The Role of EGFR Signaling in Gonadotropin-Induced Steroidogenesis

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DEDICATION

I would like to dedicate this dissertation to my loving husband for his endless support and motivation.

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by

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DISSERTATION

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I would like to acknowledge my mentor Stephen Hammes who encouraged me to become a better scientist. To all past and present members for making lab a fun place to learn and experience research to the fullest.

THE ROLE OF EGFR SIGNALING IN GONADOTROPIN-INDUCED STEROIDOGENESIS

Liliana Carbajal, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2010

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Recent evidence has demonstrated that cross talk between G protein-coupled receptors and Epidermal Growth Factor Receptor (EGFR) is critical for steroidogenesis in all three major steroid-producing tissues. We have recently characterized the intracellular signals regulating Luteinizing Hormone (LH) -induced steroid production in Leydig cells, demonstrating a linear pathway whereby LH receptor activation stimulates cAMP production and PKA signaling. Protein Kinase A (PKA) signaling then triggers EGF receptor activation, which activates the Mitogen Activated Protein Kinase (MAPK) cascade to promote steroidogenic acute regulatory (StAR) phosphorylation and translocation to the mitochondria. Interesting, PKA-mediated transactivation of the EGF receptor occurs via both intracellular, ligand-independent signaling, as well as extracellular, ligand-dependent activation that requires Matrix Metalloproteinase (MMP)-mediated release of membrane-bound EGF receptor ligands. However, only intracellular signaling is required for LH-induced steroid production. Furthermore, the LH-EGFR pathway appears to be important only for early steroidogenesis in Leydig cells, as LH-

induced steroidogenesis beyond 60 minutes no longer requires EGFR or MAPK signaling.

Here we characterize the LH-induced signals that regulate steroidogenesis in the ovary. We demonstrate that, similar to Leydig cells, activation of the EGF receptor is important for gonadotropin-induced steroid production in the ovary.

Trans-activation of the EGFR is mediated by an increase of cAMP and PKA signaling. However, steroidogenesis in the ovary is dependent on an extracellular ligand-independent mechanism, as MMPs were shown to be crucial for the cleavage and activation of membrane bound EGFR upon LH stimulation. EGFR signaling was shown to activate the MAPK cascade and lead to subsequent phosphorylation of the steroidogenic acute regulatory protein (StAR). Interestingly, EGFR and MAPK signaling, unlike that in Leydig cells, as necessary for short and long term gonadotropin-induced steroid production in the ovary. *In vivo* studies further demonstrate the importance of EGFR signaling since serum progesterone levels were significantly reduced when EGFR was inhibited.

These findings demonstrate physiologic importance and potential treatment options since women with endocrine disorder such as PCOS can be treated with EGFR antagonist to reduce excess steroid production in the ovary.

TABLE OF CONTENTS

PUBLICATIONS

Carbajal L, Prizant H. Hammes SR. EGFR trans-activation is important for regulating Gonadotropin-induced steroidogenesis in the ovary and adrenal gland.
2010. Future manuscript in preparation.

Carbajal L, Deng J, Dressing GE, Hagan CR, Lange CA, Hammes SR.
Steroids. 2009 Meeting review: Extra-nuclear steroid receptors-Integration with multiple signaling pathways. Jul; 74 (7): 551-4.

Deng J, Carbajal L, Evaul K, Rasar M, Jamnongjit M, Hammes SR. Nongenomic steroid-triggered oocyte maturation: of mice and frogs. Steroids. 2009 Jul; 74(7): 595-601.

LIST OF FIGURES

LIST OF DEFINITIONS

ACTH - Adrenocorticotropic hormone

AG1478- Tyrphostin, an EGFR inhibitor

AR – Androgen Receptor

EGFR – Epidermal Growth Factor Receptor

FSH – Follicle Stimulating Hormone

GnRH – Gonadotropin-Releasing Hormone

GPCR – G protein-coupled receptor

H89 – a PKA inhibitor

hCG – Human Chorionic Gonadotropin

HPA axis - Hypothalamic–pituitary–adrenal axis

HPG axis- Hypothalamic-pituitary-gonadal axis

LH – Luteinizing Hormone

MAPK- Mitogen Activated Protein kinase

MMP- Matrix Metalloproteinases

OGCs- oocyte-granulosa cell complexes

PCOS- Polycystic Ovary Syndrome

PKA – Protein Kinase A

PMSG- pregnant mare serum gonadotropin

PNA- PNA mice were provided prenatal androgen to induce PCOS

PR – Progesterone Receptor

StAR – Steroidogenic Acute Regulatory Protein

U0126- An inhibitor of MEK1 and MEK2

CHAPTER 1

General Introduction

1.1 HPA/HPG axis regulation

Three tissues are mainly responsible for the production of steroid hormones: the ovary, the testis, and the adrenal gland. In the testes and ovary, steroid production is regulated by the hypothalamic-pituitary-gonadal axis where the hypothalamus secrets gonadotropin releasing hormone (GnRH) that acts upon the anterior portion of the pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) (1,2). These hormones then stimulate the gonads to synthesize and release sex steroids such as testosterone, estrogen and progesterone. Similarly, adrenal steroid production is regulated by the Hypothalamic-pituitary-adrenal axis (HPA) where adrenocorticotropin-releasing hormone (CRH) is released from the hypothalamus and transported to the anterior pituitary to stimulate corticotroph cells to release adrenocorticotropic hormone (ACTH), which subsequently elicits corticosteroid production from the adrenal cortex. These pathways are essential for both the gonads and the adrenal gland to assure proper reproduction and maintain glucose homeostasis, respectively.

1.2 G protein -coupled receptors and Receptor Tyrosine Kinase Crosstalk

G protein-coupled receptors are seven-transmembrane receptors that couple to heterotrimeric G proteins and activate downstream signaling cascades via their cytoplasmic domains (3,4). Once stimulated, a conformation change occurs that allows the receptor to transduce guanine nucleotide exchange of bound GDP for GTP on the G_{α} subunit. This exchange results in the dissociation of the G alpha subunit bound to GTP. $G\alpha_s$ and $G\beta\gamma$ are then able to regulate second messengers such as adenylyl cyclase, phosphlipase C, and many others (Fig 1.1).

Trans-activation is a relatively new concept for plasma membrane receptors in which a GPCR ligand can activate a growth factor in the absence of its ligand. (5). One of the most studied examples of this process is the activation of specific receptor tyrosine kinases (RTKs) by GPCRs. GPCR agonists are able to transactivate a number of different RTKs such as the platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and the epidermal growth factor receptor (EGFR) among others. Their transactivation ultimately leads to a number of cellular processes such as proliferation, differentiation, migration and survival (6).

The mechanisms by which a GPCR can transactivate a RTK have been largely studied. Two very distinct mechanisms have been identified. The first one relies on the activation of matrix metalloprotease (MMP) by GPCR followed by MMP cleavage and shedding of RTK ectodomains that then activate a cognate receptor (7,8). This mechanism is known as ligand dependent or extracellular activation. The second mode of

activation is ligand independent, where by, RTK activation occurs via a GPCR initiated intracellular mechanism and will be further described in the next chapter.

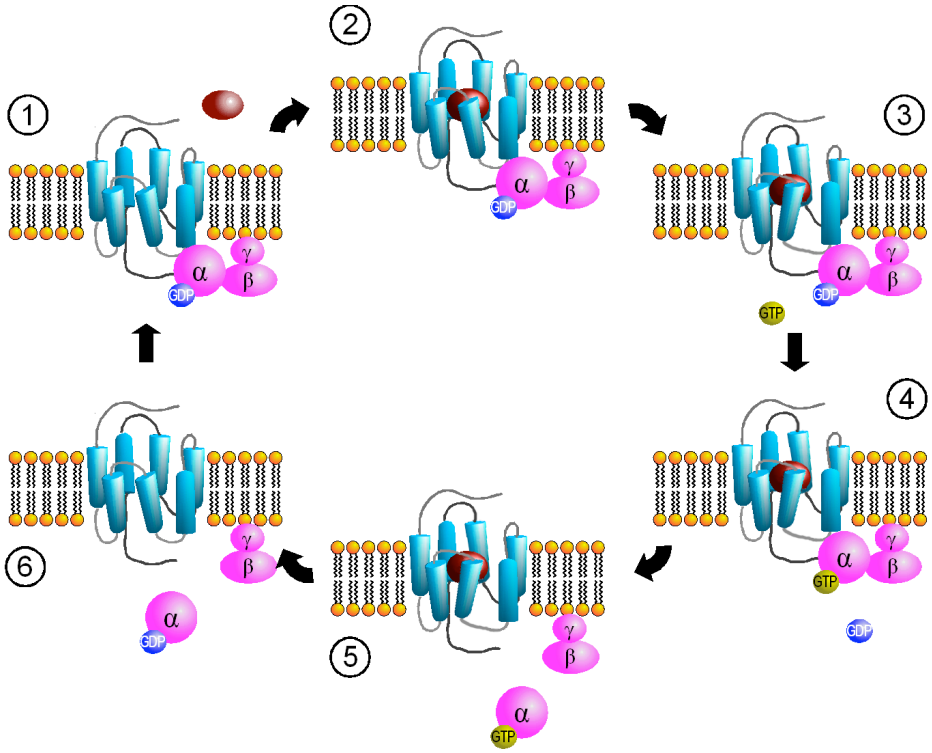

Figure 1.1 GPCR Activation. Ligand binding induces a conformation change that allows the receptor to transduce guanine nucleotide exchange of bound GDP for GTP on the G_{α} subunit. This exchange results in the dissociation of the G alpha subunit bound to GTP. G_{α} and $G_{\beta\gamma}$ are then able to regulate second messengers.

http://xray.bmc.uu.se/lars/Practicals/Signal/trans_app.html

Epidermal Growth Factor Receptor (EGFR)

As previously mentioned, the LH receptor is critical for proper male and female reproduction in that it regulates gonadal development and steroid production in both the testes and the ovaries. It is now known that LH, a GPCR agonist can transactivate the EGFR and induce oocyte maturation, ovulation and steroid production in the ovary (9,10,11). In the testes, the LH receptor has been shown to transactivate the EGFR via both mechanisms, but only the intracellular mechanism is needed to regulate steroid production (12, 42).

The EGFR is a cell surface receptor that contains an intracellular domain with tyrosine kinase activity (3). The EGFR family includes the EGFR, ERBB2, ERBB3 and ERBB4. Although the natural ligand is EGF, the following are members of the membrane-associated ligands that represent a specialized set of EGFR ligands including amphiregulin (AREG), betacellulin (BTC), heparin-binding EGF like growth factor (HBEGF), transforming growth factor alpha (TGFA), epiregulin (EREG), and epigen (EPGN). Upon ligand binding, EGFR undergoes a transformational change from an inactive monomer to an active homodimer. Once dimerization occurs, its intracellular protein-tyrosine kinase activity is activated and autophosphorylation of several tyrosine residues in the C-terminal domain take place. A vast number of proteins can then bind the tyrosine residues through their SH2 domains and elicit signal transduction cascades such as MAPK, Akt and JNK signaling (13). Disruption of EGFR signaling has been shown to have direct effects in mammalian reproduction. For example, transgenic mice with a mutant EGFR allele have been shown to have decreased LH secretion resulting in

abnormal estrus cycle. Also, pharmacological studies have shown that EGFR is necessary for proper follicular development such as steroidogenesis and oocyte maturation (9,10).

Matrix Metalloproteinase (MMP)

Matrix metalloproteinases (MMP) are a specific family of proteolytic enzymes that are known to play critical roles in many biological processes including embryonic development, organ morphogenesis, angiogenesis and recently, mammalian reproduction (14). One of their main functions is to control extracellular matrix (ECM) turnover by degrading extracellular proteins (15). The MMP family consists of 25 members that are divided into collagenases (MMPs1, 8,13), gelatinases (MMPs 2,9), stromelysins (MMPs 3,7,10,11) and membrane-type enzymes (MT-MMPs) (reviewed in 14,15). Most MMPs have an N-terminal signal sequence that directs their synthesis to the endoplasmic reticulum followed by a pro-peptide domain to maintain enzyme latency, a catalytic domain and a hampering domain connected to the catalytic domain by a linker region. The catalytic domain contains a zinc-binding region and controls cleavage- site specificity (14). Due to their ability to induce serious developmental problems and disease states, MMP activity is tightly regulated at the transcriptional and post-transcriptional level.

As previously mentioned, GPCR can trans-activate receptor tyrosine kinases such as the EGFR. This trans-activation has been shown to be MMP dependent in certain systems such as oocyte maturation and in cardiac fibroblasts. Exactly which MMPs are activated by GCPRs is still largely unknown.

1.3 The Steroidogenic pathway

Steroidogenesis is the process of steroid hormone production from cholesterol (16). This process commonly occurs in the gonads and adrenal glands. In the gonads, progesterone, estradiol, and testosterone are produced from cholesterol where corticosterone, cortisol, and aldosterone are synthesized in the adrenal gland (Figure 1.2). A number of enzymes are required for the biosynthesis of each steroid hormone and are categorized into two major classes: the cytochrome P450 heme-containing proteins, and hydroxysteroid dehydrogenases (16). The initial rate-limiting step is the transport of cholesterol from the outer to the inner mitochondrial membrane by the Steroidogenic Acute Regulatory Protein (StAR). Once cholesterol is transported, it is then metabolized to pregnenolone by CYP11A1 enzyme. Pregnenolone is then metabolized by 3ß-HSD to progesterone. In the adrenal gland, progesterone is further metabolized to corticosterone in mice, and cortisol in humans where as in the gonads; progesterone is ultimately converted to androgen and estradiol. Steroidogenesis is a tightly regulated process since the enzyme and substrate must be both present in the same cell. As will be discussed later in detail, regulation of steroid synthesis in the ovary and the testis involves a similar mechanism. However, due to the structural complexity of the ovary in comparison to the testis: paracrine signaling is essential for the ovary to produce steroid hormones such as estrogen and progesterone.

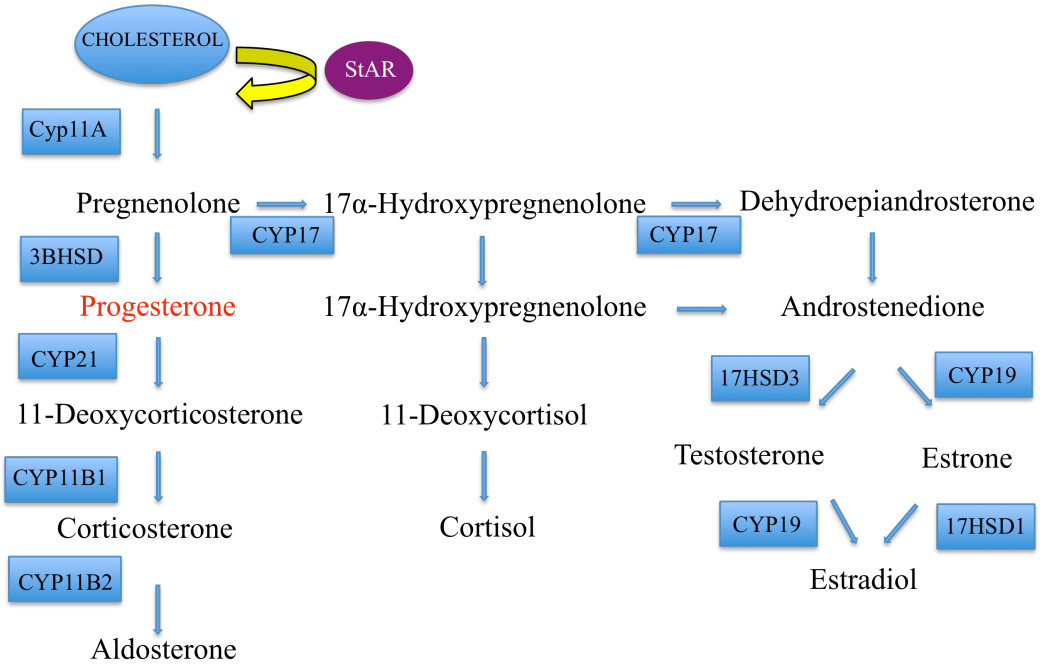

FIG. 1.2. Biosynthesis of steroid hormones in adrenal glands and gonads
Steroid byosynthesis is regulated by a number of different enzymes classified into two groups, the cytochrome P450s (Cyps) and hydroxysteroid dehydrogenases (HSDs). The end result is the production of corticosterone in the adrenal gland, testosterone in the testes, and estradiol in the ovary.

1.4 Steroidogenesis in the testes

The testes are part of the reproductive and endocrine system that are responsible for sperm production and male hormone synthesis. They are primarily made up of two cells types: Sertoli and Leydig cells. Sertoli cells help sustain spermatogenesis while Leydig cells produce and secrete androgens such as testosterone (17). Leydig cells have LH receptors that regulate steroid production via the HPG axis. As explained earlier, the LH receptor is a G protein - coupled receptor that when activated, induces cAMP and leads to downstream activation of PKA signaling. cAMP signaling and PKA activation have been shown to induce StAR activity, the rate-limiting step in steroidogenesis (18-20). Once StAR is phosphorylated, it is then believed to be activated and therefore able to translocate cholesterol to the inner mitochondrial membrane where the steroidogenic enzymes are found.

Prior work identified the EGFR as a possible regulator of steroidogenesis in the testes. EGF was shown to increase StAR expression in Leydig cells and human chorionic gonadotropin (hCG) was able to induce phosphorylation of the EGFR (18-21). Studies from the Stocco group demonstrated that EGF can promote, although at lower levels, steroidogenesis in Leydig cells (57). Even more directly, pharmacologic inhibition of the EGFR resulted in a loss of LH-induced steroidogenesis in Leydig cells (11). The mechanism that induces EGFR signaling from the LH stimulus and ultimately leads to StAR-dependent steroidogenesis was unknown until recently.

Recent work has established the mechanism by which the EGFR leads to LH-induced steroid production through a series of *in vitro* and *in vivo* experiments. First, it

was demonstrated that LH induces phosphorylation of the EGFR and that EGFR signaling is necessary for short -term but not long-term steroidogenesis in Leydig cells (12). In support of previous studies, cAMP and PKA activation were required for phosphorylation and activation of the EGFR in gonadotropin-induced steroid production. EGFR signaling induced activation of the MAPK cascade and was also shown to be important for short term and not long-term gonadotropin-induced steroid production in Leydig cells. Activation of the MAPK cascade ultimately resulted in StAR phosphorylation and mitochondrial translocation. This work also demonstrated that activation of the EGFR in Leydig cells occurs by an intracellular mechanism, since inhibition of MMPs had no affect on gonadotropin- induced steroidogenesis. In summary, EGFR/MAPK signaling in Leydig cells is important for early activation and steroid production but not late *in vitro* (Figure 1.3). However, an *in vivo* experiment where male mice were treated with the EGFR inhibitor AG1478 for 36 hours resulted in significantly reduced serum testosterone levels. These experiments again confirmed the physiological importance of LH and EGFR cross talk in regulating steroid synthesis in the testes. It is possible that long-term regulation does not require EGFR signaling since cAMP/PKA can mediate high StAR protein expression and therefore override the need for phosphorylation via MAPK signaling. However, *in vivo* experiments demonstrated that long-term inhibition of EGFR could still suppress testosterone levels. This difference may be due to normal LH cycling in males that results in a resensitized EGFR to the LH stimuli, and therefore EGFR signaling (12).

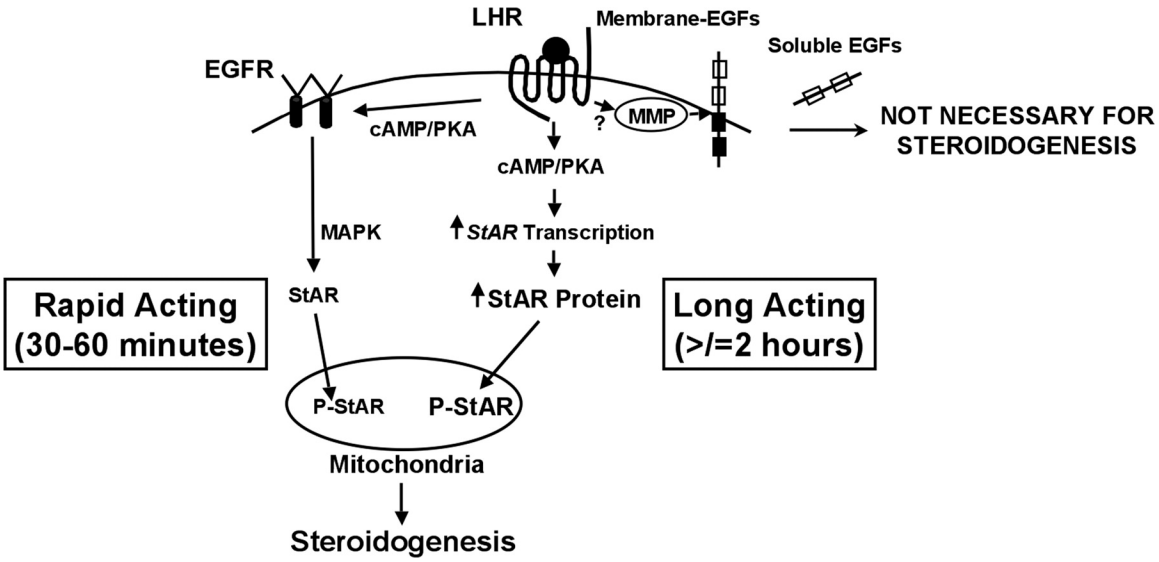

Figure 1.3. Model for gonadotropin-mediated steroidogenesis in Leydig cells. Gonadotropin (LH or hCG) activates the LH receptor, leading to a rapid and prolonged increase in cAMP levels and subsequent PKA activity. Early increases in PKA signaling trans-activate the EGFR independent of ligand (left side of figure), leading to activation of the MAPK signaling pathway. By mechanisms that are still not known, MAPK activation leads to phosphorylation and translocation of small levels of existing StAR to the mitochondria, resulting in increased steroidogenesis. Prolonged PKA signaling leads to increased transcription of StAR mRNA, followed by increased StAR protein expression (middle of figure). By 2 h, significant MAPK signaling is no longer occurring nor is it required for StAR phosphorylation and translocation to the mitochondria. Note that LH receptor activation also triggers trans-activation of the EGFR via MMP activation (right side of figure); however, this process is not necessary for steroid production at any time point.

Evaul K , Hammes S R J. Biol. Chem. 2008;283:27525-27533

1.5 Follicle Structure / Two cell model for Steroidogenesis in the ovary

The ovarian follicle is an important unit in female reproduction. It is a complex multilayer structure that encases the developing oocyte. In order to understand how oocyte maturation occurs, understanding the functions of each cell type in the follicle is important. The outer layer of the mammalian follicle is made up of theca cells, which then surround two types of granulosa cells: the mural and the cumulus cells. The cumulus cells surround the oocyte (32,33). (Figure 1.6). The LH surge initiates remodeling of all follicular cell types: mural granulosa cells differentiate into luteal cells; reentry into meiosis, and cumulus cell expansion (36). What is interesting about the LH stimuli is its LH receptor (LHR) expression. The LHR is almost exclusively expressed in theca cells during the early stages of follicular development. Later in development, LHR is expressed in mural granulosa cells, but remains absent in cumulus cells and the oocyte. Therefore, LHR can induce specific effects in cumulus cells and the oocyte despite its restricted expression. So how is this occurring? Experiments have now demonstrated that paracrine signals are responsible for transmitting the LH stimulus and will be discussed in further detail later in this chapter.

Steroidogenesis in most mammals is regulated via a two-cell/two gonadotropin model, where LH-stimulated theca cells produce androgens from cholesterol. Next, FSH stimulated granulosa cells convert androgens to estrogen. Both theca and granulosa cells express the necessary enzymes for progesterone production from cholesterol; however, granulosa cells cannot metabolize progesterone to androgen since they lack the appropriate enzyme. Consequently, the progesterone production and secretion that is required for proper ovulation is produced from granulosa cells.

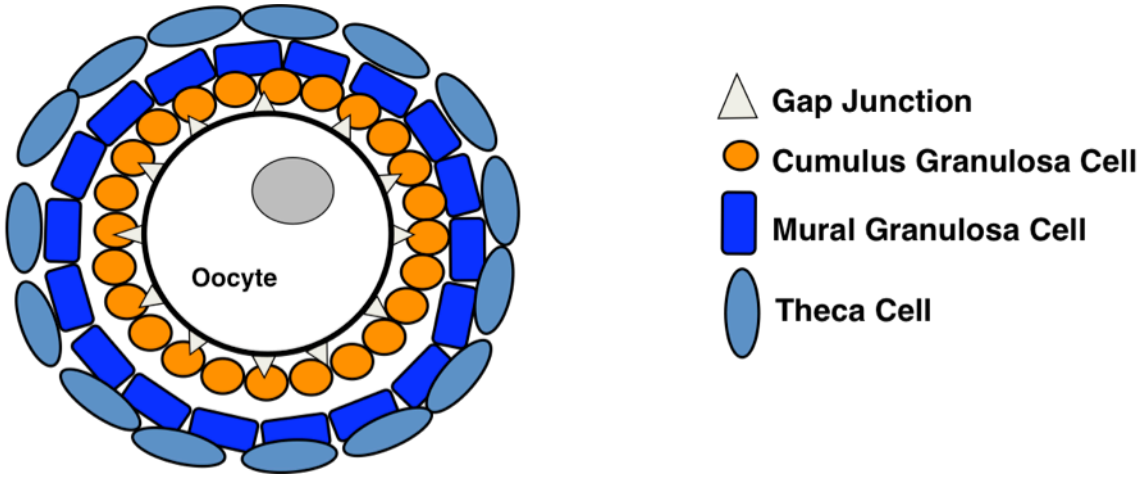

Figure 1.4. Ovarian Follicle Structure.

The ovary is a complicated system since it is made up of different layers and cell types. The immature oocyte (depicted here by the nucleus) is surrounded by cumulus granulosa cells. Cumulus cells are followed by mural granulosa cells and then by outer theca cells. While LH signaling affects remodeling of all follicular cell types, only theca cells express the LH receptor.

1.6 Ovarian Steroid Production

Ovarian steroid production begins with the transfer of cellular cholesterol located in the outer mitochondrial membrane to the inner mitochondrial membrane. This is where cytochrome P450 side chain cleavage enzyme (P450scc) converts cholesterol to pregnenolone. The rate-limiting step of steroidogenesis is the steroidogenic acute regulatory protein (StAR), since it is now known to mediate this transfer. StAR expression regulates steroid synthesis in the MA-10 leydig cell line or COS-1 cells even in the absence of hormonal stimulation. Also, humans and mice with mutations in the StAR gene are born with congenital lipid adrenal hyperplasia, a disease characterized by a rear absence of steroid hormones, and subsequent cholesterol/lipid accumulation in cells In males, StAR controls androgen production since humans lacking this gene have female characteristics. Females who are born with mutations in StAR have ovarian failure (101,102). Although the precise mechanism by which StAR regulates cholesterol transport is still controversial, several known regulators of StAR have been identified. StAR protein expression is tightly regulated in all steroidogenic tissues. As previously mentioned, the LHR is a G protein-coupled receptor that activates several downstream signaling mechanisms. These downstream messengers are known to be regulators of StAR.

The LHR coupled to $G\alpha_s$ induces adenylyl cyclase leading to increased levels of cAMP and PKA activity. PKA can then influence the activity of specific players of the steroidogenic pathway. Activating adenylyl cyclase by a number of different stimuli, such as FSH, LH or forskolin has been shown to enhance the transcription of StAR (32).

cAMP- dependent StAR expression is associated with increases of steroidogenic factor - 1 (SF-1), and CCAAT/enhancer binding protein-B activity (102-104), making them positive transcriptional regulators of StAR. PKA can also regulate StAR by phosphorylation of the serine 195 residue.

cAMP has been shown to activate the extracellular-regulated kinase (ERK) proteins (105). This family of MAPK signaling molecules is phosphorylated via a cAMP-dependent manner and has been correlated to increased StAR expression and progesterone production in porcine granulosa cells. However, studies in rat granulosa cells demonstrated a decrease in StAR and progesterone production. These varying results suggest further experiments are needed in order to understand the physiological role of the MAPK pathway in ovarian steroidogenesis (105,106).

cAMP-independent activation of steroidogenesis has also been demonstrated to occur in the ovary. For example, the LHR coupled to Gi proteins leads to activation of the phospholipase C pathway (PLC). Second messengers of this pathway include inositol-1,4,5-triphosphate (IP3), and diacylglycerol (DAG). These signal transduction pathways ultimately lead to changes in intracellular calcium and activation of protein kinase C (PKC).

Several growth factors have also been shown to modulate the actions of LH and FSH. Insulin can mediate increases of steroidogenesis by cAMP-stimulation of StAR and gonadotropin-dependent increase of P450scc in pig, bovine, and human granulosa cells. Insulin-like growth factors are thought to signal through the IGF1 receptor that may

induce activation of the PI3-K and MAPK signaling cascades (107). Finally, another growth factor has been recently implicated in ovarian steroidogenesis. The EGFR is a downstream signaling molecule that is activated by the LHR. As previously explained, the EGFR can promote steroidogenesis in Leydig cells and studies in follicles have also demonstrated its role in ovarian steroidogenesis primarily by promoting StAR expression and activity. To date, studies support a mechanism as follows; LH activates MMPs in theca cells, which in turn trigger the release of membrane bound EGF molecules. These molecules then act as paracrine signals to activate the EGFR, increasing StAR and steroid production.

Steroids are necessary for proper follicular development as well as ovulation and pregnancy. Aside from these functions, several labs have now demonstrated that steroids can promote mammalian oocyte maturation both *in vitro* and *in vivo*, the physiologic importance of steroids in this process remains uncertain. However, evidence suggests that steroid production may at least play a partial role in regulating LH-induced oocyte maturation in mice.

1.7 Lessons from Xenopus laevis

Oocyte maturation occurs when meiosis is initiated in the oocyte: resulting in a fertilizable egg. A lot of what is currently known about oocyte maturation came from studies performed using *Xenopus laevis*. It has been demonstrated that Xenopus oocytes are held in meiotic arrest by constitutive G protein signals until a stimulus initiates maturation. This stimulus in Xenopus is now known to be steroids that act via classical steroid receptors (24,25). Oocytes are held at meiotic arrest via G protein activation of adenylyl cyclase, which leads to increased intracellular cAMP (26,27). However, during ovulation, gonadotropins are secreted that result in steroid production. The steroid that has been shown to be primarily responsible for oocyte maturation in the Xenopus oocyte is testosterone (27). Maturation of the oocyte occurs when constitutive G protein signals are attenuated by activation of the androgen receptor. Once G protein signals are reduced, a drop of intracellular cAMP occurs, and downstream kinases such as the MAPK cascade are then activated (28, 31). MAPK activation along with the scaffolding protein, paxillin, allow for cell cycle regulators, such as cdc25 and CDK1, to trigger re-entry into meiosis (Figure 1.5).

In higher vertebrates, such as mammals, the stimulus that results in oocyte maturation is still controversial. However, decreased cAMP levels in most species have been shown to be necessary for maturation to initiate (29). Similar signaling pathways are also activated in the mouse oocyte at the onset of maturation. In contrast to the *Xenopus* oocyte, mouse oocytes spontaneously mature when they are removed from the ovary. These findings suggest that in mouse oocytes, the inhibitory signals come from the ovary

and not from within the oocyte (24,30). Although many studies have attempted to identify the stimulus that induced oocyte maturation in mammals, it has been difficult to identify, due to spontaneous maturation upon removal from the ovary.

Carefully executed experiments have suggested that the stimulus for mammalian oocyte maturation may also be steroids. One factor that is produced by cumulus granulosa cells in response to LH and that also regulates oocyte maturation is steroid. In fact, the same LH/EGF receptor network that regulates oocyte maturation also mediates LH-induced steroidogenesis in the ovary (11). Like oocyte maturation, EGF receptor activation is sufficient to promote rapid steroid production in oocyte-granulosa cell complexes (OGCs) and cumulus-oocyte complexes (COCs). Also similar to oocyte maturation, LH-mediated steroidogenesis is blocked by inhibitors of metalloproteinases and EGF receptor signaling, indicating that cleavage of membrane-bound EGF receptor ligands and downstream activation of the EGFR signaling in cumulus cells are critical processes regulating steroidogenesis. Several steroids were shown to promote mammalian oocyte maturation *in vitro*, with progesterone levels being the highest in most mammals at the time of the LH surge (11,41). In addition, progesterone actions via the progesterone receptor are known to be essential for ovulation, as mice lacking progesterone receptors do not ovulate in response to gonadotropin (39). These observations suggest that steroids, possibly LH-induced progesterone, may be playing at least a partial role in regulating oocyte maturation and subsequent ovulation.

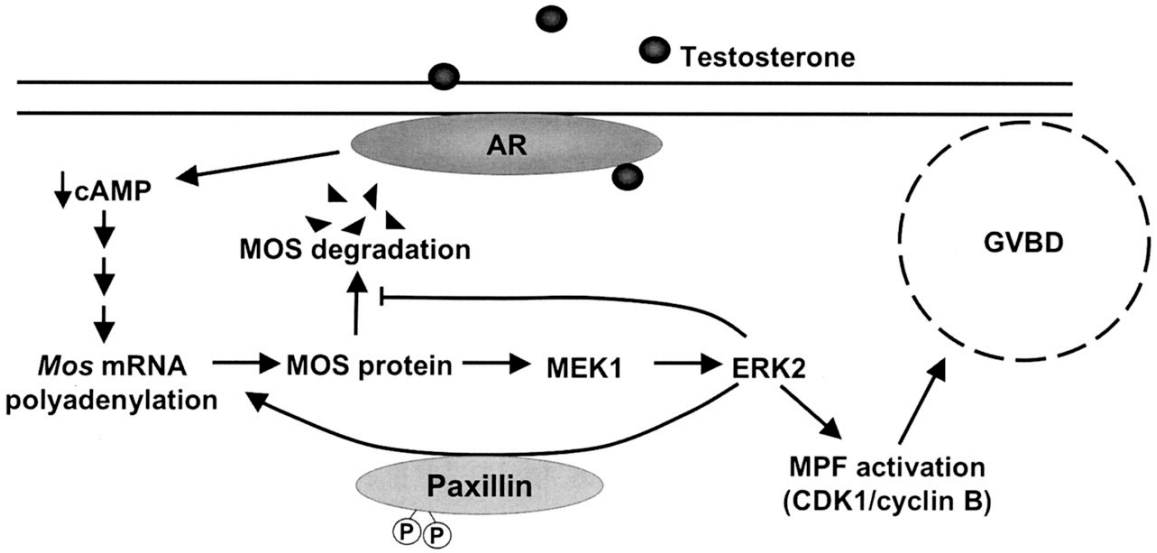

Figure 1.5. Oocyte Maturation in *the Xenopus laevis*.

Steroid stimulation of oocytes via the classic androgen receptor triggers a decrease in intracellular cAMP. Decreased cAMP results in polyadenylation of Mos mRNA leading to Mos protein stability. Mos activation then leads to activation of the MAPK pathway and the scaffolding protein paxillin. Together, this positive feedback loop allows activation of CDK1/cyclin B to drive Germinal vesicle breakdown (GVBD) or oocyte maturation.

Rasar M et al. J. Biol. Chem. 2006;281:39455-39464

1.8 Oogenesis

Oogenesis is the process in development that leads to the production of the female gametes. Oogenesis varies greatly among species. In lower species such as fish and frogs, females can produce thousands of eggs at once. Most mammals however, only produce a small number of eggs in their lifetime (22). Most oogonia die during the months of embryonic development, while the surviving oogonia enter the first meiotic division to become primary oocytes. These primary oocytes progress through the first meiotic prophase until the diplotene stage of division where they will be held at meiotic arrest during *in utero* development of the female. During sexual maturity, gonadotropins from the pituitary trigger groups of oocytes to periodically re-enter the meiotic cycle where germinal vesicle breakdown and extrusion of the first polar body occurs. These now mature oocytes are however arrested a second time at metaphase II where they await fertilization to complete meiosis II (23). Once the oocyte is fertilized, genetic material from both parents is fused resulting in a fertilized embryo (Figure 1.6).

Oogenesis

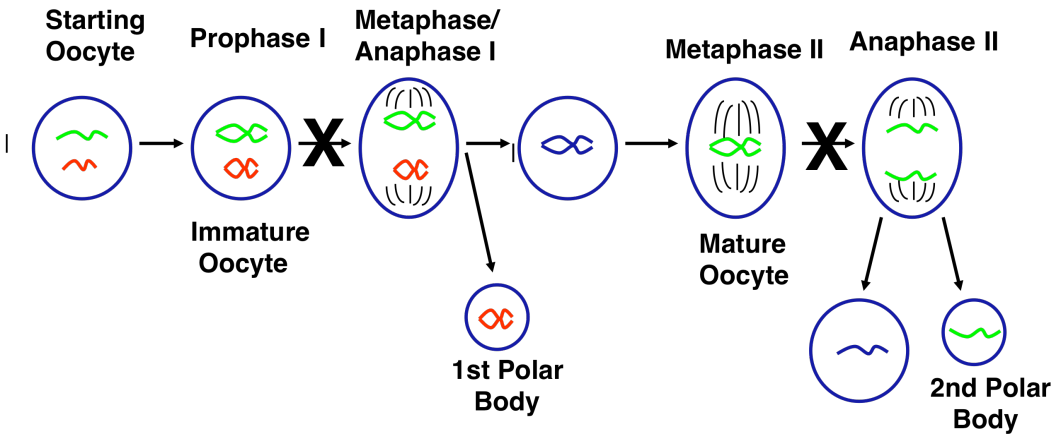

Figure 1.6 Oogenesis

Oogenesis begins with the oogonium dividing by mitosis to produce many oogonia. Oogonia then grow within the follicle where meiosis begins but stops in prophase 1. The primary oocyte develops to secondary oocytes when stimulated by gonadotropins by the pituitary. These mature oocytes are then arrested again at metaphase II until fertilization occurs.

1.9 The Adrenal Gland and Steroidogenesis

The adrenal glands are endocrine organs located on top of the kidneys. The adrenal gland is divided into two structures, the adrenal cortex and the medulla (43). The adrenal cortex produces cortisol, aldosterone, and androgens. The medulla mainly produces epinephrine and norepinephrine and is surrounded by the adrenal cortex. The hormones secreted by the medulla aid in times of physical and emotional stress (46). Hormones secreted by the pituitary and hypothalamus regulate the adrenal cortex to synthesize corticosteroid hormones from cholesterol. Similar to the testes, each cell of the adrenal gland can complete the steroidogenic pathway, and therefore does not rely on paracrine signaling as seen in ovary. The adrenal cortex is composed of three layers or zones. The outer layer is the zona glomerulosa where most of the mineralocorticoids, are produced, which participate in the regulation of blood pressure. The middle layer is the zona fasciculata, where glucocorticoids are produced. When adrenocorticotropic hormone (ACTH) is secreted from the pituitary, it stimulates this zone to produce and secrete increased concentrations of cortisol. Cortisol regulates the body's metabolism by controlling the use of fats, proteins and carbohydrates. The zona reticularis is the innermost layer of the adrenal cortex and produces weak androgens such as dehydroepiandrosterone (DHEA) and androstenedione (44,45). Each zona of the adrenal cortex has a specific function dictated by the type of steroid produced. The type of steroid produced is dictated by the compliment of enzymes present in each zone (48). For example, the enzyme required for the final step of aldosterone production is expressed exclusively in the zona glomerulosa. This type of regulation is also seen in the gonads,

where aromatase is essentially absent in the testes, making the final product testosterone and not estradiol.

Regulation of steroid synthesis in the adrenal gland has been shown to be very similar to gonadal steroidogenesis. In the adrenal gland, synthesis of adrenal steroid is initiated by ACTH activation of adenylate cyclase activity. Increases of cAMP as in the gonads, results in activation of the protein kinase A (PKA) pathway. It is uncertain what mechanism is activated downstream of PKA, but it is known that once PKA is activated, cholesterol is transported to the inner mitochondrial membrane, possibly by StAR (49-54).

1.10 Disorders of steroidogenesis

Leydig cell tumors

Testicular neoplasms can be classified into two groups: germ cell tumors or sex cord-stromal tumors that originate form either Sertoli or Leydig cells. Although Leydig cell tumors are rare, they include up to 3% of all testicular neoplasms (107). The mechanism leading to tumoriogenesis in Leydig cells is still largely unknown, however deregulation of the hypothalamic-pituitary-gonadal axis leading to excessive stimulation of Leydig cells by excess luteinizing hormone is thought to be involved. Also, structural changes in LHR and G proteins may be playing a role. Leydig cell tumors are classified as steroid-secreting tumors since they are known to produce testosterone and estrogen. Patients with Leydig cell tumors have elevated serum testosterone levels as well as elevated serum estradiol. In boys, the excess androgens cause early or advanced puberty, whereas adults with hyperandrogenism and hyperestrogenism are asymptomatic (107).

Cushing's Disease

Cushing's disease, or hypercortisolism is a hormonal disorder caused by chronic exposure to high levels of glucocorticoids. This phenotype can be due to excess production of the adrenocorticotropic hormone ACTH by a pituitary corticotroph adenoma, ectopic production of ACTH by a non-pituitary tumor, or over secretion of cortisol by the adrenal gland. Cushing's disease is relatively rare, and generally affects adults ranging from 20 to 50 yrs. Cushing's disease is an endocrine disorder with people who are obese and have type 2 diabetes at higher risk of developing the disorder (107).

Polycystic Ovary Syndrome (PCOS)

Female infertility is the inability to conceive and produce offspring. World wide, infertility is increasing and is due to a number of different causes. Among reproductive-age women, one of the leading causes of infertility is polycystic ovary syndrome (PCOS) (61,62). PCOS is defined by the presence of hyperandrogenism, anovulation, and polycystic ovaries. Along with these clinical features, insulin resistance, diabetes, and obesity have been correlated with PCOS. Improper reproductive physiology of PCOS patients includes altered gonadotrophin secretion, excessive androgen production, menstrual irregularity and abnormal ovarian morphology.

As previously mentioned, the HPG axis regulates gonadal function by allowing the secretion of GnRH, LH and FSH that ultimately lead to gonadal steroid production. In females, during the early years of life, GnRH is largely suppressed. By 10 yrs old, GnRH remains relatively low with occasional LH pulses during sleep. At the onset of puberty, an initial rise in serum FSH is followed by an increase of LH and LH pulse frequency. In contrast, girls with PCOS have elevated serum LH and LH pulse frequency compared to controls pre-and post-menarche. The underlying mechanism of this irregularity has been the subject of intense study. Recent work has indentified an important role for excess androgen production in PCOS. Normal patients treated with progesterone and or estrogen had a greater feedback suppression of LH compared to PCOS patient. However, if PCOS patients were pretreated with an androgen inhibitor, administration of progesterone and or estrogen resulted in similar LH suppression compared to normal patients (63,64). These

experiments clearly suggested that excess androgen production results in decreased sensitivity to the negative feedback loop of the HPG axis by progesterone.

Menstrual irregularity is a common symptom of PCOS. Adult patients with PCOS also exhibit chronic anovulation or lack of ovulation. Along with anovulation, PCOS patients have been shown to have bilateral ovarian enlargement, increased follicle number, and as the name implies, PCOS patients have polycystic ovaries. Follicular development is attenuated to an early antral stage.

A number of treatments are commonly used to treat PCOS patients. Studies have shown a significant positive effect by weight loss in individuals with PCOS. Weight loss has been shown to improve menstrual irregularity including ovulation and fertility (65). Weight loss has also been implicated in decreased LH pulse frequency, androgen concentration, and insulin levels. Insulin lowering drugs such as metformin have also been shown to have a positive effect in some women with PCOS, however, in the USA, metformin has not been as effective. It is now well known that lowering androgens can significantly reduce PCOS symptoms. Antiandrogen drugs such as flutamide have been shown to not only decrease androgen levels, but also improve insulin resistance in PCOS patients. Oral contraceptives have been used to treat symptoms, but are not as effective in restoring fertility. Also, many patients have adverse side affects with birth control medications and therefore must rely on alternative treatments. Lastly, clomiphene inhibits estrogen receptor (ER) action in the pituitary, and can therefore be used to stimulate the release of hormones to induce ovulation. Although this medication has been successfully

used to treat many women, Clomiphene may increase the likelihood of multiple births. We are therefore in need of more effective ways to treat both symptoms and infertility without suppressing adrenal gland function.

PCOS is a complicated disease due to its heterogenous clinical characteristics. Therefore a better understanding of its origin and mechanistic cause is crucial for improved diagnosis and treatment.

Polycystic Ovary System (PCOS): A Mouse Model

As aforementioned, the hypothalamus-pituitary-gonadal axis (HPG) regulates female fertility by modulating steroidogenesis and follicular development during the reproductive cycle. Steroids produced by the gonads feed back to regulate GnRH. Regulation of GnRH frequency is important since it dictates the release of LH versus FSH (77-79). After ovulation, which is known as the luteal phase, progesterone has a negative feedback affect to reduce GnRH, resulting in increased levels of FSH secretion. Increased FSH secretion starts the follicular phase, the pre-ovulatory stage during which follicles in the ovary mature (77). Women with PCOS have high GnRH pulse frequency, resulting in increased LH secretion that hinders FSH induced follicular maturation (80). It is believed that high androgen levels interfere with sensitivity to the progesterone negative feedback regulation on GnRH (81,82).

In the hope to understand the effects of elevated androgens on GnRH and PCOS, Dr. Moenter and colleagues developed a mouse model (PNA mice) that simulates the

reproductive endocrine phenotypes associated with PCOS (77). To achieve this, female mice were injected with dihydrotestosterone while pregnant. The female offspring of injected mice were studied once they reached adulthood. It is important to mention that this protocol to induce PCOS has also been successfully used in sheep.

A mouse estrus cycle is between 3-5 days in length, cycling between estrus, diestrus, and proestrus. In PNA mice however, the estrus cycle was lengthened from 5.9 days to 20.9 days. This was shown to be androgen receptor dependent, since treatment with an androgen receptor antagonist flutamide completely restored the estrus cycle in PNA mice. Associated with disrupted estrus cycle, PNA mice also had elevated testosterone and luteinizing hormone levels. These results suggest that androgen exposure *in utero* results in disrupted GnRH regulation, and therefore may serve as a possible mouse model to further study PCOS.

Chapter 2

Signaling Pathway for Steroidogenesis in the Ovary

Introduction:

Regulation of ovarian steroid production begins with the secretion of LH and FSH. It is well established that FSH increases expression of P450arom in the granulosa cells, while LH induces P450scc in luteal cells. This results in granulosa and luteal cell production of estrogen and progesterone, respectively (32, 83,84). Since LH and FSH are essential for proper steroid synthesis in the follicle, the downstream signaling pathways that are activated must be carefully regulated.

Normal steroidogenesis in the ovary is thought to be regulated by a two-cell/two gonadotropin model, where LH-stimulated theca cells produce androgens from cholesterol. Next, FSH stimulated granulosa cells convert androgens to estrogen. Both theca and granulosa cells express the necessary enzymes for progesterone production from cholesterol; however, granulosa cells cannot metabolize progesterone to androgen since they lack the appropriate enzyme. Consequently, the progesterone production and secretion that is required for proper ovulation is produced from granulosa cells. The focus of our current work is to identify the mechanism of LH-induced signaling that leads to progesterone production in the ovary (32,85,86).

Since the EGFR is important for oocyte maturation, steroidogenesis in the testes, and steroidogenesis in the ovary, experiments were performed to identify the mechanism downstream of EGFR activation. Steroidogenesis in the testes was found to be EGFR dependent, but MMP independent. Therefore, identifying the differences in EGFR activation in the ovary was analyzed. The MAPK pathway is known to be important for steroid production in the testes, and a recent genetic model confirmed its importance for follicular function, however, the importance of the MAPK pathway in ovarian steroidogenesis has not been identified and consequently analyzed in this chapter.

Materials and Methods:

Steroid production in Pre-ovulatory Follicles.

Three-week-old C57BL/6J female mice (The Jackson Laboratory) were primed with 5IU of PMSG (Sigma) by Intraperitoneal injection. Ovaries were harvested 40-44 hrs after PMSG injection. Follicles were isolated in M2 media (Millipore) and punctured with 30-gauge needles. Intact follicles were washed twice with M2 media (Millipore) and treated with 0.2 ug/ml LH (Sigma), 200ng/ml EGF (Invitrogen) or 20μM 22R-hydroxycholesterol (Steraloids) in M16 media (Millipore). Follicles were pre-treated with 20μM AG1478, Galardin, H89, U0126 (Calbiochem), or vehicle for 30 minutes before and during stimulation. 250μM Doxycycline (Sigma) treatment was also performed 30 minutes before stimulation. Supernatants were collected 30 minutes – 4 hrs after stimulation with LH.

Steroid Radioimmuno assays.

Progesterone concentrations from culture media or mouse serum were measured using a progesterone RIA kit (MP Biomedicals). Culture media collected from follicle culture was diluted 1:2 in diluent for all RIA assays.

StAR/MAPK Activation and Immunoblotting

Cultured follicles were washed with cold PBS after treatment and collection of media. 200ul of 1x RIPA (Santa Cruz) buffer supplemented with proteinase inhibitors, sodium orthovanadate, and PMSF was used to lyse follicles/cells. Cells were scraped and

both follicles and cells were homogenized and microcentrifuged at 14x rpm for 15 min at 4^0C. Supernatants were removed and diluted with 2X Laemmli sampler buffer containing 10% ß-mercaptoethanol (Sigma). Samples were then separated on 12.5% SDS-polyacrylamide gels and transferred to immobilon membranes (Millipore) at 100V for 60 minutes. Membranes were blocked with 5% nonfat dry milk (NFDM)in TBST (100mM NaCl, 0.1% Tween 20, 50 mM Tris pH 7.4) for 60 minutes at room temperature. Membranes were then incubated overnight at 4^0C with primary antibody in NFDM. Blots were washed three times for 10 minutes each with TBST at room temperature while shaking followed by 1 hr incubation at room temperature (1: 4000) with horseradish peroxidase-conjugated goat anti-rabbit (Bi0-Rad) in TBST and 5% NFDM. Blots were again washed three times with TBST before treatment with ECL-Plus (GE Healthcare). For StAR detection, 1:10,000 anti-StaR antiserum (provided by Doug Stocco, Texas tech). For ERK signaling, ERK1/2 (Cell Signaling) was diluted 1:4000 and total ERK (Cells Signaling) was diluted 1:4000 in TBST plus 5% milk.

In vivo Steroidogenesis Assays

Four-week-old C57BL/6J female mice (The Jackson Laboratory) were induced to superovulate by injection with 1IU of PMSG, PMSG plus 20μM AG1478 (or PMSG plus 20μM Galardin intraperitoneally in PBS. Mice were then given another injection 12 hrs later of 20μM AG1478, 20μM Galardin, or vehicle. 44 hrs after PMSG treatment, mice were given 1IU of hCG, hCG plus AG1478, hCG plus 20μM Galardin, or vehicle. 8 hrs later mice were anesthetized with avertin (Sigma) prior to blood collection via cardiac puncture. Serum was extracted using serum separator tubes (BD microtainer) by

centrifugation. Progesterone levels were also measured from isolated ovaries. Ovaries were cleaned and place overnight in 3:2 haxane/acetyl acetate. The next morning, liquid was transferred to another glass tube and dried with gas nitrogen. Steroid was dissolved in diluent from RIA kit. Progesterone levels were measured by RIA.

***In vivo* injections of inhibitors in Wild type mice**

8-week-old female mice were ordered form the Jackson laboratory. At nine weeks of age, estrus cycle was determined via vaginal smears. A 30ul PBS lavage was used to obtain cells to place on slides. Slides were air-dried, then fixed and stained with Hema 3 staining solution (Fisher). Slides were analyzed under a light microscope. Mice were followed for 2 full cycles before daily IP injections were given for 15 days of 0.01% DMSO, 20μM Galardin, 20μM AG1478, and 100μM Doxycycline. After 15 days, mice were sacrificed and ovaries collected. One ovary was used for steroid extraction and the other was sectioned and subjected to H&E staining. Sections were taken at intervals of 30 μm, and 5 μm paraffin-embedded sections were mounted on slides.

Results:

EGFR Signaling is Necessary for LH-Induced Steroidogenesis in mouse follicles and is important for MAPK activation.

Initial experiments were performed to determine the sensitivity of pre-ovulatory follicles to gonadotropin-induced steroidogenesis. Follicles were isolated and treated with increasing amounts of LH. Progesterone secreted in the media was measured at the indicated time points. As shown in figure 2.1, progesterone production was detected just 30 minutes after stimulation and continued out to 4 hrs. The EC_{50} for LH induced steroid production was about 0.2 ug/ml and therefore we used this concentration for all future studies.

Several studies have now shown the critical role epidermal growth factors (EGF) play in triggering events essential for normal follicular development (11,59, 66). We therefore analyzed EGFR signaling in LH-induced steroidogenesis in pre-ovulatory follicles using pharmacological manipulation. Follicles were pretreated for 30 minutes with the EGFR inhibitor AG1478, followed by stimulation with LH or LH plus the inhibitor. LH induced progesterone production (Figure 2.2A) as early as 30 minutes after stimulation. This LH induced progesterone production was inhibited throughout the time course of 30 minutes to 4 hours by addition of AG1478. This indicates EGFR plays an important role in LH-induced steroidogenesis in mouse follicles.

Recent work has also shown that MAPK signaling is important for steroid production in Leydig cells (12). Therefore we analyzed MAPK activation and it's

relationship to EGFR in LH-induced steroidogenesis in ovarian follicles (Figure 2.2). As expected, LH induced MAPK activation at 2hrs, but this MAPK phosphorylation was completely absent when EGFR was inhibited (Fig. 2.2B). Progesterone production showed similar changes (Fig 2.2A). These results indicate that MAPK signaling, like EGFR, is important for LH induced steroid production in follicles. These studies are consistent with recent work demonstrating that MAPK is essential in ovarian granulosa cells for the maintenance of proper female fertility (67).

We have previously shown that in male mice, treatment with AG1478 significantly reduced serum testosterone levels (12). To further confirm the importance of the EGFR in LH-induced steroid production in the ovary, we analyzed the effect of the EGFR inhibitor *in vivo*. For these experiments, female mice were super-ovulated by priming with PMSG and hCG. PMSG is a glycoprotein obtained from the serum of pregnant mares. It mimics the effects of gonadotropins secreted by the anterior pituitary. Thus PMSG stimulates development of the ovarian follicle in the female. hCG is peptide hormone produced by the embryo in the early stages of pregnancy. hCG binds and activated the LH receptor; triggering ovulation and development of the corpus luteum. As expected, females primed with PMSG and treated with hCG had increased levels of serum progesterone compared to mice only primed with PMSG and PBS (Fig. 2.2C). *In vivo* injections of AG1478 significantly reduced progesterone levels relative to hCG treated mice. These findings demonstrate the importance of the EGFR in ovarian steroid production, mirroring previous results observed in male gonadal steroidogenesis.

MAPK signaling is necessary for LH-induced steroid production in pre-ovulatory follicles and is downstream of the EGFR

To further elucidate the role of MAPK signaling in steroidogenesis of the ovary, pre-ovulatory follicles were treated with the MEK inhibitor, U0126. Inhibiting MAPK signaling significantly blocked steroid production induced by LH from 30 minutes out to 4 hours (Fig 2.3A). LH induced phosphorylation of MAPK as demonstrated in the western blot, and phosphorylation of MAPK was blocked as expected by U0126 (Fig 2.3B). To ensure that MAPK signaling is in fact downstream of the EGFR, as previously demonstrated in the testis (12), rescue experiments were performed. Follicles were co-treated with EGF in the presence of the U0126 inhibitor. As expected, EGF was not able to rescue LH induced steroid production when MAPK is blocked (Fig 2.3C). As seen previously, LH and EGF induced MAPK activation was blocked by the inhibitor. Consistent with the steroidogenesis results, EGF was not able to induce MAPK activation when rescue experiments were performed (Fig 2.3D). These results further demonstrated that MAPK signaling is occurring downstream of the EGFR and is critical for steroid production.

cAMP/PKA Signaling is Necessary for LH-Induced Steroidogenesis in mouse follicles and is found upstream of the MAPK cascade.

It has been shown that the LHR, a GPCR, can activate the EGFR by intracellular and extracellular mechanisms (68-70). Our lab has shown the important role cAMP and subsequent PKA signaling play in LH-induced steroid production in the testis (12). To analyze the role of this signal transduction pathway in ovarian signaling, PKA activity was inhibited in pre-ovulatory follicles. Follicles treated with LH had increased levels of progesterone, while follicles treated with LH and the PKA inhibitor, H89, exhibited significantly lower levels of progesterone production (Fig. 2.4A). These results suggest that similar to the testis, PKA signaling is necessary for proper steroid production in ovarian follicles. Like EGFR signaling, inhibition of PKA leads to decreased MAPK activation (Fig. 2.4B), suggesting that MAPK signaling is downstream of PKA. To ensure the specificity of the inhibitor and to analyze whether PKA signaling is upstream of the EGFR, follicles were co-treated with EGF in the presence of the H89 inhibitor. As expected, EGF was able to rescue progesterone production when PKA signaling was blocked, indicating that PKA is upstream of EGFR signaling (Fig. 2.4C). To further confirm that the MAPK pathway is downstream of EGFR and PKA signaling, western blots for pMAPK in the presence of the H89 inhibitor and the rescue experiments with EGF were analyzed. LH and EGF treatment of follicles induced MAPK activation while co-treatment with H89 significantly reduced MAPK activation (Fig. 2.4D) as previously shown in the testis. Co-treatment with EGF in the presence of H89, rescued MAPK activation compared to LH-treated follicles.

The LH receptor is coupled to Gas, which leads to increased cAMP and PKA activity. To analyze whether cAMP is sufficient to induce steroid production, pre-ovulatory follicles were treated with forskolin to increase intracellular levels of cAMP. Follicles treated with forskolin alone exhibited increased progesterone production, similar to LH stimulation (Fig. 2.5). Forskolin-induced steroid production was shown to be EGFR dependent, as co-treatment with the EGFR inhibitor AG1478 completely abolished steroid production (Fig. 2.5A). As expected, forskolin induced MAPK activation, and AG1478 abrogated activation of MAPK signaling (Fig. 2.5B).

LH induces steroidogenesis in pre-ovulatory follicles, but does not require changes in Steroidogenic Acute Regulatory protein expression.

As previously mentioned, StAR is the rate-limiting step in steroid production since it transports cholesterol to the inner mitochondrial membrane where the steroidogenic enzymes are located. It has also been shown that StAR is regulated by EGFR signaling in gonadal tissues (59,76). In Leydig cells, trans-activation of the EGFR was shown to regulate early but not prolonged phosphorylation and translocation of StAR (12). In oocyte cumulus cell cultures (OCCs), EGF–induced steroidogenesis did not require changes in Steroidogenic Acute Regulatory protein expression (11). To examine the LH effects on StAR in the intact follicle, and whether the EGFR, PKA, and MAPK pathways are involved, pre-ovulatory follicles were pre-treated with vehicle, 20μM AG1478, 20μM U0126, and 1μM H89 for 30 minutes prior to stimulation with 0.2ug/ml of LH (Figure 6). Consistent with previous results obtained in oocyte cultures, LH induced steroidogenesis does not require changes in Steroidogenic Acute Regulatory

Protein expression. Inhibition of EGFR and downstream signaling had no affect o StAR protein levels, indicating that StAR maybe regulated at the post-translational level. Preliminary results (data not shown) suggest that StAR in the ovary is also regulated by phosphorylation. However, additional experiments on intact follicles need to be performed.

Matrix metalloproteinase are important for LH-induced steroidogenesis in mouse follicles and is upstream of the EGFR.

MMP activation has been shown to be important for the shedding of EGFR ligands and for steroidogenesis in the ovary (11,29,35,37,38). Previous studies have used galardin, a broad spectrum MMP inhibitor, to demonstrate their role in ovarian steroid production (11). As previously mentioned, the MMP family consists of 25 members. We therefore, specifically analyzed the role of MMP 2/9 in ovarian steroid production. Pre-ovulatory follicles were pretreated with MMP2/9 inhibitor, or galardin inhibitor for 30 minutes before stimulation with LH. Both galardin and MMP2/9 inhibitor blocked LH – induced steroid production. (Fig.2.7A). These results indicate that specifically MMP2/9 is necessary for LH-induced steroidogenesis in pre-ovulatory follicles.

To ensure the specificity of these pharmacological agents, inhibition of MMP was achieved using an alternative approach. Doxycycline, a common antibiotic used to treat infection, has been used to decrease MMP2/9 activity in several disease states (71-74). To determine the EC_{50} of doxycycline in follicles, increasing concentrations were given to follicles for 30 minutes prior to treatment with LH. Previous *in vitro* studies have

shown doxycycline to have an EC_{50} near the 100μM rage (75). Similar results were obtained in pre-ovulatory follicles when progesterone levels were measured. Follicles co-treated with 50μM had no change in progesterone levels compared to LH treated follicles (Fig. 2.7B); however, 100μM and 250μM doxycycline treatment resulted in 50 percent and complete progesterone reduction, respectively. Since 250μM doxycycline resulted in almost complete inhibition, all future experiments were performed using this concentration. These experiments used a different and perhaps more specific mode of MMP inhibition to demonstrate that MMP activity is important for steroidogenesis in follicles. If MMP activity is upstream of the EGFR in pre-ovulatory follicles, then co-treatment with EGF and doxycycline should result in a rescue or partial rescue of steroid production. To test this theory and to demonstrate that doxycycline is specifically inhibiting MMP activity to reduce steroid production, follicles were pre-incubated with doxycycline and stimulated with LH, EGF, LH plus doxycycline, or LH plus doxycycline and EGF. As shown, EGF induced progesterone production at much lower levels compared to LH. When follicles were treated with LH and doxycycline, progesterone is significantly reduced. However, co-treatment with EGF resulted in a small but significant rescue of progesterone levels (Figure 7C). Since EGF did not induce stimulation of progesterone levels comparable to LH, rescuing with EGF is not ideal. To overcome this problem, follicles were stimulated with 22-R cholesterol. 22-R cholesterol can induce steroid production independent of StAR translocation. As shown, 22-R cholesterol was able to induce progesterone levels comparable to LH. Co-treatment with 22-R cholesterol was able to rescue decreased progesterone levels due to doxycycline treatment (Fig.

2.7D). These results demonstrate the specificity of doxycycline on inhibiting MMP activity, without being toxic to the follicular cells.

Inhibition of MMPs in vivo reduces steroidogenesis in super-ovulated mice

In vitro experiments have been able to show the important role MMP activity plays in ovarian maturation and steroidogenesis (11,35,37). However, it is still uncertain if MMP activity has a physiological role in controlling steroid production in the ovary in vivo. To answer this question, pre-pubertal female mice were super-ovulated by IP injections of PMSG and hCG. Mice treated with hCG had induced progesterone production compared to mice who only received vehicle. Mice injected with hCG and 20μM galardin demonstrated reduced serum progesterone levels (Fig. 2.8). Although progesterone levels were only partially reduced with Galardin, these experiments demonstrated that *in vivo*, galardin can have an inhibitory affect on steroidogenesis.

In vivo injection of MMP inhibitors or EGFR inhibitor does not alter the estrus cycle or follicle development of wild type mice

In vitro and *in vivo* studies have now shown the importance of MMP activity in steroidogenesis in the ovary. Since Galardin was able to restore testosterone levels and cycling of PNA mice (77), it is important to access whether MMP inhibition *in vivo* using pharmacological inhibitors will have any harmful effects in the estrus cycle and follicle development in wild type mice. To answer this question, the estrus cycle of nine-week old female mice was examined by vaginal smears through two cycles. After the cycling patterns were confirmed, IP injections of DMSO, 20μM Galardin, 20μM AG1478, or 100μM Doxycycline were given daily for a total of 15 days. As expected, Galardin and Doxycycline did not alter the estrus cycle (Fig. 2.9A). Also, mice treated with the EGFR inhibitor cycled normally compared to the vehicle group. To further analyze the estrus cycle under these conditions, the percent days in each cycle stage was determined. Under all conditions, mice spent 50% of days in diestrus, 30% in estrus, and 20% in proestrus (Fig. 2.9B). Finally, ovaries were removed after completion of treatment, sectioned and H&E stained. As expected, normal follicular development occurred even in the presence of the inhibitors (Fig. 2.9C). These experiments suggest that under normal conditions, when proper LH surge drives normal follicular development and proper steroid production, both MMP and EGFR inhibitors have no detrimental effects. However, under abnormal conditions, such as excess androgen production, MMP inhibitors can successfully reduce steroid production resulting in normal cycling.

Inhibiton of MMPs in vivo reduces steroidogenesis in the disease state

As previously stated, polycystic ovarian syndrome (PCOS) is a common disease that causes infertility in women. Patients have high androgen production partially due to increased LH frequency. Since MMP activity in the ovary is necessary for proper steroidogenesis, it is possible to expect that inhibiting MMP activity in a disease state, may lead to normal steroid levels. To address this question, PNA mice created by Dr. Moenter were used in collaboration (77). Wild type or PNA mice were given either vehicle, or 20μM Galardin IP injections for 15 days or 24 days. No changes in serum testosterone levels were detected between wild type mice given vehicle or Galardin. However, PNA mice treated with Galardin had a significant decrease in testosterone levels when compared to vehicle injected (Fig. 2.10A). The estrus cycle was analyzed under the same conditions and demonstrated that Galardin completely restores cycling of PNA mice injected with Galardin (10B). Importantly, Galardin had no negative effect on normal cycling mice. These experiments suggest that MMP inhibition may be a possible way of treating patients with high androgen levels, such as in PCOS.

Discussion:

Ovarian steroidogenesis commences with signals that originate in the hypothalamus. Hormones secreted from the pituitary travel down to the ovary and induce steroid production. The LH receptor is a GPCR that has been shown to trans-active the EGFR to drive ovarian functions, such as oocyte maturation and steroidogenesis (11,12). Although a number of mechanisms in which this cross talk is regulated have been described, the mechanism leading to steroidogenesis from the LH stimulus in the ovary has not been completely established. (95-97).

The mechanism that regulates steroid synthesis in the testis has been recently published (12). The order of signaling in the testis and ovary is conserved with PKA activation leading to EGFR signaling and subsequent MAPK activation. Despite similarities found in ovarian steroidogenesis compared to the testes, some key differences were seen. In the testes, GPCR trans-activation of the EGFR is ligand-independent; meaning that downstream signals from the LH receptor trans-active the EGFR in an intracellular fashion where MMPs are not essential. Although MMP activation and ligand dependent activation of the EGFR in the testes is thought to occur, it was not shown to be necessary for steroidogenesis (12). In the ovary, however, MMPs are necessary for proper gonadotropin-induced steroid production. This difference can be explained by the fact that in the testis, steroidogenesis occurs within a single cell type (the Leydig cell). In contrast, the ovarian follicle is composed of layers of cells in which proper steroidogenesis relies on paracrine molecules that serve as liaisons between theca and mural granulosa cells.

Although EGFR signaling is important for both the testis and the ovary, some differences were observed. EGFR signaling was shown to be necessary for short-but not long-term signaling in the testis, where in the ovary, inhibition of the EGFR only at later time points resulted in decreased steroid production. In the testis, it is possible that Leydig cells may be resensitizing to LH and reactivating the kinase cascade (12,98). It is possible that this resensitizing is not observed in the ovarian follicle due to its multi-layer complexity. In the ovary, progesterone is being made in the granulosa cells. Granulosa cells do not express the LHR, meaning that progesterone production will always rely on EGFR signaling to activate StAR in granulosa cells. In Leydig cells, cAMP/PKA eventually increases StAR to such an extent that it can activate independent of EGFR/kinase signaling.

In vivo experiments analyzing the physiological role of EGFR in both males and females resulted in decreased steroidogenesis. However, in female mice, this effect was only observed in super-ovulated mice and not normal cycling mice (Fig. 2.2). Although *in vivo* experiments demonstrated that the EGFR inhibitor could specifically reduce progesterone levels, EGFR inhibitors may not be a good alternative to treating patients with diseases such as PCOS since EGFR signaling has a number of different functions in different cell types. Cancer patients are currently being treated with EGFR inhibitors, but the impact it has on steroidogenesis has not been analyzed.

Treatment of follicles with the MAPK inhibitor, U0126 resulted in complete reduction of steroidogenesis at early and late time points (Fig. 2.3). This indicates the important role the MAPK cascade plays in regulating steroid production. In agreement with these findings, a mouse genetic model where ERK 1/2 in granulosa cells was disrupted, demonstrated that the MAPK pathway is essential for normal female fertility (67,99).

Oocyte maturation as well as Leydig cell steroidogenesis was shown to be cAMP and PKA-dependent (12, 38). Inhibition of PKA in pre-ovulatory follicles abolished steroid production (Fig. 2.4). Although previous *in vitro* experiments in follicles and Leydig cells have established PKA signaling as an important mediator, the specificity of these pharmacological experiments were still lacking. However, co-treatment of follicles with EGF in the presence of H89 completely rescued steroid production, confirming its specific role in steroidogenesis.

Understanding the mechanism(s) regulating steroid production in the ovary is important since elevated levels can lead to diseases such as PCOS. Our *in vitro* experiments demonstrating that Galardin, the broad-spectrum MMP inhibitor, MMP2/9 inhibitor as well as doxycycline, a common antibiotic, can reduce steroid levels in pre-ovulatory follicles lead to *in vivo* experiments in a PCOS mouse model. Treatment with galardin rescued the PCOS phenotype, since mice began cycling normally and testosterone levels were reduced to normal levels. In addition, galardin injections did not affect normal mice, indicating that its specificity is to the disease state.

Elevated MMP activity has been attributed to a number of diseases such as pancreatitis-associated lung injury, aortic aneurysm, and cerebral ischemia (73-75, 100). Treatment with doxycycline in these cases has been shown to decrease MMP activity and alleviate the problems associated with increased MMP activity. Our *in vitro* studies with doxycycline in pre-ovulatory follicles suggest that doxycycline may also be used for treatment of increased MMP activity in patients with PCOS. Although *in vivo* studies are still pending to further prove that doxycycline can specifically reduce steroid levels, it is exciting to imagine the use of a common antibiotic to treat patients with PCOS. This alternative treatment can regulate normal steroidogenesis, hence fertility, without the worry of secondary side affects or multiple births.

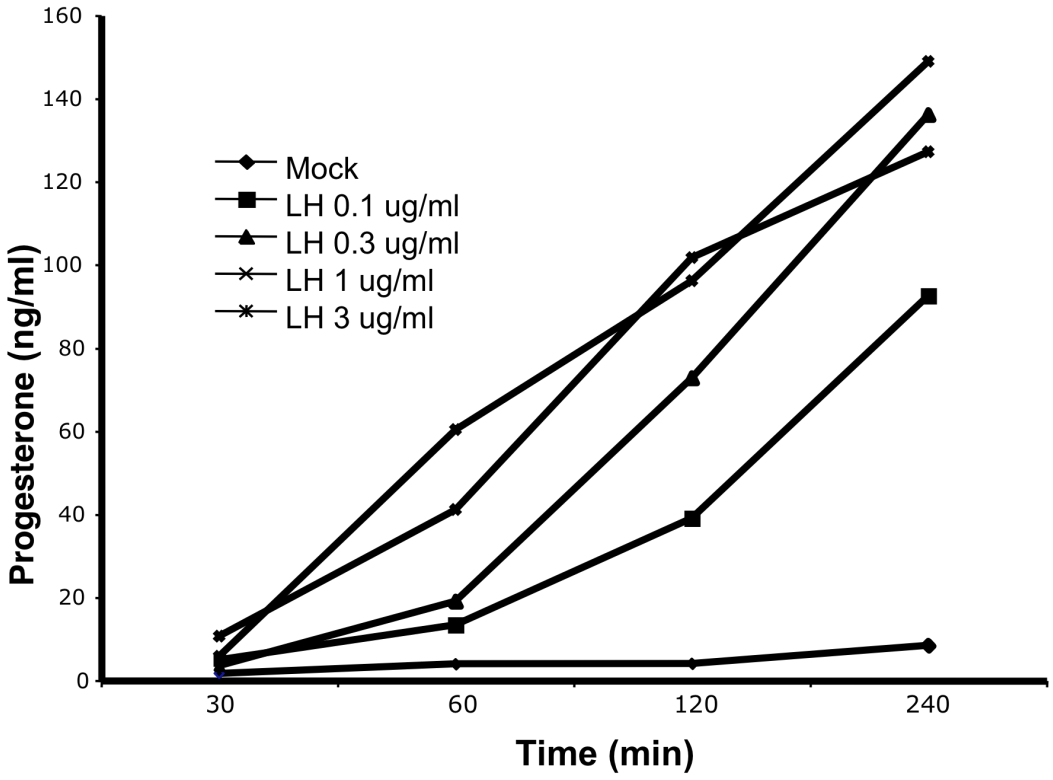

Figure 2.1: LH-induces progesterone secretion in pre-ovulatory mouse follicles in a dose and time dependent manner.

Pre-ovulatory follicles were stimulated with indicated concentration. Progesterone levels were measured in a small aliquot removed from the culture media of each well at 30, 60, 120, and 240 minutes after addition of LH. the media from 30 to 240 minutes by RIA.

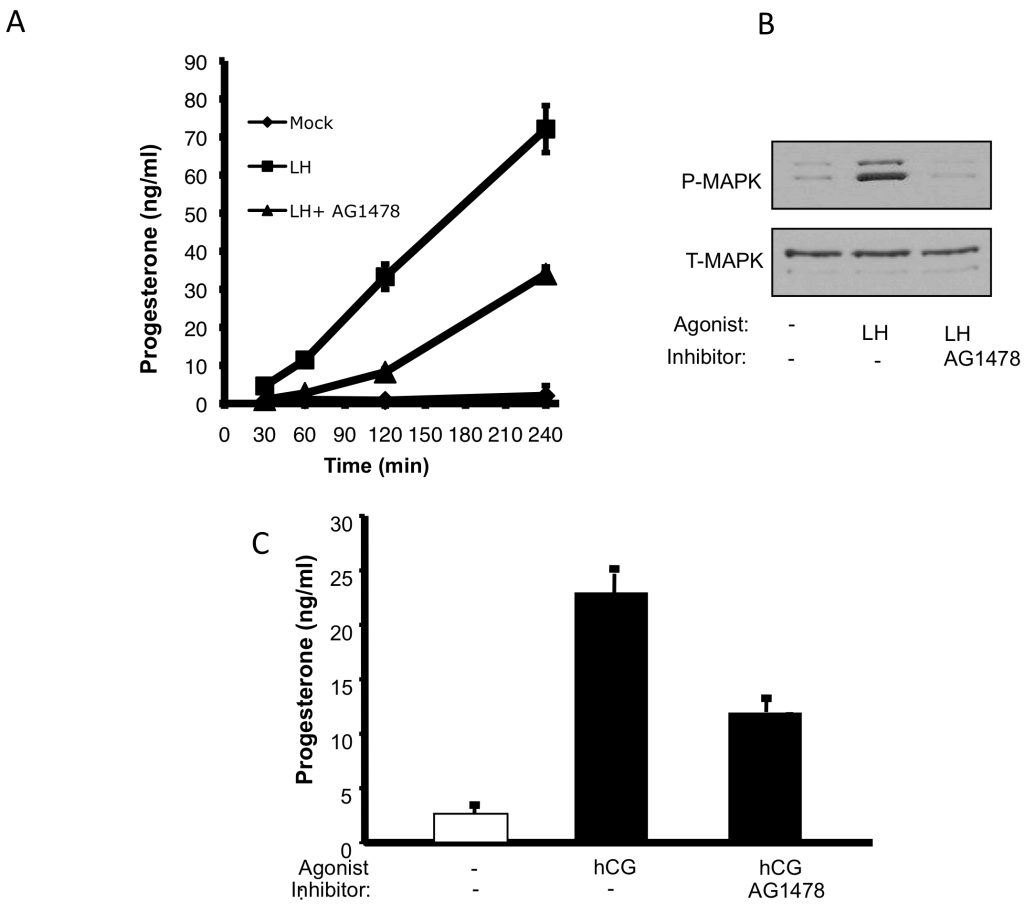

Figure 2.2: ***EGFR Signaling is Necessary for LH-Induced Steroidogenesis in mouse follicles and is important for MAPK activation.***

Pre-ovulatory follicles were pre-treated with 0.01% DMSO, or 20μM AG1478 for 30 minutes. 0.2ug/ml of LH was added to stimulate follicles for 30 minutes to 4 hrs. Progesterone concentrations were measured by RIA (A) and cell lysates were collected at 2 hrs and analyzed for phosphorylated and total MAPK (B). 3 week-old female mice were injected with PBS, 5IU of PMSG, or 5IU of PMSG plus 20μM AG1478. 12 hours later, mice were injected a second time with either PBS or AG1478. 48 hours after PMSG injection, mice were either injected with PBS, 1IU hCG, or1IU hCG plus 20μM AG1478. 8 hours after hCG injection, mice were sacrificed and serum progesterone levels were determined by RIA (C). Values depict n=15 mice for in vivo studies. Each bar represents the mean +/- S.D. (n=3) for A&B. For C, each bar represents the mean +/- S.E.M. (n=11).

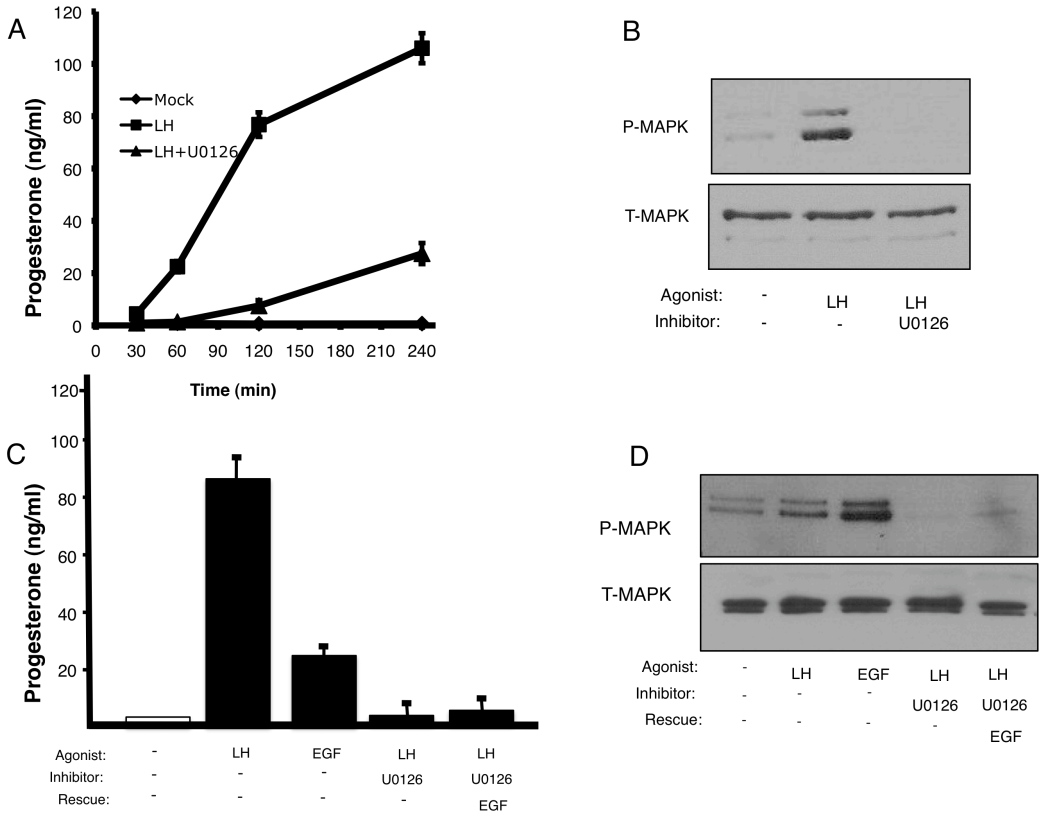

Figure 2.3 MAPK signaling is necessary for LH induced steroid production in preovulatory follicles and is downstream of the EGFR.

Pre-ovulatory follicles were pre-incubated with 0.01% DMSO, or 20μM U0126 for 30 minutes (A, B). 0.2ug/ml of LH was used to stimulate follicles for 30 minutes to 4 hrs. Progesterone concentration was measured by RIA (A), and cell lysates were collected at 2 hrs and analyzed for phosphorylated and total MAPK by western blot (B). Follicles were pretreated with 0.01% DMSO, or 20μM U0126 and stimulated with LH, 200ng EGF, or LH and 200ng EGF in the presence of the U0126 inhibitor (C, D). Progesterone concentrations were measured by RIA (C) and cell lysates were collected at 2 hrs and analyzed for phosphorylated and total MAPK (D). Each bar represents the mean +/- S.D. (n=3).

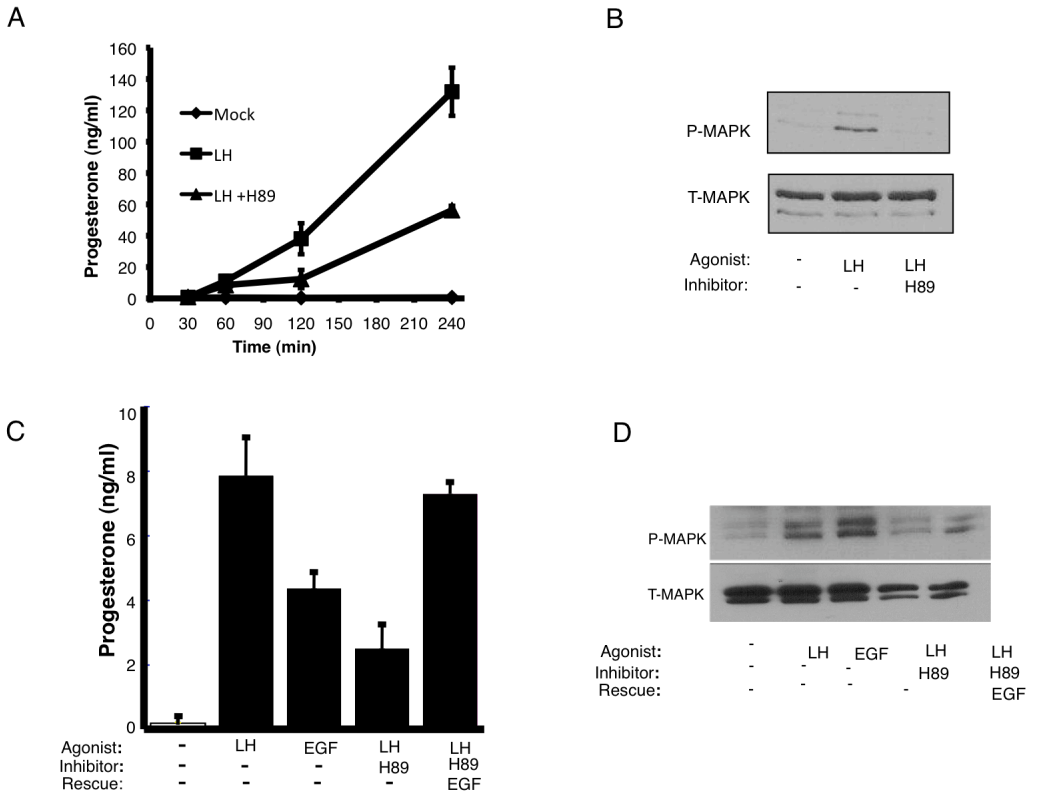

Figure 2.4 ***PKA Signaling is Necessary for LH-Induced Steroidogenesis in mouse follicles and is found upstream of the MAPK cascade***.

Pre-ovulatory follicles were pre-incubated with 0.01% DMSO, or 1μM H89 for 30 minutes (A, B). 0.2ug/ml of LH was used to stimulate follicles for 30 minutes to 4 hrs. Progesterone concentration was measured by RIA (A), and cell lysates were collected at 2 hrs and analyzed for phosphorylated and total MAPK (B). Follicles were pretreated with 0.01% DMSO, or 1μM H89 and stimulated with LH, 200ng EGF, or LH and 200ng EGF in the presence of the H89 inhibitor (C, D). Progesterone concentrations were measured by RIA (C) and cell lysates were collected at 2 hrs and analyzed for phosphorylated and total MAPK (D). Each bar represents the mean +/- S.D. (n=3).

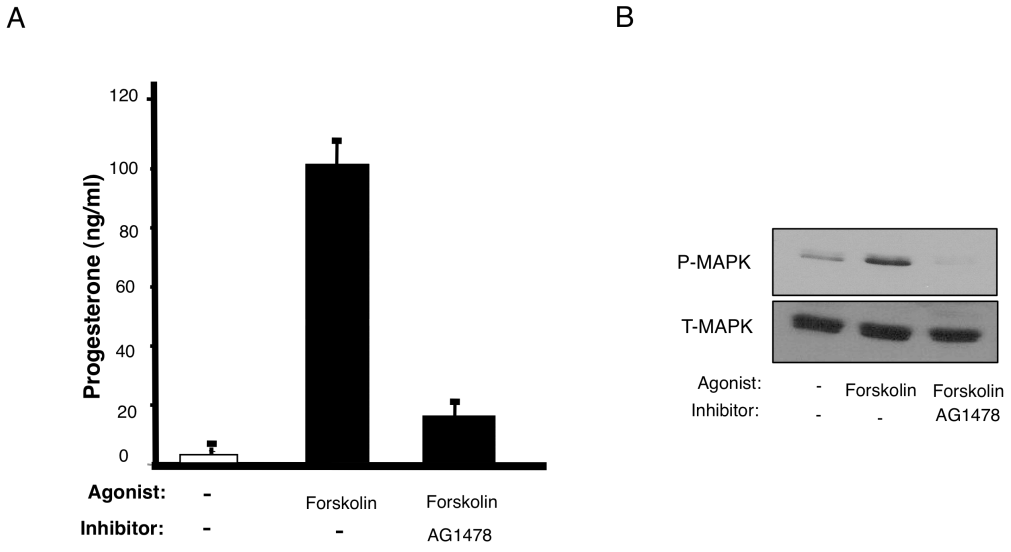

Figure 2.5 ***cAMP signaling is Necessary for LH-Induced steroidogenesis in mouse follicles and is found upstream of the MAPK cascade***.

Pre-ovulatory follicles were pretreated with 0.01% DMSO or 20μM AG1478 for 30 minutes (A, B). Follicles were then stimulated with 10μM forskolin or forskolin in the presence of the AG1478 inhibitor. Progesterone concentrations in culture media were measured by RIA (C) and cell lysates were collected at 2 hrs and analyzed for phosphorylated and total MAPK by western blot (D). Each bar represents the mean +/- S.D. (n=3).

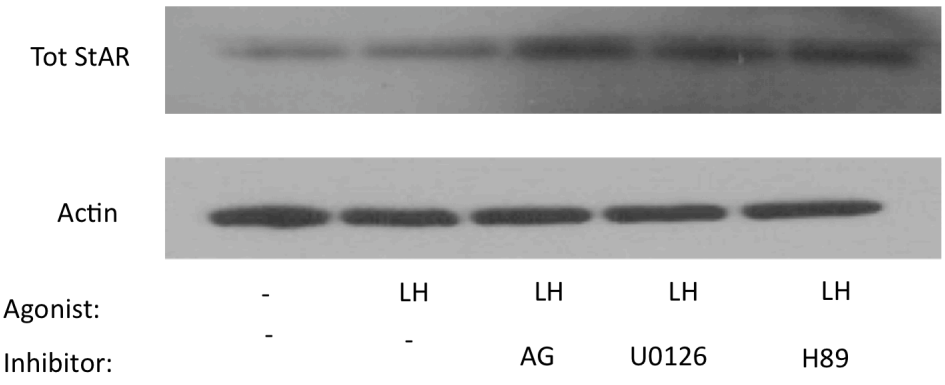

Figure 2.6 LH induces Steroidogenesis in pre-ovulatory follicles, but does not require changes in Steroidogenic Acute Regulatory protein expression.

Follicles were pretreated with 0.01% DMSO, 20μM AG1478, 20μM U0126, or 1μM H89 for 30 minutes. Follicles were then stimulated with either PBS, 0.2ug/ml of LH or LH plus inhibitors for 2 hrs. Cell lysates were collected and analyzed by western blot for total StAR and actin.

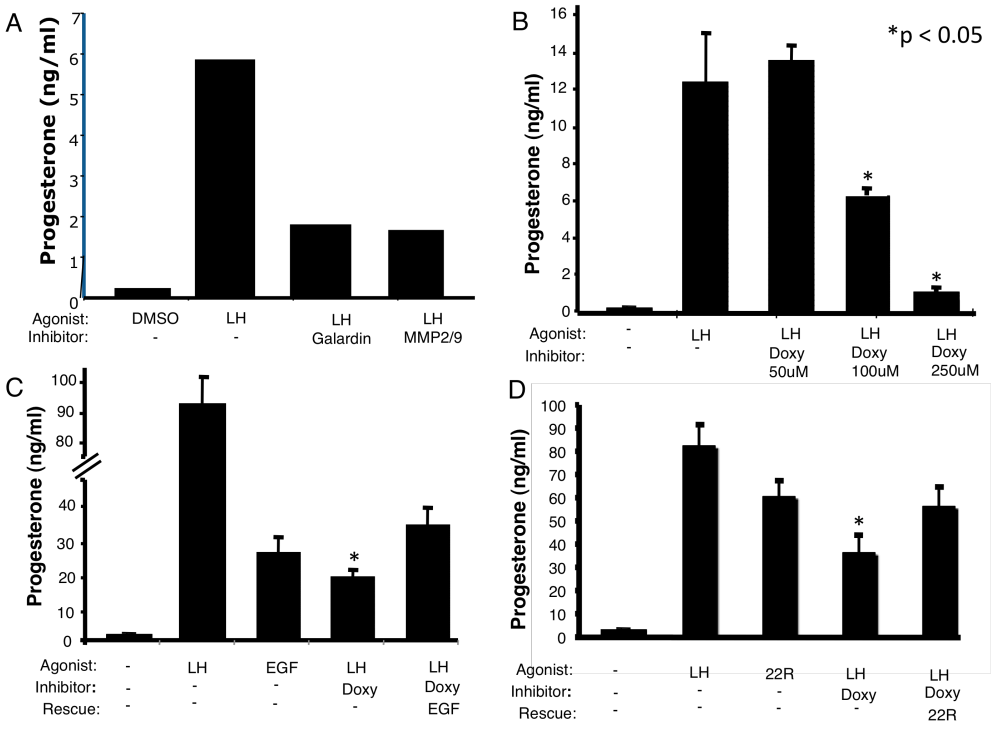

Figure 2.7 Matrix metalloproteinases are important for LH-induced steroidogenesis in mouse follicles and act upstream of the EGFR.

(A) Pre-ovulatory follicles were pretreated with 0.01% DMOS, 20μM galardin, or 20μM MMP2/9 inhibitor for 30 minutes. Follicles were stimulated with 0.2ug/ml of LH or LH plus inhibitors for 30 minutes. Progesterone levels were measured from media. (B) Follicles were pre-incubated with 0.01% DMSO, 50μM, 100μM, or 250μM doxycycline for 30 minutes. Follicles were then stimulated with PBS, or 0.2ug/ml of LH or LH plus doxycycline. Progesterone concentration was measured from the media by RIA. For rescue studies, (C) pre-ovulatory follicles were pre-incubated with 0.01% DMSO, or 250μM doxycycline for 30 minutes. (D) Follicles were stimulated with 200ng/ml EGF, or 20μM 22R-hydroxycholesterol and rescued with 20μM 22R-hydroxycholesterol (D). Each bar represent the mean +/- S.D. (n=3).

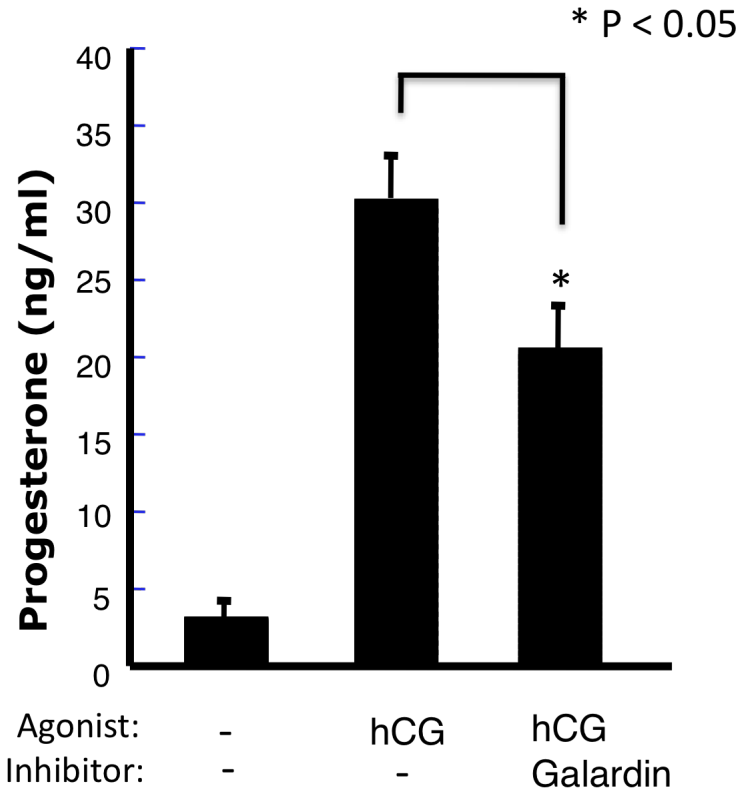

Figure 2.8. Inhibition of MMPs *in vivo* reduces steroidogenesis in super-ovulated mice.

3 week-old female mice were injected with PBS, 5IU of PMSG, or 5IU of PMSG plus 20μM Galardin. 12 hours later, mice were injected a second time with either PBS or Galardin. 48 hours after PMSG injection, mice were either injected with PBS, 1IU hCG, or1IU hCG plus 20μM Galardin. 8 hours after hCG injection, mice were sacrificed and progesterone levels were measured by RIA. Each bar represents the mean +/- S.E.M. (n=11).

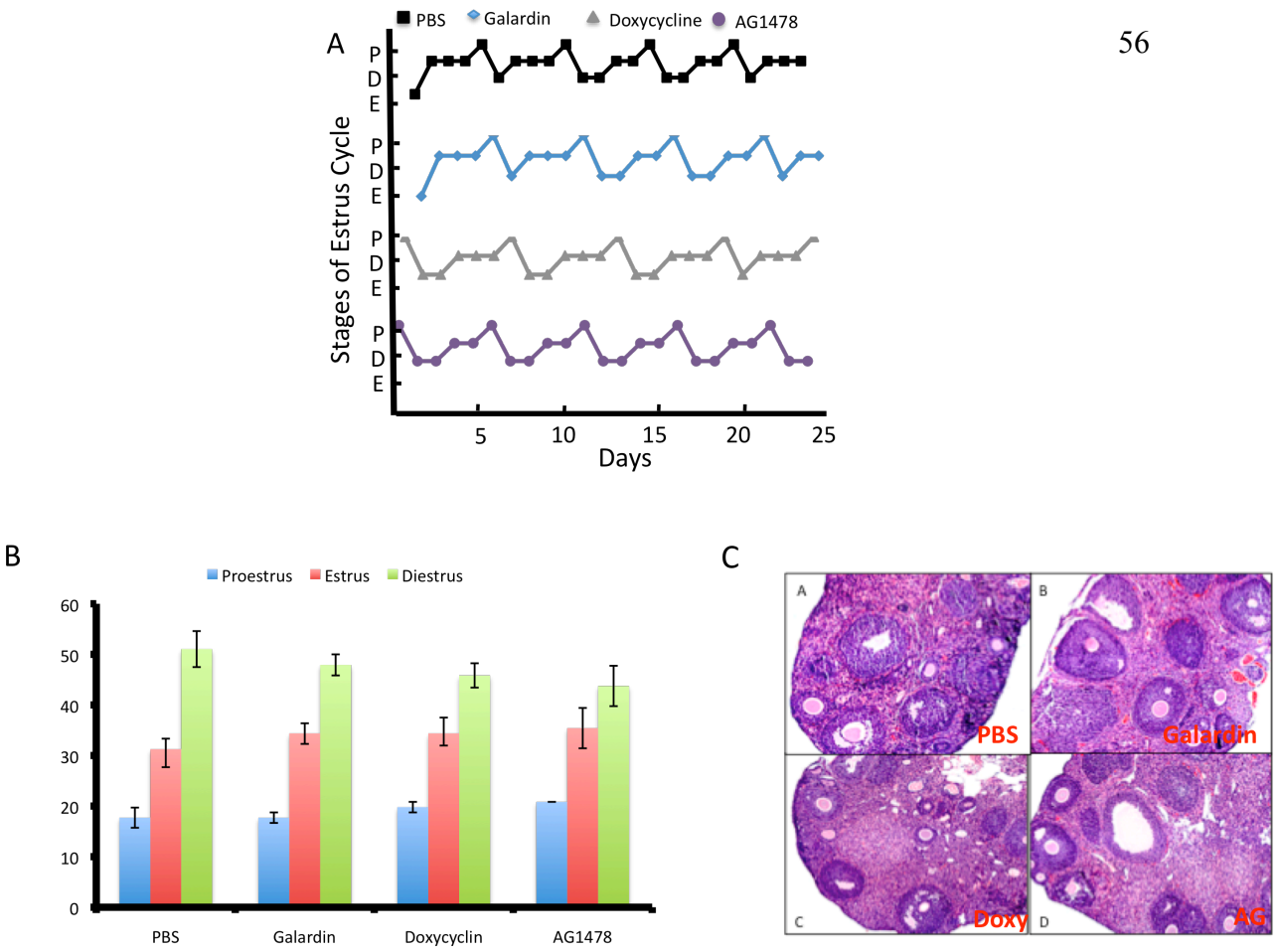

Figure 2.9 *In vivo* injection of MMP inhibitors or EGFR inhibitor does not alter the estrus cycle or follicle development of wild type mice

The estrus cycle of nice-week old female mice was analyzed by vaginal smears for two weeks. Mice were injected daily with IP injections of vehicle, 20µM Galardin, 20µM AG1478, or 100µM Doxycycline for 15 days (A, B). Percent of days spent in each estrous cycle stage (D, diestrus; P, proestrus; E, estrus). Ovaries were removed after the 15th day and stained with H&E (C). Data are represented as mean ± SE (n = 4 mice)

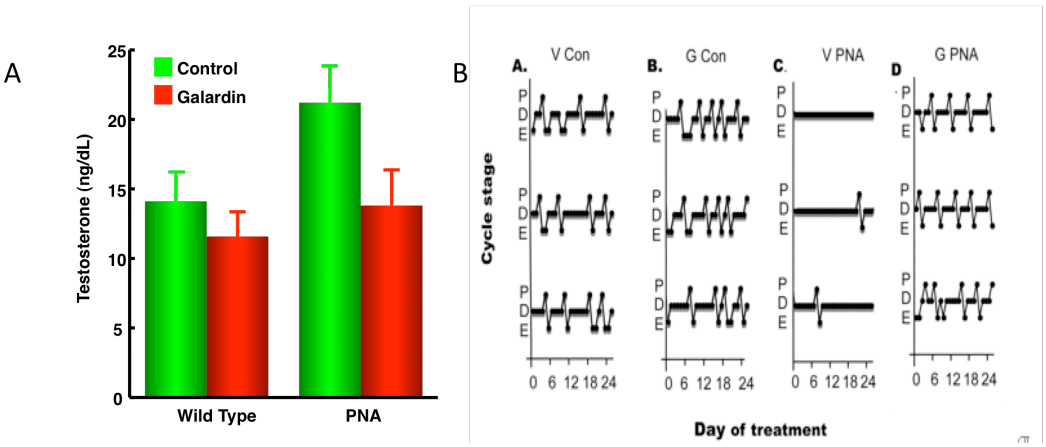

Figure 2.10 Female mice exposed to androgens in-utero exhibit elevated serum testosterone and aberrant estrus cycles.

PNA mice and wild type female mice were given daily IP injections of 20µM galardin or vehicle control for 15 days (A). Testosterone levels were measured by RIA. Wild type mice and PNA mice were IP injected with either 0.05%DMSO as vehicle, or 0.04mg/g/day of Galardin for 24 days (B). The estrus cycle of wild type and PNA mice was analyzed by vaginal smears. Three mice for each group were used.

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Chapter 3

Signaling Pathway for Steroidogenesis in the Adrenal Gland

Introduction:

The gonads and the adrenal gland have the common function of synthesizing steroids. Aside from a common function, they also have a common origin. Both the gonads and the adrenal gland originate from the adrenal-genital primordium (87-89). Differential gene expression then allows the primordial tissue to divide into two separate structures.

The adrenal cortex of the adrenal gland is responsible for synthesizing hormones such as glucocorticoids, such as cortisol (49,50). Similar to gonadal steroidogenesis, the adrenal gland synthesizes steroids under regulation of the hypothalamus and pituitary axis. ACTH is a polypeptide tropic hormone that is produced by the pituitary gland. Upon secretion, it binds and activates the ACTH receptor, a seven trans-membrane G protein-coupled receptor. Upon ligand binding, the receptor elicits a rise in cAMP and the activation of protein kinase A (PKA), leading to steroidogenesis (90). ACTH is known to stimulate steroid production by increasing cholesterol transport into the mitochondria (91). Since ACTH receptor activation is regulated in the adrenal gland by a similar mechanism as the LH receptor in the gonads, the possibility of a conserved mechanism leading to steroidogenesis was analyzed. To begin, the role of EGFR signaling in ACTH-induced steroidogenesis was analyzed by inhibition studies with the AG1478 inhibitor. In

the testes, EGFR signaling was shown to only be important for short-term but not long-term gonadotropin induced steroidogenesis (12). Similar time course studies were performed in the Y-1 cell line. Next, cAMP/MAPK signaling was blocked to identify if they are playing a similar role in steroidogenesis as observed in the testes and ovary. Finally, MMP dependent EGFR activation and subsequent steroidogenesis was examined by similar pharmacological studies.

Materials and methods:

Steroid production in Y-1 adrenal cells.

The Y-1 adrenal cell line (provided by P. Hinkle, University of Rochester) was grown in DMEM/F12 medium (GIBCO) supplemented with 15% fetal bovine serum (Invitrogen). Cells were plated in 12-well plates followed by overnight serum starvation. Experiments were also performed without serum starvation and similar results were obtained. Cells were treated with 20μM AG1478, Galardin, H89, U0126 (Calbiochem), or vehicle for 30 minutes before and during stimulation with 50nM ACTH (VWR), or EGF. Culture media from each treatment was sampled at 30 minutes and 2 hrs after ACTH stimulation.

Steroid Radioimmuno assays.

Progesterone concentrations from media were measured using a progesterone RIA kit (MP Biomedicals). For Y-1 cell experiments, media were collected at 30 minutes or 2 hrs and diluted 1:2 in kit diluent before measurement.

MAPK Activation and Immunoblotting.

Y-1 adrenal cells were washed with cold PBS after treatment and collection of supernatant. 200µl of 1x RIPA (Santa Cruz) buffer supplemented with proteinase inhibitors, sodium orthovanadate, and PMSF was used to lyse cells. Cells were scraped, homogenized and microcentrifuged at full speed for 15 min. Supernatants were removed and diluted with 2X Laemmli sampler buffer with 10% ß-mercaptoethanol (Sigma). Samples were then separated on 12.5% SDS-polyacrylamide gels and transferred to immobilon membranes (Millipore) at 100V for 60 minutes. Membranes were blocked with 5% NFDM in TBST (100mM NaCl, 0.1% Tween 20, 50 mM Tris pH 7.4) for 60 minutes. Membranes were then incubated overnight at 4^0C with primary antibody in 5% NFDM. Blots were washed three times for 10 minutes each with TBST at room temperature while shaking following 1 hr incubation at room temperature (1: 4000) with horseradish peroxidase-conjugated goat anti-rabbit (Bi0-Rad) in TBST and 5% NFDM. Blots were again washed three times with TBST before treatment with ECL-Plus (GE Healthcare). For ERK signaling, ERK1/2 (Cell Signaling) was diluted 1:4000 and total ERK (Cells Signaling) was diluted 1:4000 in TBST plus 5% milk.

Results:

ACTH promotes progesterone production in Y-1 mouse adrenal cells in a dose-dependent fashion.

To investigate the mechanism regulating steroid production in the adrenal gland, mouse Y-1 adrenal cells were used. Initial experiments were performed to determine the sensitivity of the adrenal cell line for ACTH-induced steroidogenesis. Cells were stimulated with increasing amounts of ACTH. Progesterone secreted in the media was measured 30 minutes after stimulation. Y-1 cell lines lack the enzymes required to metabolize substrate to cortisol; therefore progesterone concentrations were measured. As shown in figure 3.1, progesterone production was detected following exposure of ACTH at doses of 25ng/ml to 200ng/ml. The EC_{50} for ACTH-induced steroid production was approximately 50ng/ml, and therefore that concentration of ACTH was used for all future studies.

EGFR signaling is important for ACTH-induced steroidogenesis in adrenal cells.

Since EGFR signaling has been shown to be essential for steroidogenesis in both the testis and the ovary (11,12, 36-40), the role of EGFR signaling in the adrenal gland was analyzed. Y-1 cells were pretreated for 30 minutes with the EGFR inhibitor AG1478, followed by stimulation with ACTH or ACTH plus the inhibitor. ACTH-induced significant progesterone production (Fig. 3.2 A) as early as 30 minutes after stimulation. ACTH-induced progesterone production was inhibited by the EGFR inhibitor, indicating that EGFR plays an important role in ACTH induced steroidogenesis. Steroid induction

by ACTH and inhibition by AG1478 was also analyzed at 2 hrs. Similar to 30 minutes, AG1478 significantly reduced steroid production at 2 hrs. Surprisingly, cells treated with EGF had marginal induction of steroidogenesis both at 30 minutes and 2hrs. Recent work has previously shown that MAPK signaling is important for steroid production in Leydig cells (12), and therefore we analyzed MEK signaling in the presence of the EGFR inhibitor. ACTH induced MAPK activation at both time points and was completely absent when EGFR was inhibited (Fig. 3.2 C, D).

MAPK signaling is important for ACTH-induced steroidogenesis in adrenal cells.

To specifically examine the importance of MAPK signaling in adrenal steroidogenesis, Y-1 cells were pre-treated for 30 minutes with a MAPK inhibitor, U0126. Cells were then stimulated with ACTH for 30 minutes or 2hrs. Progesterone levels indicated the importance of MAPK signaling since blocking MAPK completely eliminated steroid production (Figure 3.3 A, B). To confirm the inhibition of MAPK, western blots were performed on cell lysates treated under these conditions. As expected, ACTH induced robust MAPK activation as early as 30 minutes but essentially absent at the 30 minute and 2 hr time point when cells were previously treated with the U0126 inhibitor (Figure 3.3 C, D). These results indicate that MAPK signaling, like EGFR, is important for ACTH-induced steroid production in the adrenal gland. Interestingly, in Leydig cells, EGFR and MAPK signaling were shown to be important for early, but not late steroid production. In contrast, steroidogenesis in the ovary required EGFR and MAPK signaling at later time-points in a manner similar to what is observed here in adrenal cells.

MMP activity is not important for ACTH-induced steroidogenesis in the adrenal gland.

EGFR signaling is dependent on matrix metalloproteinases (MMP) activity under certain conditions, such as oocyte maturation and ovarian steroid production (11,36-40). MMPs are critical for paracrine signaling in the ovary, but are not essential for steroidogenesis in the testis (12). To test the role of MMP activity in adrenal steroidogenesis, cells were pre-treated with 20μM galardin or 0.01% DMSO for 30 minutes. Cells were then stimulated with ACTH or EGF for 30 minutes or 2hrs, and progesterone levels in the media where measured. ACTH -induced steroid production was not altered after 30 minutes or 2 hrs of stimulation (Figure 3.4 A, B). These results indicate that, like Leydig cell steroidogenesis, EGFR signaling is regulated by an intracellular mechanism where MMP activity is not necessary for activation of the EGFR. Since steroidogenesis is not altered when cells are treated with an MMP inhibitor, MAPK activation should similarly be unaffected by Galardin. Cell lysates were then subjected to western blot analysis and blotted for phosphorylated and total MAPK at 2 hrs after stimulation. As expected, ACTH-induced MAPK activation was not affected by the MMP inhibitor (Figure 3.4 C).

PKA signaling is important for ACTH-induced steroidogenesis in the adrenal gland.

PKA signaling has been previously implicated in regulating EGFR signaling and steroidogenesis in other tissues. Its role in adrenal steroid production was therefore analyzed. Cells were pretreated with 20μM H89, a known PKA inhibitor or 0.01% DMSO for 30 minutes prior to stimulation with ACTH or EGF. ACTH-induced steroid

production was blocked with H89 treatment at 30 minutes and 2 hrs of stimulation (Figure 3.5 A, B). As previously seen, EGF treatment resulted in a small induction of steroidogenesis. However, a small rescue of progesterone levels were observed with co-treatment of EGF in the presence of H89. These results suggest that PKA is important for steroidogenesis, and is acting upstream of the EGFR. These results are consistent with ovarian steroidogenesis.

To analyze the role of MAPK activity in the presence of the PKA inhibitor, cells lysates were collected and analyzed for phosphorylated and total MAPK at 30 minutes and 2 hrs of stimulation. Surprisingly, MAPK activity was essentially unaffected when PKA was blocked (Figure 3.5 C, D). In the testes and ovary, the MAPK pathway was shown to be important and downstream of PKA signaling. These results suggest that another pathway is leading to activation of MAPK that is independent of steroidogenesis.

cAMP activation is important for ACTH-induced steroidogenesis in adrenal cells.

To directly increase cAMP levels and therefore analyze its role in steroidogenesis, cells were stimulated with forskolin. Forskolin acts by activating the enzyme adenylyl cyclase and increasing intracellular levels of cAMP. Cells stimulated with forskolin at 30 minutes and 2 hrs induced similar levels of progesterone seen with ACTH stimulation. Cells were pretreated with 20μM U0126, 20μM AG1478, 20μM H89, and 20μM Galardin for 30 minutes prior to stimulation. As previously seen with ACTH stimulation, the MAPK, EGFR, and PKA pathways are essential for adrenal

steroidogenesis for early and late steroidogenesis (Figure 3.6 A, B). Inhibition of MMP activity was further shown not to be essential for steroidogenesis in the adrenal gland.

Discussion:

The present work identified the mechanism regulating ACTH-induced steroid production in the adrenal gland. As expected, the mechanism regulating this process is, for the most part, conserved in all three steroidogenic tissues described. Adrenocortical hormones are synthesized from cholesterol and transported into the adrenal gland. Progesterone can then be metabolized into aldosterone or cortisol. The adrenal cortex is divided into three zones. The outermost zona glomerulosa produces aldosterone, followed by the zona fasciculata, which produces cortisol, and finally, the innermost zona reticularis that produces androgens. All of the necessary enzymes for metabolizing each steroid are found within each zone. Perhaps this can explain results obtained with the MMP inhibitor.

One major difference between testicular and ovarian steroid regulation is the involvement of MMPs. In the ovary, MMP activation was shown to be important for normal follicular development (36-39), as well as steroid production (11). However, steroidogenesis in the testes is regulated by an intracellular mechanism, where MMP activation was shown to not be essential. To address these differences in the adrenal gland, Y-1 cells were treated with the broad spectrum MMP inhibitor galardin. Results demonstrated that adrenal (Y-1 cells) steroidogenesis is regulated by a similar mechanism identified in the testis, where MMP activation is not essential for proper steroid production.

As stated in the previous chapters, EGFR signaling is important for steroid production in the ovaries and the testes (11, 12). Cell culture experiments using a mouse adrenal cells line were performed to identify the mechanism regulating ACTH-induced steroid production in the adrenal gland. Inhibition of the EGFR resulted in decreased progesterone levels, indicating that this mechanism is conserved across all three steroidogenic tissues: ovary, testes and adrenal gland.

EGFR and MAPK activation was shown to be important for proper steroid production in the adrenal gland. These results are similar to the testes with the exception that EGFR and MAPK activation was important for early and late ACTH-induced steroid production. Why is EGFR/MAPK signaling still important and therefore not turned off at later time points? This could be due to different phosphotases activated in the adrenal gland, or possibly ACTH induces higher concentrations of StAR leading to prolonged steroid production. Similarly, cAMP and subsequent PKA activation was shown to be sufficient for steroid synthesis since forskolin induced steroidogenesis. These results confirm previous experiments where ACTH induced activation of cAMP levels and PKA activation. The mechanism of ACTH-induced steroidogenesis in the adrenal gland is summarized in figure 3.7. cAMP is required for EGFR trans-activation as well StAR upregulation. Both MAPK and cAMP/PKA pathway are necessary for steroidogenesis to occur. It is still unclear how EGFR is being activated. Based on our results however, it does not seem to be activated by MMPs or PKA signaling. Perhaps Src signaling is activating the EGFR, but more studies are needed to address this possibility.

In summary of the data presented, all three steroidogenic tissues are regulated by a similar mechanism. This mechanism involves activation of the EGFR via intracellular activation seen in the testes and adrenal gland where all of the steps for steroid biosynthesis are present, and extracellular activation in the ovary, where steroidogenesis relies on paracrine signaling. These observations are important for treatment of diseases such as PCOS, where the steroid biosynthetic pathway is dysregulated in one tissue, but not the others.

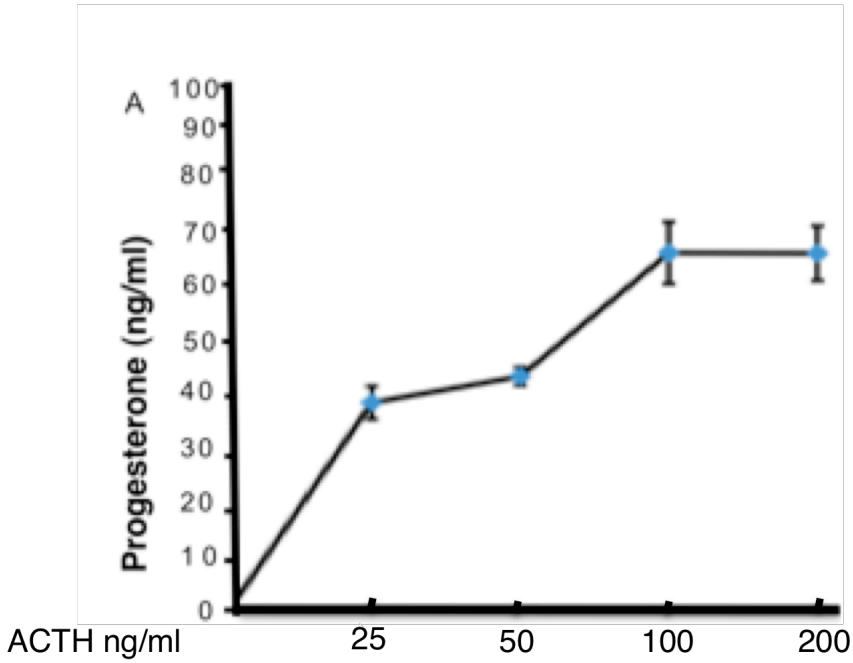

Figure 3.1*:* ACTH promotes progesterone production in Y-1 mouse adrenal cells in a dose-dependent fashion.

Y-1 cells were plated in a 12 well plate and exposed to ACTH for 30 minutes. Each point represents the mean ± S.D. (n=3). All studies were performed three times with similar results.

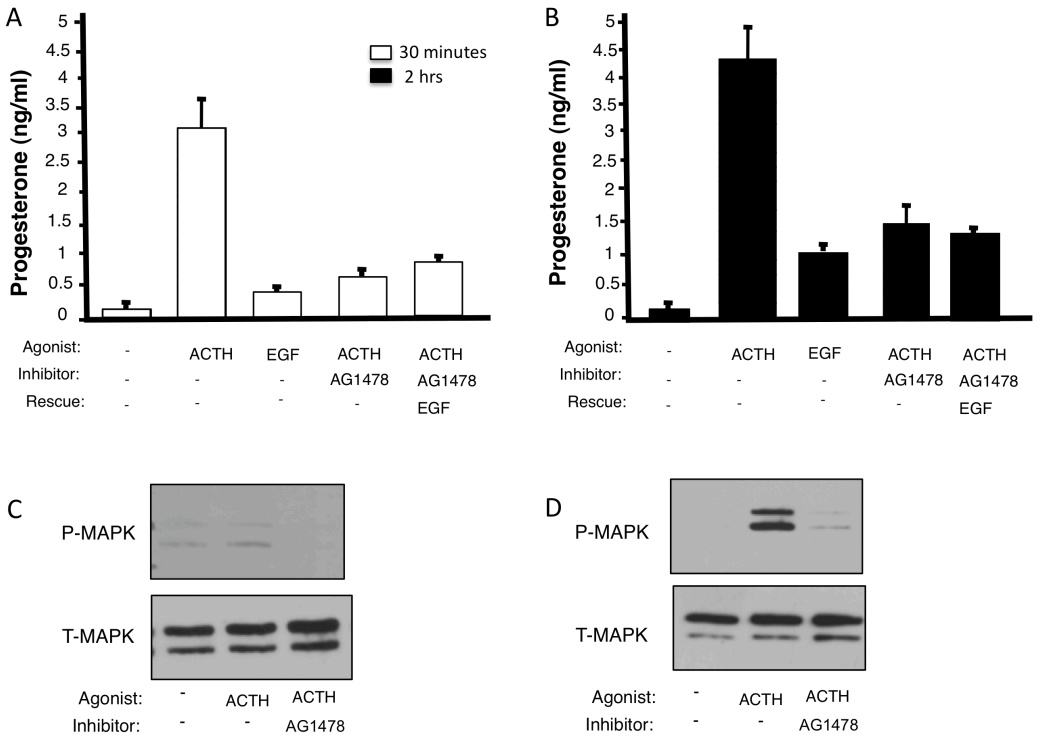

Figure 3.2: EGFR signaling is important in ACTH induced steroidogenesis in adrenal cells

Y-1 mouse adrenal cells were pre-treated with 0.01% DMSO, 20µM AG1478 for 30 minutes, prior to stimulation with 50ng/ml of ACTH or 20ng/ml EGF for 30 minutes or 2 hours. Progesterone content of the media was measured by RIA (A, B). Cell lysates were examined by Western blot for phosphorylated and total MAPK after 30minute and 2 hrs of stimulation (C, D). Each point represents the mean ± S.D. (n=3). All studies were performed three times with similar results.

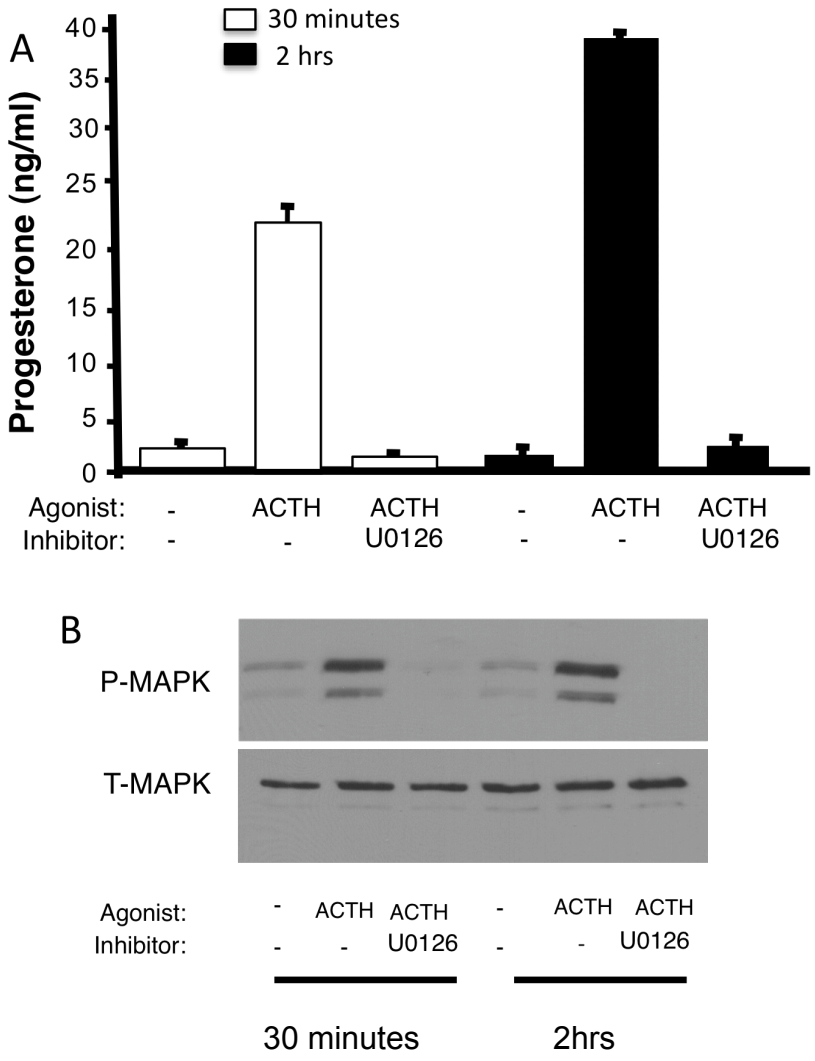

Figure 3.3: MAPK signaling is important in ACTH induced steroidogenesis in adrenal cells.

Y-1 mouse adrenal cells were pre-treated with 0.01% DMSO, or 20μM U0126, prior to stimulation with 50ng/ml of ACTH for 30 minutes or 2 hours. Progesterone content of the media was measured by RIA (A). Cell lysates were examined by Western blot for phosphorylated and total MAPK following 30 minute and 2 hrs of stimulation by agonist (B). Each point represents the mean ± S.D. (n=3). All studies were performed three times with similar results.

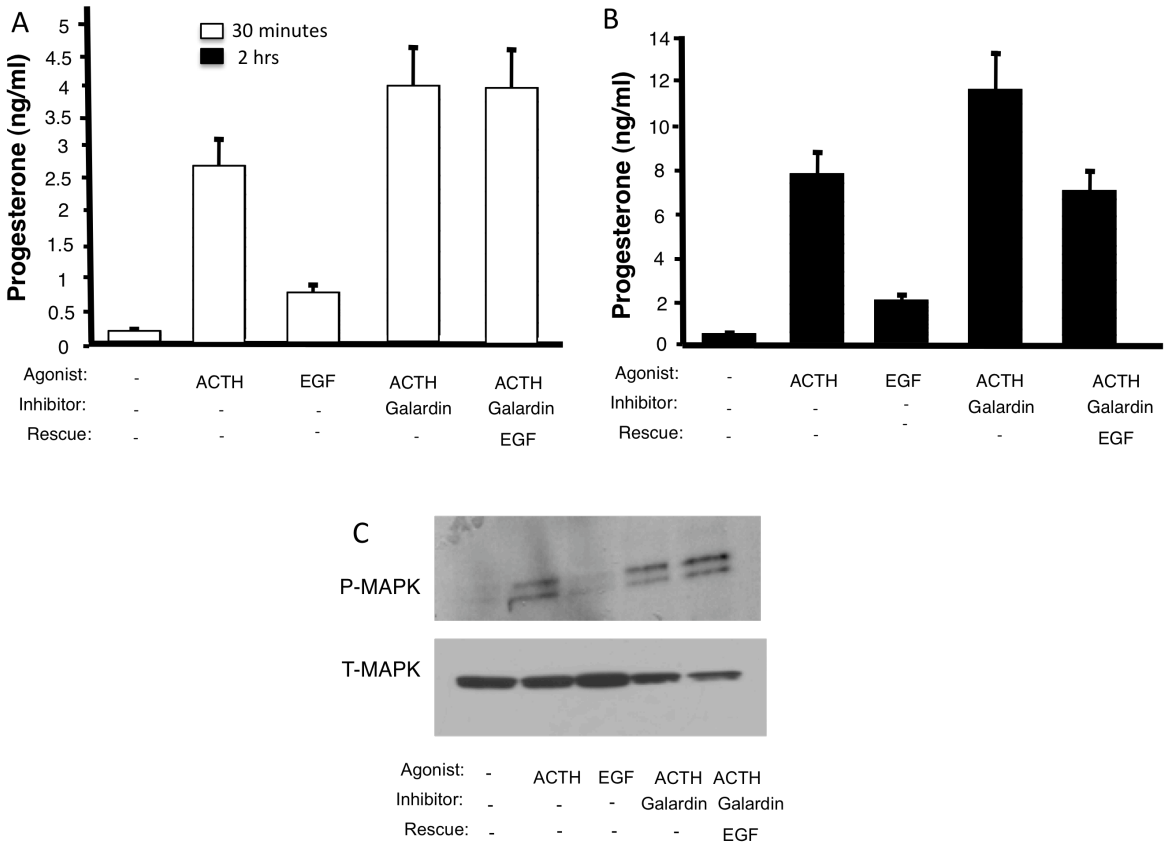

Figure 3.4: MMP activity is not important in ACTH-induced steroidogenesis in the adrenal gland

Y-1 mouse adrenal cells were pre-treated with 0.01% DMSO, or 20μM Galardin, prior to stimulation with 50ng/ml of ACTH or 20ng/ml EGF for 30 minutes or 2 hours. Progesterone content of the media was measured by RIA (A, B). Cell lysates were examined by Western blot for phosphorylated and total MAPK for 2 hrs of stimulation (C). Each point represents the mean ± S.D. (n=3). All studies were performed three times with similar results.

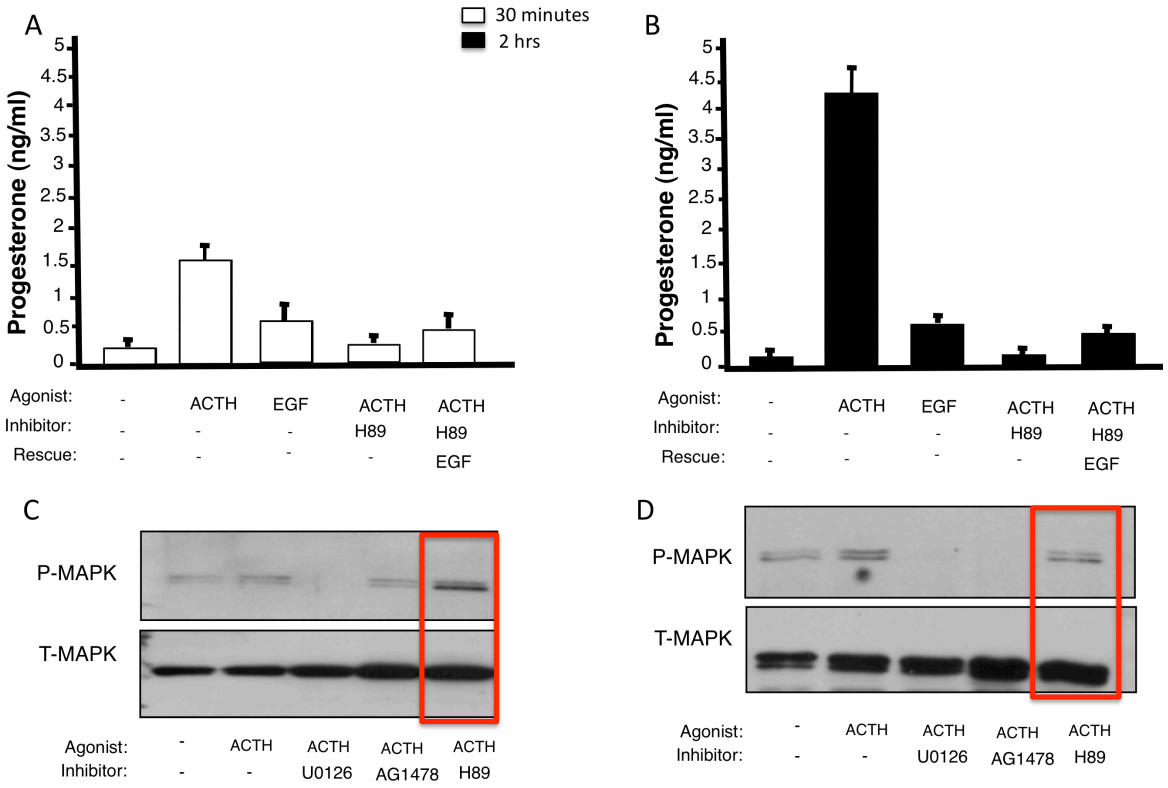

Figure 3.5: PKA signaling is important for ACTH induced steroidogenesis in the adrenal gland

Y-1 mouse adrenal cells were pre-treated with 0.01% DMSO, 20μM U0126, 20μM AG1478, or 20μM H89, prior to stimulation with 50ng/ml of ACTH or 20ng/ml EGF for 30 minutes or 2 hours. Progesterone content of the media was measured by RIA (A, B). Cell lysates were examined by Western blot for phosphorylated and total MAPK for 30 minutes and 2 hrs of stimulation (C, D). Each point represents the mean ± S.D. (n=3). All studies were performed three times with similar results.

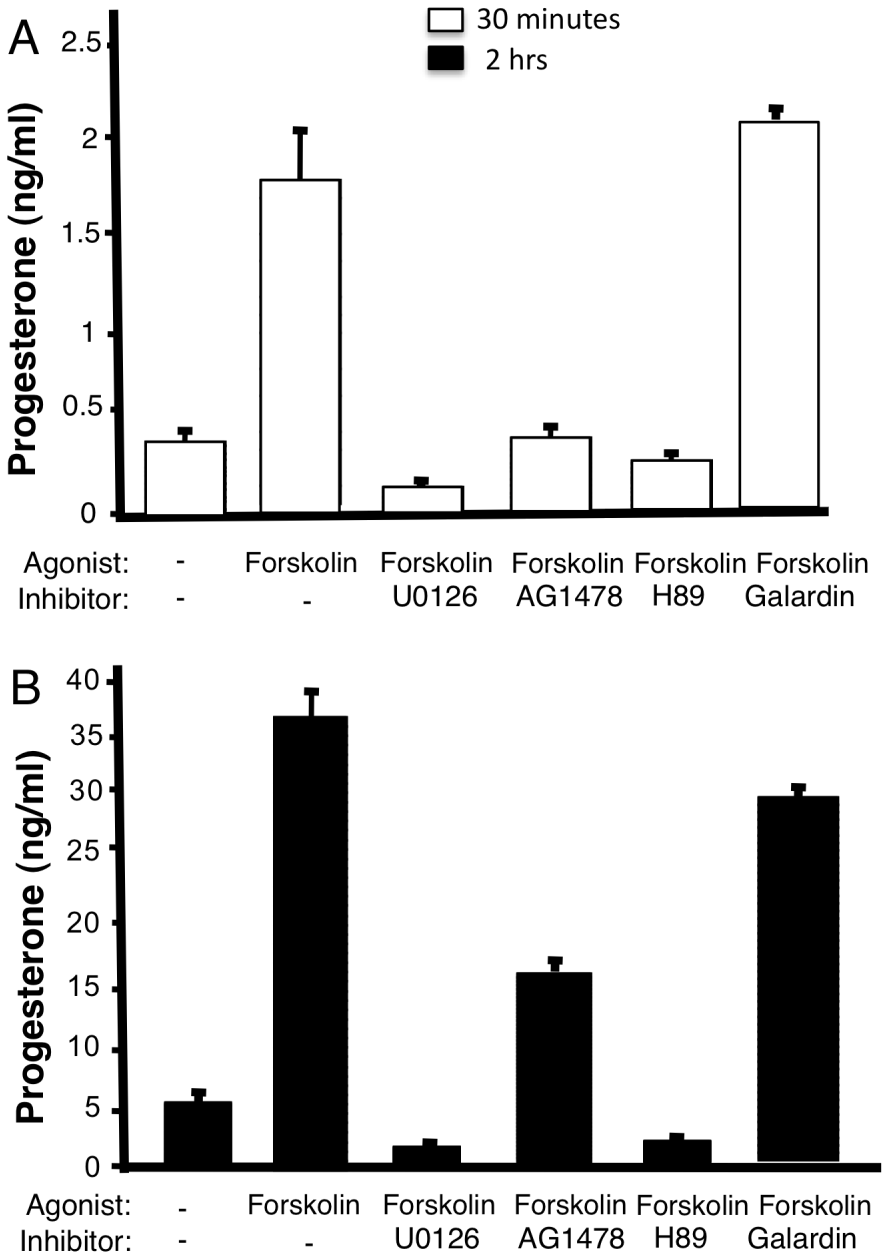

Figure 3.6: cAMP activation is important for ACTH induced steroidogenesis in adrenal cells

Y-1 mouse adrenal cells were pre-treated with 0.01% DMSO, 20μM U0126, 20μM AG1478, 20μM H89 or 20μM Galardin, prior to stimulation with 10μM Forskolin for 30 minutes or 2 hours. Progesterone content of the media was measured by RIA (A, B). Each point represents the mean ± S.D. (n=3).

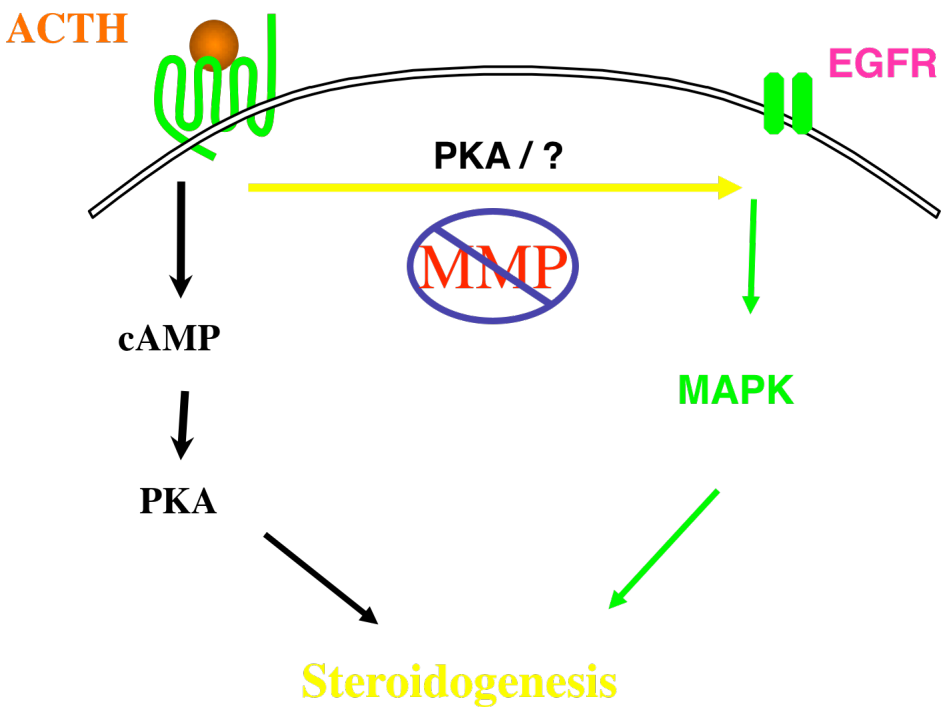

Figure 3.7: Model for ACTH-induced steroidogenesis in adrenal Y-1 Cells

ACTH binds its GPCR and induces activation of cAMP/PKA signaling to drive steroidogenesis. ACTH also drives activation of the EGFR by still an unknown mechanism. Activation of the EGFR induces MAPK signaling and therefore steroidogenesis. Both the cAMP/PKA and MAKP signaling pathways are necessary for steroidogenesis to occur.

Chapter 4

Discussion and Future Directions

Work presented in this thesis and previously published work has identified the mechanisms regulating steroid hormone synthesis in three steroidogenic tissues: the ovary, testis, and adrenal gland. In all three tissues, EGFR trans-activation followed by MAPK signaling and StAR translocation, was shown to be important for LH, and ACTH -induced steroid production (11,12). One notable difference is the mechanism in which GPCR signaling via LH or ACTH exhibits cross-talk with the EGFR. In adrenal and testes, EGFR activation is accomplished via an intracellular mechanism. The ovary is a multi-cell; complex that requires precise regulation and therefore is controlled via an extracellular approach (Figure 4.1).

Intracellular versus extracellular trans-activation of the EGFR

As detailed earlier, the ovary relies on paracrine signaling between mural and cumulus granulosa cells to induce cumulus cell expansion, oocyte maturation, and steroidogenesis (11,36-40). In contrast, steroidogenesis in the testis and adrenal gland is regulated by an intracellular mechanism (12). The mechanism leading to trans-activation of the EGFR in the ovary is mediated by matrix metalloproteinase's, however; the mechanism that regulates intracellular EGFR trans-activation and steroidogenesis in the testis and adrenal gland is still unknown. Therefore, characterization of LH or ACTH -induced EGFR phosphorylation in these cells may elucidate a possible intracellular regulatory mechanism. One possible regulator of EGFR phosphorylation is the PKA pathway. From *in vitro* data in Leydig and adrenal cells, it is clear that PKA signaling is

necessary for EGFR activation and subsequent steroid synthesis. However, data confirming that PKA directly phosphorylates the EGFR is still needed. Future *in vitro* studies can be performed to identify PKA target sites on the EGFR, and analyze whether these sites are essential for EGFR trans-activation and steroidogenesis.

Activation of Matrix Metalloproteinase

Matrix metalloproteinase's are responsible for LH –mediated ovarian steroidogenesis, since they cleave membrane bound EGF that then acts as a paracrine molecule in neighboring cells (11,36-41). Although their role in ovarian signaling has been well established, the precise MMPs that are activated by LH are still unknown. Previous *in vitro* studies have used Galardin to demonstrate their significant role in steroidogenesis. Galardin is a broad-spectrum MMP inhibitor that affects MMP1, 2, 3, 8, 9 and possibly others. Doxycycline has been shown to inhibit MMP2/9, although experiments to demonstrate its specificity have not been performed. In the PCOS mouse model, inhibition of MMPs by galardin resulted in a reduced disease state where testosterone levels were reduced and cycling was restored (77). These results suggest a possible novel treatment for PCOS patients, since galardin had no adverse side effects on wild type controls. Although these results are promising, identifying and inhibiting the MMP activated can lead to a more targeted approach to lowering testosterone levels.

Preliminary work has shed some light on which MMPs are activated in LH-mediated ovarian steroidogenesis. Pre-ovulatory follicles pre-treated with the MMP2/9 inhibitor demonstrated that blocking MMP 2/9 specifically leads to similar results as

obtained with the broad-spectrum MMP inhibitor galardin. Since MMP2 and MMP9 are both expressed in the ovary (92) and are necessary for *in vitro* steroidogenesis, MMP 2/9 might be the MMPs activated in LH-induced oocyte maturation and steroidogenesis. To confirm this hypothesis, experiments to examine fertility and steroidogenesis from MMP2, MMP9, and MMP2/9 mouse genetic knockouts should be performed.

Finally, activity-based protein profiling can be used to identify the MMPs responsible for activation of the EGFR and subsequent steroid production. In this unbiased approach, a library of probes that are recognized by active MMPs can be used to identify which MMPs are activated to drive steroidogenesis. Dr. Cravatt from Scripps Research Institute has developed a library of probes with similar structure to galardin (92,93). Initial experiments can identify a specific probe library that can block steroidogenesis in follicles treated with LH or LH plus the probes. Once a library has been identified to decrease steroid levels,
follicles can be treated with a set of probes that are covalently linked to azide-biotin, followed by UV activation to link probes to the recognized MMPs. Complexes formed can be identified by avidin affinity purification and mass spectroscopy (Figure 4.2).

In summary, identifying the specific mechanism regulating steroid production in all three steroidogenic tissues can lead to better treatment options when regulation is disturbed. For example, the finding that MMPs in the ovary can be directly inhibited to decrease testosterone levels without inhibiting normal adrenal steroid production has lead to a possible novel treatment option for women with PCOS. Specifically, the idea of

using doxycycline is intriguing since it is a common antibiotic used regularly with minimal side effects. Work presented in the previous chapters explained the use of Galardin in the PCOS mouse model. Encouraging data demonstrated the ability of galardin to correct PCOS phenotypes of increased testosterone levels and infertility due to anovulation. Even more interesting is the finding that even after galardin injections were stopped in the PNA mouse, mice continued to cycle for several weeks. These findings are fascinating due to the possibility of patients given doxycycline and then stopped treatment and allowed then to cycle normally on their own. This treatment would avoid multiple pregnancies as seen with clomiphene treatment.

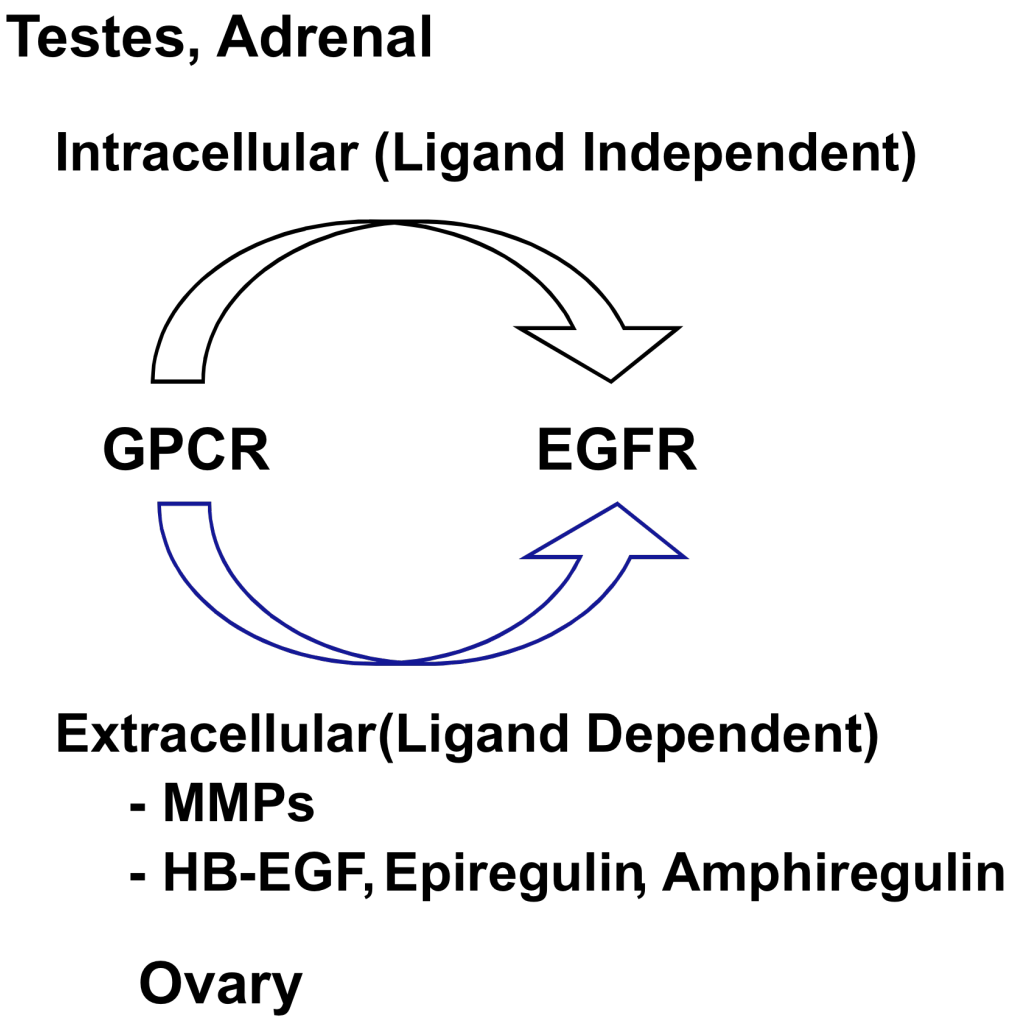

Figure 4.1 The GPCR/EGFR Pathways affecting steroidogenesis

GPCR/EGFR crosstalk is critical for gonadotropin-induced steroid production in the ovary, testis and adrenal gland. However, in the testis and adrenal, intracellular or ligand -independent regulation controls steroid production where steroidogenesis in the ovary is regulated by extracellular, ligand-dependent regulation where MMPs are necessary for activation of the EGFR.

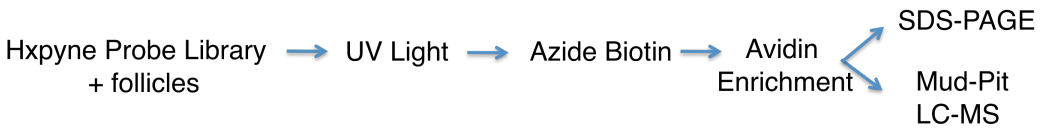

Figure 4.2 MMP active site labeling

Follicles treated with LH or DMSO will be incubated with probe libraries. Once a specific library is identified to lower steroidogenesis, probes will be covalently bound to Azide-biotin and complexes purified by mass spec.

BIBLIOGRAPHY

1. Charlton H (2008). Hypothalamic Control of Anterior Pituitary Function: History. *Journal of Neuroendocrinology*, **20**, 641-646

2. Vadakkadath Meethal ST, Liu H, Chan E, Ginsburg AC, Wilson DN, Gray RL, Bowen BK, Vonderhaar, and Atwood CS (2009). Identification of a regulatory loop for the synthesis of neurosteroids: a steroidogenic acute regulatory protein-dependent mechanism involving hypothalamic-pituitary-gonadal axis receptors. *Journal of Neurochemistry.* **110**:1014-1027

3. Hupfeld C, Olefsky JM (2007). Regulation of receptor tyrosine kinase signaling by GRKs and beta-arrestins. *Annu Rev Physiol* **69** 561-77

4. Gavi S, Shumay E, Wang H, Malbon C (2006). G protein Coupled Receptors as Substrates for Tyrosine Kinases. *Trends in Endocrinology & Metabolism.* **17** 46-52.

5. Delcourt N, Bockaert J, Marin P (2007) GPCR-jacking: from a new route in RTK signalling to a new concept in GPCR activation. *Trends in Pharmacological Sciences* **28** 602-607.

6. Shah BH, Catt KJ (2004). GPCR-mediated transactivation of RTKs in the CNS: mechanisms and consequences.*Trends in Neurosci.* **27**, 48-53

7. Wetzker R, Bohmer FD (2003). Transactivation joins multiple tracks to the MAP kinase cascade. *Nat. Rev. Mol. Cell Biol.* **4**, 651-657

8. Ohtsu H, Dempsey PJ, Eguchi S (2006). ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *Am. J. Physiol. Cell Physiol*. **291** 1-10

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

10. Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, & Tsafriri A (2005). Epidermal growth factor family members: endogenous mediators of the ovulatory response *Endocrinolog*y **146**, 77-84

11. Jamnongjit M, Gill A, Hammes SR (2005). Epidermal growth factor receptor signaling is required for normal ovarian steroidogenesis and oocyte maturation. Proc Natl Acad Sci U S A 102:16257-16261

12. Evaul K, Hammes SR (2008). Cross-talk between G protein-coupled and epidermal

growth factor receptors regulates gonadotropin-mediated steroidogenesis in Leydig cells. *J Biol Chem* **283,** 27525-27533

13. Mohammadi, M. (1983) Biochemistry. **32** 8742-48

14. Curry TE, Osteen KG. (2003). The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocrine Reviews* **24**, 428-65

15. Sternlicht MD, Werb Z. (2001) How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* **17**:463–516

16. Payne AH, Hales DB (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 2004;**25**:947-970

17. Skinner M, McLachlan R, Bremner W (1989). Stimulation of Sertoli cell inhibin secretion by the testicular paracrine factor PModS. *Mol Cell Endocrinol* **66**: 239–49

18. Clark BJ, Wells J, King SR, Stocco DM (1994). The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *The Journal of biological chemistry*. **269:** 28314-28322

19. Jo Y, Stocco DM (2004). Regulation of steroidogenesis and steroidogenic acute regulatory protein in R2C cells by DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1) *Endocrinology*. **145**: 5629-5637

20. Jo Y, King SR, Khan DA, Stocco DM (2005). Involvement of protein kinase C and cyclic adenosine 3',5'-monophosphate-dependent kinase in steroidogenic acute regulatory protein expression and steroid biosynthesis in Leydig cells. *Biol of Reproduction*. **73**: 244-5.

21. Clakr BJ, Soo SC, Caron KM, Ikeda Y, Parker KL, Stocco DM (1995). Hormonal and developmental regulation of the steroidogenic acute regulatory protein. *Mol Endocrinology*. **9**: 1346-1355

22. Pinkerton, J. H. M, McKay, D. G, Adams E C, Hertig A T. (1961). Development of the human ovary--a study using histochemical technics. *Obstet Gynecol.* **18**: 152–181

23. Albertini, D.F., Carabatsos, M.J. (1998). Comparative aspects of meiotic cell cycle control in mammals. *J Mol Med* **76**: 795-799.

24. Hammes SR. (2004). Steroids and oocyte maturation--a new look at an old story. *Mol Endocrinol*. **18:** 769-75.

25. Gill A, Jamnongjit M, Hammes SR. (2004). Androgens promote maturation and signaling in mouse oocytes independent of transcription: a release of inhibition model for mammalian oocyte meiosis. *Mol Endocrinol*.**18**: 97-104

26. Gallo CJ, Hand AR, Jones TL, Jaffe LA (1995). Stimulation of Xenopus oocyte maturation by inhibition of the G-protein alpha S subunit, a component of the plasma membrane and yolk platelet membranes. *J Cell Biol*. **130**: 275-84.

27. Lutz LB, Cole LM, Gupta MK, Kwist KW, Auchus RJ, Hammes SR (2001). Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation. *Proc Natl Acad Sci U S A*. **98:**13728-33

28. Sohaskey ML, Ferrell JE Jr (1999). Distinct, constitutively active MAPK phosphatases function in Xenopus oocytes: implications for p42 MAPK regulation In vivo.*Mol Biol Cell.* **10**:3729-43

29. Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C (2003). Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J Biol Chem.* **278**:5493-6.

30. Maller JL, Krebs EG (1980). Regulation of oocyte maturation. *Curr Top Cell Regul*. **16**:271-311

31. Rasar M, DeFranco DB, Hammes SR (2006) Paxillin regulates steroid-triggered meiotic resumption in oocytes by enhancing an all-or-none positive feedback kinase loop. *J Biol Chem.* **281**:39455-64.

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

33. Jamnongjit M, Hammes SR. (2005). Oocyte maturation: the coming of age of a germ cell. *Semin Reprod Med.* **23**:234-41

34. Hsieh M, Lee D, Panigone S, Horner K, Chen R, Theologis A, Lee DC, Threadgill DW, Conti M (2007), Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. *Mol Cell Biol* **27**:1914–1924

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

36. Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, Tsafriri A, (2005). Epidermal growth factor family members: endogenous mediators of the ovulatory response. *Endocrinology* **146**:77–84.

37. Conti M, Hsieh M, Park JY, Su YQ (2006). Role of the epidermal growth factor network in ovarian follicles. *Mol Endocrinol* **20**:715– 23

38. Panigone S, Hsieh M, Fu M, Persani L, Conti M (2008). Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. *Mol Endocrinol* **22**:924–936.

39. Conneely OM, Lydon, JP, DeMayo F,O'Malley BW (2000). Reproductive Functions of the Progesterone Receptor. Reproductive Sciences **7**:S25–S32.

40. Chaffin CL, Stouffer RL (2002). Local role of progesterone in the ovary during the periovulatory interval. *Rev Endocr Metab Disord* **3**:65–72

41. LeMaire WJ (1989). Mechanism of mammalian ovulation. *Steroids* **54**:455–469.

42. K. Shiraishi and M. Ascoli. (2006) Activation of the lutropin/ choriogonadotropin receptor in MA-10 cells stimulates tyrosine kinase cascades that activate ras and the extracellular signal regulated kinases (ERK1/2). *Endocrinology* **147**:3419–3427

43. Sapru, Hreday,Siegel A (2007) *Essential Neuroscience* 9121-9

44. Whitehead, S., Nussey, S. (2001) Endocrinology an Integrated Approach. 122-6

45. Mirilas P, Skandalakis JE, Colborn GL, Weidman TA, Foster RS, Kingsnorth A, Shandalakis LJ (2004). *Surgical Anatomy*. 399

46. Deng J, Carbajal L, Evaul K, Rasar M, Jamnongjit M, Hammes SR (2009). Nongenomic steroid-triggered oocyte maturation: of mice and frogs. *Steroids.* 74:595-601

47. Miller WL (1988). Molecular biology of steroid hormone synthesis. Endocr Rev. **9**:295-318.

48. Boyd KN, Kumar S, O'Buckley TK, Porcu P, Morrow AL (2010). Ethanol induction of steroidogenesis in rat adrenal and brain is dependent upon pituitary ACTH release and de novo adrenal StAR synthesis. J Neurochem. **3**:784-96.

49. Muller J. (1995). Aldosterone: the minority hormone of the adrenal cortex.*Steroids*. **60**:2-9

50. Rainey W, Nakamura Y (2008). Regulation of the adrenal androgen biosynthesis.*J steroid Biochem Mol Biol*. **108**:281-6

51. Clark BJ, Wells J, King SR, Stocco DM (1994). The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *The Journal of biological chemistry.* **269**:2831-28322

52. Jo Y, Stocco DM (2004) Regulation of steroidogenesis and steroidogenic acute regulatory protein in R2C cells by DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1). *Endocrinology* **145**:5629-5637

53. Stocco DM, Clark BJ, Reinhart AJ, Williams SC, Dyson M, Dassi B, Walsh LP, Manna PR, Wang XJ, Zeleznik AJ, Orly J (2001). *Molecular and cellular endocrinology* **177:**55-59

54. Morohashi K. (1997). The ontogenesis of the steroidogenic tissues. *Genes to cells*. **2**:95-106

55. Walsh LP, Webster DR, Stocco DM (2000). Dimethoate inhibits steroidogenesis by disrupting transcription of the steroidogenic acute regulatory (StAR) gene. *J Endocrinol.* **167**:253-63.

56. Gallant S, Brownie AC. (1973). The in vivo effect of indomethacin and prostaglandin E2 on ACTH and DBCAMP-induced steroidogenesis in hypophysectomized rats. *Biochem Biophys Res Commun.* **55**:831-6.

57. Stocco DM, Wang X, Jo Y, Manna PR. (2005). Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. *Mol Endocrinol*. **19**:2647-59.

58. Lin D, Sugawara T, Strauss JF 3rd, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL (1995). Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis.*Science.* **267**:1828-31.

59. Manna PR, Huhtaniemi IT, Wang XJ, Eubank DW, Stocco DM (2002). Mechanisms of epidermal growth factor signaling: regulation of steroid biosynthesis and the steroidogenic acute regulatory protein in mouse Leydig tumor cells. *Biology of reproduction* **67**:1393-1404.

60. Shiraishi K, Ascoli M (2006). Activation of lutropin/choriogonadotropin receptor in MA-10 cells stimulates tyrosine kinase cascades that activate ras and the extracellular signal regulated kinases (ERK1/2). *Endocrinology* **147**:3419-3427

61. Gambineri A, Pelusi C, Vicennati V, Pagotto U, Pasquali R (2002). Obesity and the polycystic ovary syndrome. *International Journal of Obesity*. **26,** 883-896**.**

62. Shayya, R., Chang, RJ. (2010) Reproductive endocrinology of adolescent polycystic ovary syndrome. *BJOG*.**117**, 150-155.

63. Pasto CL, Griffin-Korf ML, Aloi AJ. Evans WS, Marshall JC (1998). Polycystic ovary syndrome: evidence for reduced sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by estradiol and progesterone. *J Clin Endocrinology Metab.* **83**, 582-90.

64. Daniels TL, Berga SL (1997). Resistance of gonadotropin releasing hormone drive to sex steroid-induced suppression in hyperandrogenic anovulation. *J Clin Endocrinology Metab* **82***,* 4179-83.

65. Christman GM, Randolph JF, Kelch RP, Marshall JP (1991). Reduction of gonadotropin-releasing hormone pulse frequency is associated with subsequent selective follicle-stimulating hormone secretion in women with polycystic ovarian disease. *J Clin Endocrinology Metab.* **72**, 1278-85.

66. Ascoli, M. & Segaloff, D.L. (1989). Regulation of the differentiated functions of Leydig tumor cells by epidermal growth factor. *Ann. N.Y. Acad. Sci*. **564**, 99-115.

67. Fan, H., Liu, Z., Shimada, M., Sterneck, E., Johnson, P., Hedrick, S., Richards, J. (2009) MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility.*Science* **324**, 938-41.

68. Dufau, M. L., Tsuruhara, T., Horner, K. A., Podesta, E., and Catt, K. J. (1977). Intermediate role of adenosine 3':5'-cyclic monophosphate and protein kinase during gonadotropin-induced steroidogenesis in testicular interstitial cells. *Proc. Natl. Acad. Sci*. U.S.A. **74**, 3419-3423

69. Pierce KL, Luttrell LM, Lefkowitz RJ (2001). New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene*. **20**:1532-1539.

70. Herrlich A, Daub H, Knebel A, Herrlich P, Ullrich A, Schultz G, Gudermann T (1998). Synthesis and biological evaluation of aeroplysinin analogues: a new class of receptor tyrosine kinase inhibitors. Bioorg. Med. Chem. **6**, 1153-1162

71. Chaudhry K, Rogers R, Guo M, Lai Q, Goel G, Liebelt B, Ji X, Curry A, Carranza A, Jimenez DF, Ding Y (2010). Matrix metalloproteinase-9 (MMP-9) expression and extracellular signal-regulated kinase 1 and 2 (ERK1/2) activation in exercise-reduced neuronal apoptosis after stroke. *Neurosci Lett*. **474**:109-14

72. Lee CZ, Xu B, Hashimoto T, McCulloch CE, Yang GY, Young WL (2004). Doxycycline suppresses cerebral matrix metalloproteinase-9 and angiogenesis induced by focal hyperstimulation of vascular endothelial growth factor in a mouse model.*Stroke*. **35**:1715-9.

73. Chung AW, Yang HH, Radomski MW, van Breemen C (2008). Long-term doxycycline is more effective than atenolol to prevent thoracic aortic aneurysm in marfan syndrome through the inhibition of matrix metalloproteinase-2 and -9 *Circ Res.***102**:73-85

74. Sochor M, Richter S, Schmidt A, Hempel S, Hopt UT, Keck T (2009). Inhibition of matrix metalloproteinase-9 with doxycycline reduces pancreatitis-associated lung injury. *Digestion*. **80**:65-73.

75. Nicolescu AC, Holt A, Kandasamy AD, Pacher P, Schulz R (2009) Inhibition of matrix metalloproteinase-2 by PARP inhibitors. *Biochem Biophys Res Commun*. **387**:646-50.

76. Manna PR, Chandrala SP, Jo Y, Stocco DM (2006). cAMP-independent signaling regulates steroidogenesis in mouse Leydig cells in the absence of StAR phosphorylation. *Journal of molecular endocrinology* **37**:81-95

77. Sullivan SD, Moenter SM (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

78. Wildt L, Hausler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE, Knobil E (1981). Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: Implications for a common fertility disorder *Endocrinology* **109**:376-385.

79. Marshall JC, Griffin ML (1993). The role of changing pulse frequency in the regulation of ovulation. *Hum. Reprod*. **8:**57-61.

80. Hall JE, Taylor AE, Hayes FJ Crowley WF Jr (1998). Insights into hypothalamic-pituitary dysfunction in polycystic ovary syndrome. *Endocrinol. Invest*. **21**:602-611.

81.Marshall JC, Eagleson CA (1999). Neuroendocrine aspects of polycystic ovary

syndrome. *Endocrinol. Metab. Clin. North Am.* **28**:295-324.

82. Pastor CL, Griffin-Korf ML, Aloi JA, Evans WS, Marshall JC (1998). Polycystic Ovary Syndrome: Evidence for Reduced Sensitivity of the Gonadotropin-Releasing Hormone Pulse Generator to Inhibition by Estradiol and Progesterone[1]J. *Clin. Endocrinol. Metab.* **83**:82-590.

83. Yong EL, Hillier SG, Turner M, Baird DT, Ng SC, Bongo A, Ratnam SS (1994). Differential regulation of cholesterol side-chain cleavage (P450scc) and aromatase enzyme mRNA expression by gonadotropins and cyclic AMP in human granulosa cells. *J Mol Endocrinol.***12**:239-249

84. Leung PC, Steele GL (1992). Intracellular signaling in the gonads. *Endocr Rev* **13**:476-498

85. Havelock JC, Rainey WE, Carr BR (2004). Ovarian granulosa cell lines. *Mol Cell Endocrinology*.**228**:67-78

86. Piquette GN, LaPolt PS, Oikawa M, Hsueh AJ (1991). Regulation of luteinizing hormone receptor messenger ribonucleic acid levels by gonadotropins, growth factors, and gonadotropin-releasing hormone in cultured rat granulosa cells. *Endocrinology.***128**:2449-56

87. Hatano O, Takakusu A, Nomura M, Morohashi K (1996). Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes to Cells* **1**:663 – 671

88. MByskov AG (1986). Differentiation of mammalian embryonic gonads. Physiol. Rev. **66**: 71 – 117.

89. Dufau ML (1988). Endocrine regulation and communi-cating functions of the Leydig cell. Annu. Rev. Physiol. **50**: 483 – 508.

90. Simpson ER & Waterman MR (1988). Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu. Rev. Physiol. **50**:427 – 440

91. Jefcoate CR, DiBartolomeis MJ, Williams CA, McNamara BC (1987). ACTH regulation of cholesterol movement in isolated adrenal cells. *J Steroid Biochem.* **27**:721-9

92. Sieber SA, Niessen S, Hoover HS, Cravatt BF (2006). Proteomic profiling of metalloprotease activities with cocktails of active-site probes. *Nat Chem Biol.* **2**:274-81

93. Saghatelian A, Jessani N, Joseph A, Humphrey M, Cravatt BF (2004). Activity-based probes for the proteomic profiling of metalloproteases. *Proc Natl Acad Sci U S A*.**101**:10000-5

94. Ascoli M, Segaloff DL (1989) Regulation of the differentiated functions of Leydig tumor cells by epidermal growth factor. *Annals of the New York Academy of Sciences* **564**:99-115

95. Daaka Y, Luttrell LM, and Lefkowitz RJ (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**: 88–91

96. Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y, Lefkowitz RJ, Luttrell LM (2000). The β_2-Adrenergic Receptor Mediates Extracellular Signal-regulated Kinase Activation via Assembly of a Multi-receptor Complex with the Epidermal Growth Factor ReceptorJ. *Biol. Chem*. **275**: 9572–9580

97. Kim J, Eckhart AD, Eguchi S, Koch WJ (2002). *β-Adrenergic Receptor-mediated DNA Synthesis in Cardiac Fibroblasts Is Dependent on Transactivation of the Epidermal Growth Factor Receptor and Subsequent Activation of Extracellular Signal-regulated Kinases*. **277**:32116–32123

98. Keenan DM, Veldhuis JD (2004). Hypothesis testing of the aging male gonadal axis via a biomathematical construct Am. J. Physiol. **286**:R381–R389

99. Richards JS, Pangas SA (2010). The ovary: basic biology and clinical implications. *J Clin Invest*.**120**:963-72

100. Lee H, Park JW, Kim SP, Lo EH, Lee SR (2009). Doxycycline inhibits matrix metalloproteinase-9 and laminin degradation after transient global cerebral ischemia. *Neurobiol Dis*. **34**:189-98

101. Clark BJ, Wells J, King SR & Stocco DM (1994) The purification, cloning, and expression of a novel LH-induced mitochondrial protein in MA-10 mouse Leydig tumor cells: characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry* **269**:28314–28322.

102. Sugawara T, Holt JA, Kiriakidou M & Strauss III JF (1996) Steroidogenic factor 1-dependent promoter activity of the human steroidogenic acute regulatory protein (StAR) gene. *Biochemistry* **35** : 9052–9059.

103. Christenson LK, Johnson PF, McAllister IM, Strauss JF, III. (1999). CCAAT/enhance-binding proteins regulate expression of the human

steroidogenic acute regulatory protein (StAR) gene. *J Biol Chem* **274**:26591–26598.

104. Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane E, Strauss JF, III. (1997). Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidogenic factor1. *Steroids*. **62**:5–9.

105. Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF, III, (2001). Amsterdam A. The ERK signaling cascade inhibits gonadotropin- stimulated steroidogenesis. *J Biol Chem*. **276**:13957–13964.

106. Cameron MR, Foster JS, Bukovsky A, Wimalasena J. (1996). Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5'-monophosphates in porcine granulosa cells. *Biol Reprod*. **55**:111–119.

107. Franks S, Gilling-Smith C, Watson H, Willis D. (1999). Insulin action in the normal and polycystic ovary. *Endocrinol Metab Clin North Am*. **28**:361–378.

108. Al-Agha OM, Axiotis CA. (2007). An in-depth look at Leydig cell tumor of the testis. *Arch Path Lab Med.* **131**:311-7.

109. Xavier Bertagna, MD, PhD, Laurence Guignat, MD, Lionel Groussin, MD, PhD, Je´ ro me Bertherat, MD, PhD. (2009) Cushing's Disease. Best Practice & *Research Clinical Endocrinology & Metabolism* **23**:607–623