Chemerin and Prohibitin in the Regulation of Ovarian Follicular Development and their Potential Involvement in Polycystic Ovarian Syndrome

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ABSTRACT

Follicular growth and maturation are tightly regulated processes, which involve the participation of endocrine, autocrineparacrine factors and intracellular molecules. Due to the numerous research efforts, a large number of regulators and their mechanisms of regulation of follicular growth and differentiation have been established. Although the abnormal expression and activities of some of these regulators are believed to be associated with ovarian dysfunction diseases, such as polycystic ovarian syndrome (PCOS), the etiology and pathogenesis of this syndrome are not completely understood.

In this thesis, we have identified two novel regulators of follicular growth and differentiation and examined the cellular and molecular mechanisms that contribute to the folliculogenesis. We present here that chemerin reduces FSH-induced steroidogenic enzyme expression and steroid hormone production in follicles and granulosa cells. Prohibitin expression is upregulated by chemerin and knockdown of prohibitin attenuates the suppressive role of chemerin on steroidogenesis, an action regulated by Akt.

Using an androgenized rodent model, we also present the dysregulation of chemerin and prohibitin and their association with dysregulated follicular steroidogenesis. Our data and preliminary clinical studies demonstrate the potential involvement of chemerin and prohibitin in the etiology of PCOS. These studies significantly improve the knowledge of ovarian functions and the pathophysiology of PCOS, and provide important clues for the development of novel diagnosis biomarkers and new treatment strategies for this complex syndrome.

THESIS FORMAT

The thesis is written in the "Collection of published papers and manuscripts" format as outlined in the guidelines provided by the Faculty of Graduate and Postdoctoral Studies and the Department of Cellular and Molecular Medicine, University of Ottawa. The main body is divided as follows:

Chapter 1 (Introduction) provides a critical review of the literature in the field of ovarian function and regulation. Main topics described in this chapter are: 1) follicular development; 2) regulation of follicular function by gonadotropin, chemerin and prohibitin; 3) dysregulation of ovarian follicular development and polycystic ovarian syndrome; and how these topics are related.

Chapter 2 (Rationale of Research Program) provides the rationale for the work performed in this thesis.

Chapter 3 (Objectives and Hypotheses) offers the overall and specific objectives and hypotheses for the experiments conducted in this study.

Chapter 4 (Endocrinology, 2012, 153:5600-5611) presents the findings of the expression and role of chemerin in steroidogenesis. In this chapter, we present the evidence that chemerin suppresses FSH-induced steroidogenesis in follicles and granulosa cells. Also the abundance of chemerin and its receptor in ovarian cells from an androgenized rodent model provides an association between chemerin and the etiology of polycystic ovarian syndrome. The addendum in this chapter contains experimental data related to the manuscript and corrections/clarifications identified which could not be corrected in the manuscript due to copyright.

Chapter 5 (Endocrinology, 2013, 154: 956 - 967) provides the evidence that prohibitin and chemerin play negative roles in FSH-induced steroidogenesis. Here we present that prohibitin acts as a mediator of chemerin and suppresses FSH-induced steroidogenesis, a phenomenon regulated by Akt. The addendum in this chapter contains experimental data related to the manuscript and corrections/clarifications identified which could not be corrected in the manuscript due to copyright.

Chapter 6 (Journal of Ovarian Research, 2013, 6: 23-32) presents the observations of the regulation and function of prohibitin in follicular development. In this chapter, we present the follicular stage-dependent regulation of prohibitin and its differential roles in apoptosis and steroidogenesis. The addendum in this chapter contains corrections/clarifications identified which could not be corrected in the manuscript due to copyright.

Chapter 7 (General Discussion) consists of in-depth analysis of the data presented in the whole thesis (including addenda), describes the contributions of the findings and experimental limitations and also presents the possible questions that may be addressed in future experiments.

Chapter 8 (References) provides the source information of ideas and results cited in the present thesis.

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LIST OF ABBREVIATIONS

aa	Amino Acid
A-Akt	Constitutively Active Akt
AMH	Anti-Müllerian Hormone
ANOVA	Analysis of Variance
APAF-1	Apoptotic Protease Activating Factor-1
BAD	Bcl-2-Associated Death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra Large
BMI	Body Mass Index
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCRL2	Chemokine (C-C motif) Receptor Like 2
cDNA	complementary DNA
C/EBP	CCAAT/enhancer-binding protein
cGMP	cyclic Guanosine 3', 5'-monophosphate
CMKLR1	Chemokine-Like Receptor 1
CNP	C-type Natriuretic Peptide
CREB	cAMP-Regulatory Element Binding protein
CTL	Control
CYP17	cytochrome P450c17, 17α-hydroxylase/17, 20 lyase
Dax-1	Dosage-sensitive, sex-reversal Adrenal hypoplasia congenita critical region on the X chromosome gene 1
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHT	5α-Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
DN-Akt	Dominant Negative Akt
DNA	Deoxyribonucleic Acid
E2F1	E2F transcriptional factor 1
eCG	equine Chorionic Gonadotropin
ECL	Enhanced Chemiluminescent

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-tetraacetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ERα	Estrogen Receptor α
ERK	Extracellular Signal-Related Kinase
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FF	Follicular Fluid
FOXO1	Forkhead box O1
FSH	Follicle-Stimulating Hormone
FSHR	FSH Receptor
FSK	Forskolin
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDF9	Growth Differentiation Factor-9
GFP	Green Fluorescent Protein
Gn	Gonadotropin
GnRH	Gonadotropin Releasing Hormone
GPCR	G Protein-Coupled Receptor
GRK	G-protein-coupled Receptor Kinase
GPR-1	G-Protein coupled Receptor-1
HA	Hemagglutinin
hCG	human Chorionic Gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
IGF	Insulin-like Growth Factor
IF	Immunofluorescence
IgG	Immunoglobulin G
IL	Interleukin
IP	Immunoprecipitation
IRE	Insulin Response Element
IVF	*in vitro* Fertilization

kDa	kilodalton
LacZ	Gene for β-galactosidase
LBD	ligand binding domain
LH	Luteinizing Hormone
LHR	LH Receptor
LPS	Lipopolysaccharide
LY	LY294002
Lys	Lysine
MAPK	Mitogen-Activated Protein Kinase
α-MEM	α-Minimum Essential Medium
MEK	MAPK/ERK Kinase
M-MLV	Moloney murine leukemia virus
MOI	Multiplicity of Infection
mRNA	Messenger RNA
mTOR	mammalian Target Of Rapamycin
NIH	National Institutes of Health
NR	Nuclear Receptor
NR5a1	Nuclear Receptor subfamily 5, group A, member 1
NR5a2	Nuclear Receptor subfamily 5, group A, member 2
OD	Optical Density
O-GlcNAc	O-linked β-N-acetylglucosamine
p450scc	cytochrome P450 side-chain cleavage enzyme
PAGE	Polyacrylamide Gel Electrophoresis
PDK1	Phosphoinositide-Dependent Kinase-1
PCOS	Polycystic Ovarian Syndrome
PHB	Prohibitin
Phe	Phenylalanine
PI3K	Phosphoinositol 3-OH Kinase
PKA	Protein Kinase A
PPAR	Peroxisome Proliferator-Activated Receptor
PTEN	Phosphatase and Tensin homologue
Rb	Retinoblastoma protein
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction

s.c.	Subcutaneous
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Ser	Serine
Shp1	SH2-domain containing inositol 50-phosphatase 1
SHP	Small Heterodimer Partner
shRNA	short-hairpin RNA
Sp1	Specific protein 1
StAR	Steroidogenic Acute Regulatory protein
STAT	Signal Transducer and Activator of Transcription
STS	Staurosporine
T	Testosterone
T2D	Type II Diabetes
TBS	Tris-Buffed Saline
TBS-T	Tween-20 in TBS
TCN	Triciribine
TGF-β	Transforming Growth Factor-β
Thr	Threonine
TNF-α	Tumor Necrosis Factor-α
TNFR	TNF Receptor
TP	Testosterone Propionate
TSHR	Thyroid Stimulating Hormone Receptor
Tyr	Tyrosine
UTR	Untranslated Region
UV	Ultraviolet
XIAP	X-Linked Inhibitor of Apoptosis Protein

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CHAPTER 1: INTRODUCTION

1.1 The Ovary and Follicular Development

1.1.1 Overview

The ovary controls the growth, maturation and release of the oocyte in preparation for fertilization and ensures species propagation. It produces various steroids required for maintaining regular menstrual cycles and supporting pregnancy (Leung and Adashi, 2004). The follicle is one of the functional units in the ovary and consists of an innermost oocyte, surrounding granulosa cells and an outer layer of theca cells (McGee and Hsueh, 2000). Under the precise control of endocrine and intraovarian regulators, the follicles develop through primordial, primary and preantral stages before forming an antrum. At the antral stages, the predominant follicle is selected. Most developing follicles undergo atresia, and only a few continue to develop to preovulatory stages and eventually ovulate in response to gonadotropins (McGee and Hsueh, 2000).

1.1.2 Follicle Morphology and Growth

Follicular growth from the primordial population is termed folliculogenesis. The definition and morphology of various stages of follicles have been well established and typical follicles are presented in **Figure 1.1** (Gougeon, 2004; McGee, 2000). The primordial follicles consist of a single pre-granulosa cell (epithelium) layer surrounding each oocyte and a basal lamina. Pre-granulosa cells differentiate to form cuboidal granulosa cells in primary follicles, where a thin layer of theca cells exist outside. Preantral follicle growth is a result of granulosa cell proliferation and increased oocyte size. Preantral follicles contain three to six layers of granulosa cells and distinctive theca

layers, including an undifferentiated theca externa and steroid-secreting theca interna. With the formation of the fluid-filled antrum, the follicle growth rate increases. These follicles enlarge as a result of proliferation of granulosa cells and theca internal cells, and fluid accumulation in the antral cavity. The largest healthy follicle is selected to develop to the preovulatory stages, which exhibits accelerated growth rate and high responsiveness to follicle-stimulating hormone (FSH) (Gougeon, 2004; McGee, 2000). Upon receiving a surge of luteinizing hormone (LH), the dominant follicle ovulates to release the oocyte and the ruptured follicle develops into a corpus luteum.

1.1.3 Follicular Atresia and Apoptosis

During the dominant follicle selection, the majority of growing follicles undergo a degenerative process called atresia, a phenomenon associated with granulosa cell apoptosis and follicle degeneration (McGee and Hsueh, 2000). These atretic follicles exhibit small dense pyknotic nuclei, apoptotic granulosa cells, disrupted granulosa layer and oocyte degeneration (Gougeon, 2004; Matsuda *et al.*, 2012).

Apoptosis is a physiological programmed cell death process and serves as a protective mechanism to remove unwanted cells. Apoptotic cells exhibit cellular shrinkage, chromatin condensation and systematic DNA cleavage without inducing local inflammatory responses (Dive *et al.*, 1992; Schultz and Harrington, 2003). Inappropriate dysregulation of apoptosis contributes to the pathogenesis of human diseases, such as cancer, autoimmune diseases and degenerative disorders (Barr and Tomei, 1994; Kerr *et al.*, 1994; Thompson, 1995; Vinatier *et al.*, 1996).

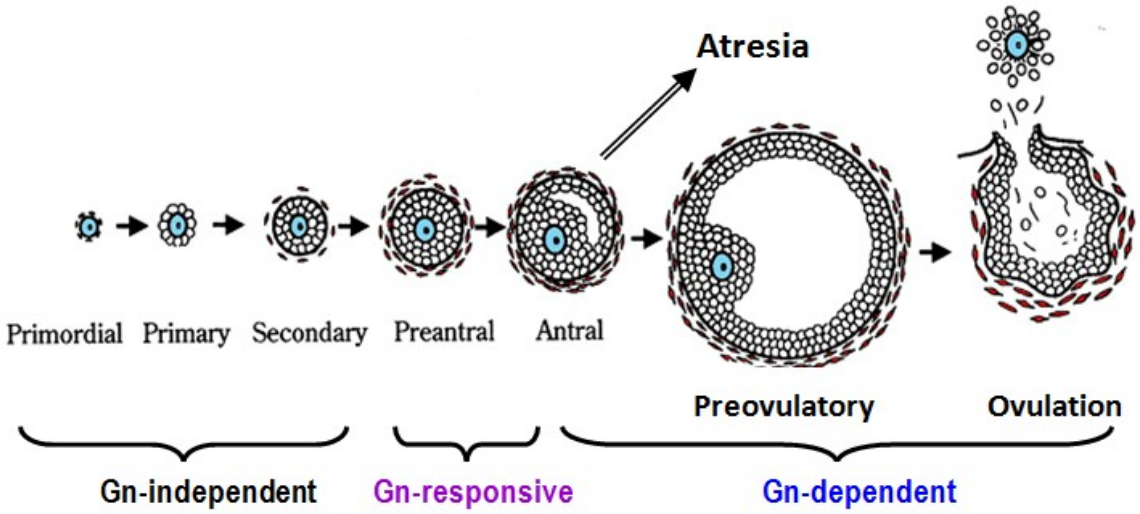

Figure 1.1 A schematic representation of ovarian follicular development

Follicles in different stages (primordial, primary, seconday, preantral, antral, preovulatory and ovulation) are shown. According to the responsiveness of follicles, the follicle development stages could be categorized as gonadotropin (Gn)-independent, Gn-responsive and Gn-dependent stages.

Two intracellular pathways are involved in the initiation of granulosa cell apoptosis: the extrinsic pathway induced by death receptor activation and the intrinsic pathway triggered by mitochondrial events (Danial and Korsmeyer, 2004; Jin and El-Deiry, 2005). In the granulosa cell death receptor-ligand system, Fas ligand (FasL) and tumor necrosis factor-α (TNF-α) are the common apoptosis inducers across species. The levels of Fas and FasL are higher in atretic subordinate follicles than in dominant follicles (Kim *et al.*, 1999; Kondo *et al.*, 1996; Lin and Rui, 2010; Porter *et al.*, 2001). The upregulation of Fas-FasL *in vitro* promotes apoptosis (Hakuno *et al.*, 1996; Rubio Pomar *et al.*, 2004; Sakamaki *et al.*, 1997), a phenomena attenuated by the presence of survival factors, such as FSH, estradiol and insulin-like growth factor (IGF) (Lin and Rui, 2010; Matsuda-Minehata *et al.*, 2007; Quirk *et al.*, 2006). The roles of TNF-α in granulosa cell proliferation and apoptosis rely on the interaction with distinct receptors TNF receptor 1 (TNFR1) and 2 (TNFR2), which may vary depending on physiological or pathological conditions. For example, binding of TNF-α to TNFR1 triggers the cell death pathway, while activation of TNFR2 stimulates anti-apoptotic gene expression (Baud and Karin, 2001; Evans *et al.*, 2004).

The intrinsic pathway is characterized by permeabilization of the outer mitochondrial membrane, loss of mitochondrial potential and release of pro-apoptotic molecules (e.g. cytochrome c) (Hussein, 2005; Jiang *et al.*, 2003). Cytochrome c binds to apoptotic protease activating factor-1 (APAF1), recruits pro-caspase-9 and forms the apoptosome complex which in turn activates caspase-3 and triggers apoptosis. The B-cell lymphoma 2 (Bcl-2) family members are the key regulators of this pathway, including both anti-apoptotic [Bcl-2 and B-cell lymphoma-extra large (Bcl-xL)] and pro-apoptotic

proteins [Bcl-2-associated death promoter (BAD) and Bcl-2-associated X protein (Bax)] (Wang, 2001), and their roles in granulosa cell apoptosis have been well established (Hsu *et al.*, 1996; Jiang *et al.*, 2003; Kaipia *et al.*, 1997; Tilly, 1996).

1.1.4 Follicular Steroidogenesis

The process of ovarian follicle maturation is coupled with the functional differentiation of the granulosa cell layer and the production of steroid hormones. Granulosa cells from preantral and earlier stage follicles are considered undifferentiated and produce minimal amounts of steroid hormones (such as progesterone and estradiol), whereas granulosa cells from preovulatory follicles and granulosa-luteal cells produce increased amounts of steroids, particularly in response to gonadotropins (Richards, 1994; Woods *et al.*, 2007).

A well coordinated biosynthesis of steroids is critical for the control of the reproductive cycle and successful ovulation. Due to the cell type-specific expression of a few steroidogenic enzymes, synergistic interaction between granulosa cells and theca cells is a prerequisite for estrogen biosynthesis and follicular development (Liu and Hsueh, 1986). Current understanding of follicular steroidogenesis is based on the "two-cell two-gonadotropin" concept (**Figure 1.2**) (Erickson, 1978; Hillier *et al.*, 1994; Wen *et al.*, 2010). The initiation and rate-limiting step in steroidogenesis is driven by the mitochondrial protein steroidogenic acute regulatory protein (StAR), which promotes the transfer of cholesterol from the outer to the inner mitochondrial membranes (Stocco, 2001), and the cytochrome P450 side-chain cleavage enzyme (p450scc, encoded by the *cyp11a1* gene) which converts cholesterol to pregnenolone, a process operational in both

granulosa cells and theca cells (Simpson, 1979). 3β-hydroxysteroid dehydrogenase (3β-HSD, encoded by *Hsd3b* gene) catalyzes the conversion of pregnenolone to progesterone, a steroid converted to androgens by cytochrome P450c17 (known as CYP17, 17α-hydroxylase/17, 20 lyase) and 17β-hydroxysteroid dehydrogenase (17β-HSD, encoded by *Hsd17b* gene) (Eimerl and Orly, 2002; Payne and Hales, 2004). Due to the absence of CYP17, granulosa cells are incapable of androgen production. Instead, they take up the androgens synthesized by the theca cells and convert them to estrogens, a reaction catalyzed by aromatase (P450arom, encoded by *cyp19* gene), a granulosa cell-specific enzyme in the ovary (Jamnongjit and Hammes, 2006; Liu and Hsueh, 1986; Wood and Strauss, 2002).

1.2 Regulation of Ovarian Follicular Development

1.2.1 Gonadotropic Regulation of Ovarian Follicular Growth and Cell Differentiation

The growth and differentiation of ovarian follicles are regulated by endocrine, paracrine and autocrine factors. The gonadotropins, including FSH, LH and human chorionic gonadotropin (hCG, analogue of LH made in placenta), are heterodimeric glycoprotein hormones with similar structures (Beck *et al.*, 1992; Pierce and Parsons, 1981). FSH is the main hormone to promote the maturation of follicles, and the preovulatory LH surge induces follicular rupture and ovulation. FSH consists of an α-subunit common to all gonadotropins and a specific β-subunit, which confers its specific biological activity (Fauser, 1996; Pierce and Parsons, 1981). FSH signals by binding to its receptor (FSHR), a member of the subfamily of G protein-coupled receptor (GPCR),

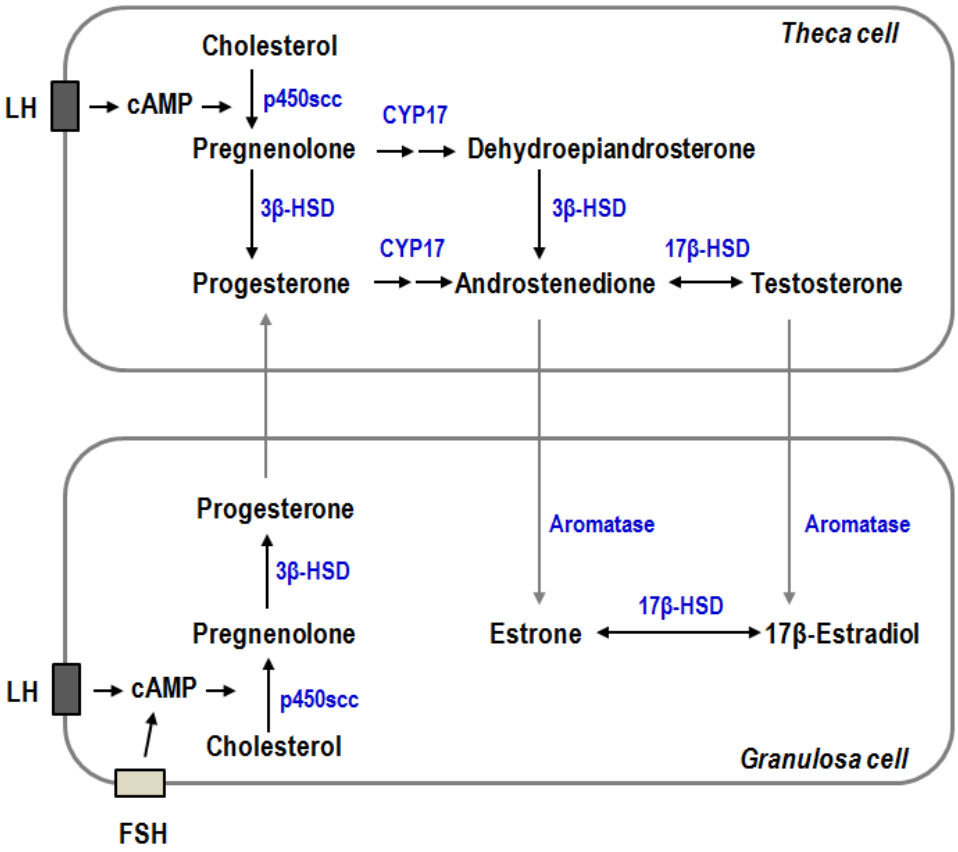

Figure 1.2 Estrogen synthesis by the "two-cell two-gonadotropin" concept

Diagramatic representation of estrogen synthesis in ovarian follicles is shown. Estradiol is synthesized in granulosa cells, demonstrating the granulosa cell dependence on theca cell testosterone production in theca cells. The expression of steroidogenic enzymes (in blue) is stimulated by the two gonadotropins (FSH and LH) in granulosa cells and theca cells (Erickson, 1978; Hillier *et al*., 1994; Wen *et al*., 2010).

and is the main hormone controlling follicular growth, selection and maturation (Bogerd, 2007; Vassart *et al.*, 2004). FSH-β or FSHR knockout mice display impaired ovarian follicular development (due to a block of antral follicle formation) and infertility (Abel *et al.*, 2000; Dierich *et al.*, 1998; Kumar *et al.*, 1997; Layman, 2000).

It is well known that FSH is critical for the continuous growth and differentiation of antral follicles, therefore this stage is considered to be a "Gn-dependent stage" (**Figure 1.1**) (Gougeon, 2004; Marshall and Kelch, 1986; McGee, 2000). The development of preantral follicles, called "Gn-responsive stage", is supported by various stimulatory and survival factors and is independent of gonadotropins, although these follicles respond to the gonadotropic stimulation (**Figure 1.1**) (Hsueh *et al.*, 2000; Marshall and Kelch, 1986; McGee *et al.*, 1997a).

The growth of antral follicles is achieved by increased granulosa cell proliferation and suppressed cell apoptosis. It has been reported that FSH activates multiple signaling pathways, increases the mRNA expression of cell cycle regulatory protein cyclin D2 and reduces the cell cycle inhibitor molecule p27kip1, leading to granulosa cell proliferation (Kayampilly and Menon, 2007; Kayampilly and Menon, 2009; Richards *et al.*, 2002). FSH also suppresses granulosa cell apoptosis by up-regulating the expression of an anti-apoptotic factor X-linked inhibitor of apoptosis proteins (XIAP) (Wang *et al.*, 2003) and decreasing the expression of cell death surface receptor Fas and its ligand FasL (Kim *et al.*, 1998; Zhang *et al.*, 2011). Gonadotropin withdrawal results in the upregulation of Fas and FasL, which is associated with follicular atresia (Chen *et al.*, 2005; Kim *et al.*, 1998).

In addition to its regulatory role in follicular growth, FSH induces granulosa cell differentiation and stimulates steroid hormone production, which is essential for

continuous development of antral follicles and subsequent successful ovulation (Hsueh *et al.*, 2000; Hunzicker-Dunn and Maizels, 2006). FSH is known to induce the expression of key steroidogenic enzymes, including aromatase, p450scc, StAR and 3β-HSD, and to increase the production of estrogen and progesterone *in vitro* (Amsterdam and Selvaraj, 1997; Eimerl and Orly, 2002; Stocco, 2001; Wayne *et al.*, 2007). FSH also upregulates the expression of differentiation-related genes, including inhibin-α, epiregulin and LH receptor (LHR) (Campbell and Baird, 2001; Wayne *et al.*, 2007), and of transcriptional factors, such as nuclear receptors (NRs) NR5a1 (known as steroidogenic factor-1), NR5a2 (known as liver receptor homolog-1), early growth response factor-1 and hypoxia -inducible factor-1α (Alam *et al.*, 2004; Russell *et al.*, 2003; Saxena *et al.*, 2007), which suggests FSH is a key growth and differentiation factor during follicular development.

LH regulates the steroidogenic activity of theca cells and stimulates the production of progesterone and androgens, such as testosterone and androstenedione (Bergh *et al.*, 1993; Fortune and Armstrong, 1977). LH stimulates the expression of steroidogenesis-related enzymes, including p450scc, CYP17, 3β-HSD and StAR (Bogovich and Richards, 1982; Magoffin and Weitsman, 1993) and triggers the expression of a number of genes involved in terminal granulosa cell differentiation, follicular rupture and ovulation, such as pro-inflammatory factor interleukin (IL)-1β, the proteases for tissue remodeling, and the progesterone receptor (Adashi, 1998; Duffy *et al.*, 1996; Hillier, 2001; Liu *et al.*, 1998).

1.2.2 Intra-ovarian Regulation of Ovarian Follicular Growth and Cell Differentiation

Follicle growth and differentiation are regulated not only by circulating gonadotropins, but also by a variety of intra-ovarian factors, including gonadal steroids, growth factors, cytokines and intracellular proteins.

IGF secreted by granulosa cells has been well characterized as a local growth factor essential for folliculogenesis. IGF-I knockout mice are infertile and exhibit arrested follicular development at the small antral stage (Baker *et al.*, 1996; Zhou *et al.*, 1997). IGF induces the expression of FSHR and LHR, increases the responsiveness of granulosa cells to FSH, and also modulates follicle steroidogenesis by enhancing aromatase expression and estradiol production, either alone or synergistically with FSH and other growth factors (Adashi *et al.*, 1985; Adashi *et al.*, 1988; Duleba *et al.*, 1997; Glister *et al.*, 2001; Kolodziejczyk *et al.*, 2003; Richards, 1994; Zhou *et al.*, 1997). In addition, IGF promotes granulosa cell proliferation and suppresses apoptosis via activating phospho-inositide 3-OH kinase (PI3K)/Akt pathway and increasing the expression of the anti-apoptotic and/or steroidogenic genes (Hu *et al.*, 2004; Mani *et al.*, 2010).

Acting as a paracrine factor, growth differentiation factor-9 (GDF9), an oocyte-derived transforming growth factor-β (TGF-β) family member, is critical for folliculogenesis since GDF9 deficiency blocks follicle growth after the primary stage and results in infertility (Dong *et al.*, 1996; Elvin *et al.*, 1999). *In vitro* data demonstrate that GDF9 promotes preantral follicle growth, increases thecal cell DNA synthesis, stimulates granulosa cell mitosis and suppresses apoptosis (Hayashi *et al.*, 1999; Hsueh *et al.*, 2000; Orisaka *et al.*, 2009; Orisaka *et al.*, 2006; Spicer *et al.*, 2008; Vitt *et al.*, 2000). GDF9 is also involved in the regulation of steroidogenesis as it stimulates basal estradiol production but suppresses FSH-induced progesterone and estradiol synthesis in rat

granulosa cells (Vitt *et al.*, 2000). GDF9 increases thecal CYP17 expression and promotes androgen production in rat and bovine follicles (Elvin *et al.*, 1999; Orisaka *et al.*, 2009; Solovyeva *et al.*, 2000). Bone morphogenetic protein (BMP)-15, another oocyte-specific molecule, stimulates granulosa cell proliferation, accelerates follicle growth in cooperation with GDF9, and inhibits FSH-induced progesterone production (McMahon *et al.*, 2008; McNatty *et al.*, 2005; Otsuka *et al.*, 2000). *In vitro* data suggest that the BMP family proteins (such as BMP4 and BMP7) not only regulate the transition stages from primordial to primary, but also promotes the action of gonadotropins on follicular growth and cell differentiation, possibly via regulating their receptor expression (Nilsson and Skinner, 2003; Shi *et al.*, 2010; Shimasaki *et al.*, 1999).

The steroids secreted by the follicles also play a role in folliculogenesis. As the follicles grow, they produce increasing amounts of estrogen, which in turn upregulates the synthesis and release of gonadotropins and promote follicle growth. A reduced production of estradiol by disruption of the *cyp19* gene *in vivo* increases follicular atresia and granulosa cell apoptosis with age, which is associated with an upregulation of the pro-apoptotic genes, p53 and Bax, in granulosa cells (Britt *et al.*, 2000; Rosenfeld *et al.*, 2001a; Toda *et al.*, 2001). *In vitro* data support an intrafollicular role of estrogen in granulosa cell survival. Estrogen inhibits granulosa cell apoptosis and increases the expression of cyclin D2, gonadotropin receptors and IGF-I (Billig *et al.*, 1993; Hsu and Hammond, 1987; Robker and Richards, 1998; Rosenfeld *et al.*, 2001b). Furthermore, androgens from the theca cells as well as cyclic guanosine 3', 5'-monophosphate (cGMP) are also important promoters of follicular growth (McGee *et al.*, 1997b; Murray *et al.*, 1998).

1.2.3 Regulation by the Adipokine Chemerin

1.2.3.1 Overview

Adipokines, a wide variety of proteins secreted from adipose tissue, act both locally and distally to regulate adipose differentiation, systemic energy balance and inflammation (Karastergiou and Mohamed-Ali, 2010). Increasing evidence indicate the important roles of adipokines (such as adiponectin, leptin and restin) in the regulation of follicular growth and steroidogenesis (Chabrolle *et al.*, 2009; Kikuchi *et al.*, 2001; Ledoux *et al.*, 2006; Spicer *et al.*, 2011).

The adipokine chemerin, also called tazarotene-induced gene 2, was first reported as a novel retinoid-responsive gene in psoriatic skin lesions (Nagpal *et al.*, 1997). It is present in serum, plasma and biological fluids (such as ascites fluid, hemofiltrate and synovial fluid) and was identified as a ligand of an orphan receptor chemokine-like receptor 1 (CMKLR1) (Bozaoglu *et al.*, 2007; Bozaoglu *et al.*, 2009; Meder *et al.*, 2003; Wittamer *et al.*, 2003; Zhao *et al.*, 2011). Chemerin is known to function as a chemoattractant that promotes the migration of immune cells to the sites of tissue injury and regulates inflammation (Berg *et al.*, 2010; Skrzeczynska-Moncznik *et al.*, 2009; Zabel *et al.*, 2005b). Recent studies demonstrate that chemerin also plays important roles in the regulation of adipogenesis (Goralski *et al.*, 2007), angiogenesis (Bozaoglu *et al.*, 2010), insulin signaling and glucose metabolism (Ernst *et al.*, 2010; Sell *et al.*, 2009; Takahashi *et al.*, 2008) and steroidogenesis (Reverchon *et al.*, 2012).

1.2.3.2 Protein Structure and Processing

Human chemerin is translated as a 163 amino acid (aa) precursor (pre-proprotein) and secreted to the circulation as a 143aa pro-chemerin (21-163aa) after removal of its N-terminal signal peptide (Ernst and Sinal, 2010; Wittamer *et al.*, 2003). Pro-chemerin has minimal biological activity and is trimmed by proteolytic processing at its C-terminus (**Figure 1.3**). The enzymes involved in coagulation, fibrinolysis and inflammation, such as plasmin, carboxypeptidases and chymase, have been shown to cleave the C-terminus of pro-chemerin to produce active and inactive chemerins *in vitro* (Du and Leung, 2009; Ernst and Sinal, 2010; Meder *et al.*, 2003; Zabel *et al.*, 2008; Zabel *et al.*, 2006a). The proteolytic processing sites depend on the location of chemerin, as the cleaved forms of chemerin found in ascites fluid, serum and hemofiltrate lack six, eight, and nine C-terminal amino acids, respectively (Du and Leung, 2009; Zabel *et al.*, 2005a; Zabel *et al.*, 2006b). Isoforms cleaved at Ala^{155} or shorter forms truncated from the active forms are inactive (Ernst and Sinal, 2010; Guillabert *et al.*, 2008). The relative amount and ratio of various forms depend on the circumstance. Under normal conditions, pro-chemerin is the dominant form and the active forms are less present; while under inflammatory or other pathological conditions, the levels of active forms chem157S and chem158K increase (Zhao *et al.*, 2011), resulting from the proteolytic processing of pro-chemerin. Therefore the proteolytic processing of chemerin may be a key regulatory mechanism which determines the local and systemic concentration of active chemerin forms and controls their physiological roles in a coordinated manner.

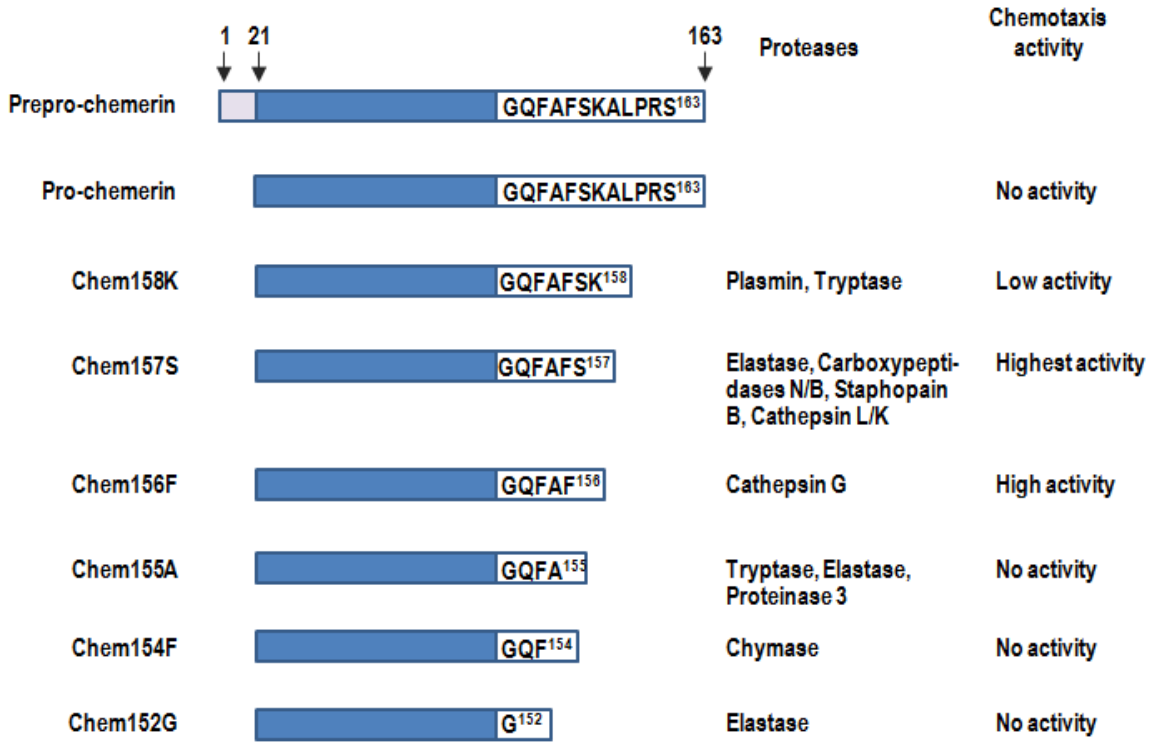

Figure 1.3 Protein structure and proteolytic processing of chemerin

A schematic representation of chemerin structure is shown. The precursor of chemerin (Prepro-chemerin, 163aa) is secreted into the circulation as pro-chemerin (143aa) after removal of its N-terminal signal peptide. The pro-chemerin is trimmed to distinct active forms by various proteases, which show the chemotaxis activities. The active forms are further proteolytically cleaved to produce the inactive forms of chemerin. The C-terminal sequences are listed using one-letter amino acid abbreviations.

A, Alanine; **G**, Glycine; **F**, Phenylalanine; **K**, Lysine; **L**, Leucine; **P**, Proline; **R**, Arginine; **Q**, Glutamine; **S**, Serine.

1.2.3.3 Tissue Distribution of Chemerin and Receptors

The chemerin mRNA has been detected by several groups in various tissues and their relative levels vary with species. In humans, chemerin is abundantly expressed in liver, lung, pituitary and ovary (Wittamer *et al.*, 2003). In murine, chemerin levels are higher in liver, fat tissue and kidney but relatively lower in the ovary (Bozaoglu *et al.*, 2007). Chemerin expression in fat tissue is lower in porcine compared with that in rodents (Huang *et al.*, 2010). These data indicate that chemerin expression in mammals is species- and tissue-dependent.

To date, chemerin has been reported to be the ligand of three GPCRs, CMKLR1, chemokine (C-C motif) receptor like 2 (CCRL2) and G-protein coupled receptor-1 (GPR-1) (Goralski *et al.*, 2007; Huang *et al.*, 2010; Wittamer *et al.*, 2003; Zabel *et al.*, 2008). The expression and tissue distribution of these receptors vary among species. In humans, CMKLR1 expression is highly expressed in spleen, lung, lymph node and dendritic cells (Wittamer *et al.*, 2003). In mouse, the expression of CMKLR1 is highest in fat tissue and lower in heart, lung and placenta in mouse (Goralski *et al.*, 2007). The relative mRNA levels of CMKLR1 and GPR-1 in porcine are differentially expressed in liver, kidney and epididymal fat tissue (Huang *et al.*, 2010), which may contribute to their distinct functions and responsiveness to chemerin in these tissues.

1.2.3.4 Functions of Chemerin

The elevated chemerin levels in tissues and fluids under chronic inflammation or pathological conditions (Nakajima *et al.*, 2010; Skrzeczynska-Moncznik *et al.*, 2009; Weigert *et al.*, 2010; Zhao *et al.*, 2011) and the expression of chemerin/CMKLR1 in

infiltrating leukocytes (Albanesi *et al.*, 2009; Zabel *et al.*, 2005b) suggest a role of chemerin in the immune system. However, our current understanding of the role of chemerin in inflammation is controversial. As a pro-inflammatory factor, chemerin promotes the migration and adhesion of macrophages and plasmacytoid dendritic cells and enhances the expression of pro-inflammatory cytokines, such as IL-6 and TNFα, in chondrocytes and fibroblast-like synoviocytes (key actors in arthiritis) (Berg *et al.*, 2010; Hart and Greaves, 2010; Kaneko *et al.*, 2011; Wittamer *et al.*, 2003). In contrast, a few reports show that chemerin is anti-inflammatory in different disease models. In a lipopolysacch-aride (LPS)-induced acute lung injury, chemerin suppresses LPS-stimulated neutrophil and macrophage infiltration as well as the secretion of IL-6 and TNF-α, an effect abolished in CMKLR1$^{-/-}$ mice (Luangsay *et al.*, 2009). In zymosan-induced peritonitis, chemerin and its C-terminal peptide C15 (A^{141}-A^{155}) inhibit macrophage activation and suppress cytokine production (Cash *et al.*, 2010; Cash *et al.*, 2008).

Recently, the functions of chemerin have been extended to the regulation of adipogenesis, energy metabolism and insulin signaling. Circulating chemerin levels are associated with body mass index (BMI) (Bozaoglu *et al.*, 2007; Hu and Feng, 2011), and its expression is higher in adipose tissue of obese and type II diabetes (T2D) patients compared with healthy subjects (Bozaoglu *et al.*, 2009; Roh *et al.*, 2007). Chemerin expression and secretion increase during adipocyte differentiation, while knockdown of chemerin or CMKLR1 impairs the differentiation of 3T3-L1 cells and reduces the expression of genes involved in glucose and lipid metabolism, such as glucose transporter-4 and adiponectin (Goralski *et al.*, 2007; Muruganandan *et al.*, 2010).

The role of chemerin in glucose homeostasis remains to be clarified due to conflicting reports. In 3T3-L1 adipocytes, chemerin has either inhibitory (Kralisch *et al.*, 2009) or stimulatory (Takahashi *et al.*, 2008) effects on insulin-stimulated glucose uptake, which may result from different chemerin dosage and various experimental models. In skeletal muscle cells, chemerin suppresses insulin-induced phosphorylation of Akt and glycogen synthase kinase 3α/β (Becker *et al.*, 2010; Sell *et al.*, 2009). Chemerin treatment *in vivo* exacerbates glucose intolerance by reducing serum insulin levels and decreasing tissue glucose uptake in obese mice but not in the lean controls (Ernst *et al.*, 2010). Moreover, chemerin knockout mice exhibit increased insulin sensitivity in skeletal muscle cells but decreased sensitivity in the liver (Takahashi *et al.*, 2011), suggesting a tissue specific role of this adipokine.

Although the expression of chemerin and its receptors (mainly CMKLR1) in the ovaries has been reported (Bozaoglu *et al.*, 2007; Goralski *et al.*, 2007; Wittamer *et al.*, 2003; Zabel *et al.*, 2005b), whether chemerin plays a role in ovarian functions remains unclear. A recent study demonstrated that chemerin inhibits IGF-I-induced steroid production and cell proliferation in luteinized granulosa cells (Reverchon *et al.*, 2012), which is the first evidence linking chemerin to follicular development.

1.2.4 Signaling Pathways Regulating Follicular Growth and Cell Differentiation

1.2.4.1 Overview

A variety of signaling pathways triggered by gonadotropins, growth factors and cytokines is involved in the regulation of follicular development. As an essential regulator of granulosa cell proliferation and differentiation, FSH binds to FSHR and leads to the rapid activation of signaling molecules that exert diverse effects in granulosa cells

(Hunzicker-Dunn and Maizels, 2006; Wayne *et al.*, 2007). Upon binding of FSH, FSHR stimulates the Gs protein, activates the membrane-associated adenylyl cyclase, and causes an elevation of intracellular cyclic adenosine monophosphate (cAMP) (Cooke, 1999). This cyclic nucleotide serves as a second messenger and quickly triggers the signaling pathways essential for cell proliferation, survival and steroidogenesis. The primary signaling cascade is the activation of adenylyl cyclase and cAMP-dependent protein kinase A (PKA), resulting in the phosphorylation of key substrates, such as cAMP-regulatory element binding protein (CREB), and facilitating the transcription of genes including aromatase and inhibin α (Gonzalez-Robayna *et al.*, 1999; Ito *et al.*, 2000; Salvador *et al.*, 2001).

Akt (known as protein kinase B), a serine/threonine kinase, is a major effector of gonadotropins and growth factors in the intracellular signaling network. Akt mediates various biological responses and regulates target genes involved in proliferation, apoptosis and differentiation (Alam *et al.*, 2004; Hu *et al.*, 2004; Johnson *et al.*, 2001; Zeleznik *et al.*, 2003). FSH-induced follicle maturation is inhibited by PI3K inhibitors or dominant negative Akt via downregulation of aromatase, inhibin-α and LHR (Zeleznik *et al.*, 2003), suggesting a critical role of PI3K/Akt pathway. Inactivation of Akt by phosphatase and tensin homologue (PTEN) attenuates IGF-I-induced cell proliferation in human granulosa cells (Goto *et al.*, 2009).

Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways are also activated by FSH to increase the mRNA expression of cyclin D2 via mammalian target of rapamycin (mTOR) signaling and to reduce the cell cycle inhibitor molecule p27kip1, which leads to proliferation (Kayampilly and Menon, 2007;

Kayampilly and Menon, 2009). The p38 MAPK inhibitor SB203580 augments FSH-induced progesterone and StAR production, but reduces estradiol production (Yu *et al.*, 2005), suggesting that p38 MAPK regulates FSH-induced progesterone and estradiol production differentially.

FSH-mediated signaling and function can be regulated via the downregulation and desensitization of FSHR. *In vivo* equine chorionic gonadotropin (eCG) treatment results in a marked increase of FSHR expression, while hCG administration decreases its expression (LaPolt *et al.*, 1992; Nakamura *et al.*, 1991). The desensitization of FSHR has been reported via phosphorylation by G-protein-coupled receptor kinases (GRKs), dissociation with Gs protein and internalization for degradation (Ascoli, 1996; Troispoux *et al.*, 1999). This process may facilitate the fine control of FSH-induced signaling pathways and prevent cells from FSH overstimulation.

1.2.4.2 Intracellular Regulator - Prohibitin

1.2.4.2.1 Prohibitin Gene and Protein

The intracellular events triggered by gonadotropins during folliculogenesis are not only regulated by kinases or phosphatases, but also by other intracellular proteins including transcriptional factors, co-activators/repressors and other molecules (such as prohibitin).

Prohibitin (PHB, also called B-cell associated protein-32) is a member of the Band-7 family of proteins sharing a conserved PHB domain (Browman *et al.*, 2007; Nijtmans *et al.*, 2002). The amino acid sequences of PHB across species are highly conserved, sharing 54 % identity between yeast and human and varying by only one

residue between rat (Tyr^{107}) and human (Phe^{107}); the sequences are identical between mice and rat (McClung *et al.*, 1995; Mishra *et al.*, 2006). In humans, the *phb* gene is located on chromosome 17q21 and consists of 7 exons with a long 3'-untranslated region (UTR), which is essential for its anti-proliferation role (Altus *et al.*, 1995; Manjeshwar *et al.*, 2003; Sato *et al.*, 1992). PHB consists of an N-terminal transmembrane domain, a PHB domain believed to be involved in protein-protein interactions, a coiled-coil domain forming an α-helix structure and a putative nuclear export sequence at its C-terminus (**Figure 1.4A**) (McClung *et al.*, 1995; Mishra *et al.*, 2006; Winter *et al.*, 2007). To date, solution or crystal structure of PHB has not been resolved. The domain structures are predicted based on the secondary structure using computer-based homology modeling techniques.

1.2.4.2.2 Functions of Prohibitin

PHB is functionally associated with multiple cellular processes such as cell-cycle regulation, immortalization, apoptosis and differentiation (Jupe *et al.*, 1995; McClung *et al.*, 1995; Roskams *et al.*, 1993). The 3'-UTR of PHB acts as a *trans*-acting regulatory RNA, inhibits DNA synthesis and suppresses cell proliferation (Jupe *et al.*, 1996; Manjeshwar *et al.*, 2003; McClung *et al.*, 1989). PHB attenuates insulin-stimulated glucose transport in adipocytes (Vessal *et al.*, 2006) and silencing of PHB reduces the expression of adipogenic markers (Liu *et al.*, 2012a), which suggests its role in adipogenesis and obesity.

To date results from studies on PHB-regulated apoptosis is controversial. PHB suppresses apoptosis induced by camptothecin (topoisomerase I inhibitor), serum/growth

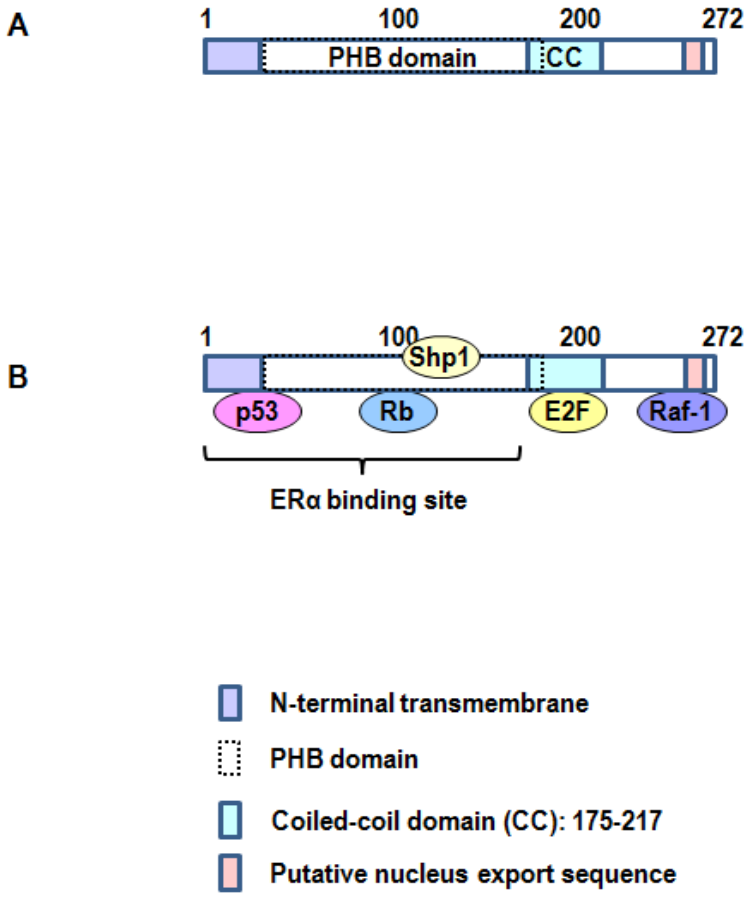

Figure 1.4 Protein structure of prohibitin and its interaction with other proteins

A. Protein structure of PHB. PHB consists of an N-terminal transmembrane domain, a PHB domain, a coiled-coil (CC) domain forming an α-helix structure and a putative nuclear export sequence at its C-terminus.

B. **The binding sites of PHB to p53, E2F1, Raf-1, Rb, ERα and Shp1 are indicated.**

factor-withdrawal, staurosporine (STS, protein kinase C inhibitor), ultraviolet (UV) B and ceramide (N-Octanoylspingosine, an apoptosis inducer) in various cell types (Chowdhury *et al.*, 2011; Chowdhury *et al.*, 2007; Fusaro *et al.*, 2003; Vander Heiden *et al.*, 2002; Wu and Wu, 2012). Its anti-apoptotic action is achieved by modulating the transcriptional activity of E2F transcriptional factor 1 (E2F1) and p53 (Fusaro *et al.*, 2003; Joshi *et al.*, 2007), or attenuating the release of cytochrome *c* into the cytocol via caspase-3 activation (Chowdhury *et al.*, 2011). To the contrary, increasing evidence indicates that PHB promotes apoptosis in cancer cells and during colitis-associated tumorigenesis, via enhancing the transcriptional activity of p53, promoting Bax expression or physically interacting with phospho-signal transducer and activator of transcription (STAT) (Joshi *et al.*, 2007; Kathiria *et al.*, 2012; Liu *et al.*, 2012b). The mechanism by which PHB differentially regulates apoptosis is unclear.

1.2.4.2.3 Expression, Post-translational Modification and Subcellular Localization of Prohibitin

PHB is widely expressed in various tissues and found in the circulation (Mengwasser *et al.*, 2004; Wang *et al.*, 2004). It is present in multiple cellular compartments, including nucleus, mitochondria and plasma membrane (Fusaro *et al.*, 2003; Kang *et al.*, 2008; Mishra *et al.*, 2005). The distinct subcellular localization of PHB potentially contributes to it multifunctional actions. PHB maintains the mitochondrial morphology, stabilizes the mitochondrial DNA and protects newly synthesized mitochondrial enzymes from degradation (Kasashima *et al.*, 2006; Nijtmans *et al.*, 2000; Steglich *et al.*, 1999). Nuclear PHB acts as a co-activator or co-repressor in the regulation

of gene expression in different cell lines, by interacting with various transcriptional factors, such as E2F, p53, Rb and estrogen receptor α (ERα) (**Figure 1.4B**) (Choi *et al.*, 2008; Fusaro *et al.*, 2003; He *et al.*, 2008; Kathiria *et al.*, 2012). Membrane-bound PHB facilitates Ras-mediated Raf-1 activation and translocates peptides to mitochondria (Chiu *et al.*, 2012; Kolonin *et al.*, 2004).

The translocation of PHB between organelles may be a precise way to switch or execute its pleiotropic functions. In response to a stress stimulus, PHB shuttles between mitochondria and nucleus, where it serves as an anti-apoptotic factor and a transcriptional co-activator/co-repressor, respectively (Sripathi *et al.*, 2011). The shuttle ability between organelles is not unique for PHB; in the presence of estradiol, mitochondrial PHB2 (a homolog of PHB) translocates to the nucleus and represses ERα-mediated transcription in cancer cells (Kasashima *et al.*, 2006). Export of PHB from nucleus to cytoplasm in breast cancer cells in response to apoptotic stimuli has also been reported (Fusaro *et al.*, 2003).

Post-translational modifications of PHB in response to upstream stimuli or pathological conditions have been reported, including phosphorylation (Ande *et al.*, 2009a; Han *et al.*, 2008; Ross *et al.*, 2008), ubiquitination (Thompson *et al.*, 2003) and O-linked β-*N*-acetylglucosamine (O-GlcNAc) modification (Ande *et al.*, 2009b), and have proved to be important in the regulation of PHB functions. Tyr^{114} phosphorylation of PHB recruits SH2-domain containing inositol 50-phosphatase (Shp1) and negatively regulates insulin-stimulated Akt phosphorylation (Ande *et al.*, 2009a). Akt phosphorylates Thr^{258} of PHB, which disrupts the interaction of PHB and Shp and in turn facilitates PI3K/Akt signaling (Ande and Mishra, 2009). Phosphorylation of PHB at

Thr^{258} is essential for Ras-mediated Raf-1 activation and enhances cell migration/ invasion via the interaction raft domain of the plasma membrane (Chiu *et al.*, 2012).

The post-translational modifications at the same or adjacent residues may affect each other, as demonstrated that Thr^{258} is either phosphorylated or modified by O-GlcNAc (Ande *et al.*, 2009b). Phosphorylation at Tyr^{114} or Tyr^{259} enhances O-GlcNAc modification at Ser^{121} and Thr^{258}, respectively, whereas O-GlcNAc modification attenuates Tyr phosphorylation (Ande *et al.*, 2009b). These modifications may recruit various interacting partners of PHB and contribute to its contradictory functions (such as pro- and anti-apoptosis) reported in the literature.

1.2.4.3 Nuclear Orphan Receptors

NR5a1 and NR5a2 belong to the nuclear receptor NR5A subfamily and play important roles in the process of follicular steroidogenesis (Boerboom *et al.*, 2000; Fayard *et al.*, 2004; Parker and Schimmer, 1997; Saxena *et al.*, 2007). NR5a1 and NR5a2 consist of a *Drosophila Fushi tarazu* factor 1 domain (specific for the NR5a subfamily) and the major structural features of all NRs: a DNA binding domain, a hinge region and a ligand binding domain (LBD) (Fayard *et al.*, 2004; Hoivik *et al.*, 2010). Unlike other NRs that are activated by hormones, phospholipids (such as phosphatidic acid and sphingolipid) bind to the LBD of NR5a1/2 and may serve as agonist or antagonist of these receptors (Krylova *et al.*, 2005; Sablin *et al.*, 2009; Urs *et al.*, 2007). The activities of NR5a1 and NR5a2 can also be regulated by a set of specific co-regulators that could repress the constitutive activity of these receptors, such as Dosage-sensitive, sex-reversal Adrenal hypoplasia congenita critical region on the X chromosome gene 1 (Dax-1) and

small heterodimer partner (SHP) (Fayard *et al.*, 2004; Lee and Moore, 2002; Saxena *et al.*, 2007).

NR5a1 is mainly expressed in the steroidogenic tissues (ovary and testis) and the tissues of hypothalamo-pituitary-adrenal axis, and regulates a large group of target genes involved in cholesterol synthesis, steroidogenesis and sex determination (Hoivik *et al.*, 2010; Parker and Schimmer, 1997; Saxena *et al.*, 2007). NR5a2 expression is confined mainly to liver, pancreas, ovary, preadipocytes and placenta (Becker-Andre *et al.*, 1993; Clyne *et al.*, 2002; Sirianni *et al.*, 2002). NR5a1 and NR5a2 are generally considered to bind to the same enhancer element in common target genes (such as StAR, aromatase, 3β-HSD and p450scc) and share similar actions on steroidogenesis during follicular development (Caron *et al.*, 1997; Hinshelwood *et al.*, 2003; Hu *et al.*, 2001; Mendelson and Kamat, 2007; Saxena *et al.*, 2007; Taniguchi *et al.*, 2009; Val *et al.*, 2003). Although both receptors are expressed in granulosa cells, the differential expression of NR5a1 and NR5a2 in other cell types, such as NR5a1 in theca cells and NR5a2 in corpus luteum (Falender *et al.*, 2003), raises a possibility that their target genes may be functional in a cell-specific manner. However, this hypothesis awaits further investigation.

1.3 Dysregulation of Ovarian Follicular Development - Polycystic Ovarian Syndrome

1.3.1 Overview

Polycystic ovarian syndrome (PCOS) is a complex and heterogeneous medical condition and affects 5-10 % women in the reproductive age (Asuncion *et al.*, 2000; Diamanti-Kandarakis *et al.*, 1999; Knochenhauer *et al.*, 1998). The patients usually

present in the clinic due to the symptoms of irregular bleeding, infertility and hirsutism. In the long-term, women with PCOS have increased risk of developing T2D, hypertension, cardiovascular diseases and endometrial cancer (Azziz *et al.*, 2006; Chittenden *et al.*, 2009; Guzick, 2004).

1.3.2 Clinical Diagnosis

To date, three criteria for clinical diagnosis of PCOS have been established since 1990 based on a combination of clinical, ultrasonographic and biochemical criteria. The 1990 National Institutes of Health (NIH) consensus workshop defined the criteria as menstrual dysfunction along with clinical and/or biochemical hyperandrogenism (Zawadski and Dunaif, 1992). Taking polycystic ovaries into consideration, the definition was revised by the 2003 Rotterdam consensus workshop that the diagnosis of PCOS should include two of the following three criteria: (1) oligo- and/or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism, (3) polycystic ovaries on ultrasonography, and exclusion of related disorders (Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop, 2004). The consensus was further modified by Androgen Excess Society with the principal conclusion that PCOS is a predominantly hyperandrogenic syndrome. These criteria defined four features of PCOS: the ovulatory dysfunction, hyperandrogenemia and polycystic ovaries, and exclusion of other androgen excess or related disorders (Azziz *et al.*, 2006).

1.3.3 Pathogenesis and Treatment of Polycystic Ovarian Syndrome

Hyperandrogenism is a well established contributor to PCOS etiology and is detected in 60-80 % of cases (Teede *et al.*, 2010). The manifestation of hyper-androgenism includes oily skin/acne and hirsutism (Guzick, 2004). Circulating free/total testosterone and androstenedione are normally elevated in PCOS patients, although the latter is not commonly considered as a required diagnostic test (Guzick, 2004). Theca cells are the major source of increased circulating androgens in PCOS women (Legro *et al.*, 1998) and exhibit increased expression and activities of CYP17, p450scc and 3β-HSD, which are the key enzymes involved in androgen production (Jakimiuk *et al.*, 2001; Nelson *et al.*, 2001; Wickenheisser *et al.*, 2000).

The steroidogenic ability of granulosa cells is also dysregulated in PCOS subjects. Individual follicles from PCOS women contain lower aromatase expression and exhibit reduced estradiol and progesterone levels in their follicular fluid compared with normally cycling women (Jakimiuk *et al.*, 1998; Welt *et al.*, 2005). Basal and hCG-stimulated 3β-HSD mRNA expression are reduced in granulosa cells from PCOS patients compared with those from normal women undergoing *in vitro* fertilization (IVF) (Doldi *et al.*, 2000). However, granulosa cells in PCOS women exhibit hyper-responsiveness to FSH (Almahbobi *et al.*, 1996; Coffler *et al.*, 2003; Mason *et al.*, 1994) that may be caused by their significantly higher FSHR expression in individual follicles (Catteau-Jonard *et al.*, 2008). Elevated circulating LH levels and LH/FSH ratio, enhanced LH pulse frequency and increased LHR expression in granulosa and theca cells have been reported in PCOS subjects (Guzick, 2004; Jakimiuk *et al.*, 2001; Wu *et al.*, 2003).

Insulin resistance is present in 50-70 % of women with this syndrome, especially in those with more severe PCOS and in women who are overweight (Dunaif, 1997; Legro

et al., 2004). Obesity enhances insulin resistance, exacerbates the reproductive and metabolic abnormalities and further increases the risk of developing T2D and cardiovascular disease (Balen *et al.*, 1995; Brassard *et al.*, 2008; Kiddy *et al.*, 1990). Increase of insulin sensitivity and reduction of insulin level improve the manifestation of hyperandrogenemia and restores ovulation (Bremer and Miller, 2008; Dunaif, 1997; Velazquez *et al.*, 1994).

The treatment of PCOS is focused on its short and long-term reproductive, metabolic and psychological features. Modification of life style, aimed at reducing weight and normalizing insulin sensitivity and androgen status, is considered the first step of the treatment strategy (Teede *et al.*, 2010). Weight loss due to diet and physical exercise in PCOS subjects has been shown to be associated with improved spontaneous ovulation rates and pregnancy as well as the reduction of circulating androgens (Moran *et al.*, 2006; Moran *et al.*, 2003; Pasquali *et al.*, 2003; Thessaloniki ESHRE/ASRM-sponsored PCOS consensus workshop, 2008). Once the weight management in obese patients has been addressed, administration of oral contraceptives is the first response as a treatment for irregular bleeding and pure anovulation, without considering hirsutism or infertility. If hirsutism is also present, spironolactone (a steroidal anti-mineralocorticoid agent with anti-androgen properties) can be used as a standard treatment (Thessaloniki ESHRE/ASRM-sponsored PCOS consensus workshop, 2008).

With regard to the ovulation induction for the treatment of infertility, the anti-estrogen clomiphene citrate remains the first-line treatment. Approximately 75 - 80 % of PCOS subjects will ovulate when on this medication (Guzick, 2004; Thessaloniki ESHRE/ASRM-sponsored PCOS consensus workshop, 2008). Moreover, the insulin-

sensitizing agents (metformin and thiazolidinediones), aromatase inhibitors (anastrozole and letrozole) and glucocorticoids (prednisone and dexamethasone) have been applied alone or in combination with clomiphene citrate for ovulation induction in PCOS subjects (Badawy and Elnashar, 2011).

1.3.4 Animal Models of Polycystic Ovarian Syndrome

To better understand the cellular and molecular mechanisms underlying the pathogenesis of PCOS and to provide clues for the identification of potential treatment targets, a range of animal models has been developed. These involve the induction by various factors including estradiol valerate and letrozole, testosterone (T), testosterone propionate (TP), dehydroepiandrosterone (DHEA), 5α-dihydrotestosterone (DHT), (Abbott *et al.*, 1998; Beloosesky *et al.*, 2004; Brawer *et al.*, 1986; Dumesic *et al.*, 2005; Kafali *et al.*, 2004; Lee *et al.*, 1998; Manneras *et al.*, 2007; Quandt and Hutz, 1993). Although these models exhibit some reproductive and/or metabolic characteristics of human PCOS, none of them reproduces all key features of PCOS.

Exposure to estrogen and the aromatase inhibitor letrozole resulted in the ovarian features of anovulation and polycystic ovaries; however, these models do not produce the metabolic features of PCOS, such as insulin resistance (Brawer *et al.*, 1986; Kafali *et al.*, 2004; Manneras *et al.*, 2007) and may be suitable only for the morphological studies of PCOS.

Prenatally androgenized sheep and monkey models display many features of human PCOS (Abbott *et al.*, 1998; Recabarren *et al.*, 2005); however they are not widely used due to the high cost and restriction on the abilities for genetic manipulation. Rodent

models provide well-characterized and stable genetic backgrounds and are low-cost. Postnatal treatment with DHEA induced most ovarian characteristics but a thin layer of theca cells and lack of vascularized theca interna, which is different from the thickened theca layer in human PCOS (Luchetti *et al.*, 2004). Prenatal exposure of T or TP does not consistently induce acyclicity and anovulation (Fels and Bosch, 1971; Slob *et al.*, 1983; Tyndall *et al.*, 2012; Walters *et al.*, 2012). Postnatal treatment of T/TP results in acyclicity, anovulation, polycystic ovaries and insulin resistance (Beloosesky *et al.*, 2004; Huffman and Hendricks, 1981; Pinilla *et al.*, 1993; Tyndall *et al.*, 2012), however the ovarian weights are reduced in these models, which is different from enlarged ovaries in human PCOS.

As DHT is a non-aromatized androgen, it has been used to develop rodent model to avoid the effect of estrogens converted from androgens. Prenatal exposure to DHT induced irregular reproductive cycles and hyperandrogenism but no polycystic ovaries (Sullivan and Moenter, 2004; Wu *et al.*, 2010). Postnatal exposure to DHT recapulates many reproductive and metabolic features of human PCOS, including acyclicity, polycystic ovaries, over-weight, increased body fat, enlarged mesenteric adipocytes and insulin resistance (Hossain *et al.*, 2013; Manneras *et al.*, 2007). Similar to other androgenized model, DHT-treated rats also exhibit reduced ovarian sizes, which is rescued by Gn administration (Hossain *et al.*, 2013).

1.3.5 Chemerin, Prohibitin and Polycystic Ovarian Syndrome

As described in earlier sections, insulin resistance and obesity occur in a high percentage of PCOS patients. Studies of chemerin in the regulation of adipogenesis and

insulin signaling have suggested a role of chemerin in the symptoms of PCOS. Clinical investigations indicated that circulating chemerin was positively associated with BMI (Bozaoglu *et al.*, 2007) and its levels elevated in obese women and in PCOS subjects compared with their controls (Bozaoglu *et al.*, 2009; Tan *et al.*, 2009). Insulin infusion *in vivo* significantly increased serum chemerin levels in human subjects and insulin stimulated the production and secretion of chemerin in adipose tissue explants (Tan *et al.*, 2009). Metformin treatment decreases serum chemerin levels in PCOS subjects (Tan *et al.*, 2009). Taken together, these findings suggest a possible correlation between chemerin and the etiology of PCOS.

The roles of PHB in the regulation of adipocyte differentiation and insulin-induced glucose metabolism have been recently reported. Ovexpression of PHB induces adipocyte differentiation (Ande *et al.*, 2011) and silencing of PHB reduces the expression of adipogenic markers (Liu *et al.*, 2012a). PHB negatively regulates insulin-stimulated Akt activation and glucose transport in a phosphorylation-dependenet manner (Ande *et al.*, 2009a; Vessal *et al.*, 2006). PHB is upregulatd by IL-6 that is known to induce insulin resistance (Theiss *et al.*, 2007). Although there is no direct evidence showing the involvement of PHB in the pathogenesis of PCOS, these data imply a link between PHB and insulin resistance and obesity.

CHAPTER 2: RATIONALE OF RESEARCH PROGRAM

Follicular development is tightly regulated by endocrine, autocrine and paracrine factors. The dysregulation of follicle growth and cell differentiation results in ovarian dysfunction related diseases, such as premature ovarian failure, poor-responsiveness to gonadotropin and PCOS. Understanding the molecular and cellular mechanisms in the control of follicular development will provide important insight into the pathophysiology of these conditions.

Despite increasing information from clinical and basic investigations on human PCOS, the etiology and pathogenesis of this syndrome are still not completed understood. An effective animal model that mimics the human situation will provide an opportunity to investigate the molecular and cellular mechanisms underlying this complex syndrome, which otherwise would be difficult due to limited access to human ovarian cells. DHT-treated rats exhibited increased body weight gain, disrupted estrus cyclicity, decreased insulin sensitivity as well as increased body fat and enlarged adipocytes (Hossain *et al.*, 2013; Manneras *et al.*, 2007). Futher investigation and manipulation of the reproductive phenotypes of the DHT-treated rat model will extend our knowledge of the pathogenesis of PCOS and facilitate the investigations into the molecular and cellular basis of PCOS.

Although a positive correlation of serum chemerin levels with obesity as well as PCOS is known to exist, whether and how chemerin contributes to the pathogenesis of PCOS, such as arrested follicular growth and dysregulated steroidogenesis, is unclear. Moreover, less is known about the function of chemerin and its receptors during follicular development, although the expression of chemerin in the ovary has been reported. Investigation of the role of chemerin in follicular growth and cell differentiation will

improve our understanding of the pathogenesis of PCOS and provide important clues on whether chemerin could be a potential diagnostic biomarker for this syndrome.

Although PHB is anti-apoptotic and functions by inhibiting caspase-3 activity and cytochrome c release in undifferentiated granulosa cells (Chowdhury *et al.*, 2011; Chowdhury *et al.*, 2007), whether it plays a role in follicular steroidogenesis and whether its functions in the ovary are follicular stage-dependent await further investigation. In addition, the findings that PHB promotes adipogenesis and attenuates insulin-stimulated glucose transport and Akt activation (Ande and Mishra, 2009; Ande *et al.*, 2012; Vessal *et al.*, 2006) suggest a possible link between PHB and glucose metabolism/adipogenesis. However, whether PHB contributes to the dysregulation of steroidogenesis, insulin resistance and obesity during the pathogenesis of PCOS is unknown.

CHAPTER 3: OBJECTIVES AND HYPOTHESES

3.1 Overall Objective and Hypothesis

The **overall objective** of the current study is to better understand the complex regulatory mechanisms involved in the control of ovarian follicular development and steroidogenesis and to gain insights into the dysregulation of these processes in the pathogenesis of PCOS. Our **overall hypothesis** is that chemerin and PHB are two regulators of follicular steroidogenesis and they contribute to PCOS in a hyperandrogenized rodent model.

3.2 Specific Hypotheses

We hypothesize that:

1. Chemerin and PHB suppress FSH-induced follicular steroidogenic enzyme expression and steroid production, which involve the nuclear receptors;
2. Chemerin and PHB contributes to the progression of PCOS in a DHT-induced rat PCOS model;
3. PHB is a mediator of the chemerin action on FSH-induced steroidogenesis
4. PHB plays a role in apoptosis during follicular development

3.3 Specific Objectives

1. To examine the expression and regulation of chemerin and PHB in the ovary;
2. To explore the roles of chemerin and PHB in FSH-induced follicular steroidogenesis;

3. To examine the molecular mechanisms by which chemerin and PHB regulate ovarian functions;
4. To investigate the potential involvement of chemerin and PHB in PCOS using a DHT-induced rat model

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Chemerin, a Novel Regulator of Follicular Steroidogenesis and its Potential Involvement in Polycystic Ovarian Syndrome

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Abbreviated title: Chemerin and steroidogenesis in rat PCOS model

Keywords: PCOS, chemerin, steroidogenesis, aromatase, p450 side-chain cleavage enzyme

Abbreviations: CMKLR1, Chemoattractant ligand for G protein-coupled receptor chemokine receptor-like 1; CTL, control; DHT, 5α-dihydrotestosterone; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HSD, hydroxysteroid dehydrogenase; MOI, multiplicity of infection; PCOS, polycystic ovarian syndrome; p450scc, p450 side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; TBST, Tween-20 in Tris and NaCl (Tris buffered saline).

Contribution of co-authors

All studies were carried out under the supervision of Dr. Benjamin K. Tsang. Qi Wang conducted all experimental work unless otherwise noted and wrote the manuscipt. Dr. Ji Young Kim was involved in the experiment of follicle culture (Figure 4A and 5A). Kai Xue was involved in the preparation of DHT-treated rats and construction of adenoviral-NR5a1/2. Both of them were consulted on the writing and editing of the manuscript and also contributed to the editing of final version. Drs. Jia-yin Liu, Arthur Leader and Benjamin K. Tsang provided input on the design of the studies and interpretation of the data, and edited the manuscript for publication.

Abstract

Polycystic ovarian syndrome (PCOS) is a heterogeneous syndrome associated with follicle growth arrest, minimal granulosa cell proliferation, dysregulated sex hormone profile, hyperthecosis, and insulin resistance. Using a 5α-dihydrotestosterone (DHT)-induced rat model that recapitulates the reproductive and metabolic phenotypes of human PCOS, we have examined the steroidogenic capability of granulosa cells from DHT-treated rats. Gene expression of several key steroidogenic enzymes including p450 side-chain cleavage enzyme (p450scc), aromatase, steroidogenic acute regulatory protein, hydroxysteroid dehydrogenase-17*β*, and hydroxysteroid dehydrogenase-3*β* were markedly lower in DHT-treated rats than the controls, although the responsiveness of their granulosa cells to FSH was higher. Expression of the adipokine chemerin and its receptor, chemokine receptor-like 1, was evident in control and DHT-treated rats, with significantly higher ovarian mRNA abundances and protein contents of chemerin and its receptor. Recombinant chemerin decreases basal estradiol secretion in granulosa cells from DHT-treated rats. When the inhibitory role of chemerin in steroidogenesis was further examined *in vitro*, chemerin suppressed FSH-induced progesterone and estradiol secretion in cultured preantral follicles and granulosa cells. Chemerin also inhibits FSH-induced aromatase and p450scc expression in granulosa cells. Overexpression of nuclear receptors NR5a1 and NR5a2 promotes p450scc and aromatase expression, respectively, which is suppressed by chemerin. These findings suggest that chemerin is a novel negative regulator of FSH-induced follicular steroidogenesis and may contribute to the pathogenesis of PCOS.

Introduction

Follicular development is tightly regulated by endocrine, autocrine, and paracrine factors, and the process of ovarian follicle maturation in both mammalian and avian species is coupled with a functional differentiation of granulosa cell layer (1). Granulosa cells from preantral or earlier stages of follicles produce minimal steroid hormones and are considered undifferentiated. By comparison, granulosa cells from preovulatory follicles secrete extensive amounts of progesterone and estradiol in response to gonadotropins (2, 3). The coordinated biosynthesis of progesterone and estradiol in the ovary is critical for normal ovary function, successful ovulation, and eventual pregnancy (4). p450 side-chain cleavage enzyme (p450scc) and aromatase are the crucial steroidogenic enzymes for progesterone and estradiol biosynthesis in granulosa cells, respectively (5, 6). Nuclear receptors NR5a1 (previously known as steroidogenic factor-1) and NR5a2 (also known as liver receptor homolog-1) are two transcriptional factors known to regulate multiple steroidogenic genes encoding aromatase, p450scc, steroidogenic acute regulatory protein (StAR), and hydroxysteroid dehydrogenase (HSD)-3β (7-11).

Polycystic ovarian syndrome (PCOS), a multifactorial heterogeneous clinical syndrome, affects up to 10 % of women of reproductive age and accounts for 75% of anovulatory infertility (12). PCOS is associated with follicle growth arrest, chronic anovulation, minimal granulosa cell proliferation, hyperthecosis with hyper-androgenemia, and insulin resistance (13). It is a disorder of reproduction and metabolism with potential systemic sequelae such as diabetes, endometrial cancer, and obesity (13). Obese women often have more severe hyperandrogenism and anovulation than normal-

weight women with PCOS (14). The pathogenesis of PCOS and the interrelationship between obesity and PCOS is complex and its etiology is not completely understood.

Chemerin is identified as a chemoattractant ligand for G protein-coupled receptor chemokine receptor-like 1 (CMKLR1) (15, 16). It was recently discovered as a novel adipokine associated with obesity and metabolic syndrome and is shown to promote adipogenesis and regulate immunity and glucose metabolism (16-20). Serum chemerin levels are higher in obese women and in PCOS subjects (21, 22), and metformin (a drug for treatment of PCOS patients with insulin resistance) decreases serum chemerin levels (21), implying a correlation between chemerin and PCOS. Although several groups reported that chemerin and CMKLR1 mRNA were expressed in the ovaries of human, rat, and mouse (16-18, 23), the role of chemerin in FSH-induced follicular development remains unclear. Moreover, whether chemerin contributes to the pathogenesis of PCOS is unknown.

In this study, a 5α-dihydrotestosterone (DHT)-induced rat model was used to mimic the reproductive and metabolic phenotypes of human PCOS following that in a recent publication (24). First, we characterized the steroidogenic capability and chemerin/CMKLR1 expression in ovarian cells from DHT-treated rats. Second, we investigated the role of chemerin in FSH-induced steroidogenesis in granulosa cells from immature rats. We proposed that chemerin is a novel negative regulator of FSH-induced follicular steroidogenesis and contributes, at least partially, to the pathogenesis of PCOS.

Materials and Methods

Reagents and antibodies

Cell culture media (M199 and α-MEM), fetal bovine serum (FBS), penicillin and streptomycin, L-glutamine, sodium pyruvate, and trypsin were purchased from Invitrogen (Burlington, Canada). Recombinant mouse active chemerin and antichemerin antibody were from R&D Systems (Minneapolis, MN). Diethylstilbestrol, HEPES, BSA, bovine insulin, transferrin, ascorbic acid, and sodium selenite anhydrous were purchased from Sigma (St. Louis, MO). Recombinant human FSH was obtained from the National Hormone and Peptide Program (Harbor-University of California, Los Angeles, Medical Center, Torrance, CA). Anti-aromatase antibody was purchased from AbD Serotec (Oxford, UK), anti-p450scc antibody from United States Biological (Swampscott, MA), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies and reagents for SDS-PAGE were supplied by Bio-Rad (Mississauga, Canada). Enhanced chemiluminescent reagent was from Thermo Fisher Scientific (Rockford, IL). QIAShredder and RNeasy minikits were purchased from QIAGEN (Mississauga, Canada). Random decamer primers were from Ambion (Austin, TX). Ribonuclease inhibitor and deoxynucleotide triphosphate were from Fermentas (Burlington, Canada). Moloney murine leukemia virus reverse transcriptase was from Promega (Madison, WI). QuantiTect SYBR Green PCR kit, phosphatase inhibitors (PhosSTOP cocktail), and protease inhibitors (Complete cocktail) were from Roche Applied Science (Québec, Canada). PCR primers were from Invitrogen. All other chemicals were of the highest analytical grade and were available from Sigma.

Animal preparation

Sprague-Dawley rats (Charles River, Montréal, Canada) were maintained on 12-h light, 12-h dark cycles and given food and water *ad libitum*. All procedures were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, Canadian Council on Animal Care, and were approved by the University of Ottawa and Ottawa Hospital Research Institute Animal Care Committee.

DHT-filled SILASTIC capsule preparation

The DHT-filled capsules were prepared as previously described (25). In brief, SILASTIC brand tubing (inner diameter 1.98mm × outer diameter 3.18 mm; Dow Corning Corp., Midland, MI), cut to an appropriate length to achieve a desired surface area of 300 mm^2, was filled with DHT (Steraloids Inc., Newport, RI) from a 1-ml syringe. The tubing was closed at each end (3 mm) with a sealant (Silicone type A; Dow Corning), ensuring that the adhesive is in contact with the tubing walls and that there are no air bubbles. Control capsules were empty with only sealant on both ends. After being left overnight to dry, the capsules were rinsed for 2 d in 3% BSA in PBS with 0.1 % NaN_3 solution, washed with PBS, and sterilized by dipping briefly in 70 % ethanol before use.

Animal surgery, DHT capsule implantation, and animal care

Female rats at 21 d of age were randomly divided into two groups (DHT *vs.* control) and implanted with a DHT-filled silicone capsules continuous releasing (83 µg/d, empty SILASTIC brand capsule as control) for 12 wk to mimic the hyperandro-

genic state in women with PCOS, whose plasma DHT levels are approximately 1.7-fold higher than those of healthy control (26, 27). Rats were monitored twice daily in the first 3 d and once daily thereafter. Animals were weighed weekly to monitor weight gain and euthanized at 12 wk after implantation.

Serum collection and chemerin level analysis

Rat blood was taken after rat euthanization and serum was collected after clotting and centrifuging (1000 × g, 10 min) at room temperature. Serum aliquots were kept in -80 C for chemerin analysis. The 0.5-µl serum sample was subjected to SDS-PAGE with 4.5 % stacking and 15 % separating gels, and chemerin levels in serum were analyzed by Western blot using anti-chemerin antibody. A pool of six mixed serum samples was loaded as an internal control for density normalization between different membranes.

Collection of ovaries from control (CTL) or DHT rats

Rat ovaries were collected after rat euthanization, and the two ovaries from the same rat were used for RNA and protein analysis, respectively. One ovary was homogenized in buffer RNeasy Lysis Buffer, and total RNA were extracted according to the manufacturer's instruction (QIAGEN RNeasy minikit). Another ovary was Dounce homogenized, and total protein was extracted on ice for 30 min in lysis buffer (10 mM Tris; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 0.5 % Igepal, pH 7.4) containing various protease/phosphatase inhibitors (Roche). Lysates were centrifuged at 13,000 × g for 10 min at 4 C, and the protein concentration in the supernatants was determined by Bradford assay (Bio-Rad Laboratories).

Follicle isolation and culture

Ovarian follicles of a diameter of 150-180 μm (large preantral/early antral follicles) were isolated from 14-d-old rats, as previously described (28). In brief, follicles were manually isolated in Leibowitz L-15 medium with BSA (0.1 %) using 28.5-gauge needles (Becton Dickinson and Co., Franklin Lakes, NJ). To minimize the experimental variation caused by damage incurred during the isolation procedures, only round and clear follicles with an intact basement membrane and theca layer were selected for the present studies. Follicles were cultured individually in a 96-well plate (Sarstedt, Newton, NC) in 100 μl α-MEM supplemented with HEPES (10 mM), 0.1 % BSA, bovine insulin (5 μg/ml), transferrin (2 μg/ml), ascorbic acid (25 μg/ml), sodium selenite anhydrous (2 ng/ml), L-glutamine (3 mM), sodium pyruvate (100 μg/ml), streptomycin (100 μg/ml), and penicillin (100 U/ml). The culture medium was changed every other day and kept in -80 C for hormone analysis.

Construction of recombinant adenovirus

Construction of recombinant adenovirus was carried out as described previously with some modifications (29). Briefly, cDNA of rat NR5a1 or NR5a2 were subcloned into the adenoviral shuttle vector pAdTrack-CMV, and the ligation product pAdTrack-NR5A was linearized and cotransformed into competent BJ5183 bacteria with adenoviral backbone plasmid. The homologous recombinant adenoviral plasmid pAd-NR5A1 was purified, linearized, and transfected to AD293 cells. After 10 d, the cells were harvested and lysed through four cycles of freeze/thaw to obtain viral supernatants. The virus was amplified by repeated infection of AD293 cells, and the resulting viral supernatants were

stored at -80 C. Control virus adenovirus-green fluorescent protein (GFP) was generated using empty pAdTrack-CMV vector with the method described above. Virus concentrations were determined by measuring the OD at 260 nm using a ratio of 1×10^{12} viral particles per 1 OD unit (30).

Primary culture of rat granulosa cells and adenoviral infection

Ovaries from diethylstilbestrol-primed immature rats (d 21, 1 mg/d, sc, for 3 consecutive days) were preincubated with 6 mM EGTA and 0.5 M sucrose (31), and granulosa cells were released by follicular puncture with a 26.5-gauge needle, washed, and centrifuged (900 × g, 5 min). Cell clumps and oocytes were removed by filtering the cell suspensions through a 40-μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ). The number of viable granulosa cells was determined by trypan blue exclusion. Granulosa cells (0.45×10^6 per well in a 12 well plate) were plated overnight in M199 with 10 % FBS under a humidified atmosphere of 95 % air and 5 % CO_2. After starving overnight in serum-free medium, granulosa cells were treated with FSH (0 - 100 ng/ml) or chemerin (0 - 100 ng/ml) for a designated time.

Granulosa cells from CTL or DHT rats were recovered in a similar but more precisely method as mentioned above. Granulosa cells from preantral/early antral follicle stages in the ovary were released by follicular puncture without touching preovulatory follicles and corpora lutea. As a low yield of granulosa cells from adult rats, granulosa cells from four to five rats were pooled and plated (0.23×10^6 per well in a 24 well plate) overnight in M199 with 10 % FBS.

For adenoviral infection, granulosa cells were cultured in serum-free M199 medium containing adenoviral particles for 24 h followed by medium change. Multiplicity of infection (MOI) and duration of infection are detailed in the figures. Equal amounts of adenovirus in each experimental group were achieved by the adjustment with an appropriate amount of adenoviral-GFP.

Reverse transcription-polymerase chain reaction

Total RNA of granulosa cells were extracted according to the manufacturer's instructions, using the QIAGEN RNeasy minikit. Two-tenths micrograms of total RNA were used to reverse transcript cDNA, and the mRNA abundances of target genes were analyzed by real-time PCR and normalized to GAPDH. Specific primer pairs using in experiments are listed in **Table 1**. Data were analyzed by the $2^{-\Delta\Delta CT}$ method (32).

Protein extraction and Western blot

Total protein extracts were prepared by adding 60 µl hot (100 C) Tris buffer (1 % sodium dodecyl sulfate, 1 mM sodium orthovanadate) to each well. The extracts were obtained by rapidly scraping, transferred to a microcentrifuge tube, and boiled for 5 min. Protein concentrations in each sample were determined by Bradford assay (Bio-Rad Laboratories). Twenty micrograms of cell lysates were subjected to SDS-PAGE with 4.5% stacking and 10 % separating gels. Proteins were electrophoretically transferred to nitrocellular membrane (Bio-Rad), blocked at room temperature with 5 % skim milk in TBST [0.05 % Tween-20 in 10 mM Tris; 0.15 M NaCl, pH 7.4 (Tris buffered saline)] for 1 h, and then incubated overnight at 4 C with 1:1000 diluted primary antibodies in TBST

Table 1 Primer pairs used for quantitative real-time PCR

Gene	Primer	Sequence (5'-3')	Accession No.
aromatase	Forward	GGCATGCACGAGAATGGCATCATA	NM_017085
	Reverse	CAGCCTGTCCAAATGCTGCTTGAT	
p450scc	Forward	ACACGACCTCCATGACTCTGCAAT	NM_017286
	Reverse	TCAGTGTCTCCTTGATGCTGGCTT	
StAR	Forward	TGTTAAGGACTGCCCACCACATCT	NM_031558
	Reverse	TGTCCTTGGCTGAAGGTGAACAGA	
HSD-3β	Forward	AGATCTGGGCTATGTGCCACTTGT	NM_001007719
	Reverse	ACCTGGTAACACCCAGAACCACAT	
HSD-17β	Forward	TGTGGGTGCTGTACTGGATGTGAA	NM_012851
	Reverse	ACTTGCTGGCACAGTACACTTCGT	
Chemerin	Forward	GGCACCTTTGTGAGGCTGGAATTT	NM_001013427
	Reverse	ACCCTGTCCAGGGCTTATTTGGAT	
CMKLR1	Forward	AAATGGCAGACAAGCAACCTGAGC	NM_022218
	Reverse	GCTGCCCTTGCAAGCAAGTTCTAT	
FSHR	Forward	TGTCCTCATCAAGCGACACCAAGA	NM_199237
	Reverse	AGGAGAATCTTGGCCTTGGACACA	
GAPDH	Forward	TGACTCTACCCACGGCAAGTTCAA	NM_017008
	Reverse	ACGACATACTCAGCACCAGCATCA	

with constant agitation. The membranes were then treated with a secondary antibody (1:5,000 to 1:50,000 based on a different primary antibody). After washing three times with TBST, immunoreactive bands were visualized with enhanced chemiluminescence according to the manufacturer's instruction. Intensity of bands of the exposed X-ray film was determined by densitometrically scanning, quantitated using AlphaEaseFC (Alpha Innotech, San Leandro, CA) and normalized with GAPDH.

Steroids secretion analysis

Spent medium of cultured granulosa cells were collected, centrifuged (900 × g, 5 min) and kept in -80 C for hormone analysis. 17β-estradiol and progesterone concentrations in spent medium were measured using enzyme immunoassay (EIA) kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instruction. The detection limitation of estradiol was 28 pg/ml, and the intra- and interassay coefficients of variation were 8 and 6 %, respectively. The detection limitation of progesterone was 8.5 pg/ml, and the intra- and interassay coefficients of variation were 7 and 6 %, respectively.

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 statistical software (San Diego, CA). The results are given as mean ± SEM of at least three independent experiments as detailed in the figures. An unpaired *t* test was used for comparing the mRNA abundances and protein contents of target genes in the ovary/granulosa cells from CTL/DHT rats or serum chemerin levels. A two-way ANOVA was used to assess the effects and

interactions of two variables and multiple comparisons were achieved by a Bonferroni *post hoc* test. Significant difference was defined at $P < 0.05$ (* or #).

Results

Dysregulated steroidogenesis in a DHT-induced rat model

To facilitate the investigations on the molecular and cellular mechanisms underlying the dysregulated reproductive and metabolic functions of PCOS, a rodent PCOS model induced by chronic DHT treatment was applied in this study (24). To assess the ovarian steroidogenic capability of DHT-treated rats, granulosa cells were collected from CTL and DHT-treated rats and mRNA levels of key steroidogenic enzymes (p450scc, aromatase, StAR, HSD-17β, and HSD-3β) were analyzed by real-time PCR. As shown in **Fig. 1A**, the mRNA abundance of p450scc ($P < 0.001$), aromatase ($P < 0.001$), StAR ($P < 0.01$), HSD-17β ($P < 0.05$), and HSD-3β ($P < 0.01$) were significantly down-regulated in granulosa cells from the DHT group compared with those from controls. The protein contents of aromatase and p450scc in granulosa cells from DHT-treated rats were also down-regulated (**Fig. 1B**), and FSH (100 ng/ml) stimulated aromatase and p450scc contents in both the CTL and DHT groups. There was significant interaction observed between FSH and DHT treatment (**Fig. 1B**, for aromatase: DHT, $P < 0.0001$; FSH, $P < 0.0001$; DHT × FSH, $P < 0.05$; for p450scc: DHT, $P < 0.0001$; FSH, $P < 0.0001$; DHT × FSH, $P < 0.01$). We also examined the steroids production in these cells in the presence or absence of FSH. Compared with CTL rats, basal levels of estradiol and progesterone in granulosa cells from DHT-treated rats was approximately 36 % (0.49 ± 0.11 vs. 1.35 ± 0.27; $P < 0.05$) and 16 % (0.18 ± 0.07 vs. 1.14 ± 0.12; $P < 0.001$), respectively. FSH

stimulated granulosa cell estradiol (**Fig. 1C**, DHT, $P < 0.05$; FSH, $P < 0.0001$; DHT × FSH, $P < 0.05$) and progesterone (**Fig. 1C**, DHT, $P < 0.001$; FSH, $P < 0.001$; DHT × FSH, $P < 0.01$) production in both CTL and DHT groups in vitro, although the levels of steroids in those from DHT group remained lower compared with the controls. Despite the similar mRNA abundances of FSHR in two groups (**Fig. 1A**, $P > 0.05$), the responsiveness of granulosa cells to FSH was higher because the level changes of estradiol and progesterone in granulosa cells from DHT rats in response to FSH were 58.5-fold (vs. 50.2 in CTL) and 6.3-fold (vs. 3.6 in CTL), respectively (**Fig. 1C**). The increases of aromatase and p450scc contents in response to FSH were similar to those of steroids production, with elevation of aromatase (6.77-fold in DHT vs. 2.36 in CTL) and p450scc (6.22-fold in DHT vs. 2.22 in CTL), as indicated in **Fig. 1B**.

Expression of chemerin and CMKLR1 in DHT-treated rat model

To examine whether the rat ovarian cells express chemerin and its receptor and whether their expression is dysregulated in the DHT-rat model, we examined the serum and ovarian chemerin levels. In DHT rats, serum chemerin levels were significantly elevated compared with CTL rats (**Fig. 2A**, $P < 0.05$). Both chemerin and its receptor CMKLR1 were expressed in adult rat ovaries and the contents of chemerin ($P < 0.05$), and CMKLR1 ($P < 0.05$) significantly increased in DHT-treated rats (**Fig. 2B**). Chronic DHT treatment also increased CMKLR1 mRNA abundance in the whole ovary (**Fig. 2C**, $P < 0.05$) and isolated granulosa cells (**Fig. 2D**, $P < 0.05$). Chemerin mRNA level in the whole ovary from DHT rats was also elevated (**Fig. 2C**, $P < 0.001$); however, its level in granulosa cells had no difference between CTL and DHT groups (**Fig. 2D**, $P > 0.05$).

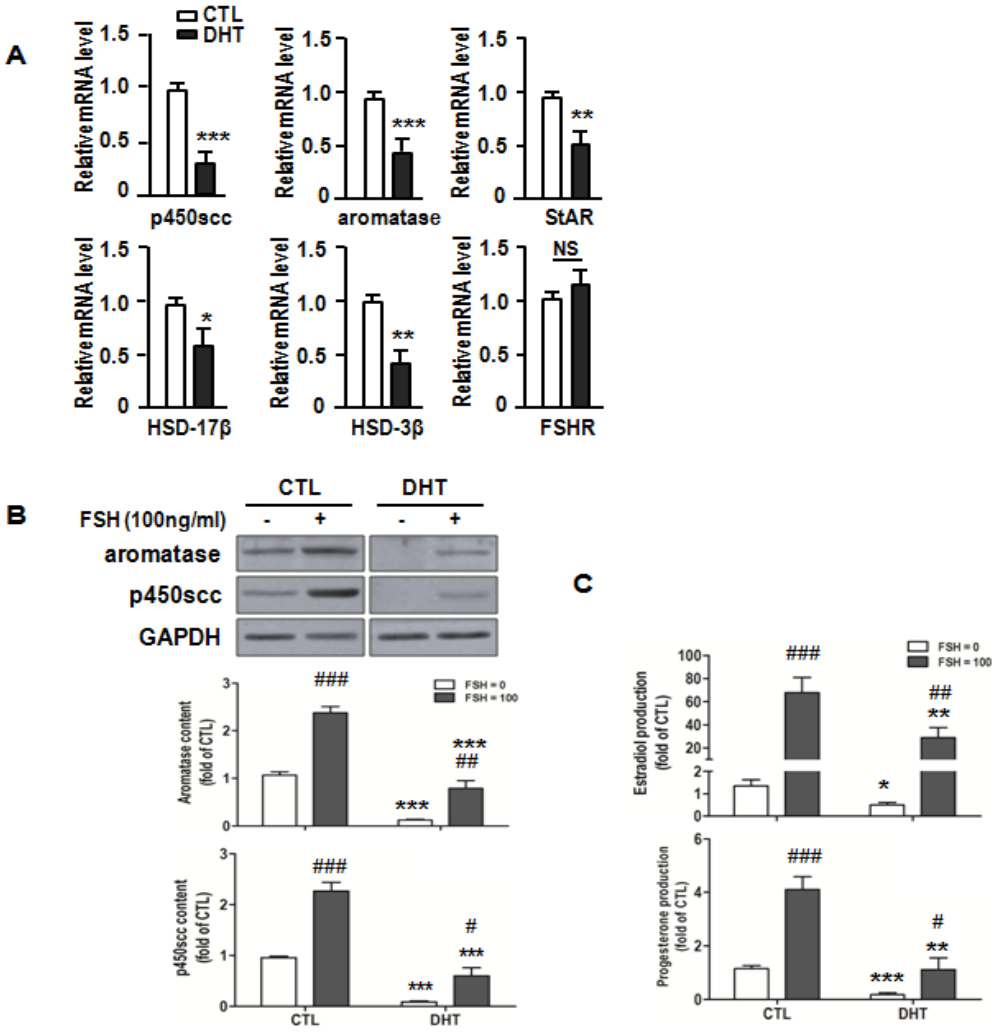

Figure 1. Steroidogenic enzyme expression and steroid production in ovarian cells from DHT-treated rats.

A, Granulosa cells were collected from CTL and DHT-treated rats and pooled for RNA extraction. The mRNA abundances of key steroidogenic enzymes and FSHR were analyzed by real-time PCR and normalized to the expression of GAPDH. Data are expressed as the fold change over that observed in control samples.

B, Granulosa cells from CTL and DHT-treated rats were cultured with or without FSH (100 ng/ml, 48 h), and protein contents of p450scc and aromatase were examined by Western blot. Immunoblots shown are representative of four independent experiments.

C, Granulosa cells from CTL and DHT-treated rats were cultured with FSH (0 - 100 ng/ml) for 48 h, and estradiol and progesterone production in spent medium was assessed by EIA. Data are expressed as the fold change in steroids production over that observed in the control samples. Data are presented as mean ± SEM of three to five independent experiments. Panel A used an unpaired student *t* test; panels B and C used two-way ANOVA followed by a Bonferroni *post hoc* test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ compared with CTL group; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$ compared with untreated control (FSH = 0). NS, Not significant

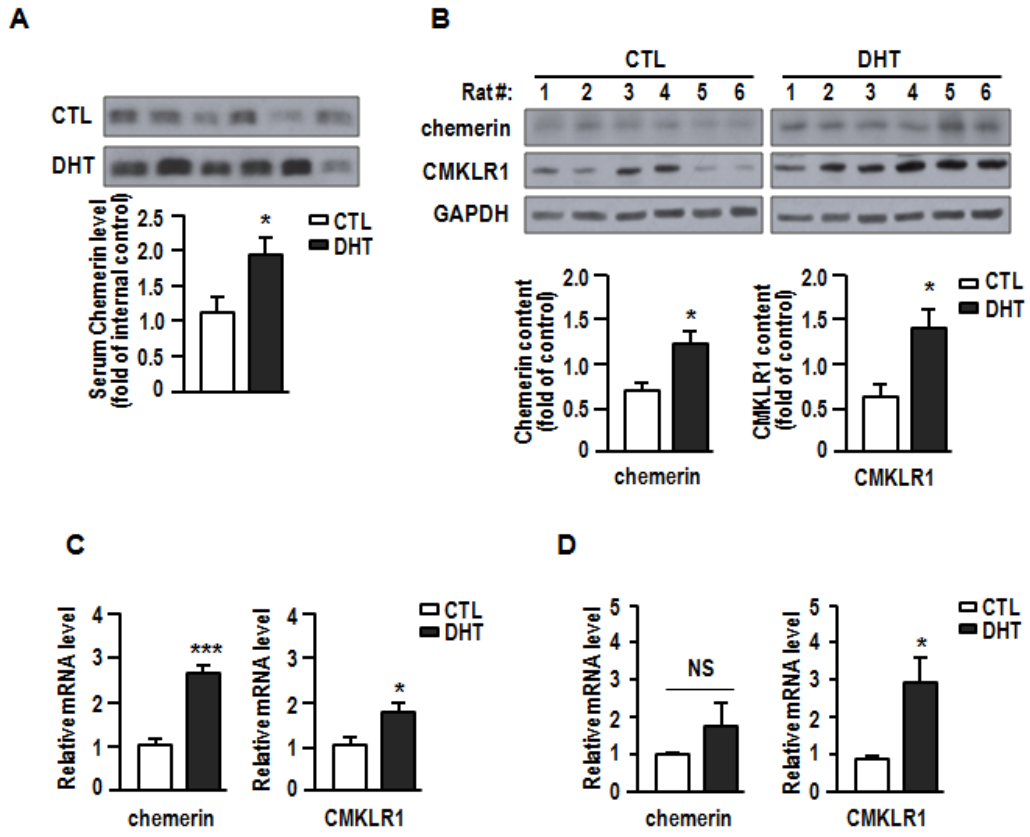

Figure 2. Chemerin levels in serum and ovarian cells in CTL and DHT-treated rats

A, Serum (0.5 μl) from CTL and DHT-treated rats was subjected to Western blot and immunoblotted with antichemerin antibody. Immunoblots shown are six representative serum samples in each experimental group, whereas each histogram shows results from 12 rats.

B, Chemerin and CMKLR1 contents in whole-ovary extracts from CTL and DHT-treated rats were analyzed by Western blot and normalized to GAPDH. Each sample was collected from one ovary of individual CTL or DHT rats (15 rats per histogram), whereas six representative samples were presented in the immunoblots.

C, Total RNA were extracted from whole ovaries of CTL and DHT-treated rats. Each sample was collected from one ovary of CTL or DHT rats (nine rats per histogram). The mRNA abundances of chemerin and CMKLR1 were analyzed by real-time PCR and normalized to the expression of GAPDH.

D, Granulosa cells were collected from four to five rats of the CTL or DHT group and pooled for total RNA extraction. The mRNA abundances of chemerin and CMKLR1 were analyzed by real-time PCR and normalized to the expression of GAPDH. Data are presented as mean ± SEM of five independent experiments. Panels A-D used an unpaired Student t test. *, $P < 0.05$; ***, $P < 0.001$ compared with CTL group. NS, Not significant

Chemerin suppresses basal estradiol secretion of granulosa cells from both CTL and DHT-treated rats

To explore the potential role of chemerin in the regulation of ovarian function, granulosa cells from CTL and DHT-treated rats were cultured in the presence of chemerin (0-100 ng/ml, 48 h), and the estradiol and progesterone levels in the spent medium were determined by EIA. Chemerin suppressed basal estradiol production (**Fig. 3**, DHT, $P < 0.01$; chemerin, $P < 0.001$; DHT × chemerin, $P < 0.05$) but had no effect on progesterone secretion (**Fig. 3**, DHT, $P < 0.001$; chemerin, $P > 0.05$; DHT × chemerin, $P > 0.05$) in granulosa cells from both CTL and DHT rats.

Chemerin inhibits FSH-induced aromatase expression and estradiol secretion in cultured follicles and granulosa cells *in vitro*

We then investigated the role of chemerin in FSH-induced estradiol production in cultured follicles and granulosa cells from immature rats. Although chemerin had minimal effect on basal steroid secretion by cultured follicles, it significantly reduced FSH-stimulated estradiol production (**Fig. 4A**, chemerin, $P < 0.05$; FSH, $P < 0.0001$; chemerin × FSH, $P < 0.05$). Chemerin also suppressed FSH-induced estradiol secretion in granulosa cells only at higher dose (100 ng/ml) (**Fig. 4B**, chemerin, $P < 0.05$; FSH, $P < 0.0001$; chemerin × FSH, $P < 0.05$).

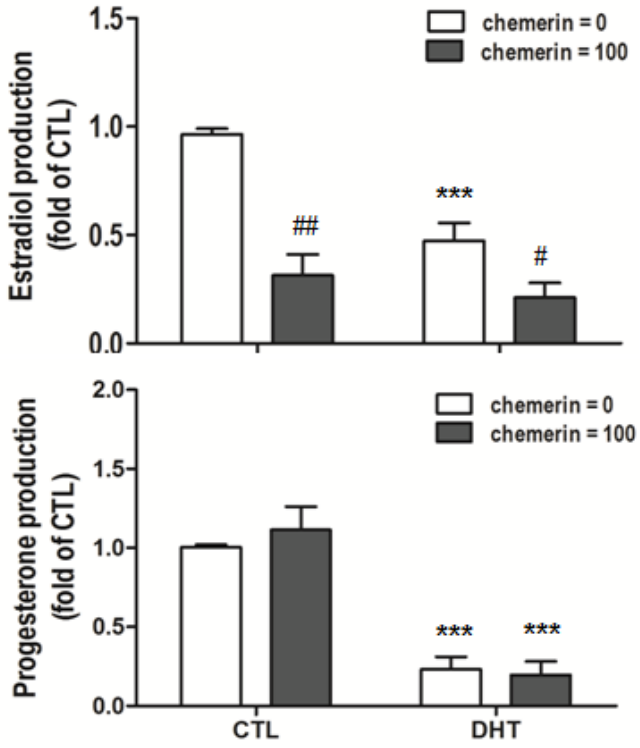

Figure 3. Effect of chemerin on steroidogenic enzyme expression and steroid production in granulosa cells from CTL and DHT-treated rats

Granulosa cells from CTL and DHT-treated rats were cultured with chemerin (0 - 100 ng/ml) for 48 h, and estradiol and progesterone levels in spent medium were assessed by EIA. Data are presented as mean ± SEM of three independent experiments and analyzed by two-way ANOVA followed by Bonferroni *post hoc* test. ***, $P < 0.001$ compared with CTL group; #, $P < 0.05$, ##, $P < 0.01$ compared with untreated control (chemerin = 0).

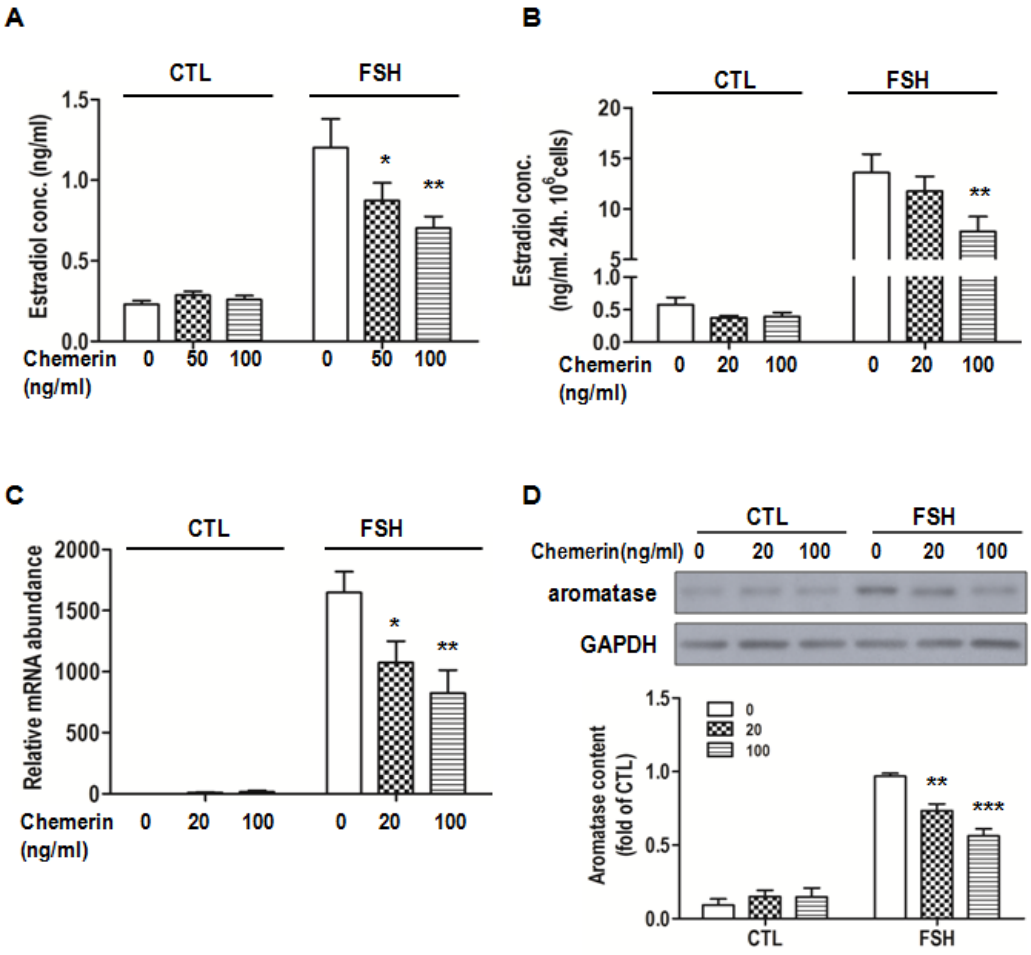

Figure 4. Effect of chemerin on FSH-induced estradiol secretion and aromatase expression in cultured follicles and granulosa cells

A, Follicles were cultured with chemerin (0 - 100 ng/ml) ± FSH (10 ng/ml) for 4 d *in vitro*, and estradiol levels in spent medium were measured by EIA. Data are presented as mean ± SEM of 26-30 follicles per group (from three independent experiments).

B-D, Granulosa cells were cultured with chemerin (0-100 ng/ml) ± FSH (100 ng/ml) for 24 h. Testosterone (0.5 μM) was added as substrate of aromatase during culture. Estradiol levels in spent medium (B) were measured by EIA. Aromatase mRNA (C) and protein levels (D) were examined by real-time PCR and Western blot, respectively. Data are presented as mean ± SEM of three to five independent experiments and analyzed by two-way ANOVA followed by Bonferroni *post hoc* test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ compared with FSH-only group

Because aromatase is the rate-limiting enzyme for estradiol synthesis, we examined whether chemerin regulates FSH-induced aromatase expression in granulosa cells. Both mRNA abundance (**Fig. 4C**, chemerin, $P < 0.05$; FSH, $P < 0.0001$; chemerin × FSH, $P < 0.05$) and protein content (**Fig. 4D**, chemerin, $P < 0.01$; FSH, $P < 0.0001$; chemerin × FSH, $P < 0.001$) of aromatase were induced by FSH and significantly down-regulated by chemerin.

Chemerin inhibits FSH-induced progesterone secretion and p450scc expression in preantral follicles and granulosa cells

We also investigated the role of chemerin in FSH-induced progesterone production in cultured follicles and granulosa cells from immature rats. As with estradiol production, FSH-induced progesterone production in preantral follicles (**Fig. 5A**, chemerin, $P < 0.01$; FSH, $P < 0.0001$; chemerin × FSH, $P < 0.01$) was significantly suppressed in the presence of chemerin. Chemerin inhibited FSH-induced progesterone in granulosa cells only at higher dose (100 ng/ml) (**Fig. 5B**, chemerin, $P < 0.0001$; FSH, $P < 0.001$; chemerin × FSH, $P < 0.05$). Although p450scc mRNA expression was dramatically stimulated by FSH and its abundance showed a trend of decrease in the presence of chemerin, it did not achieve statistical significance of chemerin effect (**Fig. 5C**, chemerin, $P > 0.05$; FSH, $P < 0.0001$; chemerin × FSH, $P > 0.05$). The content of p450scc was induced by FSH and significantly down-regulated by chemerin (**Fig. 5D**, chemerin, $P < 0.05$; FSH, $P < 0.001$; chemerin × FSH, $P < 0.05$).

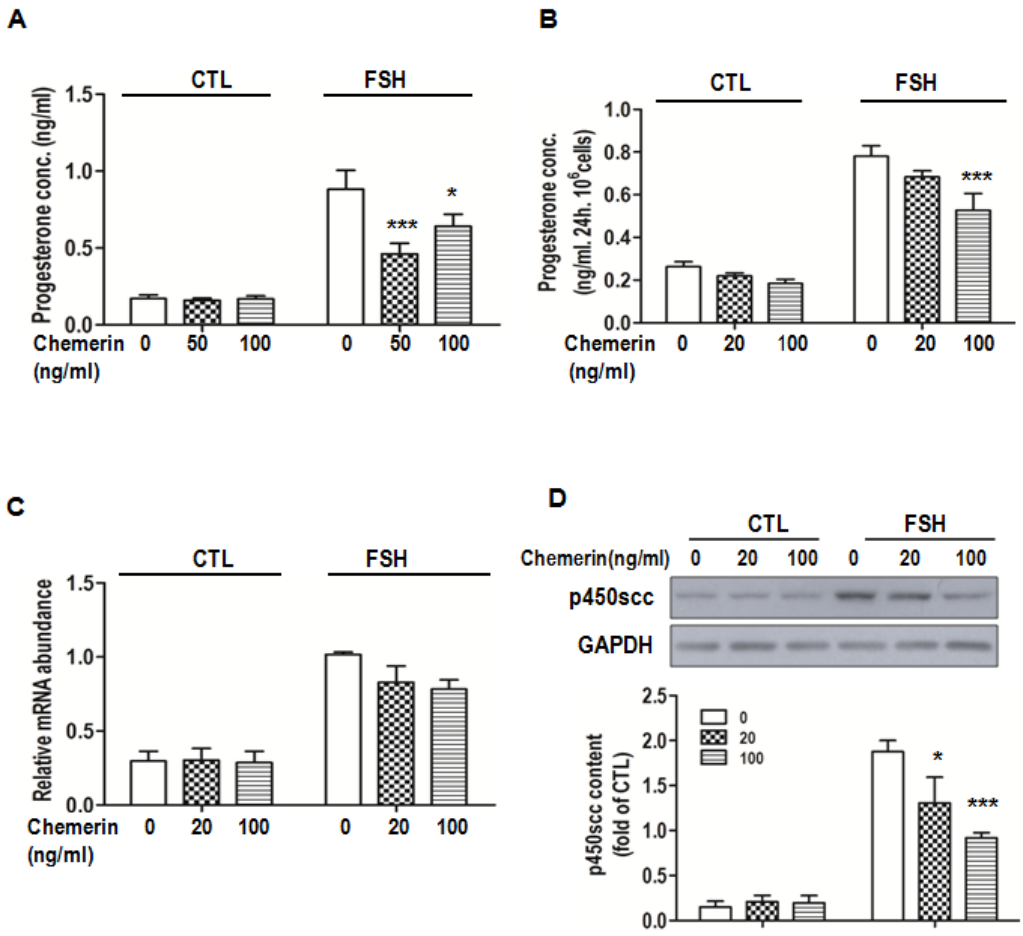

Figure 5. Effect of chemerin on FSH-induced progesterone secretion and p450scc expression in cultured follicles and granulosa cells

A, Follicles were cultured with chemerin (0 - 100 ng/ml) ± FSH (10 ng/ml) for 4 d *in vitro*, and progesterone levels in spent medium was examined by EIA. Data are presented as mean ± SEM of 26-30 follicles per group (from three independent experiments).

B-D, Granulosa cells were cultured with chemerin (0 - 100 ng/ml) ± FSH (100 ng/ml) for 24 h. Progesterone level in spent medium (B) was measured by EIA. p450scc mRNA (C) and protein levels (D) were examined by real-time PCR and Western blot, respectively. Data are presented as mean ± SEM of three to five independent experiments and analyzed by two-way ANOVA followed by a Bonferroni *post hoc* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with FSH only group

Chemerin suppresses forskolin-induced aromatase and p450scc expression

Forskolin is known to activate adenylyl cyclase and increase the intracellular level of cAMP (33). Next, we examined the effect of chemerin on forskolin-induced steroidogenesis in granulosa cells *in vitro*. Forskolin (10 μM) alone stimulated granulosa cell aromatase and p450scc expression; although pretreatment with chemerin (100 ng/ml) significantly suppressed foskolin-induced aromatase and p450scc contents (**Fig. 6A**, for aromatase: chemerin, $P < 0.05$; FSK, $P < 0.0001$; chemerin × FSK, $P < 0.01$; for p450scc: chemerin, $P < 0.05$; FSK, $P < 0.0001$; chemerin × FSK, $P < 0.05$). There was significant interaction between chemerin and FSK effect in both cases.

Chemerin suppresses NR5a1/2-induced aromatase and p450scc expression

The nuclear receptors, NR5a1 and NR5a2, have been implicated as the key transcriptional factors of multiple steroidogenic genes *in vitro* (7, 8, 10). It is possible that chemerin interferes with the transcriptional functions of NR5a1/2 on steroidogenesis. Therefore, we examined whether chemerin suppressed NR5a1/2-induced steroidogenic enzymes expression *in vitro*. Granulosa cells were infected with adenoviral NR5a1 or NR5a2 (MOI = 10, 24 h; adenoviral-GFP as control) and then cultured with chemerin (100 ng/ml) for 48 h. As shown in **Fig. 6B**, NR5a1 alone stimulated p450scc expression but not aromatase (data not shown), and addition of chemerin suppressed NR5a1-induced p450scc expression (chemerin, $P < 0.05$; NR5a1, $P < 0.001$; chemerin × NR5a1, $P < 0.01$) as well as the content of NR5a1 (chemerin, $P < 0.01$; NR5a1, $P < 0.001$; chemerin × NR5a1, $P < 0.01$). Differing from that of NR5a1, exogenous NR5a2 induced aromatase expression and chemerin suppressed this effect (**Fig. 6C**, chemerin, $P < 0.01$; NR5a2, $P <$

0.001; chemerin × NR5a2, $P < 0.05$). p450scc expression was also stimulated by NR5a2 to a lesser extent compared with those with NR5a1 because the exposure time of p450scc in Fig. 6C was much longer than that in Fig. 6B. There was no suppression effect of chemerin on NR5a2-induced p450scc expression (**Fig. 6C**, chemerin, $P > 0.05$; NR5a2, $P < 0.001$; chemerin × NR5a2, $P > 0.01$). The overexpression of NR5a2 was significantly down-regulated by chemerin (**Fig. 6C**, chemerin, $P < 0.01$; NR5a1, $P < 0.001$; chemerin × NR5a1, $P < 0.01$).

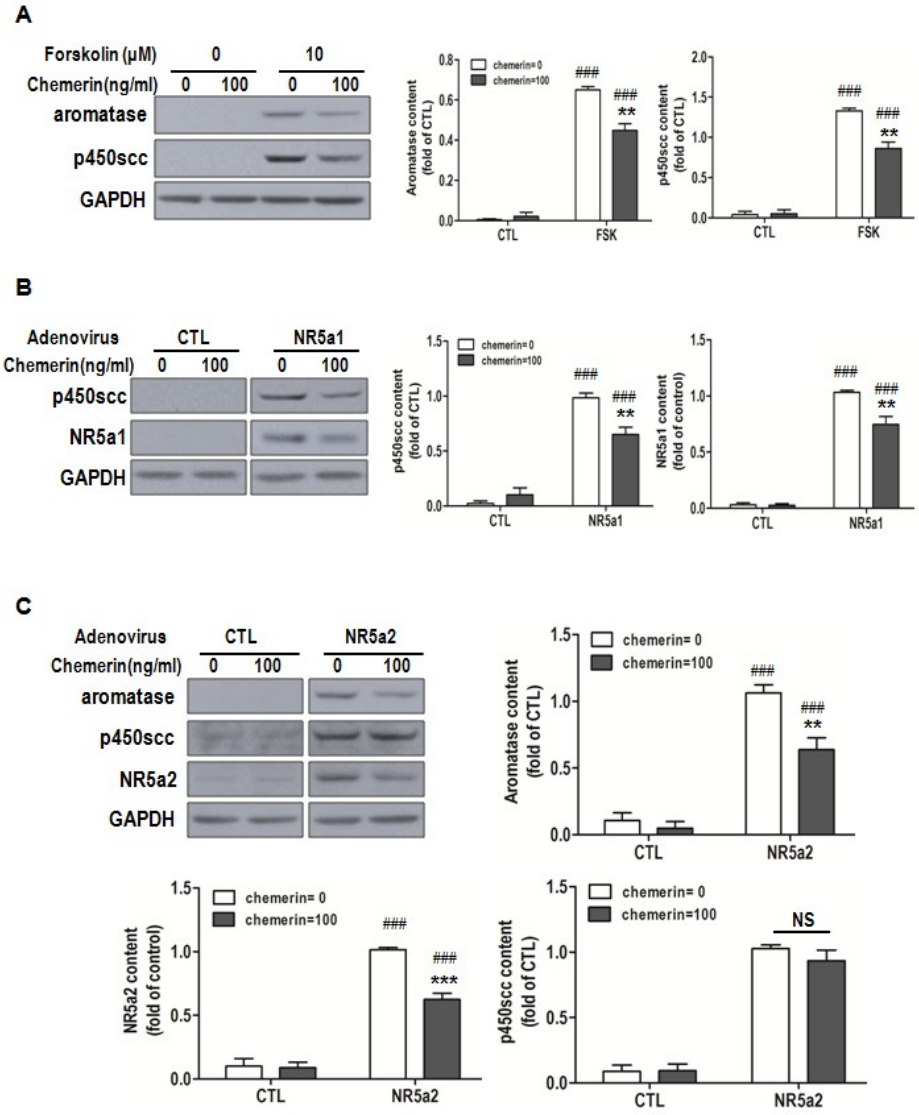

Figure 6. Effect of chemerin on forskolin- or NR5a1/2-induced aromatase and p450scc expression in granulosa cells

A, Granulosa cells were cultured with chemerin (0 and 100 ng/ml) ± forskolin (FSK; 10 μM) for 24 h, and the contents of aromatase and p450scc were examined by Western blot. Representative immunoblots and mean ± SEM of three replicates are shown. Results were analyzed by two-way ANOVA, followed by a Bonferroni *post hoc* test. **, $P < 0.01$ compared with FSK-only group; ###, $P < 0.001$ compared with CTL group (FSK = 0).

B and C, Granulosa cells were infected with adenoviral NR5a1 (B) or NR5a2 (C; MOI = 10, adenoviral-GFP as control) for 24 h and then cultured with chemerin (0 - 100 ng/ml, 48 h). The contents of aromatase, p450scc, NR5a1, and NR5a2 were examined by Western blot. Representative immunoblots and mean ± SEM of three replicates are shown. Results were analyzed by two-way ANOVA, followed by a Bonferroni *post hoc* test. **, $P < 0.01$ compared with NR5a1/2-only group; ###, $P < 0.001$ compared with CTL group. NS, Not significant

Discussion

In the present study, we have demonstrated in a hyperandrogenic rodent model that DHT-treated rats exhibit dysregulation of steroidogenic enzyme expression and aberrant serum and ovarian chemerin levels. Recombinant chemerin suppressed FSH-induced expression of steroidogenic enzymes (aromatase and p450scc) and steroid production in cultured rat preantral follicles and granulosa cells. Chemerin also reduced NR5a1/2 contents and down-regulated NR5a1-induced p450scc and NR5a2-induced aromatase expression. Our findings represent the first evidence that chemerin is a novel negative regulator of FSH-induced steroidogenesis during follicular development.

Here a DHT-induced rodent model is applied to recapitulate the reproductive and metabolic phenotypes in human PCOS. Although the basal estradiol and progesterone levels of granulosa cells from DHT-treated rats are relatively lower, their capabilities in response to FSH are higher than those from controls. Our observation is in agreement with previous *in vivo* and *in vitro* studies showing the hyperresponsiveness of granulosa cells from PCOS subjects to FSH (34-36). The enhanced responsiveness of granulosa cells may be due to either elevated FSH receptor (FSHR) expression or increased binding ability of FSH to cell surface. We observed that no obvious changes of FSHR mRNA abundance in DHT-treated rats; however, two groups recently demonstrated that FSHR expression in granulosa cell of PCOS subjects is significantly higher than women with normal ovulatory function undergoing *in vitro* fertilization (IVF) (37, 38). Gonadotropin treatment and experimental cell type may account for the difference between DHT-rat model and human PCOS. Increased binding affinity of FSH may be a possible reason in DHT-treated rats, as demonstrated in an earlier study that the binding of ^{125}I-labeled

human FSH to granulosa cells from anovulatory PCOS subjects is higher than that in controls (34). Further experiments are needed to examine this possibility.

In the DHT-treated rat model, granulosa cells exhibited reduced expression of key steroidogenic enzymes and decreased basal steroids production. Our results are in good agreement with the published data showing lower ovarian follicle aromatase expression and steroidogenesis from PCOS subjects compared with controls (39, 40). However, we observed a reduced expression of p450scc and StAR in granulosa cells from DHT-treated rats, which differs with the literature showing increased p450scc (but not StAR) expression from PCOS subjects (41, 42). Due to the heterogeneity of PCOS, there is no single model that could recapitulate all the phenotypes of human PCOS. DHT-treated rats exhibited increased body weight gain, disrupted estrus cyclicity, arrested follicle growth, decreased insulin sensitivity, and reduced ovarian weight, a phenomenon attenuated by gonadotropin administration in a time-dependent manner (Wang, Q., unpublished data). These reproductive and metabolic features in the DHT-rat model resemble the phenotypes of human PCOS, making it a useful rodent model to investigate the pathogenesis of PCOS.

We found that chemerin was expressed in rat ovary, and its serum and ovarian protein levels increased in DHT-treated rats compared with controls. It has been demonstrated that the inactive pro-form of chemerin is converted to active forms (chem158K, chem157S, and chem156F) by proteolytic cleavage at its C terminus (43-45). The double bands of serum chemerin in this study may correspond to the pro- and active forms; however, their precise identity needs to be further investigated. A recent paper reported that the active forms (chem158K+chem157S) account for approximately

18 % of total chemerin in normal human plasma, whereas it reached 50-75 % in cerebrospinal and synovial fluid samples (46), indicating that significant cleavage of chemerin occur in these inflammatory diseases. Nevertheless, the two bands observed in both CTL and DHT rats serum showed similar density with each other (by Western blot), implying that no obvious difference of chemerin processing in these rats. A more precise and sensitive assay will be helpful to determine the different forms and potential processing of chemerin in rat serum.

Using an *in vitro* preantral follicle and granulosa cell culture system, we reported for the first time that chemerin suppressed FSH- and forskolin-induced steroidogenesis in rat ovary. The suppression of steroids production and steroidogenic enzyme expression by chemerin was achieved, at least partially, via down-regulating NR5a1/2 expression and interfering with their transcriptional activity. The DNA binding domains of the nuclear receptors NR5a1 and NR5a2 are highly conserved, and the two molecules bind to the same promoter elements of common steroidogenic genes, which encode aromatase, p450scc, StAR, and HSD-3β (7-11). It is noteworthy in this study that NR5a1 and NR5a2 preferably induced p450scc and aromatase expression, respectively, in rat granulosa cells, with less induction of aromatase and p450scc expression. It may due to the sequence difference between NR5a1 and NR5a2, which results in differential recruitment of coactivators or corepressors (47). During the preparation of this manuscript, another group reported that chemerin inhibited IGF-I-induced steroidogenesis without affecting steroidogenic enzyme expression in luteinized human granulosa cells from non-PCOS infertile patients undergoing IVF (48). They demonstrated that chemerin had no effect on FSH-induced steroidogenesis, which was different from our results. The treatment of

FSH and human chorionic gonadotropin during an IVF protocol may account for the difference, as demonstrated by Falender *et al.* (9), that human chorionic gonadotropin treatment reduces NR5a1/2 expression in rat ovaries, which may minimize the effect of chemerin on steroidogenesis.

It has been reported that chemerin is present in serum/plasma (21, 22), follicular fluid (48), ascites fluid (in ovarian cancer patients) (16), and synovial fluids (in arthritic patients) (46); however, the source of chemerin protein in circulation is unclear at present. In addition to the ovary, adipose tissue, liver, and kidney also have higher chemerin mRNA expression (16-18), which may represent other sources of chemerin. It is known that serum chemerin level is elevated in PCOS subjects compared with the controls (21, 22). In this study we also demonstrated that ovarian chemerin contents increased in DHT-induced PCOS model and that exogenous chemerin suppressed FSH- and forskolin-induced steroidogenesis, suggesting that chemerin may be dysregulated in PCOS and may be important in the regulation of steroidogenesis. However, we cannot rule out the possible contribution of chemerin from extraovarian sources in this process.

In conclusion, our findings demonstrate that serum and ovarian chemerin levels are elevated in a DHT-induced rat PCOS model, which is associated with the dysregulation of steroidogenesis. We also report, for the first time, that chemerin suppresses FSH-induced estradiol and progesterone production in rat preantral follicles and granulosa cells. Chemerin inhibits FSH-induced mRNA and protein expression of aromatase and p450scc in granulosa cells. Overexpression of NR5a1 and NR5a2 promotes p450scc and aromatase expression, respectively, which is suppressed by chemerin *in vitro*. This study significantly improves our understanding of the role of

chemerin in ovarian cells. In light of these findings, our next focus will be to investigate the mechanism by which chemerin downregulates the nuclear receptors and which signaling pathway(s) is involved.

Acknowledgments

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Disclosure Summary: The authors declare no conflict of interest.

Addendum

Measurement of serum chemerin levels in adult rats

In addition to the analysis of the relative chemerin levels in serum of CTL and DHT-treated rats (**Figure 2A**), we also determined the concentration of chemerin in serum using diluted recombinant mouse chemerin as standards. Serum samples (0.5µl) from three rats in CTL group were loaded to 15 % SDS-PAGE and analyzed by Western blot (**Figure A1**). A set of diluted recombinant chemerin (0.01, 0.05, 0.1, 0.5ng) was loaded in parallel with these samples, together with a pool of serum for normalization purpose. The serum pool (Mix) was made by equally mixing serum sample from ten rats. The intensity of bands in the exposed X-ray film was determined by densitometrically scanning, quantitated using AlphaEaseFC (Alpha Innotech, San Leandro, CA) and normalized with Mix. The concentrations of rat serum samples were calculated after linear regression of the band intensity of diluted recombinant chemerin ($r^2 = 0.987$). The average concentration of chemerin in rat serum was 112.8 ± 16.72 ng/ml, which was within the range of chemerin used in experiments reported in this chapter.

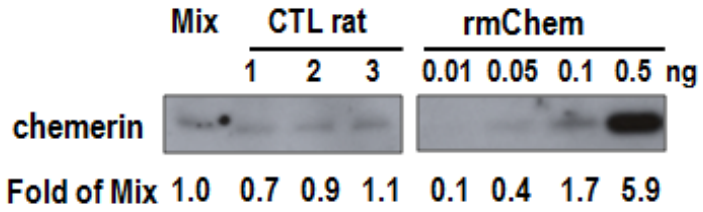

Figure A1 Estimation of serum chemerin levels in CTL rats.

0.5 µl serum samples from three control rats were subjected to SDS-PAGE and analyzed by Western blot with a set of diluted recombinant mouse chemerin (rmChem, 0.01-0.5ng). A pool of samples made from 10 equally mixed rat serum was used for normalization.

Corrections and Clarifications

Since the publication of the manuscript in this chapter, a number of typographical, grammatical or syntax errors were identified. The list below represents the corrections to be included.

Page 42, Line 14	"is" should read "was", and "are" should read "were"
Page 42, Line 22	"capsules" should read "capsule"
Page 43, Line 2	"control" should read "controls"
Page 43, Line 18	"Another" should read "The other"
Page 45, Line 1	"adenovirus" should read "adenoviral"
Page 45, Line 18	"precisely" should read "precise"
Page 46, Line 7	In the qPCR experiment, measurement of amplification efficiency of the gene of target should be considered and 90-105 % should be expected to achieve reliable qPCR results
Page 46, Line 17	"scrapping" should read "scraping"
Page 48, Line 13-14	"limitation" should read "limit".
Page 64, Line 19	"than women" should read "than in women"
Page 66, Line 17	"It may due to" should read "It may be due to"
Where applicable	"steroids production" should read "steroid production", "manufacturer's instruction" should read "manufacturer's instructions"
Figures 1, 2, 4, 5, 6	In the quantitation graph of Western blot, the label of Y axis "fold of control" should be changed to "relative content over GAPDH"
Figure 3, 6	chemerin treatment dosage "0-100ng/ml" should be changed to "0 and 100ng/ml" due to only two treatment.
Figure 6B & p.60,L19	As aromatase band was undetectable in Western blot, NR5a1 alone did not induce aromatase expression.

CHAPTER 5: ENDOCRINOLOGY, 2013, 154: 956 - 967

Inhibitory Roles of Prohibitin and chemerin in FSH - induced Rat Granulosa Cell Steroidogenesis

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Keywords: prohibitin, chemerin, steroidogenesis

Abbreviated Title: Prohibitin and chemerin suppress steroidogenesis

Abbreviations: A-Akt, active Akt; CTL, control; DES, diethylstilbestrol; DHT, ihydrotestosterone; DMSO, dimethylsulfoxide; DN, dominant negative; eCG, equine chronic gonadotropin; EIA, enzyme immunoassay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HSD-3β, hydroxysteroid dehydrogenase-3β; LY, LY294002; MEK, MAPK kinase; MOI, multiplicity of infection; PCOS, polycystic ovary syndrome; PHB, prohibitin; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; p450scc, p450 cholesterol side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; TBST, Tween-20 in Tris and NaCl (pH 7.4) (Tris buffered saline); TCN, triciribine.

Contribution of co-authors

All studies were carried out under the supervision of Dr. Benjamin K. Tsang. Qi Wang conducted all experimental work and wrote the manuscipt. Drs. Arthur Leader and Benjamin K. Tsang provided input on the design of the studies and the interpretation of the data, and edited the manuscript for publication.

Abstract:

Follicular differentiation is a tightly regulated process involving various endocrine, autocrine, and paracrine factors. The biosynthesis of progesterone and estradiol in response to FSH involves the regulation of multiple steroidogenic enzymes, such as p450 cholesterol side chain cleavage enzyme and aromatase. Here we demonstrated that prohibitin (PHB), a multifunctional protein, inhibits FSH-induced progesterone and estradiol secretion in rat granulosa cells. The mRNA abundances of *cyp11a* (coding p450 cholesterol side-chain cleavage enzyme) and *cyp19* (coding aromatase) were also suppressed by PHB in a time-dependent manner. It is known that a novel adipokine chemerin suppresses FSH-induced steroidogenesis in granulosa cells. Chemerin up-regulates the content of PHB, and PHB knockdown attenuates the suppressive role of chemerin on steroidogenesis. In addition, inhibition of phosphatidy-linositol 3-kinase/Akt pathway enhances the suppressive action of PHB, whereas expression of constitutively active Akt attenuates this response. These findings suggest that PHB is a novel negative regulator of FSH-induced steroidogenesis, and its action with chemerin may contribute to the dysregulation of steroidogenesis in the pathogenesis of polycystic ovarian syndrome.

Introduction:

Follicular growth and differentiation are tightly regulated by endocrine, autocrine and paracrine factors (1). The coordinated biosynthesis of steroids in the ovary is critical for reproductive cycle, successful ovulation and eventual pregnancy (2). FSH-induced synthesis of progesterone and estradiol involves the regulation of various steroidogenic enzymes, such as steroidogenic acute regulatory protein (StAR), p450 cholesterol side chain cleavage enzyme (p450scc), hydroxysteroid dehydrogenase-3β (HSD-3β) and aromatase (3-5). p450scc is the first and rate-limiting step in the steroidogenesis pathway in human granulosa cells and aromatase converts androstenedione and testosterone to estrone and estradiol, respectively (6, 7).

Chemerin is a chemoattractant and a recently discovered adipokine associated with obesity and the metabolic syndrome in women (8, 9). Chemerin is present in human plasma in an inactive state and its active form is released by proteolytic cleavage of the C-terminus (9-11). In addition to promoting lipogenesis, adipogenesis and angiogenesis (12-14), chemerin has been reported to act as a pro- as well as anti-inflammatory factor which regulates immunity (9, 15-17). Serum chemerin levels are higher in obese women and in PCOS subjects and metformin decreases serum chemerin level (8, 18), implying an association between chemerin and PCOS. We have previously reported that chemerin suppressed FSH-induced steroidogenesis in granulosa cells via down-regulation of the expression of nuclear receptors NR5a1/2 (19). However, the mediators and mechanism by which chemerin suppresses FSH-induced steroidogenesis remain unknown.

Prohibitin (PHB) is a multifunctional protein associated with many cellular processes such as cell cycle, proliferation, apoptosis, senescence, cellular immortalization

and differentiation (20-23). Recently PHB has been linked to adipogenesis and obesity as its expression is up-regulated in high-fat diet induced obese mice (24) and knockdown of PHB significantly reduces the expression of adipogenic markers and the accumulation of lipids (25). In the ovary, PHB is widely expressed and its expression is age and follicular stage regulated (26, 27). Although it is anti-apoptotic in granulosa cells (28) and silencing of PHB induces granulosa cell shape changes (29), our knowledge on the role and contribution of PHB to granulosa cell steroidogenesis is incomplete.

In this study, we first examined the expression of PHB and chemerin and their regulation by gonadotropins *in vitro* and *in vivo*. We also investigated the role of PHB in FSH-induced steroidogenesis in granulosa cells. We intended to demonstrate that PHB is antidifferentiative during follicular development and that it mediates the suppressive action of chemerin on FSH-induced steroidogenesis, which would suggest that PHB, together with chemerin, may contribute to the dysregulation of steroids production in the pathogenesis of ovarian diseases, such as polycystic ovaries.

Materials and Methods:

Antibodies and Reagents

Cell culture media (M199), fetal bovine serum, penicillin and streptomycin, L-glutamine, sodium pyruvate and trypsin were purchased from Invitrogen (Burlington, Canada). HEPES and equine chronic gonadotropin (eCG) were purchased from Sigma (St. Louis, MO). Recombinant mouse chemerin was obtained from R&D Systems (Minneapolis, MN) and recombinant human FSH was purchased from National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Anti-aromatase

antibody was purchased from AbD Serotec (Oxford, UK), anti-p450scc antibody from United States Biological (Swampscott, MA), anti-PHB and anti-β-actin antibodies from Abcam (Cambridge, MA), and antihemagglutinin (HA) antibody from Roche Applied Science (Quebec, Canada). Horseradish peroxidase-conjugated secondary antibodies and reagents for SDS-PAGE were supplied by Bio-Rad (Mississauga, Canada). Enhanced chemiluminescent (ECL) reagent was from Thermo Fisher Scientific (Rockford, IL). QIAShredder and RNeasy Mini kit were purchased from QIAGEN (Mississauga, Canada). Random decamer primers were from Ambion (Austin, USA). Ribonuclease (RNase) inhibitor and deoxynucleotide triphosphate were from Fermentas (Burlington, Canada). Moloney murine leukemia virus reverse transcriptase was from Promega (Madison, USA). QuantiTect SYBR Green PCR kit and phosphatase/protease inhibitors were from Roche Applied Science (Quebec, Canada). PCR primers were from Invitrogen. Adenoviral-PHB and adenoviral-short hairpin RNA targeting PHB were obtained from Dr. Winston Thompson (Morehouse School of Medicine, Atlanta, GA). Dominant negative (DN) Akt and constitutively active Akt (A-Akt) adenoviruses were generous gifts from Dr. Kenneth Walsh (Boston University School of Medicine). All chemical inhibitors were purchased from Calbiochem (Gibbstown, NJ). All other chemicals were of the highest analytical grade available from Sigma.

Animal preparation

Sprague-Dawley rats (Charles River, Montreal, Canada) were maintained on 12-h light, 12-h dark cycles and given food and water *ad libitum*. All procedures were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals,

Canadian Council on Animal Care, and were approved by the University of Ottawa and the Ottawa Hospital Research Institute Animal Care Committee.

Primary culture of rat granulosa cells and adenoviral infection

Ovaries from diethylstilbestrol (DES)-primed immature rats (D21, 1 mg/d, sc, for 3 consecutive days) were pre-incubated with 6 mM EGTA and 0.5 M sucrose (30), and granulosa cells were released by follicular puncture with a 26.5-gauge needle, washed and centrifuged (900 × g, 5 min). Cell clumps and oocytes were removed by filtering the cell suspensions through a 40-μm nylon cell strainer (BD Biosciences, Mississauga, Ontario, Canada). The number of viable granulosa cells was determined by trypan blue exclusion. Granulosa cells (0.9×10^6 per well in 6 well plate) were plated overnight in M199 with 10 % fetal bovine serum under a humidified atmosphere of 95 % air and 5 % CO_2. After culture overnight in serum-free medium, granulosa cells were treated with FSH (0 - 100 ng/mL) or chemerin (0 - 100 ng/mL) for designated duration. Unless specified, granulosa cells used in the present study were collected from large preantral and early antral follicles of DES-treated rats.

To harvest granulosa cells from follicles at different stages of development, 21-d-old immature rats were injected (sc) with eCG (10 IU), and granulosa cells were collected from the ovaries primed with eCG at 0 h (predominantly preantral and early antral follicles), 24 h (predominantly early antral follicles), or 48 h (predominantly large antral and preovulatory follicles), as previously described (31).

For adenoviral infection, granulosa cells were cultured in serum-free M199 medium containing adenoviral particles for 24 h followed by medium change. Multi-

plicity of infection (MOI) and duration of infection are detailed in the figures. Equal amounts of adenovirus in each experimental group were achieved by the adjustment with an appropriate amount of adenoviral-LacZ (negative control for adenoviral-PHB, DN-Akt and A-Akt) or adenoviral-shNeg (negative control for shPHB).

Preparation of dihydrotestosterone (DHT)-treated rats

The DHT-filled capsules were prepared as previously described (32). In brief, SILASTIC brand tubing (Dow Corning, Midland, MI; inner diameter 1.98 mm × outer diameter 3.18 mm;), cut to an appropriate length to achieve a desired surface area of 300 mm^2, was filled with DHT powder (Steraloids Inc, Newport) from 1-mL syringe. The tubing was closed at each end (3 mm) with a sealant (Silicone Type A, Dow Corning), ensuring that adhesive is in contact with tubing walls and that there are no air bubbles. Control (CTL) capsules were empty with sealant on both ends. After being left overnight to dry, the capsules were rinsed for 2 days in 3 % BSA in PBS with 0.1 % NaN_3 solution, washed with PBS, and sterilized by dipping briefly in 70 % ethanol before use.

Female rats at 21 days of age were divided into two groups (DHT vs. CTL) and implanted with a DHT-filled silicone capsule continuous-releasing (83 μg per day, empty SILASTIC capsule as control; Dow Corning) for 12 weeks to mimic the hyperandrogenic state in women with PCOS, whose plasma DHT levels are approximately 1.7-fold higher than those of healthy control (33, 34). Rats were monitored twice daily in the first 3d and once daily thereafter. Animals were weighed weekly to monitor weight gain and euthanized at 12 weeks post-implantation.

Isolation of granulosa cells from CTL or DHT rats

Rat ovaries were collected after rat euthanization and granulosa cells from CTL or DHT rats were recovered in a similar but more precise method than that mentioned above. Granulosa cells from preantral/early antral follicle stages in the ovary were released by follicular puncture but the preovulatory follicles and corpora lutea were spared. A pool of granulosa cells collected from 5 rats of each group was used for mRNA and protein analysis.

Reverse transcription-polymerase chain reaction

Total RNAs of granulosa cells were extracted according to the manufacturer's instruction, using the QIAGEN RNeasy Mini kit. Then 0.2 μg total RNAs was used to reverse transcribe cDNAs and the mRNA abundance of target genes was analyzed by real-time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specific primer pairs used in the experiments are listed in **Table 1**. Data were analyzed by $2^{-\Delta\Delta CT}$ method (35).

Protein extraction and Western blot

Total protein extracts were prepared by adding 100 μl hot (100 °C) Tris buffer (10 mM Tris, pH 7.4; 1 % sodium dodecyl sulfate; 1mM sodium orthovanadate) to each well. The extracts were obtained by rapidly scraping, transferred to a microcentrifuge tube, and boiled for 5 minutes. Protein concentrations in each sample were determined by a Bradford assay (Bio-Rad Laboratories). Twenty-microgram cell lysates were subjected to

Table 1 Primer pairs used for quantitative real-time PCR

Gene	Primer	Sequence (5'-3')	Accession No.
Cyp19	Forward	GGCATGCACGAGAATGGCATCATA	NM_017085
	Reverse	CAGCCTGTCCAAATGCTGCTTGAT	
Cyp11a	Forward	ACACGACCTCCATGACTCTGCAAT	NM_017286
	Reverse	TCAGTGTCTCCTTGATGCTGGCTT	
StAR	Forward	TGTTAAGGACTGCCCACCACATCT	NM_031558
	Reverse	TGTCCTTGGCTGAAGGTGAACAGA	
HSD-3β	Forward	AGATCTGGGCTATGTGCCACTTGT	NM_001007719
	Reverse	ACCTGGTAACACCCAGAACCACAT	
PHB	Forward	ACCGATTCTGTGGCATACAGGACA	XM_001053030
	Reverse	TGGTCGAGAGCAGCAGTCAAAGAT	
Chemerin	Forward	GGCACCTTTGTGAGGCTGGAATTT	NM_001013427
	Reverse	ACCCTGTCCAGGGCTTATTTGGAT	
GAPDH	Forward	TGACTCTACCCACGGCAAGTTCAA	NM_017008
	Reverse	ACGACATACTCAGCACCAGCATCA	

SDS-PAGE with 4.5% stacking and 10% separating gels. Proteins were electrophoretically transferred to nitrocellular membrane (Bio-Rad Laboratories), blocked at room temperature with 5 % skim milk in TBST [0.05 % Tween-20 in 10 mM Tris, 0.15 M NaCl, pH7.4 (Tris buffered saline)] for 1 h, and then incubated overnight at 4 °C with diluted primary antibodies (1:1000 to 1:10,000 according to manufactory instruction) in TBST with constant agitation. The membranes were then treated with a secondary antibody (1:5000 to 1:50,000 based on different primary antibodies). After washing three times with TBST, immunoreactive bands were visualized with an enhanced chemiluminescent agent according to the manufacturer's instruction. Intensity of bands of the exposed X-ray film was determined by densitometrically scanning, quantified using AlphaEaseFC (Alpha Innotech, San Leandro, CA) and normalized with β-actin.

Steroids secretion analysis

Spent medium of cultured granulosa cells were collected, centrifuged (900 × g, 5 min) and kept in -80 °C for hormone analysis. 17β-estradiol and progesterone concentrations in spent medium were measured using enzyme immunoassay (EIA) kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instruction. The detection limitation of 17β-estradiol was 28 pg/ml, and the intra- and inter-assay coefficients of variation were 8 % and 6 %, respectively. The detection limitation of progesterone was 8.5 pg/ml, and the intra-and inter-assay coefficients of variation were 7 % and 6 %, respectively.

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 statistical software (San Diego, CA). Results are expressed as mean ± SEM of at least 3 independent experiments as detailed in the figures. Unpaired *t* test was used for comparing the mRNA abundance of target genes in granulosa cells treated with FSH, or mRNA/protein level of targets in granulosa from CTL/DHT rats. One-way and two-way ANOVA were used to assess the effects and interactions of 1 or 2 variables, and multiple comparisons were achieved by a Bonferroni *post hoc* test. Significant difference was defined at $p < 0.05$ (*, or #).

Results:

Gonadotropin down-regulates the mRNA abundance of chemerin and PHB in granulosa cells

It has been reported that chemerin and PHB are expressed in ovarian cells (9, 27). To determine whether their expression is dependent on the stage of follicular development, granulosa cells were collected from the ovaries primed with eCG at 0 h (predominantly preantral and early antral follicles), 24 h (predominantly early antral follicles), or 48 h (predominantly large antral and preovulatory follicles), and mRNA abundance of chemerin and PHB were analyzed by real-time PCR. As shown in **Figure 1A**, mRNA expression of both chemerin ($p < 0.001$) and PHB ($p < 0.001$) in granulosa cells significantly decreased at late stages of follicular development (gonadotropin 24 h and 48 h).

We also examined whether FSH down-regulates the expression of chemerin and PHB *in vitro*. **Figure 1B** indicated that both chemerin ($p < 0.001$) and PHB ($p < 0.01$) mRNA levels were decreased by FSH. As gonadotropin similarly regulates the

expression of chemerin and PHB, we then examined whether chemerin regulates PHB expression. As shown in **Figure 1C**, treatment with chemerin significantly increased PHB content in granulosa cells ($p < 0.05$).

Exogenous PHB suppressed FSH-induced estradiol and progesterone secretion

Our previous results indicated that chemerin suppressed FSH-induced granulosa cell steroidogenesis (19). Because chemerin up-regulates PHB content in granulosa cells, it is possible that the suppressive role of chemerin may be mediated by PHB. To test this possibility, we first examined whether PHB overexpression suppresses FSH-induced progesterone and estradiol production. As observed in **Figure 2A**, FSH markedly stimulated progesterone secretion in granulosa cells, a response significantly suppressed by exogenous PHB (FSH, $p < 0.001$; PHB, $p < 0.05$; FSH × PHB, $p < 0.05$). Similarly, PHB also suppressed FSH-induced estradiol secretion (FSH, $p < 0.001$; PHB, $p < 0.01$; FSH × PHB, $p < 0.01$). In addition, the increase of aromatase and p450scc contents induced by FSH was suppressed by exogenous PHB (**Figure 2B**; for p450scc: FSH, $p < 0.001$; PHB, $p < 0.001$; FSH × PHB, $p < 0.001$; for aromatase: FSH, $p < 0.001$; PHB, $p < 0.001$; FSH × PHB, $p < 0.01$).

We then examined whether PHB knockdown attenuates the suppression of steroid-genesis by chemerin. Granulosa cells were infected with adenoviral-shPHB (MOI = 10, shNeg as control) to knock down PHB and then cultured with chemerin (0 - 100 ng/mL) in the presence of FSH (100 ng/mL, 24 h). As shown in **Figure 2C**, the suppression of p450scc and aromatase expression by chemerin was attenuated by PHB knockdown (for p450scc: chemerin, $p < 0.05$; shPHB, $p > 0.05$; chemerin × shPHB, $p > 0.05$; for aromatase: chemerin, $p < 0.05$; shPHB, $p > 0.05$; chemerin × shPHB, $p > 0.05$).

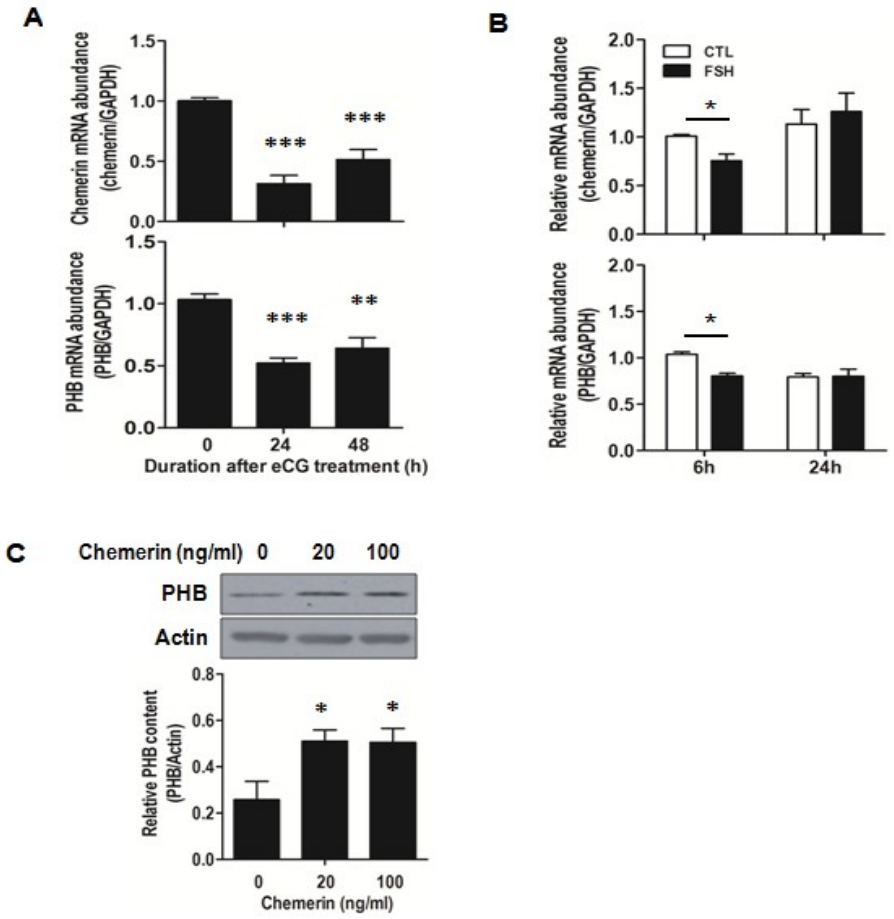


Figure 1. Expression of chemerin and PHB and their regulation by gonadotropin

(A) Granulosa cells were isolated from follicles at different stages of development in immature rats injected with eCG (0 h, preantral; 24 h, early antral; 48 h, late antral and preovulatory follicles). The mRNA abundances of chemerin and PHB were analyzed by real-time PCR and normalized to the *GAPDH* mRNA.

(B) Granulosa cells from DES-treated rats were cultured with FSH (100 ng/mL) for designated time point (6 and 24 h) and the mRNA abundance of chemerin and PHB was analyzed by real-time PCR and normalized to *GAPDH* mRNA.

(C) Granulosa cells from DES-treated rats were treated with chemerin (0-100 ng/mL, 24h) and the content of PHB was assessed by Western blot. Shown are representative immunoblots and data are presented as mean ± SEM of three independent experiments. A and C, one-way ANOVA followed by Bonferroni *post hoc* test; B, unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with eCG 0 h or untreated control (FSH = 0 or chemerin = 0).

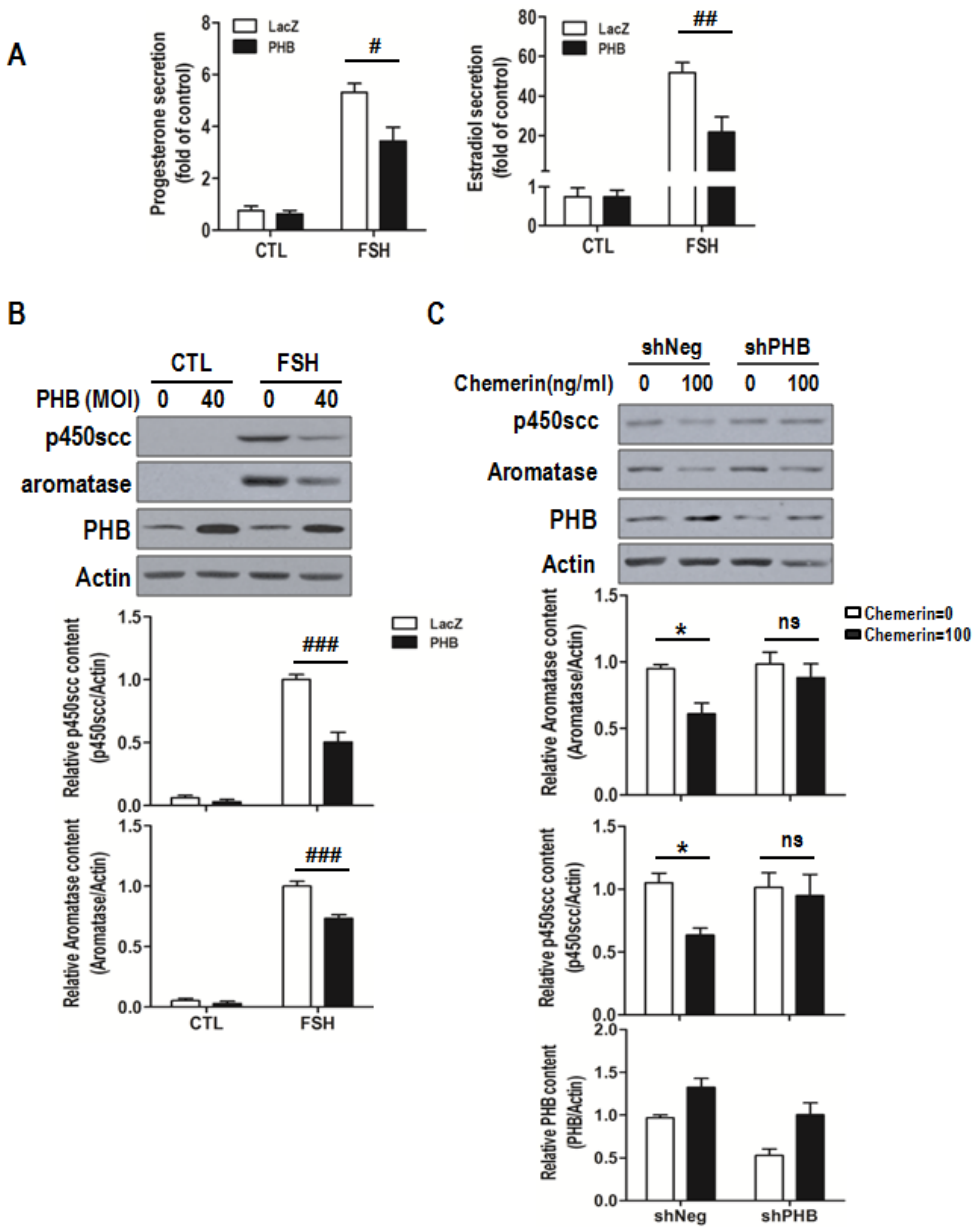


Figure 2. Role of PHB in FSH-induced, chemerin-mediated steroidogenesis

(A) Granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-LacZ as control) for 24 h and then cultured with FSH (100 ng/mL, 24 h). Testosterone (0.5 μM) was added to serve as substrate of aromatase during culture. Progesterone and estradiol secreted into the medium was measured by EIA. Data are presented as mean ± SEM of 4 independent experiments, analyzed by 2-way ANOVA and subsequently by Bonferroni *post hoc* test. #, $p < 0.05$; ##, $p < 0.01$ compared with LacZ

(B) Granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-LacZ as control) for 24 h and then cultured with FSH (100 ng/mL, 24 h). The contents of p450scc and aromatase were examined by Western blot. Shown are representative immunoblots. Data are presented as mean ± SEM of 4 independent experiments and analyzed by 2-way ANOVA and subsequently by Bonferroni *post hoc* test. ###, $p < 0.001$ compared with LacZ

(C) Granulosa cells were infected with adenoviral-shPHB (MOI = 10, adenoviral-shNeg as control) for 48 h and then treated with chemerin (0-100 ng/mL, 24 h) in the presence of FSH (100 ng/mL). The contents of p450scc and aromatase were examined by Western blot. Data are presented as mean ± SEM of 4 independent experiments and analyzed by 2-way ANOVA and subsequently by Bonferroni *post hoc* test. *, $p < 0.05$ compared with control (chemerin = 0); #, $p < 0.05$ compared with shNeg; NS, not significant

PHB suppressed FSH-induced steroidogenesis in a time-dependent manner in granulosa cells

Next we examined whether the suppression of FSH-induced steroidogenesis by PHB is time dependent and whether steroidogenic enzymes expression is regulated at the transcriptional level. As shown in **Figure 3A**, FSH gradually increased estradiol production, starting at 6 h and reaching significance after 18 h. Exogenous PHB suppressed FSH-induced estradiol production in a time-dependent manner (PHB, $p < 0.001$; Time, $p < 0.0001$; PHB × Time, $p < 0.0001$). PHB had no effect on FSH-induced estradiol secretion at an earlier time point but showed inhibition action at 18 and 24 h. The mRNA abundance of *cyp19* rapidly increased 50 - 350 folds (3-12 h) and reached significance at 18 h with elevated level of about 2200-fold in the presence of FSH (**Figure 3A**). The effect of PHB on *cyp19* expression showed a similar trend as that of estradiol production, with dramatical down-regulation at 18 and 24 h (PHB, $p < 0.001$; Time, $p < 0.001$; PHB × Time, $p < 0.001$).

In contrast to FSH-stimulated estradiol secretion, FSH rapidly increased progesterone secretion at 3 h, maintained higher level until 12 h and then a greater increase in progesterone production at 18 h and 24 h (**Figure 3B**). Although PHB had minimum or no effect on progesterone levels at an early time point (0 - 18 h), it was inhibitory at 24 h (PHB, $p < 0.05$; Time, $p < 0.001$; PHB × Time, $p < 0.05$). Although progesterone production quickly and significantly increased at 3 h in response to FSH, there was only a slightly increase of *cyp11a* expression until 12 h (**Figure 3B**, 3.03 ± 0.24-fold at 3 h and 7.76 ± 1.01-fold at 12 h vs. 1.03 ± 0.03 at 0h). Obvious elevation of *cyp11a* expression

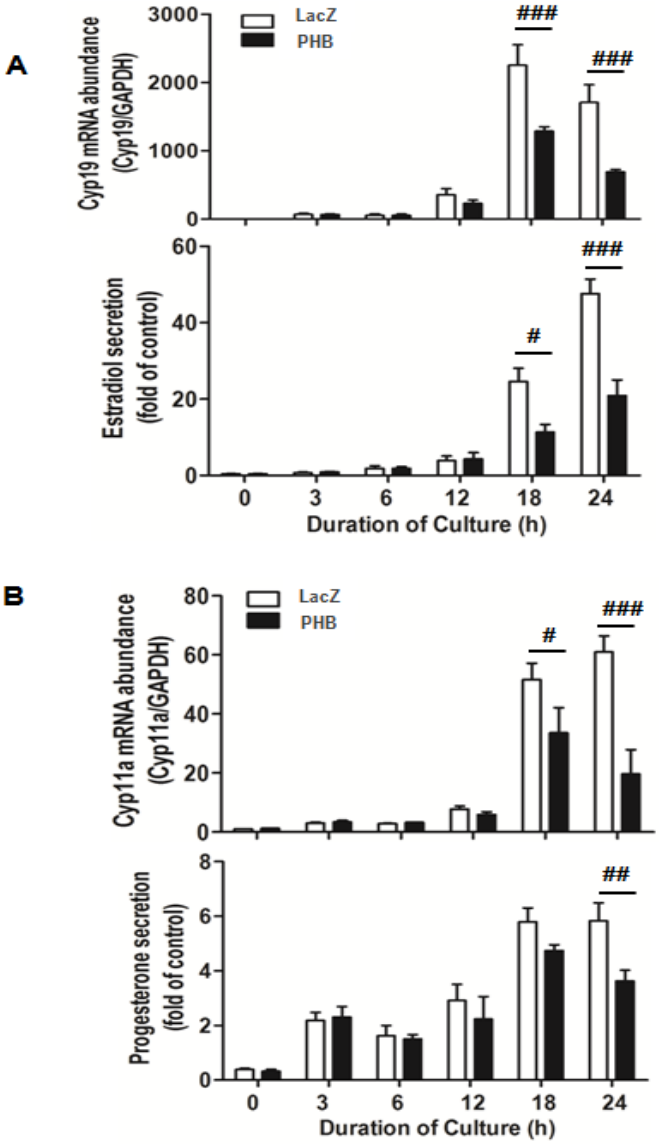


Figure 3. **Influence of PHB on FSH-induced steroid production and steroidogenic enzyme expression**

Granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-LacZ as control) for 24 h and then cultured with FSH (100 ng/mL) at designated duration (0-24 h). Testosterone (0.5 μM) was added to serve as substrate of aromatase during culture. Estradiol (A) and progesterone (B) secreted into the medium were measured by EIA. The mRNA abundance of *cyp19* (A) and *cyp11a* (B) was determined by real-time PCR. Data are presented as mean ± SEM of 4 independent experiments and analyzed by 2-way ANOVA and subsequently by Bonferroni *post hoc* test. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with LacZ

induced by FSH occurred at 18 h (51.66 ± 5.54-fold) and 24 h (60.99 ± 5.45-fold), which was significantly suppressed by PHB (PHB, $p < 0.001$; Time, $p < 0.0001$; PHB × Time, $p < 0.0001$). However, PHB had no significant effect on FSH-induced *StAR* and *HSD-3β* expression (**Supplement Figure S1**).

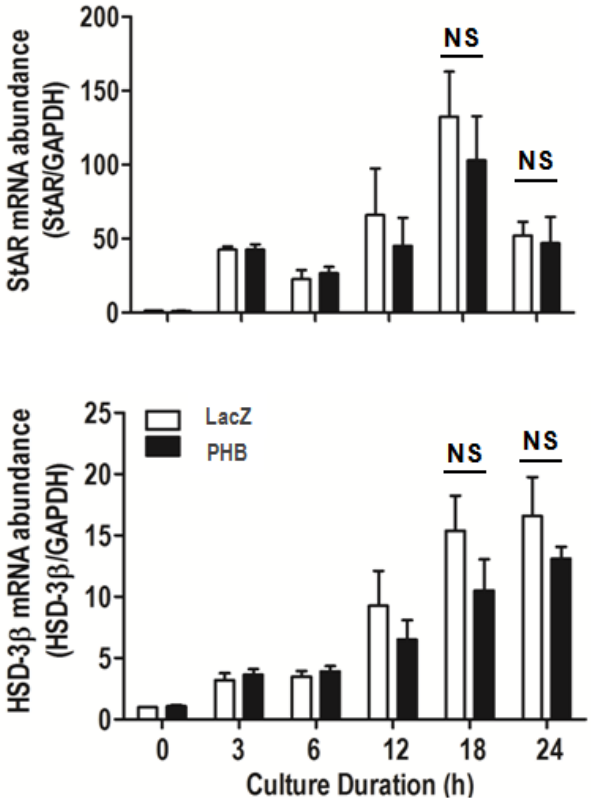
Phosphatidylinositol 3-kinase (PI3K)/Akt pathway negatively regulates the expression of PHB

Akt activation is an essential for granulosa cell differentiation (36-38). We therefore determined whether the PI3K/Akt pathway modulates the expression and function of PHB. Specific inhibitor of PI3K [LY294002 (LY), 10μM; dimethylsulfoxide (DMSO) as control] and of Akt [triciribine (TCN), 5 μM; DMSO as control] were added to the granulosa cell cultures, and Western blot results indicated that inhibition of the PI3K/Akt pathway significantly increased the expression of PHB (**Figure 4A**, $p < 0.001$).

We further examined whether activation of Akt affects the level of PHB using adenoviral constitutively A-Akt (containing a HA tag) and adenoviral DN-Akt. As shown in **Figures 4, B and C**, PHB content was down-regulated by the activation of Akt ($p < 0.05$) and up-regulated when Akt function was down-regulated by DN-Akt ($p < 0.001$).

PHB knockdown up-regulates Akt phosphorylation

We next examined whether PHB regulates Akt content and phosphorylation in granulosa cells. Granulosa cells were infected with adenoviral-shPHB, and the contents of phosphorylated and total Akt were examined. As shown in **Figure 4D**, knockdown of



Supplement Figure S1. PHB has no effect on FSH-induced StAR and HSD-3β expression in granulosa cells

Granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-LacZ as control) for 24 h and then cultured with FSH (100 ng/mL) at designated duration (0 - 24 h). Testosterone (0.5 μM) was added to serve as substrate of aromatase during culture. The mRNA abundance of *StAR* and *HSD-3β* was determined by real-time PCR. Data are presented as mean ± SEM of 4 independent experiments and analyzed by 2-way ANOVA and subsequently by Bonferroni *post hoc* test. NS, not significant

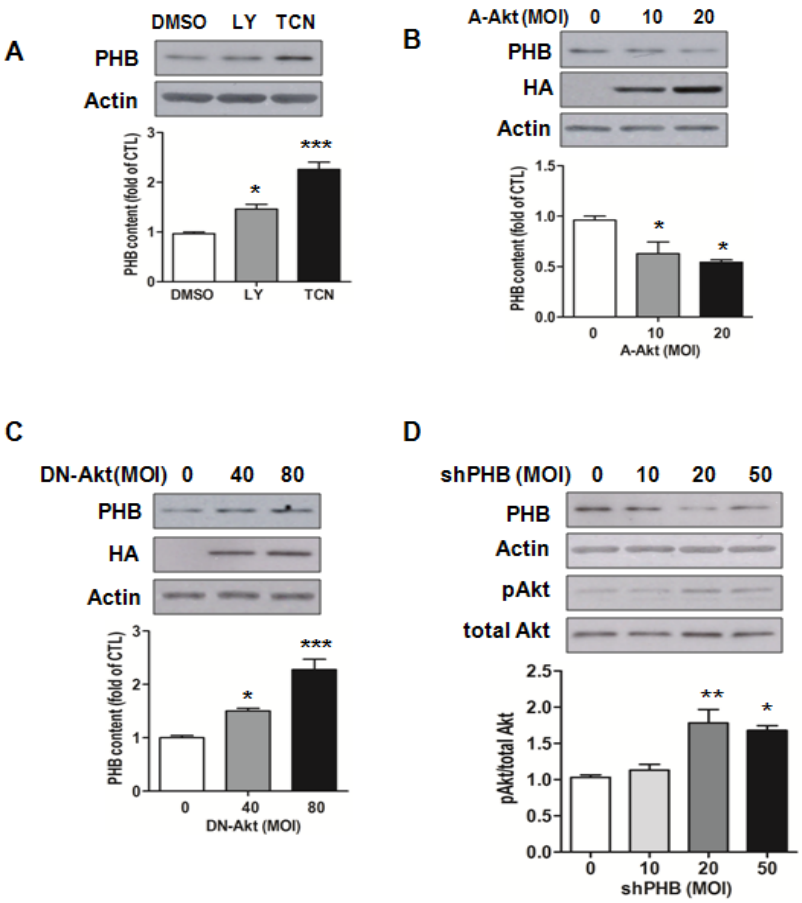


Figure 4. A-C, The influence of modulation of the PI3K/Akt pathway on granulosa cell PHB expression

(A) Granulosa cells were treated with PI3K inhibitor LY (10 μM, DMSO as control) or Akt inhibitor TCN (5μM) for 24h and the content of PHB was examined by Western blot. **(B and C)** Granulosa cells were infected with adenoviral-constitutive A-Akt (MOI=0-20, 24h, adenoviral-LacZ as control) or adenoviral-DN-Akt (MOI = 40-80, 48 h) and PHB content was determined by Western blot. Both adenoviral constructs contain an HA tag.

(D) Effect of PHB knockdown on pAkt content.

Granulosa cells were infected with adenoviral-shPHB (MOI = 0 - 50, 24 h, adenoviral-shNeg as negative control), and the contents of PHB, pAkt and total Akt were examined by Western blot. Representative immunoblots are shown in the figure. Data are presented as mean ± SEM of 3 independent experiments and analyzed by 1-way ANOVA and subsequently by Bonferroni *post hoc* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with DMSO or adenoviral control.

PHB significantly increased phosphorylated Akt levels ($p < 0.01$) at higher doses (MOI = 20 and 50).

Inhibition of PI3K/Akt pathway enhanced the suppressive role of PHB on steroidogenesis

Next we assessed whether modulation of PI3K/Akt pathway alters the steroidogenic response to PHB. As shown in **Figure 5A**, PHB alone suppressed FSH-induced aromatase expression (22 %) and estradiol secretion (31 %). The effect of PHB was enhanced in the presence of LY, exhibiting approximate 75 % suppression of aromatase expression and 82 % inhibition of estradiol secretion. Although pre-treatment of LY alone suppressed FSH-induced steroidogenesis, there was significant interaction between PHB and LY treatment (treatment, $p < 0.001$; PHB, $p < 0.001$; PHB × treatment, $p < 0.01$). PHB was less effective on TCN treatment group because the Akt inhibitor alone blocked greater than 95 % steroidogenesis induced by FSH. These inhibitors had similar effect on p450scc expression and secretion of progesterone (data not shown).

Activation of PI3K/Akt pathway attenuated the suppressive role of PHB in steroidogenesis

In addition, the experiments using adenoviral A-Akt were also performed to examine whether expression of constitutively active Akt attenuates the action of PHB on FSH-induced steroidogenesis. **Figure 5B** indicated that forced expression of the A-Akt in granulosa cells prior to treatment with FSH and LY significantly attenuated the PHB-induced down-regulation of aromatase expression and estradiol secretion (A-Akt, $p <$

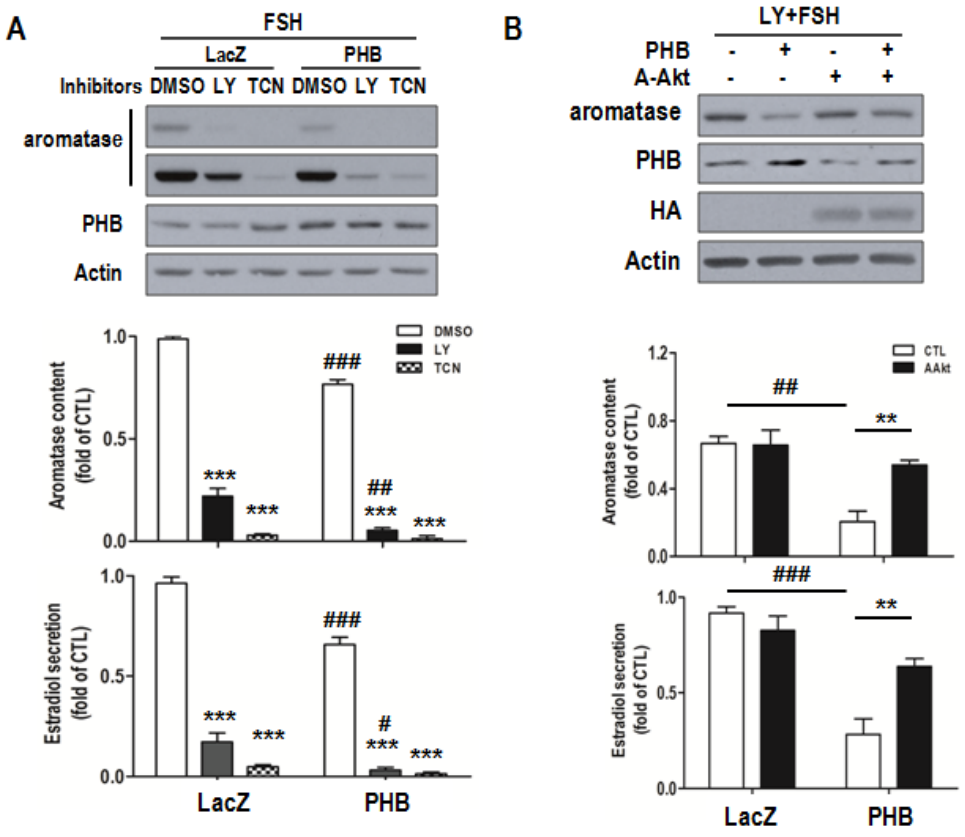


Figure 5. **The modulatory role of PI3K/Akt pathway on the suppression of FSH-induced steroidogenesis by PHB**

(A) Granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-LacZ as control) for 24 h and treated LY (10 μM) or Akt inhibitor TCN (5 μM) for 1 h prior to culture with FSH (100 ng/mL, 24 h). **(B)** Granulosa cells were co-infected with adenoviral-PHB (MOI = 40) and constitutively A-Akt (with HA tag, MOI = 10) for 24 h and then treated with LY in the presence of FSH. Testosterone (0.5 μM) was added to serve as substrate of aromatase during culture. Estradiol secreted into the medium (EIA) and the contents of aromatase, PHB, and HA (Western blot) were analyzed. Shown are representative immunoblots. The 2 blots for aromatase show the signals with shorter (top panel) and longer (bottom panel) exposure time. Data are presented as mean ± SEM of 3 independent experiments and analyzed by 2-way ANOVA and subsequently by Bonferroni *post hoc* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with DMSO. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with adenovirus control (LacZ or Neg)

0.05; PHB, $p < 0.001$; PHB × A-Akt, $p < 0.01$). These inhibitors had similar effect on the granulosa cell p450scc mRNA abundance and progesterone secretion (data not shown).

Elevated expression of PHB in a DHT-induced PCOS rat model

We have reported that serum and ovarian chemerin levels increased in a DHT-induced PCOS rat model (19). Because PHB might be a mediator in the suppressive role of chemerin in steroidogenesis, it is possible that the increase in chemerin content in ovarian cells in DHT-treated rats may be associated with an increase of PHB expression. To test this possibility, granulosa cells were collected from CTL and DHT-rats and the levels of PHB (mRNA and protein) were examined. As predicted, both mRNA abundance (**Figure 6A**, $p < 0.05$) and protein level (**Figure 6B**, $p < 0.05$) of PHB were significantly higher in granulosa cells from DHT-treated rats compared with their controls.

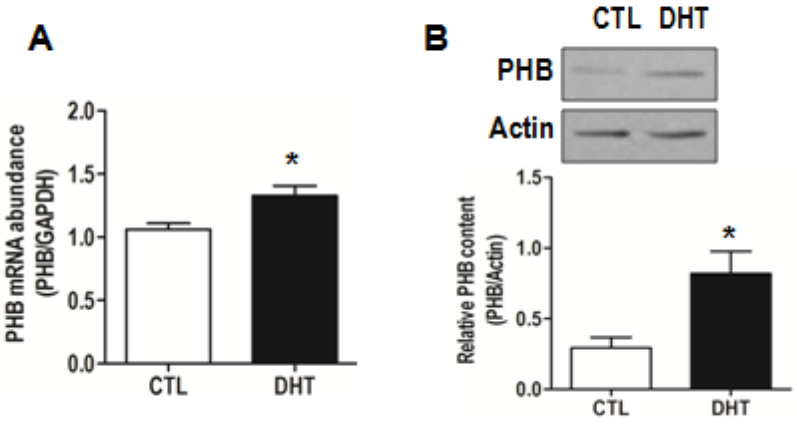


Figure 6. **Expression of PHB in granulosa cells from CTL and DHT-treated rats.**

Granulosa cells were collected from 5 rats of the CTL or DHT group and pooled for mRNA and protein analysis. The mRNA abundance (A) and protein content (B) of PHB were measured by real-time PCR and Western blot, respectively. Data are presented as mean ± SEM of 3 independent experiments and analyzed by 1-way ANOVA and subsequently by Bonferroni *post hoc* test. *, $p < 0.05$ compared with CTL.

Discussion:

In the present study, we have demonstrated that PHB is a novel negative regulator of FSH-induced steroidogenesis in rat granulosa cells and mediates the suppressive action of chemerin on steroid production. Forced expression of PHB suppresses FSH-induced expression of steroidogenic enzymes (p450scc and aromatase) at both transcriptional and translational levels and progesterone and estradiol secretion in granulosa cells. The suppressive role of chemerin in FSH-induced p450scc and aromatase expression is attenuated by PHB knockdown. The inhibition of the PI3K/Akt pathway increases PHB content and enhances its regulatory role on aromatase expression, whereas induction of constitutively active Akt rescues this response. Elevated expression of PHB in granulosa cells from a chronic DHT-induced PCOS model also suggests that PHB may contribute to the dysregulation of steroids production during the pathogenesis of PCOS.

In the present study, we observed that gonadotropin down-regulated the expression of PHB *in vivo* and *in vitro*. The regulation of PHB mRNA abundance by FSH *in vitro* was time dependent, with down-regulation evident at 6 h but not at 24 h, which might be due to reduced basal level of PHB at the latter time point. This would explain the observation that FSH failed to reduce PHB protein content after 24 h treatment (**Figure 2B**). However, our *in vivo* results were not in agreement with earlier observations that gonadotropin treatment either had no effect on the mRNA/protein levels (26) or increased PHB protein level (27). Although the reasons for these apparent differences are not immediately obvious, it is possible that this could be due to differences in gonadotropin dosage used and techniques in granulosa cell isolation and

mRNA detection. A high dose (50 IU) of gonadotropin could have minimized its effect on PHB expression via receptor down-regulation/desensitization.

Although the multifunctional role of PHB has been demonstrated in various cell types, how the PHB gene is regulated is not completely understood. Here, we show, in addition to FSH, chemerin may be a novel regulator of PHB in granulosa cells. However, the mechanism by which chemerin up-regulates PHB expression is unknown. In addition to putative binding sites for CCAAT/enhancer-binding protein and insulin response element in the promoter of PHB (39), there are other elements such as proliferator-activated receptor, activating protein 2, estrogen receptor, and specificity protein-1 based on the prediction using the transcription element search system. Whether chemerin regulates the expression of PHB via modulating these transcriptional factors requires further investigation.

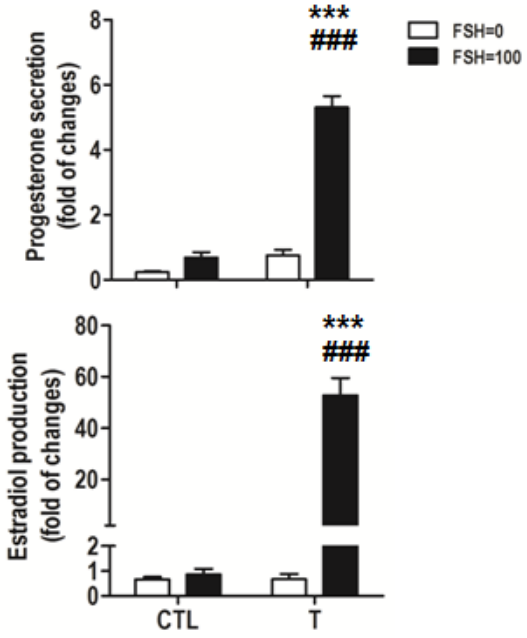
The observations that chemerin and PHB were regulated by FSH in a similar pattern and that chemerin up-regulates PHB content in granulosa cells suggest that PHB may act as a downstream molecule in granulosa cells. We have demonstrated that chemerin plays an inhibitory role in FSH-induced steroidogenesis in follicle and granulosa cell cultures (19) and that exogenous PHB suppressed FSH-induced steroid-genesis (present study). These findings are further supported by the loss-of-function study showing that PHB knockdown attenuated chemerin-suppressed granulosa cell steroidogenesis. However, the mechanism by which PHB regulates steroidogenesis is unclear. Because chemerin suppresses the nuclear receptor NR5a1/2-mediated steroid-genesis (19) and PHB is shown to act as a co-activator or co-repressor of various transcr-

iptional factors (e.g. E2F transcriptional factor 1 or p53) (40, 41), it is possible that PHB acts as a co-repressor of NR5a1/2 to regulate granulosa cell steroidogenesis.

PCOS is commonly associated with obesity, insulin resistance, dysregulation of steroids, and polycystic ovaries (42). Using a chronic DHT-induced PCOS rat model as previously reported (43), we have demonstrated that serum and ovarian chemerin levels are elevated in DHT-treated rats compared with controls (19). The observations that serum chemerin levels are elevated in obese women and in PCOS subjects (8), that chemerin is important in adipogenesis, and that it suppresses FSH-induced steroidogenesis (19) are consistent with a possible involvement of chemerin in the pathogenesis of PCOS. In addition, our finding that both mRNA and protein levels of PHB increase in DHT-treated rats, together with its suppressive role in steroidogenesis, also raises the interesting possibility of an association between PHB and PCOS. Recently PHB has been linked to adipogenesis and obesity because its expression is up-regulated in high-fat diet induced obese mice, and PHB knockdown significantly reduces the expression of adipogenic markers (24, 25, 39). Both chemerin and PHB could regulate insulin signaling because chemerin induces insulin resistance in skeletal muscle *in vivo* (44), metformin treatment reduces serum chemerin level (18), and PHB attenuates insulin-stimulated glucose transport (45, 46). The up-regulation of PHB by chemerin, as well as their suppressive role in steroidogenesis and insulin signaling, raises a possibility that chemerin may contribute to the pathogenesis of PCOS, particularly in the phenotype of obesity, insulin resistance, and dysregulation of steroidogenesis in a PHB-mediated way.

In our culture system, granulosa cells have a good steroidogenic response to FSH, which is in good agreement with well-established observations that FSH stimulates

cyp19/cyp11a expression and steroid production (5, 47, 48). Testosterone was added in the culture as the substrate of aromatase, which is commonly used in the studies of FSH-induced estrogen production (38, 48, 49). We also observed that the presence of testosterone augmented the effect of FSH on the production of progesterone and estradiol in granulosa cells (**Supplement Figure S2**), which was in agreement with the literature (38, 50, 51). It is noteworthy that exogenous PHB suppresses FSH-induced steroidogenic enzyme expression at transcriptional and translational levels. The inhibitory role of PHB in *cyp19* expression and estradiol production occurs at 18 and 24h after an FSH challenge. Although there is a rapid increase of progesterone production at 3 h, exogenous PHB suppresses *cyp11a* expression and progesterone secretion only at 24 h under current experimental conditions. The increase of progesterone secretion is likely due to rapid increase in *StAR* expression (42-fold) known to facilitate cholesterol transport into the inner mitochondrial membrane for steroidogenesis (48, 52). However PHB has no suppression on *StAR* and *HSD-3β* expression. Because PHB is reported to serve as a co-activator or co-repressor of distinct transcriptional factors (estrogen receptor-α, p53, E2F family of DNA-binding transcription factors, etc,) (40, 53, 54), we hypothesize that PHB suppresses FSH-induced steroidogenesis via acting as a co-repressor of transcriptional factors targeting steroidogenic enzymes, such as NR5a1, NR5a2, CCAAT/enhancer-binding protein, *et al* (55-58). The different effect of PHB on *cyp11a*, *cyp19*, *StAR* and *HSD-3β* may result from the affinity or specificity of PHB to various transcriptional factors. Further studies on the promoter activity and loss in function by mutagenesis are needed to test this possibility and to explore the interaction region between these molecules.



Supplement Figure S2. **Effect of FSH on steroids production in granulosa cells**

Granulosa cells were cultured with FSH (100 ng/mL) ± testosterone (0.5 μM) for 24h and the levels of progesterone and estradiol in the spent medium were measured by EIA. Data are presented as mean ± SEM of 4 independent experiments and analyzed by 2-way ANOVA and subsequently by Bonferroni test. ***, $p < 0.001$ compared with FSH = 0. ###, $p < 0.001$ compared with T only

The PI3K/Akt signaling pathway plays an essential role in the regulation of granulosa cell survival and differentiation during follicular development (36-38). Using specific inhibitors and a dominant negative Akt construct, we observed that active Akt negatively regulates PHB expression and its suppressive role in granulosa cells. Although PHB is reported to be a substrate of Akt and the insulin receptor (59, 60), its phosphorylation status is not affected by PI3K inhibitor in granulosa cells (29). Nevertheless, recent data show that overexpression of PHB decreases phospho-Akt (S^{473} and T^{308}) levels in adipocytes and MCF-7 cells (59, 61), suggesting an inverse relationship between PHB and phosphorylated Akt. We have observed that knockdown of PHB increased the level of phospho-Akt (S^{473}) in granulosa cells, suggesting that a negative feedback regulation on the Akt phosphorylation by PHB. Another example of a negative regulation loop is reported between the up-regulation of PHB by insulin and an inhibitory effect of PHB overexpression on insulin-induced adipogenesis (39). In addition, a positive regulatory loop is observed between PHB and MAPK kinase (MEK)-1, demonstrating that PHB is required for MEK1 activity, while being a potential target of MEK1 (29). These regulatory loops, whether positive or negative, may represent a common mechanism for the precise control of PHB function and regulation of granulosa cell differentiation.

In addition to regulating the PI3K/Akt pathways, FSH is known to activate the protein kinase A (PKA) pathway in ovarian cells (62-64). We have demonstrated that chemerin suppressed forskolin-induced steroidogenesis, possible via decreasing the transcriptional activity of NR5a1/2 (19) and that PHB inhibited Akt phosphorylation. Whether chemerin/PHB interacts with the PKA pathway is unknown. Besides the

regulation of NR5a1/2, it is also possible that chemerin binds to its Gi protein-coupled receptor and negatively regulates PKA signaling; however this possibility needs to be further investigated.

In this study, the presence of PI3K inhibitor LY dramatically reduced FSH-induced steroidogenesis. Although constitutively active Akt amplified FSH-induced granulosa cell differentiation (38), it alone had no significant influence on the action of FSH in the presence of PI3K inhibitor LY (**Figure 5B**). A possible reason is that the downstream molecules of PI3K other than Akt (such as PKA and protein kinase C) are also required in the regulation of steroidogenesis. Apart from phosphorylating Akt at Thr^{308}, 3'-phosphoinositide-dependent kinase-1 (PDK1) phosphorylates the equivalent residues on PKA, protein kinase C, and p70-S6 Kinase, kinases in the same AGC family as Akt (65-68). Suppression of PI3K activity may affect PKA signaling, and constitutively active Akt may be unable to compensate for the inhibition of PKA signaling by inhibitors on FSH-induced steroidogenesis, as reported previously that constitutively active Akt does not overcome the inhibitory effect of PKA inhibitor H89 on FSH-induced estradiol production (38).

In conclusion, our findings demonstrate that PHB is a novel negative regulator on FSH-induced steroidogenesis and mediates the action of chemerin on steroid production. As shown in the hypothetical model (**Figure 7**), chemerin up-regulates PHB expression which inhibits FSH-induced steroidogenesis. Knockdown of PHB attenuates the suppressive effect of chemerin on FSH-induced p450scc and aromatase expression. PI3K/Akt activation negatively regulates the expression and function of PHB; whereas inhibition of PI3K pathway enhances the suppressive role of PHB. The increase of PHB

expression in granulosa cells from DHT-treated rats suggests that, as in the case of chemerin, PHB may be involved in the dysregulation of steroids production in PCOS. This study significantly improves our understanding of the role of PHB in ovarian cells and its potential contribution to the pathogenesis of PCOS.

Acknowledgement:

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Disclosure Summary: The authors declare no conflict of interest.

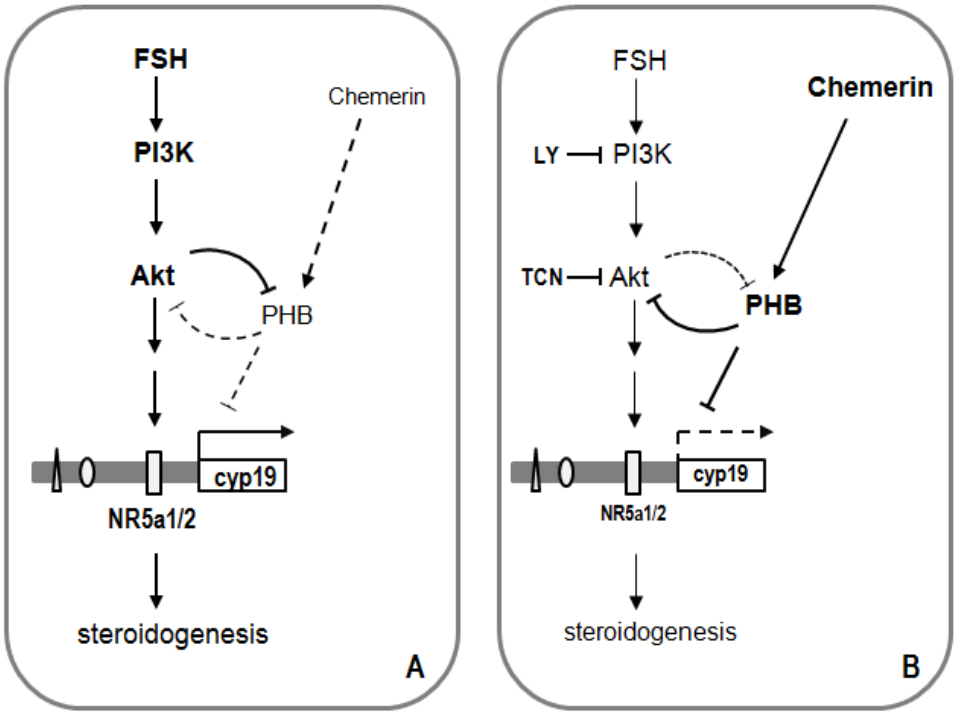

Figure 7. **A hypothetical model depicting PHB and chemerin as negative regulators of FSH-induced steroidogenesis**

In the absence of chemerin (A), FSH stimulates PI3K/Akt signaling, induces the expression of steroidogenic enzymes (e.g. *cyp19*), and promotes steroid production. Akt negatively regulates PHB expression and function; NR5a1 and NR5a2 are the main transcriptional factors involved in this process. When extracellular chemerin level increases (B), chemerin up-regulates PHB expression, which suppresses FSH-induced steroidogenesis via modulating the expression and activity of NR5a1/2. Inhibition of PI3K/Akt signaling enhances the suppressive role of PHB due to reduced feedback inhibition.

Addendum

Expression and regulation of chemerin receptors in rat ovary

To date three chemerin receptors (CMKLR1, CCRL2 and GPR-1) have been identified and their tissue distribution varies from species to species (Goralski *et al.*, 2007; Huang *et al.*, 2010; Wittamer *et al.*, 2003; Zabel *et al.*, 2008). Differential expression of CMKLR1 and GPR-1 has been reported in porcine tissues, especially in liver, kidney and epididymal fat (Huang *et al.*, 2010). One group analyzed the distribution of all three receptors in several mouse tissues except the ovary (Takahashi *et al.*, 2011). The tissue-specific expression of these receptors may correspond to their distinct functions. Therefore, it is of interest to explore the relative expression of three receptors in the ovary.

To examine the gene expression of the chemerin receptors, granulosa cells were collected from immature rats and total RNAs were extracted using Qiagen RNeasy Mini kit following manufacturer's instructions. The mRNA abundance of CMKLR1, CCRL2 and GPR-1 was analyzed by qPCR. The CCRL2 primers were a 5' forward primer (5'-CGGCTTTGGCAAACCTCACTTCTT-3') and a 3' reverse primer (5'-AAGTTCTG CGCTGTCCTCATTTGC-3'). The GPR-1 primers were a 5' forward primer (5'-ACTGG CTTGGCCTTCCTCAATAGT-3') and 3' reverse primer (5'-AGAGACAGGCTCTTGG TTTCAGCA-3'). The CMKLR1 and GAPDH primers are shown in **Table 1**.

As shown in **Figure A2 (A)**, rat granulosa cells express predominantly CMKLR1, low level of CCRL2 and undetectable level of GPR-1. Therefore the action of chemerin in the regulation of ovarian function may be mediated by CMKLR1.

In this chapter, we have demonstrated that chemerin expression is downregulated by gonadotropin *in vivo* (**Figure 1A**). Since CMKLR1 and CCRL2 are expressed in ovarian cells and either one or both receptors could mediate the action of chemerin in the ovary, we examined the mRNA abundance of CMKLR1 and CCRL2 in the same experiment. As shown in **Figure A2 (B)**, eCG administration significantly downregulated the mRNA abundance of CMKLR1 ($p < 0.01$). Although there was a trend of decrease of CCRL2 mRNA at 24 h post-eCG treatment, it did not reach statistical significance ($p > 0.05$). These results suggest that the gene expression of CMKLR1, as in the case of chemerin, is altered during follicular development. Whether the change of CMKLR1 mRNA abundance corresponds to the alteration of its protein level as well as its function, need to be explored with further experiments.

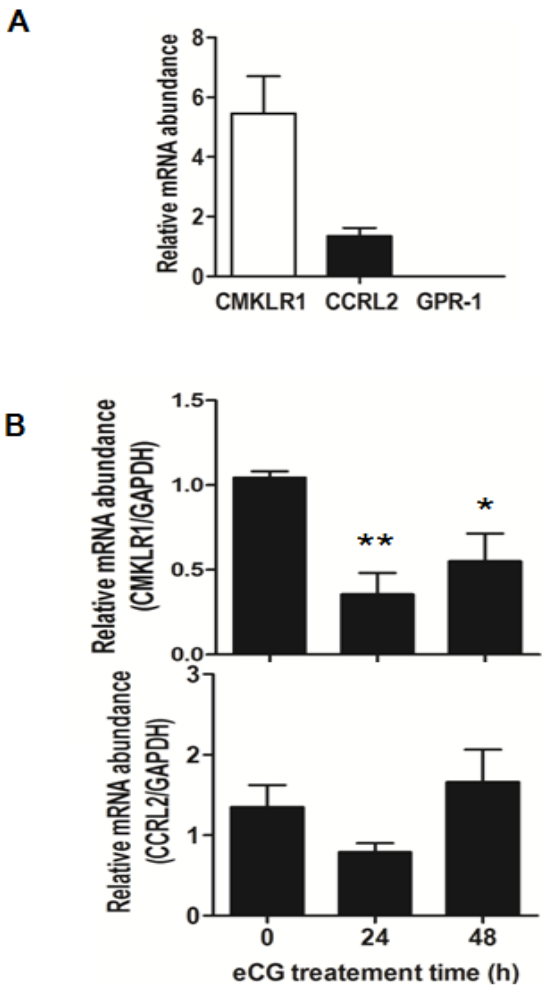

Figure A2 Expression and regulation of the chemerin receptors in the ovary

(A) The mRNA abundance of CMKLR1, CCRL2 and GPR-1 in granulosa cells.

Granulosa cells were collected by follicle puncture from immature rats (Day 23) and the mRNA abundance of CMKLR1, CCRL2 and GPR-1 was analyzed by quantitative real-time PCR.

(B) Effect of gonadotropin on the expression of CMKLR1 and CCRL2 *in vivo*.

Granulosa cells were isolated from follicles at different stages of development in immature rats injected with eCG (0 h, preantral; 24 h, early antral; 48 h, late antral and preovulatory follicles). The mRNA abundance of CMKLR1 and CCRL2 was analyzed by qPCR and normalized to the *GAPDH* mRNA. Data are presented as mean ± SEM of four independent experiments and analyzed by one-way ANOVA and subsequently by Bonferroni *post hoc* test. *, $p < 0.05$; **, $p < 0.01$ compared with eCG at 0h.

Corrections and Clarifications

Since the publication of the manuscript in this chapter, a number of typographical, grammatical or syntax errors were identified. The list below represents the corrections to be included.

Page 76, Line 5	"in granulosa cells" should read "in undifferentiated granulosa cells"
Page 76, Line 6	"our knowledge on" should read "our knowledge of"
Page 78, Line 14	"Unless specified" should read "Unless otherwise specified"
Page 79, Line 12	"is" should read "was", and "are" should read "were"
Page 82, Line 2	"nitrocellular membrane" should read "nitrocellulose membrane"
Page 82, Line 18-19	"limitation" should read "limit"
Page 88, Line 4	Since the mRNA level of a gene results from gene transcription and mRNA degradation, we measured the mRNA abundance but not gene transcription. Therefore, "whether steroidogenic enzymes expression is regulated at the transcriptional level" should be changed to "whether mRNA levels of steroidogenic enzyme expression is regulated"
Page 88, Line 9	"inhibition" should read "inhibitory"
Page 88, Line 17	"minimum" should read "minimal"
Page 88, Line 20	"slightly" should read "slight"
Page 90, Line 8	"is an essential" should read "is essential"
Page 97, Line 5	"at both transcriptional and translational levels" should read "at both mRNA and protein levels"
Page 100, Line 14	"PHB has no suppression on" should read "PHB does not suppress"
Page 100, Line 22	"in" should read "of"
Page 102, Line 21	"possible" should read "possibly"
Page 103, Line 17	"on" should read "of"
Where applicable	"steroids production" should read "steroid production", "manufacturer's instruction" should read "manufacturer's instructions"
Page 96, Figure 6	"analyzed by 1-way ANOVA and subsequently by Bonferroni post hoc test." should be changed to "analyzed by student t test"

CHAPTER 6: JOURNAL OF OVARIAN RESEARCH, 2013, 6: 23-32

Follicular Stage-dependent Regulation of Apoptosis and Steroidogenesis by Prohibitin in Rat Granulosa Cells

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Keywords: prohibitin, apoptosis, steroidogenesis

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Abstract

Background: Follicular growth and atresia are tightly regulated processes, which involve the participation of endocrine, autocrine and paracrine factors at the cellular level. Prohibitin (PHB) is a multifunctional intracellular protein playing an important role in the regulation of proliferation, apoptosis and differentiation. Here we examined the expression of PHB and its regulation by FSH *in vitro* and studied the role of PHB in the regulation of apoptosis and steroidogenesis in response to the apoptosis inducer staurosporine (STS) and to FSH, respectively.

Methods: Undifferentiated and differentiated granulosa cells were collected from diethylstilbestrol (DES)- and equine chronic gonadotropin (eCG)-primed immature rats, respectively and then cultured with various treatments (FSH, adenovirus infection, STS) according to experimental design. The apoptosis rate, the production of estradiol and progesterone, and the expression of distinct proteins (PHB, caspase-3, phospho- and total Akt) were assessed.

Results: PHB is anti-apoptotic and its action is dependent on the differentiated state of the granulosa cells. Data from gain- and loss-of-function experiments demonstrate that PHB inhibited STS-induced caspase-3 cleavage and apoptosis in undifferentiated granulosa cells, but was ineffective in differentiated cells. In contrast, PHB suppresses FSH-induced steroidogenesis and this response is evident irrespective of the differentiated state of granulosa cells.

Conclusion: These findings suggest that PHB regulates granulosa cell apoptosis and steroidogenesis in a follicular stage-dependent manner and that the dysregulation of PHB expression and action may be relevant to ovarian dysfunction.

Backgroud

The destiny of the growing follicles (ovulation or atresia) is dependent on the fate of the cells within them (proliferation, differentiation or apoptosis) and is tightly regulated by endocrine, autocrine and paracrine factors [1]. During follicular development, a large number of follicles undergo atresia, a process tightly controlled by the cross-talk of cell death and survival signals [1, 2]. The dominant follicles continue to develop to preovulatory stages, producing appreciable amounts of steroid hormones which are critical for the reproductive cycle and successful ovulation [3, 4].

Prohibitin (PHB) is a multifunctional protein highly conserved in various species, with identical amino acid sequences in mouse and rat and only one residue differing from that in human [5]. It is present in multiple cellular compartments, including nucleus, mitochondria, plasma membrane and lipid droplets, as well as in the circulation [6-10]. The subcellular localization of PHB may contribute to its diverse functions in the regulation of proliferation, apoptosis, senescence and differentiation [11-14]. For example, mitochondrial PHB facilitates the maintenance of mitochondrial morphology and stabilizes newly synthesized mitochondrial enzymes [15, 16]. Nuclear PHB has been implicated in the regulation of gene expression by interacting with various transcriptional factors, such as E2F, p53 and estrogen receptor α (ERα) [7, 17, 18].

PHB is widely expressed in the ovary and is anti-apoptotic during staurosporine (STS)- and ceramide-induced apoptosis in undifferentiated granulosa cells [19-21]. However, it is unknown whether PHB performs similar roles during follicular development. Although recent data indicated that silencing of PHB induced granulosa cell shape changes [22] and PHB suppressed steroidogenesis in undifferentiated

granulosa cells [23], our knowledge on the role and contribution of PHB to granulosa cell differentiation is incomplete.

In this study, we first examined the expression of PHB and their regulation by FSH in vitro. Using differentiated and undifferentiated granulosa cells from distinct stages of follicular development, we compared its responsiveness to the apoptosis inducer STS and to FSH, a differentiation inducer, and also examined the role of PHB by gain- and loss-of function experiments. We also studied if the roles of PHB in the regulation of apoptosis and steroidogenesis are follicular stage-dependent.

Materials and Methods

Antibodies and Reagents

Cell culture media (M199), fetal bovine serum (FBS), penicillin and streptomycin, L-glutamine, sodium pyruvate and trypsin were purchased from Invitrogen (Burlington, Canada). HEPES, Hoechst 33258, Equine chronic gonadotropin (eCG), and diethylstilbestrol (DES) were purchased from Sigma (St. Louis, MO). Recombinant human FSH was purchased from National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Anti-caspase-3 antibody (recognizing both intact and active caspase-3), anti-phospho-Akt (S^{473}) and anti-Akt antibodies were purchased from Cell signaling (Danvers, MA), anti-PHB and anti-β-Actin antibodies were from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies and reagents for SDS-PAGE were supplied by Bio-Rad (Mississauga, Canada). Enhanced chemiluminescent (ECL) reagent was from Thermo Fisher Scientific (Rockford, IL). Adenoviral-PHB, shPHB and their control particles were obtained from Dr. Winston

Thompson (Morehouse School of Medicine, Atlanta). QIAShredder and RNeasy minikits were purchased from QIAGEN (Mississauga, Canada). Random decamer primers were from Ambion (Austin, TX). Ribonuclease inhibitor and dNTP were from Fermentas (Burlington, Canada). Moloney murine leukemia virus reverse transcriptase was from Promega (Madison, WI). PCR primers were from Invitrogen. All chemical inhibitors were purchased from Calbiochem (Gibbstown, NJ). All other chemicals were of the highest analytical grade available from Sigma.

Animal preparation

Twenty one days old Sprague-Dawley rats (Charles River, Montreal, Canada) were maintained on 12-h light, 12-h dark cycles and given food and water *ad libitum*. All procedures were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, Canadian Council on Animal Care, and were approved by the University of Ottawa and the Ottawa Hospital Research Institute Animal Care Committee.

Primary culture of rat granulosa cells and adenoviral infection

Granulosa cells from eCG-primed immature rats (10 IU, 48h, s.c.; considered as differentiated granulosa cells) and DES-injected control rats (1 mg/day for 3 consecutive days, s.c.; considered as undifferentiated granulosa cells) were pre-incubated with 6 mM EGTA and 0.5 M sucrose [24] and were released by follicular puncture with a 26.5-gauge needle, washed and centrifuged (900 × g, 5 min). Cell clumps and oocytes were removed by filtering the cell suspensions through a 40-μm nylon cell strainer (BD Biosciences). The viability of granulosa cells was determined by trypan blue exclusion.

Granulosa cells (0.9×10^6 per well in 6-well plate) were plated overnight in M199 with 10% FBS under a humidified atmosphere of 95% air and 5% CO2. After culture overnight in serum-free medium, granulosa cells were treated with FSH (0–200 ng/ml) or STS (1 μM) for a designated duration.

For adenoviral infection, granulosa cells were cultured in serum-free M199 medium containing adenoviral particles for 24 h followed by medium change. Multiplicity of infection (MOI) and duration of infection are detailed in the figures. Equal amounts of adenovirus in each experimental group were achieved by adjusting with an appropriate amount of adenoviral-LacZ (negative control for adenoviral-PHB) or adenoviral-shNeg (negative control for shPHB).

RT-PCR

Total RNA of granulosa cells was extracted according to the manufacturer's instruction, using the QIAGEN RNeasy Mini kit. Two hundred ng total RNA were used to reverse transcribe cDNAs and the mRNA abundance of target genes was analyzed by PCR. The PHB primers used for amplification were a 5' forward primer (5'-TGGCAGC-CTGAGTAGACCTT-3') and a 3' reverse primer (5'-TCACGGTTAAGAGGGAATGG-3'). The p450scc primers were a 5' forward primer (5'-ACCCTGAGTCCCAGCGGTTC-3') and a 3' reverse primer (5'-CACCCCTCCTGCCAGCATCT-3'). The aromatase primers were a 5' forward primer (5'-TGGTCCCGGAAACTGTGCCT-3') and a 3' reverse primer (5'-CCACGCTTGCTGCCGAATCT-3'). The actin primer were (5'-CGT-CCACCCGCGAGTACAAC-3') and a 3' reverse primer (5'-GCCTCTCTTGCTCTGGG-CCT-3'). The thermal cycling conditions were comprised of an initial denaturation step at

95 C for 10 min followed by 30 cycles amplification for PHB, p450scc and aromatase (20 cycles for actin) at 95 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec. The PCR products were subjected to 2 % ethidium bromide-containing agarose gel and visualized under UV light.

Protein extraction and Western blot

At the end of the culture period, floating cells and attached cells (recovered by 0.05% trypsin treatment) were pooled and centrifuged (1000 × g, 10 min). For protein extraction, cell pellets were resuspended in a lysis buffer (PBS, pH 7.4) containing NaCl (150 mM), SDS (0.1%), sodium deoxycholate (0.5%), Nonidet P-40 (1%), and the protease inhibitor cocktail (Sigma) and kept on ice for 30 min. Cell lysates were sonicated and centrifuged (12,000 × g, 5 min, 4°C) to remove insoluble material. Supernatant was recovered and stored at -20°C until further processing. Protein concentrations in each sample were determined by the Bradford assay (Bio-Rad Laboratories). Twenty μg of protein of cell lysates were subjected to SDS-PAGE with 4.5% stacking and 15% separating gels. Proteins were electrophoretically transferred onto nitrocellular membrane (NC, Bio-Rad), blocked at room temperature with 5% skim milk in TBST [0.05% Tween-20 in Tris (10 mM) and NaCl 181 (0.15 M), pH7.4 (TBS)] for 1 h and then incubated over-night at 4°C with diluted primary antibodies (1:1000) in TBST with constant agitation. The membranes were then treated with a secondary antibody (1:2000 to 1:10,000 based on different primary antibody). After washing three times with TBST, immunoreactive bands were visualized with ECL according to the manufacturer's instruction. Intensity of bands of the exposed X-ray film was determined by densitome-

trically scanned, quantified, using AlphaEaseFC (Alpha Innotech, CA) and normalized with β-Actin.

Assessment of Apoptosis

Apoptotic cells were identified morphologically by Hoechst-33258 (bisBenzimide, Sigma) staining as previously reported [25]. At the end of the culture period, suspended cells were collected by centrifugation and attached cells were trypsinized. The two cell fractions were then pooled, pelleted, and suspended in 10% phosphate-buffered formalin containing Hoechst 33258 (6.25 μg/ml; room temperature, 2 h), Cells were then spotted on slides and assessed for typical apoptotic nuclear morphology. To quantify the number of apoptotic cells, healthy and apoptotic cells were counted (counter was "blinded to sample identity") and the apoptotic cells were expressed as a percentage of total cells. A minimum of 400 cells were counted in each treatment group.

Steroids secretion analysis

Spent medium from granulosa cell cultures were collected, centrifuged (900 × g, 5 min) and kept in −80°C for hormone analysis. 17β-estradiol and progesterone concentrations in spent medium were measured using enzyme immunoassay kit (EIA; Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instruction. The detection limitation of estradiol was 28 pg/ml, and the intra- and inter-assay coefficients of variation were 8 and 6%, respectively. The detection limitation of progesterone was 8.5 pg/ml, and the intra- and inter-assay coefficients of variation were 7 and 6%, respectively.

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 statistical software (San Diego, CA). Results are expressed as mean ± SEM of at least three independent experiments as detailed in the figures. One-way and two-way ANOVA were used to assess the effects and interactions of one or two variables and multiple comparisons were achieved by a Bonferroni *post hoc* test. Significant difference was defined at $p < 0.05$ (*, or #).

Results:

PHB is differentially expressed in granulosa cells from different follicular stages

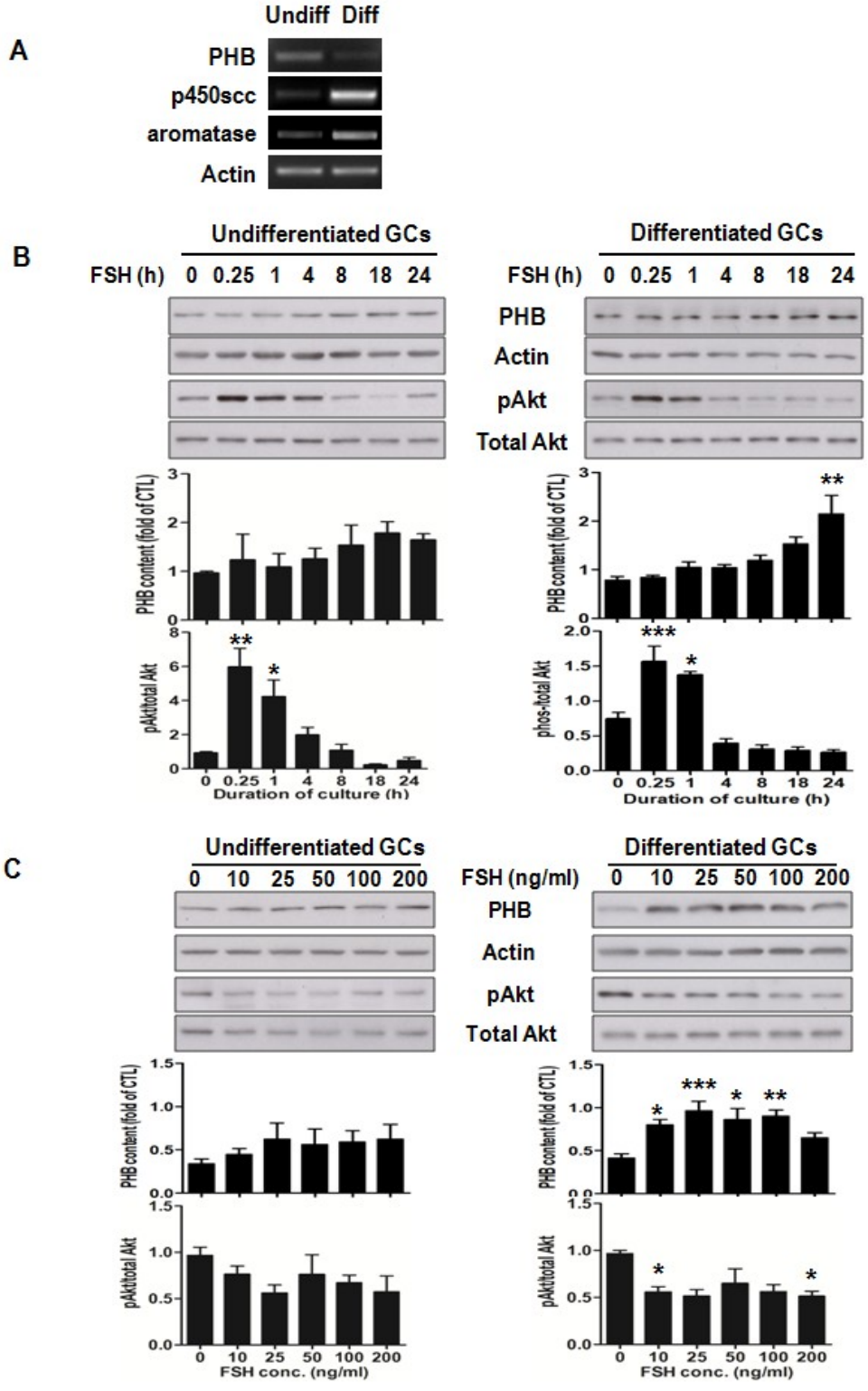
To examine whether PHB is expressed *in vivo* in a follicular stage-dependent manner, differentiated and undifferentiated granulosa cells were collected and the gene expression of PHB was analyzed by traditional PCR. As shown in **Figure 1A**, differentiated granulosa cells exhibited increased expression of *p450scc* and *aromatase*, the steroidgenic enzymes known to be associated with granulosa cell differentiation. The *PHB* expression in differentiated granulosa cells was lower than that in undifferentiated granulosa cells.

FSH upregulates PHB content in differentiated but not undifferentiated granulosa cells

To explore whether FSH regulates PHB expression *in vitro*, undifferentiated and differentiated granulosa cells were cultured with FSH (100 ng/ml) for designated time (0–24 h) and the contents of PHB in two types of cells were examined. As shown in **Figure 1B**, FSH had no effect on PHB content in undifferentiated granulosa cells ($p > 0.05$). In the contrast, FSH up-regulated PHB expression in differentiated granulosa cells ($p <$

0.001). While there was an apparent gradual increase in PHB content with the duration of culture in the presence of FSH, significant upregulation was not evident until 24 h. Both undifferentiated ($p < 0.0001$) and differentiated ($p < 0.001$) granulosa cells exhibited a rapid increase in phosphorylated Akt content; however, Akt activation in the former was stronger (6-fold vs. 2-fold change at 0.25h) and sustained longer (over basal level at 4-8h) compared with that in the latter.

We then examined whether FSH regulates PHB contents in differentiated granulosa cells in a concentration-dependent manner. Undifferentiated and differentiated granulosa cells were cultured with different FSH concentrations (0-200 ng/ml) for 24 h and the content of PHB at the two follicular stages was examined. As shown in **Figure 1C**, FSH had no effect on PHB content in undifferentiated granulosa cells ($p > 0.05$); however FSH significantly up-regulated the content of PHB ($p < 0.001$) at a concentration range of 10–100 ng/ml in differentiated granulosa cells. There was no significant difference between the FSH (200 ng/ml) and the control groups. Since we have reported that Akt and PHB could regulate each other [23], we also assessed the phosphorylated and total Akt levels in this experiment. As shown in **Figure 1C**, although there was a trend of decrease, FSH didn't significantly reduce Akt phosphorylation in undifferentiated granulosa cells ($p > 0.05$). In the contrast, phosphorylated Akt levels in granulosa cells in differentiated granulosa cells decreased in the presence of FSH (**Figure 1C**, $p < 0.05$), which was reversely related to PHB contents.


A
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Differentiated GCs
FSH (h) 0 0.25 1 4 8 18 24
PHB
Actin
pAkt
Total Akt
PHB content (fold of CTL)
pAkt/total Akt
phos-total Akt
Duration of culture (h)
C
Undifferentiated GCs
Differentiated GCs
FSH (ng/ml) 0 10 25 50 100 200
PHB
Actin
pAkt
Total Akt
PHB content (fold of CTL)
pAkt/total Akt
FSH conc. (ng/ml)

Figure 1. **The regulation of PHB by gonadotropin**

(A) Total RNAs of undifferentiated and differentiated granulosa cells were extracted and mRNA abundance of PHB was analyzed by PCR. p450scc and aromatase mRNA levels were assessed as differentiated marker and Actin was used as loading control.

(B) Undifferentiated and differentiated granulosa cells were cultured with FSH (100 ng/ml) for designated time period and the contents of PHB, pAkt and total Akt were examined by Western blot.

(C) Undifferentiated and differentiated granulosa cells were cultured with FSH (0 - 200 ng/ml) for 24 h. PHB, pAkt and total Akt contents were examined by Western blot. Representative immunoblots are shown and data are presented as mean ± SEM of three independent experiments. B-C, one-way ANOVA, followed by Bonferroni test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (compared with FSH = 0 or FSH at 0 h).

Exogenous PHB suppresses staurosporine-induced caspase-3 cleavage and apoptosis in undifferentiated, but not differentiated, granulosa cells

Although PHB has been reported to be anti-apoptotic in undifferentiated granulosa cells [19, 20], whether it plays a similar role in granulosa cells after differentiation is not known. To test this question, comparison experiments were performed using undifferentiated and differentiated granulosa cells. Granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-LacZ as control, 24 h) to over-express or knockdown PHB, and then cultured with the apoptosis inducer staurosporine (STS, 1 μM, 2 h). As shown in **Figure 2A**, whereas PHB over-expression had no effect on basal level of apoptosis, it suppressed STS-induced caspase-3 cleavage (**Figure 2A**, PHB, $p < 0.05$; STS, $p < 0.0001$; PHB × STS, $p < 0.05$) and apoptosis (PHB, $p < 0.05$; STS, $p < 0.0001$; PHB × STS, $p < 0.05$). In contrast, overexpression of PHB in differentiated granulosa cells had no obvious effect on STS-induced caspase-3 cleavage and apoptosis (**Figure 2B**), suggesting that PHB is anti-apoptotic during preantral follicular growth but this property is lost as the cells differentiate during follicle transition into the antral stage.

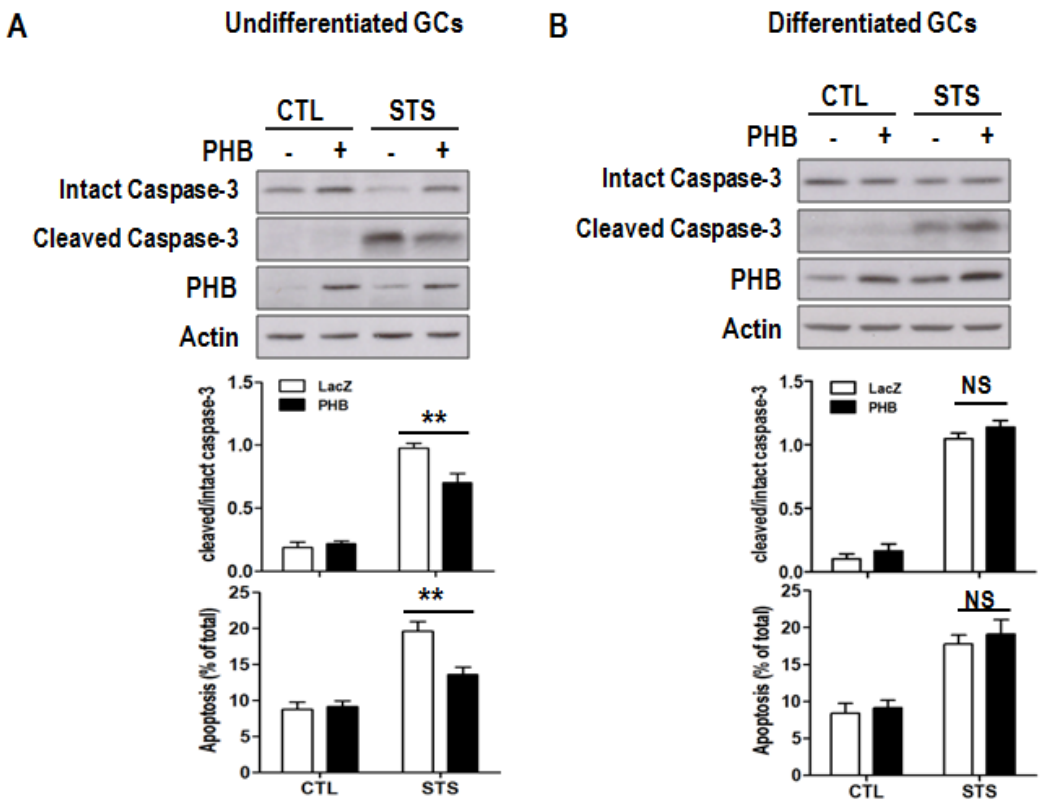


Figure 2. Effect of exogenous PHB on STS-induced caspase-3 cleavage and apoptosis

Undifferentiated (**A**) and differentiated (**B**) granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-lacZ as control) for 24 h and then cultured with STS (1 μM, 2 h). The contents of cleaved, intact caspase-3 and PHB were examined by Western blot and the apoptosis rate of granulosa cells were assessed by nuclear morphology (Hoechst staining). Data are presented as mean ± SEM of three independent experiments and analyzed by two-way ANOVA and subsequently by Bonferroni test. **, $p < 0.01$ (compared with LacZ). NS: not significant

Knockdown of PHB increases staurosporine-induced caspase-3 cleavage and apoptosis in undifferentiated, but not differentiated, granulosa cells

Similar experiments were performed using adenoviral-shRNA to knockdown PHB in granulosa cells derived from two preparations. As shown in **Figure 3**, STS-induced caspase-3 cleavage and apoptosis were enhanced after knockdown of PHB in undifferentiated granulosa cells (**Figure 3A**, for cleaved caspase-3, shPHB, $p < 0.01$; STS, $p < 0.0001$; shPHB × STS, $p < 0.05$. For apoptosis, shPHB, $p < 0.01$; STS, $p < 0.0001$; shPHB × STS, $p < 0.05$). However, there was no effect of PHB knockdown on STS-induced caspase-3 cleavage and apoptosis in differentiated granulosa cells (**Figure 3B**).

Differentiated granulosa cells are more responsive to FSH in steroids production compared with undifferentiated granulosa cells

We have previously reported that PHB suppressed FSH-induced estradiol and progesterone secretion and p450scc/aromatase expression in undifferentiated granulosa cells [23]. As the regulatory role of PHB in granulosa cell apoptosis is dependent on the state of cellular differentiation, we then examined whether it regulates steroidogenesis differently in differentiated granulosa cells by comparing the steroidogenic responsiveness of granulosa cells at the two differentiative states. Undifferentiated and differentiated granulosa cells were cultured with FSH (0-100 ng/ml) ± testosterone (T, 0.5 μM, 24 h), which served as a substrate of aromatase, and then the levels of estradiol and progesterone in spent medium were measured. We have previously demonstrated that T enhanced FSH-induced progesterone and estradiol secretion in undifferentiated granulosa

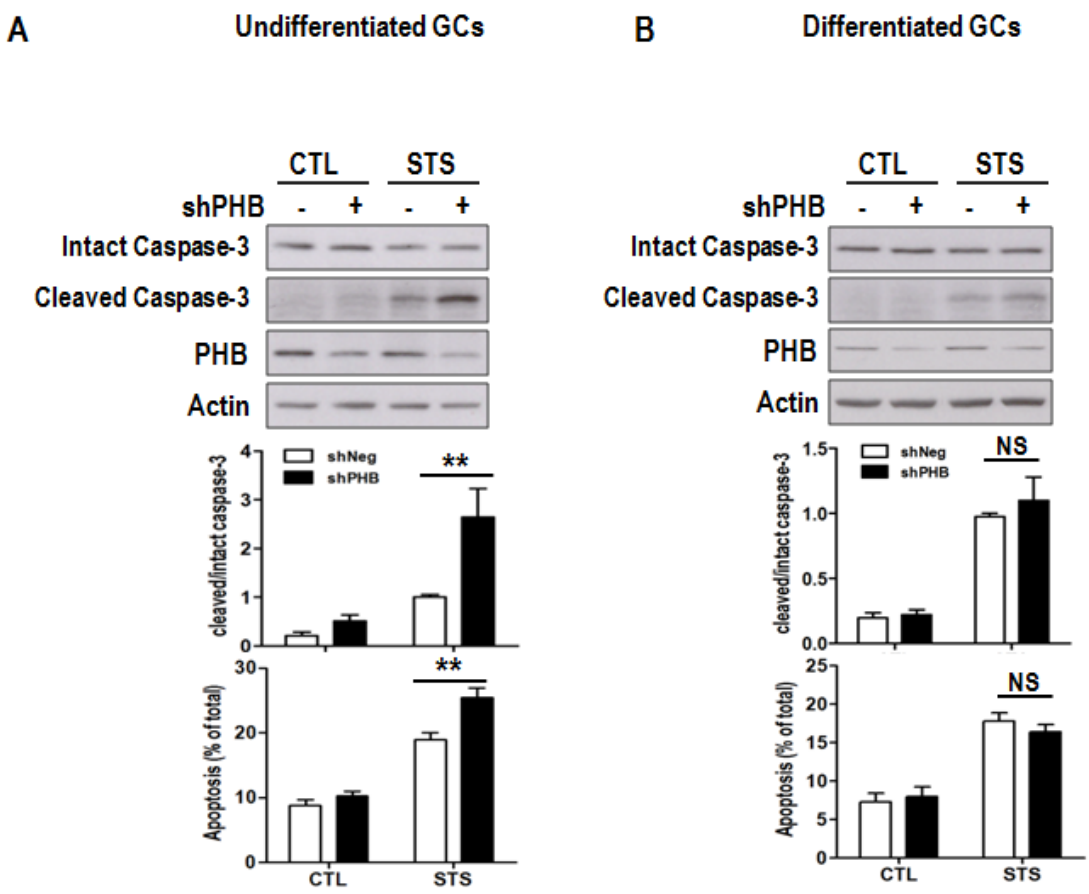


Figure 3. Effect of PHB knockdown on STS-induced caspase-3 cleavage and apoptosis

Undifferentiated (**A**) and differentiated (**B**) granulosa cells were infected with adenoviral-shPHB (MOI = 10, adenoviral-shNeg as control) for 48 h and then cultured with STS (1μM, 2 h). The contents of cleaved, intact caspase-3 and PHB were examined by Western blot and the apoptosis rate of granulosa cells were assessed by nuclear morphology (Hoechst staining). Data are presented as mean ± SEM of three independent experiments and analyzed by two-way ANOVA and subsequently by Bonferroni test. **, $p < 0.01$ (compared with LacZ). NS: not significant

cells [23]. We observed that FSH-induced progesterone production in these cells was dramatically increased in the presence of T (5.49 ± 0.4 vs. 0.63 ± 0.13 ng/ml) and the estradiol secretion exhibited similar effect (48.7 ± 5.7 vs. 0.91 ± 0.16 ng/ml). We concomitantly tested the effect of T and FSH on differentiated cells (**Figure 4A**) and observed that T also synergistically enhanced FSH-stimulated progesterone (7.79 ± 0.63 vs. 1.22 ± 0.98 ng/ml) and estradiol secretion (94.4 ± 10.23 vs. 0.88 ± 0.14 ng/ml) in differentiated granulosa cells (**Figure 4A**, for progesterone: FSH, $p < 0.001$; T, $p < 0.001$; FSH × T, $p < 0.001$. for estradiol: FSH, $p < 0.001$; T, $p < 0.001$; FSH × T, $p < 0.001$). The basal levels of estradiol and progesterone at the two cellular differentiative states were similar; however production of these steroids in the presence of FSH and T was about 2-fold higher in differentiated granulosa cells than in undifferentiated granulosa cells.

PHB suppresses FSH-induced steroid production in both undifferentiated and differentiated granulosa cells

Next we examined whether PHB suppresses FSH-induced steroidogenesis in granulosa cells and if its response is dependent on the differentiated state of the cells. Undifferentiated and differentiated granulosa cells were infected with adenoviral-PHB (adenoviral-LacZ as control) and then cultured with FSH (0-100 ng/ml) in the presence of T (0.5 μM) for 24 h. The levels of estradiol and progesterone in spent medium were measured by EIA. Exogenous PHB suppressed FSH-induced steroid production and the expression of steroidogenic enzymes p450scc and aromatase in undifferentiated granulosa cells [23]. FSH-induced progesterone production in these cells was dramatically suppressed by exogenous PHB (5.45 ± 0.47 vs. 3.14 ± 0.35 ng/ml) and the

estradiol secretion exhibited similar effect (55.6 ± 5.0 vs. 21.76 ± 7.72 ng/ml). Concomitant studies with differentiated granulosa cells indicate that PHB similarly inhibited FSH-induced progesterone (7.70 ± 0.52 vs. 4.70 ± 0.78 ng/ml) and estradiol (95.56 ± 11.17 vs. 48.96 ± 6.7 ng/ml) secretion (**Figure 4B**, for progesterone: PHB, $p < 0.01$; FSH, $p < 0.001$; PHB × FSH, $p < 0.01$. for estradiol: PHB, $p < 0.05$; FSH, $p < 0.001$; PHB × FSH, $p < 0.05$) *in vitro*. The contents of p450scc and aromatase induced by FSH were also suppressed by PHB (**Figure 4C**).

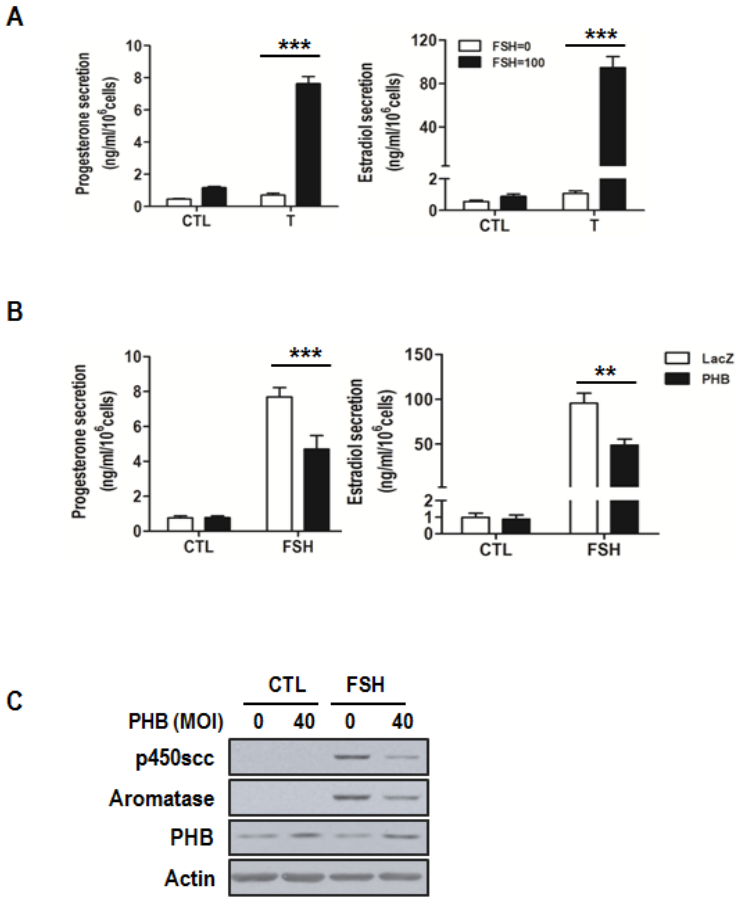


Figure 4. **Influence of PHB on FSH-induced steroid production**

(A) Differentiated granulosa cells were cultured with FSH (100 ng/ml) ± T (0.5 μM) for 24 h and the levels of progesterone and estradiol in the spent medium were measured by EIA. Data are presented as mean ± SEM of four independent experiments and analyzed by two-way ANOVA and subsequently by Bonferroni post hoc test. ***, $p < 0.001$ (compared with LacZ).

(B-C) Differentiated granulosa cells were infected with adenoviral-PHB (MOI = 40, adenoviral-LacZ as control) for 24 h and then cultured with FSH (100 ng/ml, 24 h) in the presence of T (0.5 μM). Progesterone and estradiol in the spent medium (B) were measured by EIA. Data are presented as mean ± SEM of four independent experiments and analyzed by two-way ANOVA and subsequently by Bonferroni post hoc test. **, $p < 0.01$; ***, $p < 0.001$ (compared with CTL). (C) The expression of p450scc and aromatase was examined by Western blot. Representative immunoblots from three replicate experiments are shown.

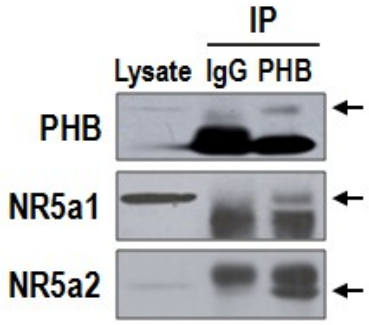
Discussion:

In the present study, we have demonstrated the distinct roles of PHB in STS-induced apoptosis and FSH-induced steroidogenesis in granulosa cells at different stages of follicular development and thus at different cellular differentiative states. FSH regulates PHB expression in differentiated but not undifferentiated granulosa cells *in vitro*. PHB is anti-apoptotic and a suppressor of steroidogenesis in undifferentiated granulosa cells, while it loses its role in regulating apoptosis but maintains its latter action as the cells differentiate. These findings suggest that suppressive roles of PHB in apoptosis and steroidogenesis are precisely regulated in a follicular stage-dependent manner. The role of PHB in the regulation of apoptosis, steroidogenesis as well as proliferation [11,12], the dysregulation of ovarian follicular growth and steroidogenesis in polycystic ovarian syndrome (PCOS) [26-28], together with the observations that the expression of PHB increased in a chronically androgenized rat PCOS model [23] suggest a possibility that dysregulation of PHB may be relevant to the etiology of this syndrome.

The main observation in present study is that the anti-apoptotic action of PHB is evidenced at the preantral follicle stage of development, one often referred to as the "penultimate stage" when the destiny of the follicle (continued growth versus atresia) is determined. This observation also raise the interesting possibility that PHB may play an important role in deciding the fate of the granulosa cells as the follicles transition from the preantral to early antral stage. With regard to the regulation of apoptosis, PHB may execute its anti-apoptotic role by downregulating caspase-3 cleavage and inhibiting cytochrome c release from mitochondria [20]. The loss of inhibition on apoptosis in differentiated granulosa cells may due to increased X-linked inhibitor of apoptosis

protein (XIAP) expression and elevated Akt phosphorylation in these cells [29] known to inhibit apoptosis, which may modulate the action of PHB. However, we cannot rule out the possibility that PHB inhibits apoptosis via regulating the activity of transcriptional factors involved in apoptosis, as demonstrated by Fusaro *et al* that PHB protects cancer cells from camptothecin-induced apoptosis via suppressing E2F1-mediated transcriptional activity [30]. While the different transcriptional factors targeted by PHB may switch off its anti-apoptotic function, this hypothesis needs further investigation.

In contrast, the participation of PHB in the control of steroidogenesis in both undifferentiated and differentiated granulosa cells is consistent with important intra-ovarian regulatory role of steroids during follicular development and the involvement of PHB in the control of the steroidogenic processes. The mechanism by which PHB acts as a steroidogenesis-suppressor or anti-apoptotic factor during follicular development and the physiological signal that drives these responses are unknown. PHB is shown to be regulated by the novel adipokine chemerin *in vitro* and it mediates the suppressive role of chemerin on FSH-induced steroidogenic enzyme expression in undifferentiated granulosa cells [23]. Because PHB is a co-activator or co-repressor of distinct transcriptional factors (p53, E2F, ERα) [7,17,18] and we have observed an interaction of PHB and NR5a1/2 in freshly isolated rat granulosa cells (Additional file 1: **Figure S1**), it is possible that PHB suppresses steroidogenesis via acting as a co-repressor of transcriptional factors targeting steroidogenic enzymes, such as nuclear receptors NR5a1/NR5a2, C/EBP [31-34]. Further studies on promoter activity, DNA mutagenesis and protein-protein interaction assay are needed to test this possibility. However, we cannot rule out the possibility that PHB may regulate the mRNA expression of FSH receptor.



Additional file 1:

Figure S1. Interaction of PHB and NR5a1/2.

Granulosa cells were collected from eCG-primed rats and then lyzed in IP lysis buffer. Endogenous PHB in 500 μg cell lysate was immunoprecipitated with 2 μg mouse anti-PHB antibody (normal mouse IgG as control), subjected to 15 % SDS-PAGE and probed with the antibodies targeting PHB, NR5a1 and NR5a2, respectively. Darker bands in Western blot indicate the heavy or light chain of IgG.

Besides PHB, other proteins have also been reported to be differentially regulated and play various functions in ovarian cells from different follicular stages. Plasminogen activator plays a crucial role in the dynamic tissue remodeling during follicular development and ovulation [35,36]. Its activity is increased by FSH, and inhibited by transforming growth factor α, in undifferentiated granulosa cells but decreased in differentiated ones [37,38]. Another example is C-type natriuretic peptide (CNP), which binds to its receptor and promotes preantral follicle growth via stimulating the cGMP release in undifferentiated granulosa cells; however CNP is unable to increase cGMP level in differentiated granulosa cells [39]. The cell differentiation-dependent regulation of intra-ovarian and intracellular factors may facilitate the precise control of granulosa cell fate and function during follicular development.

Although the functions of PHB are reported to regulate many cellular processes in various cell types, how its expression is regulated is largely unknown. The regulation of PHB by gonadotropin in the literature is controversial. We demonstrated that gonadotropin *in vivo* reduced PHB mRNA abundance; however others reported that PHB mRNA levels and protein contents are not altered [40] or higher after gonadotropin treatment [21]. While the reasons for these apparent differences are not immediately clear, whether differences in the dosages of gonadotropin used and/or in the methods of granulosa cell isolation could account for the different outcomes, remains to be determined. In this context, our results also indicated that high concentration of FSH (200 ng/ml) failed to increase PHB expression in vitro whereas lower concentration did (**Figure 1C**). The effect of high doses of FSH or other molecules on gene expression has been well documented and an effective negative feedback mechanism to precisely control

gene expression may be operational. FSH receptor is desensitized and down-regulated by long exposure of high dose of FSH [41-43], accompanied by reduced cyp19 expression and estradiol production in granulosa cells [43]. In addition, anti-Müllerian hormone, known to inhibit FSH-induced aromatase expression, is also up-regulated by low but down-regulated by high dose of FSH [43]. Low dose of AMH stimulates inhibin B level in human granulosa cells while the opposite was true with higher dosages [44].

The mechanism by which FSH regulates PHB expression is unclear. It is known that FSH acts through multiple signaling pathways, including cAMP/PKA, PI3K/Akt and MAPK, and via various transcriptional factors, such as forkhead box O1, cAMP regulatory element binding protein and specific protein 1 (Sp1) [45-48]. In addition to putative binding sites for CCAAT/enhancer-binding protein (C/EBP) and insulin response element in the promoter of PHB [49], there are other DNA binding elements such as E2F, GATA, ER and Sp1 as predicted by the transcription element search system. It is possible that the transcription factors maintaining PHB expression in undifferentiated granulosa cells is switched to others in differentiated granulosa cells due to the distinct cellular contents. However, the particular transcription factor involved in the regulation of PHB in granulosa cells at the two stages of cellular differentiation needs to be further investigated.

It is well known that FSH stimulates Akt phosphorylation in ovarian cells [45]. Our present studies extent these findings and show that the rapid increase of phosphorylated Akt content in response to FSH is dependent on the state of cellular differentiation. Since the phosphorylated Akt content is a consequence of both kinase and phosphatase activities [50], it was of interest to determine whether this signal is removed

with a different efficiency between different state of differentiation and whether the decreased pAkt levels are correlated with increased PHB contents. Our results indicated that the efficiency at which the PI3K-Akt signaling pathway is turned on and off by FSH is also dependent on the state of cellular differentiation and may be related to the action of PHB. The reverse correlation of PHB and pAkt content was supported by a recent finding that PHB and Akt could regulate the expression of each other [23].

In our culture system, both undifferentiated and differentiated granulosa cells exhibited a robust steroidogenic response to FSH. Basal and FSH-induced estradiol secretion in undifferentiated granulosa cells were lower than those in differentiated granulosa cells in the presence of testosterone, which could be due to higher basal levels of p450scc and aromatase induced by gonadotropin with increased granulosa cell differentiation (this study, [51]). Testosterone was added in the culture as the substrate of aromatase, which is commonly used in the studies on FSH-induced estrogen production [45,52,53]. It is also possible that testosterone not only acted as a substrate in granulosa cells, but also augmented the action of FSH on the production of progesterone and estradiol [52,54,55].

Conclusion

Our findings demonstrate that PHB expression is regulated by FSH in a follicular stage-dependent manner in vitro and the roles of PHB as an anti-apoptotic factor and in the regulation of steroidogenesis are dependent on the differentiation status of granulosa cells. It is an inhibitor of steroidogenesis in both undifferentiated and differentiated granulosa cells, but is anti-apoptotic in undifferentiated granulosa cells. This study

significantly improves our understanding of the role of PHB in the ovary although the mechanism by which PHB suppresses apoptosis and steroidogenesis and the factors involved in the regulation of PHB remains to be investigated.

Abbreviation

PHB, prohibitin; STS: staurosporine; eCG: equine chronic gonadotropin; DES: diethylstilbestrol; ECL: enhanced chemiluminescent; MOI: multiplicity of infection; EIA: enzyme immunoassay; T: testosterone; p450scc: p450 side-chain cleavage enzyme

Competing interests

The authors declare that they have no competing interests

Authors' contributions

QW performed the experiments, prepared the data and drafted the manuscript. AL and BKT are co-mentors, provided input of studies and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgement

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Addendum

Since the publication of the manuscript in this chapter, a number of typographical, grammatical or syntax errors were identified. The list below represents the corrections to be included.

Title	A more accurate title of the manuscript could be "Role of PHB in the regulation of granulosa cell steroidogenesis and apoptosis during follicular development"
Page 113, Line 3	"their" should be "its"
Page 113, Line 5	"its" should read "their"
Page 116, Line 1	"cycles amplification" should read "cycles of amplification"
Page 116, Line 15	"of" should read "from"
Page 116, Line 23	"densitometrically scanned" should read "densitometrical scanning"
Page 117, Line 15	"Steroids secretion analysis" should read "Steroid assays"
Page 117, Line 20-21	"limitation" should read "limit"
Page 118, Line 23	"In the contrast" should read "In contrast"
Page 119, Line 7	"contents" should read "content"
Page 119, Line 20	"reversely" should read "inversely"
Page 122, Line 7	"or knockdown" should be deleted
Page 126, Line 16	"differentiated" should read "differentiation"
Page 129, Line 18	"raise" should read "raises"
Page 132, Line 10	"of" should read "by"
Page 133, Line 2	"exposure of" should read "exposure to"
Page 133, Line 6	"was" should read "is"
Where applicable	"differentiative states" should read "states of differentiation",
Figure 1	In the legend (A), "differentiated marker" should be changed to "differentiation markers"
Figure 4	In the legend (A), "LacZ" should be changed to "FSH=0"; (B), "CTL" should be changed to "LacZ"

CHAPTER 7: GENERAL DISCUSSION

7.1 Overview and Significance

Ovarian follicular development is a process precisely controlled by both systemic factors (gonadotropins) and intraovarian regulators including steroids, growth factors, cytokines and intracellular proteins. Due to numerous research efforts, a large number of regulators and their mechanisms of regulation of follicular growth, atresia and differentiation have been established. Although the abnormal expression and activities of some of these regulators are believed to be associated with ovarian dysfunction diseases, such as PCOS, the etiology and pathogenesis of this syndrome are not completely understood. As obesity is highly associated with this syndrome, whether adipokines secreted from the fat tissue affect the physiological function of the ovarian cells remains largely unknown.

In this thesis, we have examined the molecular mechanisms that contribute to the folliculogenesis and the development of PCOS. We present here, for the first time, that two regulators chemerin and PHB play important roles in FSH-induced steroidogenesis, and address the mechanisms regulating granulosa cell fate. We also present the evidence of the dysregulation and potential involvement of these factors in the pathogenesis of PCOS based on an androgenized rodent model. These findings significantly improve our knowledge of ovarian functions and the pathophysiology of PCOS, and provide important clues for the development of novel diagnosis biomarkers and new treatment strategies for this complex syndrome.

7.2 Role of Chemerin in the regulation of ovarian function

During follicular development, granulosa cell fate (proliferation, apoptosis or differentiation) is regulated by various intraovarian and intracellular factors. A number of adipokines secreted by adipose tissue, including leptin, adiponectin and IL-6, have been shown to target the ovary and regulate ovarian function (Chabrolle *et al.*, 2009; Kikuchi *et al.*, 2001; Ledoux *et al.*, 2006; Maeda *et al.*, 2007; Spicer *et al.*, 2011). After the mRNA expression of chemerin was demonstrated in the ovary in various species, Reverchon *et al.* reported that chemerin regulates follicular steroidogenesis (Reverchon *et al.*, 2012). Chemerin suppressed IGF-I (but not FSH)-induced steroidogenesis and cell proliferation in human granulosa cells *in vitro* (Reverchon *et al.*, 2012). They also demonstrated that chemerin reduced the phosphorylation of IGFR, ERK1/2 and Akt induced by IGF-I (Reverchon *et al.*, 2012). In contrast to their observations, the present study indicates a negative effect of chemerin on FSH-induced steroidogenesis. The hormone treatment before cell collection (FSH/hCG vs. DES), the differentiation status of cells (luteinized vs. undifferentiated), and experimental species (human vs. rat) may contribute to the difference.

The current study shows that chemerin suppresses NR5a1/2-mediated steroidogenic enzyme expression and down-regulates the expression of NR5a1/2, however the signaling pathways involved are unknown. Chemerin has recently been identified as a target gene of farnesoid X receptor (FXR) and a FXR element in chemerin promoter is essential for this process (Deng *et al.*, 2013). Since FXR activation induces the expression of the NR5a1/2 suppressor SHP (Fayard *et al.*, 2004; Goodwin *et al.*, 2000; Lee and Moore, 2002; Saxena *et al.*, 2007), it is possible chemerin inhibits NR5a1/2-induced gene transcription of steroidogenic enzyme by increasing SHP

expression. In addition to the suppressors, the activity of NR5a1/2 can also be regulated by post-translational modifications (phosphorylation, acetylation and SUMOylation) (Chen *et al.*, 2004; Hammer *et al.*, 1999; Jacob *et al.*, 2001; Komatsu *et al.*, 2004; Yang *et al.*, 2009) and NR5a1 undergos polyubiquitination for proteasomal degradation (Chen *et al.*, 2007).

The binding of PHB and NR5a1/2 (by IP) and the up-regulation of PHB by chemerin suggest the possibility that chemerin may increase the interaction of PHB-NR5a1/2 which in turn interferes with the steroidogenic gene transcription and/or their degradation. Since we used a CMV promoter-drived vector to overexpress NR5a1/2 in vitro, we cannot rule out the possibility that chemerin directly regulates the activity of CMV promoter.

As an ovarian paracrine/autocrine factor, chemerin could not only regulate granulosa cell steroidogenesis but also play a role in the control of follicular growth and atresia. A recent paper indicated an inhibitory role of chemerin in IGF-induced thymidine incorporation in human luteinzed granulosa cells (Reverchon *et al.*, 2012). Our unpublished data demonstrate that chemerin inhibits the basal and FSH-induced rat preantral follicle growth and reduces the level of GDF9 (Kim *et al.*, 2013), an oocyte-specific molecule known to promote preantral follicle growth, increase thecal cell DNA synthesis and suppress apoptosis (Hayashi *et al.*, 1999; Orisaka *et al.*, 2009; Orisaka *et al.*, 2006; Vitt *et al.*, 2000). In addition, chemerin has also been shown to induce granulosa cell apoptosis, possibly via downregulating Akt phosphorylation and XIAP expression (Kim *et al.*, 2013). These findings extend the role of chemerin as a paracrine/autocrine regulator of ovarian function.

7.3 Prohibitin as a regulator of granulosa cell fate

While PHB is functionally associated with multiple cellular processes such as cell-cycle regulation, proliferation, apoptosis and differentiation (Jupe *et al.*, 1995; McClung *et al.*, 1995; Roskams *et al.*, 1993), studies on its functions in the ovary are limited. PHB is known to inhibit apoptosis in undifferentiated granulosa cells by reducing cytochrome c release and caspase-3 activation (Chowdhury *et al.*, 2011; Chowdhury *et al.*, 2007). By comparing undifferentiated and differentiated granulosa cells, we have demonstrated the follicular stage-dependent role of PHB in the regulation of granulosa cell apoptosis. Our findings also indicate that PHB is a potential mediator of the chemerin action and suppresses FSH-induced steroidogenesis, which is attenuated by constitutively active Akt. These observations raise an interesting possibility that PHB may play an important role in determining the fate of the granulosa cells as the follicles transition from the preantral to early antral stage. However, the mechanism by which the function of PHB is switched between proliferation, apoptosis and differentiation is unclear.

Besides its direct role in caspase-3 activation and cytochrome c release from mitochondria in response to apoptotic stimulus (Chowdhury *et al.*, 2007), PHB also serves as a co-activator or co-repressor of various transcriptional factors, such as p53 and E2F1, and modulates the apoptotic gene expression in cancer cells (Choi *et al.*, 2008; Fusaro *et al.*, 2003; He *et al.*, 2008). The evidence that PHB binds to NR5a1/2 suggests a possible mechanism by which PHB regulates FSH-induced steroidogenesis in granulosa cells. Additionally, two negative co-regulators Dax-1 and SHP have been shown to suppress the transcriptional activity of NR5a members (Fayard *et al.*, 2004; Lee and

Moore, 2002; Saxena *et al.*, 2007). It is possible that PHB may also interact with these regulators and interfere with the transcriptions of key steroidogenic enzymes. Further experiments, using IP assay and site mutagenesis, will be useful to test this possibility.

It is well known that PI3K/Akt pathway is activated by gonadotropins and growth factors and plays a critical role in the regulation of proliferation, apoptosis and differentiation (Alam *et al.*, 2004; Hu *et al.*, 2004; Johnson *et al.*, 2001; Zeleznik *et al.*, 2003). Although phosphorylation of PHB by Akt (at Thr) and insulin receptor (at Tyr) has been reported in adipocytes and pancreatic cells (Ande *et al.*, 2009a; Han *et al.*, 2008), PI3K/Akt inhibitor does not block the phosphorylation of PHB in granulosa cells (Chowdhury *et al.*, 2012), suggesting that PHB is not a substrate for PI3K/Akt and its phosphorylation may be mediated by other kinases. In the present study, we observed that PHB and Akt regulate each other. Overexpression of A-Akt reduced PHB content and knockdown of PHB increased the level of phospho-Akt (Ser^{473}) in granulosa cells. The latter observation is in agreement with the findings that exogenous PHB decreases insulin-induced phospho-Akt (Ser^{473} and Thr^{308}) levels in adipocytes and MCF-7 cells, while overexpression of mutant Tyr^{114}Phe of PHB abolishes the suppression (Ande *et al.*, 2009a; Ande and Mishra, 2009). As Akt is a major signaling molecule involved in many cellular processes, the negative regulation ofAkt phosphorylation by PHB may contribute to the multiple functions of PHB. In addition, a positive regulatory loop between PHB and MEK-1 has been suggested since PHB is required for MEK1 activity, while serving as a potential target of MEK1 (Chowdhury *et al.*, 2012). These regulatory loops, whether positive or negative, may represent a common mechanism for the precise control of PHB in the regulation of multiple cellular processes.

7.4 DHT-treated rats as an effective model for the study of polycystic ovarian syndrome

A range of animal models has been developed using various inducers to investigate the cellular and molecular mechanisms in the etiology of PCOS (Abbott *et al.*, 1998; Beloosesky *et al.*, 2004; Brawer *et al.*, 1986; Dumesic *et al.*, 2005; Kafali *et al.*, 2004; Lee *et al.*, 1998; Manneras *et al.*, 2007; Quandt and Hutz, 1993). However, none of the current models could ideally reproduce all the phenotypes of PCOS. The recently established DHT-treated PCOS model displays many ovarian and metabolic features of PCOS (Manneras *et al.*, 2007). Low frequency physical exercise of these rats enhances insulin sensitivity, modulates adipose tissue gene expression and improves the metabolic disturbances (Manneras *et al.*, 2008), indicating that the first choice of weight loss in the treatment of human PCOS also applies to that of DHT-treated rats.

In the present study, DHT-induced rat PCOS model was used in our studies and it exhibited increased body weight gain, disrupted estrus cyclicity and insulin resistance (Hossain *et al.*, 2013), which were consistent with an earlier report (Manneras *et al.*, 2007). With regard to the steroidogenic capability of PCOS ovaries, we have demonstrated a reduced expression of steroidogenic enzymes and increased responsiveness of granulosa cells to FSH in DHT-treated rats. In addition, reduced ovarian sizes in DHT-treated rats were normalized upon gonadotropin administration *in vivo (Hossain et al.,* 2013), implying that the follicle growth arrest in PCOS is gonadotropin-responsive. As shown in **Table 7.1**, many features are in good agreement with the clinical phenotypes in PCOS subjects, with a few exceptions.

Table 7.1 Comparison of DHT-induced model with human PCOS

	PCOS vs. healthy women	DHT vs. control animals
Body weight	↑	↑
Obesity	↑	↑
Insulin sensitivity	↑	↑
Ovarian size	↑	↓
Irregular estrus cycle	Yes	Yes
Arrested follicle growth	Yes	Yes
Fasting Insulin conc.	↑	↑
Leptin level	↑	↑
Adiponectin level	↓	↓
Hormones (serum/plasma)	P4↓; E2—; T↑; DHT↑	P4↓; E2—; T—; DHT↑/—
Chemerin level	↑ (serum)	↑ (serum/ovarian)
Responsiveness to FSH	↑	↑
Steroidogenic enzymes (GC)	Arom↓; p450scc↑; 3β-HSD↓; StAR—	Arom↓; scc↓; 3β-HSD↓; StAR↓
FSHR expression	Expression↑; binding activity↑	Expression —

(Jakimiuk *et al*., 2001; Mannerås *et al*., 2007; Keller *et al*.,2011; van Houten, *et al*. 2012; Wang *et al*, 2012; Jakimiuk *et al*., 1998; Doldi *et al*., 2000; Almahbobi *et al*., 1996; Tan et al., 2009; Catteau-Jonard *et al*., 2008; Walters *et al*., 2012; Kim *et al*., unpublished data; Hossain *et al*., unpublished data)

The reduction of ovarian size in DHT-treated rats is a commonly observed phenomenon in androgenized and estrogenized rodent models, which differ from the human phenotype. Investigation of ovarian morphology in these rats demonstrated lack of follicular fluid, loss of granulosa cells and shrinkage of antral follicles, which are associated with the reduction of cytoskeleton proteins (Kim *et al.*, 2013). These may account for the smaller ovarian size of DHT-treated rats.

Plasma testosterone and DHT levels in DHT-treated rats are similar to those in controls. One possible explanation of this is that the exogenous administration of DHT might have reduced endogenous androgen production, as demonstrated by Cooper et al. that androgen injections in men reduce endogenous androgen production (Cooper *et al.*, 1998). Another possibility is that the excess androgens could have been sequestered in the fat pads (Azziz, 1989; Feher and Bodrogi, 1982), as these DHT-treated rats are obese.

Despite these inconsistencies, our findings provide additional information that extends the work of Manneras *et al*, especially the reproductive phenotype, indicating that DHT-treated rat model is a useful model for PCOS. The application of this model will facilitate future investigations into the molecular and cellular basis of PCOS, exploiting gene manipulation approaches *in vitro* and *in vivo*.

7.5 Chemerin and prohibitin as potential contributors of polycystic ovarian syndrome

Since human PCOS normally presents with arrested follicle growth, dysregulated steroidogenesis and insulin resistance, our studies of the roles of chemerin and PHB in the ovary have provided evidence linking these molecules to PCOS. The present study

has demonstrated that the elevated serum chemerin level and increased ovarian expression of chemerin, CMKLR1 and PHB in DHT-treated rats are associated with the reduced steroidogenic enzyme expression, suggesting their potential involvement in the dysregulation of steroidogenesis in PCOS. This is supported by our *in vitro* data that both chemerin and PHB suppress FSH-induced steroid production and steroidogenic enzyme expression (Wang *et al.*, 2012; Wang *et al.*, 2013).

Recent reports indicate that the cystic follicles display a weak proliferation rate and low apoptosis frequency in rat, bovine and porcine (Isobe and Yoshimura, 2007; Salvetti *et al.*, 2009; Sun *et al.*, 2012), which maintains a static condition without degeneration and results in the slow or arrested follicular growth. It is known that PHB is an anti-proliferation and anti-apoptotic factor in various cell types (including our data) (Chowdhury *et al.*, 2011; Jupe *et al.*, 1996; McClung *et al.*, 1989) and chemerin inhibits basal preantral follicle growth *in vitro* (Kim *et al.*, 2013). These data, together with the increased ovarian expression of chemerin and PHB in DHT-treated rats, suggest a possible contribution of the two molecules in the formation of follicle cysts, although this notion requires confirmation.

Chemerin and PHB are involved in the regulation of adipogenesis and glucose metabolism. They promote adipocyte differentiation (Goralski *et al.*, 2007; Liu *et al.*, 2012a) and inhibit insulin-stimulated glucose uptake and/or Akt phosphorylation in skeletal muscle cells and adipocytes (Ande and Mishra, 2009; Becker *et al.*, 2010; Kralisch *et al.*, 2009; Sell *et al.*, 2009). As chemerin level increases in DHT-treated rats and in PCOS subjects, it is possible that it may contribute to the metabolic phenotypes such as obesity and insulin resistance. In addition, we cannot rule out the potential

involvement of other adipokines, such as TNF-α, IL-6 and adiponectin, which are known to regulate insulin sensitivity (Hillenbrand *et al.*, 2012; Pedersen and Febbraio, 2007).

7.6 Future directions

Manipulation of chemerin and PHB genes in vivo

We have examined the reproductive phenotypes of DHT-treated rats and demonstrated the dysregulation of steroidogenic features in ovarian cells. Increased ovarian levels of chemerin and PHB in DHT-treated rats indicate their potential involvement in the etiology and pathogenesis of PCOS. To further investigate this possibility, gene manipulation in follicles collected from DHT-treated rats with adenoviral-shRNA targeting chemerin or PHB could be an effective strategy using our *in vitro* follicle culture system. The steroidogenic capability of these follicles after knockdown of chemerin or PHB could be assessed. The improvement of follicle steroid-genesis after gene knockdown would support the notion that chemerin and/or PHB contribute to the dysregulation of follicle differentiation in DHT-treated rats.

Moreover, the ovary-specific conditional knockout system has been used to investigate the functions of various genes involved in follicle growth and steroidogenesis, such as NR5a1 and smad4 (Jeyasuria *et al.*, 2004; Pangas *et al.*, 2006). The depletion of chemerin and/or PHB gene by conditional knockout in the ovary could be performed to explore the phenotypes of these animals and their ovarian steroidogenic capability. These studies will facilitate the understanding of the roles of chemerin and PHB in the ovary.

Clinical studies

While the DHT-treated rat model provides a mechanistic insight into the patho-physiology of PCOS, these findings need to be validated in human. This study should be extended to clinical investigation in order to examine the association of chemerin with PCOS and/or obesity. As chemerin in the circulation may come from different sources, including adipose tissue, liver, ovary etc., analysis of chemerin levels in follicular fluid may be more reflective of the dysregulated growth and function at the follicle level in PCOS. A recent study has reported the presence of chemerin in human follicular fluid (Reverchon *et al.*, 2012). The relationship between chemerin and the reproductive and metabolic parameters related to PCOS and obesity (such as sex hormone levels, sex hormone binding globulin, and free androgen index) could be analyzed. These studies would validate the mechanistic findings carried out in the rodent PCOS model and provide important physiologic and pathophysiologic insights into PCOS.

As insulin resistance is a common feature in PCOS women, treatment with insulin-sensitizing agents improves the manifestation of hyperandrogenemia and restores ovulation (Dunaif, 1997; Katsiki and Hatzitolios, 2010). As the serum chemerin levels decrease in metformin-treated PCOS subjects (Tan *et al.*, 2009), it will be of interest to investigate the relationship between chemerin and ovarian dysfunction in PCOS following metformin treatment. Follicular fluid levels of chemerin and steroid hormone as well as follicle growth could be examined in PCOS subjects before and after metformin treatment to shed light on these relationships. Granulosa cells could be analysed for steroidogenic enzyme expression. If metformin treatment decreases the chemerin levels in follicular fluid and restores the steroidogenic capability of granulosa

cells, it will offer additional insights in the involvement of chemerin in the etiology of PCOS.

7.7 Conclusions

This thesis demonstrates two novel regulators in follicular development and steroidogenesis and provides significant insights into the cellular and molecular mechanisms involved in the regulation of folliculogenesis and the development of PCOS. Specifically, we have demonstrated that chemerin and PHB negatively regulate FSH-induced steroid production and steroidogenic enzyme expression. The inhibitory role of chemerin is via decreasing NR5a1/2-mediated transcription. PHB is a potential intracellular mediator of chemerin action and plays an important role in granulosa cell apoptosis and steroidogenesis. Using a rodent PCOS model, we present the evidence for the potential involvement of chemerin and PHB in PCOS.

Taken together, these findings improve our understanding of granulosa cell fate regulation and ovarian follicle growth, and provide important clues for the investigation of etiology of PCOS. A better understanding of the ovarian function and PCOS would ultimately facilitate the development of new treatment strategies for this complex syndrome.

CHAPTER 8: REFERENCES

8.1 General References

Abbott D. H., Dumesic D. A., Eisner J. R., Colman R. J., Kemnitz J.W. (1998). Insights into the development of polycystic ovary syndrome (PCOS) from studies in prenatally androgenised female rhesus monkeys. *Trends Endocrinol Metab* **9:** 62-67.

Abel M. H., Wootton A. N., Wilkins V., Huhtaniemi I., Knight P. G., Charlton H. M. (2000). The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* **141:** 1795-803.

Adashi E. Y. (1998). The potential role of interleukin-1 in the ovulatory process: an evolving hypothesis. *Mol Cell Endocrinol* **140:** 77-81.

Adashi E. Y., Resnick C. E., D'Ercole A. J., Svoboda M. E., Van Wyk J. J. (1985). Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocr Rev* **6:** 400-20.

Adashi E. Y., Resnick C. E., Hernandez E. R., May J. V., Knecht M., Svoboda M. E., Van Wyk J. J. (1988). Insulin-like growth factor-I as an amplifier of follicle-stimulating hormone action: studies on mechanism(s) and site(s) of action in cultured rat granulosa cells. *Endocrinology* **122:** 1583-91.

Alam H., Maizels E. T., Park Y., Ghaey S., Feiger Z. J., Chandel N. S., Hunzicker-Dunn M. (2004). Follicle-stimulating hormone activation of hypoxia-inducible factor-1 by the phosphatidylinositol 3-kinase/AKT/Ras homolog enriched in brain (Rheb)/mammalian target of rapamycin (mTOR) pathway is necessary for induction of select protein markers of follicular differentiation. *J Biol Chem* **279:** 19431-40.

Albanesi C., Scarponi C., Pallotta S., Daniele R., Bosisio D., Madonna S., Fortugno P., Gonzalvo-Feo S., Franssen J. D., Parmentier M., De Pita O., Girolomoni G., Sozzani S. (2009). Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* **206:** 249-58.

Almahbobi G., Anderiesz C., Hutchinson P., McFarlane J. R., Wood C., Trounson A. O. (1996). Functional integrity of granulosa cells from polycystic ovaries. *Clin Endocrinol (Oxf)* **44:** 571-80.

Altus M. S., Wood C. M., Stewart D. A., Roskams A. J., Friedman V., Henderson T., Owens G. A., Danner D. B., Jupe E. R., Dell'Orco R. T., et al. (1995). Regions of evolutionary conservation between the rat and human prohibitin-encoding genes. *Gene* **158:** 291-4.

Amsterdam A., Selvaraj N. (1997). Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes. *Endocr Rev* **18:** 435-61.

Ande S. R., Gu Y., Nyomba B. L., Mishra S. (2009a). Insulin induced phosphorylation of prohibitin at tyrosine 114 recruits Shp1. *Biochim Biophys Acta* **1793:** 1372-8.

Ande S. R., Mishra S. (2009). Prohibitin interacts with phosphatidylinositol 3,4,5-triphosphate (PIP3) and modulates insulin signaling. *Biochem Biophys Res Commun* **390:** 1023-8.

Ande S. R., Moulik S., Mishra S. (2009b). Interaction between O-GlcNAc modification and tyrosine phosphorylation of prohibitin: implication for a novel binary switch. *PLoS One* **4:** e4586.

Ande S. R., Xu Z., Gu Y., Mishra S. (2011). Prohibitin has an important role in adipocyte differentiation. *Int J Obes (Lond)***:** 1-9.

Ande S. R., Xu Z., Gu Y., Mishra S. (2012). Prohibitin has an important role in adipocyte differentiation. *Int J Obes (Lond)* **36:** 1236-1244.

Ascoli M. (1996). Functional consequences of the phosphorylation of the gonadotropin receptors. *Biochem Pharmacol* **52:** 1647-55.

Asuncion M., Calvo R. M., San Millan J. L., Sancho J., Avila S., Escobar-Morreale H. F. (2000). A prospective study of the prevalence of the polycystic ovary syndrome in unselected Caucasian women from Spain. *J Clin Endocrinol Metab* **85:** 2434-8.

Azziz R. (1989). Reproductive endocrinologic alterations in female asymptomatic obesity. *Fertil Steril* **52:** 703-25.

Azziz R., Carmina E., Dewailly D., Diamanti-Kandarakis E., Escobar-Morreale H. F., Futterweit W., Janssen O. E., Legro R. S., Norman R. J., Taylor A. E., Witchel S. F. (2006). Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. *J Clin Endocrinol Metab* **91:** 4237-45.

Badawy A., Elnashar A. (2011). Treatment options for polycystic ovary syndrome. *Int J Womens Health* **3:** 25-35.

Baker J., Hardy M. P., Zhou J., Bondy C., Lupu F., Bellve A. R., Efstratiadis A. (1996). Effects of an Igf1 gene null mutation on mouse reproduction. *Mol Endocrinol* **10:** 903-18.

Balen A. H., Conway G. S., Kaltsas G., Techatrasak K., Manning P. J., West C., Jacobs H. S. (1995). Polycystic ovary syndrome: the spectrum of the disorder in 1741 patients. *Hum Reprod* **10:** 2107-11.

Barr P. J., Tomei L. D. (1994). Apoptosis and its role in human disease. *Biotechnology (N Y)* **12:** 487-93.

Baud V., Karin M. (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* **11:** 372-7.

Beck S., Hanson I., Kelly A., Pappin D. J., Trowsdale J. (1992). A homologue of the Drosophila female sterile homeotic (fsh) gene in the class II region of the human MHC. *DNA Seq* **2:** 203-10.

Becker-Andre M., Andre E., DeLamarter J. F. (1993). Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences. *Biochem Biophys Res Commun* **194:** 1371-9.

Becker M., Rabe K., Lebherz C., Zugwurst J., Goke B., Parhofer K. G., Lehrke M., Broedl U. C. (2010). Expression of human chemerin induces insulin resistance in the skeletal muscle but does not affect weight, lipid levels, and atherosclerosis in LDL receptor knockout mice on high-fat diet. *Diabetes* **59:** 2898-903.

Beloosesky R., Gold R., Almog B., Sasson R., Dantes A., Land-Bracha A., Hirsh L., Itskovitz-Eldor J., Lessing J. B., Homburg R., Amsterdam A. (2004). Induction of polycystic ovary by testosterone in immature female rats: Modulation of apoptosis and attenuation of glucose/insulin ratio. *Int J Mol Med* **14:** 207-15.

Berg V., Sveinbjornsson B., Bendiksen S., Brox J., Meknas K., Figenschau Y. (2010). Human articular chondrocytes express ChemR23 and chemerin; ChemR23 promotes inflammatory signalling upon binding the ligand chemerin(21-157). *Arthritis Res Ther* **12:** R228.

Bergh C., Carlsson B., Olsson J. H., Selleskog U., Hillensjo T. (1993). Regulation of androgen production in cultured human thecal cells by insulin-like growth factor I and insulin. *Fertil Steril* **59:** 323-31.

Billig H., Furuta I., Hsueh A. J. (1993). Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology* **133:** 2204-12.

Boerboom D., Pilon N., Behdjani R., Silversides D. W., Sirois J. (2000). Expression and regulation of transcripts encoding two members of the NR5A nuclear receptor subfamily of orphan nuclear receptors, steroidogenic factor-1 and NR5A2, in equine ovarian cells during the ovulatory process. *Endocrinology* **141:** 4647-56.

Bogerd J. (2007). Ligand-selective determinants in gonadotropin receptors. *Mol Cell Endocrinol* **260-262:** 144-52.

Bogovich K., Richards J. S. (1982). Androgen biosynthesis in developing ovarian follicles: evidence that luteinizing hormone regulates thecal 17 alpha-hydroxylase and C17-20-lyase activities. *Endocrinology* **111:** 1201-8.

Bozaoglu K., Bolton K., McMillan J., Zimmet P., Jowett J., Collier G., Walder K., Segal D. (2007). Chemerin is a novel adipokine associated with obesity and metabolic syndrome. *Endocrinology* **148:** 4687-94.

Bozaoglu K., Curran J. E., Stocker C. J., Zaibi M. S., Segal D., Konstantopoulos N., Morrison S., Carless M., Dyer T. D., Cole S. A., Goring H. H., Moses E. K., Walder K., Cawthorne M. A., Blangero J., Jowett J. B. (2010). Chemerin, a novel adipokine in the regulation of angiogenesis. *J Clin Endocrinol Metab* **95:** 2476-85.

Bozaoglu K., Segal D., Shields K. A., Cummings N., Curran J. E., Comuzzie A. G., Mahaney M. C., Rainwater D. L., VandeBerg J. L., MacCluer J. W., Collier G., Blangero J., Walder K., Jowett J. B. (2009). Chemerin is associated with metabolic syndrome phenotypes in a Mexican-American population. *J Clin Endocrinol Metab* **94:** 3085-8.

Brassard M., AinMelk Y., Baillargeon J. P. (2008). Basic infertility including polycystic ovary syndrome. *Med Clin North Am* **92:** 1163-92, xi.

Brawer J., Munoz M., Farookhi R. (1986). Development of the polycystic ovarian condition (PCO) in the estradiol valerate-treated rat. *Biol Reprod* **35:** 647-55.

Bremer A.A., Miller W. L. (2008). The serine phosphorylation hypothesis of polycystic ovary syndrome: a unifying mechanism for hyperandrogenemia and insulin resistance. *Fertil Steril* **89:** 1039-48.

Britt K. L., Drummond A. E., Cox V. A., Dyson M., Wreford N. G., Jones M. E., Simpson E. R., Findlay J. K. (2000). An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene. *Endocrinology* **141:** 2614-23.

Browman D. T., Hoegg M. B., Robbins S. M. (2007). The SPFH domain-containing proteins: more than lipid raft markers. *Trends Cell Biol* **17:** 394-402.

Campbell B. K., Baird D. T. (2001). Inhibin A is a follicle stimulating hormone-responsive marker of granulosa cell differentiation, which has both autocrine and paracrine actions in sheep. *J Endocrinol* **169:** 333-45.

Caron K. M., Ikeda Y., Soo S. C., Stocco D. M., Parker K. L., Clark B. J. (1997). Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. *Mol Endocrinol* **11:** 138-47.

Cash J. L., Christian A. R., Greaves D. R. (2010). Chemerin peptides promote phagocytosis in a ChemR23- and Syk-dependent manner. *J Immunol* **184:** 5315-24.

Cash J. L., Hart R., Russ A., Dixon J. P., Colledge W. H., Doran J., Hendrick A. G., Carlton M. B., Greaves D. R. (2008). Synthetic chemerin-derived peptides suppress inflammation through ChemR23. *J Exp Med* **205:** 767-75.

Catteau-Jonard S., Jamin S. P., Leclerc A., Gonzales J., Dewailly D., di Clemente N. (2008). Anti-Mullerian hormone, its receptor, FSH receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **93:** 4456-61.

Chabrolle C., Tosca L., Rame C., Lecomte P., Royere D., Dupont J. (2009). Adiponectin increases insulin-like growth factor I-induced progesterone and estradiol secretion in human granulosa cells. *Fertil Steril* **92:** 1988-96.

Chen Q., Yano T., Matsumi H., Osuga Y., Yano N., Xu J., Wada O., Koga K., Fujiwara T., Kugu K., Taketani Y. (2005). Cross-Talk between Fas/Fas ligand system and nitric oxide in the pathway subserving granulosa cell apoptosis: a possible regulatory mechanism for ovarian follicle atresia. *Endocrinology* **146:** 808-15.

Chen W. Y., Lee W. C., Hsu N. C., Huang F., Chung B. C. (2004). SUMO modification of repression domains modulates function of nuclear receptor 5A1 (steroidogenic factor-1). *J Biol Chem* **279:** 38730-5.

Chen W. Y., Weng J. H., Huang C. C., Chung B. C. (2007). Histone deacetylase inhibitors reduce steroidogenesis through SCF-mediated ubiquitination and degradation of steroidogenic factor 1 (NR5A1). *Mol Cell Biol* **27:** 7284-90.

Chittenden B. G., Fullerton G., Maheshwari A., Bhattacharya S. (2009). Polycystic ovary syndrome and the risk of gynaecological cancer: a systematic review. *Reprod Biomed Online* **19:** 398-405.

Chiu C. F., Ho M. Y., Peng J. M., Hung S. W., Lee W. H., Liang C. M., Liang S. M. (2012). Raf activation by Ras and promotion of cellular metastasis require phosphorylation of prohibitin in the raft domain of the plasma membrane. *Oncogene*.

Choi D., Lee S. J., Hong S., Kim I. H., Kang S. (2008). Prohibitin interacts with RNF2 and regulates E2F1 function via dual pathways. *Oncogene* **27:** 1716-25.

Chowdhury I., Branch A., Olatinwo M., Thomas K., Matthews R., Thompson W. E. (2011). Prohibitin (PHB) acts as a potent survival factor against ceramide induced apoptosis in rat granulosa cells. *Life Sci* **89:** 295-303.

Chowdhury I., Garcia-Barrio M., Harp D., Thomas K., Matthews R., Thompson W. E. (2012). The emerging roles of prohibitins in folliculogenesis. *Front Biosci (Elite Ed)* **4:** 690-9.

Chowdhury I., Xu W., Stiles J. K., Zeleznik A., Yao X., Matthews R., Thomas K., Thompson W. E. (2007). Apoptosis of rat granulosa cells after staurosporine and serum withdrawal is suppressed by adenovirus-directed overexpression of prohibitin. *Endocrinology* **148:** 206-17.

Clyne C. D., Speed C. J., Zhou J., Simpson E. R. (2002). Liver receptor homologue-1 (LRH-1) regulates expression of aromatase in preadipocytes. *J Biol Chem* **277:** 20591-7.

Coffler M. S., Patel K., Dahan M. H., Malcom P. J., Kawashima T., Deutsch R., Chang R. J. (2003). Evidence for abnormal granulosa cell responsiveness to follicle-stimulating hormone in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **88:** 1742-7.

Cooke B. A. (1999). Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. *Mol Cell Endocrinol* **151:** 25-35.

Cooper C. S., Perry P. J., Sparks A. E., MacIndoe J. H., Yates W. R., Williams R. D. (1998). Effect of exogenous testosterone on prostate volume, serum and semen prostate specific antigen levels in healthy young men. *J Urol* **159:** 441-3.

Danial N. N., Korsmeyer S. J. (2004). Cell death: critical control points. *Cell* **116:** 205-19.

Deng Y., Wang H., Lu Y., Liu S., Zhang Q., Huang J., Zhu R., Yang J., Zhang R., Zhang D., Shen W., Ning G., Yang Y. (2013). Identification of Chemerin as a Novel FXR Target Gene Down-Regulated in the Progression of Nonalcoholic Steatohepatitis. *Endocrinology* **154:** 1794-801.

Diamanti-Kandarakis E., Kouli C. R., Bergiele A. T., Filandra F. A., Tsianateli T. C., Spina G. G., Zapanti E. D., Bartzis M. I. (1999). A survey of the polycystic ovary syndrome in the Greek island of Lesbos: hormonal and metabolic profile. *J Clin Endocrinol Metab* **84:** 4006-11.

Dierich A., Sairam M. R., Monaco L., Fimia G. M., Gansmuller A., LeMeur M., Sassone-Corsi P. (1998). Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci U S A* **95:** 13612-7.

Dive C., Gregory C. D., Phipps D. J., Evans D. L., Milner A. E., Wyllie A. H. (1992). Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta* **1133:** 275-85.

Doldi N., Grossi D., Destefani A., Gessi A., Ferrari A. (2000). Polycystic ovary syndrome: evidence for reduced 3 beta-hydroxysteroid dehydrogenase gene expression in human luteinizing granulosa cells. *Gynecol Endocrinol* **14:** 32-7.

Dong J., Albertini D. F., Nishimori K., Kumar T. R., Lu N., Matzuk M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383:** 531-5.

Du X. Y., Leung L. L. (2009). Proteolytic regulatory mechanism of chemerin bioactivity. *Acta Biochim Biophys Sin (Shanghai)* **41:** 973-9.

Duffy D. M., Molskness T. A., Stouffer R. L. (1996). Progesterone receptor messenger ribonucleic acid and protein in luteinized granulosa cells of rhesus monkeys are regulated in vitro by gonadotropins and steroids. *Biol Reprod* **54:** 888-95.

Duleba A. J., Spaczynski R. Z., Olive D. L., Behrman H. R. (1997). Effects of insulin and insulin-like growth factors on proliferation of rat ovarian theca-interstitial cells. *Biol Reprod* **56:** 891-7.

Dumesic D. A., Schramm R. D., Abbott D. H. (2005). Early origins of polycystic ovary syndrome. *Reprod Fertil Dev* **17:** 349-60.

Dunaif A. (1997). Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. *Endocr Res* **18:** 774-800.

Eimerl S., Orly J. (2002). Regulation of steroidogenic genes by insulin-like growth factor-1 and follicle-stimulating hormone: differential responses of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, and 3beta-hydroxysteroid dehydrogenase/isomerase in rat granulosa cells. *Biol Reprod* **67:** 900-10.

Elvin J. A., Yan C., Wang P., Nishimori K., Matzuk M. M. (1999). Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. *Mol Endocrinol* **13:** 1018-34.

Erickson G. F. (1978). Normal ovarian function. *Clin Obstet Gynecol* **21:** 31-52.

Ernst M. C., Issa M., Goralski K. B., Sinal C. J. (2010). Chemerin exacerbates glucose intolerance in mouse models of obesity and diabetes. *Endocrinology* **151:** 1998-2007.

Ernst M. C., Sinal C. J. (2010). Chemerin: at the crossroads of inflammation and obesity. *Trends Endocrinol Metab* **21:** 660-7.

Evans A. C., Ireland J. L., Winn M. E., Lonergan P., Smith G. W., Coussens P. M., Ireland J. J. (2004). Identification of genes involved in apoptosis and dominant follicle development during follicular waves in cattle. *Biol Reprod* **70:** 1475-84.

Falender A. E., Lanz R., Malenfant D., Belanger L., Richards J. S. (2003). Differential expression of steroidogenic factor-1 and FTF/LRH-1 in the rodent ovary. *Endocrinology* **144:** 3598-610.

Fauser B. C. (1996). Interference with follicle stimulating hormone regulation of human ovarian function. *Mol Hum Reprod* **2:** 327-34.

Fayard E., Auwerx J., Schoonjans K. (2004). LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. *Trends Cell Biol* **14:** 250-60.

Feher T., Bodrogi L. (1982). A comparative study of steroid concentrations in human adipose tissue and the peripheral circulation. *Clin Chim Acta* **126:** 135-41.

Fels E., Bosch L. R. (1971). Effect of prenatal administration of testosterone on ovarian function in rats. *Am J Obstet Gynecol* **111:** 964-9.

Fortune J. E., Armstrong D. T. (1977). Androgen production by theca and granulosa isolated from proestrous rat follicles. *Endocrinology* **100:** 1341-7.

Fusaro G., Dasgupta P., Rastogi S., Joshi B., Chellappan S. (2003). Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. *J Biol Chem* **278:** 47853-61.

Glister C., Tannetta D. S., Groome N. P., Knight P. G. (2001). Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells. *Biol Reprod* **65:** 1020-8.

Gonzalez-Robayna I. J., Alliston T. N., Buse P., Firestone G. L., Richards J. S. (1999). Functional and subcellular changes in the A-kinase-signaling pathway: relation to aromatase and Sgk expression during the transition of granulosa cells to luteal cells. *Mol Endocrinol* **13:** 1318-37.

Goodwin B., Jones S. A., Price R. R., Watson M. A., McKee D. D., Moore L. B., Galardi C., Wilson J. G., Lewis M. C., Roth M. E., Maloney P. R., Willson T. M., Kliewer S. A. (2000).

A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol Cell* **6:** 517-26.

Goralski K. B., McCarthy T. C., Hanniman E. A., Zabel B. A., Butcher E. C., Parlee S. D., Muruganandan S., Sinal C. J. (2007). Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J Biol Chem* **282:** 28175-88.

Goto M., Iwase A., Harata T., Takigawa S., Suzuki K., Manabe S., Kikkawa F. (2009). IGF1-induced AKT phosphorylation and cell proliferation are suppressed with the increase in PTEN during luteinization in human granulosa cells. *Reproduction* **137:** 835-42.

Gougeon A. (2004). Dynamics of human follicular growth: Morphologic, dynamic, and functional aspects. A chapter in The Ovary, second edition, by Peter Leung.**:** 21-43.

Guillabert A., Wittamer V., Bondue B., Godot V., Imbault V., Parmentier M., Communi D. (2008). Role of neutrophil proteinase 3 and mast cell chymase in chemerin proteolytic regulation. *J Leukoc Biol* **84:** 1530-8.

Guzick D. S. (2004). Polycystic ovary syndrome. *Obstet Gynecol* **103:** 181-93.

Hakuno N., Koji T., Yano T., Kobayashi N., Tsutsumi O., Taketani Y., Nakane P. K. (1996). Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. *Endocrinology* **137:** 1938-48.

Hammer G. D., Krylova I., Zhang Y., Darimont B. D., Simpson K., Weigel N. L., Ingraham H. A. (1999). Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. *Mol Cell* **3:** 521-6.

Han E. K., McGonigal T., Butler C., Giranda V. L., Luo Y. (2008). Characterization of Akt overexpression in MiaPaCa-2 cells: prohibitin is an Akt substrate both in vitro and in cells. *Anticancer Res* **28:** 957-63.

Hart R., Greaves D. R. (2010). Chemerin contributes to inflammation by promoting macrophage adhesion to VCAM-1 and fibronectin through clustering of VLA-4 and VLA-5. *J Immunol* **185:** 3728-39.

Hayashi M., McGee E. A., Min G., Klein C., Rose U. M., van Duin M., Hsueh A. J. (1999). Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology* **140:** 1236-44.

He B., Feng Q., Mukherjee A., Lonard D. M., DeMayo F. J., Katzenellenbogen B. S., Lydon J. P., O'Malley B. W. (2008). A repressive role for prohibitin in estrogen signaling. *Mol Endocrinol* **22:** 344-60.

Hillenbrand A., Weiss M., Knippschild U., Wolf A. M., Huber-Lang M. (2012). Sepsis-Induced Adipokine Change with regard to Insulin Resistance. *Int J Inflam* **2012:** 972368.

Hillier S. G. (2001). Gonadotropic control of ovarian follicular growth and development. *Mol Cell Endocrinol* **179:** 39-46.

Hillier S. G., Whitelaw P. F., Smyth C. D. (1994). Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol Cell Endocrinol* **100:** 51-4.

Hinshelwood M. M., Repa J. J., Shelton J. M., Richardson J. A., Mangelsdorf D. J., Mendelson C. R. (2003). Expression of LRH-1 and SF-1 in the mouse ovary: localization in different cell types correlates with differing function. *Mol Cell Endocrinol* **207:** 39-45.

Hoivik E. A., Lewis A. E., Aumo L., Bakke M. (2010). Molecular aspects of steroidogenic factor 1 (SF-1). *Mol Cell Endocrinol* **315:** 27-39.

Hossain M.M., Cao M., Wang Q., Kim J. Y., Schellander K., Tesfaye D., Tsang B. K. (2013). Altered expression of miRNAs in a dihydrotestosterone-induced rat PCOS model. *J Ovarian Res.***:** Under Revision.

Hsu C. J., Hammond J. M. (1987). Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells in vitro. *Endocrinology* **120:** 198-207.

Hsu S. Y., Lai R. J., Finegold M., Hsueh A. J. (1996). Targeted overexpression of Bcl-2 in ovaries of transgenic mice leads to decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis. *Endocrinology* **137:** 4837-43.

Hsueh A. J., McGee E. A., Hayashi M., Hsu S. Y. (2000). Hormonal regulation of early follicle development in the rat ovary. *Mol Cell Endocrinol* **163:** 95-100.

Hu C. L., Cowan R. G., Harman R. M., Quirk S. M. (2004). Cell cycle progression and activation of Akt kinase are required for insulin-like growth factor I-mediated suppression of apoptosis in granulosa cells. *Mol Endocrinol* **18:** 326-38.

Hu M. C., Hsu N. C., Pai C. I., Wang C. K., Chung Bc (2001). Functions of the upstream and proximal steroidogenic factor 1 (SF-1)-binding sites in the CYP11A1 promoter in basal transcription and hormonal response. *Mol Endocrinol* **15:** 812-8.

Hu W., Feng P. (2011). Elevated serum chemerin concentrations are associated with renal dysfunction in type 2 diabetic patients. *Diabetes Res Clin Pract* **91:** 159-163.

Huang J., Zhang J., Lei T., Chen X., Zhang Y., Zhou L., Yu A., Chen Z., Zhou R., Yang Z. (2010). Cloning of porcine chemerin, ChemR23 and GPR1 and their involvement in regulation of lipogenesis. *BMB Rep* **43:** 491-8.

Huffman L., Hendricks S. E. (1981). Prenatally injected testosterone propionate and sexual behavior of female rats. *Physiol Behav* **26:** 773-8.

Hunzicker-Dunn M., Maizels E. T. (2006). FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. *Cell Signal* **18:** 1351-9.

Hussein M. R. (2005). Apoptosis in the ovary: molecular mechanisms. *Hum Reprod Update* **11:** 162-77.

Isobe N., Yoshimura Y. (2007). Deficient proliferation and apoptosis in the granulosa and theca interna cells of the bovine cystic follicle. *J Reprod Dev* **53:** 1119-24.

Ito M., Park Y., Weck J., Mayo K. E., Jameson J. L. (2000). Synergistic activation of the inhibin alpha-promoter by steroidogenic factor-1 and cyclic adenosine 3',5'-monophosphate. *Mol Endocrinol* **14:** 66-81.

Jacob A. L., Lund J., Martinez P., Hedin L. (2001). Acetylation of steroidogenic factor 1 protein regulates its transcriptional activity and recruits the coactivator GCN5. *J Biol Chem* **276:** 37659-64.

Jakimiuk A. J., Weitsman S. R., Brzechffa P. R., Magoffin D. A. (1998). Aromatase mRNA expression in individual follicles from polycystic ovaries. *Mol Hum Reprod* **4:** 1-8.

Jakimiuk A. J., Weitsman S. R., Navab A., Magoffin D. A. (2001). Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. *J Clin Endocrinol Metab* **86:** 1318-23.

Jamnongjit M., Hammes S. R. (2006). Ovarian steroids: the good, the bad, and the signals that raise them. *Cell Cycle* **5:** 1178-83.

Jeyasuria P., Ikeda Y., Jamin S. P., Zhao L., De Rooij D. G., Themmen A. P., Behringer R. R., Parker K. L. (2004). Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Mol Endocrinol* **18:** 1610-9.

Jiang J. Y., Cheung C. K., Wang Y., Tsang B. K. (2003). Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. *Front Biosci* **8:** d222-37.

Jin Z., El-Deiry W. S. (2005). Overview of cell death signaling pathways. *Cancer Biol Ther* **4:** 139-63.

Johnson A. L., Bridgham J. T., Swenson J. A. (2001). Activation of the Akt/protein kinase B signaling pathway is associated with granulosa cell survival. *Biol Reprod* **64:** 1566-74.

Joshi B., Rastogi S., Morris M., Carastro L. M., DeCook C., Seto E., Chellappan S. P. (2007). Differential regulation of human YY1 and caspase 7 promoters by prohibitin through E2F1 and p53 binding sites. *Biochem J* **401:** 155-66.

Jupe E. R., Liu X. T., Kiehlbauch J. L., McClung J. K., Dell'Orco R. T. (1995). Prohibitin antiproliferative activity and lack of heterozygosity in immortalized cell lines. *Exp Cell Res* **218:** 577-80.

Jupe E. R., Liu X. T., Kiehlbauch J. L., McClung J. K., Dell'Orco R. T. (1996). The 3' untranslated region of prohibitin and cellular immortalization. *Exp Cell Res* **224:** 128-35.

Kafali H., Iriadam M., Ozardali I., Demir N. (2004). Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. *Arch Med Res* **35:** 103-8.

Kaipia A., Hsu S. Y., Hsueh A. J. (1997). Expression and function of a proapoptotic Bcl-2 family member Bcl-XL/Bcl-2-associated death promoter (BAD) in rat ovary. *Endocrinology* **138:** 5497-504.

Kaneko K., Miyabe Y., Takayasu A., Fukuda S., Miyabe C., Ebisawa M., Yokoyama W., Watanabe K., Imai T., Muramoto K., Terashima Y., Sugihara T., Matsushima K., Miyasaka N., Nanki T. (2011). Chemerin activates fibroblast-like synoviocytes in patients with rheumatoid arthritis. *Arthritis Res Ther* **13:** R158.

Kang X., Zhang L., Sun J., Ni Z., Ma Y., Chen X., Sheng X., Chen T. (2008). Prohibitin: a potential biomarker for tissue-based detection of gastric cancer. *J Gastroenterol* **43:** 618-25.

Karastergiou K., Mohamed-Ali V. (2010). The autocrine and paracrine roles of adipokines. *Mol Cell Endocrinol* **318:** 69-78.

Kasashima K., Ohta E., Kagawa Y., Endo H. (2006). Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2. *J Biol Chem* **281:** 36401-10.

Kathiria A. S., Neumann W. L., Rhees J., Hotchkiss E., Cheng Y., Genta R. M., Meltzer S. J., Souza R. F., Theiss A. L. (2012). Prohibitin attenuates colitis-associated tumorigenesis in mice by modulating p53 and STAT3 apoptotic responses. *Cancer Res* **72:** 5778-89.

Katsiki N., Hatzitolios A. I. (2010). Insulin-sensitizing agents in the treatment of polycystic ovary syndrome: an update. *Curr Opin Obstet Gynecol* **22:** 466-76.

Kayampilly P. P., Menon K. M. (2007). Follicle-stimulating hormone increases tuberin phosphorylation and mammalian target of rapamycin signaling through an extracellular signal-regulated kinase-dependent pathway in rat granulosa cells. *Endocrinology* **148:** 3950-7.

Kayampilly P. P., Menon K. M. (2009). Follicle-stimulating hormone inhibits adenosine 5'-monophosphate-activated protein kinase activation and promotes cell proliferation of primary granulosa cells in culture through an Akt-dependent pathway. *Endocrinology* **150:** 929-35.

Kerr J. F., Winterford C. M., Harmon B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer* **73:** 2013-26.

Kiddy D. S., Sharp P. S., White D. M., Scanlon M. F., Mason H. D., Bray C. S., Polson D. W., Reed M. J., Franks S. (1990). Differences in clinical and endocrine features between obese and non-obese subjects with polycystic ovary syndrome: an analysis of 263 consecutive cases. *Clin Endocrinol (Oxf)* **32:** 213-20.

Kikuchi N., Andoh K., Abe Y., Yamada K., Mizunuma H., Ibuki Y. (2001). Inhibitory action of leptin on early follicular growth differs in immature and adult female mice. *Biol Reprod* **65:** 66-71.

Kim J. M., Boone D. L., Auyeung A., Tsang B. K. (1998). Granulosa cell apoptosis induced at the penultimate stage of follicular development is associated with increased levels of Fas and Fas ligand in the rat ovary. *Biol Reprod* **58:** 1170-6.

Kim J. M., Yoon Y. D., Tsang B. K. (1999). Involvement of the Fas/Fas ligand system in p53-mediated granulosa cell apoptosis during follicular development and atresia. *Endocrinology* **140:** 2307-17.

Kim J. Y., Xue K., Cao M., Wang Q., Liu J. Y., Leader A., Han J. Y., Tsang B. K. (2013). Chemerin suppresses Ovarian Follicular Development and Its Potential Involvement in Follicular Arrest in a Chronically Androgenized Rat Model for Polycystic Ovarian Syndrome. *Endocrinology***:** Accepted.

Knochenhauer E. S., Key T. J., Kahsar-Miller M., Waggoner W., Boots L. R., Azziz R. (1998). Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study. *J Clin Endocrinol Metab* **83:** 3078-82.

Kolodziejczyk J., Gertler A., Leibovich H., Rzasa J., Gregoraszczuk E. L. (2003). Synergistic action of growth hormone and insulin-like growth factor I (IGF-I) on proliferation and estradiol secretion in porcine granulosa and theca cells cultured alone or in coculture. *Theriogenology* **60:** 559-70.

Kolonin M. G., Saha P. K., Chan L., Pasqualini R., Arap W. (2004). Reversal of obesity by targeted ablation of adipose tissue. *Nat Med* **10:** 625-32.

Komatsu T., Mizusaki H., Mukai T., Ogawa H., Baba D., Shirakawa M., Hatakeyama S., Nakayama K. I., Yamamoto H., Kikuchi A., Morohashi K. (2004). Small ubiquitin-like modifier 1 (SUMO-1) modification of the synergy control motif of Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) regulates synergistic transcription between Ad4BP/SF-1 and Sox9. *Mol Endocrinol* **18:** 2451-62.

Kondo H., Maruo T., Peng X., Mochizuki M. (1996). Immunological evidence for the expression of the Fas antigen in the infant and adult human ovary during follicular regression and atresia. *J Clin Endocrinol Metab* **81:** 2702-10.

Kralisch S., Weise S., Sommer G., Lipfert J., Lossner U., Bluher M., Stumvoll M., Fasshauer M. (2009). Interleukin-1beta induces the novel adipokine chemerin in adipocytes in vitro. *Regul Pept* **154:** 102-6.

Krylova I. N., Sablin E. P., Moore J., Xu R. X., Waitt G. M., MacKay J. A., Juzumiene D., Bynum J. M., Madauss K., Montana V., Lebedeva L., Suzawa M., Williams J. D., Williams S. P., Guy R. K., Thornton J. W., Fletterick R. J., Willson T. M., Ingraham H. A. (2005). Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. *Cell* **120:** 343-55.

Kumar T. R., Wang Y., Lu N., Matzuk M. M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* **15:** 201-4.

LaPolt P. S., Tilly J. L., Aihara T., Nishimori K., Hsueh A. J. (1992). Gonadotropin-induced up- and down-regulation of ovarian follicle-stimulating hormone (FSH) receptor gene expression in immature rats: effects of pregnant mare's serum gonadotropin, human chorionic gonadotropin, and recombinant FSH. *Endocrinology* **130:** 1289-95.

Layman L. C. (2000). Mutations in the follicle-stimulating hormone-beta (FSH beta) and FSH receptor genes in mice and humans. *Semin Reprod Med* **18:** 5-10.

Ledoux S., Campos D. B., Lopes F. L., Dobias-Goff M., Palin M. F., Murphy B. D. (2006). Adiponectin induces periovulatory changes in ovarian follicular cells. *Endocrinology* **147:** 5178-86.

Lee G. Y., Croop J. M., Anderson E. (1998). Multidrug resistance gene expression correlates with progesterone production in dehydroepiandrosterone-induced polycystic and equine chorionic gonadotropin-stimulated ovaries of prepubertal rats. *Biol Reprod* **58:** 330-7.

Lee Y. K., Moore D. D. (2002). Dual mechanisms for repression of the monomeric orphan receptor liver receptor homologous protein-1 by the orphan small heterodimer partner. *J Biol Chem* **277:** 2463-7.

Legro R. S., Castracane V. D., Kauffman R. P. (2004). Detecting insulin resistance in polycystic ovary syndrome: purposes and pitfalls. *Obstet Gynecol Surv* **59:** 141-54.

Legro R. S., Spielman R., Urbanek M., Driscoll D., Strauss J. F., 3rd, Dunaif A. (1998). Phenotype and genotype in polycystic ovary syndrome. *Recent Prog Horm Res* **53:** 217-56.

Leung P. C., Adashi E. Y. (2004). The Ovary (second edition). Chapter 2, Academic Press.

Lin P., Rui R. (2010). Effects of follicular size and FSH on granulosa cell apoptosis and atresia in porcine antral follicles. *Mol Reprod Dev* **77:** 670-8.

Liu D., Lin Y., Kang T., Huang B., Xu W., Garcia-Barrio M., Olatinwo M., Matthews R., Chen Y. E., Thompson W. E. (2012a). Mitochondrial dysfunction and adipogenic reduction by prohibitin silencing in 3T3-L1 cells. *PLoS One* **7:** e34315.

Liu K., Wahlberg P., Ny T. (1998). Coordinated and cell-specific regulation of membrane type matrix metalloproteinase 1 (MT1-MMP) and its substrate matrix metalloproteinase 2 (MMP-2) by physiological signals during follicular development and ovulation. *Endocrinology* **139:** 4735-8.

Liu Y. H., Peck K., Lin J. Y. (2012b). Involvement of prohibitin upregulation in abrin-triggered apoptosis. *Evid Based Complement Alternat Med* **2012:** 605154.

Liu Y. X., Hsueh A. J. (1986). Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera. *Biol Reprod* **35:** 27-36.

Luangsay S., Wittamer V., Bondue B., De Henau O., Rouger L., Brait M., Franssen J. D., de Nadai P., Huaux F., Parmentier M. (2009). Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. *J Immunol* **183:** 6489-99.

Luchetti C.G., Solano M.E., Sander V., Barreiro-Arcos M.L., Gonzalez C., Girolamo G., Chiocchio S., Cremaschi G., Motta A. B. (2004). Effects of dehydroepiandrosterone on ovarian cystogenesis and immune function. *J Reprod Immunol* **64:** 59-74.

Maeda A., Inoue N., Matsuda-Minehata F., Goto Y., Cheng Y., Manabe N. (2007). The role of interleukin-6 in the regulation of granulosa cell apoptosis during follicular atresia in pig ovaries. *J Reprod Dev* **53:** 481-90.

Magoffin D. A., Weitsman S. R. (1993). Differentiation of ovarian theca-interstitial cells in vitro: regulation of 17 alpha-hydroxylase messenger ribonucleic acid expression by luteinizing hormone and insulin-like growth factor-I. *Endocrinology* **132:** 1945-51.

Mani A. M., Fenwick M. A., Cheng Z., Sharma M. K., Singh D., Wathes D. C. (2010). IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. *Reproduction* **139:** 139-51.

Manjeshwar S., Branam D. E., Lerner M. R., Brackett D. J., Jupe E. R. (2003). Tumor suppression by the prohibitin gene 3'untranslated region RNA in human breast cancer. *Cancer Res* **63:** 5251-6.

Manneras L., Cajander S., Holmang A., Seleskovic Z., Lystig T., Lonn M., Stener-Victorin E. (2007). A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. *Endocrinology* **148:** 3781-91.

Manneras L., Jonsdottir I. H., Holmang A., Lonn M., Stener-Victorin E. (2008). Low-frequency electro-acupuncture and physical exercise improve metabolic disturbances and modulate gene expression in adipose tissue in rats with dihydrotestosterone-induced polycystic ovary syndrome. *Endocrinology* **149:** 3559-68.

Marshall J. C., Kelch R. P. (1986). Gonadotropin-releasing hormone: role of pulsatile secretion in the regulation of reproduction. *N Engl J Med* **315:** 1459-68.

Mason H. D., Willis D. S., Beard R. W., Winston R. M., Margara R., Franks S. (1994). Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentrations of gonadotropins and sex steroids in follicular fluid. *J Clin Endocrinol Metab* **79:** 1355-60.

Matsuda-Minehata F., Goto Y., Inoue N., Sakamaki K., Chedrese P. J., Manabe N. (2007). Anti-apoptotic activity of porcine cFLIP in ovarian granulosa cell lines. *Mol Reprod Dev* **74:** 1165-70.

Matsuda F., Inoue N., Manabe N., Ohkura S. (2012). Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *J Reprod Dev* **58:** 44-50.

McClung J. K., Danner D. B., Stewart D. A., Smith J. R., Schneider E. L., Lumpkin C. K., Dell'Orco R. T., Nuell M. J. (1989). Isolation of a cDNA that hybrid selects antiproliferative mRNA from rat liver. *Biochem Biophys Res Commun* **164:** 1316-22.

McClung J. K., Jupe E. R., Liu X. T., Dell'Orco R. T. (1995). Prohibitin: potential role in senescence, development, and tumor suppression. *Exp Gerontol* **30:** 99-124.

McGee E. A. (2000). The regulation of apoptosis in preantral ovarian follicles. *Biol Signals Recept* **9:** 81-6.

McGee E. A., Hsueh A. J. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* **21:** 200-14.

McGee E. A., Perlas E., LaPolt P. S., Tsafriri A., Hsueh A. J. (1997a). Follicle-stimulating hormone enhances the development of preantral follicles in juvenile rats. *Biol Reprod* **57:** 990-8.

McGee E., Spears N., Minami S., Hsu S. Y., Chun S. Y., Billig H., Hsueh A. J. (1997b). Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3',5'-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endocrinology* **138:** 2417-24.

McMahon H. E., Hashimoto O., Mellon P. L., Shimasaki S. (2008). Oocyte-specific overexpression of mouse bone morphogenetic protein-15 leads to accelerated folliculogenesis and an early onset of acyclicity in transgenic mice. *Endocrinology* **149:** 2807-15.

McNatty K. P., Juengel J. L., Reader K. L., Lun S., Myllymaa S., Lawrence S. B., Western A., Meerasahib M. F., Mottershead D. G., Groome N. P., Ritvos O., Laitinen M. P. (2005). Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function. *Reproduction* **129:** 473-80.

Meder W., Wendland M., Busmann A., Kutzleb C., Spodsberg N., John H., Richter R., Schleuder D., Meyer M., Forssmann W. G. (2003). Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23. *FEBS Lett* **555:** 495-9.

Mendelson C. R., Kamat A. (2007). Mechanisms in the regulation of aromatase in developing ovary and placenta. *J Steroid Biochem Mol Biol* **106:** 62-70.

Mengwasser J., Piau A., Schlag P., Sleeman J. P. (2004). Differential immunization identifies PHB1/PHB2 as blood-borne tumor antigens. *Oncogene* **23:** 7430-5.

Mishra S., Murphy L. C., Murphy L. J. (2006). The Prohibitins: emerging roles in diverse functions. *J Cell Mol Med* **10:** 353-63.

Mishra S., Murphy L. C., Nyomba B. L., Murphy L. J. (2005). Prohibitin: a potential target for new therapeutics. *Trends Mol Med* **11:** 192-7.

Moran L. J., Brinkworth G., Noakes M., Norman R. J. (2006). Effects of lifestyle modification in polycystic ovarian syndrome. *Reprod Biomed Online* **12:** 569-78.

Moran L. J., Noakes M., Clifton P. M., Tomlinson L., Galletly C., Norman R. J. (2003). Dietary composition in restoring reproductive and metabolic physiology in overweight women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **88:** 812-9.

Murray A. A., Gosden R. G., Allison V., Spears N. (1998). Effect of androgens on the development of mouse follicles growing in vitro. *J Reprod Fertil* **113:** 27-33.

Muruganandan S., Roman A. A., Sinal C. J. (2010). Role of chemerin/CMKLR1 signaling in adipogenesis and osteoblastogenesis of bone marrow stem cells. *J Bone Miner Res* **25:** 222-34.

Nagpal S., Patel S., Jacobe H., DiSepio D., Ghosn C., Malhotra M., Teng M., Duvic M., Chandraratna R. A. (1997). Tazarotene-induced gene 2 (TIG2), a novel retinoid-responsive gene in skin. *J Invest Dermatol* **109:** 91-5.

Nakajima H., Nakajima K., Nagano Y., Yamamoto M., Tarutani M., Takahashi M., Takahashi Y., Sano S. (2010). Circulating level of chemerin is upregulated in psoriasis. *J Dermatol Sci* **60:** 45-7.

Nakamura K., Minegishi T., Takakura Y., Miyamoto K., Hasegawa Y., Ibuki Y., Igarashi M. (1991). Hormonal regulation of gonadotropin receptor mRNA in rat ovary during follicular growth and luteinization. *Mol Cell Endocrinol* **82:** 259-63.

Nelson V. L., Qin K. N., Rosenfield R. L., Wood J. R., Penning T. M., Legro R. S., Strauss J. F., 3rd, McAllister J. M. (2001). The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome. *J Clin Endocrinol Metab* **86:** 5925-33.

Nijtmans L. G., Artal S. M., Grivell L. A., Coates P. J. (2002). The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. *Cell Mol Life Sci* **59:** 143-55.

Nijtmans L. G., de Jong L., Artal Sanz M., Coates P. J., Berden J. A., Back J. W., Muijsers A. O., van der Spek H., Grivell L. A. (2000). Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. *EMBO J* **19:** 2444-51.

Nilsson E. E., Skinner M. K. (2003). Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biol Reprod* **69:** 1265-72.

Orisaka M., Jiang J. Y., Orisaka S., Kotsuji F., Tsang B. K. (2009). Growth differentiation factor 9 promotes rat preantral follicle growth by up-regulating follicular androgen biosynthesis. *Endocrinology* **150:** 2740-8.

Orisaka M., Orisaka S., Jiang J. Y., Craig J., Wang Y., Kotsuji F., Tsang B. K. (2006). Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. *Mol Endocrinol* **20:** 2456-68.

Otsuka F., Yao Z., Lee T., Yamamoto S., Erickson G. F., Shimasaki S. (2000). Bone morphogenetic protein-15. Identification of target cells and biological functions. *J Biol Chem* **275:** 39523-8.

Pangas S. A., Li X., Robertson E. J., Matzuk M. M. (2006). Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. *Mol Endocrinol* **20:** 1406-22.

Parker K. L., Schimmer B. P. (1997). Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr Rev* **18:** 361-77.

Pasquali R., Pelusi C., Genghini S., Cacciari M., Gambineri A. (2003). Obesity and reproductive disorders in women. *Hum Reprod Update* **9:** 359-72.

Payne A. H., Hales D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* **25:** 947-70.

Pedersen B. K., Febbraio M. A. (2007). Point: Interleukin-6 does have a beneficial role in insulin sensitivity and glucose homeostasis. *J Appl Physiol* **102:** 814-6.

Pierce J. G., Parsons T. F. (1981). Glycoprotein hormones: structure and function. *Annu Rev Biochem* **50:** 465-95.

Pinilla L., Trimino E., Garnelo P., Bellido C., Aguilar R., Gaytan F., Aguilar E. (1993). Changes in pituitary secretion during the early postnatal period and anovulatory syndrome induced by neonatal oestrogen or androgen in rats. *J Reprod Fertil* **97:** 13-20.

Porter D. A., Harman R. M., Cowan R. G., Quirk S. M. (2001). Relationship of Fas ligand expression and atresia during bovine follicle development. *Reproduction* **121:** 561-6.

Quandt L. M., Hutz R. J. (1993). Induction by estradiol-17 beta of polycystic ovaries in the guinea pig. *Biol Reprod* **48:** 1088-94.

Quirk S. M., Cowan R. G., Harman R. M. (2006). The susceptibility of granulosa cells to apoptosis is influenced by oestradiol and the cell cycle. *J Endocrinol* **189:** 441-53.

Recabarren S.E., Padmanabhan V., Codner E., Lobos A., Duran C., Vidal M., Foster D.L., Sir-Petermann T. (2005). Postnatal developmental consequences of altered insulin sensitivity in

female sheep treated prenatally with testosterone. *Am J Physiol Endocrinol Metab* **289:** E801-6.

Reverchon M., Cornuau M., Rame C., Guerif F., Royere D., Dupont J. (2012). Chemerin inhibits IGF-1-induced progesterone and estradiol secretion in human granulosa cells. *Hum Reprod* **27:** 1790-1800.

Richards J. S. (1994). Hormonal control of gene expression in the ovary. *Endocr Rev* **15:** 725-51.

Richards J. S., Russell D. L., Ochsner S., Hsieh M., Doyle K. H., Falender A. E., Lo Y. K., Sharma S. C. (2002). Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. *Recent Prog Horm Res* **57:** 195-220.

Robker R. L., Richards J. S. (1998). Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Mol Endocrinol* **12:** 924-40.

Roh S. G., Song S. H., Choi K. C., Katoh K., Wittamer V., Parmentier M., Sasaki S. (2007). Chemerin--a new adipokine that modulates adipogenesis via its own receptor. *Biochem Biophys Res Commun* **362:** 1013-8.

Rosenfeld C. S., Roberts R. M., Lubahn D. B. (2001a). Estrogen receptor- and aromatase-deficient mice provide insight into the roles of estrogen within the ovary and uterus. *Mol Reprod Dev* **59:** 336-46.

Rosenfeld C. S., Wagner J. S., Roberts R. M., Lubahn D. B. (2001b). Intraovarian actions of oestrogen. *Reproduction* **122:** 215-26.

Roskams A. J., Friedman V., Wood C. M., Walker L., Owens G. A., Stewart D. A., Altus M. S., Danner D. B., Liu X. T., McClung J. K. (1993). Cell cycle activity and expression of prohibitin mRNA. *J Cell Physiol* **157:** 289-95.

Ross J. A., Nagy Z. S., Kirken R. A. (2008). The PHB1/2 phosphocomplex is required for mitochondrial homeostasis and survival of human T cells. *J Biol Chem* **283:** 4699-713.

Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop (2004). Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril* **81:** 19-25.

Rubio Pomar F. J., Roelen B. A., Slot K. A., van Tol H. T., Colenbrander B., Teerds K. J. (2004). Role of Fas-mediated apoptosis and follicle-stimulating hormone on the developmental capacity of bovine cumulus oocyte complexes in vitro. *Biol Reprod* **71:** 790-6.

Russell D. L., Doyle K. M., Gonzales-Robayna I., Pipaon C., Richards J. S. (2003). Egr-1 induction in rat granulosa cells by follicle-stimulating hormone and luteinizing hormone: combinatorial regulation by transcription factors cyclic adenosine 3',5'-monophosphate regulatory element binding protein, serum response factor, sp1, and early growth response factor-1. *Mol Endocrinol* **17:** 520-33.

Sablin E. P., Blind R. D., Krylova I. N., Ingraham J. G., Cai F., Williams J. D., Fletterick R. J., Ingraham H. A. (2009). Structure of SF-1 bound by different phospholipids: evidence for regulatory ligands. *Mol Endocrinol* **23:** 25-34.

Sakamaki K., Yoshida H., Nishimura Y., Nishikawa S., Manabe N., Yonehara S. (1997). Involvement of Fas antigen in ovarian follicular atresia and luteolysis. *Mol Reprod Dev* **47:** 11-8.

Salvador L. M., Park Y., Cottom J., Maizels E. T., Jones J. C., Schillace R. V., Carr D. W., Cheung P., Allis C. D., Jameson J. L., Hunzicker-Dunn M. (2001). Follicle-stimulating hormone stimulates protein kinase A-mediated histone H3 phosphorylation and acetylation leading to select gene activation in ovarian granulosa cells. *J Biol Chem* **276:** 40146-55.

Salvetti N. R., Panzani C. G., Gimeno E. J., Neme L. G., Alfaro N. S., Ortega H. H. (2009). An imbalance between apoptosis and proliferation contributes to follicular persistence in polycystic ovaries in rats. *Reprod Biol Endocrinol* **7:** 68.

Sato T., Saito H., Swensen J., Olifant A., Wood C., Danner D., Sakamoto T., Takita K., Kasumi F., Miki Y., et al. (1992). The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. *Cancer Res* **52:** 1643-6.

Saxena D., Escamilla-Hernandez R., Little-Ihrig L., Zeleznik A. J. (2007). Liver receptor homolog-1 and steroidogenic factor-1 have similar actions on rat granulosa cell steroidogenesis. *Endocrinology* **148:** 726-34.

Schultz D. R., Harrington W. J., Jr. (2003). Apoptosis: programmed cell death at a molecular level. *Semin Arthritis Rheum* **32:** 345-69.

Sell H., Laurencikiene J., Taube A., Eckardt K., Cramer A., Horrighs A., Arner P., Eckel J. (2009). Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. *Diabetes* **58:** 2731-40.

Shi J., Yoshino O., Osuga Y., Nishii O., Yano T., Taketani Y. (2010). Bone morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating hormone (FSH) receptor in human granulosa cells. *Fertil Steril* **93:** 1273-9.

Shimasaki S., Zachow R. J., Li D., Kim H., Iemura S., Ueno N., Sampath K., Chang R. J., Erickson G. F. (1999). A functional bone morphogenetic protein system in the ovary. *Proc Natl Acad Sci U S A* **96:** 7282-7.

Simpson E. R. (1979). Cholesterol side-chain cleavage, cytochrome P450, and the control of steroidogenesis. *Mol Cell Endocrinol* **13:** 213-27.

Sirianni R., Seely J. B., Attia G., Stocco D. M., Carr B. R., Pezzi V., Rainey W. E. (2002). Liver receptor homologue-1 is expressed in human steroidogenic tissues and activates transcription of genes encoding steroidogenic enzymes. *J Endocrinol* **174:** R13-7.

Skrzeczynska-Moncznik J., Wawro K., Stefanska A., Oleszycka E., Kulig P., Zabel B. A., Sulkowski M., Kapinska-Mrowiecka M., Czubak-Macugowska M., Butcher E. C., Cichy J. (2009). Potential role of chemerin in recruitment of plasmacytoid dendritic cells to diseased skin. *Biochem Biophys Res Commun* **380:** 323-7.

Slob A. K., den Hamer R., Woutersen P. J., van der Werff ten Bosch J. J. (1983). Prenatal testosterone propionate and postnatal ovarian activity in the rat. *Acta Endocrinol (Copenh)* **103:** 420-7.

Solovyeva E. V., Hayashi M., Margi K., Barkats C., Klein C., Amsterdam A., Hsueh A. J., Tsafriri A. (2000). Growth differentiation factor-9 stimulates rat theca-interstitial cell androgen biosynthesis. *Biol Reprod* **63:** 1214-8.

Spicer L. J., Aad P. Y., Allen D. T., Mazerbourg S., Payne A. H., Hsueh A. J. (2008). Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of follicle size on responses to GDF9. *Biol Reprod* **78:** 243-53.

Spicer L. J., Schreiber N. B., Lagaly D. V., Aad P. Y., Douthit L. B., Grado-Ahuir J. A. (2011). Effect of resistin on granulosa and theca cell function in cattle. *Anim Reprod Sci* **124:** 19-27.

Sripathi S. R., He W., Atkinson C. L., Smith J. J., Liu Z., Elledge B. M., Jahng W. J. (2011). Mitochondrial-nuclear communication by prohibitin shuttling under oxidative stress. *Biochemistry* **50:** 8342-51.

Steglich G., Neupert W., Langer T. (1999). Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. *Mol Cell Biol* **19:** 3435-42.

Stocco D. M. (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol* **63:** 193-213.

Sullivan S. D., Moenter S. M. (2004). Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. *Proc Natl Acad Sci U S A* **101:** 7129-34.

Sun Y. L., Zhang J., Ping Z. G., Wang C. Q., Sun Y. F., Chen L., Li X. Y., Li C. J., Zhu X. L., Liu Z., Zhang W., Zhou X. (2012). Relationship between apoptosis and proliferation in granulosa and theca cells of cystic follicles in sows. *Reprod Domest Anim* **47:** 601-8.

Takahashi M., Okimura Y., Lguchi G., Nishizawa H., Yamamoto M., Suda K., Kitazawa R., Fujimoto W., Takahashi K., Zolotaryov F. N., Hong K. S., Kiyonari H., Abe T., Kaji H., Kitazawa S., Kasuga M., Chihara K., Takahashi Y. (2011). Chemerin regulates β-cell function in mice. *Scientific Reports* **1:** Article number: 123.

Takahashi M., Takahashi Y., Takahashi K., Zolotaryov F. N., Hong K. S., Kitazawa R., Iida K., Okimura Y., Kaji H., Kitazawa S., Kasuga M., Chihara K. (2008). Chemerin enhances insulin signaling and potentiates insulin-stimulated glucose uptake in 3T3-L1 adipocytes. *FEBS Lett* **582:** 573-8.

Tan B. K., Chen J., Farhatullah S., Adya R., Kaur J., Heutling D., Lewandowski K. C., O'Hare J. P., Lehnert H., Randeva H. S. (2009). Insulin and metformin regulate circulating and adipose tissue chemerin. *Diabetes* **58:** 1971-7.

Taniguchi H., Komiyama J., Viger R. S., Okuda K. (2009). The expression of the nuclear receptors NR5A1 and NR5A2 and transcription factor GATA6 correlates with steroidogenic gene expression in the bovine corpus luteum. *Mol Reprod Dev* **76:** 873-80.

Teede H., Deeks A., Moran L. (2010). Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. *BMC Med* **8:** 41.

Theiss A. L., Obertone T. S., Merlin D., Sitaraman S. V. (2007). Interleukin-6 transcriptionally regulates prohibitin expression in intestinal epithelial cells. *J Biol Chem* **282:** 12804-12.

Thessaloniki ESHRE/ASRM-sponsored PCOS consensus workshop (2008). Consensus on infertility treatment related to polycystic ovary syndrome. *Hum Reprod* **23:** 462-77.

Thompson C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456-62.

Thompson W. E., Ramalho-Santos J., Sutovsky P. (2003). Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biol Reprod* **69:** 254-60.

Tilly J. L. (1996). Apoptosis and ovarian function. *Rev Reprod* **1:** 162-72.

Toda K., Takeda K., Okada T., Akira S., Saibara T., Kaname T., Yamamura K., Onishi S., Shizuta Y. (2001). Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. *J Endocrinol* **170:** 99-111.

Troispoux C., Guillou F., Elalouf J. M., Firsov D., Iacovelli L., De Blasi A., Combarnous Y., Reiter E. (1999). Involvement of G protein-coupled receptor kinases and arrestins in desensitization to follicle-stimulating hormone action. *Mol Endocrinol* **13:** 1599-614.

Tyndall V., Broyde M., Sharpe R., Welsh M., Drake A. J., McNeilly A. S. (2012). Effect of androgen treatment during foetal and/or neonatal life on ovarian function in prepubertal and adult rats. *Reproduction* **143:** 21-33.

Urs A. N., Dammer E., Kelly S., Wang E., Merrill A. H., Jr., Sewer M. B. (2007). Steroidogenic factor-1 is a sphingolipid binding protein. *Mol Cell Endocrinol* **265-266:** 174-8.

Val P., Lefrancois-Martinez A. M., Veyssiere G., Martinez A. (2003). SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nucl Recept* **1:** 8.

Vander Heiden M. G., Choy J. S., VanderWeele D. J., Brace J. L., Harris M. H., Bauer D. E., Prange B., Kron S. J., Thompson C. B., Rudin C. M. (2002). Bcl-x(L) complements Saccharomyces cerevisiae genes that facilitate the switch from glycolytic to oxidative metabolism. *J Biol Chem* **277:** 44870-6.

Vassart G., Pardo L., Costagliola S. (2004). A molecular dissection of the glycoprotein hormone receptors. *Trends Biochem Sci* **29:** 119-26.

Velazquez E. M., Mendoza S., Hamer T., Sosa F., Glueck C. J. (1994). Metformin therapy in polycystic ovary syndrome reduces hyperinsulinemia, insulin resistance, hyperandrogenemia, and systolic blood pressure, while facilitating normal menses and pregnancy. *Metabolism* **43:** 647-54.

Vessal M., Mishra S., Moulik S., Murphy L. J. (2006). Prohibitin attenuates insulin-stimulated glucose and fatty acid oxidation in adipose tissue by inhibition of pyruvate carboxylase. *FEBS J* **273:** 568-76.

Vinatier D., Dufour P., Subtil D. (1996). Apoptosis: a programmed cell death involved in ovarian and uterine physiology. *Eur J Obstet Gynecol Reprod Biol* **67:** 85-102.

Vitt U. A., Hayashi M., Klein C., Hsueh A. J. (2000). Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biol Reprod* **62:** 370-7.

Walters K. A., Allan C. M., Handelsman D. J. (2012). Rodent models for human polycystic ovary syndrome. *Biol Reprod* **86:** 149, 1-12.

Wang P., Mariman E., Keijer J., Bouwman F., Noben J. P., Robben J., Renes J. (2004). Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines. *Cell Mol Life Sci* **61:** 2405-17.

Wang Q., Kim J. Y., Xue K., Liu J. Y., Leader A., Tsang B. K. (2012). Chemerin, a novel regulator of follicular steroidogenesis and its potential involvement in polycystic ovarian syndrome. *Endocrinology* **153:** 5600-11.

Wang Q., Leader A., Tsang BK. (2013). Inhibitory roles of prohibitin and chemerin on FSH-induced rat granulosa cell steroidogenesis. *Endocrinology* **154:** 956-67.

Wang X. (2001). The expanding role of mitochondria in apoptosis. *Genes Dev* **15:** 2922-33.

Wang Y., Rippstein P. U., Tsang B. K. (2003). Role and gonadotrophic regulation of X-linked inhibitor of apoptosis protein expression during rat ovarian follicular development in vitro. *Biol Reprod* **68:** 610-9.

Wayne C. M., Fan H. Y., Cheng X., Richards J. S. (2007). Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation. *Mol Endocrinol* **21:** 1940-57.

Weigert J., Obermeier F., Neumeier M., Wanninger J., Filarsky M., Bauer S., Aslanidis C., Rogler G., Ott C., Schaffler A., Scholmerich J., Buechler C. (2010). Circulating levels of chemerin and adiponectin are higher in ulcerative colitis and chemerin is elevated in Crohn's disease. *Inflamm Bowel Dis* **16:** 630-7.

Welt C. K., Taylor A. E., Fox J., Messerlian G. M., Adams J. M., Schneyer A. L. (2005). Follicular arrest in polycystic ovary syndrome is associated with deficient inhibin A and B biosynthesis. *J Clin Endocrinol Metab* **90:** 5582-7.

Wen X., Li D., Tozer A. J., Docherty S. M., Iles R. K. (2010). Estradiol, progesterone, testosterone profiles in human follicular fluid and cultured granulosa cells from luteinized pre-ovulatory follicles. *Reprod Biol Endocrinol* **8:** 117.

Wickenheisser J. K., Quinn P. G., Nelson V. L., Legro R. S., Strauss J. F., 3rd, McAllister J. M. (2000). Differential activity of the cytochrome P450 17alpha-hydroxylase and steroidogenic acute regulatory protein gene promoters in normal and polycystic ovary syndrome theca cells. *J Clin Endocrinol Metab* **85:** 2304-11.

Winter A., Kamarainen O., Hofmann A. (2007). Molecular modeling of prohibitin domains. *Proteins* **68:** 353-62.

Wittamer V., Franssen J. D., Vulcano M., Mirjolet J. F., Le Poul E., Migeotte I., Brezillon S., Tyldesley R., Blanpain C., Detheux M., Mantovani A., Sozzani S., Vassart G., Parmentier M., Communi D. (2003). Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J Exp Med* **198:** 977-85.

Wood J. R., Strauss J. F., 3rd (2002). Multiple signal transduction pathways regulate ovarian steroidogenesis. *Rev Endocr Metab Disord* **3:** 33-46.

Woods D. C., Haugen M. J., Johnson A. L. (2007). Actions of epidermal growth factor receptor/mitogen-activated protein kinase and protein kinase C signaling in granulosa cells from Gallus gallus are dependent upon stage of differentiation. *Biol Reprod* **77:** 61-70.

Wu Q., Wu S. (2012). Lipid rafts association and anti-apoptotic function of prohibitin in ultraviolet B light-irradiated HaCaT keratinocytes. *Exp Dermatol* **21:** 640-2.

Wu X. K., Zhou S. Y., Liu J. X., Pollanen P., Sallinen K., Makinen M., Erkkola R. (2003). Selective ovary resistance to insulin signaling in women with polycystic ovary syndrome. *Fertil Steril* **80:** 954-65.

Wu X. Y., Li Z. L., Wu C. Y., Liu Y. M., Lin H., Wang S. H., Xiao W. F. (2010). Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. *Endocr J* **57:** 201-9.

Yang F. M., Pan C. T., Tsai H. M., Chiu T. W., Wu M. L., Hu M. C. (2009). Liver receptor homolog-1 localization in the nuclear body is regulated by sumoylation and cAMP signaling in rat granulosa cells. *FEBS J* **276:** 425-36.

Yu F. Q., Han C. S., Yang W., Jin X., Hu Z. Y., Liu Y. X. (2005). Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differentially. *J Endocrinol* **186:** 85-96.

Zabel B. A., Allen S. J., Kulig P., Allen J. A., Cichy J., Handel T. M., Butcher E. C. (2005a). Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. *J Biol Chem* **280:** 34661-6.

Zabel B. A., Nakae S., Zuniga L., Kim J. Y., Ohyama T., Alt C., Pan J., Suto H., Soler D., Allen S. J., Handel T. M., Song C. H., Galli S. J., Butcher E. C. (2008). Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. *J Exp Med* **205:** 2207-20.

Zabel B. A., Ohyama T., Zuniga L., Kim J. Y., Johnston B., Allen S. J., Guido D. G., Handel T. M., Butcher E. C. (2006a). Chemokine-like receptor 1 expression by macrophages in vivo: regulation by TGF-beta and TLR ligands. *Exp Hematol* **34:** 1106-14.

Zabel B. A., Silverio A. M., Butcher E. C. (2005b). Chemokine-like receptor 1 expression and chemerin-directed chemotaxis distinguish plasmacytoid from myeloid dendritic cells in human blood. *J Immunol* **174:** 244-51.

Zabel B. A., Zuniga L., Ohyama T., Allen S. J., Cichy J., Handel T. M., Butcher E. C. (2006b). Chemoattractants, extracellular proteases, and the integrated host defense response. *Exp Hematol* **34:** 1021-32.

Zawadski J.K., Dunaif A. (1992). Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif A, Givens JR, haseletine FP, Merriam GR, editors. Polycystic ovary syndrome. Boston (MA): Blackwell Scientific Publications. *1992***:** 377-84.

Zeleznik A. J., Saxena D., Little-Ihrig L. (2003). Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. *Endocrinology* **144:** 3985-94.

Zhang C., Xia G., Tsang B. K. (2011). Interactions of thyroid hormone and FSH in the regulation of rat granulosa cell apoptosis. *Front Biosci (Elite Ed)* **3:** 1401-13.

Zhao L., Yamaguchi Y., Sharif S., Du X. Y., Song J. J., Lee D. M., Recht L. D., Robinson W. H., Morser J., Leung L. L. (2011). Chemerin158K protein is the dominant chemerin isoform in synovial and cerebrospinal fluids but not in plasma. *J Biol Chem* **286:** 39520-7.

Zhou J., Kumar T. R., Matzuk M. M., Bondy C. (1997). Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Mol Endocrinol* **11:** 1924-33.

8.2 Endocrinology, 2012, 153:5600-5611

1. **McGee EA, Hsueh AJ** 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-214
2. **Woods DC, Haugen MJ, Johnson AL** 2007 Actions of epidermal growth factor receptor/mitogen-activated protein kinase and protein kinase C signaling in granulosa cells from Gallus gallus are dependent upon stage of differentiation. Biol Reprod 77:61-70
3. **Richards JS** 1994 Hormonal control of gene expression in the ovary. Endocr Rev 15:725-751
4. **Liu YX, Hsueh AJ** 1986 Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera. Biol Reprod 35:27-36
5. **Yong EL, Hillier SG, Turner M, Baird DT, Ng SC, Bongso A, Ratnam SS** 1994 Differential regulation of cholesterol side-chain cleavage (P450scc) and aromatase (P450arom) enzyme mRNA expression by gonadotrophins and cyclic AMP in human granulosa cells. J Mol Endocrinol 12:239-249
6. **Sher N, Yivgi-Ohana N, Orly J** 2007 Transcriptional regulation of the cholesterol side chain cleavage cytochrome P450 gene (CYP11A1) revisited: binding of GATA, cyclic adenosine 3',5'-monophosphate response element-binding protein and activating protein (AP)-1 proteins to a distal novel cluster of cis-regulatory elements potentiates AP-2 and steroidogenic factor-1-dependent gene expression in the rodent placenta and ovary. Mol Endocrinol 21:948-962
7. **Boerboom D, Pilon N, Behdjani R, Silversides DW, Sirois J** 2000 Expression and regulation of transcripts encoding two members of the NR5A nuclear receptor subfamily of orphan nuclear receptors, steroidogenic factor-1 and NR5A2, in equine ovarian cells during the ovulatory process. Endocrinology 141:4647-4656
8. **Hinshelwood MM, Repa JJ, Shelton JM, Richardson JA, Mangelsdorf DJ, Mendelson CR** 2003 Expression of LRH-1 and SF-1 in the mouse ovary: localization in different cell types correlates with differing function. Mol Cell Endocrinol 207:39-45
9. **Falender AE, Lanz R, Malenfant D, Belanger L, Richards JS** 2003 Differential expression of steroidogenic factor-1 and FTF/LRH-1 in the rodent ovary. Endocrinology 144:3598-3610
10. **Saxena D, Escamilla-Hernandez R, Little-Ihrig L, Zeleznik AJ** 2007 Liver receptor homolog-1 and steroidogenic factor-1 have similar actions on rat granulosa cell steroidogenesis. Endocrinology 148:726-734
11. **Taniguchi H, Komiyama J, Viger RS, Okuda K** 2009 The expression of the nuclear receptors NR5A1 and NR5A2 and transcription factor GATA6 correlates with steroidogenic gene expression in the bovine corpus luteum. Mol Reprod Dev 76:873-880
12. **Franks S** 1995 Polycystic ovary syndrome. N Engl J Med 333:853-861
13. **Dunaif A** 1997 Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr Rev 18:774-800
14. **Gambineri A, Pelusi C, Vicennati V, Pagotto U, Pasquali R** 2002 Obesity and the polycystic ovary syndrome. Int J Obes Relat Metab Disord 26:883-896
15. **Meder W, Wendland M, Busmann A, Kutzleb C, Spodsberg N, John H, Richter R, Schleuder D, Meyer M, Forssmann WG** 2003 Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23. FEBS Lett 555:495-499
16. **Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, Brezillon S, Tyldesley R, Blanpain C, Detheux M, Mantovani A, Sozzani S, Vassart G, Parmentier M, Communi D** 2003 Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. J Exp Med 198:977-985

17. **Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, Walder K, Segal D** 2007 Chemerin is a novel adipokine associated with obesity and metabolic syndrome. Endocrinology 148:4687-4694
18. **Goralski KB, McCarthy TC, Hanniman EA, Zabel BA, Butcher EC, Parlee SD, Murugan-andan S, Sinal CJ** 2007 Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. J Biol Chem 282:28175-28188
19. **Roh SG, Song SH, Choi KC, Katoh K, Wittamer V, Parmentier M, Sasaki S** 2007 Chemerin-a new adipokine that modulates adipogenesis via its own receptor. Biochem Biophys Res Commun 362:1013-1018
20. **Ernst MC, Sinal CJ** 2010 Chemerin: at the crossroads of inflammation and obesity. Trends Endocrinol Metab 21:660-667
21. **Tan BK, Chen J, Farhatullah S, Adya R, Kaur J, Heutling D, Lewandowski KC, O'Hare JP, Lehnert H, Randeva HS** 2009 Insulin and metformin regulate circulating and adipose tissue chemerin. Diabetes 58:1971-1977
22. **Bozaoglu K, Segal D, Shields KA, Cummings N, Curran JE, Comuzzie AG, Mahaney MC, Rainwater DL, VandeBerg JL, MacCluer JW, Collier G, Blangero J, Walder K, Jowett JB** 2009 Chemerin is associated with metabolic syndrome phenotypes in a Mexican-American population. J Clin Endocrinol Metab 94:3085-3088
23. **Zabel BA, Silverio AM, Butcher EC** 2005 Chemokine-like receptor 1 expression and chemerin-directed chemotaxis distinguish plasmacytoid from myeloid dendritic cells in human blood. J Immunol 174:244-251
24. **Manneras L, Cajander S, Holmang A, Seleskovic Z, Lystig T, Lonn M, Stener-Victorin E** 2007 A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology 148:3781-3791
25. **Brawer J, Schipper H, Robaire B** 1983 Effects of long term androgen and estradiol exposure on the hypothalamus. Endocrinology 112:194-199
26. **Silfen ME, Denburg MR, Manibo AM, Lobo RA, Jaffe R, Ferin M, Levine LS, Oberfield SE** 2003 Early endocrine, metabolic, and sonographic characteristics of polycystic ovary syndrome (PCOS): comparison between nonobese and obese adolescents. J Clin Endocrinol Metab 88:4682-4688
27. **Fassnacht M, Schlenz N, Schneider SB, Wudy SA, Allolio B, Arlt W** 2003 Beyond adrenal and ovarian androgen generation: Increased peripheral 5 alpha-reductase activity in women with polycystic ovary syndrome. J Clin Endocrinol Metab 88:2760-2766
28. **Kobayashi N, Orisaka M, Cao M, Kotsuji F, Leader A, Sakuragi N, Tsang BK** 2009 Growth differentiation factor-9 mediates follicle-stimulating hormone-thyroid hormone interaction in the regulation of rat preantral follicular development. Endocrinology 150:5566-5574
29. **Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC** 2007 A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. Nat Protoc 2:1236-1247
30. **Mittereder N, March KL, Trapnell BC** 1996 Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 70:7498-7509
31. **Campbell KL** 1979 Ovarian granulosa cells isolated with EGTA and hypertonic sucrose: cellular integrity and function. Biol Reprod 21:773-786
32. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408
33. **Hedin L, Rosberg S** 1983 Forskolin effects on the cAMP system and steroidogenesis in the immature rat ovary. Mol Cell Endocrinol 33:69-80
34. **Almahbobi G, Anderiesz C, Hutchinson P, McFarlane JR, Wood C, Trounson AO** 1996 Functional integrity of granulosa cells from polycystic ovaries. Clin Endocrinol (Oxf) 44:571-580

35. **Mason HD, Willis DS, Beard RW, Winston RM, Margara R, Franks S** 1994 Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentrations of gonadotropins and sex steroids in follicular fluid. J Clin Endocrinol Metab 79:1355-1360
36. **Coffler MS, Patel K, Dahan MH, Malcom PJ, Kawashima T, Deutsch R, Chang RJ** 2003 Evidence for abnormal granulosa cell responsiveness to follicle-stimulating hormone in women with polycystic ovary syndrome. J Clin Endocrinol Metab 88:1742-1747
37. **Catteau-Jonard S, Jamin SP, Leclerc A, Gonzales J, Dewailly D, di Clemente N** 2008 Anti-Mullerian hormone, its receptor, FSH receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. J Clin Endocrinol Metab 93:4456-4461
38. **Gonzalez-Fernandez R, Pena O, Hernandez J, Martin-Vasallo P, Palumbo A, Avila J** 2011 Patients with endometriosis and patients with poor ovarian reserve have abnormal follicle-stimulating hormone receptor signaling pathways. Fertil Steril 95:2373-2378
39. **Jakimiuk AJ, Weitsman SR, Brzechffa PR, Magoffin DA** 1998 Aromatase mRNA expression in individual follicles from polycystic ovaries. Mol Hum Reprod 4:1-8
40. **Welt CK, Taylor AE, Fox J, Messerlian GM, Adams JM, Schneyer AL** 2005 Follicular arrest in polycystic ovary syndrome is associated with deficient inhibin A and B biosynthesis. J Clin Endocrinol Metab 90:5582-5587
41. **Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA** 2001 Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. J Clin Endocrinol Metab 86:1318-1323
42. **Wickenheisser JK, Quinn PG, Nelson VL, Legro RS, Strauss JF, 3rd, McAllister JM** 2000 Differential activity of the cytochrome P450 17alpha-hydroxylase and steroidogenic acute regulatory protein gene promoters in normal and polycystic ovary syndrome theca cells. J Clin Endocrinol Metab 85:2304-2311
43. **Yamaguchi Y, Du XY, Zhao L, Morser J, Leung LL** 2011 Proteolytic cleavage of chemerin protein is necessary for activation to the active form, Chem157S, which functions as a signaling molecule in glioblastoma. J Biol Chem 286:39510-39519
44. **Du XY, Leung LL** 2009 Proteolytic regulatory mechanism of chemerin bioactivity. Acta Biochim Biophys Sin (Shanghai) 41:973-979
45. **Zabel BA, Allen SJ, Kulig P, Allen JA, Cichy J, Handel TM, Butcher EC** 2005 Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. J Biol Chem 280:34661-34666
46. **Zhao L, Yamaguchi Y, Sharif S, Du XY, Song JJ, Lee DM, Recht LD, Robinson WH, Morser J, Leung LL** 2011 Chemerin158K protein is the dominant chemerin isoform in synovial and cerebrospinal fluids but not in plasma. J Biol Chem 286:39520-39527
47. **Fayard E, Auwerx J, Schoonjans K** 2004 LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. Trends Cell Biol 14:250-260
48. **Reverchon M, Cornuau M, Rame C, Guerif F, Royere D, Dupont J** 2012 Chemerin inhibits IGF-1-induced progesterone and estradiol secretion in human granulosa cells. Hum Reprod 27:1790-1800

8.3 Endocrinology, 2013, 154: 956 - 967

1. **McGee EA, Hsueh AJ** 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-214
2. **Jamnongjit M, Hammes SR** 2006 Ovarian steroids: the good, the bad, and the signals that raise them. Cell Cycle 5:1178-1183
3. **Yong EL, Hillier SG, Turner M, Baird DT, Ng SC, Bongso A, Ratnam SS** 1994 Differential regulation of cholesterol side-chain cleavage (P450scc) and aromatase (P450arom) enzyme mRNA expression by gonadotrophins and cyclic AMP in human granulosa cells. J Mol Endocrinol 12:239-249
4. **Sher N, Yivgi-Ohana N, Orly J** 2007 Transcriptional regulation of the cholesterol side chain cleavage cytochrome P450 gene (CYP11A1) revisited: binding of GATA, cyclic adenosine 3',5'-monophosphate response element-binding protein and activating protein (AP)-1 proteins to a distal novel cluster of cis-regulatory elements potentiates AP-2 and steroidogenic factor-1-dependent gene expression in the rodent placenta and ovary. Mol Endocrinol 21:948-962
5. **Eimerl S, Orly J** 2002 Regulation of steroidogenic genes by insulin-like growth factor-1 and follicle-stimulating hormone: differential responses of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, and 3beta-hydroxysteroid dehydrogenase/isomerase in rat granulosa cells. Biol Reprod 67:900-910
6. **Irving-Rodgers HF, Harland ML, Sullivan TR, Rodgers RJ** 2009 Studies of granulosa cell maturation in dominant and subordinate bovine follicles: novel extracellular matrix focimatrix is co-ordinately regulated with cholesterol side-chain cleavage CYP11A1. Reproduction 137:825-834
7. **Hong Y, Yu B, Sherman M, Yuan YC, Zhou D, Chen S** 2007 Molecular basis for the aromatization reaction and exemestane-mediated irreversible inhibition of human aromatase. Mol Endocrinol 21:401-414
8. **Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, Walder K, Segal D** 2007 Chemerin is a novel adipokine associated with obesity and metabolic syndrome. Endocrinology 148:4687-4694
9. **Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, Brezillon S, Tyldesley R, Blanpain C, Detheux M, Mantovani A, Sozzani S, Vassart G, Parmentier M, Communi D** 2003 Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. J Exp Med 198:977-985
10. **Zabel BA, Allen SJ, Kulig P, Allen JA, Cichy J, Handel TM, Butcher EC** 2005 Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. J Biol Chem 280:34661-34666
11. **Zhao L, Yamaguchi Y, Sharif S, Du XY, Song JJ, Lee DM, Recht LD, Robinson WH, Morser J, Leung LL** 2011 Chemerin158K protein is the dominant chemerin isoform in synovial and cerebrospinal fluids but not in plasma. J Biol Chem 286:39520-39527
12. **Bozaoglu K, Curran JE, Stocker CJ, Zaibi MS, Segal D, Konstantopoulos N, Morrison S, Carless M, Dyer TD, Cole SA, Goring HH, Moses EK, Walder K, Cawthorne MA, Blangero J, Jowett JB** 2010 Chemerin, a novel adipokine in the regulation of angiogenesis. J Clin Endocrinol Metab 95:2476-2485
13. **Huang J, Zhang J, Lei T, Chen X, Zhang Y, Zhou L, Yu A, Chen Z, Zhou R, Yang Z** 2010 Cloning of porcine chemerin, ChemR23 and GPR1 and their involvement in regulation of lipogenesis. BMB Rep 43:491-498
14. **Goralski KB, McCarthy TC, Hanniman EA, Zabel BA, Butcher EC, Parlee SD, Muruganandan S, Sinal CJ** 2007 Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. J Biol Chem 282:28175-28188
15. **Luangsay S, Wittamer V, Bondue B, De Henau O, Rouger L, Brait M, Franssen JD, de Nadai P, Huaux F, Parmentier M** 2009 Mouse ChemR23 is expressed in dendritic cell

subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. J Immunol 183:6489-6499
16. **Bondue B, Wittamer V, Parmentier M** 2011 Chemerin and its receptors in leukocyte trafficking, inflammation and metabolism. Cytokine Growth Factor Rev 22:331-338
17. **Cash JL, Hart R, Russ A, Dixon JP, Colledge WH, Doran J, Hendrick AG, Carlton MB, Greaves DR** 2008 Synthetic chemerin-derived peptides suppress inflammation through ChemR23. J Exp Med 205:767-775
18. **Tan BK, Chen J, Farhatullah S, Adya R, Kaur J, Heutling D, Lewandowski KC, O'Hare JP, Lehnert H, Randeva HS** 2009 Insulin and metformin regulate circulating and adipose tissue chemerin. Diabetes 58:1971-1977
19. **Wang Q, Kim JY, Xue K, Liu JY, Leader A, Tsang BK** 2012 Chemerin, a novel regulator of follicular steroidogenesis and its potential involvement in polycystic ovarian syndrome. Endocrinology 153:5600-5611
20. **Nuell MJ, Stewart DA, Walker L, Friedman V, Wood CM, Owens GA, Smith JR, Schneider EL, Dell' Orco R, Lumpkin CK, et al.** 1991 Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. Mol Cell Biol 11:1372-1381
21. **McClung JK, Jupe ER, Liu XT, Dell'Orco RT** 1995 Prohibitin: potential role in senescence, development, and tumor suppression. Exp Gerontol 30:99-124
22. **Rastogi S, Joshi B, Dasgupta P, Morris M, Wright K, Chellappan S** 2006 Prohibitin facilitates cellular senescence by recruiting specific corepressors to inhibit E2F target genes. Mol Cell Biol 26:4161-4171
23. **Roskams AJ, Friedman V, Wood CM, Walker L, Owens GA, Stewart DA, Altus MS, Danner DB, Liu XT, McClung JK** 1993 Cell cycle activity and expression of prohibitin mRNA. J Cell Physiol 157:289-295
24. **Oh TS, Kwon EY, Choi JW, Choi MS, Yun JW** 2011 Time-dependent hepatic proteome analysis in lean and diet-induced obese mice. J Microbiol Biotechnol 21:1211-1227
25. **Liu D, Lin Y, Kang T, Huang B, Xu W, Garcia-Barrio M, Olatinwo M, Matthews R, Chen YE, Thompson WE** 2012 Mitochondrial dysfunction and adipogenic reduction by prohibitin silencing in 3T3-L1 cells. PLoS One 7:e34315
26. **Thompson WE, Powell JM, Whittaker JA, Sridaran R, Thomas KH** 1999 Immunolocalization and expression of prohibitin, a mitochondrial associated protein within the rat ovaries. Anat Rec 256:40-48
27. **Thompson WE, Asselin E, Branch A, Stiles JK, Sutovsky P, Lai L, Im GS, Prather RS, Isom SC, Rucker E, 3rd, Tsang BK** 2004 Regulation of prohibitin expression during follicular development and atresia in the mammalian ovary. Biol Reprod 71:282-290
28. **Chowdhury I, Xu W, Stiles JK, Zeleznik A, Yao X, Matthews R, Thomas K, Thompson WE** 2007 Apoptosis of rat granulosa cells after staurosporine and serum withdrawal is suppressed by adenovirus-directed overexpression of prohibitin. Endocrinology 148:206-217
29. **Chowdhury I, Garcia-Barrio M, Harp D, Thomas K, Matthews R, Thompson WE** 2012 The emerging roles of prohibitins in folliculogenesis. Front Biosci (Elite Ed) 4:690-699
30. **Campbell KL** 1979 Ovarian granulosa cells isolated with EGTA and hypertonic sucrose: cellular integrity and function. Biol Reprod 21:773-786
31. **Wang H, Jiang JY, Zhu C, Peng C, Tsang BK** 2006 Role and regulation of nodal/activin receptor-like kinase 7 signaling pathway in the control of ovarian follicular atresia. Mol Endocrinol 20:2469-2482
32. **Brawer J, Schipper H, Robaire B** 1983 Effects of long term androgen and estradiol exposure on the hypothalamus. Endocrinology 112:194-199
33. **Silfen ME, Denburg MR, Manibo AM, Lobo RA, Jaffe R, Ferin M, Levine LS, Oberfield SE** 2003 Early endocrine, metabolic, and sonographic characteristics of polycystic

ovary syndrome (PCOS): comparison between nonobese and obese adolescents. J Clin Endocrinol Metab 88:4682-4688
34. **Fassnacht M, Schlenz N, Schneider SB, Wudy SA, Allolio B, Arlt W** 2003 Beyond adrenal and ovarian androgen generation: Increased peripheral 5 alpha-reductase activity in women with polycystic ovary syndrome. J Clin Endocrinol Metab 88:2760-2766
35. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408
36. **Mani AM, Fenwick MA, Cheng Z, Sharma MK, Singh D, Wathes DC** 2010 IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. Reproduction 139:139-151
37. **Alam H, Maizels ET, Park Y, Ghaey S, Feiger ZJ, Chandel NS, Hunzicker-Dunn M** 2004 Follicle-stimulating hormone activation of hypoxia-inducible factor-1 by the phosphatidylinositol 3-kinase/AKT/Ras homolog enriched in brain (Rheb)/mammalian target of rapamycin (mTOR) pathway is necessary for induction of select protein markers of follicular differentiation. J Biol Chem 279:19431-19440
38. **Zeleznik AJ, Saxena D, Little-Ihrig L** 2003 Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. Endocrinology 144:3985-3994
39. **Ande SR, Xu Z, Gu Y, Mishra S** 2011 Prohibitin has an important role in adipocyte differentiation. Int J Obes (Lond):1-9
40. **Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S** 2003 Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. J Biol Chem 278:47853-47861
41. **Joshi B, Rastogi S, Morris M, Carastro LM, DeCook C, Seto E, Chellappan SP** 2007 Differential regulation of human YY1 and caspase 7 promoters by prohibitin through E2F1 and p53 binding sites. Biochem J 401:155-166
42. **Yildiz BO, Azziz R** 2010 Ovarian and adipose tissue dysfunction in polycystic ovary syndrome: report of the 4th special scientific meeting of the Androgen Excess and PCOS Society. Fertil Steril 94:690-693
43. **Manneras L, Cajander S, Holmang A, Seleskovic Z, Lystig T, Lonn M, Stener-Victorin E** 2007 A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology 148:3781-3791
44. **Becker M, Rabe K, Lebherz C, Zugwurst J, Goke B, Parhofer KG, Lehrke M, Broedl UC** 2010 Expression of human chemerin induces insulin resistance in the skeletal muscle but does not affect weight, lipid levels, and atherosclerosis in LDL receptor knockout mice on high-fat diet. Diabetes 59:2898-2903
45. **Brasaemle DL, Dolios G, Shapiro L, Wang R** 2004 Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. J Biol Chem 279:46835-46842
46. **Vessal M, Mishra S, Moulik S, Murphy LJ** 2006 Prohibitin attenuates insulin-stimulated glucose and fatty acid oxidation in adipose tissue by inhibition of pyruvate carboxylase. FEBS J 273:568-576
47. **Miller WL** 1988 Molecular biology of steroid hormone synthesis. Endocr Rev 9:295-318
48. **Wayne CM, Fan HY, Cheng X, Richards JS** 2007 Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation. Mol Endocrinol 21:1940-1957
49. **Escamilla-Hernandez R, Little-Ihrig L, Zeleznik AJ** 2008 Inhibition of rat granulosa cell differentiation by overexpression of Galphaq. Endocrine 33:21-31
50. **Armstrong DT, Dorrington JH** 1976 Androgens augment FSH-induced progesterone secretion by cultured rat granulosa cells. Endocrinology 99:1411-1414

51. **Harlow CR, Shaw HJ, Hillier SG, Hodges JK** 1988 Factors influencing follicle-stimulating hormone-responsive steroidogenesis in marmoset granulosa cells: effects of androgens and the stage of follicular maturity. Endocrinology 122:2780-2787
52. **Pescador N, Houde A, Stocco DM, Murphy BD** 1997 Follicle-stimulating hormone and intracellular second messengers regulate steroidogenic acute regulatory protein messenger ribonucleic acid in luteinized porcine granulosa cells. Biol Reprod 57:660-668
53. **He B, Feng Q, Mukherjee A, Lonard DM, DeMayo FJ, Katzenellenbogen BS, Lydon JP, O'Malley BW** 2008 A repressive role for prohibitin in estrogen signaling. Mol Endocrinol 22:344-360
54. **Choi D, Lee SJ, Hong S, Kim IH, Kang S** 2008 Prohibitin interacts with RNF2 and regulates E2F1 function via dual pathways. Oncogene 27:1716-1725
55. **Boerboom D, Pilon N, Behdjani R, Silversides DW, Sirois J** 2000 Expression and regulation of transcripts encoding two members of the NR5A nuclear receptor subfamily of orphan nuclear receptors, steroidogenic factor-1 and NR5A2, in equine ovarian cells during the ovulatory process. Endocrinology 141:4647-4656
56. **Hinshelwood MM, Repa JJ, Shelton JM, Richardson JA, Mangelsdorf DJ, Mendelson CR** 2003 Expression of LRH-1 and SF-1 in the mouse ovary: localization in different cell types correlates with differing function. Mol Cell Endocrinol 207:39-45
57. **Reinhart AJ, Williams SC, Stocco DM** 1999 Transcriptional regulation of the StAR gene. Mol Cell Endocrinol 151:161-169
58. **Andrieu T, Pezzi V, Sirianni R, Le Bas R, Feral C, Benhaim A, Mittre H** 2009 cAMP-dependent regulation of CYP19 gene in rabbit preovulatory granulosa cells and corpus luteum. J Steroid Biochem Mol Biol 116:110-117
59. **Ande SR, Mishra S** 2009 Prohibitin interacts with phosphatidylinositol 3,4,5-triphosphate (PIP3) and modulates insulin signaling. Biochem Biophys Res Commun 390:1023-1028
60. **Han EK, McGonigal T, Butler C, Giranda VL, Luo Y** 2008 Characterization of Akt overexpression in MiaPaCa-2 cells: prohibitin is an Akt substrate both in vitro and in cells. Anticancer Res 28:957-963
61. **Ande SR, Gu Y, Nyomba BL, Mishra S** 2009 Insulin induced phosphorylation of prohibitin at tyrosine 114 recruits Shp1. Biochim Biophys Acta 1793:1372-1378
62. **Salvador LM, Park Y, Cottom J, Maizels ET, Jones JC, Schillace RV, Carr DW, Cheung P, Allis CD, Jameson JL, Hunzicker-Dunn M** 2001 Follicle-stimulating hormone stimulates protein kinase A-mediated histone H3 phosphorylation and acetylation leading to select gene activation in ovarian granulosa cells. J Biol Chem 276:40146-40155
63. **DeManno DA, Cottom JE, Kline MP, Peters CA, Maizels ET, Hunzicker-Dunn M** 1999 Follicle-stimulating hormone promotes histone H3 phosphorylation on serine-10. Mol Endocrinol 13:91-105
64. **Hunzicker-Dunn M, Maizels ET** 2006 FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. Cell Signal 18:1351-1359
65. **Vanhaesebroeck B, Alessi DR** 2000 The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 346 Pt 3:561-576
66. **Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ** 1998 Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science 281:2042-2045
67. **Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, Thomas G** 1998 Phosphorylation and activation of p70s6k by PDK1. Science 279:707-710
68. **Cheng X, Ma Y, Moore M, Hemmings BA, Taylor SS** 1998 Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. Proc Natl Acad Sci U S A 95:9849-9854

8.4 Journal of Ovarian Research, 2013, 6: 23 - 32

1. McGee EA, Hsueh AJ. **Initial and cyclic recruitment of ovarian follicles**. Endocr Rev 2000; **21**: 200-214.
2. Craig J, Orisaka M, Wang H, Orisaka S, Thompson W, Zhu C, Kotsuji F, Tsang BK. **Gonadotropin and intra-ovarian signals regulating follicle development and atresia: the delicate balance between life and death**. Front Biosci 2007; **12**: 3628-3639.
3. Woods DC, Haugen MJ, Johnson AL. **Actions of epidermal growth factor receptor/mitogen-activated protein kinase and protein kinase C signaling in granulosa cells from Gallus gallus are dependent upon stage of differentiation**. Biol Reprod 2007; **77**: 61-70.
4. Richards JS. **Hormonal control of gene expression in the ovary**. Endocr Rev 1994; **15**: 725-751.
5. Mishra S, Ande SR, Nyomba BL. **The role of prohibitin in cell signaling**. FEBS J 2010; **277**: 3937-3946.
6. Mishra S, Murphy LC, Nyomba BL, Murphy LJ. **Prohibitin: a potential target for new therapeutics**. Trends Mol Med 2005; **11**: 192-197.
7. Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S. **Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling**. J Biol Chem 2003; **278**: 47853-47861.
8. Brasaemle DL, Dolios G, Shapiro L, Wang R. **Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes**. J Biol Chem 2004; **279**: 46835-46842.
9. Mengwasser J, Piau A, Schlag P, Sleeman JP. **Differential immunization identifies PHB1/PHB2 as blood-borne tumor antigens**. Oncogene 2004; **23**: 7430-7435.
10. Kang X, Zhang L, Sun J, Ni Z, Ma Y, Chen X, Sheng X, Chen T. **Prohibitin: a potential biomarker for tissue-based detection of gastric cancer**. J Gastroenterol 2008; **43**: 618-625.
11. Nuell MJ, Stewart DA, Walker L, Friedman V, Wood CM, Owens GA, Smith JR, Schneider EL, Dell' Orco R, Lumpkin CK, et al. **Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells**. Mol Cell Biol 1991; **11**: 1372-1381.
12. Roskams AJ, Friedman V, Wood CM, Walker L, Owens GA, Stewart DA, Altus MS, Danner DB, Liu XT, McClung JK. **Cell cycle activity and expression of prohibitin mRNA**. J Cell Physiol 1993; **157**: 289-295.
13. McClung JK, Jupe ER, Liu XT, Dell'Orco RT. **Prohibitin: potential role in senescence, development, and tumor suppression**. Exp Gerontol 1995; **30**: 99-124.
14. Rastogi S, Joshi B, Dasgupta P, Morris M, Wright K, Chellappan S. **Prohibitin facilitates cellular senescence by recruiting specific corepressors to inhibit E2F target genes**. Mol Cell Biol 2006; **26**: 4161-4171.
15. Kasashima K, Ohta E, Kagawa Y, Endo H. **Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2**. J Biol Chem 2006; **281**: 36401-36410.
16. Nijtmans LG, de Jong L, Artal Sanz M, Coates PJ, Berden JA, Back JW, Muijsers AO, van der Spek H, Grivell LA. **Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins**. EMBO J 2000; **19**: 2444-2451.
17. Choi D, Lee SJ, Hong S, Kim IH, Kang S. **Prohibitin interacts with RNF2 and regulates E2F1 function via dual pathways**. Oncogene 2008; **27**: 1716-1725.
18. He B, Feng Q, Mukherjee A, Lonard DM, DeMayo FJ, Katzenellenbogen BS, Lydon JP, O'Malley BW. **A repressive role for prohibitin in estrogen signaling**. Mol Endocrinol 2008; **22**: 344-360.

19. Chowdhury I, Branch A, Olatinwo M, Thomas K, Matthews R, Thompson WE. **Prohibitin (PHB) acts as a potent survival factor against ceramide induced apoptosis in rat granulosa cells**. Life Sci 2011; **89**: 295-303.
20. Chowdhury I, Xu W, Stiles JK, Zeleznik A, Yao X, Matthews R, Thomas K, Thompson WE. **Apoptosis of rat granulosa cells after staurosporine and serum withdrawal is suppressed by adenovirus-directed overexpression of prohibitin**. Endocrinology 2007; **148**: 206-217.
21. Thompson WE, Asselin E, Branch A, Stiles JK, Sutovsky P, Lai L, Im GS, Prather RS, Isom SC, Rucker E, 3rd, Tsang BK. **Regulation of prohibitin expression during follicular development and atresia in the mammalian ovary**. Biol Reprod 2004; **71**: 282-290.
22. Chowdhury I, Garcia-Barrio M, Harp D, Thomas K, Matthews R, Thompson WE. **The emerging roles of prohibitins in folliculogenesis**. Front Biosci (Elite Ed) 2012; **4**: 690-699.
23. Wang Q, Leader A, Tsang B. **Inhibitory roles of prohibitin and chemerin on FSH-induced rat granulosa cell steroidogenesis**. Endocrinology 2013; **154**: 956-967.
24. Campbell KL. **Ovarian granulosa cells isolated with EGTA and hypertonic sucrose: cellular integrity and function**. Biol Reprod 1979; **21**: 773-786.
25. Wang H, Jiang JY, Zhu C, Peng C, Tsang BK. **Role and regulation of nodal/activin receptor-like kinase 7 signaling pathway in the control of ovarian follicular atresia**. Mol Endocrinol 2006; **20**: 2469-2482.
26. Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. **Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries**. J Clin Endocrinol Metab 2001; **86**: 1318-1323.
27. Jonard S, Dewailly D. **The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest**. Hum Reprod Update 2004; **10**: 107-117.
28. Wang Q, Kim JY, Xue K, Liu JY, Leader A, Tsang BK. **Chemerin, a novel regulator of follicular steroidogenesis and its potential involvement in polycystic ovarian syndrome**. Endocrinology 2012; **153**: 5600-5611.
29. Asselin E, Wang Y, Tsang BK. **X-linked inhibitor of apoptosis protein activates the phosphatidylinositol 3-kinase/Akt pathway in rat granulosa cells during follicular development**. Endocrinology 2001; **142**: 2451-2457.
30. Fusaro G, Wang S, Chellappan S. **Differential regulation of Rb family proteins and prohibitin during camptothecin-induced apoptosis**. Oncogene 2002; **21**: 4539-4548.
31. Boerboom D, Pilon N, Behdjani R, Silversides DW, Sirois J. **Expression and regulation of transcripts encoding two members of the NR5A nuclear receptor subfamily of orphan nuclear receptors, steroidogenic factor-1 and NR5A2, in equine ovarian cells during the ovulatory process**. Endocrinology 2000; **141**: 4647-4656.
32. Hinshelwood MM, Repa JJ, Shelton JM, Richardson JA, Mangelsdorf DJ, Mendelson CR. **Expression of LRH-1 and SF-1 in the mouse ovary: localization in different cell types correlates with differing function**. Mol Cell Endocrinol 2003; **207**: 39-45.
33. Reinhart AJ, Williams SC, Stocco DM. **Transcriptional regulation of the StAR gene**. Mol Cell Endocrinol 1999; **151**: 161-169.
34. Andrieu T, Pezzi V, Sirianni R, Le Bas R, Feral C, Benhaim A, Mittre H. **cAMP-dependent regulation of CYP19 gene in rabbit preovulatory granulosa cells and corpus luteum**. J Steroid Biochem Mol Biol 2009; **116**: 110-117.
35. Ny T, Wahlberg P, Brandstrom IJ. **Matrix remodeling in the ovary: regulation and functional role of the plasminogen activator and matrix metalloproteinase systems**. Mol Cell Endocrinol 2002; **187**: 29-38.
36. Reich R, Miskin R, Tsafriri A. **Intrafollicular distribution of plasminogen activators and their hormonal regulation in vitro**. Endocrinology 1986; **119**: 1588-1593.

37. Karakji EG, Tsang BK. **Follicular stage-dependent regulation of rat granulosa cell plasminogen activator system by transforming growth factor-alpha in vitro**. Biol Reprod 1995; **52**: 411-418.
38. Karakji EG, Tsang BK. **Tumor necrosis factor alpha inhibits rat granulosa cell plasminogen activator activity in vitro during follicular development**. Biol Reprod 1995; **52**: 745-752.
39. Noubani A, Farookhi R, Gutkowska J. **B-type natriuretic peptide receptor expression and activity are hormonally regulated in rat ovarian cells**. Endocrinology 2000; **141**: 551-559.
40. Thompson WE, Powell JM, Whittaker JA, Sridaran R, Thomas KH. **Immunolocalization and expression of prohibitin, a mitochondrial associated protein within the rat ovaries**. Anat Rec 1999; **256**: 40-48.
41. Simoni M, Gromoll J, Nieschlag E. **The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology**. Endocr Rev 1997; **18**: 739-773.
42. Minegishi T, Tano M, Kishi H, Kameda T, Miyamoto K. **Follicle-stimulating hormone regulation on its receptor messenger ribonucleic acid levels in cultured rat granulosa cells**. Biochim Biophys Acta 1997; **1359**: 165-173.
43. Scheetz D, Folger JK, Smith GW, Ireland JJ. **Granulosa cells are refractory to FSH action in individuals with a low antral follicle count**. Reprod Fertil Dev 2012; **24**: 327-336.
44. Pellatt L, Rice S, Dilaver N, Heshri A, Galea R, Brincat M, Brown K, Simpson ER, Mason HD. **Anti-Mullerian hormone reduces follicle sensitivity to follicle-stimulating hormone in human granulosa cells**. Fertil Steril 2011; **96**: 1246-1251 e1241.
45. Wayne CM, Fan HY, Cheng X, Richards JS. **Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation**. Mol Endocrinol 2007; **21**: 1940-1957.
46. Fang WL, Lee MT, Wu LS, Chen YJ, Mason J, Ke FC, Hwang JJ. **CREB coactivator CRTC2/TORC2 and its regulator calcineurin crucially mediate follicle-stimulating hormone and transforming growth factor beta1 upregulation of steroidogenesis**. J Cell Physiol 2012; **227**: 2430-2440.
47. Mayr B, Montminy M. **Transcriptional regulation by the phosphorylation-dependent factor CREB**. Nat Rev Mol Cell Biol 2001; **2**: 599-609.
48. Saxena D, Escamilla-Hernandez R, Little-Ihrig L, Zeleznik AJ. **Liver receptor homolog-1 and steroidogenic factor-1 have similar actions on rat granulosa cell steroidogenesis**. Endocrinology 2007; **148**: 726-734.
49. Ande SR, Xu Z, Gu Y, Mishra S. **Prohibitin has an important role in adipocyte differentiation**. Int J Obes (Lond) 2011: 1-9.
50. Bayascas JR, Alessi DR. **Regulation of Akt/PKB Ser473 phosphorylation**. Mol Cell 2005; **18**: 143-145.
51. Liu WK, Bousfield GR, Moore WT, Jr., Ward DN. **Priming procedure and hormone preparations influence rat granulosa cell response**. Endocrinology 1985; **116**: 1454-1459.
52. Zeleznik AJ, Saxena D, Little-Ihrig L. **Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation**. Endocrinology 2003; **144**: 3985-3994.
53. Escamilla-Hernandez R, Little-Ihrig L, Zeleznik AJ. **Inhibition of rat granulosa cell differentiation by overexpression of Galphaq**. Endocrine 2008; **33**: 21-31.
54. Armstrong DT, Dorrington JH. **Androgens augment FSH-induced progesterone secretion by cultured rat granulosa cells**. Endocrinology 1976; **99**: 1411-1414.
55. Harlow CR, Shaw HJ, Hillier SG, Hodges JK. **Factors influencing follicle-stimulating hormone-responsive steroidogenesis in marmoset granulosa cells: effects of androgens and the stage of follicular maturity**. Endocrinology 1988; **122**: 2780-2787.