation, inhibiting apoptosis of follicular cells, and maintaining steroidogenesis [70, 71]. ANGPTs and VEGF have synergistic functions to control the stability of blood vessels. Although ANGPT-1 and -2 have similar binding affinities to TIE-2 receptor, they exert opposing functions following receptor binding. The binding of ANGPT-1 to Tyrosine kinase with Immunoglobulin-like and EGF-like domain-2 (TIE-2) induces receptor phosphorylation, resulting in the recruitment of pericytes and smooth muscle cells stabilizing the new vasculature formed by VEGF [72, 73], However, ANGPT-2 functions as an antagonist by blocking the binding of ANGPT-1 to TIE-2 receptor [73, 74]. Therefore, these results suggest that VEGF expression or the ratio of ANGPT-1 and -2 expression may be important in the regulation of follicular angiogenesis. In other words, a deficiency or disrupted expression of angiogenic factors disrupts angiogenesis, resulting in severe defects during the development of ovarian follicles. Despite accumulating data regarding the importance of angiogenesis in the development of mammalian ovaries, the relationship between ovarian follicle development and angiogenesis has not been studied in chicken ovarian follicles. Considering that hen follicle growth is dependent upon acquiring growth factors, hormone, and

large amounts of nutrients through blood vessels, it is proposed that GCs regulate angiogenesis by secreting angiogenic factors (*e.g.*, VEGF and ANGPT) and increased vasculature development is required for rapid follicle growth beginning at follicle selection.

Taken together, studies of cellular mechanisms regulating FSH- and VIP-mediated cell signaling in GCs can provide fundamental knowledge to understand mechanisms related to follicle selection in vertebrates. Although the mechanisms regulating receptor-responsiveness are extended to the study of circadian biological rhythms and angiogenesis as they pertain to GC differentiation during hen follicle development, these concepts may be applicable to understanding the function of GPCRs in GCs from mammalian and non-mammalian species. In some domesticated species an improved regulation of follicle selection may contribute to enhancing the production of domestic animals by increasing the number and quality of selected follicles as well as enhancing ovulation rate.

HYPOTHESES AND OBJECTIVES

1. FSHR is desensitized in undifferentiated GCs prior to follicle selection and follicle development is initiated by FSHR re-sensitization at follicle selection

1-1. The lack of cAMP signaling through FSHR in undifferentiated GCs results in the absence of progesterone production.

Since it is well known that the interaction of FSH with FSHR induces cAMP signaling and FSHR is highly expressed in undifferentiated GCs, FSH-induced cAMP and progesterone production will be measured after short-term incubation of GCs from prehierarchal (6-8 mm) and the most recently selected (9-12 mm) follicles using an enzyme immunoassay (EIA). To confirm these results, cAMP-mediated STAR protein expression will be analyzed by western blot. To

determine if FSH-induced cAMP is absent specifically within GCs, theca layers from 6-8 mm follicles will be incubated with rhFSH then *STAR* mRNA expression will be measured.

1-2. FSHR in undifferentiated GCs is desensitized by a βARRESTIN-mediated event.

To test this hypothesis, primary GCs will be isolated from prehierarchal follicles (6-8 mm) and used for RNA interference (RNAi) experiments to significantly reduce endogenous βARRESTIN expression. rhFSH (10 ng/ml) will be added to cultured GCs previously transfected with *βARRESTIN1*- and scrambled-siRNA. FSHR desensitization will be verified by measuring cAMP accumulation (in the presence of 3-isobutyl-1-methylxanthine; IBMX) and progesterone. To confirm results from cAMP and progesterone assays, STAR protein will be determined by western blot analysis [18, 75]

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1-3. Overexpression of βARRESTIN1 suppresses FSH-induced cAMP accumulation in actively differentiating GCs.

Immediately subsequent to follicle selection, GCs initially acquire FSHR-responsiveness and become competent to initiate cAMP signaling. Thus, to induce receptor desensitization, actively differentiating GCs from 9-12 mm follicle will be transfected with *βARRESTIN1* and *GRK2* or empty vector (pcDNA3.1) as previously described [76]. Accumulation of cellular cAMP (in the presence of IBMX) and media progesterone production will be measured by EIA following a challenge with rhFSH (10 ng/ml) for 3 h.

2. VIP contributes to promoting GC differentiation and entrains clock gene expression within GCs of chicken ovarian follicles

2-1. Undifferentiated GCs fail to induce cAMP-mediated signaling in response to chVIP.

Since *VPAC1* and *VPAC2* mRNA expression has not previously been evaluated in chicken ovarian follicles, GCs and theca cells (TCs) at various stages of follicle development will be used to determine their expression levels by quantitative real time-polymerase chain reaction (qRT-PCR). In addition, freshly collected GCs from 3-5 mm, 6-8 mm, 9-12 mm and fourth largest (F4) follicles will be isolated and incubated with chVIP (1 μM) for 4 h. To assess chVIP-induced signaling, the amount of cAMP accumulation (in the presence of IBMX) and progesterone production will be measured by EIA. To confirm these results, cAMP-mediated STAR protein expression will be analyzed by western blot.

2-2. VPACs in undifferentiated GCs are desensitized by a βARRESTIN-mediated event.

Undifferentiated GCs from 6-8 mm follicles will be transiently transfected with *βARRESTIN1*- or Scrambled-siRNA to examine the effects of reduced βARRESTIN expression on VPAC-mediated signaling. siRNA-transfected GCs will subsequently be incubated with or without chVIP (1 μM) for 4 h. VPAC-responsiveness will be identified by measuring cAMP accumulation and progesterone production. To confirm results from cAMP and progesterone assays, chVIP-induced STAR protein expression will be analyzed by western blot.

2-3. VIP-induced signaling regulates clock gene expression in hen GCs.

Initially, the expression of clock genes (*BMAL1*, *CLOCK*, and *PER2*) will be determined in GCs and TCs from different follicle stages of development (3-5 mm, 6-8 mm, 9-12 mm and F4) by qRT-PCR method. To examine if clock gene expression is regulated by VIP signaling, primary GCs will be isolated from different stages of development and incubated with chVIP (1 μM) for 4 h. RNAs will be isolated and used to measure the levels of clock gene expression using qRT-PCR.

2-4. Clock gene expression prior to follicle selection is precluded by a βARRESTIN-mediated event.

Previous studies demonstrated that clock gene expression has essentially no rhythmicity in immature and undifferentiated GCs in both mammalian and avian ovarian follicles [47, 77, 78]. To assess whether clock gene expression prior to follicle selection is precluded by a βARRESTIN-mediated event, undifferentiated GCs will be transiently transfected with *βARRESTIN1*-or Scrambled-siRNA. siRNA-transfected GCs will be subsequently treated with chVIP (1 μM) for 4 h. RNAs isolated from each group will be used to analyze the levels of clock gene expression.

To begin to assess whether a rhythmicity of clock gene expression is initiated at follicle selection, undifferentiated GCs from 6-8 mm follicles will be cultured into two groups; 1) transforming growth factor alpha (TGFα) (10 ng/ml) treatment group to maintain cells in an undifferentiated state [30, 79]; and 2) control group without TGFα treatment to initiate spontaneous differentiation of GCs. Cells will be collected at 6 h intervals over a 30 h period and RNA samples will be used to monitor the expression patterns of *BMAL1*, *PER2*, *CLOCK* and *STAR* expression using qRT-PCR.

3. Angiogenic factors secreted by GCs facilitate follicle development by inducing blood vessel formation in the hen ovary

3-1. VEGF and ANGPTs expression are regulated by gonadotropins in hen GCs.

Since *VEGF* and *ANGPTs* have not previously been studied in hen ovarian follicles, primary GCs and TCs from different stages of development will be isolated and the expression of these genes will be assessed. To examine if expression of the *VEGF* and *ANGPTs* genes are regulated by gonadotropins, GCs from 6-8 mm, 9-12 mm, and F4 follicles will be incubated with rhFSH (10 ng/ml) for 4 h. GCs from F3 follicles will be isolated and incubated with oLH (10

ng/ml) for 4 h. Finally, RNAs will be used to measure the levels of *VEGF* and *ANGPTs* gene expression using qRT-PCR.

3-2. VEGF and ANGPTs expression are regulated by TGFβ superfamily members in hen GCs

Considering the absence of FSH responsiveness in undifferentiated GCs, it is speculated that there exist additional signaling pathways independent of FSH-mediated cAMP signaling that maintain a minimal level of vasculature prior to follicle selection. There are currently a few reports demonstrating that TGFβ superfamily members induce VEGF expression in bovine and human GCs [80-82]. To test this in avian follicles, undifferentiated GCs from 6-8 mm follicles will be isolated and treated with rhTGFβ1 (10 ng/ml) and rh BONE MORPHOGENETIC PROTEIN 4 (BMP4) (10 ng/ml) for 4 h. Messenger RNA from each group will be collected to measure the levels of *VEGF* and *ANGPTs* gene expression using qRT-PCR.

Chapter 2

REVIEW OF LITERATURE

FEMALE REPRODUCTIVE PHYSIOLOGY IN CHICKENS

Anatomy

The avian reproductive system consists of two separate components; the ovary and the oviduct. Although ovaries and oviduct exist as paired structures during development in most avian species, when female chicks hatch, unlike mammals, only the left ovary and its oviduct continues to grow and reach sexual maturity [83-85]. During early embryogenesis, active aromatase enzyme induces the synthesis of estrogen and estrogen receptor (ER) which is exclusively detected on the left, but not in the right, gonad in the female chick embryo [85-90]. It is known that estrogen-induced signaling is related with the inactivation of anti-müllerian hormone (AMH), resulting in developing the left ovary and regressing the right ovary [87]. The left ovary grows rapidly 16 weeks after hatching, becoming functional just prior to the onset of ovulation. Pullet chicks hatch with tens of thousands of potential ova that potentially could be ovulated. However, most of these are inactivated and approximately 250-500 eggs are typically produced during a hen's life. The remaining follicles will undergo atresia via the process of apoptosis [91-94].

The chicken ovarian follicle consists of multiple functional layers surrounding the oocyte and yolk; 1) the oocyte plasma membrane, 2) the perivitelline membrane, 3) GCs, 4) basement membrane, and 5) the theca layer (interna and externa) (**Figure 2.4**). The small white spot visible on the yolk is the germinal disc, where genetic material exists and the blastoderm develops after

fertilization. The second major part is the oviduct, a long convoluted tube approximately 25-27 inches long, is divided into five functionally distinct sections; 1) infundibulum (or funnel), 2) magnum, 3) isthmus, 4) shell gland (uterus) and 5) vagina (**Figure 2.1**). As compared to the mammalian counterparts, a unique morphological and functional characteristic of the single avian ovary is that ovarian follicles at all stages of development from resting primordial and primary follicles to the fully differentiated preovulatory state exist simultaneously during egg-laying (**Figure 2.2**). This is a significant advantage in using the chicken as an animal model to study female reproductive biology.

Ovarian follicle development in the hen

The process of follicle development can be divided into the following phases depending on the size and degree of yolk formation: 1) the organization and early growth of primordial follicles (0.08~1 mm in diameter), 2) the recruitment of primary follicles into the slow growth stage of prehierarchal follicles (6-8 mm in diameter), 3) follicle selection into the preovulatory hierarchy (9-12 mm in diameter), and 4) the subsequent rapid growth and ovulation of preovulatory follicles (up to 50 mm in diameter). Primordial follicles are formed with primary oocytes enclosed with a single GC layer and stay in an arrested state of development [14]. The transition of primordial follicle growth to the primary follicle is initiated with the recruitment of theca cells (from the stromal layers), which forms a layer separated from the granulosa layer by the basal lamina [14, 95]. Further development of the slow growing, prehierarchal follicles (1 to 8 mm) accumulate a small amount of lipoprotein-rich white yolk and accompany the differentiation of theca into interna and externa layers [14]. At this stages, vasculature and nervous innervation are established into the theca layer [14]. Following follicle selection into the preovulatory hierarchy, follicles grow rapidly, accompanying with the proliferation and differentiation of

granulosa and theca cells initiated by gonadotropin-responsiveness and transforming growth factor beta (TGFβ) superfamily members (TGFβ1, BMPs, AMH, Inhibins and Activins). The largest preovulatory follicle (F1) begins to incorporate large amounts of yolk containing VTG and VLDL, and will be eventually ovulated at the region of the stigma, an avascular area along the surface of ovum (**Figure 2.1**).

Ovarian follicle development in chickens includes not only proliferation and differentiation of follicular cells but also a tremendous increase in oocyte size due to massive yolk uptake. Yolk proteins mainly consist of very low density lipoprotein (VLDL) and vitellogenin (VTG) and are synthesized in and secreted from the liver, where their formation is primarily regulated by gonadotropin and steroid hormones [96-98]. Yolk precursors are transported into the ovary via blood vessels and pass through the basement membrane and gap juctions between GCs [13, 14]. This process is a receptor-mediated mechanism using oocyte-specific low density lipoprotein (LDL) receptor family [97, 99] and is required for DNA and protein synthesis, achieved by using glucose, fatty acids, and steroid hormones during the rapid follicle development. This suggests that follicle development is accomplished by divergent changes in gene expression and regulated by numerous intra and extra-factors in the hen ovary [100, 101].

Ovarian follicle selection in the hen

Follicle selection is an important process characterized by the emergence of one dominant follicle or several "healthy" follicles per ovulation cycle and reflects the reproductive capacity of female vertebrates. Unlike mammals where a cohort of gonadotropin-dependent follicles ("dominant follicles") are selected by a "follicular wave" during the estrous cycle [102-104], follicle selection in the laying hen occurs from a cohort of prehierarchal follicles with a 6-8

mm in diameter (**Figure 2.3**). These different mechanisms in follicle selection are likely related to the constant level of physiological FSH concentration in the chicken ovary [105, 106], suggesting that all prehierarchal follicles have an equal chance to be selected into the preovulatory hierarchy. However, only a limited number of these follicles will be selected to ovulate and many follicles will undergo atresia via the apoptosis signaling-induced by oxidative stress and activation of apoptotic factors [91-94]. The viability of follicles is primarily dependent upon survival factors as well as gonadotropins (FSH and LH), paracrine/autocrine factors produced by follicular cells, and neuronal hormones synthesized by the nervous system [29, 32, 107, 108].

The process of ovarian follicle selection is tightly associated with the transition of GCs from an undifferentiated to differentiated state in both mammals and birds [19, 109, 110]. Like other types of cells that change their shape by modulating DNA synthesis or protein expression, this transition of GCs also entails morphological changes from flat to cuboidal shape by modulating intracellular junctions and cytoskeleton protein and regulates gonadotropin-responsiveness [14, 111-113]. In particular, GCs from hierarchal follicles are considered differentiated, because they are responsible for producing progesterone in response to luteinizing hormone (LH) due to the high expression of LH receptor (LHR), P450 cholesterol side-chain cleavage (P450scc/CYP11A) enzyme activity and steroidogenic acute regulatory (STAR) protein [18, 27]. However, GCs from prehierarchal follicles with a 6-8 mm diameter are mitotically active, but incapable of producing any steroids (*e.g.*, progesterone). Moreover, it is known that GCs from prehierarchal follicles express extremely low to nondetectable levels of P450scc and STAR protein (**Figure 2.5**) [18, 32]. Based on the above criteria, GCs from prehierarchal follicles are defined as being maintained in an undifferentiated state.

Previously, it was demonstrated that *FSHR* mRNA is expressed in undifferentiated GCs from prehierarchal follicles (6-8 mm in diameter) and its expression is typically highest in one of them, suggesting that it may be the next follicle to be selected [19]. Following follicle selection,

the expression of *FSHR* mRNA decreases in the recently selected follicle (9-12 mm in diameter) and subsequently the expression of *LHR* mRNA significantly increases, suggesting that the transition of gonadotropin from FSH to LH promotes GC differentiation with LH-dependent signaling [103, 114]. Moreover, there is evidence that paracrine/autocrine factors and cell signaling pathways are associated with the initiation of GC differentiation. For example, undifferentiated GCs treated with BMP4 and/or BMP6 maintain *FSHR* mRNA levels and induce FSH-induced cAMP and progesterone production [29, 30]. As transcriptional regulators of *FSHR* mRNA, ID proteins were also assessed to understand the mechanism of follicle selection at the transcriptional level [76]. The expression level of ID2 protein is associated with increased LHR expression and gonadotropin-induced differentiation in GCs [76]. As mentioned above, it has been proposed that the undifferentiated state of GCs is maintained by active MAPK signaling induced by epidermal growth factor receptor ligands (EGFRLs) by inhibiting FSHR transcription [19, 29, 30, 34]. Furthermore, BMP2 induced mRNA expression of EGFRLs (*e.g.*, betacellulin and EGF) and ID proteins (ID1, ID3, and ID4) resulted in the inhibition of TGFβ and FSH-induced FSHR expression and progesterone production [76, 115]. Together, these results suggest that follicle selection is accompanied with the GC differentiation regulated by follicular cell-derived factors and initiated by obtaining FSH-responsiveness. Nonetheless, it is still unclear how GCs are maintained in an undifferentiated state and how they escape from inhibitory mechanisms at follicle selection. The consideration of these issues is required to understand follicle selection mechanisms in vertebrates.

Steroidogenesis

Steroidogenesis is the process by which cholesterol acquired from either intracellular (*de novo*) or circulating forms (LDLs) is transformed into biologically active steroid hormones (four

main families; progestagens, androgens, estrogens and corticosteroids) under the influence of multiple enzymes [116]. The process largely occurs in the various endocrine tissues including the adrenal glands, ovary and testis [116]. In ovarian follicles, steroidogenesis is mainly confined to granulosa and theca cells under the regulatory signaling of FSH and LH via AC/cAMP signaling pathway (**Figure 2.6**) [117, 118].

In chicken GCs, steroidogenic capabilities are directly associated with the transition of prehierarchal into preovulatory follicles, which leads to follicle growth, maturation, and ovulation. According to the classical three cell model in avian species, progesterone produced by GCs is used by theca interna to convert into androgen, which is subsequently metabolized to estrogen by theca externa (**Figure 2.5**) [119]. Steroidogenesis is traditionally thought to be under the influence of FSH and LH in prehierarchal and hierarchal follicles, respectively [120, 121]. Previous work has found that GCs from prehierarchal follicles are incapable of producing steroids [19, 27, 122], because GCs from prehierarchal follicles express extremely low level of P450scc enzyme (CYP11A) and STAR protein [27, 122]. Thus, theca cells in prehierarchal follicles are the main source of ovarian steroids and produce basal levels of progesterone, androstenedione and estradiol through Δ5 pathway (**Figure 2.5**) [16, 123]. In contrast to prehierarchal follicles, GCs from preovulatory follicles predominantly produce progesterone through Δ4 pathway, which is used for testosterone and androstenedione production in the theca cells. Since both granulosa and theca cells from preovulatory follicles have elevated levels of LHR expression and decreased expression of FSHR, steroidogenesis is predominantly dependent upon LH-responsiveness [17, 124]. Similar to mammals, the first rate-limiting step in steroidogenesis in the chicken ovary is the delivery of cholesterol from the outer membrane to the inner membrane of mitochondria through the action of STAR protein. Once cholesterol is translocated into the inner mitochondrial membrane, it is converted into pregnenolone by P450scc and pregnenolone is converted into progesterone by 3-β-HYDROXYSTEROID DEHYDROGENASE (3βHSD) enzyme [116].

Importantly, each of these steroidogenic factors are predominantly dependent upon cAMP signal transduction. Thus, the binding of gonadotropins to their own receptors, which belong to a family of GPCRs, activates AC activity, resulting in the production and accumulation of cAMP that initiates transcription of steroidogenic factors (*e.g*., *STAR* and *CYP11A*).

FOLLICLE STIMULATING HORMONE IN CHICKEN OVARIAN FOLLICLES

Follicle stimulating hormone (FSH)

FSH is a gonadotropic hormone synthesized and secreted by gonadotrope cells of the anterior pituitary gland. Like LH, FSH is a dimeric glycoprotein composed a common α-subunit and unique β-subunit. FSH is necessary for reproduction in vertebrates, and the requirement for FSH has been demonstrated for oocyte maturation and ovarian development using *FSH*- or *FSHR*-knockout animal models [125, 126]. As in mammals, FSH facilitates follicle development in the avian ovary by inducing steroidogenic factors [29, 30, 76]. Additionally, both follicle viability and GC differentiation at selection are thought to be primarily FSH-dependent in the chicken ovary, whereas preovulatory follicles are preferentially dependent upon LH for ovulation. Specifically, as follicles develop subsequent to selection, *FSHR* mRNA expression decreases and *LHR* mRNA expression increases [17, 19, 127].

In mammals, FSH synthesis is positively and negatively regulated by Gonadotropin Releasing Hormone (GnRH) and activins, and by inhibins and follistatin, respectively [128-130]. GnRH, a decapeptide, is secreted in a pulsatile fashion from neurons within the hypothalamus, and stimulates gonadotrope cells of the anterior pituitary to initiate transcription of FSHβ and LHβ via MAPK cascades [131, 132]. In particular, the frequency of GnRH pulses is able to synthesis and secretion of FSH and LH. Slow pulses per 2-4 hours prefer to stimulate FSH

secretion which leads to ovarian follicle development, whereas rapid and high amplitude pulses every 30 minutes is required for LH surge to induce ovulation [133-135]. Activins enhance FSHβ subunit transcription by stimulating Smad-dependent intracellular signaling, while inhibins and follistatins suppress FSH synthesis and secretion by binding to activin receptors rather than inhibiting intracellular signaling.

Although the complementary DNA sequences of the chicken gonadotropins have been characterized [136, 137], mechanisms regulating the synthesis and secretion of FSH have not been extensively studied. Unlike mammals that demonstrate a midcycle increase in circulating FSH pulse during the estrous cycle, there are no cycle-related fluctuations in plasma FSH in chickens [105, 106]. Nonetheless, recent studies have reported that gonadotropin inhibitory hormone (GnIH) from hypothalamus inhibits FSH and LH secretion and decreases *FSH* mRNA expression in the pituitary gland of adult cockerels [138, 139]. Since GnRH synthesis is promoted by melatonin synthesized in the dark, melatonin and GnIH may play a role in regulating FSH synthesis and secretion in the domestic chicken.

Follicle stimulating hormone receptor (FSHR)

The mature chicken FSHR protein is composed of 693 amino acids and has similar size and sequence homology to mammalian FSHR (678 amino acids in the human, 675 amino acids in the rat and 677 amino acids in the equine) [127, 140-142]. Therefore, the molecular weights based on the cDNA sequence are predicted to have ranges between 74 kDa and 76 kDa depending on the different species. Like other gonadotropin receptors, FSHR is also a member of GPCRs which induce signal transduction pathway by coupling G protein subunits. Unlike the other GPCRs, it is known that the FSHR has extended N-terminal extracellular domains with leucine-rich repeats which provide for ligand affinity and specificity [143].

FSHR is predominantly expressed in granulosa and theca cells in the ovary and Sertoli cells in the testis [35]. Although it is considered that expression of FSHR is specific to the gonads, recent work has demonstrated multiple localizations of FSHR expression, including the prostate, bone, and ovarian surface epithelial cells [144-146]. This suggests different physiological functions for FSH-FSHR depending on the localization. For example, the disruption of FSH signaling in mice lacking FSHR attenuates bone loss both by inhibiting bone resorption by osteoclasts and by stimulating bone formation [147, 148]. Particularly, the interaction of FSH and FSHR induces the production of estradiol and progesterone which facilitate follicular growth and the differentiation of GCs [122]. Since FSH secreted from the anterior pituitary gland exerts its biological activity by binding to FSHR, the expression of FSHR may contribute to both FSH responsiveness and target cell identity. In addition, the signaling generated by the interaction of FSH and FSHR is essential for a variety of transcriptional, metabolic, and hormonal reactions that are essential for follicular growth and oocyte maturation [149, 150]. For example, it was demonstrated that female mice with *FSHR* knocked out or mice with FSHR mutations have significantly smaller ovaries and were infertile [151].

To date, it has been demonstrated that FSH stimulates the expression of *FSHR* mRNA/protein, indicating FSH-induced cAMP regulates the level of receptor expression [152-156]. Additionally, *FSHR* transcription has been shown to be positively regulated by TGFβ superfamily members such as TGFβ1, BMP4 and BMP6 in a paracrine and/or autocrine manner and inhibited by EGFR-mediated MAPK signaling in GCs from chicken ovary [19, 29, 30, 34]. Although it was demonstrated that *FSHR* mRNA levels significantly decrease while *LHR* mRNA increases as follicles develop in the chicken ovary [103, 114], it is still unclear what factors inhibit transcription of *FSHR* at follicle selection in the hen ovary.

FSH-FSHR signal transduction

1) Protein kinase A (PKA) signaling pathway

FSH cell signaling is exclusively initiated by the interaction with FSHR, which is preferentially coupled to Gαs subunit. When FSH binds to FSHR, activated Gαs subunit stimulates membrane-associated adenylyl cyclase, resulting in the accumulation of intracellular cAMP which acts as a secondary messenger to amplify and activate the downstream signaling such as PKA [157, 158]. Subsequently, activated PKA phosphorylates transcriptional factors such as cAMP response-element binding protein (CREB)/cAMP response-element modulator (CREM), which binds to cAMP response-elements (CRE) on the promoter region [159-161]. Depending on the developmental stages or physiological situations, alternatively spliced forms of CREB and CREM are differentially expressed, and these lead to activation or inhibition of gene expression (**Figure 2.6**).

CREB in the nucleus is rapidly phosphorylated by PKA in response to FSH in GCs. Phosphorylated CREB can initiate transcription of cAMP-mediated steroidogenic factors such as STAR and CYP11A required for progesterone production [162, 163]. However, CREM is highly homologous to CREB and has same efficacy and affinity for the CRE as CREB. CREM produces the inducible cAMP early repressor (ICER) which has four CRE binding sites, indicating that ICER inhibits transcription of cAMP responsive genes by binding to CRE regions within those promoters [164]. In conclusion, FSH-induced cAMP activates PKA that leads to phosphorylation of transcriptional factors to regulate steroidogenesis.

2) Protein kinase C (PKC) signaling pathway

Although it has been well established that FSHR is preferentially coupled to Gαs subunit to induce cAMP-PKA signaling, it was demonstrated that there are alternative pathways

modulating the production of steroids through FSHR in both mammalian and avian model systems. FSH binding is also associated with the induction of intracellular calcium uptake which subsequently activates the PKC signaling pathway in Sertoli cells [167-171]. The relationship between FSH and calcium signaling has been largely investigated in spermatogenesis. It is not known if FSH stimulates PKC activation in female reproductive system.

It has been demonstrated that PKC activated by the chemical agonist, phorbol 12-myristate 13-acetate (PMA), inhibits STAR protein expression, whereas inhibition of the PKC activity using pharmacological inhibitors (*e.g.*, staurosporine, GF109203X) significantly increases gonadotropin-induced STAR protein expression and progesterone production in rat and chicken GCs [27, 28, 163]. In porcine luteal cells, PKC enhances LH-induced cAMP formation [172]. In the laying hen, it has been previously reported that PKC acts primarily as an inhibitory signaling in undifferentiated GCs, whereas it is necessary to maintain steroidogenesis in differentiated GCs [34].

Regulation of FSH-FSHR signal transduction

The regulatory mechanisms of FSH-responsiveness have been largely investigated in testicular Sertoli cells [164, 173-176]. According to those results, repeated or high doses of FSH produce less amount of cAMP by up-regulating phosphodiesterase activity and by down-regulating FSHR expression [177-179]. This strongly supports the hypothesis that FSHR is a member of GPCR family which has conventional regulatory mechanisms mediated by arrestins. Significantly, it has been shown that GRKs and arrestins can desensitize FSHR in Sertoli cells of the testis using both *in vitro* and *in vivo* models [175, 176, 180].

Despite evidence in the male reproductive system, the regulatory mechanisms of FSH-responsiveness remain unclear in the female reproductive system. However, a few studies have

shown that LHR is desensitized by adenosine diphosphate (ADP) ribosylation factors (ARFs) in mammalian GCs [181-183]. Recently, a single nucleotide or amino acid variation has been reported to influence FSHR protein expression, FSH-responsiveness, and consequently, ovarian functions, suggesting genetic FSHR polymorphisms may contribute to the regulation of FSH-induced signaling [184-187]. It has been also shown that AMH inhibits FSH-induced cAMP formation, estradiol production, and aromatase expression in human granulosa-lutein cells [188].

In the laying hen, GCs from prehierarchal follicles are steroidogenically inactive, because of extremely low to nondetectable levels of cAMP-dependent steroidogenic factors, even though *FSHR* mRNA and protein is highly expressed when compared to GCs from hierarchal follicles. Although it is known that BMPs induce AMH expression in chicken GCs, there is a research barrier to understand the mechanisms due to the lack of chicken AMHRs sequence information [29, 30]. Moreover, GnIH, a hypothalamic inhibitory peptide, has also been identified to inhibit AC activity via Gαi subunit-mediated signaling in the avian model [108, 139]. Nonetheless, the regulatory mechanisms of FSH-responsiveness have not been well studied in any female reproductive system.

VASOACTIVE INTESTINAL PEPTIDE IN CHICKEN OVARIAN FOLLICLES

Vasoactive intestinal peptide (VIP)

VIP is a neuropeptide composed of 28 amino acids that was first isolated from porcine duodenum in the early 1970s [189] and subsequently isolated from the gut of the chicken [190]. VIP protein sequence is highly conserved in vertebrates (**Table 2.1**) [191, 192]. VIP is produced in various types of tissues including the pancreas, heart, lung, hypothalamus, and suprachiasmatic nucleus (SCN), which enables VIP to have a diverse range of physiological functions including

relaxation of smooth muscle, vasodilation, and hormonal regulation [193-195]. In addition, there is growing evidence that VIP plays pivotal roles in the female reproductive system such as in the regulation of steroidogenesis and the development of follicles by activating cAMP-dependent signaling pathway [31, 38, 196]. It was reported that nerve fibers producing VIP are found in ovaries and innervates follicles at all stages of follicle development in rodents [197] and avian [37] species. VIP cDNAs were also characterized in both the chicken and turkey [198]. Furthermore, previous studies proposed that VIP-encoding mRNA was found in human, rat, and bovine ovaries [199-201]. It was demonstrated that VIPergic nerve is localized in the theca layer and adjacent to the granulosa layer in chicken ovarian follicles, the localization of VIP in the hen ovary is unclear [37]. Most research has been limited to identifying the relationship of hypothalamic VIP and PRL regulation in avian species [202-204]. According to the studies of PRL regulation, *VIP* mRNA levels are variable during the photo-induced reproduction cycle, implicating that VIP expression is regulated in a circadian fashion with the brain. To date, several circadian rhythm of VIP have been studied in mammalian systems, whereas no work has been done in birds. Therefore, I propose to study the regulatory mechanisms of VIP as related to circadian cycles, and the influence of VIP on circadian clock proteins.

VIP Receptors (VPACs)

Like VIP, VPAC1 and VPAC2 are largely tissue specific, and their distribution is widely expressed in vertebrates. For example, although both are found in cerebral cortex, suprachiasmatic nucleus (SCN), and hippocampus in the central nervous system (CNS) [205], VPAC1 is mainly localized in breast, kidney, liver, lung, prostate, and spleen [206], while VPAC2 is predominantly found in adrenal medulla, blood vessels, pancreas, smooth muscle, and thyroid [207]. Since *VPAC* cDNAs were characterized in both mammals and non-mammals [198,

208, 209], it has been well accepted that VPACs are found in the ovary from different species including rodents, human, and avians [200, 207, 210, 211]. Similar to other peripheral tissues in mammals, previous studies have found that VPAC1 is abundantly expressed more than VPAC2 in mouse ovaries [212]. In the rat preovulatory follicles, GCs express VPAC2 and theca cells exclusively express VPAC1 [213]. Although it has been identified that VIP stimulates steroidogenesis in preovulatory granulosa and theca cells [31, 37], there is no direct evidence to prove the localization, regulation and other physiological functions of VPACs in the chicken ovary. Therefore, it is necessary to establish the expression levels of VPACs to understand the neuroendocrine-mediated mechanisms in the development of chicken ovarian follicles.

VIP signaling pathway and functions in ovarian follicles

Like other agonists of GPCRs, the interaction of VIP and VPACs initiates the activation and dissociation of Gα subunits (Gαs, Gαi, and Gαq) to induce downstream signaling [38, 45, 203, 214]. With recently cloned and sequenced data, VPACs are preferentially coupling to the Gαs subunit which stimulates AC leading to the activation of cAMP and PKA signaling pathways [215]. For examples, VIP-induced cAMP promotes cell proliferation and differentiation as shown in cancer and immune cells [216-218]. A few studies have also reported that VIP activates phospholipase C (PLC) triggering inositol triphosphate (IP3)/PKC signaling pathway [202, 215, 219, 220]. However, it is currently uncertain which receptor is specific for each Gα subunit. Considering the wide distribution of VPACs, the signaling pathway might be dependent upon different physiological environments.

It has been observed that testosterone levels in VIP-deficient mice are lower compared to wild type, accompanied by reduced expression of STAR and 3βHSD in testes, suggesting VIP is important in the regulation of male reproduction [221]. In the female reproductive system, VIP-

induced cAMP stimulates FSHR and P450 aromatase (*CYP19*) expression when the ovary becomes responsive to gonadotropins in cultured rat ovaries [222] This suggests that VIP provides additional cAMP signaling to promote FSHR-mediated responsiveness during the estrous cycle in rats. VIP also stimulates STAR expression by activating PKA and PKC signaling pathway in mouse primary GCs [38]. These results indicate that VIP-VPAC system plays critical roles in steroidogenesis leading to follicle development and maturation in mammalian ovaries.

It has been demonstrated that the physiological role of VIP is involved in steroidogenesis by inducing AC/cAMP system in granulosa and theca cells from the largest preovulatory F1 follicle of the hen, suppressing apoptosis and promoting differentiation in GCs from 6-8 mm follicles [31, 37, 223]. In goose GCs, VIP maintains cell survival by up-regulating *B-cell lymphoma-2* (*BCL-2*) gene expression which has anti-apoptotic functions [40]. Additionally, VIP influences egg production and broodiness traits by regulating secretion and gene expression of prolactin (PRL) in chickens and turkeys [203, 204, 224-226]. In particular, it was demonstrated that immunization against VIP terminates incubation behavior by inhibiting PRL secretion in chickens [227]. These results demonstrate that VIP is involved not only in the process of follicle development and cell differentiation, but also in the regulation of behavioral patterns.

DESENSITIZATION OF G PROTEIN-COUPLED RECEPTORS

G protein-coupled receptors (GPCRs)

GPCRs, also known as seven-transmembrane (7-TM) receptors, represent a receptor family that transduces extracellular stimuli into intracellular signaling by coupling to guanine nucleotide binding protein (G protein) [228]. GPCRs are a large family of surface receptors and responsible for divergent processes ranging from the perception of light, smell, taste, and pain, to

the release of neurotransmitters and the regulation of hormones [229]. G proteins consist of 3 subtypes (α, β, and γ) and form heterotrimeric structures which are in an inactive state. When a signaling molecule binds to the receptor, the conformational change triggers the dissociation of Gα subunit from Gβγ dimer by replacing guanosine diphosphate (GDP) bound to the alpha subunit with guanosine triphosphate (GTP). Finally, this activated Gα subunit can generate downstream signaling by activating secondary messengers (*e.g.*, cAMP, IP3, Calcium, etc) [230, 231]. However, there are different types of Gα subunits; Gs, Gi/0, and Gq/11, which means downstream signaling pathway can be primarily influenced by each particular Gα subunit.

GPCRs that increase intracellular cAMP levels are coupled to Gαs subunit, which stimulate the enzyme AC activity. cAMP is synthesized from adenosine triphosphate (ATP) through cyclization process by removing two phosphate groups in a reaction catalyzed by the activated AC. It has been well established that most hormone-induced responses are mediated by cAMP. For examples, the synthesis and secretion of progesterone by the ovary are mediated by cAMP in response to FSH and LH [19, 29, 30, 120, 127, 143, 159, 172]. Moreover, thyroid-stimulating hormone (TSH) and adrenocorticotrophic hormone (ACTH) increase cAMP concentration and induce secretion of thyroid hormone and cortisol in thyroid gland and adrenal cortex, respectively [232-236].

Functions of $G_{i/0}$ subunits can be regulated by bacterial toxins. For example, Pertussis toxin catalyzes the ADP ribosylation of the Gα subunit, resulting in inhibiting Gα from interacting with receptors [237, 238]. As a result, the G protein maintains its GDP-bound form, which is inactive state (Gi).

Gq subunits stimulate the enzyme activity of PLC which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP3), resulting in the production of IP3 and membrane-anchored diacylglycerol (DAG) as secondary messengers. IP3 stimulates the release of calcium ions from the smooth endoplasmic reticulum, whereas DAG activates PKC with calcium ions.

Previously, it was reported that overexpressed Gq protein inhibit FSH-induced estradiol and progesterone production and mRNA level of steroidogenic factors as well in rat GCs [239]. Although this group didn't show PKC activation by overexpressed G_q, these results imply that PKC is possibly involved in the regulation of FSH responsiveness in GCs. It has been previously reported that PKC signaling inhibits differentiation of GCs from prehierarchal follicles in the hen ovary [34]. Besides coupling to G protein, GPCRs can specifically interact with βARRESTIN to terminate their signaling through receptor desensitization and subsequent internalization, both of which are initiated upon the phosphorylation of carboxy terminus domain in receptor by GPCR kinases (GRKs) [240-244].

Beta-ARRESTIN

Arrestins are a family of molecules that bind to phosphorylated intracellular C-terminal domain of GPCRs and interfere with G protein coupling. There are four different members of the arrestin family; 1) Visual arrestin (arrestin 1) in retinal rods and cones, 2) X-arrestin (arrestin 4) in retinal cones only, 3) β-arrestin 1 (arrestin 2), and 3) β-arrestin 2 (arrestin 3). In mammals, arrestin 1 and 4 are largely expressed in photoreceptors, whereas β-arrestin 1 and 2 are ubiquitously found and their sequence similarity is highly conserved across species [245]. After the complex of arrestin and GPCR is formed, arrestins can also mediate receptor internalization, leading to receptor degradation, receptor recycling, or the generation of signaling as scaffold proteins (**Figure 2.7**). During this process, the complex of arrestin and GPCR promotes the translocation of endocytosis-related components such as adaptor protein-2 (AP-2) and clathrin, N-ethylmaleimide-sensitive fusion protein (NSF), resulting in facilitating the internalization of the receptor [246-248].

In chickens, there is less information available about *βARRESTIN* sequences, although βARRESTINs were identified in divergent species including quails, frogs, and eels [249-251]. A partial sequence of *Gallus βARRESTIN1* was generated and reported to GenBank (**JX088657**) (**Figure 2.8).** *Gallus* βARRESTIN1 was found to have high similarity of nucleotide (87.5%) (**Figure 2.8**) and amino acid sequences (97%) (**Figure 3.5**) compared to that of cattle. The discovery of *βARRESTIN1* in chicken GCs provides the potential for arrestin-mediated mechanisms regulating in physiological events in the hen ovary.

GPCR kinases (GRKs)

GRKs are a family of kinases that phosphorylate intracellular loops and C-terminus of GPCRs when GPCRs are exposed to high concentrations of ligands [252]. Phosphorylation of the receptor recruits the arrestin family of proteins that inhibit signal transduction by uncoupling the Gα subunit (**Figure 2.7**). Thus, GRKs play important roles in regulating GPCR cell signaling by βARRESTINs. Seven GRKs have been identified in mammals [242, 253]. While most of the GRK isoforms are ubiquitously expressed in a variety of tissues, GRK1 and 7 are mainly expressed in the retina and GRK4 is specifically expressed in the testes [242, 253, 254]. However, GRKs have different levels of protein expression and are involved in distinct signaling pathways depending upon tissues or cell types [253]. Particularly, it has been found that GRK2 is required in heart development, because mice lacking GRK2 die at embryonic stages due to severe myocardial hypoplasia and failure of heart development [255].

Similar to other types of kinases, the activity and function of GRKs are regulated by other kinases which transfer phosphate groups [253]. PKA can phosphorylates serine residues of GRK2, which increases the binding affinity for Gβγ dimers and the recruitment of GRK2 to the plasma membrane [256]. These results demonstrate that PKA indirectly induces the desensitization of

GPCRs by phosphorylating GRK2. As shown in the case of PKA, PKC-induced phosphorylation also increases the activity of GRK2 to regulate GPCR signal transduction. It has been demonstrated that GRK2 is redistributed from the cytosol to the plasma membrane in cells treated with PMA, a PKC activator [257], suggesting that PKC may also induce receptor desensitization by regulating GRK2 activity.

Receptor desensitization

Desensitization is a well-established regulatory mechanism mediated by arrestins and GRKs [243, 244, 253, 258, 259]. Active GRKs first phosphorylate serine and/or threonine residues on internal loops and the C-terminal domain of receptors. This phosphorylation induces the translocation of βARRESTIN from cytoplasmic regions to the membrane, which uncouples Gα subunit and terminates signal transduction (**Figure 2.7**). This is different from an cAMP degradation facilitated by active phosphodiesterase and an early event after receptor activation [177, 260]. The receptor/arrestin complex is linked to several endocytotic proteins (*e.g.*, clathrin and adapter proteins) and subsequently initiates the process of receptor internalizaition into cytoplasmic region [246, 247]. This internalized receptor/arrestin complex is known to activate MAPKs (*e.g.*, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 3 (JNK3), and p38 MAPK), to undergo degradation process accompanied with ubiquitination and to be recycled for receptor re-sensitization [242, 259, 261]. Considering cell defense mechanisms, the signal termination by desensitization may be a protective mechanism of cells from overstimulation of ligands by regulating the availability and functionality of their receptors.

It has been previously shown that gonadotropin receptors can be desensitized by βARRESTINs and GRKs in mammalian cell lines [180, 262]. When Sertoli cells from the testes were incubated with FSH in a time- and concentration-dependent manner, FSH-responsive AC

was desensitized, resulting in decreasing cAMP production [36]. In the laying hen, it has been previously reported that undifferentiated GCs from prehierarchal follicles are unable to produce cAMP in response to FSH stimulation, despite of high levels of *FSHR* mRNA and protein [19]. These results suggest that there is an inhibitory mechanism in the regulation of FSHR-mediated signaling in undifferentiated GCs prior to follicle selection. However, to date there is no evidence that FSHR-mediated signaling is regulated by a βARRESTIN-mediated mechanism in ovarian follicles from any vertebrate. Therefore, the initial study in this dissertation was performed to identify whether FSHR-mediated signaling is regulated by a βARRESTIN-mediated event and if this mechanism serves to initiate follicle growth and differentiation beginning at the time of follicle selection.

CIRCADIAN RHYTHMS

Overview of circadian rhythms

As a consequence of daily variations of environmental circumstances, such as the cycle of light and dark, most animals adapt their physiological and behavioral activities during the day and relative to season. A circadian rhythm refers to the physiological process depending on the approximate 24 hr cycle of light and dark in most living organisms from cyanobacteria to humans [263, 264]. This rhythm regulates many biological behaviors and physiological processes, including sleep/wake, feeding, body temperature, hormone secretion, and other metabolisms [264-268]. Typically, light is a representative source to synchronize oscillators and trigger biological rhythms by activating the SCN, a pair of distinct regions located in the anterior hypothalamus of the brain [43, 44, 269]. The SCN consists of 20,000 neurons and can receive photic input through retino-hypothalamic tract and transfer this photic information for the

entrainment of circadian rhythm as an output. Previous studies reported the requirement of SCN to regulate circadian rhythm by demonstrating that transplantation of SCN grafts into SCN-ablated animals can restore physiological and behavioral rhythmicity [266, 269]. In mammals, a dominant circadian rhythm is concentrated within the SCN via the retina of the eyes [268, 270]. In addition to the SCN, circadian oscillators are found in a variety of peripheral tissues [271]. The avian circadian system is more complex than in mammals, because the pineal gland and retina can also produce circadian rhythmicity [270, 272-276]. Similar to mammals, a variety of peripheral tissues contain the circadian oscillators in avian species as well, and their circadian rhythm is regulated not by light source, but by humoral factors such as hormones and glucose [270, 274, 277-281].

Clock genes

Circadian clock genes interact with each other to generate a feedback loop and regulate gene expression related to physiological processes. This mechanism is highly conserved across species and regulated by clock genes (**Figure 2.9**). Two basic helix-loop-helix (bHLH) transcriptional activator, BMAL and CLOCK form heterodimers in the cytoplasm and subsequently enter the nucleus to initiate the transcription of additional clock genes (*PER* and *CRY*) following binding to conserved E-box sequences (*e.g.*, -CACGTG- in chicken) within the promoter regions of *PER* and *CRY* [282]. When the concentration of PER and CRY is increased, they form heterodimers in the cytoplasm. Subsequently, the complex of PER and CRY proteins translocates back to the nucleus and inhibits the transcription of *CLOCK/BMAL* [54, 282, 283]. It is known that this feedback loop is regulated by post-translational modification such as phosphorylation. For example, casein kinase ε (CKε) phosphorylates PER protein to induce its degradation via ubiquitin-mediated proteolysis [284-286].

As mentioned above, the circadian system in birds is known to be more complex than that of mammals, because birds can perceive photic information through three extraocular photoreceptors in functionally and anatomically distinct areas; 1) the retina of the eyes [274, 287, 288], 2) the SCN [289, 290], and 3) the pineal gland which synthesizes and secretes melatonin [274, 291, 292] The photosensitive clock cells in those three areas have opsin-type photoreceptive molecules [276, 293, 294] that initiate G protein-mediated signal transduction pathways [275, 295]. In birds, two different structures have been associated with SCN function: the medial SCN (mSCN) and the visual SCN (vSCN) [296, 297]. However, they have different functions. Since the vSCN exclusively expresses melatonin receptor, melatonin functions regulating metabolic activity are shown in the vSCN, but not in the mSCN [273, 298]. By comparison, the rhythmicity of clock genes is found at the mSCN only in Japanese quail [299]. In addition, the function and the significance of the pineal gland has been extensively investigated in chickens, because the chicken pinealocyte contains its own circadian oscillator which regulates photic-input and melatonin-output pathway [276, 279, 300]. So far, orthologous genes from the mammal clock genes have been identified in chicken; chicken *PER2 (cPER2), cPER3, cBMAL1*, *cBMAL2, cCRY1, cCRY2,* and *cCLOCK* [301-304]. A *PER1* gene has not yet been identified in birds [305]. The sequence similarity of clock proteins found in chickens was compared to mammalian species and shown in **Table 2.1**. In both mammal and avian species, the SCN organizes the circadian rhythm of multiple organs by synchronizing peripheral clock systems [268, 306, 307]. However, it is still unknown how multiple pacemakers in the brain lead tissue specific autonomous rhythms to coordinate peripheral circadian rhythms. Moreover, peripheral circadian rhythms have not been investigated in birds as extensively as in mammals, although bird behavior and cognitive skills are controlled by biological clock genes. The present studies attempted to investigate the relationship of circadian clock genes to the process of GC differentiation in the chicken ovary.

Functions of clock genes in ovarian follicles

Recent studies demonstrated that autonomous rhythms exist in ovaries, and clock genes are expressed in ovarian follicular cells to influence physiological functions [48, 308-310]. The disruption of circadian rhythm causes not only a wide range of physiological problems such as aberrations in locomotive activity, feeding, and sleeping, but also reproductive problems including irregular estrous cycle and infertility [57, 265, 306, 307]. In particular, the importance of clock genes was described in both male and female reproductive system using transgenic animal models [53, 56, 265, 311]. *PER1/2* knockout mice show reduced fertility [51] and *CLOCK*-mutant animals have irregular estrous cycles because of abnormal rhythms of vasopressin expression and reduced receptor in SCN neurons and target regions in the hypothalamus, respectively [52, 53, 312]. In addition, reduced expression of STAR protein has been observed in *BMAL1* knockout female mice, resulting in reduced progesterone production and abnormal steroidogenesis from luteal cells [55]. In the avian system, several experiments demonstrated that the cycle of oviposition and ovulation in the hen and Japanese quail is regulated by a central and ovulatory clock entrained to the daily light and dark cycle [47, 313] which results in eggs typically being laid in the early morning for chickens and middle of the afternoon for quail [47, 48, 278, 314].

Although gene knock-out studies demonstrated the essential roles of clock genes in female reproductive system, it is not clearly defined how clock gene expressions are synchronized or what specific factors regulate the circadian rhythm in the ovary. It has been documented that neuroendocrine factors regulate steroidogenesis via the regulation of clock gene expression. For example, VIP deficient mice show reduced rhythmic expressions of clock genes in the SCN and peripheral organs [44, 315, 316]. In addition, mice lacking VPAC2 displayed a loss of clock gene rhythms in adrenal gland and accompanied no rhythmicity of *STAR* mRNA expression [317].

These knockout studies strongly suggest that VIP-mediated signaling is necessary to maintain clock gene rhythms that might affect steroidogenesis. Therefore, the present studies were conducted to evaluate whether VIP, a neuroendocrine factor, regulates the expression of clock genes related to follicle development in the hen ovary.

ANGIOGENESIS IN THE CHICKEN OVARY

Overview of angiogenesis

Angiogenesis is the process of blood vessel formation from pre-existing blood vessels and is essential for normal tissue growth and development [318]. This is different from vasculogenesis, which is the *de novo* formation from endothelial precursor cells (angioblasts) [319-321]. Since blood vessels are part of the circulation system to transport blood including oxygen throughout the body, deliver growth factors and nutrients, and remove wastes from cells, angiogenesis is necessary in normal physiological mechanisms. The angiogenic process is regulated by proangiogenic and antiangiogenic factors, which indicates that angiogenesis is controlled by "angiogenic balance" between stimulatory and inhibitory signals for blood vessel formation [318]. In normal physiological environments, angiogenesis plays critical roles in tissue growth and repair [322, 323], suggesting that inappropriate blood vessel formation is related to various pathological symptoms including tumor growth, psoriasis, diabetic retinopathy, and rheumatoid arthritis [324-327].

Angiogenesis in the development of chicken ovarian follicles

It is well established that angiogenesis plays critical roles in the female reproductive system of mammals including the formation of corpus luteum, ovulation, and menstruation [62]. However, most research has been largely studied with regard to ovarian cancer in the chicken model [328, 329], primarily because it is well emphasized that angiogenesis is a process required to initiate tumor metastasis [318, 320]. Although there is no evidence for the role and function of angiogenesis in the hen ovary, it was preliminarily studied that in 1987, Japanese group found that acid mucopolysaccharide, one of fractionized factors from hen ovarian extracts, showed neovascularization [330]. Additionally, components of the basal lamina in chicken ovarian follicles were analyzed and it was determined that this layer contains not only different types of growth factors, but also angiogenic factors such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [95, 331]. In chicken ovarian follicles, it was observed that there is an unequal formation of a number of blood vessels on the surface of preovulatory follicles and prehierarchal follicles (**Figure 2.10**). Given that angiogenesis is a critical process that maintains and supports the tissue development, these preliminary results suggest that angiogenesis regulated by angiogenic factors from within ovarian follicles is associated with the differentiation and proliferation of ovarian follicular cells.

Vascular endothelial growth factor (VEGF) and Receptors

VEGF is a potent stimulator of angiogenesis by inducing the permeability [332, 333], proliferation [334, 335], and migration of endothelial cells (ECs) [336, 337]. So far, the VEGF family has been identified to have at least six members (VEGF-A, -B, -C, -D, -E and -F) [338, 339]. In particular, VEGF-A has been widely investigated with regards to follicle development in

mammals and generates four different isoforms by the alternative mRNA splicing (VEGF121, 165, 189, and 206) [340, 341]. It was demonstrated that the expression of mRNA and protein of VEGF is found in ovarian follicular cells (*e.g.*, granulosa and theca cells) and levels increase depending upon the stage of ovarian follicle development in mammals [342, 343]. By comparison, the effects of VEGF have been undefined in the avian reproductive system, except for a few studies related to ovarian cancer [344-346].

There are three major types of VEGFRs identified as VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4) [347, 348]. All have tyrosine kinase activity which can transfer phosphate groups to targeted proteins for initiating signal transduction and regulating cellular functions [347-349]. Each receptor has a different affinity for the VEGF isoforms, resulting in the activation of various cellular signaling. For examples, VEGFR1 expressed on ECs and monocytes regulates cell migration and most of isoforms are not membrane bound forms [350]. The vascular permeability induced by the proliferation and migration of ECs is preferentially mediated though VEGFR2 [351, 352]. Lastly, VEGFR3 is proposed to control lymphangiogenesis [353, 354]. It was identified that the *Gallus VEGFR1* gene sequence is highly conserved compared to mammals [355] while VEGFR2 was identified in chorioallantoic membrane, heart, and the lens of the eye in chickens [356-358]. However, VEGFR3 has not been well studied in chickens.

Angiopoietins (ANGPTs) and Receptors

ANGPTs are approximately 70 kDa glycoproteins involved in the development and stability of blood vessels [359, 360]. There are four identified ANGPTs (ANGPT-1, -2, -3 and -4) and most studies have demonstrated that ANGPT-1 and -2 plays critical roles in the migration and sprouting of ECs and the remodeling of the blood vessel wall [73, 361, 362]. It is known that

ANGPTs form multimers to exert their functions. For instance, multimeric forms of ANGPT-1 is critical to phosphorylate TIE-2 receptor [360, 363].

ANGPTs have their own receptors, TIE-1 and -2, both of which are receptor tyrosine kinases [364, 365]. Since TIE-1 is an orphan receptor with unidentified ligands, most studies have largely focused on TIE-2 [366]. While all ANGPTs bind to TIE-2 receptor, they function as either an agonist (ANGPT-1 and -4) or an antagonist (ANGPT-2 and -3) of each other [74, 361, 367, 368] (**Figure 2.10**). In chickens, ANGPT-1 and -2 are identified in the endometrium on the chorioallantoic membrane [369] and testes [370], but TIE-1/-2 are unidentified. Furthermore, the role of both ANGPTs and TIEs is completely unclear in the hen ovary.

ANGPT-1 is mainly expressed in perivascular cells surrounding blood vessels, such as pericytes and vascular smooth muscle cells [367, 371, 372] and binds to TIE-2 receptor that are subsequently activated by auto-phosphorylation within intracellular domains of the receptor [373, 374]. The interaction of ANGPT-1 and TIE-2 stimulates the phosphainositide3-kinase (PI3K)/protein kinase B (PKB/AKT) and MAPK pathways which promote EC survival and nitric oxide synthesis [373, 374]. *ANGPT-1* deficient mice have severe abnormalities in vascular formation, showing disrupted interactions between ECs and peri-ECs and causing embryonic death [361, 367]. In addition, ANGPTs can bind integrins that allow ANGPTs to stimulate signaling within non-ECs that lack TIE-2 expression, such as cardiac and skeletal myocytes [375, 376]. By comparison, ANGPT-2 is primarily produced in ECs and has low agonist activity for TIE-2 and acts as a natural antagonist by inhibiting the binding of ANGPT-1 to TIE-2 and inducing weak phosphorylation [377, 378]. Therefore, the ratio of ANGPT-1/-2 and the oligomeric state may be important for regulating the process of angiogenesis.

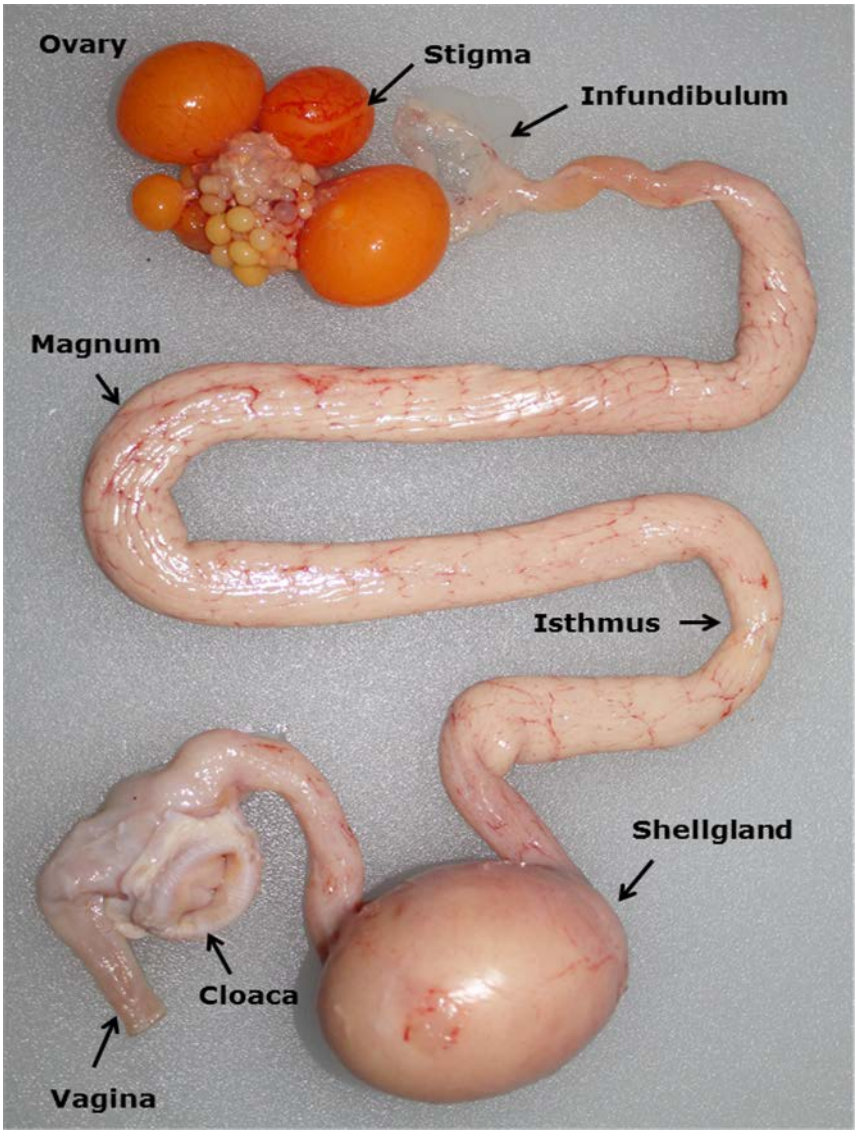


Figure 2.1. The female reproductive system of the chicken.

The female reproductive system of the chicken is divided into two separate parts: 1) ovary and 2) oviduct. In most species of birds, only the left ovary and oviduct are matured and functional. The ovary is a cluster of developing follicles. The oviduct is a long tube divided into five major parts; infundibulum, magnum, isthmus, uterus, and vagina. The stigma is a nonvascularized surface of ovarian follicle where final ovulation occurs.

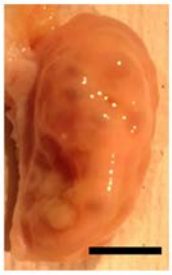
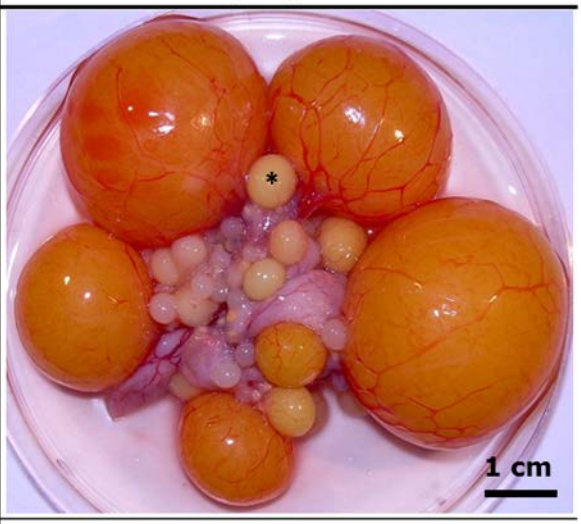
	Mouse	Cattle	Laying hen
Ovary	 0.3 cm		 1 cm
Ovulation Cycles	4 days	21 days	***1 day (~27 hours)***
Ovary Length	2-4 mm	30 mm	**~ 80 mm**
Follicle Size	<0.5 mm	<20 mm	**40-50 mm**
# of Granulosa Cells	~0.1x10^6	~0.5x10^6	***~1x10^6 in 6-8 mm follicle****

Figure 2.2. Ovarian follicles in the hen as a model system.

Compared to mice (up to 0.4 cm) and cattle (up to 3 cm), follicle size is the largest in chickens (up to 4 cm). The number of GCs in a 6-8 mm (prehierarchal) follicle is approximately 1 x 10^6. Importantly, ovulation cycles of chickens are approximately 1 per day, which is considerably shorter than mice and cattle. Importantly, all different stages of follicle development, from primordial to preovulatory phase, are found within the chicken ovary at the same time.

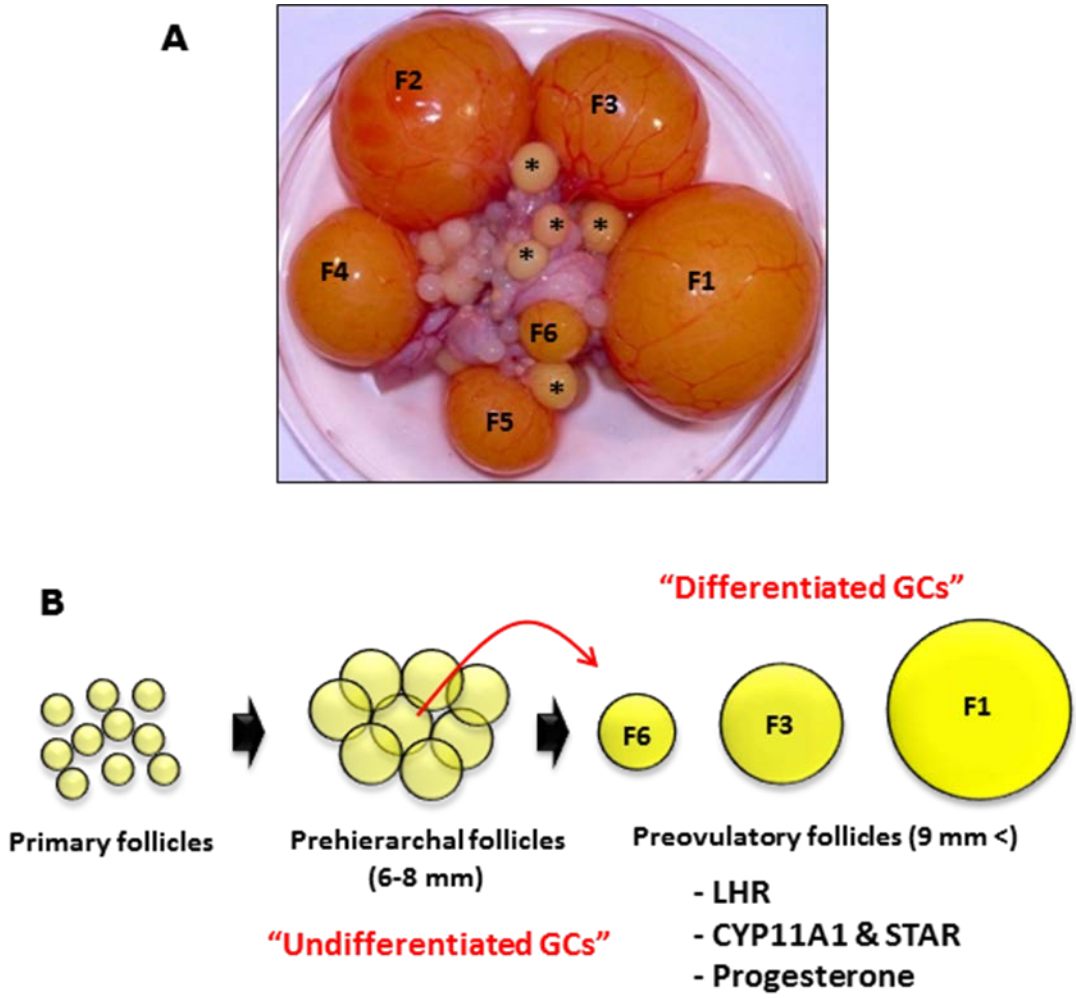


Figure 2.3. Ovarian follicle selection in the hen.

A. F1-F5 represent preovulatory follicles in which GCs are actively differentiating or have terminally differentiated; F6 represents the most recently selected follicle with a 9-12 mm diameter, which contains actively differentiating GCs, * follicles are prehierarchal follicles (6-8 mm) which have not yet been selected and contain undifferentiated GCs. **B**. In the laying hen, one prehierarchal follicle is selected per day then rapidly grows in preparation for final ovulation. Only following follicle selection are steroidogenic factors (*e.g.*, CYP11A and STAR) and LHR expressed at functional levels.

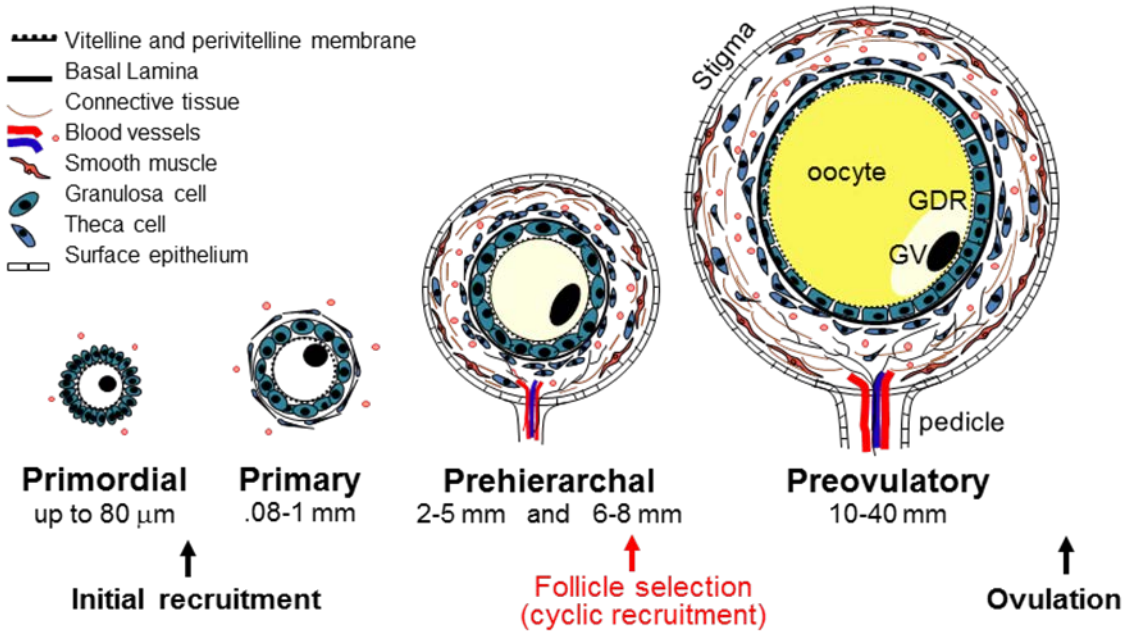


Figure 2.4. Structure and development of ovarian follicles in the hen.

There are 4 major stages during follicle development in chickens. Primordial follicles represent the early growth stage and are up to 80 µm in diameter. Primary oocytes are surrounded by a vitelline membrane and presumptive granulosa cells. The perivitelline membrane is subsequently formed by GCs. Primary follicles (0.08-1 mm) include the recruitment of theca cells from mesenchymal cells and have initiated the slow growth phase. Prehierarchal follicles (2-5 and 6-8 mm) initiate the accumulation of white yolk and the differentiation of the theca into interna and externa layers. Notably, the vascular and nervous systems are found surrounding and within the theca layer, and a single follicle selection occurs from the 6-8 mm stage of development. Once a follicle is selected, follicles rapidly grow from 9 to 40 mm) due to the uptake of significant amounts of yellow yolk. Eventually, the largest preovulatory follicle is ovulated with the germ cell released at the site of the avascular stigma ([14]; Johnson, A.L. and D.C. Woods (2007) Chapter 6. Ovarian dynamics and follicle development. In: Reproductive Biology and Phylogeny of Aves, B.G.M. Jamieson, Ed., Science Publishers, Inc., pp. 243-277).

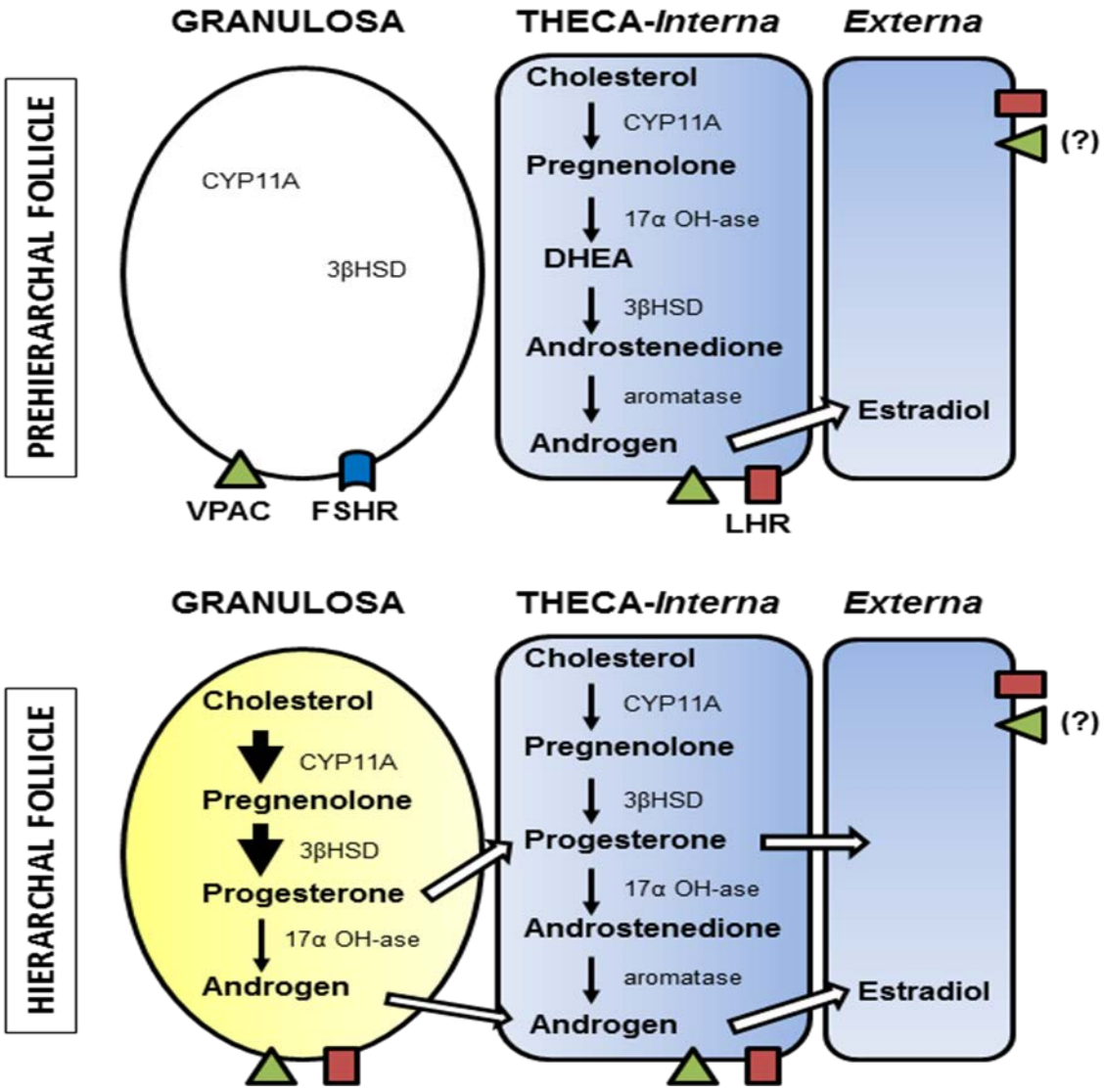


Figure 2.5. Steroidogenesis in granulosa and theca cells from ovarian follicles in the hen.

While a three-cell model of steroidogenesis is proposed for hierarchal follicles, GCs in prehierarchal follicles are unable to produce steroid due to negligible levels of steroidogenic factors, this despite of VPAC and FSHR expression. However, the theca layer of prehierarchal follicles is steroidogenically functional. Thus, the theca layer represents the primary tissue for steroidogenesis in prehierarchal follicles [13].

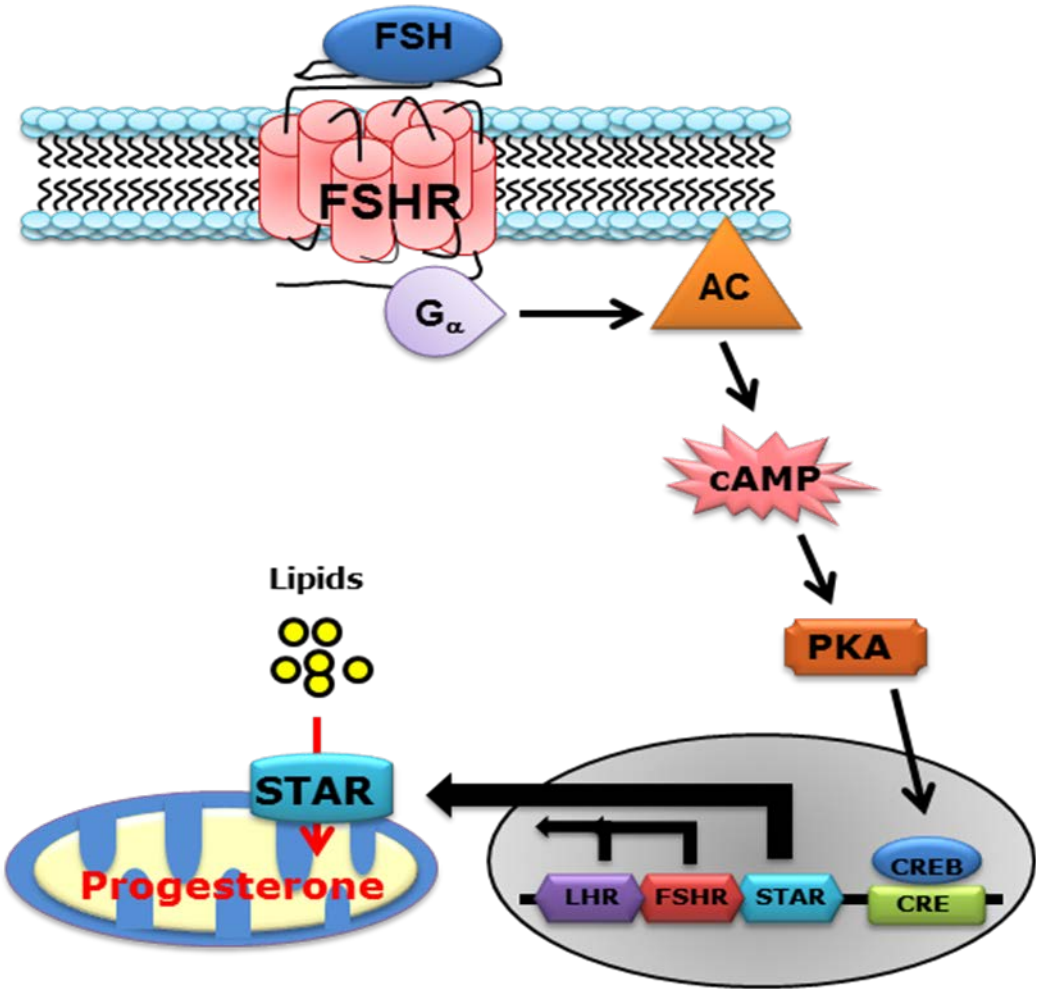


Figure 2.6. FSH-FSHR signaling in hen granulosa cells.

In differentiated GCs from selected follicles, FSH-activated Gα subunit stimulates adenylyl cyclase activity that leads to intracellular cAMP formation. Following cAMP formation, activated PKA induces translocation of CREB from the cytoplasm to nucleus that, in turn, initiates the transcription of steroidogenic factors and LHR. STAR, a rate-limiting factor, is a transporter protein which deliver cholesterol into mitochondria where cholesterol is transformed into pregnenolone and finally progesterone [157].

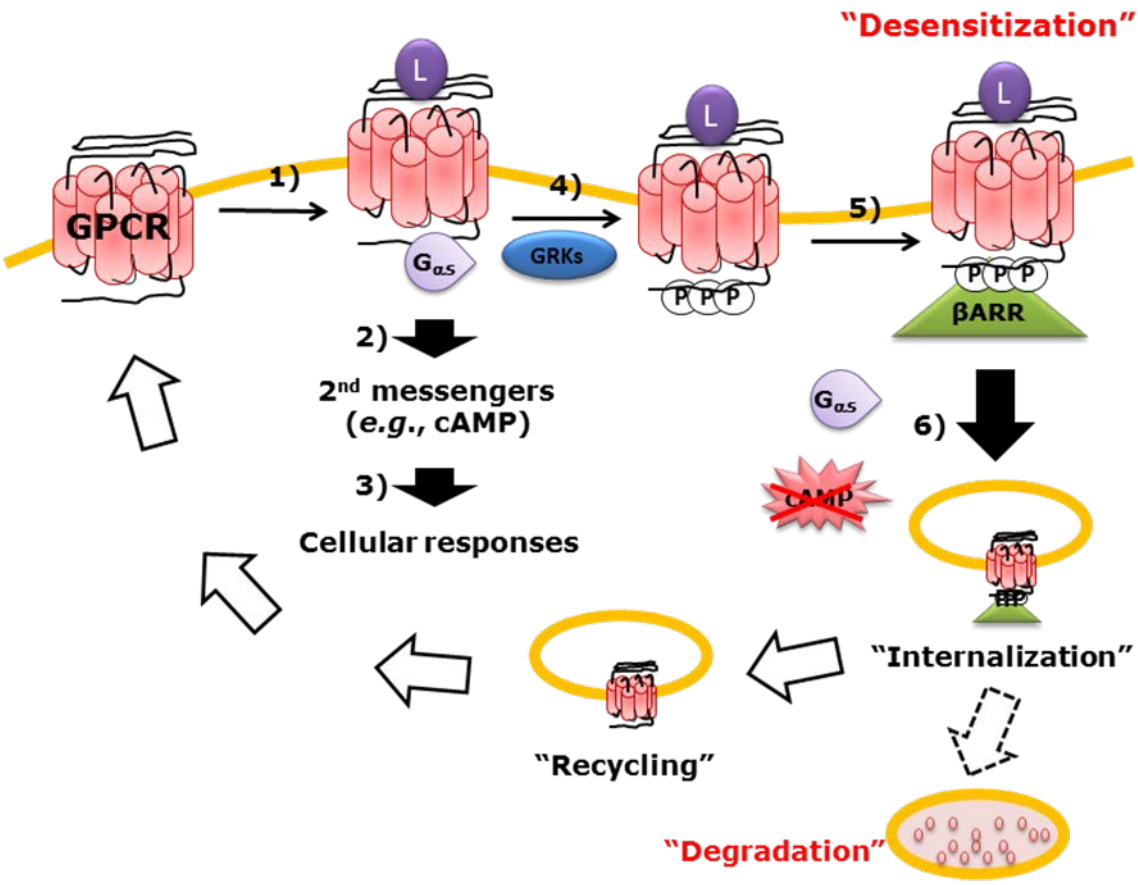


Figure 2.7. Conventional G protein-coupled receptor (GPCR) signaling mechanisms.

Binding of an agonist (L) (1) to a GPCR activates G protein ($G_{\alpha s}$) that in turn stimulates adenylyl cyclase to produce cAMP (2) and induce cellular responses (3). High concentrations or continuous exposure of agonists can lead to conformational changes of GPCRs and subsequently the phosphorylation (P) of C-terminal domain by GRK-2 (4) that results in the recruitment of βARRESTIN (βARR) (5) and uncoupling of the $G_{\alpha s}$ subunit. Finally GPCRs become desensitized and no longer initiate cell signaling. Desensitized GPCRs are internalized by clathrin-mediated endocytosis (6). Once internalized, receptors are recycled (solid arrow) back to the membrane surface or degraded by lysosomes (dashed arrow) (adapted from [228]).

```
Gallus gallus     2  GGACCTGAGGACACAGGGAAGGCCTGCGGTGTGGACTATGAGGTCAAAGCTTTCTGCGCG   61
Bos taurus      449  GGACCTGAAGATACAGGGAAGGCCTGCGGTGTGGACTACGAAGTGAAAGCCTTCTGTGCG  508

Gallus gallus    62  GAGAACCTGGAGGAGAAAATCCATAAGAGGAACTCTGTTCGCTTGGTCATCCGCAAGGTG  121
Bos taurus      509  GAGAACCTGGAGGAGAAGATCCACAAGCGGAATTCTGTGCGCCTGGTCATCCGGAAGGTT  568

Gallus Gallus   122  CAGTACGCCCCGGAGCGCCCCGGCCCCCAACCCATGGCAGAGACCACCAGGCAGTTCCTC  181
Bos taurus      569  CAGTATGCCCCAGAGAGGCCTGGCCCCCAGCCCACGGCCGAGACCACCAGGCAGTTCCTC  628

Gallus gallus   182  ATGTCGGACAAACCGC-TGCACCTGGAGGCGTCCCTGGACAAAGAGATCTACTACCACGG  240
Bos taurus      629  ATGTCAGACAAGCC-CTTGCATCTGGAGGCCTCCCTGGACAAGGAGATCTACTACCACGG  687

Gallus gallus   241  GGAACCCATCAGCGTCAACGTCCACGTCACCAACAACACCAACAAGACGGTGAAGAAGAT  300
Bos taurus      688  AGAACCCATCAGTGTCAACGTCCATGTCACCAACAACACCAACAAGACGGTGAAGAAGAT  747

Gallus gallus   301  CAAGATCTCAGTGCGCCAATACGCCGACATCTGCCTCTTCAACACGGCCCAGTACAAGTG  360
Bos taurus      748  CAAGATCTCGGTGCGCCAGTATGCAGACATCTGTCTGTTCAACACAGCCCAGTACAAGTG  807

Gallus gallus   361  TCCC-GTGGCTGTGGAGGACGCCGATGACATGGTGGCTCCGAGCTCAACGTTCTGCAAAG  419
Bos taurus      808  -CCCTGTGGCCATGGAAGAGGCTGATGACACAGTGGCACCCAGCTCTACGTTCTGCAAGG  866

Gallus gallus   420  TCTACACCCTGACCCCCTTCCTTGCCAACAACCGGGAGAAACGCGGG  466
Bos taurus      867  TCTACACGCTGACCCCCTTCCTGGCCAACAATCGAGAGAAGCG-GGG  912
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Figure 2.8. Partial cDNA sequences of *βARRESTIN1* in *Gallus gallus*

Partial cDNA sequence of *Gallus βARRESTIN1* was cloned and sequenced and this sequence has been deposited in GenBank (Accession#: JX088657.1). The sequence homology of *Gallus βARRESTIN1* is 87.5% compared to *Bos taurus*. Letters in red define mismatched sequences between *Gallus gallus* and *Bos taurus*.

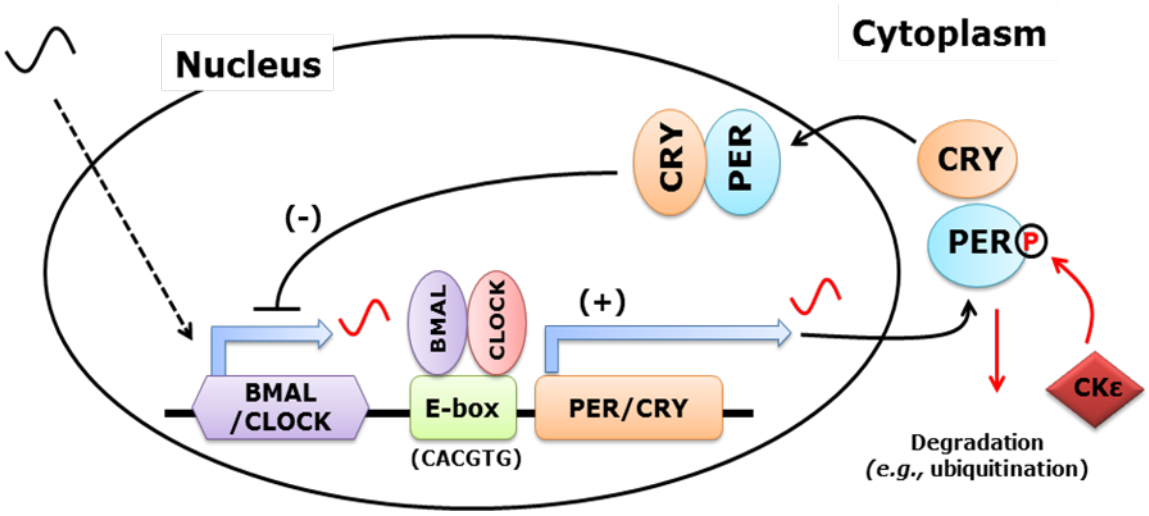

Figure 2.9. Regulatory mechanisms of clock genes.

Two transcriptional activators, BMAL and CLOCK, form heterodimers in the cytoplasm and enter the nucleus to bind E-box sequences (*e.g.*, CACGTG) in the promoter regions of PER and CRY genes leading to initiate their expression (positive feedback). PER and CRY form a dimer in the cytoplasm and translocate to the nucleus to inhibit the transcription of BMAL and CLOCK (negative feedback). Especially, casein kinase ε (CKε) phosphorylates PER protein for degradation (red arrow), resulting in resetting the circadian cycle by reducing levels of PER/CRY heterodimers.

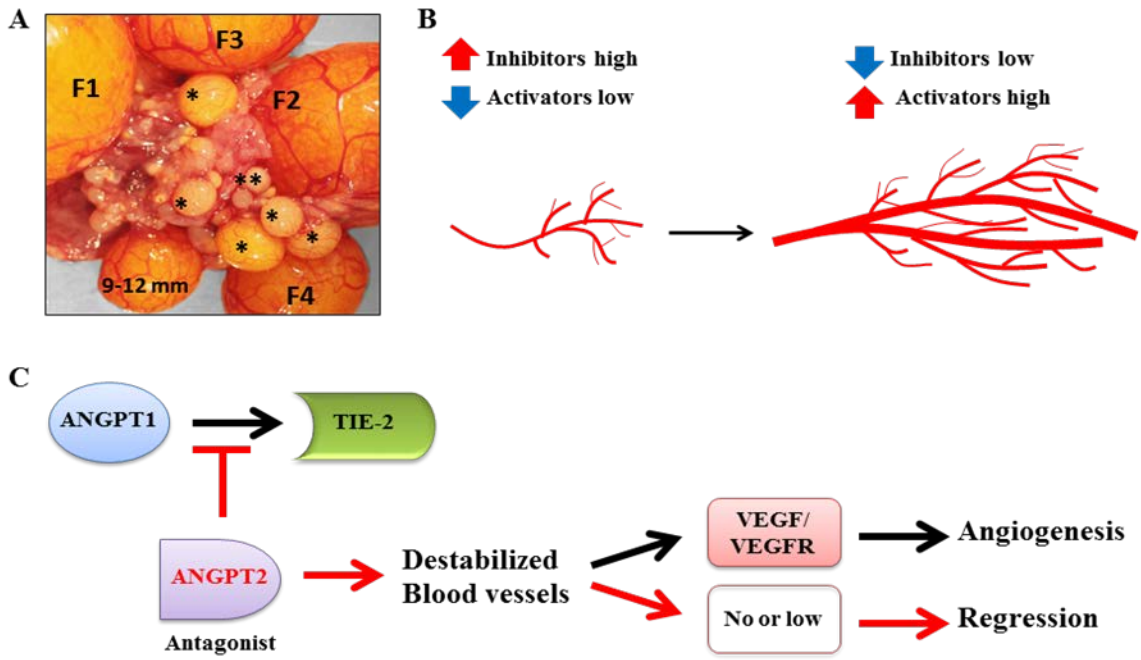

Figure 2.10. Angiogenesis and distribution of vasculature on the surface of ovarian follicles in the hen.

A. Blood vessels found during follicle development in the chicken ovary. While there are a limited number of blood vessels found in 6-8 mm follicles prior to follicle selection, a significant increase of blood vessels occurs in preovulatory follicles. B. Process of angiogenesis in normal tissues. Angiogenesis is the process of new blood vessel formation from the pre-existing vasculature and is regulated by the balance between activators and inhibitors. C. Mechanisms of angiopoietins and VEGF during angiogenesis. ANGPT1 binds to TIE-2 receptor and plays a role in maintaining the structure of blood vessels. However, ANGPT-2 as an antagonist inhibits the binding of ANGPT-1 to TIE-2 receptor. This results in separating mural cells (*e.g.*, pericytes and smooth muscle cells) from endothelial cells and inducing destabilized blood vessels. ANGPT-mediated signaling is dependent on VEGF signaling to initiate angiogenesis. Therefore, together with VEGF signaling, the balance between ANGPT-1 and -2 regulates angiogenesis.

Table 2.1. Sequence homology of *Gallus* proteins presented compared to mammalian species.

Protein	Description	Accession #	Homology to *Gallus* (%)		
			Human	Bovine	Mouse
ANGPT1	Angiopoietin 1	NP_001186376.1	92	91	90
ANGPT2	Angiopoietin 2	NP_990148.1	86	85	83
βARRESTIN1	Arrestin, Beta 1 (partial)	AFN89792.1	97	97	*N.A.*
BMAL1	Brain and muscle ARNT-like1	NP_001001463.1	93	92	92
BMP4	Bone morphogenetic protein 4	NP_990568.3	84	83	84
CLOCK	Clock Circadian Regulator	NP_989505.2	83	83	82
P450scc	Cytochrome P450 Cholesterol side chain cleavage enzyme	NP_001001756.1	54	52	54
FSHB	Follicle stimulating hormone beta	AAL99279.1	68	68	67
FSHR	FSH receptor	NP_990410.1	74	75	72
GRK2	G-protein-coupled receptor kinase 2	NP_001026524.1	92	92	92
Id2	Inhibitor of DNA binding 2	NP_990333.1	94	93	93
LH	Luteinizing hormone	Not found			
LHR	LH receptor	NP_990267.1	71	73	69
PER2	Period circadian clock2	NP_989593.1	58	54	57
STAR	Steroidogenic acute regulator	NP_990017.1	72	69	71
TGFβ	Transforming growth factor beta	Not found			
TIE-2	TEK tyrosine kinase	XP_424944.3	74	74	73
VEGF-A	Vascular endothelial growth factor A	NP_990373.1	75	67	77
VEGFR1	VEGF receptor1	NP_989583.1	69	66	65
VEGFR2	VEGF receptor2	NP_001004368.1	73	73	72
VIP	Vasoactive intestinal peptide	NP_990697.2	60	55	55
VPAC1	Vasoactive intestinal peptide receptor 1	NP_001090992.1	66	67	67
VPAC2	Vasoactive intestinal peptide receptor 2	NP_001014970.1	73	73	72

N.A. : Not available

Chapter 3

FSH Receptor-Responsiveness in Undifferentiated Hen Granulosa Cells is Precluded by a βARRESTIN-Mediated Mechanism

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Key Words: Granulosa cells, FSH receptor, βARRESTIN, G Protein-Coupled Receptor, hen

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ABSTRACT

In the ovary of the domestic hen, the selection of a single follicle each ovulatory cycle occurs from a small cohort of prehierarchal follicles measuring 6-8 mm in diameter. The present studies investigate cellular events that: 1) maintain granulosa cells (GCs) from prehierarchal follicles in an undifferentiated state prior to selection; and 2) subsequently initiate GC differentiation at follicle selection. Specifically, we focus on the regulation of cell signaling via the FSH receptor (FSHR), a G-Protein Coupled Receptor, by a βARRESTIN-mediated mechanism. Despite the finding that undifferentiated GCs express the highest levels of *FSHR* mRNA and membrane-localized protein during follicle development, freshly collected cells from prehierarchal follicles fail to initiate cAMP signaling following a 1 h challenge with FSH. Consequently, these cells remain in an undifferentiated state and demonstrate virtually no capacity for steroidogenesis due to the absence of cAMP-dependent STAR protein and *CYP11A1* gene expression. Knockdown of *βARRESTIN1* mRNA in cultured, undifferentiated GCs using small interfering RNA (siRNA) facilitated FSH-induced cAMP, STAR expression and progesterone production. Furthermore, over-expression of bovine βARRESTIN1 and G PROTEIN-COUPLED RECEPTOR KINASE 2 proteins in actively differentiating GCs significantly decreased cAMP accumulation and progesterone production following a challenge with FSH. These findings indicate that a βARRESTIN-mediated mechanism is both sufficient and necessary to maintain FSHR desensitization in undifferentiated GCs from prehierarchal follicles. We conclude that prior to follicle selection FSHR desensitization within the GC layer of prehierarchal follicles prevents premature GC differentiation and propose that this desensitization is maintained, at least in part, by MAPK signaling.

INTRODUCTION

In the ovary of the domestic hen, the selection of a single follicle for final growth and differentiation prior to ovulation occurs on a near-daily basis from a small cohort of undifferentiated, 6-8 mm (prehierarchal) follicles. Importantly, the maintenance of this prehierarchal cohort in a viable, undifferentiated state is required in order to insure the production of one ovulation each day for the duration of a laying sequence. Prior to follicle selection freshly collected granulosa cells (GCs) from such follicles fail to produce cAMP when challenged with FSH [122], this despite expression of the highest levels of FSH receptor (*FSHR*) mRNA observed during follicle development [19, 127]. As a consequence, these cells express near-undetectable levels of the cAMP-dependent genes encoding the cholesterol side-chain cleavage enzyme (CYP11A), Steroidogenic Acute Regulatory Protein (STAR), as well as the LH receptor (LHR) [17, 27, 379]. Accordingly, at this stage of development these GCs are considered steroidogenically incompetent and undifferentiated. At follicle selection GCs acquire FSHR-responsiveness and become competent to initiate FSH-induced cAMP formation, progesterone production and LHR expression [27, 30]. Subsequent to selection, these preovulatory follicles rapidly grow due to increased yolk incorporation and produce increasingly greater amounts of progesterone production in preparation for ovulation [23, 380].

To date, research has focused on factors that influence *FSHR* mRNA expression in GCs from prehierarchal follicles [19, 32, 34, 76, 381]. For instance, several growth factors from the Transforming Growth Factor beta (TGFβ) family, including TGFβ1, Activin A and Bone Morphogenetic Protein (BMP)-4 and -6 [19, 29, 30, 32], have been reported to promote and maintain *FSHR* mRNA expression, plus initiate FSH-responsiveness in GCs after culture for 18 h or longer [19, 29, 30, 115, 382]. Without growth factor support, levels of *FSHR* mRNA expression in undifferentiated GCs rapidly and progressively decrease when cultured in media containing 2.5% fetal bovine serum but without such growth factors [19]. On the other hand,

enhanced Mitogen-Activated Protein Kinase (MAPK) signaling induced by Epidermal Growth Factor Receptor Ligands (EGFRLs) blocks TGFβ- and BMP-induced *FSHR* mRNA expression and prevents the initiation of GC differentiation in cultured cells [28, 34]. We have previously proposed that the initiation of GC differentiation in the single selected follicle results from a reduction of MAPK signaling [79]. Nevertheless, cellular mechanisms regulating FSH-responsiveness surrounding the time of follicle selection have yet to be characterized.

The FSHR is a member of the G Protein-Coupled Receptor (GPCR) family that signals, at least in part, via the stimulatory G-protein subunit, $G\alpha_s$, to activate adenylyl cyclase and increase cAMP production [177-179]. In turn, cAMP acts as a second messenger to activate protein kinase A (PKA) and affect gene transcription. In hen granulosa and theca cells this canonical signaling pathway is required to initiate the transcription of *STAR*, *CYP11A*, the aromatase enzyme (*CYP19*) and *LHR* [16, 30, 37, 120, 379]. Significantly, desensitization and internalization of the FSHR via a βARRESTIN-mediated event has been documented in rat Sertoli cells [164, 173, 175, 176]. Typically, GPCR desensitization is initiated as a mechanism to modulate signal transduction following exposure to its ligand for a prolonged time and/or in an excessively high dose [240, 383]. A prerequisite for GPCR desensitization is the phosphorylation of the intracellular (C-terminal) domain by a G Protein-Coupled Receptor Kinase (GRK) [243, 244, 253, 258, 259]. GRKs are serine/threonine protein kinases that preferentially recognize and phosphorylate activated GPCRs. Alternatively, GRK activity can be regulated independent of an activated GRCR by a phosphorylation event induced by protein kinase C (PKC) or c-Src tyrosine kinase [384-388]. Either of these signaling pathways can be activated downstream of Epidermal Growth Factor (ERBB) receptors. As one example, Chen *et al.* have reported that ligand activation of the ERBB receptor induces GRK2 phosphorylation [387]. Via either pathway, this phosphorylation initiates the recruitment of βARRESTIN and prevents the $G\alpha_s$ subunit from initiating GPCR-mediated signaling [389]. Although FSHR desensitization has been documented

in rat Sertoli cells (25-28) as well as various mammalian granulosa cell lines [180, 262, 390, 391], there is currently no evidence for a relationship between FSHR desensitization and follicle selection and differentiation in the ovary of any vertebrate. The present studies were initiated to identify the cellular events that desensitize FSHR signaling and maintain GCs from hen prehierarchal follicles in an undifferentiated state prior to selection.

MATERIALS AND METHODS

Animals and reagents

Single-comb White Leghorn hens 34-55 weeks of age and laying sequences of 5 or more eggs were used in the studies described. Hens were housed individually in laying batteries, with free access to feed and water, under a controlled photoperiod of 15 h light, 9 h dark (lights on at 01:30 h). Hens were euthanized by cervical dislocation 5-10 h following a mid-sequence ovulation at which time the ovary was removed and placed in ice-cold sterile 1% NaCl solution. All procedures described herein were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committees, and were performed in accordance with The Guiding Principles for the Care and Use of Laboratory Animals.

Recombinant human (rh) transforming growth factor α (TGFα), TGFβ, and BMP4 proteins were obtained from PeproTech (Rocky Hill, NJ) while rhFSH was provided by the National Hormone and Pituitary Program (Torrance, CA). Collagenase type II was from Worthington Biochemical Corporation (Lakewood, NJ), forskolin and the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), were from Sigma-Aldrich (St. Louis, MO).

Tissue collection and culture

Ovarian follicles from individual hens were grouped by developmental stage, and GCs were isolated and dispersed for short-term (1-3 h) incubation or culture as previously described [19, 122]. Briefly, for short-term incubations GC layers were collected and immediately dispersed into small pieces by gentle, repeated pipetting, then aliquoted in equal proportions into 12 x 75 mm polypropylene culture tubes containing 1 ml of Dulbecco's Modified Eagle Medium (DMEM; HyClone, Thermo Scientific) containing 2.5% FBS (PAA Laboratories, Piscataway, NJ), 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY) and non-essential amino acids (Gibco). Culture tubes were incubated in a shaking water bath at 40 °C in absence or presence of FSH (10 ng/ml) for 1 h (for cAMP) or 3 h (for progesterone) [30]. For cell cultures, GC layers were dispersed using 0.3% collagenase to achieve a single cell suspension, then cultured in 12-well polystyrene culture plates (Beckton Dickinson, Franklin Lakes, NJ) containing 1 ml of DMEM plus 2.5% FBS, 0.1 mM non-essential amino acids, and 1% antibiotic-antimycotic mixture (Invitrogen Life Technologies, Carlsbad, CA) at 40 °C in an atmosphere of 95% air: 5% CO_2. In some experiments cells were cultured in presence of either BMP4 or TGFβ (10 ng/ml) to maintain *FSHR* mRNA expression [19, 115], and used for transfecting recombinant plasmids and siRNAs. Theca layers collected from 6-8 mm and 9-12 mm follicles were washed free of any remaining GCs and separately diced into small pieces using a scalpel blade [16]. Layers were washed with DMEM to remove any remaining yolk and incubated for 3 h in DMEM containing 2.5% FBS in a shaking water bath without or with FSH (10 or 100 ng/ml).

Transfection of βARRESTIN1 and GRK2 plasmids and small interfering RNAs (siRNAs)

Bovine *βARRESTIN1* and *GRK2* plasmids were kindly provided by Dr. Jeffrey L. Benovic (Thomas Jefferson Medical School). Double stranded siRNA specific for *Gallus βARRESTIN1* was generated using SciTools online software (Integrated DNA Technologies,

Coralville, IA) and purchased from Integrated DNA Technologies, Inc (**Table 3.2**). Non-targeting (scrambled sequence) siRNA was used as a control. Transient transfections were performed on primary GCs grown to 50-60% confluence in 12-well plates with 2 µg of *βARRESTIN1* and *GRK2* plasmids (or the empty vector as controls) or 100 nM of siRNA:

(*βARRESTIN1* Set #1: **Sense,** 5'-CCACCAGGCAGUUCCUCAUGUCGGA-3';

Antisense, 5'-UCCGACAUGAGGAACUGCCUGGUGGUC-3';

Set #2: **Sense,** 5'-CCAACAAGACGGUGAAGAAGAUCAA-3';

Antisense, 5'-UUGAUCUUCUUCACCGUCUUGUUGGUG-3';

or scrambled sequence: **Sense,** 5'-CGUUAAUCGCGUAUAAUACGCGUAT-3';

Antisense, 5'-AUACGCGUAUUAUACGCGAUUAACGAC-3')

Transfections were accomplished using Lipofectamine 2000 (Invitrogen) at a 1:2 ratio as previously reported [76]. Western blot or quantitative RT-PCR was utilized to assess the extent of protein over-expression or mRNA knock-down, respectively.

RNA isolation and quantitative real time PCR

Total RNA was isolated from cells using Trizol reagent (Life Technologies, Grand Island, NY) and dissolved in nuclease-free water. The purity and concentration of final RNA pellets were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, West Palm Beach, FL). Random-primed, reverse transcribed cDNA reactions were performed using the Promega RT System (Promega), according to the conditions described by the manufacturer. For negative RT samples, all components of cDNA synthesis were used, but lacked the reverse transcriptase enzyme to insure the lack of genomic DNA contamination. For water control samples, all components of the PCR or real-time PCRs were added, but water was substituted for the template to check for primer contamination.

Forward and reverse primers encoding *Gallus βARRESTIN1*, four *GRK* isoforms (*GRK2,3,5* and *6*), *FSHR*, *STAR*, *CYP11A1*, and *18S* rRNA mRNA were generated using SciTools online software (Integrated DNA Technologies, Coralville, IA) (**Table 3.1**). Final concentrations of the sense and antisense primers were determined for each primer pair based upon optimal amplification efficiency. For real-time PCR, primers and 50 ng cDNA template were added to 10 ml total reaction volume using the reagents provided in the PerfeCTa Sybr Green FastMix Low Rox (Quanta Biosciences, Inc., Gaithersburg, MD, USA). Reactions were completed on the AB 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were conducted with the following conditions: 30 s at 95 °C, followed by 40 cycles each for 3 s at 95 °C, 1 min at 56 °C, and 30 s at 72 °C. For primer validation, melting curves were generated for each run. Initially, amplified products were visualized by ethidium bromide after running on a 1.5% agarose (Invitrogen) gel then sequenced by Pennsylvania State University Genomics Core Facility. The Ct (cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction (run in triplicate) using the 7500 software (v.2.0.4) and quantification accomplished using the ΔΔCt method [392]. Target values were standardized to *18S* rRNA. Results were expressed as fold-difference compared with an appropriate control tissue or treatment.

Western blot analysis

Western blot analysis was conducted as previously described [29, 30, 76, 115] and used to evaluate the extent of βARRESTIN1 and GRK2 over-expression, plus levels of FSHR and STAR protein in GCs. Briefly, cells were harvested and lysed in RIPA buffer (Santa Cruz) containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). To assess FSHR, membrane proteins were extracted using the Mem-PER^{TM} Plus Membrane Protein Extraction Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Protein concentrations were

assessed by the BCA method (BioRad, Hercules). Gel electrophoresis and blotting were conducted according to manufacturer's recommendations using the NuPAGE® SDS-PAGE gel system (Invitrogen). Following electrophoresis and transfer, blots were incubated in SuperBlock® TBS blocking buffer (Pierce) for 3 h, followed by incubation with antibody. An affinity-purified goat GRK2 (sc-18409, Santa Cruz) and rabbit polyclonal βARRESTIN1 (sc-28869, Santa Cruz) antibodies were used at 1:200 dilutions. The STAR antiserum was generously provided by Dr. Buck Hales [393] and was used at a 1:5,000 dilution. For GRK2, mouse anti-goat IgG conjugated with HRP (Pierce) was incubated with 1:10,000 dilution and for βARRESTIN and STAR, goat anti-rabbit IgG conjugated with HRP (Pierce) was incubated with 1:10,000 dilution for 1 h at room temperature. The *Gallus* anti-FSHR polyclonal, affinity-purified serum was generated in a rabbit against the C-terminal peptide sequence, Ac-RFGPVENEFDYGLC-amide (GenBank NP_990410), and used for Western blotting at a dilution of 1:5000. Preliminary studies conducted to establish antiserum specificity determined that: 1) co-incubation of the antiserum with differentiated GCs completely blocked FSH-induced progesterone production; and 2) preabsorption of the antiserum with the immunogen completely eliminated a signal corresponding to the predicted 78 kDa *Gallus* FSHR protein by Western blot. βActin (antiserum from Sigma, used at a 1:10,000 dilution) was utilized as a loading control to assess levels of membrane-associated [394] and total protein. All Western blots were developed with ECL™ Plus Chemiluminescence Detection Reagent (Pierce). Chemiluminescent signals were detected using the Storm 860 optical scanner (Amersham Biosciences), and signals analyzed using Image Quant TL™ software (Amersham Biosciences).

Progesterone assay

Progesterone concentrations in culture medium samples were measured using two different enzyme immunoassay (EIA) kits. Samples collected from 6-8 mm follicle GCs were

assayed using a high sensitivity EIA from Cayman Chemical Co. (Ann Arbor, MI) while samples from 9-12 mm and F4 follicle GCs were measured with an EIA from Oxford Biomedical Research (Rochester Hills, MI) as previously described [30]. Final concentrations of progesterone were normalized to total amount of RNA, and expressed as ng per µg of RNA or in some instances as a % increase compared to the appropriate control.

cAMP assay

Intracellular cAMP levels were evaluated by EIA (Cayman Chemical Co.) as previously described [30]. Briefly, GCs transfected with over-expression constructs or siRNA were pretreated with 10 µM of IBMX for 20 min and challenged with FSH (10 ng/ml) for 1 h. Cells were lysed in 0.1 N HCl for 20 min at room temperature by repeated pipetting then centrifuged at 1,000 x *g* for 10 min. Supernatants were collected and used to measure the accumulation of intracellular cAMP. Final cAMP levels were normalized to total protein and expressed as fmol per µg of protein or as a % increase compared to control.

Data Analysis

Replicate experiments were independently conducted a minimum of three times. Data from all experiments were compared with an appropriate control group and subjected to a Student's t-test or one-way ANOVA using the general linear model (GLM) procedure (SAS Institute Inc., Cary, NC). Least square mean values between or among groups were separated using a least square means test. A probability level of $p \leq 0.05$ was considered statistically significant.

RESULTS

Absence of FSH-induced cAMP in undifferentiated GCs

As previously reported [19, 127], levels of *FSHR* mRNA expression are not different in undifferentiated GCs from prehierarchal (6-8 mm) follicles compared to actively differentiating GCs from the most recently selected (9-12 mm) follicle (**Fig. 3.1A**). Moreover, despite elevated levels of membrane-localized FSHR protein, undifferentiated GCs from prehierarchal follicles failed to produce cAMP in response to a 1 h incubation with 10 ng FSH/ml (**Fig. 3.1B**). This contrasts to a significant accumulation of cAMP in actively differentiating GCs from the most recently selected follicle ($P<0.001$). Importantly, incubation of undifferentiated GCs for 1 h with forskolin (20 μM), a cell-permeable and direct activator of adenylyl cyclase activity, induced a very high level of cAMP accumulation in undifferentiated GCs, demonstrating the potential for functional intracellular signaling downstream of the FSHR (**Fig. 3.1C**). These findings suggest that the absence of FSHR signaling in undifferentiated GCs is due to some form of receptor desensitization.

Absence of cAMP signaling in undifferentiated GCs results in the absence of steroidogenesis

Basal expression of STAR protein (**Fig. 3.2A**, -FSH) and *CYP11A* mRNA (**Fig. 3.2B**) is very low to non-detectable in GCs from 6-8 mm follicles. STAR protein expression is induced by a 3 h incubation with FSH (10 ng/ml) in actively differentiating GCs from 9-12 mm and F4 follicles but not in undifferentiated GCs (**Fig. 3.2A**). Consequently, GCs from prehierarchal (6-8 mm) follicles produce non-detectable levels of progesterone in response to 10, 33 or 100 ng FSH/ml (**Fig. 3.2C**).

To establish whether the absence of FSH-responsiveness is restricted to the GC layer, theca layers from 6-8 mm follicles were incubated for 3 h with 0, 10 or 100 ng FSH/ml, and *STAR* mRNA expression was subsequently quantified. In contrast to the lack of response by

undifferentiated GCs from 6-8 mm follicles (**Fig. 3.2C**), the adjacent theca layer demonstrated increased *STAR* mRNA expression in response to a challenge with 100 ng/ml FSH (**Fig. 3.3**). Collectively, results from the first three studies indicate that FSHR signaling via cAMP is absent specifically in GCs from prehierarchal follicles and is initiated within the GC layer at follicle selection.

Active MAPK signaling inhibits FSH-responsiveness in undifferentiated GCs

Previous studies have demonstrated that MAPK signaling induced by EGFR ligands blocks TGFβ- and BMP4-induced *FSHR* mRNA expression, plus prevents FSH-induced *STAR* plus *CYP11A* mRNA expression in cultured GCs from prehierarchal follicles [18, 19, 30, 34]. Thus, we speculated that MAPK signaling plays a direct role in inhibiting cellular accumulation FSH-induced cAMP at the level of the FSHR. Undifferentiated GCs were isolated from 6-8 mm follicles and cultured with BMP4 (10 ng/ml) overnight to maintain *FSHR* mRNA expression [30]. These cells were then treated without or with TGFα (10 ng/ml) for 6 h and subsequently challenged with 10 ng FSH/ml for 1 h in the presence of 10 μM IBMX. While accumulation of intracellular cAMP levels following a 1 h treatment with FSH was increased in control cultured cells, the 6 h pretreatment with TGFα completely prevented FSH-induced cAMP accumulation (**Fig. 3.4**). We conclude that undifferentiated GCs cultured overnight in DMEM containing FBS begin to acquire of the capacity for FSHR cell signaling via cAMP (*e.g.*, initiate differentiation in culture), but that a subsequent activation of MAPK signaling is capable of reestablishing receptor desensitization.

Expression of βARRESTIN1 and GRKs in hen GCs

Given that the FSHR is a member of the GPCR family of seven-transmembrane domain receptors, we predicted that FSHR cell signaling could be regulated by βARRESTIN-mediated

desensitization. Messenger RNA for both *βARRESTIN1* and *GRKs* (*GRK2, -3, -5 and -6*) are expressed in hen GCs from prehierarchal (6-8 mm) follicles, the most recently selected (9-12 mm) follicle and the fifth largest (F5) preovulatory follicle (**Fig. 3.5**). Each of the predicted protein sequences is highly homologous (≥87%) to their bovine counterparts.

Knock-down of βARRESTIN1 expression increases FSH-responsiveness in undifferentiated GCs

Undifferentiated GCs from 6-8 mm follicles were cultured in the presence of TGFβ and transiently transfected with *Gallus βARRESTIN1*-siRNA (βAsiR) to evaluate the effects of decreasing βARRESTIN expression on FSHR signaling. The knock-down efficiency of *βARRESTIN* mRNA was determined by quantitative RT-PCR, and both siRNAs were determined to be equally effective in decreasing mRNA expression by 62+/-7% (mean +/- SEM) at 48 h-post transfection compared to cells transfected with scrambled sequence (Scr) (**Fig. 3.6A**). FSHR responsiveness was evaluated after a 1 h challenge without or with 10 ng FSH/ml by measuring intracellular cAMP. Accumulation of cAMP was increased in cultures treated with *βARRESTIN1*-siRNA transfected cells compared to Scr-transfected cells (**Fig. 3.6B**). Moreover, both FSH-induced STAR protein expression (**Fig. 3.6C**) and progesterone production (**Fig. 3.6D**) were significantly increased in *βARRESTIN1*-siRNA transfected cells compared to Scr-transfected cells. These findings provide evidence that βARRESTIN is necessary to maintain FSHR desensitization in undifferentiated GCs. It is also noted that there was a significant increase in cAMP accumulation in the Scr + FSH treated cells compared to Scr-transfected cells (**Fig. 3.6B**), a noticeable presence of STAR protein expression (**Fig. 3.6C**), and a significant increase in progesterone production (**Fig. 3.6D**). We attribute this level of FSHR-responsiveness to an initiation of GC differentiation during culture as demonstrated in **Fig. 3.4**.

Co-transfection with βARRESTIN1 and GRK2 suppresses FSH-induced cAMP accumulation in actively differentiating GCs

Actively differentiating GCs from 9-12 mm follicles were cultured with TGFβ (10 ng/ml, to maintain *FSHR* expression; 3) and transfected with Bovine *βARRESTIN1* and *GRK2* (CoT) or empty vector (EMT) as previously described (11) (**Fig. 3.7A**). Cultures were subsequently challenged without or with 10 ng FSH/ml for 1 h (for cAMP accumulation) or 3 h (for media levels of progesterone). Co-transfection of *βARRESTIN1* plus *GRK2* suppressed both FSH-induced cAMP accumulation (**Fig. 3.7B**) and progesterone production (**Fig. 3.7C**) compared to empty vector-transfected cells. These data indicate that a βARRESTIN-mediated event is sufficient to promote FSHR desensitization in hen GCs.

DISCUSSION

To our knowledge, this is the first study to demonstrate a relationship between FSHR desensitization and either GC differentiation or follicle growth and development in any vertebrate ovary. The present results provide evidence that a βARRESTIN-mediated event is both sufficient and necessary to mediate FSHR desensitization (*e.g*., preclude signaling via cAMP) in GCs from hen prehierarchal follicles. In the absence of cAMP signaling, GCs are maintained in an undifferentiated state, as indicated by the inability to produce steroids. In almost all avian species the maintenance of follicles within this prehierarchal cohort in a viable, undifferentiated state is prerequisite to provide for the selection and subsequent ovulation of one follicle each day for the duration of a laying sequence. Furthermore, we propose that prior to follicle selection FSHR desensitization is maintained by inhibitory MAPK signaling. Our working model (**Summary model 1**) predicts that some reduction in MAPK signaling, by reduced EGFRL expression [34], MAPK activity or enhanced phosphatase activity [33], occurs at follicle selection to initiate GC

differentiation. Overall, this strategy for ovarian follicle selection and subsequent growth plus differentiation contrasts with that of mammals. Specifically, in mammals a new cohort of follicles is recruited each ovulatory cycle and eventually all non-selected (subdominant) follicles undergo atresia [91-94].

Our initial studies confirmed that while *FSHR* mRNA is expressed at similar levels in GCs from 6-8 mm follicles compared to the two most recently selected follicles (**Fig. 3.1A)** [19, 395], undifferentiated GCs from prehierarchal follicles produce a negligible amount of cAMP in response to a FSH challenge (**Fig. 3.1B**) [122]. This lack of responsiveness cannot be attributed to the absence of membrane-localized FSHR protein (**Fig. 3.1A**). Moreover, treatment with forskolin, a direct activator of adenylyl cyclase, potently induces cAMP accumulation in undifferentiated GCs (**Fig. 3.1C**). Taken together, these results provide evidence that the absence of FSH-responsiveness in undifferentiated GCs is mediated by FSHR desensitization. It is well established that FSH binding to the FSHR (a member of GPCR family) can activate the $G\alpha_s$ subunit and increase intracellular concentrations of cAMP [16, 30, 382, 396-400]. On the other hand, excessive or prolonged exposure to FSH initially results in βARRESTIN-mediated desensitization and the loss of cAMP signaling, followed by receptor internalization [401-403]. While events occurring subsequent to receptor desensitization were not examined in the present studies, the potential for this process and the extent of receptor internalization in undifferentiated GCs are currently under investigation.

The expression of both *CYP11A* and *STAR* mRNA in hen GCs is dependent upon cAMP/PKA signaling to induce gene transcription [27, 37, 120, 379]. Accordingly, in the absence of FSH-induced cAMP there exist essentially undetectable levels of endogenous STAR protein and *CYP11A1* mRNA (**Figs. 3.2A and 3.2B**), thus non-detectable levels of progesterone (**Fig. 3.2C**) generated by GCs from prehierarchal follicles. Neither the exact timing of follicle selection during the approximate 24 h ovulation cycle of the hen nor the proximal signal initiating follicle

selection is currently known. Nevertheless, it is evident that the GC layer from the most recently selected, 9-12 mm follicle has already acquired FSH-responsiveness (**Fig. 3.1**) and is steroidogenically competent (**Fig. 3.2**). While the GC layer from prehierarchal follicles expresses *FSHR*, but not *LHR*, the adjacent theca layer expresses both gonadotropin receptors at this stage of follicle development [16, 17, 127, 404]. Previous studies have reported that intact, 1-10 mm follicles produce ng quantities of androstenedione in response to a 3 h challenge with ovine LH [405]. The present studies determined that the isolated theca layer from 6-8 mm follicles is equally responsive to FSH when compared to that from 9-12 mm follicles (**Fig. 3.3**). Collectively, these results demonstrate that the process of follicle selection in the hen ovary is closely associated with the functional differentiation of the GC layer.

Although freshly collected GCs from prehierarchal follicles are unresponsive to a short-term challenge with FSH (1-3 h; **Figs. 3.1 and 3.2**), there are a number of published studies demonstrating that these cells begin to acquire the capacity to produce cAMP in response to FSH when placed in culture for 6 h or longer (*e.g.*, CYP11A and STAR) [27, 75, 120, 379, 406, 407]. Furthermore, co-culture with a MAPK inhibitor (U0126) potentiates this FSH-responsiveness [19, 27, 407]. On the other hand, following overnight culture, during which FSH-responsiveness was initiated, a 6 h pretreatment with TGFα was determined to reestablish FSHR desensitization, as indicated by the absence of FSH-induced cAMP accumulation (**Fig. 3.4**). Based upon previous results in which TGFα treatment promotes extracellular signal-regulated kinase (ERK) phosphorylation (49) we speculate that FSHR desensitization in undifferentiated GCs is regulated, at least in part, by MAPK signaling.

A number of previous studies have demonstrated a role for βARRESTIN-mediated desensitization of gonadotropin receptors, including the FSHR, in mammalian GCs and Sertoli cells [175, 176, 181-183, 408]. At the time the present studies were initiated, there was no annotated sequence for a *Gallus βARRESTIN* described within GenBank. Thus, primer pairs for

βARRESTIN1 were generated based on the bovine *βARRESTIN1* sequence, and subsequently a 465 base-pair cDNA was amplified from hen GCs. The deduced partial amino acid sequences for *Gallus βARRESTIN1* was submitted to GenBank (ID: JX088657). Both βARRESTIN1 and GRK2 proteins, together with GRK3, GRK5 and GRK6, show a high degree of homology between *Bos taurus* and *Gallus gallus* (**Fig. 3.5**).

In light of evidence that the inhibition of FSH-induced cAMP accumulation occurs prior to the activation of adenylyl cyclase (**Fig. 3.1C**), siRNA directed specifically against the *Gallus βARRESTIN* sequence was utilized to significantly reduce endogenous levels of its expression in undifferentiated GCs (**Fig. 3.6A**). Despite the predicted high level of homology between the bovine and chicken βARRESTIN proteins (**Fig. 5**), levels of endogenous expression were not detectable by Western blot analysis (see **Fig. 3.7A, EMT *versus* CoT**). Nevertheless, siRNA-transfected cells challenged with FSH for 3 h produced significantly increased levels of cAMP, STAR expression and progesterone production (**Fig. 3.6**). Moreover, over-expression of βARRESTIN was able to re-establish a significant level of FSHR desensitization in actively differentiating GCs from the most recently selected (9-12 mm) follicle (**Fig. 3.7**). Taken together, these two experiments provide compelling evidence for FSHR desensitization in undifferentiated GCs from prehierarchal follicles being mediated via βARRESTIN.

Our working model depicts EGFRL acting via ERBB receptors as a primary means by which MAPK is activated; this, in turn, is proposed to phosphorylate and activate GRK (**Summary model 1**). Nevertheless, GPCRs are also capable of forming complexes with a variety of accessory proteins including the clathrin adaptor protein, AP-2 [246, 259, 409]. Such GPCR complexes have been demonstrated not only to induce receptor internalization, but also to activate MAPK signaling [259, 410, 411]. Thus, it is possible that βARRESTIN, as one such scaffold protein, may by itself or together with EGFRLs help maintain FSHR desensitization by

supporting inhibitory MAPK signaling [410, 412, 413]. Because the initiation of GC differentiation at the time of follicle selection is dependent upon cell signaling via cAMP, additional studies are required to identify the most proximal cellular event(s) that initiate and terminate βARRESTIN-mediated desensitization.

In summary, we conclude that prior to follicle selection FSHR desensitization within specifically the GC layer of prehierarchal follicles precludes FSH-induced cAMP signaling and prevents premature GC differentiation. Moreover, we propose that this desensitization is maintained, at least in part, by MAPK signaling. It is important to note, however, that together with the gonadotropin receptors at least two additional GPCRs that signal via cAMP are expressed by GCs from hen prehierarchal follicles, including the vasoactive intestinal peptide (VIP) receptors, VPAC1 and VPAC2 (unpublished data). Our current studies of these receptors are designed to assess the capacity for VIP-induced receptor signaling within the GC layer relative to stage of follicle development. Finally, it is also noted that LH-induced cAMP accumulation in fully differentiated GCs from preovulatory follicles is significantly reduced (by 30-40%) following pretreatment with either EGF or TGFα [395, 414]. It will be of interest to assess whether this inhibitory effect in fully differentiated GCs is also attributed to βARRESTIN-mediated desensitization.

ACKNOWLEDGEMENTS

We thank Dr. Jeffrey L. Benovic (Thomas Jefferson Medical School) for the bovine *βARRESTIN1* and *GRK2* plasmids, and Dr. Buck Hales (Southern Illinois University School of Medicine) for providing the avian-specific STAR antiserum.

Table 3.1. Sequences of forward and reverse primers used for real-time PCR, together with the NCBI nucleotide accession number.

Gene	Accession #	Sequence
β-ARRESTIN 1	JX088657	FWD 5'-AGGCAGTTCCTCATGTCGGACAAA-3' REV 5'-TTGTTGGTGTTGTTGGTGACGTGG-3'
GRK2	NM_001031353	FWD 5'-TGACATTGGCTCCTTTGACGAGGA-3' REV 5'-CCAGCTGCTTGTTCTTGGCTTTCT-3'
GRK3	XM_415195	FWD 5'-ACGCTCACCGTGAATGTGGAGTTA-3' REV 5'-TCACTTCTTGTGCACTCCTCCCTT-3'
GRK5	XM_421789	FWD 5'-AGGAAAGGCGAATCCATGGCACTA-3' REV 5'-ACCCGGGTTTCCCATGTTGTAGAT-3'
GRK6	XM_414676	FWD 5'-AGTTTGCTACAGGAAGCGTACCCA-3' REV 5'-TGGAGTAACCCTTTCTTGGGCTGT-3'
FSHR	NM_205079	FWD 5'-TTAATTCCTGTGCTAACCCTTTCC-3' REV 5'-CCAAACTTGCTCAACAGAATGAAG-3'
CYP11A1	NM_001001756	FWD 5'-ACTTCAAGGGACTGAGCTTTGGGT-3' REV 5'-AGTTCTCCAGGATGTGCATGAGGA-3'
STAR	NM_204686	FWD 5'-TGCCTGAGCAGCAGGGATTTATCA-3' REV 5'-TGGTTGATGATGGTCTTTGGCAGC-3'
18S rRNA	AF173612	FWD 5'-TTAAGTCCCTGCCCTTTGTACAC-3' REV 5'-CGATCCGAGGAACCTCACTAAAC-3'

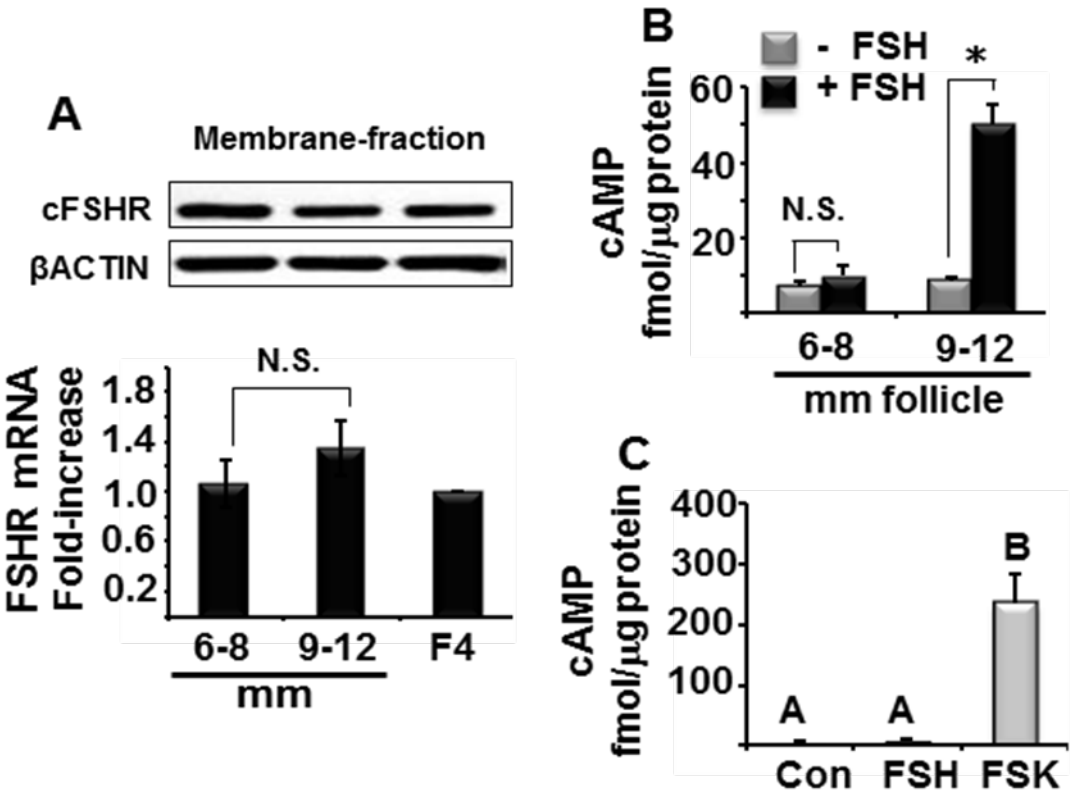


Figure 3.1. Absence of FSHR responsiveness in undifferentiated GCs.

A. FSHR membrane-localized protein (FSHR; ~78kDa) in GCs from 6-8 mm, 9-12 mm and F4 follicles. This blot was replicated with similar results. *FSHR* mRNA expression in prehierarchal (6-8 mm) and the most recently selected (9-12 mm) follicles is expressed as a fold-increase compared to the fourth largest preovulatory (F4) follicle (N.S., P=0.366; n=4 replicate experiments). **B**. Comparison of FSH (10 ng/ml)-induced accumulation of intracellular cAMP between undifferentiated (6-8 mm) and actively differentiating (9-12 mm) GCs following a 1 h incubation (N.S., P>0.3; *, P<0.001, n=4). **C**. Forskolin (20 µM)-induced intracellular cAMP in undifferentiated GCs. Data represent the mean ± SEM. A,B: P<0.003 by ANOVA and Tukey's multiple range test (n=3-6).

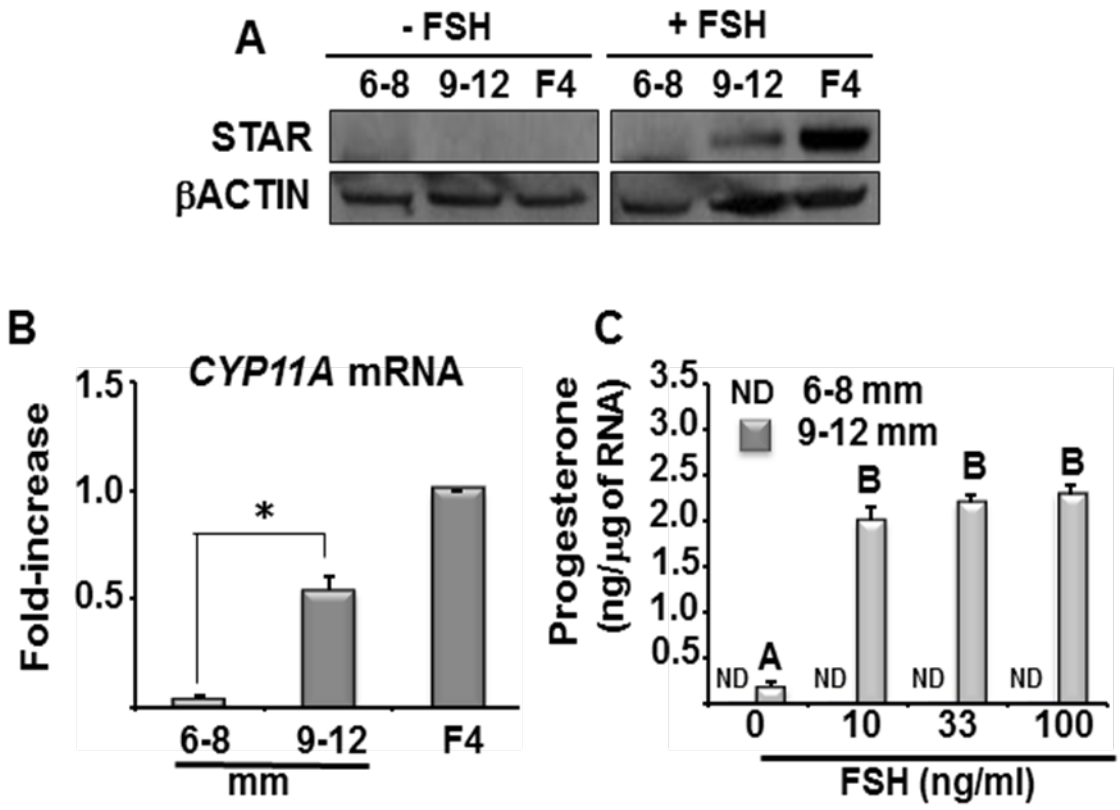


Figure 3.2. Absence of steroidogenesis from undifferentiated GCs.

A. Western blot of STAR protein expression (30 kDa) following a 3 h incubation with FSH (10 ng/ml) in freshly isolated GCs from 6-8 mm and actively differentiating 9-12 mm follicles compared to the differentiated GCs from the fourth largest (F4) preovulatory follicle. This blot is representative of replicate experiments. **B**. Expression levels of endogenous *CYP11A1* mRNA in incubated GCs from 6-8 mm and 9-12 mm follicles relative to the F4 follicle (*, $P< 0.001$ by Student's *t* test, n=4). **C**. Media levels of progesterone in GCs from 6-8 and 9-12 mm follicles induced by 10, 33 and 100 ng FSH/ml after a 3 h incubation. ND, not detected; A,B: $P<0.0001$ by ANOVA and Tukey's multiple range test; n=3-6.

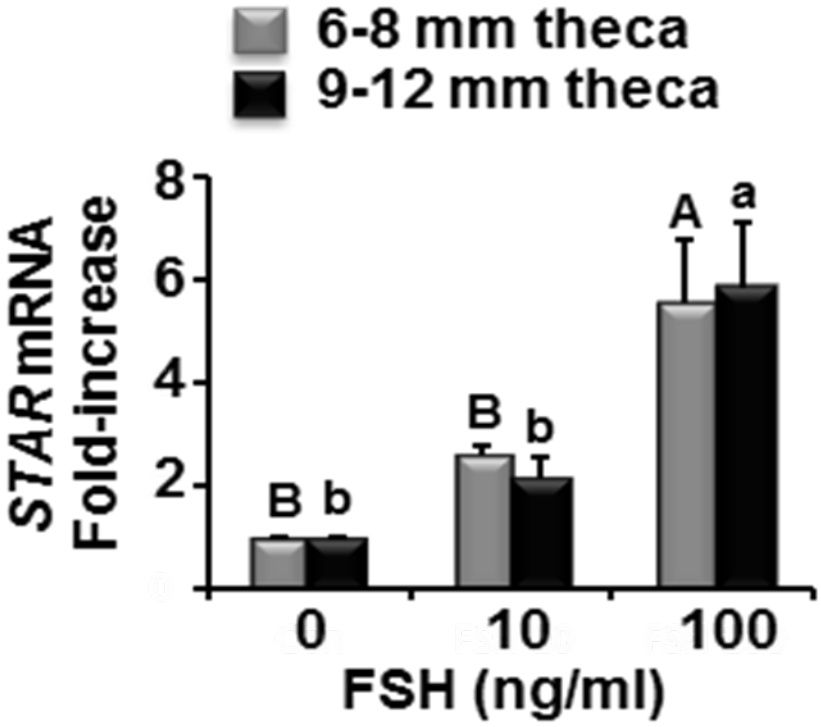


Figure 3.3. FSH-induced *STAR* mRNA expression in theca tissue from 6-8 mm and 9-12 mm follicles following incubation with FSH (10 and 100 ng/ml) for 3 h. A,B, a,b: $P<0.02$ by ANOVA; n=4.

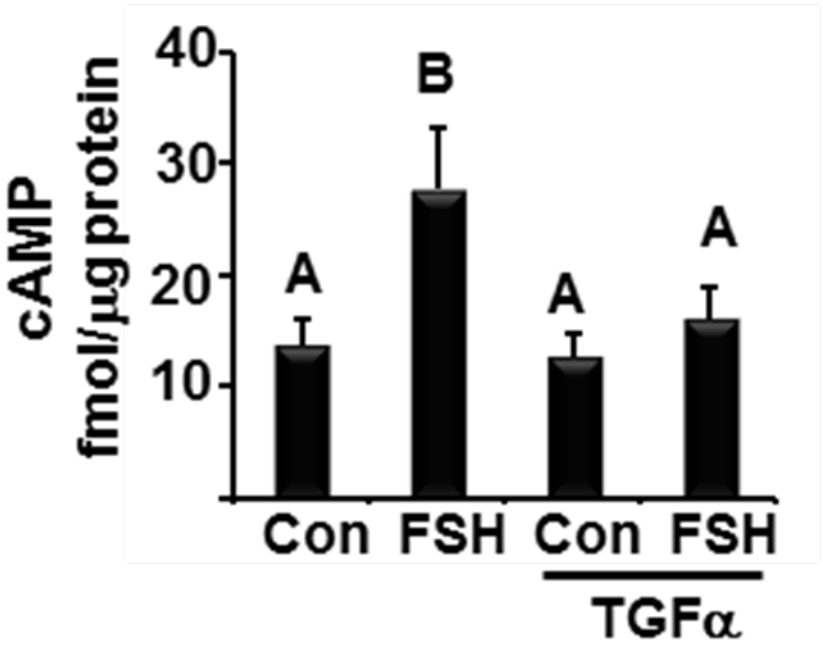
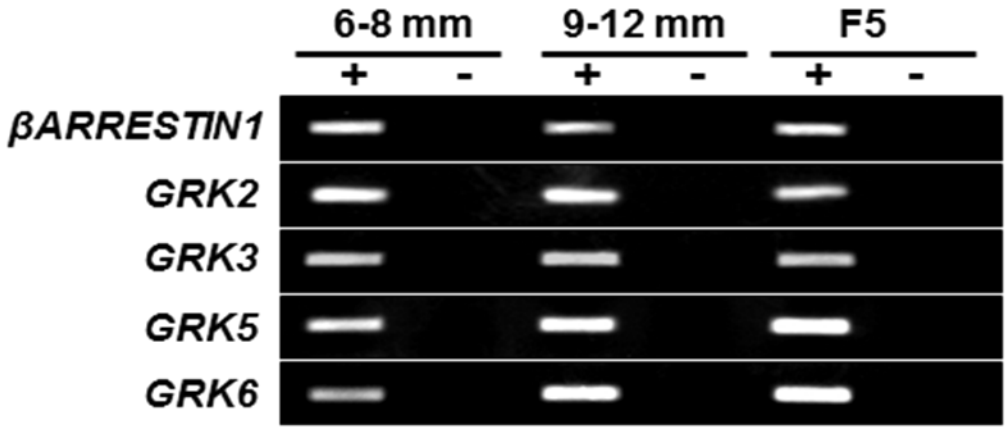


Figure 3.4. TGFα treatment reestablishes FSHR desensitization and prevents FSH-induced cAMP accumulation in early differentiating GCs. Undifferentiated GCs were cultured in 12-well plates overnight with DMEM containing 2.5% FBS then subsequently cultured for 6 h in the absence or presence of TGFα (10 ng/ml), followed by a 1 h challenge with FSH (10 ng/ml). A,B: $P<0.05$ by ANOVA and Duncan's multiple range test. Data represent the mean ± SEM from 4 replicate experiments**.**



Gene	*Gallus gallus* Accession #	*Bos taurus* Accession #	Homology of protein to *Bos* (%)
β-ARRESTIN1	JX088657	NP 776668.1	97%
GRK2	NP_001026524.1	NP 777135.1	92%
GRK3	XP_415195.3	NP 776925.1	94%
GRK5	XP_421789.2	NP 776756.1	90%
GRK6	XP_414676.3	NP_001192319.1	87%

Figure 3.5. Top. Expression of *Gallus βARRESTIN1* and *GRK*s at different stages of follicle development. *βARRESTIN1, GRK2, GRK3, GRK5, and GRK6* gene expression was identified in undifferentiated GCs (6-8 mm; prior to follicle selection) and actively differentiating GCs (9-12 mm and F5; subsequent to selection). PCR amplification was accomplished using primer pairs described in Table 1. +, - represents presence and absence of reverse transcriptase, respectively. **Bottom**. Sequence homology between the *Gallus* and *Bos* proteins.

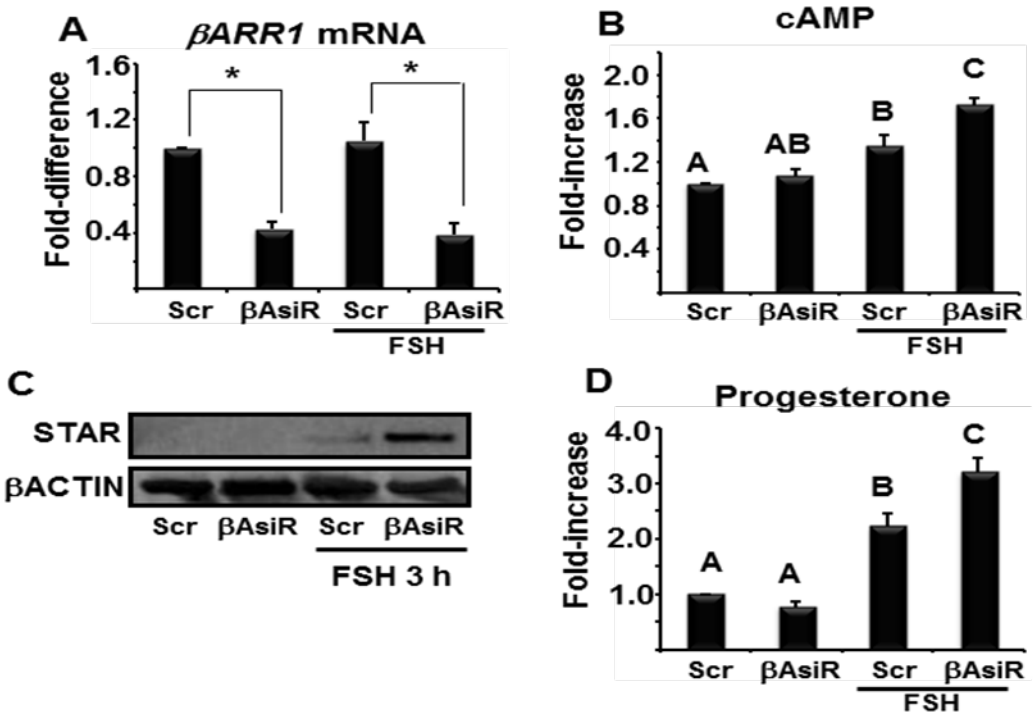


Figure 3.6. Transfection with *Gallus βARRESTIN1*-siRNA initiates FSH-induced cAMP accumulation in undifferentiated GCs.

A. Levels of *βARRESTIN1* mRNA expression after transfection for 48 h with scrambled sequence (Scr) siRNA or *βARRESTIN1*-siRNA (βAsiR) in undifferentiated GCs. Cells were challenged without or with FSH (10 ng/ml) for 3 h after transfection. Data represent the mean ± SEM from 4 replicate experiments relative to Scr siRNA (*, P<0.005 by Student's *t* test). **B**. cAMP accumulation in undifferentiated GCs following FSH (10 ng/ml) for 1 h. Data are expressed as a fold-increase compared to Scr siRNA transfected cells following FSH challenge and analyzed by ANOVA and Tukey's multiple range test (A,B,C: P<0.01, n=4). **C**. Western blot for STAR protein levels after transfection of Scr or *βARRESTIN1*-siRNA in undifferentiated GCs. This blot was repeated once with similar results. **D**. Progesterone production in *βARRESTIN1*-siRNA transfected GCs following FSH (10 ng/ml) for 3 h compared to Scr siRNA transfected cells. Data are expressed as fold-increase compared to Scr siRNA transfected cells and analyzed by ANOVA and Tukey's multiple range test (A,B,C: P<0.03, n=4).

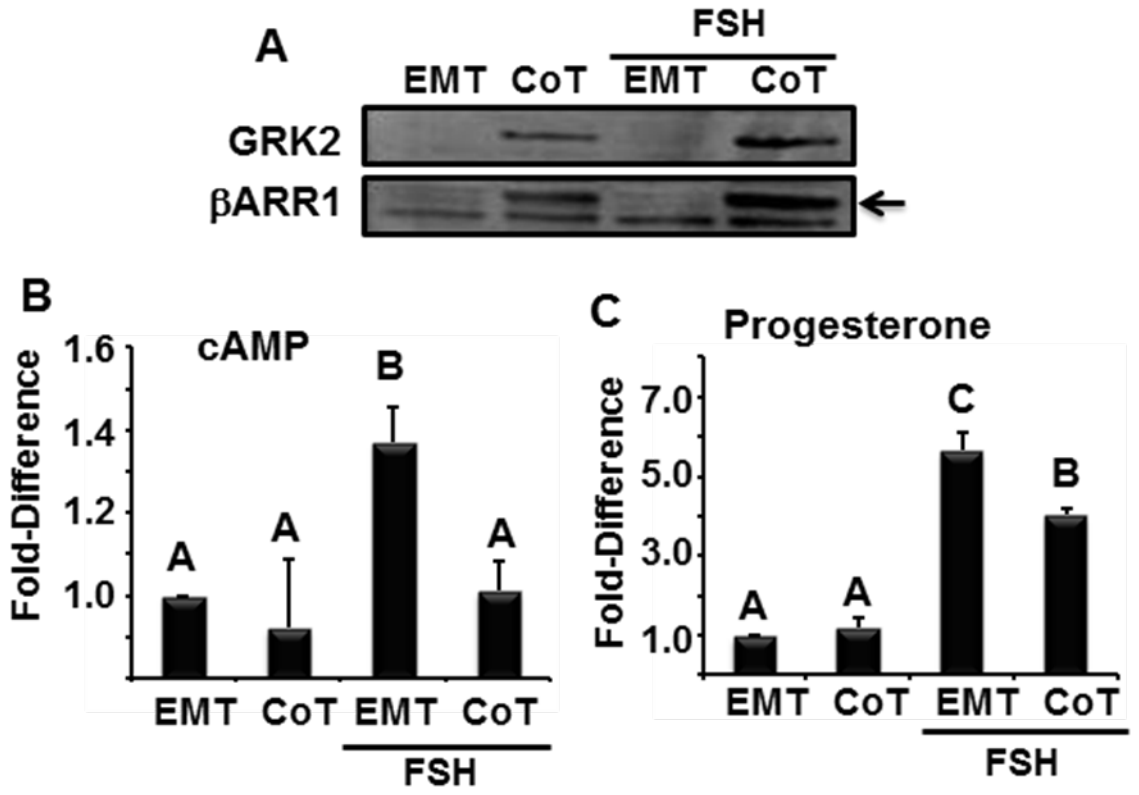


Figure 3.7. Co-transfection (CoT) of bovine βARRESTIN1 (βARR1) and GRK2 suppresses FSH-induced signaling in differentiating GCs.

A. Western blot of βARRESTIN1 (~50 kDa) and GRK2 (~80 kDa; arrow) in co-transfected GCs from actively differentiating GCs from 9-12 mm follicles compared to the empty vector (EMT). This blot was replicated with similar results. **B**. cAMP accumulation following FSH (10 ng/ml) for 1 h in GCs transfected with pcDNA-*βARRESTIN1* and *GRK2* compared with control pcDNA3-transfected cells (EMT). Data are expressed as the mean ± SEM, and analyzed by ANOVA and Tukey's multiple range test (A,B: $P<0.03$, N=3-5). **C**. Progesterone production after transfection with pcDNA3 (EMT) or pcDNA- *βARRESTIN1* and pcDNA-*GRK2* followed by FSH treatment (10 ng/ml) for 3 h. Data are expressed as the mean ± SEM, and analyzed by ANOVA and Tukey's multiple range test (A,B,C: $P<0.02$, N=3).

Chapter 4

Acquisition of VIP Responsiveness Promotes Granulosa Cell Differentiation and Regulates Clock Gene Expression in Hen Granulosa Cells

Authors : Dongwon Kim and Alan L. Johnson

Key Words: Granulosa cells, Vasoactive Intestinal Peptide, VPAC, βARRESTIN, Circadian rhythm

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ABSTRACT

Vasoactive intestinal peptide (VIP) signaling via cyclic adenosine monophosphate (cAMP) promotes cell viability and steroidogenesis in ovarian granulosa cells (GC) from a variety of vertebrate species, including the domestic hen. It was recently established in the hen ovary that prior to follicle selection FSH-induced cAMP signaling in undifferentiated GC is absent due to FSH receptor (FSHR) desensitization. Given that VIP receptors (VPAC1 and VPAC2), like the FSHR, represent G protein-coupled receptors that signal via cAMP, we predicted that such VIP signaling in GC from prehierarchal follicles is also absent, and that this desensitization is mediated by βARRESTIN. Initial studies established that mRNA encoding both *VPAC1* and *VPAC2* are expressed within the GC layer throughout follicle development. Nevertheless, undifferentiated GC from prehierarchal (6-8 mm) follicles do not accumulate cAMP in response to a 4 h incubation with chicken VIP, and receptor signaling in response to VIP is attained only in actively differentiating GC collected from follicles already selected into the preovulatory hierarchy. Moreover, expression of mRNA encoding the *Gallus* clock protein, *BMAL1*, significantly increased after treatment with VIP, but only in GC from selected follicles. Significantly, undifferentiated GC transfected with *βARRESTIN1*-siRNA, *in vitro,* enhanced VIP-induced cAMP production, STAR protein expression and progesterone production and promoted *BMAL1* expression. These findings provide evidence that at follicle selection the acquisition of VPAC responsiveness helps initiate and promote the process of GC differentiation. Furthermore, we propose that VIP signaling via cAMP may serve to phase a rhythmic expression of clock genes in GCs from preovulatory follicles.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is a member of the secretin/pituitary adenylate cyclase-activating polypeptide (PACAP) family of neuropeptide hormones, and in its mature form is composed of 28 amino acids [415]. VIP protein is found in various peripheral tissues such as the gut, pancreas, blood vasculature and some sympathetic nerves such as those that innervate the ovary, as well as the suprachiasmatic nuclei (SCN) of the hypothalamus [416-418]. The biological effects of VIP are mediated via two receptors, VPAC1 and VPAC2, both of which are members of the secretin family of G protein-coupled receptors (GPCR) [201, 419]. Within the ovary, immunoreactive VIP neuronal fibers are found dispersed throughout the theca layer of rat [197], pig [420], cow [199] and chicken [37] follicles, whereas VPAC receptors are expressed within both the granulosa and theca layers of the rat [213]. Among the various physiological actions previously ascribed to VIP within the vertebrate ovary are enhanced expression of cytochrome P450 cholesterol side-chain cleavage (*CYP11A*), 17 alpha-hydroxylase (*CYP17*) and aromatase (*CYP19*) mRNA expression [31, 37, 421], STAR protein expression and steroidogenesis [31, 38], primordial follicle development [39], enhanced plasminogen activator activity [31, 196], and the suppression of apoptosis within the granulosa layer [223, 422].

Prior to follicle selection, granulosa cells (GC) from hen prehierarchal follicles are maintained in an undifferentiated state, and initiate differentiation only in the single follicle at the time of selection. It has been previously established that GC from prehierarchal follicles remain undifferentiated due, at least in part, to the absence of FSH receptor signaling via cAMP [396]. Moreover, the absence of FSHR responsiveness is mediated by βARRESTIN-mediated desensitization [19, 28, 34], **Chapter 3**]. While VPAC receptors belong to the same GPCR family that signals via cAMP, it has not yet been established whether VPAC receptors, like FSHR, remain in a desensitized state prior to follicle selection.

In mammals the absence of VIP/VPAC2 signaling within the SCN results in the loss of both

synchronized circadian rhythms [423, 424] and the inability to appropriately encode information provided by photoperiod [425]. The SCN is proposed to encode daily and seasonal information by regulating clock gene expression via VIP and GPCR/cAMP signaling [426]. Input from the SCN to the ovary is predicted to occur via both hormonal and neural signals that, in turn, phase clock gene expression. With regards to neuronal input, there is evidence for direct hypothalamic control of VIP levels in the immature rat ovary such that the transection of ovarian extrinsic innervation or lesions within the preoptic-anterior hypothalamic area reduces ovarian VIP to undetectable levels [427].

In the domestic hen, the timing of ovulation, and presumably follicle selection, is controlled by both photoperiod and an inherent open period for LH release [428]. Although it is well established that photoperiod directly influences the timing of LH surge to induce ovulation, mechanisms that influence the timing of follicle selection have apparently not been studied. Unlike preovulatory follicles, GC from prehierarchal follicles cannot directly respond to the preovulatory LH surge due to an apparent absence of LH receptor expression [17, 27, 429]. In addition, there are no detectable changes [430] or no consistent changes [105, 106] reported for circulating levels of FSH during the ovulatory cycle that could potentially serve to time follicle selection. Accordingly, fundamental questions to be addressed pertain to if and how follicle selection is dependent upon a circadian or ovulatory cycle-related rhythm and how this rhythm is directly or indirectly phased by photoperiod.

There is already considerable evidence for the regulation of ovarian function by circadian clock genes (*BRAIN AND MUSCLE ARYLHYDROCARBON RECEPTOR* [*BMAL1*], *CLOCK*, *PERIOD2* [*PER2*], *CRYPTOCHROME* [*CRY*]). For instance, rhythmic clock gene expression has been described in the ovary of rodents [77, 309, 310] and the domestic hen [47, 48], and these rhythms have been linked to timing steroid synthesis, follicle growth and differentiation plus ovulation [47, 310]. In the hen ovary, the role of clock genes has largely been studied in

differentiated GCs from preovulatory follicles [47, 48]. For instance, Nakao *et al.*, reported that in quail maintained under a 16 h light, 8 h dark (16L:8D) photoperiod, GCs collected from preovulatory follicles over a 24 h interval demonstrate a rhythmic pattern of expression [47]. By comparison, undifferentiated GCs collected from prehierarchal follicles fail to express a detectable rhythm of clock gene expression, *in vitro*. Accordingly, objectives of the present studies were to: 1) assess the capacity for VIP-mediated signaling via cAMP in undifferentiated GCs from prehierarchal follicles compared to actively differentiated GC from selected follicles; and 2) determine whether VIP can induce and potentially phase clock gene expression in cultured GCs.

MATERIALS AND METHODS

Animals and reagents

Single-comb White Leghorn hens 34-55 weeks of age and laying sequences of 5 or more eggs were used for all studies described. Hens were housed individually in laying batteries, with free access to feed and water, under a controlled photoperiod of 15 h light, 9 h dark, (lights on at 01:30h). Hens were euthanized by cervical dislocation 5-10 h following a mid-sequence ovulation and the ovary was immediately removed and transferred into ice-cold sterile 1% NaCl solution until GCs were collected. All procedures described herein were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committees, and were performed in accordance with The Guiding Principles for the Care and Use of Laboratory Animals.

Recombinant human (rh) TGFβ1 and TGFα were purchased from PeproTech (Rocky Hill, NJ), while rhFSH was provided by the National Hormone and Pituitary Program (Torrance, CA). Chicken (ch) VIP was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA).

Collagenase type II was from Worthington Biochemical Corporation (Lakewood, NJ) and 3-isobutyl-1-methylxanthine (IBMX) was from Sigma-Aldrich (St. Louis, MO).

Tissue collection, short-term incubations and culture

Theca layers were collected from 3-5 mm, 6-8 mm, 9-12 mm and the fourth largest (F4) preovulatory follicles, rinsed vigorously to avoid contamination with GCs, and used to characterize expression of *VPAC* receptor mRNA. GC layers were collected from the same developmental stages, and processed for *VPAC* receptor expression, or dispersed for short term (4 h) incubation or culture as previously described [19, 30]. Briefly, for short term incubation GC layers were immediately dispersed by gentle, repeated pipetting, then evenly aliquoted into 12 x 75 mm polypropylene culture tubes with 1 ml of Dulbecco's Modified Eagle Medium (DMEM; HyClone, Thermo Scientific) containing 2.5% FBS (PAA Laboratories, Piscataway, NJ), 1% antibiotic-antimycotic solution (Invitrogen Life Technologies, Carlsbad, CA) and 0.1 mM non-essential amino acids (Gibco). GC were incubated in a shaking water bath at 40 C with IBMX (10 μM) in the absence or presence of chVIP for 4 h. Subsequently, GCs were collected and the assayed for intracellular levels of cAMP while the incubation medium was assayed for progesterone.

For cell cultures, fresh GC layers were dispersed into single cells using 0.3% collagenase, then cultured in 12-well polystyrene culture plates (Beckton Dickinson, Franklin Lakes, NJ) containing 1 ml DMEM plus 2.5% FBS, 0.1 mM non-essential amino acids (Gibco), and 1% antibiotic/antimycotic mixture (Invitrogen Life Technologies) at 40 C in an atmosphere of 95% air : 5% CO_2 [19].

RNA isolation and quantitative real time PCR

Total RNA was extracted from incubated and cultured cells using Trizol Reagent (Life Technologies, Grand Island, NY) and dissolved in nuclease-free water. The purity and concentration of final RNA pellets were determined using a NanoDrop 2000 spectrophotometer (Thermo Science, West Palm Beach, FL). Isolated RNAs (1.5 μg per reaction) were treated with DNase I (Promega, Madison, WI) to eliminate contaminating genomic DNA. These were subsequently used for reverse transcriptase reactions using random primers, a deoxynucleoside triphosphate mix, and M-MLV reverse transcriptase (Promega). For negative RT samples, all components of cDNA synthesis were used, but lacked the reverse transcriptase enzyme to insure the lack of genomic DNA contamination.

Forward and reverse primers encoding *Gallus VPAC1* and *VPAC2*, *INHIBITOR OF DIFFERENTIATION 2* (*ID2*) [76], *BMAL1, PER2, CLOCK* and *18S* rRNA [[30], **Chapter 3**] mRNA were generated using SciTools online software (Integrated DNA Technologies, Coralville, IA) (**Table 4.1**). Final concentrations of the sense and antisense primers were determined for each primer pair based upon optimal amplification efficiency. For real-time PCR, 50 ng of cDNA template was added to 10 μl total reaction mixture including appropriate primers and PerfeCTa Sybr Green FastMix with Low Rox (Quanta Biosciences, Inc., Gaithersburg, MD). Reactions were performed on the AB7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following conditions: 30s at 95 °C, followed by 40 cycles each for 3s at 95 °C, 1 min at 56 °C, and 30s at 72 °C. For water control samples, all components of the PCR or real-time PCRs were added, but water was substituted for the template to check for primer contamination. For primer validation, melting curves were generated for each run and amplified products were sequenced by the Pennsylvania State University Genomics Core Facility. The C_t value (cycle number at which the fluorescence exceeds a threshold level) was determined for triplicate reactions using the 7500 software (v.2.0.4) and quantified by normalizing to *18S* rRNA

using the $\Delta\Delta C_t$ method [392]. Results were expressed as fold-difference compared to an appropriate control tissue or treatment.

Transfection of small interfering RNA (siRNA)

Double stranded siRNA specific for *Gallus βARRESTIN1* was generated using SciTools online software (Integrated DNA Technologies, Coralville, IA) and purchased from Integrated DNA Technologies, Inc. (Table 2). Non-targeting (scrambled) sequence siRNA was used as a control [**Chapter 3**]. Transient transfections were performed on primary GCs grown to 60-70% confluence in 12-well plates with 100 nM of *βARRESTIN1* and scrambled sequence siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a 1:2 ratio as previously reported. The knock-down efficiency of *βARRESTIN* mRNA was determined by quantitative RT-PCR, and both siRNAs were determined to be equally effective in decreasing mRNA expression by 62+/-7% (mean +/- SEM) at 48 h-post transfection compared to cells transfected with scrambled sequence (Scr) [**Chapter 3**].

Western blot analysis

Western blot analysis was performed as previously described [30] to identify STAR protein in GC stimulated with chVIP. Briefly, *βARRESTIN1* siRNA-transfected cells were harvested following a control or chVIP treatment for 4 h, then lysed in RIPA buffer (Santa Cruz, Dallas, TX) including a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Protein concentrations of lysates were assessed by the BCA method (BioRad, Hercules). Gel electrophoresis and protein transfer was conducted according to the protocol of NuPAGE® SDS-PAGE gel system (Invitrogen). Subsequently, blots were incubated in SuperBlock™ TBS blocking solution (Pierce) for 3 h at room temperature, followed by incubation with antibody. The STAR antiserum was generously provided by Dr. Buck Hales [393] and was incubated in a

1:5,000 dilution overnight at 4 °C. Goat anti-rabbit IgG secondary antiserum conjugated with HRP (Pierce) was incubated at a 1:10,000 dilution for 1 h at room temperature. βActin (Sigma) was used at a 1:10,000 dilution as a loading control to assess levels of total protein, and was detected by incubation with goat anti-mouse-horse radish peroxidase (1:10,000). Western blots were developed with ECL™ Plus Chemiluminescence Detection Reagent (Pierce). Chemiluminescent signals were detected using the Storm 860 optical scanner (Amersham Biosciences) and images were analyzed using Image Quant TL™ software (Amersham Biosciences).

Progesterone Enzyme Immunoassay

Culture medium samples were assayed for progesterone using an enzyme immunoassay (EIA). Samples for GCs collected from 6-8 mm follicles were assayed using a high sensitivity EIA kit from Cayman Chemical Co. (Ann Arbor, MI), whereas samples from GCs of 9-12 mm and F4 follicle were measured with a high range EIA kit from Oxford Biochemical Research (Rochester Hills, MI) as previously described [30]. Final concentrations of progesterone were normalized to total amount of RNA or Protein and expressed as pg per μg of RNA or Protein.

cAMP Enzyme Immunoassay

Intracellular accumulation of cAMP was quantitated by EIA kit (Cayman Chemical Co.) as previously described [30]. Briefly, freshly isolated GCs or siRNA-transfected GCs were pretreated with 10 μM of IBMX for 20 min and incubated with chVIP (1 μM) for 4 h. Cells were lysed in 0.1 N HCl for 20 min at room temperature by repeated pipetting and centrifuged at 1,000x*g* for 10 min. Supernatants were harvested and used to measure the accumulation of intracellular levels of cAMP.

Data Analysis

Replicate experiments were conducted a minimum of three times. Data are expressed as fmol cAMP or pg progesterone per μg RNA or protein then subjected to one-way ANOVA using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Data expressed as fold-increase compared to an appropriate control (set to 1.0) and compared among follicle sizes were analyzed by ANOVA without including the control (standardized to 1.0) group. Least square mean values between groups are partitioned using an appropriate least square means test (Tukey or Duncan). Within each follicle size, control versus chVIP treated cells were analyzed by Student's t-test. A probability level of $p \leq 0.05$ was considered statistically significant.

RESULTS

Absence of chVIP-induced cAMP signaling in undifferentiated GCs

Readily detectable levels of *VPAC1 and VPAC2* mRNA were amplified from GC (**Figure 4.1A**) and the theca layer (**Figure 4.1B**) at each stage of follicle development investigated. Levels of *VPAC1* mRNA expression are increased in actively differentiating GC from the most recently selected (9-12 mm) follicle compared to undifferentiated GCs from prehierarchal (6-8 mm) follicles (**Figure 4.1C**). By comparison, levels of *VPAC2* decrease in the granulosa layer and increase in the theca layer with differentiation (**Figure 4.1D**). Actively differentiating GC from recently selected (9-12 mm plus F4) follicles were incubated for 4 h without or with 1, 10, 100 and 1000 nM chVIP then evaluated for levels of *STAR* mRNA and progesterone plus *VPAC1* and *VPAC2* (**Figure 4.2**). Results established that a 1 μM dose of chVIP represented an effective stimulatory dose for *STAR*, progesterone and *VPAC1* without any apparent receptor desensitizing effects.

While incubation for 4 h with chVIP (1 μM) significantly increased cAMP accumulation, progesterone production and STAR protein expression in actively differentiating GC from the most recently selected (9-12 mm) follicle, undifferentiated GC from prehierarchal follicles failed to accumulate a significant increase in cAMP (**Figure 4.3A**). The lack of chVIP-induced cAMP in undifferentiated GCs was reflected by non-detectable levels of progesterone production (**Figure 4.3B**) and STAR protein expression (**Figure 4.3C**). These findings demonstrate that, as recently reported for the FSHR [**Chapter 3**], VPAC signaling via cAMP is absent in undifferentiated GC from prehierarchal follicles.

βARRESTIN1-siRNA treatment increases VIP-responsiveness in undifferentiated GCs

Undifferentiated GCs from 6-8 mm follicles were transiently transfected with *Gallus βARRESTIN1*-siRNA to examine the effects of reduced βARRESTIN expression on VPAC signaling. Scrambled sequence (Scr)- and siRNA-transfected GCs were subsequently treated without or with chVIP (1 μM) for 4 h to evaluate VPAC responsiveness by measuring intracellular accumulation of cAMP. As previously reported with respect to FSH [**Chapter 3**], both Scr and βARRESTIN siRNA-transfected cells acquired some level of responsiveness to treatment with VIP, and this is attributed to the initiation of differentiation when GC from prehierarchal follicles are cultured in the absence of inhibitory MAP kinase signaling [30, 79]. Importantly, however, chVIP-induced accumulation of cAMP was significantly increased in *βARRESTIN1*-siRNA transfected cells compared to Scr-transfected cells (**Figure 4.4A**). Both chVIP-induced STAR protein expression (**Figure 4.4B**) and progesterone production (**Figure 4.4C**) were significantly increased in *βARRESTIN1*-siRNA transfected cells. Moreover, levels of *ID2* mRNA, a marker of GC differentiation [76], were significantly increased in *βARRESTIN1*-siRNA transfected cells without VIP treatment and increased further when challenged with VIP

(**Figure 4.4D**). These findings provide evidence that βARRESTIN, at least in part, mediates VPAC desensitization in undifferentiated GCs.

Regulation of clock gene expression in hen GCs

BMAL1, *CLOCK*, and *PER2* are each expressed in hen GC both prior and subsequent to follicle selection (**Figure 4.5**), however, only levels of *BMAL1* and *CLOCK* showed an increase associated with follicle selection. To examine whether clock gene expression can be induced by VIP signaling, freshly GC were isolated from different stages of follicles and incubated with or without chVIP (1 μM) for 4 h. While *BMAL1* mRNA expression was increased in response to chVIP in actively differentiating (9-12 mm) and differentiated (F4) GC (**Figure 4.6**), *CLOCK* and *PER2* mRNA expression were not significantly altered. Significantly, undifferentiated GC failed to respond to a chVIP challenge with any increase in *BMAL1* gene expression. These results demonstrate that *BMAL1* expression within the granulosa layer can be modulated by VIP signaling, but only after follicle selection.

Knock-down of ARRESTIN1 expression enhances BMAL1 expression in undifferentiated GC.

To assess whether clock gene expression prior to selection is precluded by a βARRESTIN-mediated mechanism, undifferentiated GCs were transiently transfected with *βARRESTIN1*-siRNA or a scrambled sequence as described above. While *PER2* and *CLOCK* mRNA expression was unaffected by treatment, *BMAL1* mRNA expression was significantly increased in *βARRESTIN1*-siRNA transfected cells challenged with chVIP compared to Scr-siRNA transfected cells (**Figure 4.7**).

Clock gene expression in cultured GC

Undifferentiated GC from prehierarchal follicles were seeded into 12-well plates and maintained in an undifferentiated state by treatment with TGFα (10 ng/ml) [30, 79] throughout the culture period. Additional GCs were seeded into plates without adding TGFα to initiate the process of differentiation. Cells from each plate were collected at plating (0 h) and at 6 h intervals thereafter over a 30 h period to monitor the expression of *BMAL1*, *PER2*, *CLOCK* and *STAR* expression. *BMAL1*, *CLOCK* and *STAR* gene expression showed no significant change in undifferentiated GCs during the culture interval, while *PER2* expression levels declined over time (**Figure 4.8**). By comparison, GC that initiate differentiation in the absence of TGFα treatment showed an increase in both *BMAL1* and *CLOCK* expression that peaked at 24 h of culture. While mean levels of *STAR* expression were highest at 18 h of culture, the variability in levels (81.4-fold to 486.1–fold 0 h) precluded statistical significance.

DISCUSSION

Results from the present studies provide support for a model in which the GPCRs, VPAC1 and/or VPAC2, in undifferentiated GC from prehierarchal follicles remain in a desensitized state prior to follicle selection (**Summary model 2**). These findings are similar to those from a previous report in which the absence of FSH responsiveness (*e.g.*, signaling via cAMP) prior to follicle selection is, at least in part, attributed to βARRESTIN-mediated desensitization [**Chapter 3**]. Accordingly, we propose that prior to follicle selection the absence of GPCR-mediated cAMP formation within the GC layer results in non-detectable to very low levels of *CYP11A* [30, 396], *STAR* [18], and *ID2* [76] expression as well as the absence of progesterone production. At follicle selection the acquisition of FSHR- and VPAC-mediated signaling via cAMP, specifically within the GC layer, initiates or dramatically enhances expression for each of these genes, and eventually initiates *LH RECEPTOR* (*LHR*) expression

[17]. The undifferentiated state of the GC layer from prehierarchal follicles contrasts with the adjacent theca layer, as both *FHSR* and *LHR* are highly expressed within the theca at the 3-5 mm and 6-8 mm stages of follicle development [17, 127], and each gonadotropin receptor is responsive to its respective ligand [[431], **Chapter 3**].

Previous studies established that VIPergic neurons radiate through the theca layer of hen prehierarchal follicles with some immunoreactive terminals localized adjacent to the basement membrane [37]. Although the sequence of chicken VPAC cDNA and its localization have been identified [210], we demonstrate for the first time that both VIP receptors, VPAC1 and VPAC2, are expressed at varying levels in both hen GC and theca during follicle development (**Figure 4.1**). Treatment of hen GC from preovulatory follicles with VIP induces cAMP accumulation, promotes steroidogenesis and enhances plasminogen activator activity [31, 37]. Preliminary studies reported herein reestablished that a 1 μM dose of chVIP reliably induces cAMP accumulation [37], *STAR* and *VPAC1* mRNA expression plus progesterone production in GC from the most recently selected (9-12 mm and F4) follicles (**Figure 4.2**). While apparently neither circulating nor neuronal levels of VIP have been reported for the hen, this dose of VIP is the same established by Kowalewski *et al*. for immortalized (KK1) and primary rodent GCs [38, 432].

For the present experiments with undifferentiated GC, we initially hypothesized that due to FSHR desensitization [**Chapter 3**], VIP/VPAC signaling via cAMP may play a role in initiating the process of GC differentiation at follicle selection. Nevertheless, similar to FSH [**Chapter 3**], a challenge of freshly collected cells from prehierarchal follicles with 1 μM chVIP failed to promote cAMP formation, *STAR* expression or progesterone production (**Figure 4.3**). Similar to results reported for the FSHR [**Chapter 3**], siRNA-mediated knock-down of *βARRESTIN* mRNA expression (by 62+/- 6.9%), significantly enhanced the ability of VIP/VPAC signaling to promote each of these markers of GC differentiation, including *ID2* expression [76].

We have previously reported that the culture of undifferentiated hen GC rapidly results in the acquisition of FSH-responsiveness and the initiation of GC differentiation. By contrast, supplementing the culture medium with an epidermal growth factor receptor ligand (*e.g.*, transforming growth factor α, betacellulin) prevents differentiation [19, 30, 76]. In the present studies, undifferentiated GC cultured for 48 h with the control siRNA (Scr) sequence (**Figure 4.4**) resulted in a significant increase of chVIP-induced cAMP, STAR expression and progesterone production, plus *ID2* expression. This response contrasts with the absence of a VIP-induced response following a 4 h challenge of freshly collected undifferentiated GC (**Figure 4.3**). Collectively, these data provide additional support for our working model that follicle selection and the subsequent initiation of GC differentiation in the hen only occurs subsequent to a release from MAPK and βARRESTIN-mediated inhibitory signaling (**Summary model 2**). Only then is GC responsiveness attained for both the FSHR [**Chapter 3**] and VPAC, followed by FSH- and VIP-mediated differentiation. The most proximal mechanism mediating the removal of inhibitory signaling within the selected follicle is currently under investigation. It is worthy of note that this model for the process of ovarian follicle selection in the hen contrasts with monovulatory mammals in that the follicle that attains dominance is proposed to be selected on the basis of an enriched follicular fluid microenvironment mediated by enhanced FSH responsiveness [433].

In light of these data, our alternate hypothesis was that immediately subsequent to follicle selection, VIP derived from either a neuronal [37, 434] or humoral [435-437] origin may serve to regulate expression of clock genes within the GC layer. It has previously been established that the quail and chicken ovary represents a clock-dependent peripheral organ whose phase is regulated by one or more factors produced by a central clock within the neuroendocrine system [47, 48]. For instance, the expression of clock genes in freshly collected GC layers from the largest (F1) preovulatory follicle of quail [47] and the domestic hen [48] held under a 16 h L : 8 h D photoperiod shows significant variations over a 24 h sampling interval. This rhythmicity is

proposed to be regulated by the preovulatory LH surge [47]. In turn, CLOCK/BMAL1 heterodimers can bind to one or more E-box within the *STAR* promoter to up-regulate *STAR* mRNA expression [47]. Both LH and FSH play a role in regulating a circadian clock in the mammalian ovary [77, 308, 310]. While the preovulatory LH surge (acting via cAMP signaling) can provide a mechanistic link for communicating circadian information from the central pacemaker to both avian and mammalian preovulatory follicles [47, 48, 308, 310], there are very low levels of *LHR* mRNA expressed in GC from 9-12 mm hen follicles [17, 429]. Furthermore, LH treatment fails to induce any significant increase in cAMP accumulation at the early stage of GC differentiation [122]. In addition, there are no apparent photoperiod- or ovulatory cycle-related fluctuations of circulating FSH detected in laying hens [105, 106]. Thus, we speculated that VIP reaching the ovary via a neural and/or humoral route may serve to promote clock gene expression in GC immediately subsequent to follicle selection.

In the present study, the expression of *BMAL1*, *CLOCK* and *PER2* was readily detected in GC from prehierarchal follicles (**Figure 4.5**), yet a 4 h challenge with chVIP initiated a significant increase in *BMAL1* only in GC from selected (9-12 mm and F4) follicles (**Figure 4.6**). By comparison, chVIP induced a lesser increase in *PER2* expression in actively differentiating GC and no significant increase in levels of *CLOCK*. A role for VIP to enhance *BMAL1* expression is supported by the finding that siRNA-mediated knock-down of βARRESTIN results in increased levels of *BMAL1*, but not *CLOCK* or *PER2* (**Figure 4.7**). From these results it is predicted that cAMP-mediated expression of BMAL1 may represent the rate-limiting step to regulating clock gene rhythmicity in actively differentiating hen GC. Interestingly, a previous study reported that the LH surge, *in vivo*, increased levels of both *BMAL1* and *PER2* in GC collected from the largest (F1) follicle [48]. Given that there is typically an anti-phase relationship between *BMAL1-CLOCK* and *PER2-CRY*) expression [438], reasons for the apparent difference in results between these two studies are not clear.

Previous results from quail housed under a 16 h L: 8 h D photoperiod demonstrated that GC collected from preovulatory follicles (F1, F2 and F3 follicles) showed significant variations in PER2 and PER3 expression over a 24 h interval. By comparison, quail GC from prehierarchal follicles showed no rhythmicity of clock gene expression over a 24 h interval [47]. Consistent with the latter results, undifferentiated GC from hen prehierarchal follicles cultured in the presence of TGFα (to maintain an undifferentiated state) over a 30 h interval also failed to demonstrate any significant changes in *BMAL1* or *CLOCK* expression. Significantly, undifferentiated GC cultured in the absence of TGFα (*e.g*., actively differentiating cells) showed significant differences in *BMAL1* and *CLOCK* expression over the same interval (**Figure 4.8**). Occurring coincident with increasing levels of *BMAL1* and *CLOCK* are levels of *STAR*. As noted above, *STAR* gene expression is initially up-regulated in GC at follicle selection by FSHR- and VPAC-induced cAMP [27] and can be mediated at the transcriptional level by BMAL1/CLOCK heterodimers [47, 438].

In summary, results from the present studies provide evidence that, as previously reported for the FSHR, the VIP receptor(s), VPAC1 and/or VPAC2, expressed within the granulosa layer remain in a desensitized state and fail to initiate signaling via cAMP until the follicle has been selected into the preovulatory hierarchy. At follicle selection the capacity for VPAC signaling helps initiate and promote both the process of GC differentiation and rapid growth and maturation in preparation for ovulation. Our results also provide evidence that VIP signaling via cAMP can promote *BMAL1* expression and may serve to initiate and subsequently phase the rhythmic expression of clock genes in GCs from preovulatory follicles.

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Table 4.1. Sequences of *Gallus*-specific forward and reverse primers used for real-time PCR, together with the NCBI nucleotide accession number.

Gene	Accession #	Sequence
VPAC1	NM_001097523.1	FWD 5'-GGGTGCTGGGAGGAAATAATAG-3' REV 5'-CGGATGATGCAAATGAAGAGAATG-3'
VPAC2	NM_001014970.1	FWD 5'-GTTTCCTGACCGCAGTTCCAACAA-3' REV 5'-CTTCCAGCACAAACTCCGCCATTT-3'
STAR	NM_204686	FWD 5'-TGCCTGAGCAGCAGGGATTTATCA-3' REV 5'-TGGTTGATGATGGTCTTTGGCAGC-3'
BMAL1	AF205219.1	FWD 5'-AACGAGCCAGATAACGAGGGTTGT-3' REV 5'-AGGTTTCACCCTGATCTCACCGTT-3'
CLOCK	AF246959.1	FWD 5'-TATGGGAAAGGGAAGTCATGTT-3' REV 5'-CCACTGGTGGTACGTGATATAG-3'
PER2	AF246956.1	FWD 5'-GCAAGCCTGAAAGTGTTGTATC-3' REV 5'-CTCCACTTGGACCATCTTCTATC-3'
ID2	AF068831	FWD 5'-GCTCAACACAGACATCAGCATCC-3' REV 5'-GACTCCCCTAAAGAACTGAACGC-3'
18S rRNA	AF173612	FWD 5'-TTAAGTCCCTGCCCTTTGTACAC-3' REV 5'-CGATCCGAGGAACCTCACTAAAC-3'

Table 4.2. siRNA Sequences of *Gallus βARRESTIN1* and non-targeting control.

Target	Sequence
βARRESTIN1	Sense 5'- CCAACAAGACGGUGAAGAAGAUCAA-3' Antisense 5'- UUGAUCUUCUUCACCGUCUUGUUGGUG-3'
Scrambled	Sense 5'- CGUUAAUCGCGUAUAAUACGCGUAT-3' Antisense 5'- AUACGCGUAUUAUACGCGAUUAACGAC-3'

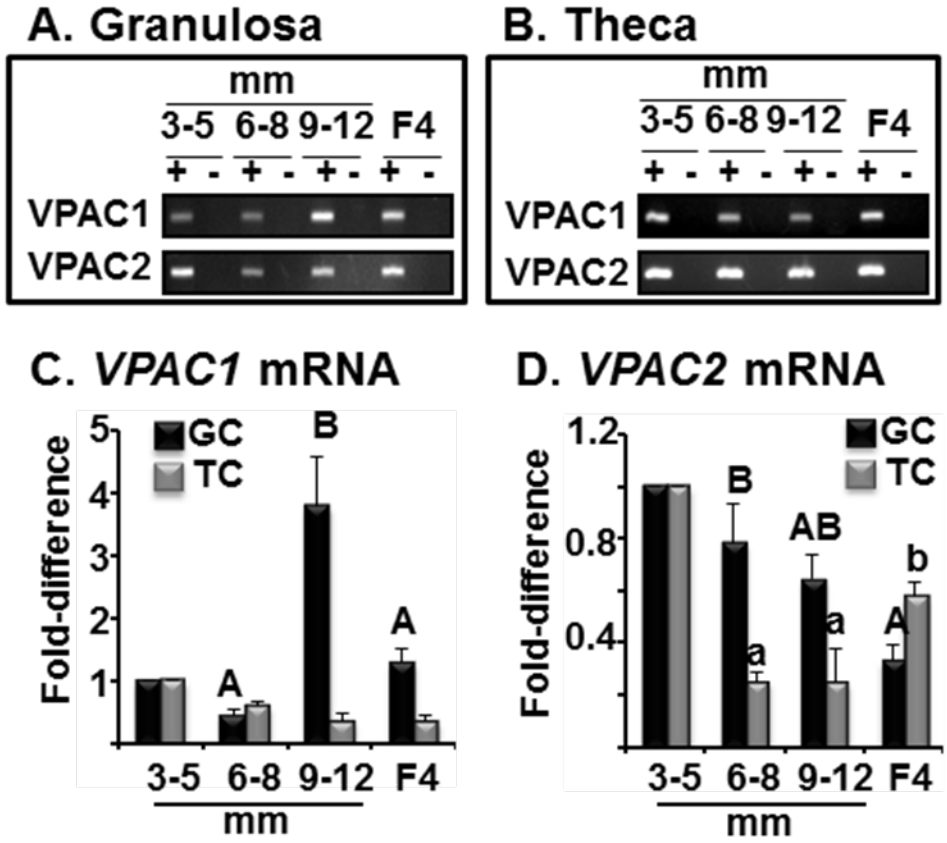

Figure. 4.1. The VIP receptors, VPAC1 and VPAC2, are expressed in granulosa (GC) and theca (TC) cells prior and subsequent to follicle selection.

A, **B**: *VPAC* expression GC and TC tissues collected from 3-5 mm and 6-8 mm prehierarchal follicles, plus 9-12 mm and the F4 preovulatory follicles as determined by polymerase chain reaction (PCR) amplification. +, - Represents the presence or absence of reverse transcriptase. **C**, **D**: Expression of *VPAC1* and *VPAC2* mRNA in follicles collected prior to selection (3-5 and 6-8 mm) and after selection (9-12 mm and F4). Data are expressed as a fold-difference compared to levels in 3-5 mm follicles. N=4-6 replicates; a,b; A,B: $P<0.05$.

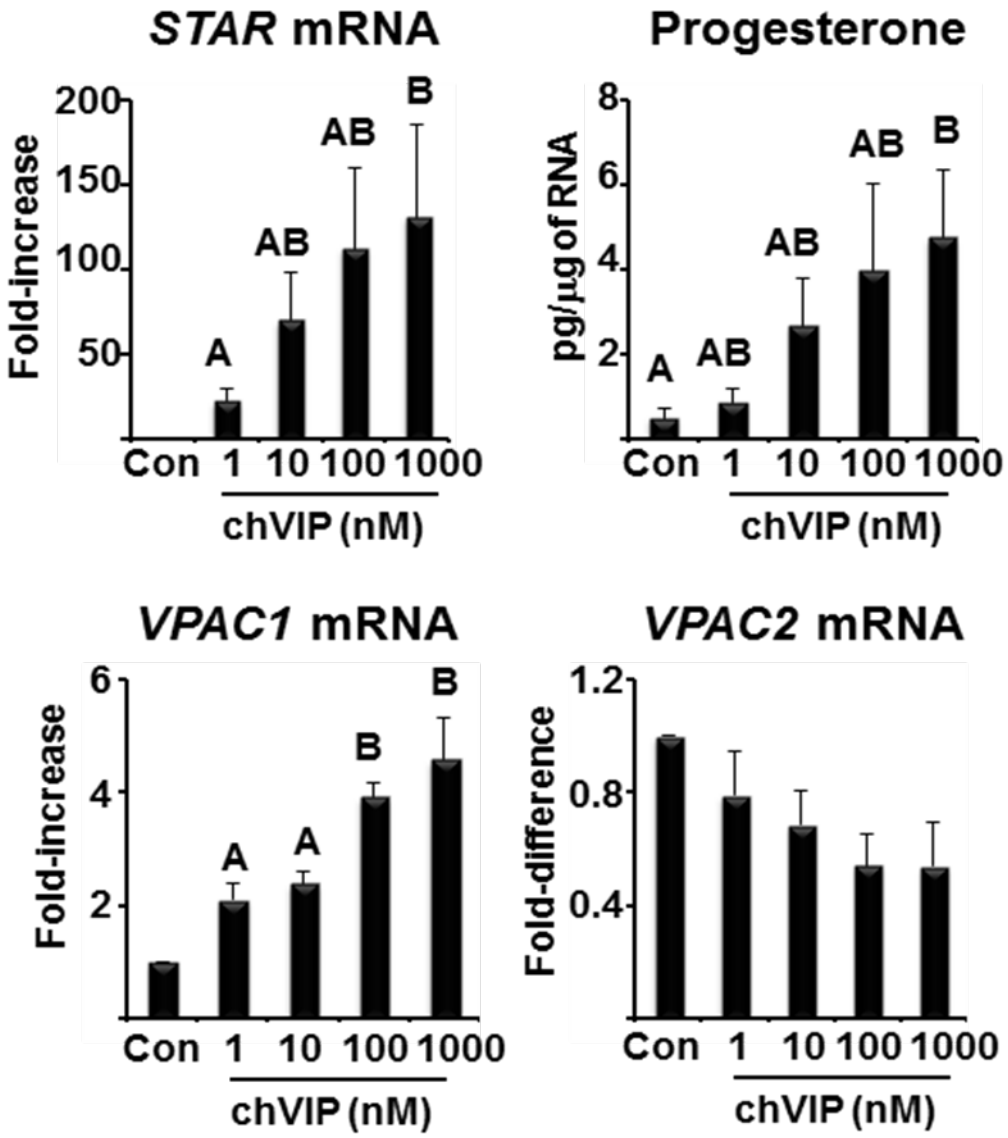

Figure 4. 2. Response of actively differentiating GC (from 9-12 mm and F4 follicles) to 1 to 1000 nM chVIP measured by *STAR, VPAC1* and *VPAC2* mRNA expression and progesterone production. N= 4 replicate experiments. A,B: P<0.05.

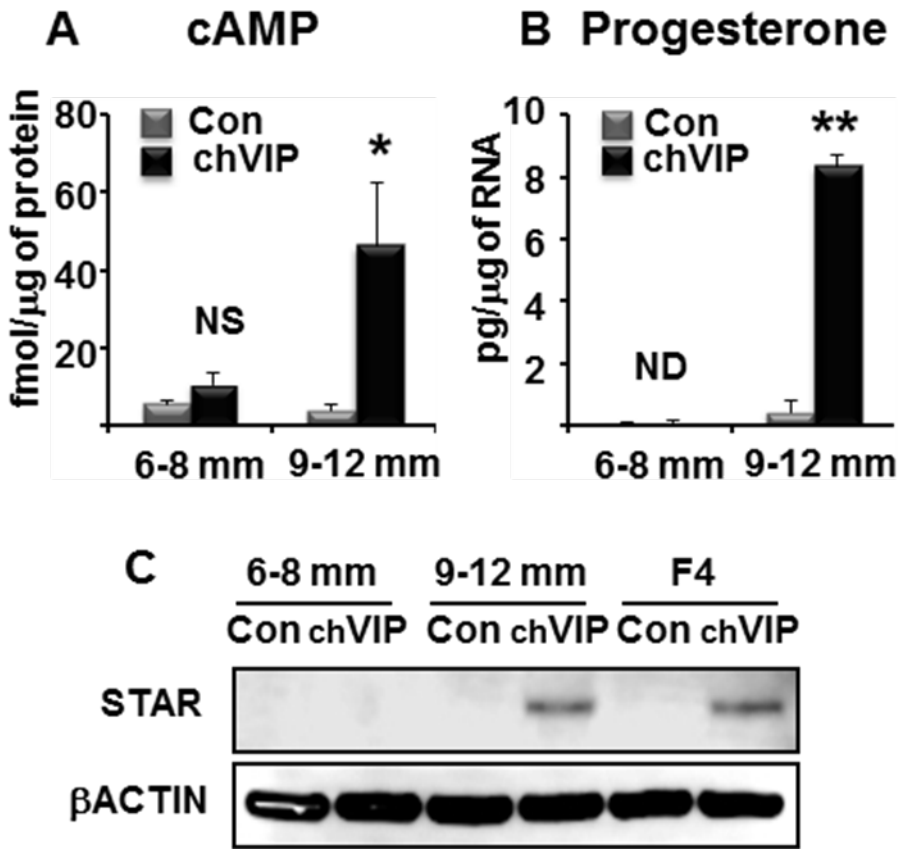

Figure 4.3. Absence of VIP signaling via cAMP in undifferentiated GC following a 4 h incubation with 1 µM chVIP.

Compared to actively differentiating GC from 9-12 mm follicles, undifferentiated GC from 6-8 mm prehierarchal follicles cultured in the presence of 10 µM IBMX failed to initiate any significant cAMP accumulation (**A**). As a consequence, GC from 6-8 mm follicles do not initiate progesterone production (**B**) or STAR protein expression (**C**). NS: $P>0.13$; *: $P<0.05$; **: $P<0.001$ by t-test; ND, below assay sensitivity; N= 4 (panel A) or 3 (panel B) replicate experiments. The STAR Western blot was replicated twice with similar results.

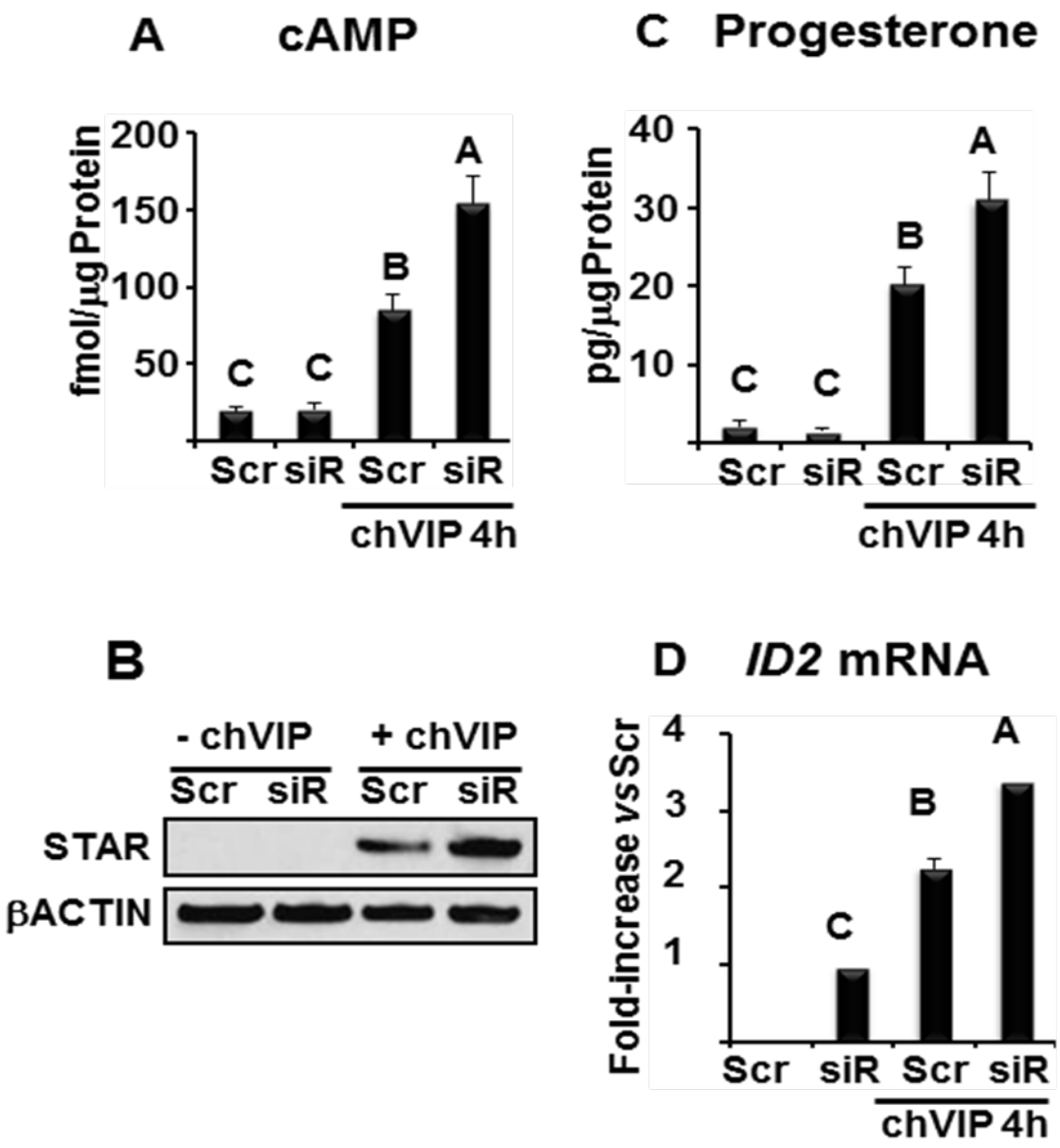

Figure 4. 4. Transfection of undifferentiated GC with *Gallus βARRESTIN1*-siRNA (siR) enhances chVIP (1 µM for 4 h)-induced cAMP accumulation (**A**), progesterone production (**B**), STAR protein expression (**C**) and *ID2* expression (**D**) compared to the scrambled sequence (Scr)-plus-VIP treatment control. Data are expressed as fmol/µg protein (cAMP), pg/µg protein (progesterone) or as a fold-increase compared to the Scr control (N= 4-6 replicate experiments). A,B,C: $P<0.05$. The STAR Western blot was replicated once with similar results.

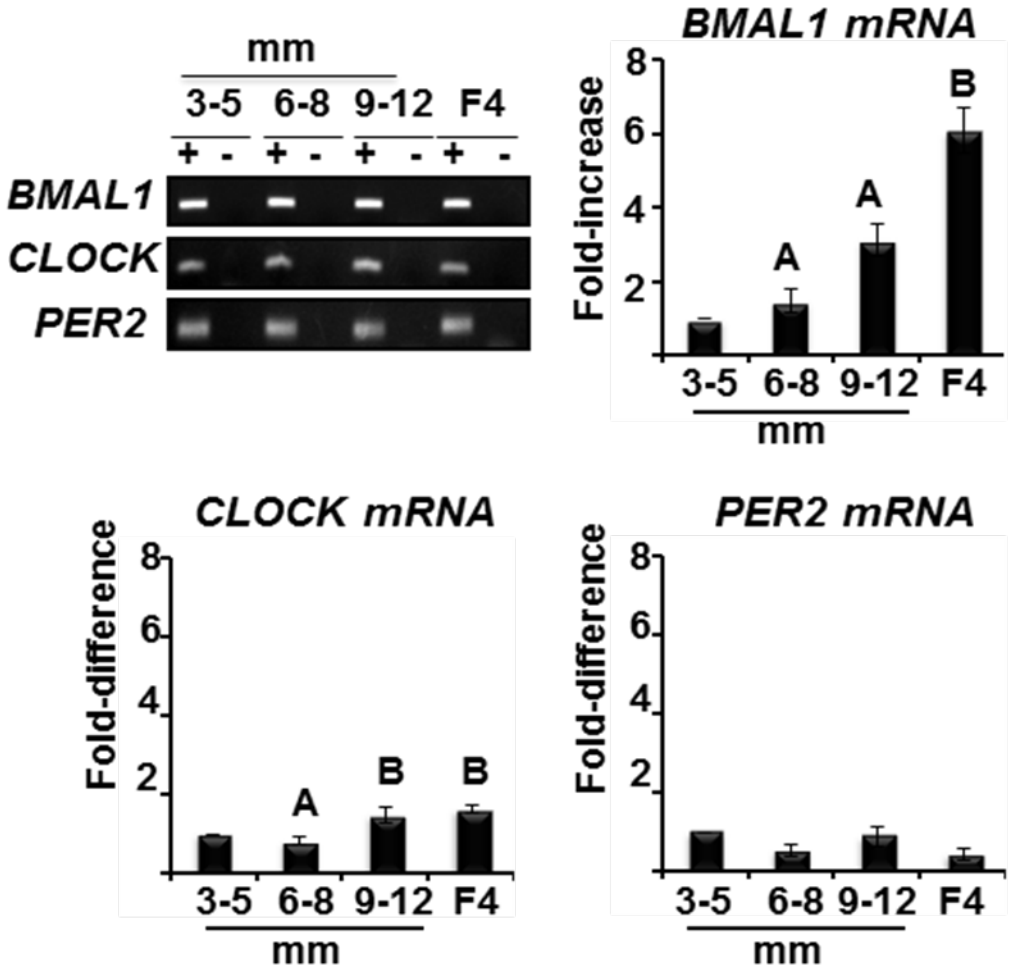

Figure 4.5. Clock gene expression in undifferentiated GC from prehierarchal (3-5 mm and 6-8 mm) follicles, and actively differentiating GC from the most recently selected (9-12 mm) and fourth largest (F4) preovulatory follicle. Data are expressed as fold-difference compared to the 3-5 mm GC layer. +, - Represents the presence or absence of reverse transcriptase. A, B: $P<0.05$, N=3-4 replicate experiments.

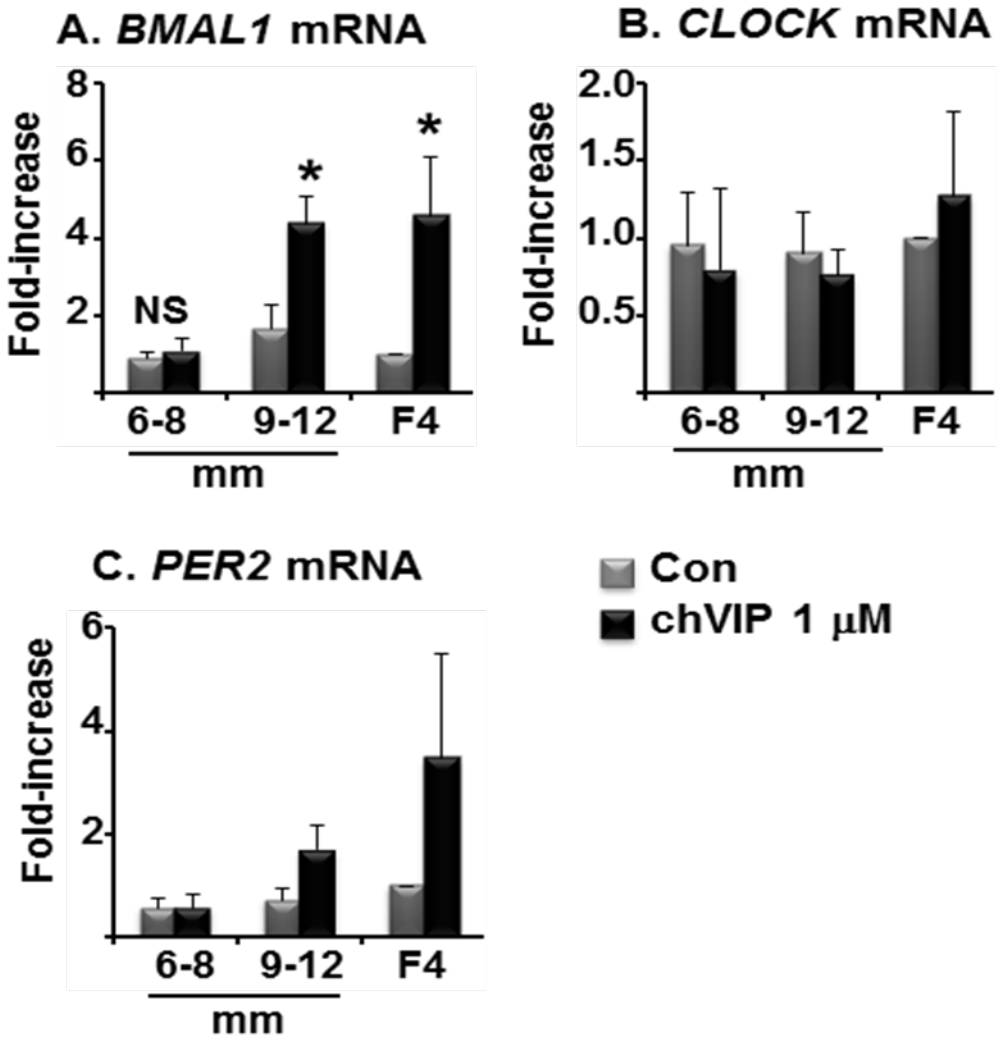

Figure 4.6. chVIP (1 μM)-induced clock gene expression is absent in undifferentiated GC from 6-8 mm (prehierarchal) follicles after a 4 h incubation. By comparison, chVIP induced a significant increase in *BMAL1* (**A**), but not *CLOCK* (**B**) or *PER2* (**C**), expression in actively differentiating GCs from recently selected (9-12 and F4) follicles. *: $P<0.05$ by unpaired t-test, N=3 replicate experiments.

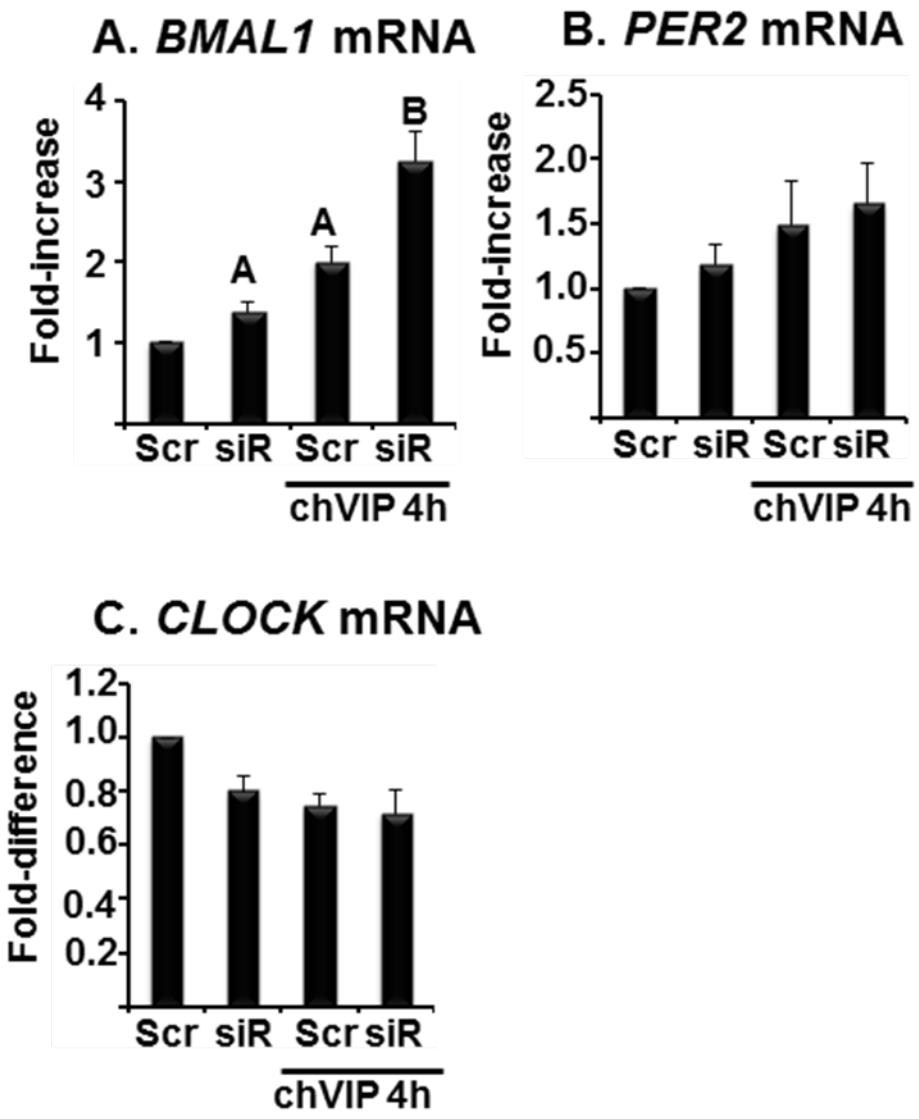

Figure 4.7. Effect of transfection with *Gallus βARRESTIN1*-siRNA (siR) on *BMAL1* (**A**), *PER2* (**B**) and *CLOCK* (**C**) mRNA expression in GCs from 6-8 mm follicles treated without or with chVIP (1 μM) for 4 h (N= 3-5 replicate experiments). Data are expressed as a fold-difference compared to the scrambled sequence (Scr) control. A,B: $P<0.05$.

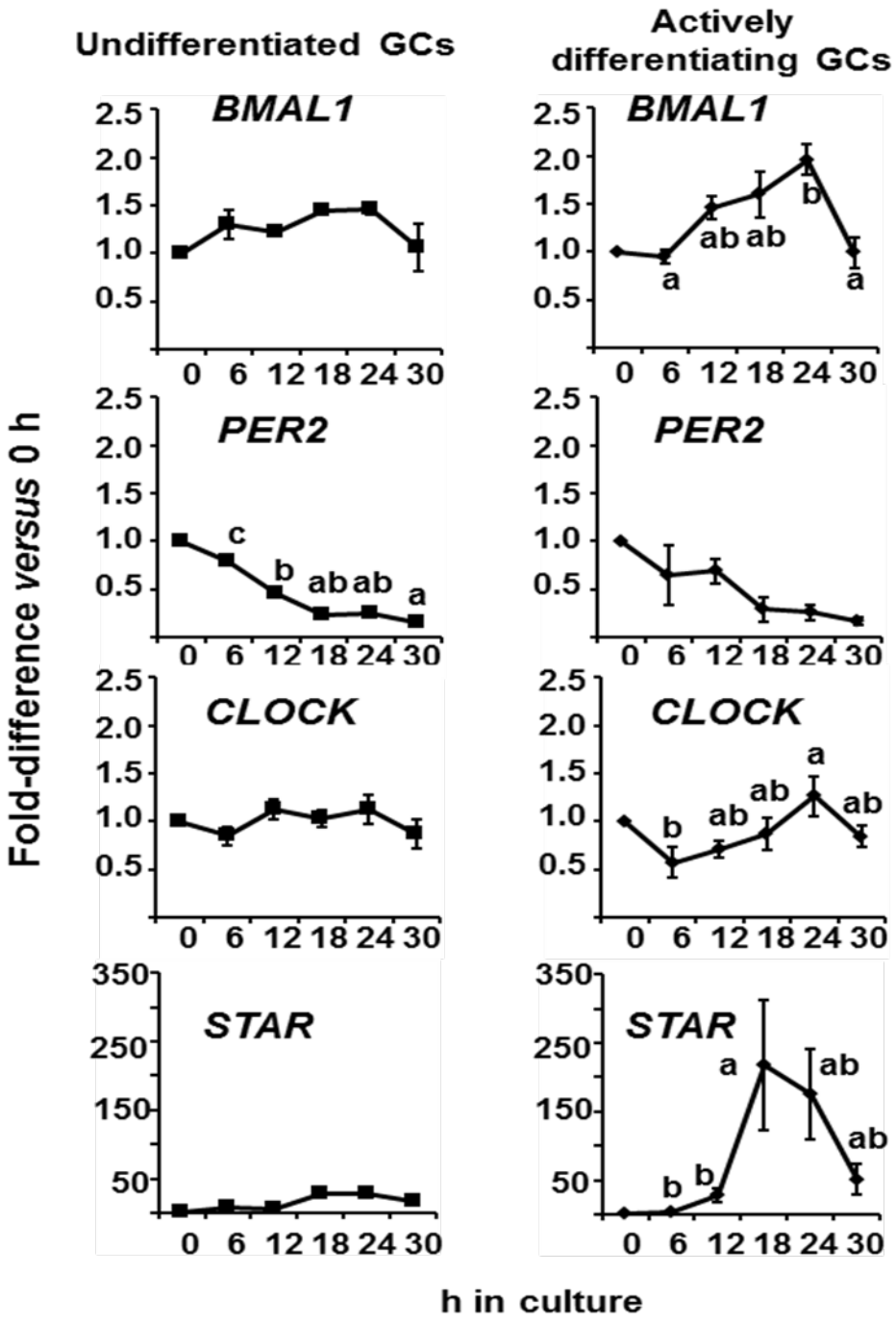

Figure 4.8. Clock gene expression in GCs from 6-8 mm follicles maintained in an undifferentiated state (*e.g.*, cultured in the presence of 10 ng TGFα/ml) compared to actively differentiating (cultured without TGFα) for 0 to 30 h. Note that mRNA levels are expressed as fold-difference compared to 0 h cells. a,b,c: $P<0.05$; N=4 replicate experiments.

Chapter 5

The Expression and Regulation of Vascular Endothelial Growth Factor and Angiopoietin during Follicle Development in The Hen Ovary

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Key Words : Granulosa cells, Angiogenesis, VEGF, Angiopoietin,

ABSTRACT

Ovarian follicle development requires a network of blood vessels that is initiated and supported by the actions of angiogenic factors. Within the avian ovary such factors enable follicles to acquire increasingly greater amounts of oxygen, nutrients and various hormones beginning at the time of follicle selection. Although angiogenic factors and their receptors have been well studied in mammalian ovaries, there have been essentially no studies conducted in chicken ovarian follicles. Hen prehierarchal follicles (*e.g.*, prior to follicle selection) are visibly less vascularized than are preovulatory follicles subsequent to selection, and the increase in the number and size of blood vessels observed immediately following follicle selection is required for rapid follicle growth and dramatically increased yolk incorporation. The present studies investigate levels of *VEGF*, *ANGPT-1* and *ANGPT-2* mRNAs together with their receptors (*VEGFR and TIE-2*, respectively), together with endocrine, paracrine and autocrine factors that regulate angiogenesis during follicle development. Levels of *VEGF* mRNA gradually increase while levels of *ANGPT-2* decrease in granulosa cells (GCs) during follicle development. By comparison, levels of *VEGF* and *VEGFR* together with *ANGPT-1*, *ANGPT-2* and *TIE-2* mRNA in the theca layer were not significantly changed prior to *versus* following selection. Both gonadotropins (FSH and LH) and TGFβ superfamily members (TGFβ1 and BMP4) stimulate *VEGF* and inhibit *ANGPT-2* mRNA expression in actively differentiating GCs from 9-12 mm and F4 follicles. By comparison, only BMP4 and TGFβ1, and not FSH, increased levels of *VEGF* in undifferentiated GCs from prehierarchal follicles. Accordingly, it is concluded that prior to follicle selection VEGF and ANGPT-2 are preferentially regulated by TGFβ1/BMP4. This level of signaling is sufficient to maintain minimal levels of vascularization. At follicle selection, actively differentiating GCs increase levels of *VEGF* mRNA expression and decrease *ANGPT-2* expression initially in response to FSH and later by LH. These events are predicted to rapidly and dramatically enhance vascular formation within the theca layer.

INTRODUCTION

Angiogenesis represents the process of new blood vessel formation from pre-existing vasculature and is strongly associated with physiological events such as embryonic development, tumor growth, wound healing, and inflammation [439]. In particular, the presence of blood vessels is required for the maintenance of various female reproductive processes such as ovarian follicle maintenance, development and ovulation [63, 440-443]. It is well established that angiogenesis occurs throughout ovarian follicle development, supplying nutrients and hormones and providing for follicle development, oocyte maturation, and corpus luteum formation [70, 444]. A number of studies have demonstrated that angiogenic factors secreted from GCs play critical roles in the maintenance of follicle survival and growth by inducing angiogenesis [68, 80, 445]. Any misregulation of angiogenesis can contribute to disorders such as infertility, anovulation, polycystic ovary syndrome (PCOS) and ovarian cancer [61, 446-449]. Within the chicken ovary, blood vessels are not uniformly distributed throughout the stages of follicle development. In particular, numerous large blood vessels are visible on the exterior surface of preovulatory follicles, compared to a sparse population of small diameter vessels surrounding prehierarchal follicles. Whereas it has been estimated that the two largest preovulatory follicles each receive 12.4% to 17.4% of the total blood flow to the ovary, blood flow to all the prehierarchal follicles combined is estimated to total 12.6% [450]. Nonetheless, there is essentially no information currently available regarding the hormonal regulation of angiogenesis within the hen ovary.

As ovarian follicle development progresses, the theca layer of growing follicles establish a network of blood vessels regulated by angiogenic factors that include vascular endothelial growth factor (VEGF) and angiopoietin (ANGPT) [63, 447, 451]. In particular, VEGF produced by vascular endothelial cells is a strong mitogen for vascular endothelial cells and a potent agonist of angiogenesis for a variety of physiological events that include embryogenesis, tissue

development, and tumor growth [62, 348]. Moreover, VEGF has been implicated in female reproductive physiology including follicle development, menstruation cycles, and the formation of corpus luteum [440, 447, 452]. These findings indicate that VEGF plays a significant role in critical ovarian functions such as steroidogenesis and follicle maturation. The VEGF family is composed of six members (VEGF-A, -B, -C, -D, -E, and -F), of which VEGF-A (referred to herein as VEGF), has been well studied in mammalian ovarian follicles [453, 454]. VEGF-A is comparatively less expressed during early development and this expression significantly increases within the granulosa and theca layers according to the developmental stages of follicles in mammalian ovaries [343, 455-458]. VEGF-A is comparatively less expressed during early development and this expression significantly increases within the granulosa and theca layers according to the developmental stages of follicles in mammalian ovaries [343, 455-458]. The VEGF family exerts their biological effects by binding to three structurally related receptor tyrosine kinases (RTKs), VEGF receptors (VEGFR-1, -2, and -3). VEGF-A and -B bind to VEGFR-1 (also known as Flt-1) that has positive regulator of monocytes and macrophages [459-461]. However, secreted VEGFR-1, which lacks the transmembrane portion of the receptor, works as a negative regulator with the high affinity to VEGF-A by preventing binding to the VEGFR-2 [462-465]. VEGF-A, -C, and -D are ligands of VEGFR-2 and promote the migration and proliferation of endothelial cells via VEGFR-2 (also known as KDR/Flk-1) expressed at high levels in endothelial cells [466-468]. VEGF-C and -D bind to VEGFR-3 expressed at high levels in lymphatic endothelial cells and associated with lymphangiogenesis [469-471].

Although VEGF is the major initiator of angiogenesis, newly established capillaries become structurally and functionally matured by pericytes and smooth muscle cells. ANGPTs are known to function in modulating vessel remodeling and maturation by inducing the migration of pericytes and smooth muscle cells [364, 472, 473]. While there are four structurally related isoforms (ANGPT-1 to -4), ANGPT-1 and -2 are mostly studied in angiogenesis. Similar to

VEGF family, ANGPT-1 and -2 also exert their functions via TIE-2 receptor with tyrosine kinase activity [365, 474-476]. While ANGPT-1 binds to TIE-2 receptor and induces phosphorylation of receptor to initiate downstream signaling pathways, including the phosphoinositide 3-kinase (PI3K) pathway [477], ANGPT-2 with a similar affinity for TIE-2 antagonizes ANGPT-1-induced phosphorylation of TIE-2 [74, 378]. In female reproductive system, ANGPT-1, ANGPT-2, and TIE-2 are found in the ovarian follicle and corpus luteum [478-480]. It has been demonstrated that disrupted balance of ANGPT-1 and ANGPT-2 by injecting ANGPT-2 inhibits follicular angiogenesis, ovulation, and the formation of corpus luteum in primates [481]. Therefore, the ratio of ANGPT-1 to -2 is critical to regulating maintenance and stability of blood vessels. Previous studies have provided evidence that follicle development is dependent upon VEGF production and increased blood supply in mammalian ovaries [456, 482-484]. One implication is that the appropriate acquisition of vasculature is an important factor regulating follicle selection. In contrast, follicular atresia is closely associated with reduced and inadequate development of vasculature surrounding and within the theca layer [65, 485]. Gonadotropins induce expression and secretion of angiogenic factors that include VEGF, platelet-derived growth factor (PDGF) and ANGPT in GCs [486-489]. Moreover, several members of the transforming growth factor beta (TGFβ) superfamily have been demonstrated to induce VEGF expression in bovine and human GCs [80, 81, 488]. Despite considerable evidence demonstrating the importance of angiogenic factors in the ovarian follicle development of mammals [452, 489-492], the expression and mechanisms regulating angiogenic factors are not defined within the chicken ovary. Specifically, prior to follicle selection hen prehierarchal follicles are sparsely populated with small blood vessels. Immediately following selection the number and size of blood vessels servicing each preovulatory follicle are visibly increased, which provides for rapid follicle growth and dramatically increased yolk incorporation. Accordingly, it is hypothesized that this increase in vasculature is mediated by angiogenic factors secreted within theca and granulosa layers.

Accordingly, the objectives of the present study were to establish levels of *VEGF/VEGFR* and *ANGPTs/TIE-2* expression during hen follicle development and to establish autocrine/paracrine mechanisms by which *VEGF* and *ANGPT* expression are regulated.

MATERIALS AND METHODS

Animals and reagents

Single-comb White Leghorn hens 34-55 weeks of age and laying sequences of 5 or more eggs were used in the studies described. Animals were housed individually in laying batteries, with free access to feed and water, under a controlled photoperiod of 15 h light, 9 h dark, (lights on at 01:30h). Hens were euthanized by cervical dislocation 5-10 h following a mid-sequence ovulation and the ovary was immediately removed and transferred into ice-cold sterile 1% NaCl solution until GCs were isolated. All procedures represented herein were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committees, and were performed in accordance with The Guiding Principles for the Care and Use of Laboratory Animals.

Recombinant human (rh) TGFβ1 and rhBMP4 were purchased from PeproTech (Rocky Hill, NJ), while rhFSH and ovine (o) LH were provided by the National Hormone and Pituitary Program (Torrance, CA). Collagenase type II was from Worthington Biochemical Corporation (Lakewood, NJ).

Tissue collection and culture

Ovarian follicles from individual hens were grouped by developmental stage, and GCs were isolated and dispersed for short-term (1 or 3 h) incubation or culture as previously described [19, 122]. Briefly, for short-term incubations GC layers were collected and immediately dispersed

into small pieces by gentle, repeated pipetting, then aliquoted in equal proportions into 12 x 75 mm polypropylene culture tubes containing 1 ml of Dulbecco's Modified Eagle Medium (DMEM; HyClone, Thermo Scientific) containing 2.5% FBS (PAA Laboratories, Piscataway, NJ), 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY) and non-essential amino acids (Gibco). Culture tubes were incubated in a shaking water bath at 40 °C in absence or presence of gonadotropins (FSH and LH) and TGFβ superfamily (TGFβ1 and BMP4) for 4 h. For cell cultures, granulosa layers were dispersed using 0.3% collagenase to achieve a single cell suspension, then cultured in 12-well polystyrene culture plates (Beckton Dickinson, Franklin Lakes, NJ) containing 1 ml of DMEM plus 2.5% FBS, 0.1 mM non-essential amino acids (NEAA), and 1% antibiotic-antimycotic mixture (Invitrogen Life Technologies, Carlsbad, CA) at 40 °C in an atmosphere of 95% air: 5% CO_2. Theca layers collected from 3-5 mm, 6-8 mm, 9-12 mm, and F4 follicles were washed with DMEM to remove any remaining yolk or GCs [16].

RNA isolation and quantitative real time PCR

Total RNA was extracted from isolated and cultured cells using Trizol reagent (Life Technologies, Grand Island, NY) and dissolved in nuclease-free water. The purity and concentration of final RNA pellets were determined by NanoDrop 2000 spectrophotometer (Thermo Science, West Palm Beach, FL). The isolated RNAs (1.5 μg per reaction) were treated with DNase I (Promega, Madison, WI) to eliminate contaminating genomic DNA. Subsequently, these samples were used for reverse transcriptase reactions using the combination of random primers, deoxynucleoside triphosphaste mix, and M-MLV reverse transcriptase (Promega). Forward and reverse primers encoding *Gallus VEGF*, *VEGFRs* (*VEGFR-1* and *-2*), *ANGPTs* (*ANGPT-1* and *-2*), *TIE-2*, and *18S* rRNA mRNA were generated by SciTools online software (Integrated DNA Technologies, Coralville, IA). For real-time PCR, 50 ng of cDNA template was added to 10 μl total reaction mixture including appropriate primers and PerfeCTa Sybr Green

FastMix Low Rox (Quanta Biosciences, inc., Gaithersburg, MD). Reactions were performed on the AB 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following conditions: 30s at 95 °C, followed by 40 cycles each for 3s at 95 °C, 1 mi at 56 °C, and 30s at 72 °C. For primer validation, melting curves were generated for each run and amplified products were sequenced by the Pennsylvania State university Genomics Core Facility. The C_t value (cycle number at which the fluorescence exceeds a threshold level) was determined for triplicate reactions using the 7500 software (v.2.0.4) and quantified by normalizing to *18S* rRNA using the $\Delta\Delta C_t$ method [392]. Results were expressed as fold-difference compared with an appropriate control tissue or treatment.

Western blot analysis

Western blot analysis was performed as previously described [30] to identify endogenous levels of VEGF protein in GCs from different stages of ovarian follicles. Briefly, fresh isolated GCs from 3-5 mm, 6-8 mm, 9-12 mm, and next largest follicle (F4) were lysed in RIPA buffer (Santa Cruz, Dallas, TX) including a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO) and protein concentrations of lysates were assessed by BCA method (BioRad, Hercules, CA). Gel electrophoresis and blotting were conducted according to the protocol of NuPAGE® SDS-PAGE gel system (Invitrogen). Following electrophoresis and membrane transfer, blots were incubated in 5% Non-fat dry milk in TBST as a blocking solution for 3 h at room temperature, followed by incubation with primary antibody. Rabbit anti-VEGF antibody (sc-507) was used at a 1:200 dilution overnight at 4 °C. As a secondary antibody, goat anti-rabbit IgG conjugated with HRP (Pierce, Rockford, IL) was incubated with 1:10,000 dilution for 1 h at room temperature. βActin (Sigma) was used at a 1:10,000 dilution as a loading control to assess levels of total protein, followed by the incubation of 1:10,000 dilution of goat anti-

mouse IgG conjugated with HRP for 1 h at room temperature (Pierce). All western blots were developed with WesternSure™ Premium Chemiluminescence Substrate (LI-COR, Lincoln, NE). Chemiluminescent signals were detected using the LI-COR C-DiGit™ Blot Scanner (LI-COR) and images were represented as JPEG files.

Data Analysis

Replicate experiments were conducted a minimum of three times and data are presented as a fold-difference compared with the control group. Data were analyzed by Student *t* test to determine significant difference between control versus treatment and subjected to one-way ANOVA with Tukey's test for multiple comparisons using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). A probability level of $p \leq 0.05$ was considered statistically significant.

RESULTS

The expression of VEGF and VEGFRs mRNA in hen ovarian follicles

Compared to prehierarchal follicles in which the number and size of blood vessels is limited, the most recently selected (9-12 mm) and preovulatory (*e.g.*, F2 and F4) follicles are well vascularized (**Fig. 5.1**). GCs from the most recently selected (9-12 mm) and fourth largest (F4) follicles showed increased *VEGF* mRNA (**Fig. 5.2A**) and VEGF protein expression (**Fig. 5.2B**) compared to GCs from 6-8 mm prehierarchal follicles (6-8 mm). *VEGFR1* and *VEGFR2* mRNA expression in the granulosa was essentially non-detectable (data not shown). By comparison, levels of *VEGF* and *VEGFR* mRNA within the theca layer were not different among the different stages of follicle development investigated (**Figs. 5.2C-E**). These findings suggest that angiogenesis associated with the outer theca layer can be enhanced by increasing levels of VEGF produced by the GC layer at follicle selection.

The expression of ANGPTs and TIE-2 mRNA in hen ovarian follicles

While *AGNPT-2* mRNA was preferentially detected in GCs (Ct value of *ANGPT-1* in GCs: 32±0.5) (**Figure 5.3A**), both *ANGPT-1* and *-2* mRNA are expressed in the theca layer (**Figure 5.3B and C**). The relative increase of *ANGPT-2* mRNA was more pronounced particularly in GCs from prehierarchal follicles (6-8 mm; about 2-fold) compared to GCs from the 9-12 mm and F4 follicle. Levels of *ANGPT-1* and *-2* mRNA in the theca layer were not significantly different among the different stages of follicle development. *TIE-2* mRNA was exclusively found in the theca layer and the relative expression was similar during the follicle development (**Figure 5.3D**).

Given that levels of ANGPT-2 are essential to function as an antagonist against ANGPT-1 [481], these results suggest that the relatively high expression of ANGPT-2 may induce inhibitory signaling that results in establishing a limited number of blood vessels in prehierarchal follicles.

VEGF and VEGFR expression are decreased in atretic follicle

Previous studies demonstrated an inhibition of VEGF expression facilitates the disruption of angiogenesis, and results in follicle atresia in mammalian species [69, 493, 494]. Normal and atretic follicles (3-5 mm) were collected for RNA isolation and levels of *VEGF* and *VEGFRs* were compared using qRT-PCR. To characterize normal and atretic follicles, B-cell lymphoma-extra large (*BCL-XL*) gene, an anti-apoptotic cell survival factor, was used [495] and expressed in normal follicles at a level higher than in atretic follicles (**Figure 5.4A**). Finally, it was determined that atretic follicles showed low levels of *VEGF* and *VEGFR* expression, compared to those of normal follicles (**Figure 5.4B-D**). These results suggest that reduced expression of VEGF and VEGFRs is related to follicle atresia

Gonadotropins induce VEGF and decrease ANGPT-2 expression in GCs

GCs were isolated from different stages of follicles and incubated with rhFSH (10 ng/ml) or oLH (10 ng/ml) for 4 h. FSH- or LH-induced *VEGF* mRNA expression was increased in GCs from 9-12 mm, F4, and F3 follicle, compared to GCs from 6-8 mm follicle that demonstrated no significant increase (**Figure 5.5A and B**). We also determined that FSH or LH-treated GCs from selected follicles showed significantly less expression of *ANGPT-2* mRNA (**Figure 5.5C and D**), compared to undifferentiated GCs from prehierarchal follicles without any differences, suggesting that gonadotropin-induced cAMP inhibits *ANGPT-2* transcription. These findings indicate that *VEGF* and *ANGPT-2* are cAMP-dependent genes and gonadotropin-induced cAMP differentially regulate *VEGF* and *ANGPT-2* expression in GCs. Additionally, it was found that *VEGF* and *ANGPT-2* expression was not changed with FSH or LH treatment, suggesting that their expression may be precluded in undifferentiated GCs from prehierarchal follicles (6-8 mm) because of FSHR desensitization [**Chapter 3**].

TGFβ superfamily members induce VEGF and decrease ANGPT-2 expression in GCs

Despite an inability to respond to a FSH challenge, a small number of blood vessels are found on prehierarchal follicles. It has previously been determined that TGFβ superfamily members can also up-regulate the expression of angiogenic factors including VEGF [488, 496-498]. The expression of *VEGF* mRNA was stimulated with rhTGFβ1 and rhBMP4 treatment (**Figure 5.6A**), indicating that VEGF transcription is up-regulated by both Smad2/3 and Smad1/5/8 signaling in GCs. Additionally, it was determined that BMP4, but not TGFβ1, inhibits *ANGPT-2* mRNA expression in undifferentiated GCs (**Figure 5.6B**), indicating that *ANGPT-2* transcription is inhibited by BMP4-induced Smad1/5/8 signaling.

DISCUSSION

Previous studies in mammals have reported that comparatively more vasculature is associated with the dominant follicle compared to other antral follicles [485, 493, 499], and that increased blood vessels play critical roles in the growth of ovarian follicles [63, 500, 501]. Although many studies support the importance of angiogenesis in mammalian female reproductive system, no evidence exists in chicken ovarian follicles. Therefore, this is the first study to establish that VEGF, ANGPTs, and their receptors are produced by GCs and TCs from chicken ovarian follicles, and their expressions are differently regulated by gonadotropins and TGFβ superfamily in GCs.

Since GCs are closely associated with the theca layer that contains the network of blood vessels, it was speculated that follicular angiogenesis is regulated by various angiogenic factors (*e.g.,* VEGF and ANGPTs) produced from GCs [67, 445, 502]. Thus, our initial studies confirmed the expression levels of VEGF, ANGPTs, and receptors in GCs and TCs from different stages of follicles. Although *VEGF, ANGPTs,* and their respective receptors are found in the theca layer, expression was not changed (**Figure 5.2C-E and 5.3B-D**). By comparison, *VEGFR* mRNAs were not detected in GCs, whereas the expression of *VEGF* mRNA is increased according to the stage of follicle development (**Figure 5.2A**). In chicken GCs, VEGF protein was detected as a dimer (42 kDa), known to be the active form (**Figure 5.2B**) [62, 503-505]. Additionally, we determined that the expression of *ANGPT-2* mRNA is decreased in GCs according to the stages of follicle development (**Figure 5.3A**), opposite to the increase in *VEGF* mRNA expression (**Figure 5.2A**) and *ANGPT-1* and *TIE-2* were not detected in GCs (data not shown). Given that VEGF acts as a strong angiogenic factor and ANGPT-2 acts as an antagonist to ANGPT-1, these results suggest that follicular angiogenesis in the hen ovary is regulated by both VEGF and the ratio of ANGPT-1 and ANGPT-2 expression produced by GCs [481]. Each of

these is provided to the theca layer in a paracrine fashion to stimulate the establishment and maintenance of blood vessels.

During follicle development, gonadotropins together with local ovarian growth factors activate intracellular signaling pathways to inhibit follicle atresia mediated via apoptosis [223, 506-509]. It was previously reported that follicle atresia in the hen ovary is mediated via apoptosis initiated within the GC layer [510-512]. Moreover, a recent publication demonstrated that inhibition of follicular angiogenesis promotes follicle atresia by inducing GC apoptosis [513]. Given that angiogenesis plays a role in the follicle survival, it was hypothesized that decreased levels of angiogenic factors are associated with follicle atresia. Results from the present study demonstrate that the relative levels of *VEGF* and its receptors mRNA in atretic follicles are lower than in normal follicles (**Figure 5.4**). These data provide evidence that a decrease of *VEGF* expression in apoptotic GCs leads to an insufficient network of blood vessels in and surrounding the theca layer. Subsequently the inefficient supply of nutrients, hormones, and growth factors for follicle growth eventually results in follicle atresia in the hen ovary.

Gonadotropins (*e.g.*, FSH and LH) known to inducer cAMP formation induce *VEGF* mRNA expression in GCs [514-516]. Consistent with evidence from mammals, *VEGF* mRNA expression was induced in actively differentiating (9-12 mm) and differentiated (F3) GCs in response to FSH or LH (**Figure 5.5A and 5.5B**), respectively. Additionally, it was demonstrated that FSH or LH inhibits *ANGPT-2* mRNA expression in actively differentiating and differentiated GCs (**Figure 5.5C and 5.5D**). However, undifferentiated GCs failed to increase *VEGF* (**Figure 5.5A**) or to decrease *ANGPT-2* (**Figure 5.5C**) mRNA expression in response to FSH. Previously, we have demonstrated that FSHR in undifferentiated GCs is desensitized by a βARRESTIN-mediated mechanism, resulting in inactivated cAMP-mediated signaling [**Chapter 3**]. Therefore, no changes in *VEGF* and *ANGPT-2* expression can be attributed to FSHR desensitization in GCs prior to follicle selection.

Although preovulatory follicles demonstrate extensive development of blood vessels in the hen ovary, very few are still visible on the surface of prehierarchal follicles prior to follicle selection (**Figure 1**). Considering FSHR desensitization in undifferentiated GCs, it is speculated that there exist additional signaling pathways independent of FSH-mediated cAMP signaling. Supportively, there are a few studies demonstrating that TGFβ superfamily members induce VEGF expression in bovine and human GCs [80-82]. The present studies demonstrate that TGFβ1 and BMP4 can increase *VEGF* mRNA expression in undifferentiated GCs (**Figure 6A**), suggesting that transcriptional and translational regulation of VEGF is mediated via a SMAD-binding element (SBE) within the promoter region [517]. Although VEGF expression is regulated by TGFβ1 or BMP4-mediated signaling in undifferentiated GCs, there is no evidence currently available to demonstrate the underlying mechanisms regulating ANGPT expression via Smad-signaling. The present study also demonstrated that BMP4 inhibits *ANGPT-2* mRNA expression in undifferentiated GCs (**Figure 5B**), suggesting the ANGPT-2 expression is regulated by BMP4-mediated Smad1/5/8 signaling. Collectively, these findings provide evidence that weak angiogenesis is preferentially maintained by one or more members of the TGFβ superfamily in GCs from prehierarchal follicles.

We conclude that both gonadotropins and TGFβ superfamily members differentially regulate the expression of VEGF and ANGPT-2 in GCs from ovarian follicles in the hen. Prior to follicle selection, *VEGF* mRNA expression is positively regulated by TGFβ1 and BMP4-mediated Smad signaling, while *ANGPT-2* mRNA expression is negatively regulated by BMP4-mediated signaling in undifferentiated GCs from prehierarchal follicles. Once follicle selection occurs, actively differentiating GCs increase *VEGF* and decrease *ANGPT-2* mRNA expression by the synergic effects of both FSH and BMP4-meidated signaling, allowing follicles to rapidly enhance angiogenesis (**Summary model 3**). Finally we propose that relatively high levels of ANGPT-2 compared to ANGPT-1 induce limited angiogenesis in prehierarchal follicles, and

angiogenesis regulated by the balance of angiogenic factors may be related to the process of follicle selection.

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Table 5.1. Sequences of forward and reverse primers used for real-time PCR, together with the NCBI nucleotide accession number.

Gene	Accession #	Sequence
ANGPT-1	NM_001199447.1	FWD 5'-TGGAGGATGGACAGTTAT-3' REV 5'-TCATTTCCCAGCCAGTGTTC-3'
ANGPT-2	NM_204817.1	FWD 5'-ATGCTTGTGGTCCTTCTAACC-3' REV 5'-TCCTGAGCCTTTCCAGTAGTA-3'
VEGF	AB011078.1	FWD 5'-CGGAAGCCCAACGAAGTTA-3' REV 5'-ACACAGGATGGCCTGAATATG-3'
VEGFR1	NM_204252.1	FWD 5'-TGGATGTGGTATCCCTGTAGA-3' REV 5'-GCTCTGGATCCTGTTTCCTATG-3'
VEGFR2	NM_001004368.1	FWD 5'-CGTCCGTCAGGATGGTAAAG-3' REV 5'-AGGTGAAGTTGGGAGAGAGA-3'
TIE-2	XM_424944.4	FWD 5'-GTTCTGCCTACCAGATCCATAC-3' REV 5'-TGCAGTCTGACCCATAATAACC-3'
BCL-XL	U26645.1	FWD 5'-TTGGATGACCACGTACTTGAC-3' REV 5'-ACAGCGTTGTTCCCATACAGA-3'
18S rRNA	AF173612	FWD 5'-TTAAGTCCCTGCCCTTTGTACAC-3' REV 5'-CGATCCGAGGAACCTCACTAAAC-3'

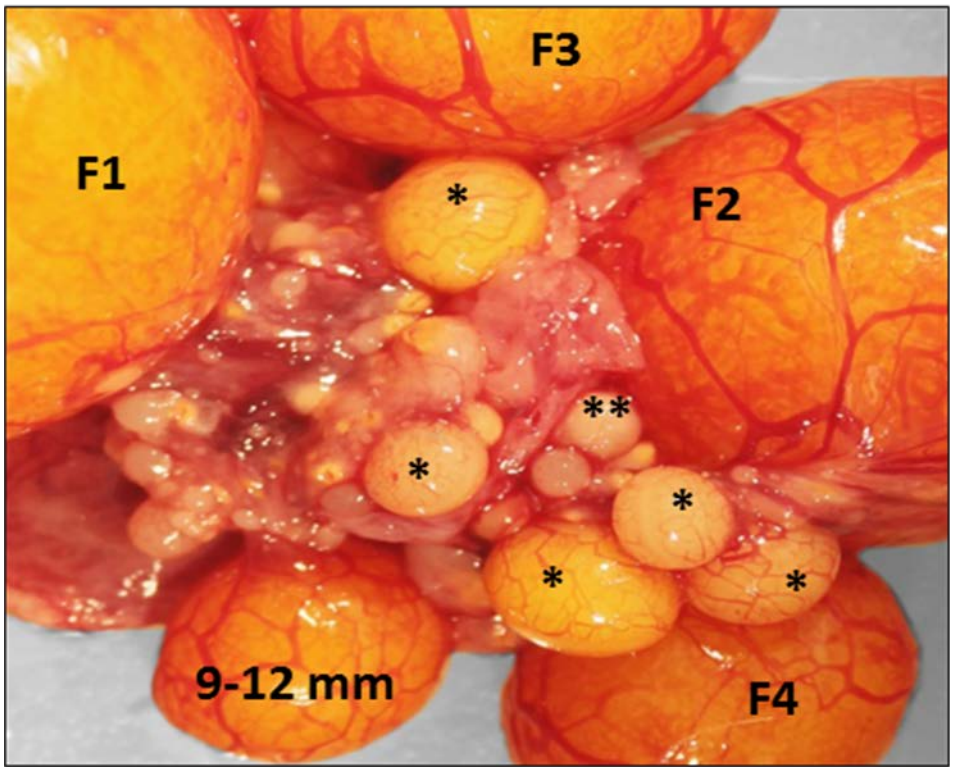


Figure 5.1. Vascularized ovarian follicles in the hen.

Preovulatory follicles (F1-F4 and 9-12 mm) show numerous large and small blood vessels, compared to prehierarchal follicles (*). *: 6-8 mm follicle; **: 3-5 mm follicle.

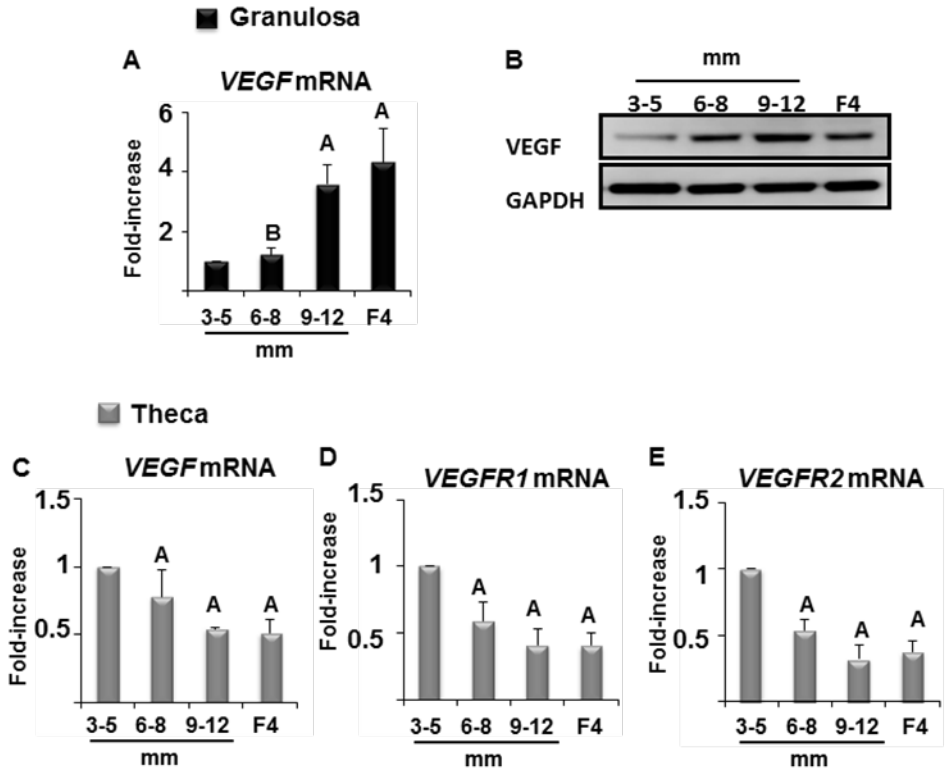


Figure 5.2. Expression of *VEGF* and *VEGFRs* mRNA in the hen ovary.

A, Endogenous expression of *VEGF* mRNA in GCs from follicles prior to (3-5 and 6-8 mm) and after (9-12 mm and F4) follicle selection. **B**, Expression of VEGF protein in GCs from different stages of follicle development was analyzed by western blot. Results are representative of two replicate experiments. **C**, Endogenous expression of *VEGF* mRNA in theca layers at different stages of follicle development. Levels of *VEGF* mRNA expression were not significantly different (P>0.05) among theca layers from prehierarchal and preovulatory follicles. Expression of *VEGFR-1* (**D**) and *-2* (**E**) mRNA in theca layers from different stages of follicle development. *VEGFR-1* and *-2* mRNA were detected in theca layers, but not in GCs (data not shown). The relative levels of *VEGFR-1* and *-2* mRNA were not different (P>0.05) during follicle development (N=3). Data are expressed as a fold-difference ± SEM compared to the 3-5 mm follicle and were analyzed by ANOVA and Tukey's multiple range test (N=3-4 replicates, A,B: P<0.05).

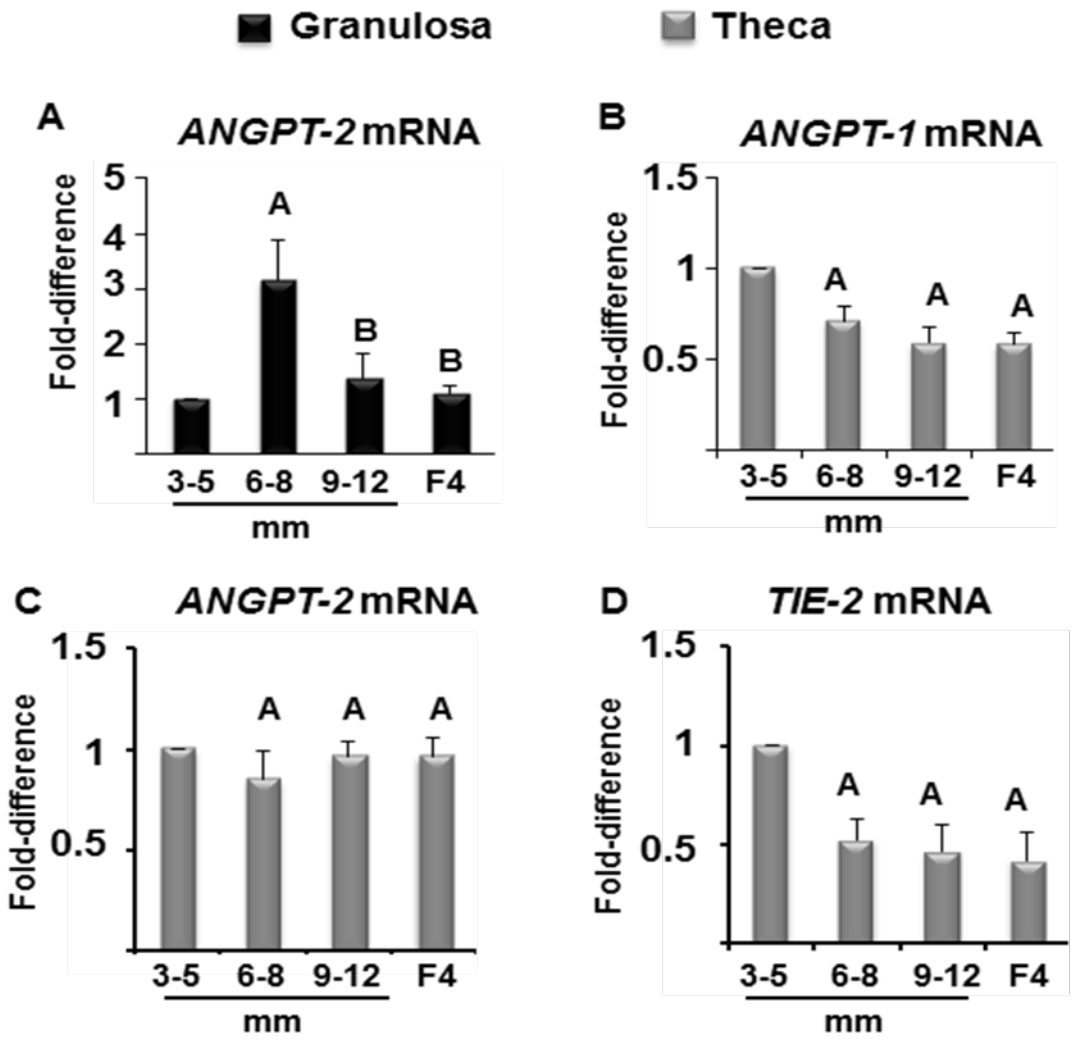


Figure 5.3. Expression of *ANGPT-1*, *ANGPT-2* and *TIE-2* mRNA in hen ovary.

A, Endogenous expression of *ANGPT-2* mRNA in GCs from different stages of follicle development. Levels of *ANGPT-2* mRNA are increased in undifferentiated GCs from prehierarchal (6-8 mm) compared to actively differentiating (9-12 mm) and differentiated (F4) follicles. **B-D**, There are no differences in the endogenous expression of *ANGPT-1/-2* and *TIE-2* mRNA in the theca layer from different stages of follicle development ($P>0.05$). Data represent the mean± SEM and were analyzed by ANOVA and Tukey's multiple range test (N=3-5 replicates, A,B: $P<0.05$).

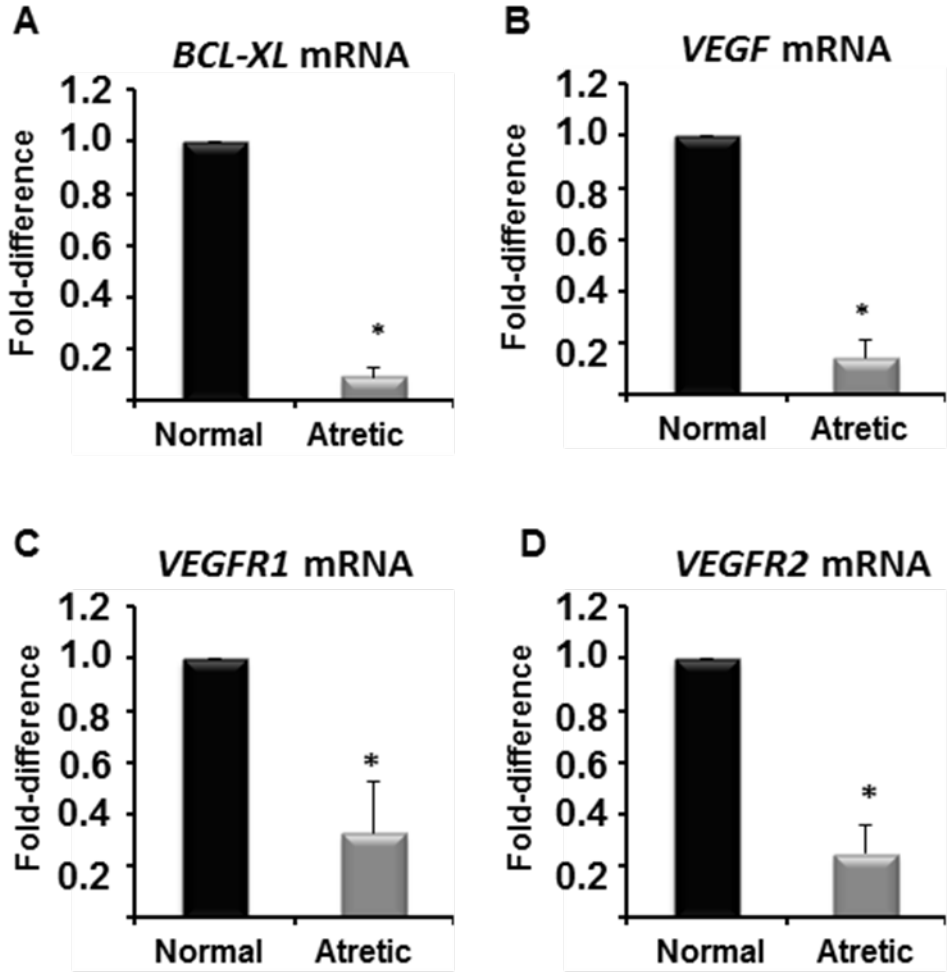


Figure 5.4. Levels of *VEGF* and *VEGFR* mRNA in atretic follicles.

Levels of *BCL-XL* (**A**), *VEGF* (**B**), *VEGFRs* (**C,D**) mRNA were analyzed in atretic *versus* normal follicles (3-5 mm prehierarchal follicles) by quantitative real-time PCR. Data represent the mean± SEM from 3-4 replicate experiments and are analyzed by unpaired t-test. (*P<0.05)

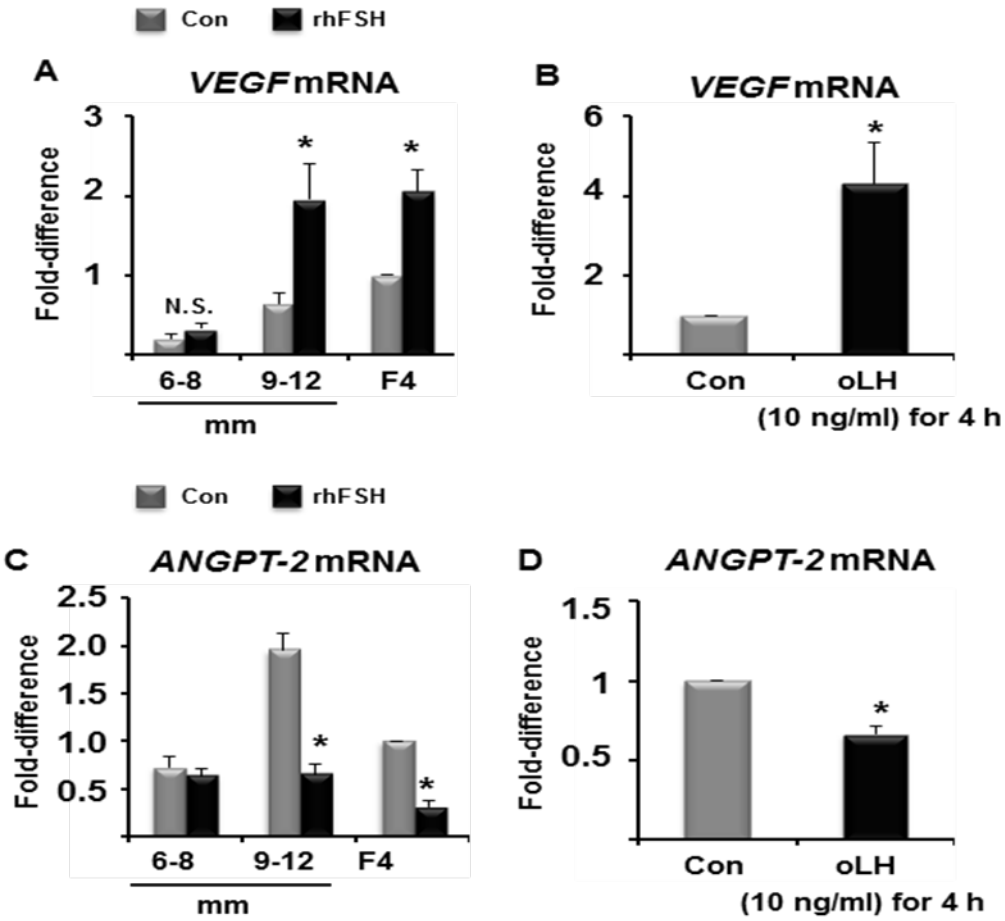


Figure 5.5. Regulation of *VEGF* and *ANGPT-2* transcription by gonadotropins in GCs.

A, FSH-induced *VEGF* mRNA expression in GCs from different stages of follicle development. *VEGF* mRNA expression was significantly increased in actively differentiating (9-12 mm) and differentiated (F4) GCs in response to a challenge with rhFSH (10 ng/ml) for 4 h, whereas undifferentiated GCs failed to increase *VEGF* mRNA expression. **B**, *VEGF* mRNA expression was increased by a 4 h challenge with LH (10 ng/ml). **C**, *ANGPT-2* mRNA expression was decreased by a 4 h challenge with rhFSH in GCs from actively differentiating (9-12 mm and F4) GCs, but not undifferentiated GCs (n=4). **D**, *ANGPT-2* mRNA expression was inhibited by a 4 h challenge with oLH (10 ng/ml) in GCs from the third largest preovulatory F3 follicle. Data represent the mean± SEM from 3-7 replicate experiments and are analyzed by unpaired t-test (*P<0.05).

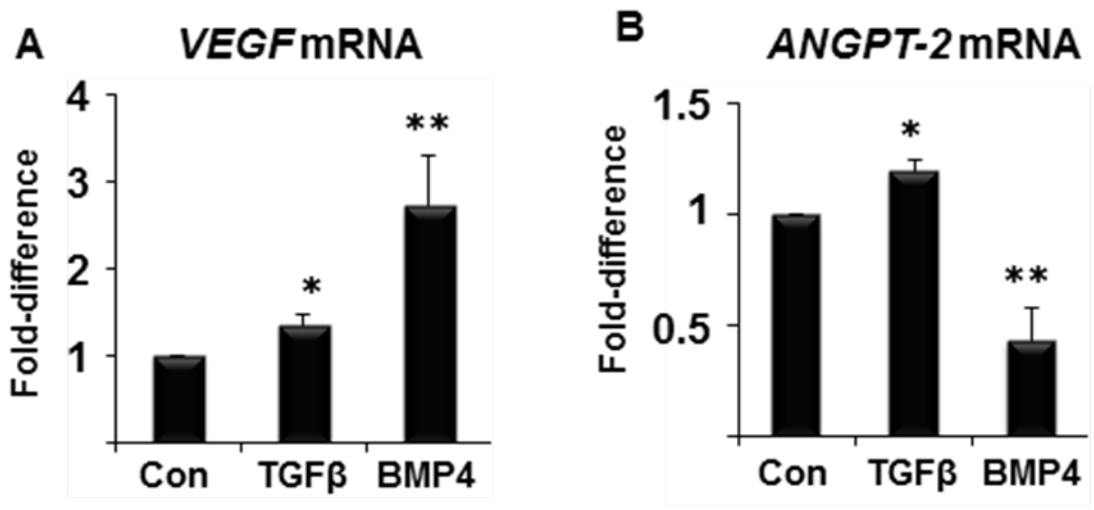


Figure 5.6. Regulation of *VEGF* and *ANGPT-2* transcription by TGFβ superfamily members in GCs from prehierarchal (6-8 mm) follicles.

A, *VEGF* mRNA expression was increased in undifferentiated GCs from prehierarchal follicles by a 4 h challenge with rhTGFβ1 (10 ng/ml) and rhBMP4 (10 ng/ml). **B**, *ANGPT-2* mRNA expression was increased by TGFβ1 after a 4 h challenge of undifferentiated GCs, but increased by BMP4. Data represent the mean± SEM from 3-4 replicate experiments and are analyzed by unpaired t-test (*P<0.05)

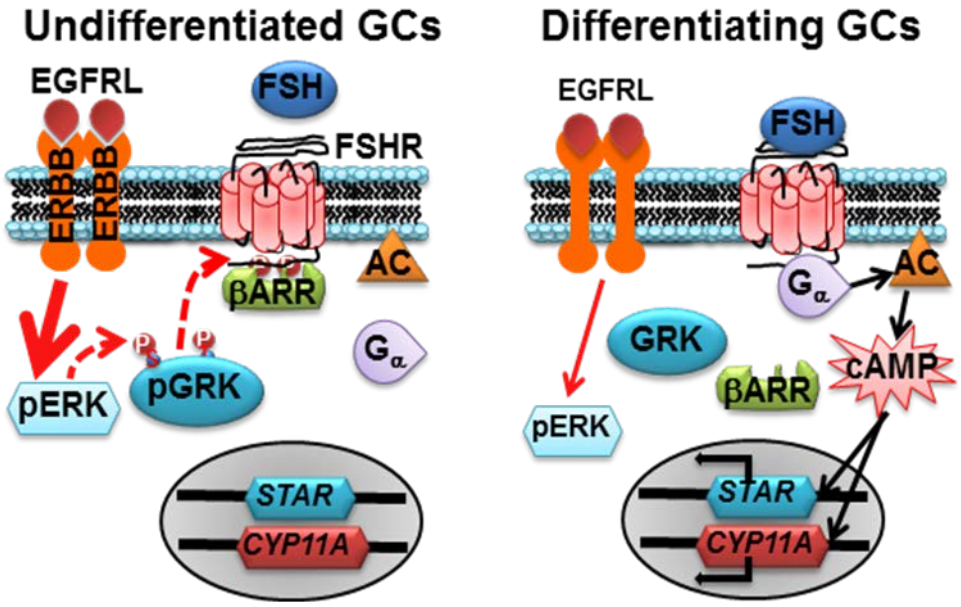
Summary

In monovulatory vertebrate species, ovarian follicle selection occurs on each ovulation cycle for follicle development, maturation and ovulation. Follicle selection is closely associated with the functional differentiation of GCs [19, 109, 110]. Subsequent to selection the transition of dominant responsiveness from FSH to LH represents a mechanism through which continued maturation of the selected follicles occurs in vertebrates [103, 114]. In the laying hen, a single follicle is selected from the cohort of resting prehierarchal follicles via the transition of GCs from an undifferentiated to a differentiated state, resulting in the initiation of steroidogenesis [18, 27, 28]. However, undifferentiated GCs from prehierarchal follicles have a chance to undergo apoptosis, which leads to follicle atresia [12, 495]. Therefore, the two global questions that remain unanswered are: 1) how undifferentiated GCs initiate differentiation within the single follicle selected; and 2) how GCs from selected follicles escape atresia. The present studies were conducted to provide and identify mechanisms associated with follicle selection in the chicken ovary.

1. Model for FSHR desensitization in GCs from prehierarchal follicles

It is well documented in the literature that prior to follicle selection, undifferentiated GCs from prehierarchal follicles fail to induce cAMP-mediated signaling in response to FSH, despite the high levels of FSHR expression [122]. The present studies demonstrated that unlike results demonstrating a lack of FSH responsiveness in fresh isolated undifferentiated GCs, FSH-induced cAMP was significantly increased in undifferentiated GCs transfected with *βARRESTIN1*-siRNA,

compared to GCs transfected with scrambled-siRNA. Moreover, cAMP-mediated STAR protein expression and progesterone production were increased. These data were confirmed using actively differentiating GCs that have already acquired FSH-responsiveness. Over-expression of βARRESTIN1 and GRK2 proteins re-established desensitization of the FSHR in actively differentiating GCs. It is concluded that FSHR in undifferentiated GCs from prehierarchal follicles is desensitized by a βARRESTIN-mediated event prior to follicle selection. However, it is also known that GCs are maintained in an undifferentiated state by inhibitory MAPK signaling induced by EGFR ligands (*e.g.*, TGFα and Betacellulin) [19, 29, 30, 34]. In these studies, it was demonstrated that undifferentiated GCs cultured overnight *in vitro* acquire of the capacity for FSHR-mediated cell signaling via cAMP (*e.g.*, initiate differentiation in culture). However, even after the initiation of differentiation a subsequent activation of MAPK signaling is capable of reestablishing receptor desensitization.



Summary model 1. Proposed model for FSHR desensitization in GCs from prehierarchal follicles. EGFRL: epidermal growth factor receptor ligand, ERBB: epidermal growth factor receptor, FSH: follicle stimulating hormone, FSHR: FSH receptor, βARR: beta ARRESTIN, AC: adenylyl cyclase, pERK:

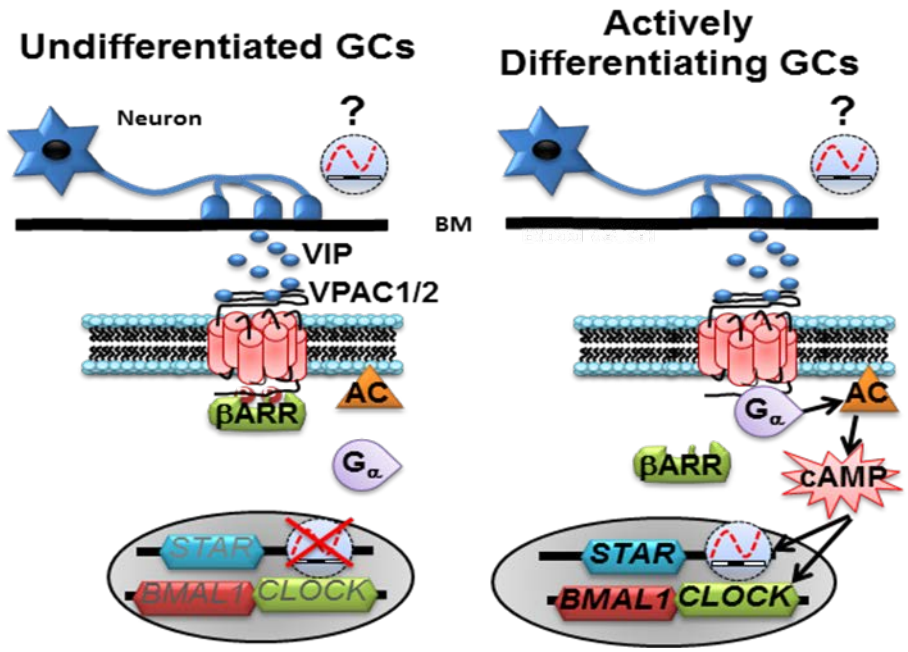
phosphorylated extracellular signal-related kinase, pGRK: phosphorylated G protein-coupled receptor kinase, Gα: guanine-nucleotide binding protein alpha, STAR: steroidogenic acute regulatory, CYP11A: cytochrome P450 side-chain cleavage enzyme, cAMP: cyclic 3,5-adenosine monophosphate.

Prior to follicle selection FSH fails to induce cAMP signaling due to a GRK and βARRESTIN-mediated event. It is proposed that FSHR-desensitization is maintained, at least in part, by ERBB receptor/MAPK-induced phosphorylation and the activation of GRK (dashed red arrows). Uncoupling of the Gα subunit to FSHR is induced by the recruitment of βARRESTIN. As a result, FSH fails to increase cAMP production. Once follicle selection occurs, actively differentiating GCs from the most recently selected follicle (9-12 mm) become responsive to FSH by reducing or eliminating βARRESTIN-mediated desensitization. In turn, this mechanism promotes expression of cAMP-dependent genes (*e.g.*, *STAR* and *CYP11A*). We speculate that this is due to a reduction in inhibitory MAPK signaling and/or increased phosphatase activity.

2. Model for VPAC-mediated signaling in phasing the rhythmicity of clock genes

VIP plays a critical role in female reproductive system and exerts its function by binding to VPACs belonging to the GPCR family. It is hypothesized that prior to follicle selection, VPAC receptors remain in a desensitized state due to a βARRESTIN-mediated event. Consistent with the previous study [**Chapter 3**], freshly isolated undifferentiated GCs fail to respond to chVIP, compared to the significant increase of cAMP-mediated signaling in GCs from selected follicles after VIP treatment. Transfection of *βARRESTIN1*-siRNA results in inducing VIP-mediated cAMP formation, STAR protein expression and progesterone production in undifferentiated GCs These results provide support for the model that VPACs are desensitized by βARRESTIN-mediated events in undifferentiated GCs prior to follicle selection.

Previous results from quail demonstrate that GCs from prehierarchal follicles showed no rhythmic expression of clock genes over a 24 h interval [47] compared to GCs from preovulatory follicles that show significant variations in clock gene expression. Consistent with these results, the present studies demonstrate that *BMAL1*, *CLOCK*, and *PER2* mRNA expression is not induced by VIP treatment in undifferentiated GCs because of the lack of VIP-responsiveness. Moreover, it was confirmed that undifferentiated GC cultured with TGFα (to maintain cells in an undifferentiated state) over a 30 h interval also failed to demonstrate any significant changes in *BMAL1* or *CLOCK* expression. Significantly, undifferentiated GC cultured in culture media alone (*e.g.*, which results in actively differentiating cells) showed significant differences in *BMAL1* and *CLOCK* expression over the same interval.



Summary model 2. Proposed model for VIP actions in undifferentiated *versus* actively differentiating GCs from hen ovarian follicles. VIP: vasoactive intestinal peptide, VPAC1/2: VIP receptor-1/-2, βARR: beta arrestin, AC: adenylyl cyclase, Gα: guanine-nucleotide binding protein alpha, cAMP: cyclic 3,5-adenosine monophosphate. STAR: steroidogenic acute regulatory, BMAL1: brain and muscle ARNT-like 1, CLOCK: circadian locomotor output cycles kaput, BM: basement membrane.

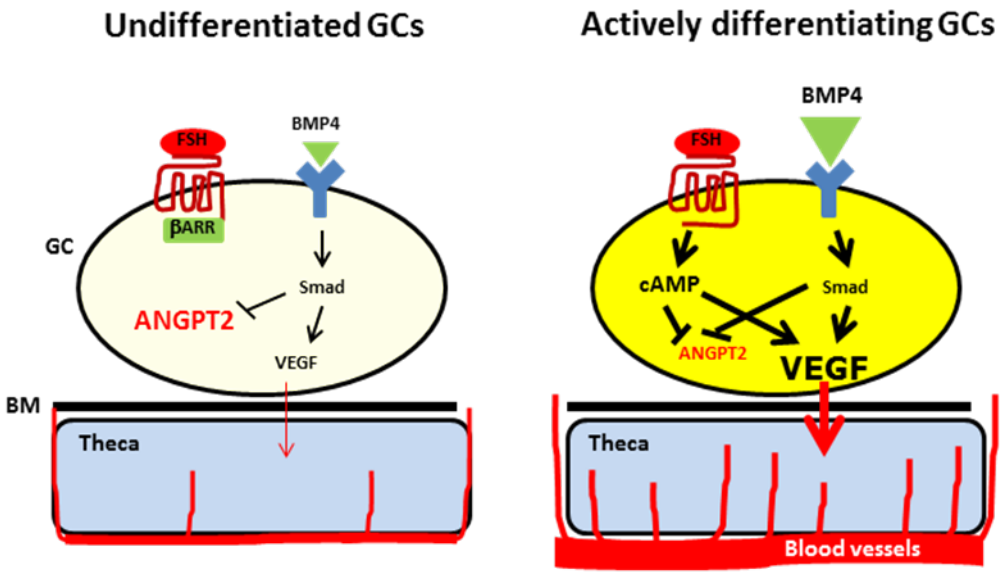
Collectively, prior to follicle selection, undifferentiated GCs are unresponsive to VIP (of neuronal and/or vascular origin), which leads to the absence of receptor-mediated signaling via cAMP. To this end, clock gene (*BMAL1*) expression remains comparatively low and without inherent rhythmicity. By comparison, at follicle selection the acquisition of VIP/VPAC signaling via cAMP begins to induce *BMAL1* expression and potentially phase a rhythmic expression of *BMAL1* in actively differentiating/differentiated GC from preovulatory follicles. Furthermore, although *STAR* mRNA expression is primarily regulated by cAMP signaling, its expression may be also modulated by clock proteins (*e.g*., BMAL1/CLOCK heterodimers) [47].

3. Model for expression and regulation of angiogenic factors in GCs during follicle development

Ovarian follicle development requires a network of vasculature that surrounds and penetrates the theca layer. In the hen ovary, prehierarchal follicles have minimal vasculature. Upon follicle selection, a large increase in both number and size of vasculature is observed in preovulatory follicles. Although angiogenic factors and their receptors have been well studied in mammalian ovaries [61, 70, 447], very little is known of these factors in chicken ovarian follicles. The aim of this study is to investigate the ratio of *VEGF/VEGFR* and *ANGPTs/TIE-2* mRNAs and the regulatory mechanisms supported by endocrine, paracrine and autocrine factors during follicle development.

In the hen ovary, the expression of *VEGF* mRNA in GCs during follicle development gradually increases while the expression of *ANGPT-2* mRNA decreases. By comparison, the ratio of *VEGF/VEGFRs* and *ANGPT-1/-2/TIE-2* mRNAs were not changed in the theca layer, suggesting the network of blood vessels in the theca layer is preferentially influenced by angiogenic factors secreted from GCs. Importantly, both gonadotropins (FSH and LH) and TGFβ superfamily members (TGFβ1 and BMP4) regulate *VEGF* and *ANGPT-2* mRNA expression in

actively differentiating GCs. However, only TGFβ1 and BMP4, but not FSH, up-regulated the expression of *VEGF* in undifferentiated GCs. As previously discussed [**Chapter 3**], it is likely that VEGF expression is precluded in undifferentiated GCs as a result of FSHR desensitization in undifferentiated GCs. Thus, it is suggested that TGFβ superfamily-mediated Smad signaling maintains a minimum amount of vascularization in prehierarchal follicles. At follicle selection, actively differentiating GCs acquire FSHR-responsiveness which, in concert with BMP4-mediated signaling, promotes increased *VEGF* expression and decreased *ANGPT-2* expression.



Summary model 3. Proposed model for the regulation of follicular angiogenesis in undifferentiated *versus* actively differentiating GCs from the hen ovary. FSH: follicle stimulating hormone, BMP4: bone morphogenetic protein 4, βARR: beta arrestin, ANGPT2: angiopoietin-2, VEGF: vascular endothelial growth factor, cAMP: cyclic 3,5-adenosine monophosphate, GC: granulosa cell, BM: basement membrane.

Accordingly, prior to follicle selection, undifferentiated GCs express relatively higher levels of ANGPT-2 and lower levels of VEGF expression in response to BMP4-mediated Smad signaling, because FSHR is desensitized by a βARRESTIN-mediated event [**Chapter 3**]. To this

end, there are a small number of blood vessels in prehierarchal follicles. At follicle selection, the acquisition of FSH/FSHR signaling [**Chapter 3**] and the increased expression of BMP4 [30] in GCs initiates an increase VEGF expression and decrease ANGPT-2 expression with enhanced BMP4-mediated Smad signaling. These events are predicted to rapidly enhance vascular formation within the theca layer in preovulatory follicles.

Taken together, prior to follicle selection, undifferentiated GCs are unresponsive to GPCRs-mediated (*e.g.*, FSHR and VPACs) signaling via cAMP, because GPCRs are desensitized, at least in part, by a βARRESTIN-mediated event. Results from the present studies provide evidence that receptor-desensitization is initiated and maintained by the MAPK signaling pathway through ERBB receptors in undifferentiated GCs. Thus, this receptor-desensitization contributes to the inactivation of steroidogenesis, arrhythmic expression of clock genes, and the comparatively low level of angiogenesis in prehierarchal follicles. At follicle selection, undifferentiated GCs escape from receptor desensitization mediated by MAPK signaling and βARRESTIN, and then initiate the process of differentiation. Actively differentiating GCs from the most recently selected follicle initially acquire FSHR-responsiveness and can induce ligand-induced cAMP formation, resulting in the initiation of steroid hormones and angiogenic factors. In addition to FSH, actively differentiating GCs also respond to VIP, a factor proposed to be delivered to the ovary from the central nerve system (CNS) and/or the blood vasculature and to phase clock gene expression according to the prevailing photoperiod.

Future Studies

1. Model for FSHR desensitization in GCs from prehierarchal follicles

Although it is proposed that FSHR-desensitization is initiated and maintained by MAPK signaling, the present studies suggest, but do not confirm, the effect of MAPK signaling on the

initiation of receptor-desensitization in GCs. Moreover, the mechanisms by which undifferentiated GCs escape receptor desensitization have yet to be defined. As proposed in the summary model 1, increased phosphatase activity may inhibit MAPK-mediated GRK2 activation to cause receptor desensitization. Thus, phosphatase activity may be evaluated between undifferentiated and differentiating GCs.

2. Model for VPAC-mediated signaling in phasing the rhythmicity of clock genes

Currently, ovarian clock genes have been determined to play a role in the timing of ovulation, steroidogenesis, and follicle development [310, 518, 519]. While it is currently proposed that the timing of ovulation in birds is dependent primarily upon the preovulatory LH surge [47, 48, 314] further studies are required to establish mechanisms that time the process of follicle selection in the hen ovary.

3. Model for the regulation and expression of angiogenic factors in GCs during follicle development

In the present study, only two angiogenic factors, VEGF and ANGPT, were investigated in chicken ovarian follicles. Other potential factors (*e.g.*, FGF, PDGF, and MMPs) need to be investigated in order to understand the interactions of mechanisms regulating angiogenesis during the development of ovarian follicles in chickens. Unlike the mammalian ovary, chicken ovarian follicles contain a stigma where the germ cell is released. The vasculature on all developing follicles is found throughout the follicle surface except for the stigma region (see Figure 2.1). Notably, there is no explanation as to how the stigma remains void of blood vessels. Results of such studies are not just related to understanding the formation of the stigma in the hen ovary, but could be applied to physiological events related to abberrent angiogenesis, including that with occurs in ovarian cancers.

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Honors and Awards

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Publications

1. Lack of FSH-responsiveness in granulosa cells from hen prehierarchal follicles is mediated by β-Arrestin. **DW Kim**, OM Ocón-Grove, AL Johnson. (*In revision*)
2. Vasoactive intestinal peptide (VIP) promotes rhythms of clock genes and clock-related genes in granulosa layer after follicle selection. **DW Kim**, AL Johnson. (*Submitted*)
3. Bone Morphogenetic Protein 4 (BMP4) supports the initial differentiation of hen (*Gallus gallus*) granulosa cells. **DW Kim**, OM Ocón-Grove, AL Johnson. *Biol Repro* 2013; 88(6):161-167
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