THE ROLE OF EGFR SIGNALING IN GONADOTROPIN-INDUCED STEROIDOGENESIS AND OOCYTE MATURATION

APPROVED BY SUPERVISORY COMMITTEE

Stephen Hammes, M.D., Ph.D.

Carole Mendelson, Ph.D.

Philip Shaul, M.D.

David Garbers, Ph.D.

Dedicated To My Parents

For their constant love and encouragement

# EGFR SIGNALING IS NECESSARY FOR GONADOTROPIN-INDUCED STEROIDOGENESIS AND OOCYTE MATURATION

by

MICHELLE THUC-QUYEN JAMNONGJIT

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Michelle Thuc-Quyen Jamnongjit, PhD.

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Supervising Professor: Stephen R Hammes M.D., PhD.

The mid-cycle luteinizing hormone (LH) surge triggers ovarian processes that are critical for normal female fertility, including steroidogenesis, oocyte maturation, and subsequent ovulation. Studies were designed to examine the LH-induced signaling pathways that regulate ovarian steroidogenesis, as well as the subsequent role of steroids and their receptors on oocyte and follicle development. Initial work focused on transcription-independent, or nongenomic, steroid-induced maturation of oocytes. We demonstrated for the first time that steroids trigger oocyte maturation in several different mouse models, activating G protein signaling, as well as MAPK and CDK1/cdc2 intracellular signaling cascades. Pharmacologic studies using receptor antagonists as well as transgenic mouse models suggested that steroid-induced maturation is mediated through classical steroid receptors. Subsequent studies focused on LH-induced signals that regulate steroidogenesis in

the mouse ovary. We found that EGF triggered steroid production in three separate models of follicle culture and surprisingly, EGF receptor signaling was absolutely necessary for LH-induced steroidogenesis in an intact follicle. In addition, studies performed in testicular Leydig cells and adrenal cells revealed that EGF receptor signaling is required for normal steroid production, making this a universal signaling pathway in steroid physiology. In summary, our data suggest a model whereby stimulation of LH receptors in theca and mural granulosa cells activates matrix metalloproteinases (MMP) to cleave membrane-bound EGF moieties. The soluble EGF molecules then stimulate EGFRs on granulosa cells (and possibly theca cells), leading to activation of StAR and increased steroidogenesis. The steroids in turn act upon the oocyte to promote maturation, allowing for ovulation to occur. Notably, MMPs appear to be important only for ovarian steroid production, offering us a novel target for specifically regulating steroidogenesis in the ovary. Accordingly, in cycling and superovulated mice, we were able to show that treatment with the broad-spectrum MMP inhibitor Galardin *in vivo*, decreased ovarian steroid production without affecting adrenal steroidogenesis. Given these findings we propose that MMP inhibitors may be useful to specifically down-regulate excess ovarian steroid production in diseases such as polycystic ovarian syndrome (PCOS), the leading cause of infertility in women of reproductive age.

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## LIST OF DEFINITIONS

AR- androgen receptor

BMP15- bone morphogenic protein 15

CDK- cyclin dependent kinase

Cx43- connexin 43

dbcAMP- dibutyryl, 3'-5'-cyclic adenosine monophosphate

EGFR- epidermal growth factor receptor

ER- estrogen receptor

GDF9 - growth differentiation factor

GPCR- G protein-coupled receptor

GVBD- germinal vesicle breakdown

HB-EGF – heparin bound epidermal growth factor

hCG- human chorionic gonadotropin

HPG- hypothalamic/pituitary/gonadal

IBMX- isobutyl methyl xanthine

LH- luteinizing hormone

MAPK- mitogen activated protein kinase

MAS- meiosis activating sterol

MMP- matrix metalloproteinases

OCCs- oocyte-cumulus cell complexes

OGCs- oocyte-granulosa cell complexes

PCOS- polycystic ovarian syndrome

PDE- phosphodiesterase

PMSG- pregnant mare serum gonadotropin

PR- progesterone receptor

Pyk2 – protein tyrosine kinase 2

Src- cellular sarcoma virus

StAR- steroidogenic acute regulatory protein

# Chapter 1

## General Introduction

### 1.1 Oogenesis

During human fetal development, primordial germ cells originate in the dorsal endoderm of the yolk sac, close to the allantoic evagination. By weeks 4-5, these cells migrate through the hindgut before settling in the urogenital ridge (diZerega and Hodgen, 1981). By week 25 of gestation, approximately 7 million oogonia have been formed by mitosis and developed into primary oocytes (Griffin and Ojeda, 2000). At this stage, a single layer of flattened epithelial cells surrounds the primary oocyte to form a primordial follicle. The first steps of meiosis I occur in the primordial follicle, including the replication of DNA and arrest in the diplotene state of prophase I. Controversy has existed for many decades regarding the status of the oocyte population within the mammalian ovary after birth. The more traditional conviction has been that mammals are born with their full contingent of oocytes that are held in meiotic arrest until puberty, when small populations of growing follicles are triggered to mature during each cycle (Mandl and Zuckerman, 1951). In contrast, recent evidence suggests that, at least in mice, oocytes may be steadily destroyed and then regenerated from a small population of germline stem cells throughout the early and reproductive years of mammalian life (Johnson et al., 2004). Although still controversial with regard to mammals, this latter hypothesis is consistent with the ovarian biology of lower vertebrates, such as *Xenopus laevis*, where evidence is relatively strong that new oocytes are

constantly developing from oogonia throughout most of the lifespan of the female frog (Hausen, 1991; Keem, 1979; Smith, 1955).

### 1.2 Meiotic Arrest

Before discussing the external triggers and intracellular events that promote oocyte maturation, the signals that hold oocytes in meiotic arrest must be described. These inhibitory signals appear to originate from the surrounding ovary, as removal of mammalian oocytes from the ovary results in spontaneous maturation within hours (Edwards, 1965a; Edwards, 1965b). The un-prompted re-entry into meiosis that occurs in denuded mammalian oocytes contrasts with lower vertebrates such as frogs, where signals maintaining meiotic arrest appear to be endogenous to the oocytes themselves. For example, isolated amphibian oocytes remain locked in meiotic arrest indefinitely until triggered by exogenous addition of steroids and other compounds (Maller and Krebs, 1980; Smith and Ecker, 1971).

One of the most important intracellular signaling molecules believed to be responsible for maintaining meiotic arrest is cAMP (Conti et al., 2002). In general, intracellular cAMP homeostasis is regulated by two important groups of enzymes: the adenylyl cyclases (ACs), which generate cAMP; and the phosphodiesterases (PDEs), which metabolize cAMP. Most of the well-characterized adenylyl cyclases are regulated by G proteins that either promote ($G\alpha_s$) or inhibit ($G\alpha_i$) their activity (Freissmuth et al., 1989). In contrast, the mechanisms that control PDE activity are less well understood, but may involve short-term activation in response to protein kinase A (PKA)-mediated phosphorylation, as well as long-term regulation that entails changes in mRNA and protein expression (Mehats et al., 2002). cAMP within oocytes appears to act as an inhibitor of oocyte maturation, perhaps

through activation of PKA. Evidence supporting this claim include the following: 1) Elevated intracellular cAMP levels in denuded mouse oocytes prevent spontaneous maturation. For example, isolated oocytes can be held in meiotic arrest by incubation with cAMP analogues such as dibutyryl cAMP or PDE antagonists such as 3-isobutyl-1-methylxanthine (IBMX) or milrinone (Conti et al., 2002; Conti et al., 1998); 2) Meiotic arrest of mouse oocytes within follicles can be released by injecting antibodies targeted against $G\alpha_s$ into oocytes (Mehlmann et al., 2002), and oocyte maturation can be blocked *in vivo* by feeding mice PDE inhibitors prior to and during ovulation (Wiersma et al., 1998); 3) Intracellular cAMP appears to drop rapidly under some conditions at the start of mammalian oocyte maturation (Conti et al., 2002).

Interestingly, similar studies whereby cAMP levels in frog oocytes were artificially elevated resulted in inhibition of steroid-induced maturation, and likewise a drop in cAMP upon activation of meiosis by progesterone has been observed (Morrill et al., 1977; Sadler and Maller, 1981; Sadler and Maller, 1985). In addition, detailed signaling studies using frog oocytes have produced a "release of inhibition" model whereby constitutive $G\beta\gamma$ and/or $G\alpha_s$ signaling within the oocyte appears to hold them in meiotic arrest, perhaps by stimulating adenylyl cyclase to produce cAMP (Gallo et al., 1995; Lutz et al., 2001; Sheng et al., 2001). Triggers of maturation, such as steroids, appear to overcome this inhibitory signal, releasing meiotic arrest and allowing maturation to occur (Hammes, 2003; Hammes, 2004; Maller and Krebs, 1980; Smith and Ecker, 1971). Perhaps these inhibitory G protein-mediated signals have evolved from being constitutively active within the oocytes of lower, egg-laying vertebrates to being activated by factors outside the oocyte in mammalian follicles. Notably,

however, while increased intracellular cAMP clearly inhibits maturation, whether a decrease in intracellular cAMP is either necessary or sufficient to promote oocyte maturation remains unknown (Eppig and Downs, 1988; Faure et al., 1998; Gelerstein et al., 1988).

Finally, one of the earliest candidates to be considered an inhibitor of meiosis in mammalian oocytes was the purine hypoxanthine. Hypoxanthine appears to be produced in the follicle, and inhibits *in vitro* meiosis of oocytes that are either denuded or encased in follicles (Shim et al., 1992). Hypoxanthine functions as a phosphodiesterase inhibitor that might prevent metabolism of cAMP, thus maintaining meiotic-arresting levels of cAMP within the oocyte (Downs et al., 1989); however, the physiologic importance of hypoxanthine still remains uncertain (Eppig and Downs, 1987).

### 1.3 Follicle structure and the gap junction

Appreciation of the structural relationships between oocytes and their surrounding follicle cells is important for understanding the mechanisms regulating meiosis in oocytes (Fig. 1.1A). The mammalian follicle consists of several outer layers of theca cells that play a prominent role in ovarian steroidogenesis. The theca cells surround layers of outer mural granulosa cells, which in turn encompass layers of inner cumulus granulosa cells. These cumulus granulosa cells then form close contacts with the oocytes via gap junctions. Gap junctions are composed of proteins from the connexin family, with Connexin 43 (Cx43) being the primary member in the ovary. Cx43-containing gap junctions appear to be necessary for expansion of granulosa cells during follicular growth (Ackert et al., 2001), as well as for oocyte development. In lower vertebrates, gap junctions play important roles in

supplying nutrition to the oocytes, as they regulate the transport of yolk proteins from the blood to the oocyte (Browne et al., 1979). Likewise, the role of gap junctions in transferring nutrients and metabolic precursors to mammalian oocytes is well established (Herlands and Schultz, 1984).

Interestingly, gap junctions may also be important for maintaining meiotic arrest. Oocytes surrounded primarily by cumulus granulosa cells remain in meiotic arrest when cultured *in vitro*, while disruption of oocyte-granulosa cell contacts during cumulus cell expansion (Fig. 1.1A) coincides with oocyte maturation (Eppig, 1994). These observations suggest that gap junctions may therefore be serving as conduits to transport meiotic inhibitory factors such as cAMP from granulosa cells to oocytes. However, whether disruption of gap junctions actually precedes the initiation of oocyte maturation, or whether disturbance of gap junction integrity is necessary for maturation, remain uncertain; thus, this hypothesis has yet to be verified.

## 1.4 Signals that activate oocyte maturation

### Growth Factors

Although exposure of ovarian follicles to LH leads to oocyte maturation, neither oocytes nor surrounding cumulus granulosa cells contain LH receptors. Instead, the theca and mural granulosa cells express LH receptors, indicating that some paracrine factor(s) must be released by these outer cells in response to LH to promote cumulus cells expansion and oocyte maturation (Peng et al., 1991). The identity of at least some of these paracrine markers has been elucidated, and they appear to be members of the epidermal growth factor

(EGF) family. While the ability of growth factors to promote cumulus cell expansion and subsequent oocyte maturation in follicle cultures has been long established (Downs et al., 1988), their physiologic role in regulating meiosis and ovulation had been uncertain. Studies have now confirmed that the growth factors amphiregulin and epiregulin, and perhaps others, appear to stimulate oocyte maturation and ovulation via the following model (Fig. 1.1B) (Park et al., 2004): First, in addition to promoting steroid production, LH triggers the release of growth factors from the theca and/or mural granulosa cells. These growth factors then act in a paracrine fashion to stimulate inner cumulus granulosa cell expansion, which is followed by disruption of oocyte-granulosa cell contacts, oocyte maturation, and eventual ovulation. Growth factors also stimulate cumulus granulosa cells to produce steroids (Jamnongjit et al., 2005), and though still not proven, follicular fluid-derived meiosis-activating sterol (FF-MAS), and possibly other factors that may play a role in promoting oocyte maturation.

In addition to promoting cumulus cell expansion, EGF-mediated signaling in cumulus granulosa cells might play a direct role in promoting the breakdown of cumulus cell-oocyte gap junctions. EGF treatment of granulosa cells leads to activation of mitogen-activated protein kinase (MAPK), which in turn phosphorylates connexin 43 (Cx43) (Kanemitsu and Lau, 1993). Phosphorylation of Cx43 appears to destabilize gap junctions, which disrupts intercellular junctional communication (Lampe and Lau, 2004) and might therefore prevent the aforementioned transport of meiotic inhibitory factors to the oocyte.

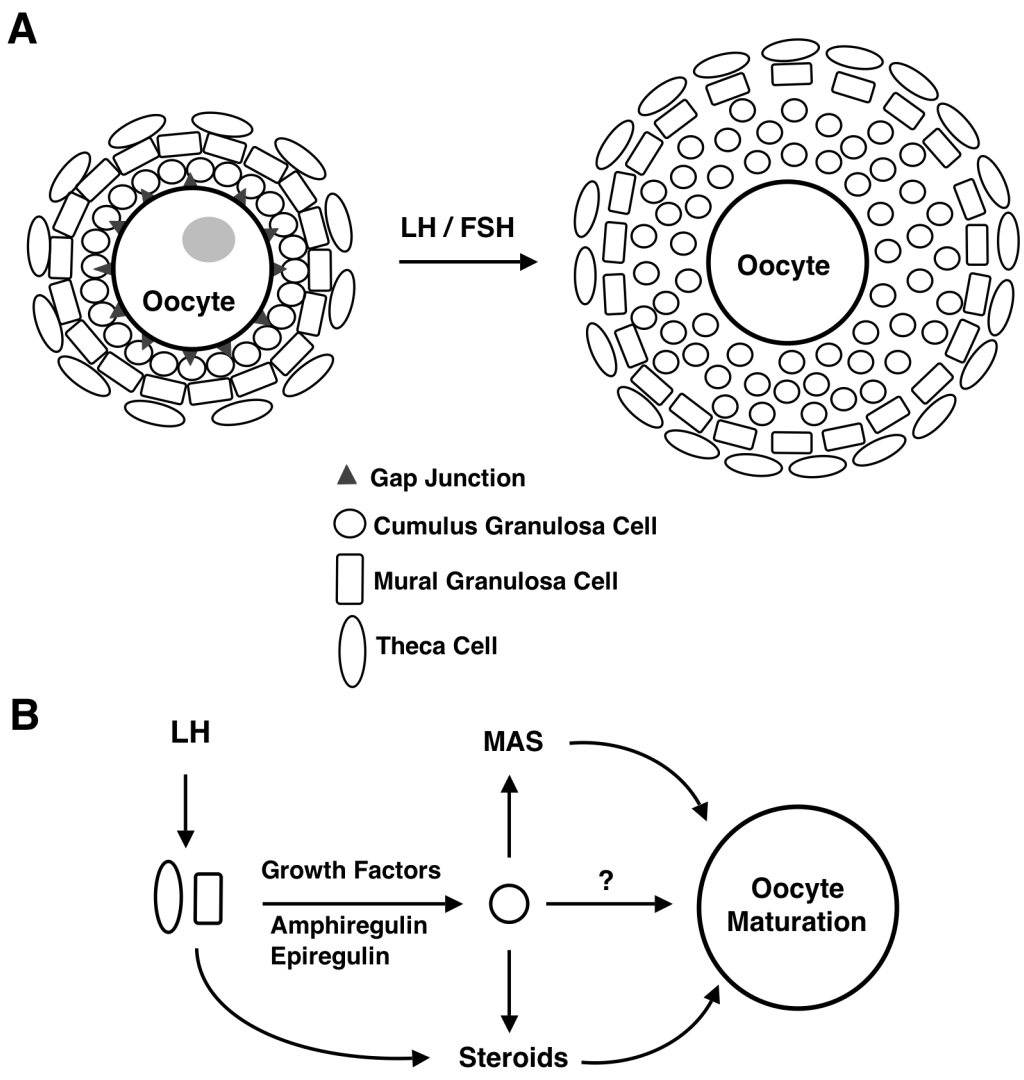


**Figure 1. 1 Follicle structure and EGF mediators.** A. Schematic model of follicle structure and gonadotropin-mediated cumulus cell expansion. Oocytes are directly surrounded by cumulus granulosa cells. These in turn are surrounded by mural granulosa cells, followed by layers of theca cells. LH and FSH stimulate expansion of cumulus granulosa cells, loss of gap junctions, and oocyte maturation, (depicted by loss of gray nucleus). B. Model for gonadotropin-induced oocyte maturation. LH stimulates both steroid and growth factor production by theca and mural granulosa cells. These growth factors, including amphiregulin and epiregulin, then act in a paracrine fashion on cumulus granulosa cells to produce steroids, and possibly MAS, or other unknown factors that can promote oocyte maturation.

**Meiosis-Activating Sterol (MAS)**

As mentioned, one of the factors that might be released from cumulus granulosa cells to promote oocyte maturation is the meiosis-activating $C_{29}$ sterol, FF-MAS (Fig. 2). FF-MAS is an intermediate along the biosynthetic pathway from lanosterol to cholesterol that has been shown to promote maturation of either isolated oocytes held in meiotic arrest or oocytes in cumulus cell cultures (Byskov et al., 1997; Xia et al., 1994). FF-MAS is found in ovarian follicular fluid, and may be secreted by granulosa cells. T-MAS is a metabolite of FF-MAS that has been isolated from bull testis and also appears to promote oocyte maturation *in vitro*. While FF-MAS clearly triggers maturation *in vitro*, some controversy still exists concerning it physiologic importance. Evidence supporting a physiologic role for FF-MAS in regulating meiosis is mainly based on studies demonstrating that inhibition of FF-MAS production or metabolism reduces or enhances, respectively, maturation in cumulus oocyte complexes *in vitro* (Byskov et al., 1995; Lu et al., 2000; Xie et al., 2004). In addition, FF-MAS production has been shown to increase in response to gonadotropins. For example, injection of female rats with pregnant mare's serum gonadotropin (PMSG) stimulates a 3-fold increase in the activity of P450 14α-demethylase, the rate-limiting enzyme in the conversion of lanosterol to FF-MAS (Fig. 1.3), after 48 hours (Yoshida et al., 1996). Furthermore, ovarian FF-MAS levels appear to rise after LH-stimulation in rabbit follicles (Grondahl et al., 2003), which would be consistent with the timing of maturation and ovulation.

In contrast, other studies using similar inhibitors of FF-MAS production do not support its physiologic role in regulating maturation. Furthermore, some laboratories have demonstrated that the time course of FF-MAS-mediated maturation and activation of MAPK, as well as the conditions whereby FF-MAS-induced maturation occur, differ from spontaneous or gonadotropin-induced maturation, suggesting that the sterol is unlikely to be the primary mediator of oocyte maturation *in vivo* (Downs et al., 2001; Faerge et al., 2001; Gross et al., 1993; Tsafriri et al., 2002; Tsafriri et al., 1998; Yamashita et al., 1995). Finally, micromolar amounts of MAS are required to promote maturation, raising concerns that such high sterol concentrations may be having nonspecific effects on cellular membranes (Vaknin et al., 2001). While the physiologic importance of FF-MAS-induced oocyte maturation remains controversial, recent work has demonstrated that, in addition to enhancing meiotic progression from metaphase I to metaphase II, the sterol may play a role in stabilizing oocytes in metaphase II arrest. In fact, treatment of oocytes with FF-MAS seems to markedly improve their ability to be successfully fertilized *in vitro* (Cavilla et al., 2001; Marin Bivens et al., 2004), suggesting that FF-MAS may eventually prove useful as an adjunct during *in vitro* fertilization.

**Steroids**

Steroids have been known to promote oocyte maturation in lower vertebrates such as fish and frogs for many decades. Interestingly, this steroid-mediated maturation occurs independent of transcription, and may be regulated by steroid receptors located at the plasma membrane of cells (Maller and Krebs, 1980; Smith and Ecker, 1971; Smith et al., 1968). One of the best-studied models for oocyte maturation comes from the frog *Xenopus laevis*.

Several steroids are known to promote *Xenopus* oocyte maturation, including progestins, glucocorticoids, and androgens, with the latter being the most potent (Le Goascogne et al., 1985; Lutz et al., 2001; Smith and Ecker, 1971). Evidence has suggested that the membrane receptors regulating progestin- and androgen-triggered maturation might in fact be their classical receptors that are traditionally thought to be located in the nucleus or cytoplasm (Bayaa et al., 2000; Lutz et al., 2001; Lutz et al., 2003; Tian et al., 2000). For example, elimination of the classical *Xenopus* androgen receptor (AR) by RNA interference, or inhibition studies using AR antagonists, significantly and specifically reduced androgen-mediated maturation, as well as androgen-induced activation of accompanying MAPK and CDK1 signals. Furthermore, blockade of androgen production in intact ovaries reduced hCG-induced oocyte maturation, suggesting that androgens are playing an important physiologic role in the regulation of gonadotropin-induced oocyte maturation in *Xenopus laevis* (Lutz et al., 2001; Lutz et al., 2003). Finally, selective androgen receptor modulators (SARMs) have been characterized that specifically regulate genomic versus nongenomic AR-mediated signaling, and therefore may prove useful for specifically modulating oocyte maturation *in vivo* (Lutz et al., 2003).

Experiments aimed at determining the effects of steroids on mammalian oocyte maturation have been difficult to interpret. Several studies have attempted to examine the role of steroidogenesis in regulating meiosis in cultured oocytes by using ketoconazole or other compounds to block sterol production. The results of these studies were variable (Downs et al., 2001; Lu et al., 2000; Tsafriri et al., 1998; Vaknin et al., 2001; Yamashita et al., 2003), perhaps due to the non-specific nature of the inhibitor's effects. Ketoconazole

blocks the activity of nearly all cytochrome P450 enzymes (designated CYP in Fig. 1.2), and is therefore a potent inhibitor of steroid, FF-MAS, as well as cholesterol production. As such, ketoconazole can have profound effects on cholesterol content and transport within cells. Since recent evidence indicates that intracellular cholesterol may play a critical role in maturation of frog oocytes (Sadler and Jacobs, 2004), ketoconazole may not be the most appropriate inhibitor of steroidogenesis to use when studying oocyte maturation in any species.

In addition to blocking steroidogenesis, several studies have directly examined steroid effects on the spontaneous maturation of isolated mammalian oocytes, finding that micromolar amounts of some steroids slowed maturation (Eppig et al., 1983; Rice and McGaughey, 1981; Schultz et al., 1983; Smith and Tenney, 1980). However, these studies were hampered in that they examined steroid-mediated changes in oocyte maturation using oocytes that were already spontaneously maturing rather than studying steroid-triggered maturation of oocytes arrested in prophase I. Furthermore, the high concentrations of steroids may have been toxic to the oocytes, thus complicating interpretation of the results.

Steroids may be tertiary messengers that, along with FF-MAS and perhaps other substances, are secreted by cumulus granulosa cells in response to growth factors to promote maturation (Fig. 1.1B). Detailed studies using follicle cultures and *in vivo* models will be necessary to confirm the role of steroids in oocyte maturation; however, injection of progestins and androgens has recently been shown to enhance oocyte maturation in monkey models (Borman et al., 2004), suggesting that, as in lower vertebrates, steroids may indeed be playing a physiologic role in the regulation of meiosis in mammalian oocytes.

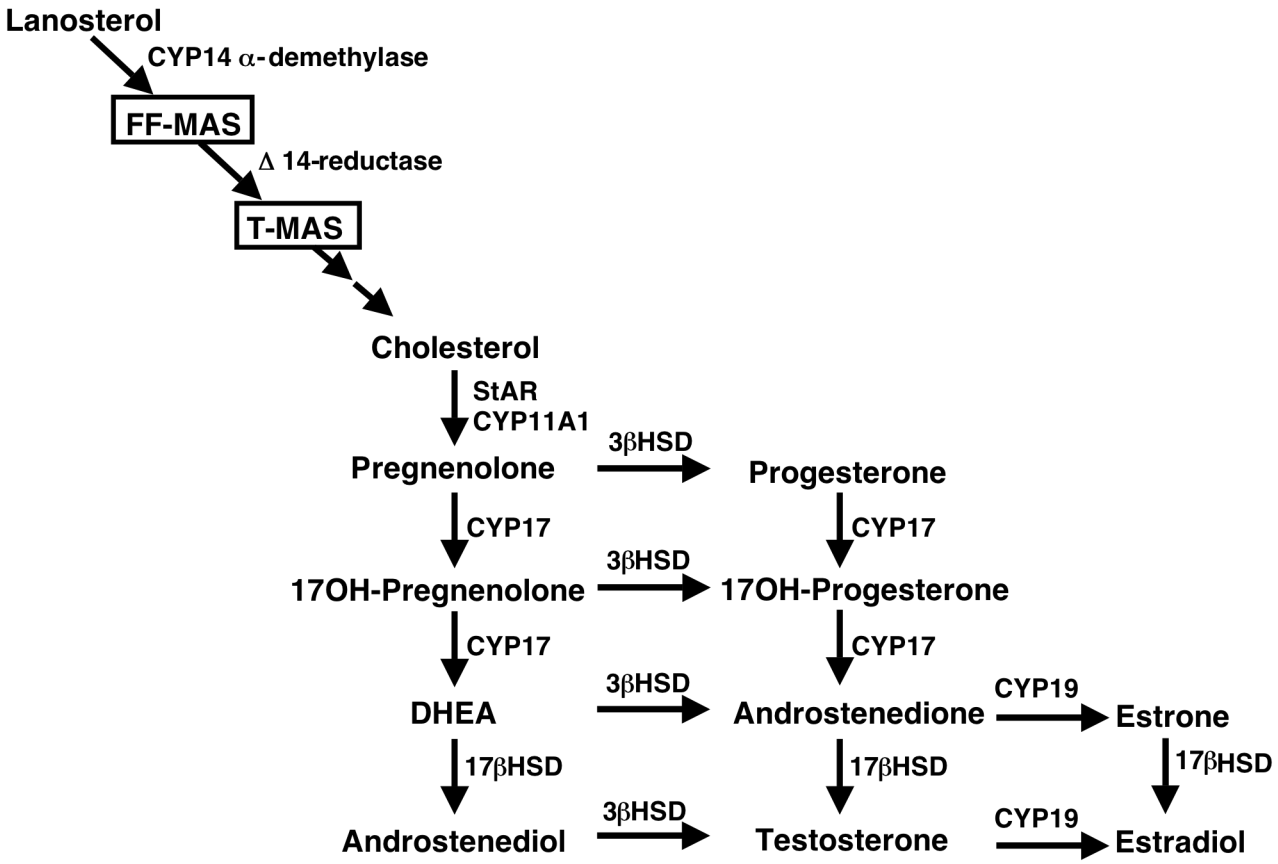


**Figure 1.2. Steroidogenic pathway from the cholesterol precursor lanosterol to the sex steroids testosterone and estradiol.** Sterols that have previously been shown to promote mouse oocyte maturation *in vitro* are boxed.

## 1.5 Physiologic steroid signaling in the ovary

Ovarian steroid levels vary depending upon the day on which they are measured. In mammals, the ovarian steroid content usually mirrors serum steroid levels, with androgen, estrogen, and progesterone levels rising during ovulation, and estrogen and progesterone levels reaching even higher levels during the luteal phase. However, intriguingly, ovarian steroid content is still quite high even at baseline, with follicular fluid containing micromolar amounts of steroid (Enien et al., 1998). What then prevents these saturating amounts of steroid from constantly signaling via their cognate receptors, which bind ligand in the nanomolar range? Possible explanations for this conundrum may include the following. First, the intracellular steroid concentrations might be far lower than the extracellular amounts, as steroid metabolism may be occurring in follicular cells to inactivate the steroid hormones before they can bind to their receptors. Second, follicular fluid contains high amounts of sex hormone binding globulin, as well as other proteins that could potentially sequester steroids (Ben-Rafael et al., 1986). Thus, while the total steroid content may be high, the bioavailable steroid amounts may be relatively low.

### Progesterone Actions

Direct measurement of ovarian steroid content during the LH surge in many different mammals reveals a large and rapid rise in progesterone production (Enien et al., 1998), suggesting that progesterone may play an important role at the time of ovulation. Accordingly, progesterone signaling in the ovary seems to be essential for normal gonadotropin-induced ovulation, as injection of RU486, a classical PR antagonist, blocks ovulation in rodents (Loutradis et al., 1991). Furthermore, knockout mice lacking functional

PRs, fail to ovulate in response to gonadotropins. Histology in these null mice reveals normal development of intraovarian follicles to the tertiary follicle stage, with luteinization of granulosa cells and the production of oocytes that are competent for in vitro fertilization (Lydon et al.). In fact, the major defect appears to be the complete elimination of LH-induced follicle rupture. Additional studies have revealed the abnormal expression of ADAMTS-1 and cathepsin L, two proteases known to be involved in follicle rupture (Robker et al., 2000), indicating that trascriptional targets of the PR may be involved in the progesterone-mediated signaling cascade that triggers ovulation.

**Estradiol Actions**

Estradiol signals primarily via two classical receptors: ERα and ERβ. Granulosa cells mainly express ERβ, suggesting that this receptor may regulate estradiol-mediated stimulation of granulosa cell growth and modulation of FSH action. Accordingly, follicles from ERβ knockout mice have a diminished response to exogenous estradiol, with a reduced cumulus cell mass and a decreased rate of ovulation (Krege et al., 1998). This defect in granulosa cell growth results in reduced female fertility, with fewer and smaller litters than wild-type mice, despite normal sexual behavior.

In contrast to ERβ, ERα is expressed in every location of the hypothalamic/pituitary/gonadal (HPG) axis, and ERα null mice are completely infertile. Interestingly, early ovarian development is unimpaired in the ERα knockout mice, and primordial and primary follicles are indistinguishable from those of wild-type females. However, concomitant with elevated serum gonadotropin levels, follicular development is markedly accelerated in the null relative to wild-type mice around day 20 of life. Follicles

develop to the late antral stage but fail to progress to final maturation, often containing hemorrhagic and atretic cysts (Schomberg et al.). This pathology may in part be explained by a lack of negative feedback by estradiol on the pituitary, leading to excess gonadotropin production and subsequent signaling in the follicular cells.

Similar to the ER knockout mice, elimination of CYP19 aromatase expression results in excess gonadotropin secretion due to the lack of estradiol-mediated negative feedback (Huynh et al., 2004). The hyperstimulation of ovaries by high gonadotropin levels results in the failure to ovulate. In fact, human females with CYP19 deficiency usually have ovarian failure with multicystic ovaries and mild virilization, most likely due the inability to metabolize androgens (Conte et al., 1994).

**Androgen Actions**

Theca cells are stimulated by LH to produce androgens, which then act as paracrine regulators within the ovarian cell types. One function of androgens is to modulate FSH signaling by acting through ARs on granulosa cells to amplify cAMP levels (Hillier and de Zwart, 1982). Interestingly, AR mRNA and protein levels are appear to be down-regulated during late preovulatory development, which may serve to desensitize granulosa cells until exposure to the LH surge (Hillier and Tetsuka, 1997). The importance of the AR in ovarian function has been further examined in AR null mice, where females are subfertile with reduced litter size (Hu et al., 2004; Yeh et al., 2002). Interestingly, older females develop premature ovarian failure (Shiina et al., 2006), further implicating the AR as a mediator of normal follicle development.

## 1.6 Ovarian Steroidogenesis

Ovarian steroid production is critical for the normal development and function of several distant tissues in women, including the uterus, breast, skeleton, and brain. Importantly, steroids produced from the ovary also have autocrine effects that are essential for normal ovarian physiology. While normal ovarian steroid production is critical for the maintenance of standard ovarian development and function, excess ovarian steroid secretion and subsequent local steroid signaling can lead to significant ovarian pathology. The intracellular signals that regulate ovarian steroid production and the physiologic and pathologic consequences of increased steroid signaling in the ovary will be discussed.

### The Two Cell Model

Normal ovarian steroid production is a tightly regulated and complex process that involves several different signaling pathways in multiple kinds of cells (Fig 1.3). A typical mammalian ovarian follicle consists of at least four cell types. Each follicle contains an oocyte surrounded by cumulus granulosa cells. These cells are then bounded by outer mural granulosa cells, which in turn are surrounded by theca cells (Havelock et al., 2004; Wood and Strauss, 2002). Steroidogenesis in most mammals (including humans and mice), appears to occur via the two cell/two gonadotropin model whereby androgens are synthesized from cholesterol in LH-stimulated theca cells, followed by conversion to estrogens in FSH-stimulated granulosa cells. LH receptors and the enzyme CYP17, which converts pregnenolone and progesterone to DHEA and androstenedione, respectively, are expressed primarily in theca cells, while FSH receptors and aromatase (CYP19), which converts androgens to estrogens, are expressed mainly in granulosa cells. Notably, LH receptors are

not exclusively found in theca cells, as their expression levels in mural granulosa cells has been noted to increase just prior to the LH surge (Piquette et al., 1991).

Interestingly, both theca and granulosa cells express steroidogenic acute regulatory protein (StAR, see below), CYP11A1 (side chain cleavage enzyme), and 3β-HSD. Both of these cell types are therefore capable of making pregnenolone and/or progesterone from cholesterol. However, in the follicular phase, the relatively avascularized granulosa cells see limited oxygen or cholesterol, thus very few of these steroids are produced. After exposure to gonadotropins, the granulosa cells become "luteinized" and form part of the corpus luteum, where they then synthesize large amounts of pregnenolone and progesterone from cholesterol (Havelock et al., 2004; Wood and Strauss, 2002). Notably, since granulosa cells lack CYP17, they cannot metabolize progesterone to androgens as would occur in CYP17-expressing theca cells. Thus, most ovarian progesterone production likely comes from granulosa rather than theca cells. Since progesterone is necessary for normal ovulation (Conneely et al., 2000), understanding how LH-induced signaling in theca cells leads to progesterone production in granulosa cells is essential.

## StAR Protein

As mentioned, both theca and granulosa cells express StAR protein and are therefore capable of making steroid from cholesterol precursors (Kiriakidou et al., 1996). StAR is the primary transporter of cholesterol from the outer to inner mitochondrial membrane, where most of the other steroidogenic enzymes are located. Thus StAR plays a key regulatory role in steroid production in the gonads and adrenal glands. StAR is essential for corticosteroid production in the adrenal gland, as humans or mice lacking StAR activity are born with

adrenal insufficiency requiring corticosteroid replacement (Bose et al., 2000; Hasegawa et al., 2000). Similarly StAR is critical for normal androgen production in the testes, as male humans and mice lacking active StAR have external female genitalia due to insufficient androgen production in utero. However, males still retain their internal Wolffian remnants, and young male mice produce low levels of testosterone, suggesting that small amounts of StAR-independent steroidogenesis may still be occurring in the testes during embryogenesis and in early life. As observed in the testes, ovarian steroid production is also partially intact in female mice and humans lacking functional StAR. Low plasma sex steroid levels are present in early pubertal knockout female mice, but eventually decline after puberty (Hasegawa et al., 2000). In addition, some human females lacking StAR activity progress through puberty and even cycle for a short period of time before amenorrhea ensues (Bose et al., 2000).

A "two hit" model has been described to explain the variable steroidogenic organ failure in animals and people lacking functional StAR (Bose et al., 2000). This model proposes that low-level StAR-independent steroidogenesis initially occurs in all steroidogenic tissue, perhaps via precursors other than cholesterol that can be transported to the inner mitochondria in the absence of StAR. With time, however, these cells accumulate cholesterol that cannot be appropriately metabolized, resulting in the eventual destruction of the gland. With regards to the adrenal gland, this "second hit" primarily occurs during fetal development, as fetal adrenal tissue makes steroids and therefore accumulate significant lipids during embryogenesis. Similarly, the testes make steroids both in utero and just after birth; thus, Leydig cell damage occurs during these early periods. In contrast, the ovary

remains relatively dormant until puberty; thus this gland is often preserved until that time. Once steroidogenesis begins, however, the ovary rapidly takes up cholesterol and is destroyed like the adrenal gland and testes. Whether early StAR-independent steroid production in the ovary is sufficient to regulate relatively normal steroid-mediated processes such as follicular growth and oocyte maturation, has yet to be determined.

**LH-induced signaling molecules that may regulate ovarian steroidogenesis**

StAR protein expression and activity is regulated by many factors in steroidogenic tissue, starting with activation of the LH receptor in theca cells. LH-triggered signaling via its G protein-coupled receptor induces several important second messengers that have been implicated as regulators of StAR activity and/or steroidogenesis in general, including the following: (Fig 1.3)

**cAMP and PKA**

While many intracellular signaling pathways have been implicated as regulators of LH-induced ovarian steroid production; gonadotropin-induced elevation of cAMP is one of the few such pathways that has been definitively proven to be important. Both the FSH and LH receptors couple to $G\alpha_s$ when activated by their respective ligands. $G\alpha_s$ in turn stimulates adenylyl cyclase, leading to an elevation in intracellular cAMP levels and subsequent activation of protein kinase A (PKA) (Wood and Strauss, 2002). Elevation of intracellular cAMP in theca or granulosa cells by gonadotropins, forskolin, or membrane-permeable dibutryl cAMP (dbcAMP) promotes steroidogenesis by increasing expression of StAR as well as by increasing StAR activity via phosphorylation on serine 195 (Strauss et al., 1999; Wood and Strauss, 2002). Furthermore, cAMP regulates CYP19 (aromatase) and

CYP17 expression and activity in granulosa and theca cells, respectively, which contributes to estrogen and androgen production (Havelock et al., 2004).

**Phospholipase C (PLC)**

Although primarily coupling with $G\alpha_s$, evidence suggests that the LH and FSH receptors can also activate Gq and $G\alpha_i$ in response to ligand. Gq and $G\beta\gamma$ (whose activity accompanies $G\alpha_i$ and likely $G\alpha_s$ signaling) are both known to activate PLC, which then triggers the production of second messengers inositol-1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG). These molecules in turn increase intracellular calcium and activate PKC, respectively. Under some conditions, PKC and calcium may inhibit adenylyl cyclase and reduce steroidogenesis in mice (Leung and Steele, 1992); however, the precise role of PLC and its downstream signals in regulating ovarian steroidogenesis remains uncertain.

**Src and Extracellular-regulated kinases (ERKs)**

Activation of $G\beta\gamma$ by G protein-coupled receptors activates Src, which in turn triggers the Ras/Raf/MEK/MAPK/ERK (or MAPK) pathway. LH and FSH receptors increase activation of ERKs in some cells lines (Cameron et al., 1996; Seger et al., 2001), and ERK activation increases StAR expression in Y1 adrenal cells (Gyles et al., 2001). In contrast, Src may inhibit steroid production in cultured theca cells by decreasing activity of CYP17 and other steroidogenic enzymes (Chaturvedi et al., 2004). Similarly, MAPK signaling has been shown to decrease CYP17 expression and activity in theca cells, and decreases in overall MAPK signaling may in fact be contributing to elevated CYP17 activity and subsequent androgen production in PCOS (Nelson et al., 2001). Given these conflicting results in the literature, more experiments in better models are necessary to clearly elucidate the

physiologic role of the LH-induced Src and MAPK signaling in the regulation of ovarian steroidogenesis.

**Epidermal Growth Factor (EGF) Receptor.**

Another potentially important signaling molecule that is activated downstream of the LH receptor is the EGF receptor. G protein-coupled receptors (GPCRs) are known to activate EGF receptor signaling by several mechanisms (Herrlich et al., 1998; Pierce et al., 2001). For example, stimulation of the $G\alpha_s$-coupled β2-adrenergic receptor activates membrane-bound matrix metalloproteinases (MMPs, see below) that cleave heparin-bound EGF (HB-EGF) from the cell surface to auto-activate the EGF receptor (Maudsley et al., 2000). Interestingly, several studies have demonstrated that EGF receptor signaling in response to its ligand can promote steroidogenesis in gonadal cells, including Leydig cell lines and ovarian follicles (Ascoli and Segaloff, 1989; Jezova et al., 2001; Manna et al., 2002). Like LH, EGF appears to be promoting steroidogenesis primarily via activation of StAR. In Leydig cells, EGF stimulates StAR production. These observations suggest that, in addition to cAMP, EGF receptor signaling might also be contributing to StAR-mediated steroidogenesis in response to LH.

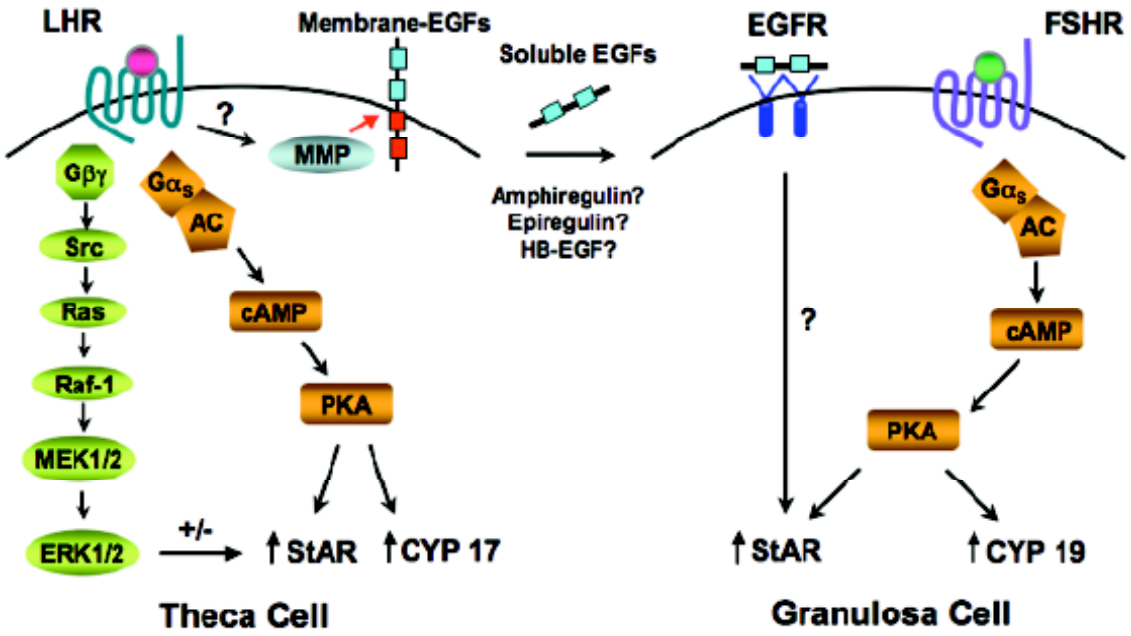


Figure 1.3. **Gonadotropins trigger multiple signals that regulate steroidogenesis**. LH-induced activation of the LH receptor on theca, and likely mural granulosa, cells triggers several signaling pathways that can regulate steroid production. $G\alpha_s$-mediated increases in cAMP lead to elevated PKA activity, which in turn can increase StAR and CYP17 expression and activity via mechanisms that are still not well understood. Concomitant $G\beta\gamma$-mediated signaling can activate the Src/Ras/MAPK signaling cascade, which in turn has been shown to both increase and decrease StAR and CYP17 activity, depending upon the cell culture system. Finally, through yet unknown mechanisms, LH receptor signaling can trigger activation of MMPs, which in turn may cleave membrane-EGF moieties to release soluble EGFs (possibly amphiregulin, epiregulin, and/or HB-EGF). These soluble EGFs can then activate the EGF receptor on granulosa, and possibly theca, cells to increase StAR activity via mechanisms that have yet to be characterized. Finally, similar to the LH receptor, activation of the FSH receptor on granulosa cells increases intracellular cAMP and activates PKA, which in turn can increase StAR and CYP19 (aromatase) expression and activity.

## 1.7 Pathologic steroid signaling in the ovary – Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is the primary cause of infertility in young adults, with approximately 10% of all women being affected. It is associated with high ovarian androgen production in conjunction with anovulation; however, many patients with PCOS also have features of the metabolic syndrome, including obesity, hyperlipidemia, and insulin resistance (Dunaif, 1997; Dunaif et al., 1989; Ehrmann et al., 1995). The architecture of the ovaries in PCOS patients is abnormal, consisting of multiple medium-sized follicles, many of which have degenerated and contain nonfunctioning oocytes. This ovarian anatomy is consistent with unregulated follicular growth resulting in the absence of dominant follicle formation that is necessary for normal ovulation.

It is important to note that while PCOS is complicated by a high incidence of insulin resistance and metabolic syndrome (Dunaif, 1997; Dunaif et al., 1989; Ehrmann et al., 1995), abnormal follicle growth occurs under any conditions whereby androgens are in excess, including in women taking exogenous androgens (Pache et al., 1991), women with congenital adrenal hyperplasia (Speiser, 2001), and women with aromatase deficiency (Ito et al., 1993). While the ovaries of PCOS patients contain degenerated follicles with abnormal oocytes, the maturation status of these oocytes is not certain. Further translational studies linking laboratory research to the infertility clinic will lead to new and exciting advances towards improving fertility in women.

# CHAPTER TWO

## Steroids promote maturation in mouse oocytes

### Introduction

In the female embryo germ cells mitotically divide to produce large pools of oogonia which then develop into primary oocytes. These oocytes enter prophase I of meiosis, replicate their DNA and are arrested in state from before birth until puberty. Just prior to ovulation, gonadotropins stimulate ovarian follicular development, which in turn promotes oocytes to reenter the meiotic cycle, or mature, until they arrest again in metaphase II of meiosis. These mature oocytes are then competent for ovulation and subsequent fertilization (Albertini and Carabatsos, 1998). Little is known about the ovarian signals that promote oocyte maturation in mammals; however, studies performed in *Xenopus laevis* over the past four decades has served as one of the best models for examining meiosis (Maller and Krebs, 1980). Xenopus oocytes remain in meiotic arrest after removal from the ovary, and can be induced *in vitro* to mature by addition of various steroids, including progesterone, corticosteroids, and androgens. Meiotic arrest appears to be maintained in part by constitutive Gβγ- and/or Gαs-mediated signaling that maintain high intracellular cAMP levels (Lutz et al., 2000 ; Sheng et al.. 2001). Steroids promote maturation through a release of inhibition mechanism whereby steroids overcome constitutive inhibitory signals, allowing meiosis to progress. Maturation is accompanied by several signaling events, including activation of cyclin-dependent kinase 1 (CDK1), or cdc2, and the MAPK cascade (Maller and Krebs, 1980; Shibuya et al.). Steroids induce all of these processes in a transcription-

independent, or nongenomic, fashion. Furthermore, evidence suggests that androgens may be the primary physiologic mediators of *Xenopus* oocyte maturation *in vivo*, and are likely signaling in part through the classical androgen receptor (AR) expressed in oocytes (Lutz et al., 2001).

Importantly, regulation of mammalian oocyte maturation is remarkably similar to that of *Xenopus laevis*. For example, meiotic arrest of mouse oocytes also appears to be regulated by constitutive signals that elevate intracellular cAMP, and the same signaling pathways are activated once mouse oocytes mature (Conti et al., 2002; Mehlmann et al., 2002). Notably, in contrast to *Xenopus*, mouse oocytes spontaneously mature when removed from the ovary, suggesting that the primary signal maintaining meiotic arrest of mouse oocytes comes from the ovary rather than being endogenous to the oocyte itself. The spontaneous maturation of mouse oocytes upon removal from the ovary has complicated efforts to determine the ovarian factors that can promote maturation in mice and other mammals; thus, the precise role of steroids in mediating mammalian oocyte maturation has yet to be determined. In light of the similarities of signaling pathways associated with meiotic arrest and maturation in frogs and mice, we tested if steroids may be signaling and promoting maturation by a release of inhibition as seen in frogs.

We found that isolated mouse oocytes responded to steroids similarly to frog oocytes. Initial experiments showed that progesterone, testosterone, and estradiol induced maturation of mouse oocytes arrested in prophase I in a dose-dependent fashion. This steroid-induced maturation appeared to occur in the absence of transcription, and was accompanied by activation of MAPK and CDK1/cdc2 signaling, suggesting that the mechanisms of

steroid-induced oocyte maturation are conserved from lower to high vertebrate. Importantly, classical receptor antagonists attenuated steroid-mediated signaling indicating that steroid actions are occurring at least in part via classical receptors. Additional evidence for the involvement of classical receptors comes from the inability of oocytes from progesterone receptor null mice to mature in response to progestin despite maturating in response to an alternative steroid. Finally directly measuring ovarian steroid content during the estrus cycle showed an increase in steroid levels prior to ovulation providing physiologic support for the role of steroids in normal oocyte maturation.

## Materials and Methods

### Oocyte Isolation

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ovaries were removed directly from 4- week old mice and placed in M2 medium (Cell and Molecular Technologies, Lavallette, NJ) containing 200 μM IBMX (Calbiochem, La Jolla, CA). Ovaries were punctured and oocytes removed from follicles using 30-gauge needles. Oocytes were denuded by gentle pipetting with pulled glass pasteur pipettes, washed with M2 medium, and placed in M16 medium (Sigma, St. Louis, MO) containing 200 μM IBMX (M16/IBMX) for maturation and signaling assays.

### Oocyte Maturation, MAPK, and CDK1/cdc2 Assays

Equal numbers of isolated and washed oocytes (approximately 50–60) were placed in M16/IBMX and incubated at 37 C with either ethanol or steroids (Sigma, St. Louis, MO, and Steraloids, Newport, RI) at the concentrations described in figure legends. Ethanol concentrations were kept at 0.4% in all samples. An equal number of oocytes were also placed in M16 media lacking IBMX as a positive control for maturation and signaling. After 16 h, oocytes were examined under a dissecting microscope and the number of oocytes with GVBD was determined. All oocytes were then placed in 2X sodium dodecyl sulfate (SDS)-sample buffer and phosphorylation of p42/p44 was detected by Western Blot analysis as described (Lutz et al., 2000). After immunoblotting for phosphorylated p42/p44, blots were stripped and reprobed with antitotal p42/p44 as a control for gel loading. The strength of the signals were then determined using the National Institutes of Health Image program (Lutz et al.,2002).

For the actinomycin D experiments, oocytes were treated as above, only they were preincubated for 4–5 h with either ethanol or 10 μg/ml actinomycin D (Sigma) followed by addition of steroid in the continued presence of ethanol or actinomycin D. For other experiments, oocytes were preincubated for 30 min with ethanol, 10 μM flutamide (a gift from Dr. M. McPhaul, University of Texas Southwestern Medical Center at Dallas, Dallas, TX (UTSW), 3 μM R1881 (NEN Life Science Products, Boston, MA), or 10 μM ICI 182, 780 (AstraZeneca, London, UK), before addition of steroids. In order to measure CDK1/cdc2 activity, 12 oocytes per condition were treated with steroid as above for 7 h at 37 C. Oocytes were then lysed and extracts examined for their ability to phosphorylate histone H1 (Sigma) in vitro as described (Liu, 1998). Reactions were run for 30 min and stopped by addition of 2X SDS-sample buffer. Extracts were then resolved on 12% polyacrylamide gels and inspected by autoradiography.

**Immunofluorescence**

Immunofluorescence was performed as described (Brown et al., 2002). Briefly, denuded oocytes from 4 week old C57BL/6 mice were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Primary antibodies concentrations were 0.4 mg/ml for rabbit anti-progesterone receptor (C-19) and rabbit anti-androgen receptor (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA), and 2 mg/ml rabbit for the anti-estrogen receptor beta (Affinity Bioreagents, Golden, CO). For controls, the anti-androgen and progesterone receptor antibodies were pre-incubated with their target peptides for 16 hours. The secondary antibody, anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR) was used

at 8 mg/ml. Images were visualized using an Optiphot microscope (Nikon, Melville, NY) equipped with a UV light source and filter for fluorescein visualization.

**Oocyte-cumulus cell complex isolation and maturation.**

Ovaries from unprimed immature 21-to 24-day-old (C57BL/6J x SJL/J) $F_1$ or PRKO mice (a gift from Dr. O. Conneely, Baylor College of Medicine) were isolated and placed into M2 medium (Chemicon, Temecula, CA) containing 200 μM IBMX (Calbiochem, San Diego, CA). Large follicles were punctured with 30 gauge needles and oocyte-cumulus cell complexes (OCCs) were harvested by gentle aspiration with pulled-glass pasteur pipettes. OCCs were washed with M2 medium, allocated into various conditions in four-well plastic culture dishes (Nunclon, Nunc, Copenhagen, Denmark) containing 500 μl of M16/IBMX, and incubated at 37 °C. OCCs were pretreated with classical steroid receptor antagonists, or equal amounts of vehicle for 30 minutes before and after the addition of stimulators. Concentrations of dimethysulfoxide (DMSO) or ethanol were 0.1% in all samples. Maturation, or GVBD, was scored after 8 hrs by gently denuding the oocytes.

**Ovarian steroid levels**

Steroid levels from mature mice were measured in 8 week-old C57BL/6 mice maintained on a 12:12 light/dark cycle and tracked by vaginal cytology through a minimum of 2 complete estrus cycles. Approximately 100 μl of methylene blue dye (Sigma St. Louis, MO) was flushed into the vagina with an eye dropper. Twenty μl of dye was then examined under a microcope. Diestrus was characterized by an abundance of leukocytes, nucleated, and cornified cells. Proestrus contained clumped nucleated cells and estrus was marked by sparse nucleated and cornified cells. Animals were sacrificed 2 hours prior to the start of the

dark cycle, and the ovaries were removed and placed in 3:2 ethyl acetate: hexane. The tubes were then rocked for 14 hrs at room temperature. Solvent was removed and evaporated, steroids were resuspended in reaction buffer from kits, and RIAs were performed.

## Results

### Testosterone triggers maturation and activation of MAPK and CDK1/cdc2 signaling in mouse oocytes arrested in prophase I of meiosis

To determine whether androgens could promote mouse oocyte maturation, spontaneous maturation was inhibited *in vitro* by raising intracellular cAMP levels using the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), and the ability of testosterone to overcome this inhibition was examined. Oocytes were removed from 4-week-old prepubertal mice ovaries to ensure minimal exposure to steroids before our studies. In the absence of IBMX, nearly 100% of isolated oocytes underwent spontaneous maturation, exhibiting germinal vesicle breakdown (GVBD) (Fig. 2.1, *left*). IBMX concentrations were then titrated to inhibit maturation by approximately 60% as determined by visual examination of GVBD. Interestingly, addition of testosterone to IBMX-treated cells completely reversed IBMX-mediated inhibition of maturation, resulting in nearly 100% of the oocytes reaching GVBD (Fig. 2.1A, left). Similar results were observed using milrinone, an alternative phosphodiesterase inhibitor, or the cAMP analogue dbcAMP (data not shown) to hold oocytes in meiotic arrest. These results confirmed that androgens were indeed capable of overcoming the inhibitory effects of elevated intracellular cAMP to promote mouse oocyte maturation *in vitro*.

It is well established in frogs that oocyte maturation is accompanied by activation of the MAPK and CDK1/cdc2 signaling cascades. Therefore, as a more objective measurement of steroid-mediated signaling in mouse oocytes, testosterone-induced activation of the

MAPK cascade and CDK1/ cdc2 were examined. Spontaneous maturation resulted in activation of the MAPK cascade, as demonstrated by increased phosphorylation of p42/p44 (Fig. 2.1B, *top panel*). IBMX significantly attenuated p42/p44 phosphorylation in mouse oocytes, whereas addition of testosterone in the presence of IBMX led to an increase in p42/p44 phosphorylation. The levels of total p42/p44 were relatively similar in each lane, indicating equal sample loading for each condition (Fig. 2.1C, *bottom panel*). Similarly, CDK1/ cdc2 activity, as measured by the ability of ooctye extracts to phosphorylate histone H1, was elevated in untreated oocytes, inhibited by IBMX, and increased by adding testosterone (Fig. 2.1D). These results indicate that in addition to promoting mouse oocyte maturation as assessed by GVBD, testosterone activates signaling pathways known to accompany maturation in frogs.

**Testosterone-mediated events in mouse oocytes occur independent of transcription**

To test whether steroid-induced maturation of mouse oocytes was occurring independent of transcription as seen in *Xenopus* (Lutz et al., 2003; Maller and Krebs, 1980), oocytes were pretreated with the potent transcriptional inhibitor actinomycin D before addition of steroid. Actinomycin D was added at a concentration known to significantly reduce transcription in mouse (Huarte et al., 1985) and *X. laevis* oocytes (Lutz et al.). To confirm that transcription was inhibited, radiolabeled d-UTP was added to untreated mouse oocytes or those treated with actinomycin D and testosterone. Incorporation of radiolabeled d-UTP was undetectable (Fig.2.1B) in oocytes treated with actinomycin D indicating that this concentration was sufficient to halt transcription. Both spontaneous and testosterone-mediated oocyte maturation and activation of the MAPK signaling pathway were completely

unaffected by actinomycin D (Fig. 2.1, A, *right*, and C, *right*, respectively), suggesting that, as with frog oocytes, androgen-induced signaling and maturation in mouse oocytes appears to occur transcription independently.

**Steroids activate MAPK in a dose dependent manner**

To examine the effects of ovarian steroids on oocyte maturation in more detail, we began to test the ability of progestins and estrogens, in addition to androgens, to activate MAPK signaling. Estradiol was a weaker activator of the MAPK cascade in oocytes when compared with testosterone (Fig. 2.2A) whereas progesterone appeared to be a moderate activator (Fig.2.2B). Using 3 different concentrations of each steroid revealed a dose dependent increase in p42/p44 phosphorylation.

**Classical steroid receptors may play a partial role in nongenomic steroid-mediated signaling in mouse oocytes**

Recent work by several laboratories has shown that many nongenomic steroid-induced events are mediated by classical steroid receptors signaling in the plasma membrane (Bayaa et al., 2000; Kousteni et al., 2001; Lutz et al., 2003; Shaul, 2002; Tian et al., 2000). To begin to address the possibility that the nongenomic effects of androgens in mouse oocytes might be regulated through classical ARs, mouse oocytes were treated with the classical AR antagonist flutamide before addition of testosterone. Addition of 10 μM flutamide consistently reduced testosterone- mediated phosphorylation of p42/p44 by 50–70%, as determined by densitometry (Fig. 2.3A, *upper panel*), whereas total p42/p44 levels were similar (Fig. 2.3A, *lower panel*). This result confirms that the classical AR may be playing at least a partial role in testosterone-mediated signaling and maturation of mouse

oocytes. Similar results were obtained using hydroxyflutamide (data not shown). The lack of complete inhibition by flutamide was most likely due to its lower affinity for and higher dissociation rate from the AR relative to testosterone; thus, a 40-fold excess of flutamide may not have been sufficient to completely block testosterone binding over the course of the MAPK assays. Flutamide concentrations significantly above 10 μM appeared to be toxic to oocytes, and therefore could not be used for these studies (data not shown).

Interestingly, R1881 the more potent promoter of AR-mediated transcription than testosterone, induced little to no phosphorylation of p42/p44 in oocytes. In fact, as seen with *Xenopus* oocytes (Lutz et al., 2003), 3 μM R1881 consistently attenuated testosterone-induced activation of MAPK by approximately 65% (Fig. 2.3B), but not spontaneous maturation. Again, complete inhibition would be unexpected with only 10-fold excess of R1881 over the course of a 16-h experiment. These results indicate that, regardless of the species, R1881 appears to act as a selective AR modulator that promotes genomic, but not nongenomic, AR-mediated signaling.

Similar to the results seen with flutamide inhibition of testosterone-mediated maturation, estradiol-induced phosphorylation of p42/p44 was attenuated by addition of the antiestrogen ICI 182,780 (faslodex), a non-selective ERα and ERβ antagonist (Fig. 2.3A), as was estradiol-induced activation of CDK1/cdc2 (data not shown). These results suggest that, similar to testosterone, estradiol-mediated signaling in mouse oocytes may be mediated at least in part through its classical receptor.

The ability of classical receptor antagonists to attenuate steroid-mediated activation of MAPK suggested that classical receptors were present in mouse oocytes. To provide further

evidence, steroid receptor expression was examined in denuded oocytes using immunofluorescence. Intracellular progesterone, androgen, and estrogen beta-receptors were detected throughout oocytes, as indicated by green fluorescence (Fig 2.4A). In contrast, the alpha-form of the estrogen receptor was undetectable using two different antibodies (data not shown), indicating that the beta-form predominates in oocytes. Pretreatment of the antibody preparation with the peptide to which the antibody was directed completely abolished staining of the oocytes, demonstrating the specificity of this result (Fig. 2.4B).

After confirming the expression of classical steroid receptors in mouse oocytes, pharmacologic experiments were performed in a more complex model to determine whether these receptors mediate steroid-induced maturation in oocyte-cumulus cell cultures (OCCs). OCCs are isolated from 21-24 day old mice and contain 1-3 layers of surrounding cumulus cells. Care was taken to use agonists that could not be metabolized. First, the synthetic progestin promegestone (RU5020), an agonist for the progesterone receptor, potently promoted oocyte maturation, and its effects were reduced by high concentrations of the progesterone receptor antagonist mifepristone (RU486) (Table 1). Estradiol rescued this inhibition at a concentration that was 10% that of the antagonist mifepristone, indicating that the antagonist blocked the progesterone receptor specifically. Second, faslodex (ICI, 182, 780), blocked estradiol-mediated maturation, and this inhibition was rescued by the progesterone receptor agonist promegestone. Finally, the classical androgen receptor antagonist flutamide blocked maturation induced by dihydrotestosterone, and this inhibition was rescued by estradiol. These pharmacologic experiments support the concept that classical intracellular steroid receptors mediate steroid-triggered oocyte maturation.

**Progesterone receptor null mice do not mature in response to progestin**

After confirming the expression of the progesterone receptor on the oocyte and the sensitivity of progestin-induced maturation to mifepristone (RU486), we wanted to provide conclusive evidence that this effect was in fact mediated through the classical progesterone receptor. We obtained progesterone receptor null mice (PRKO) and isolated follicles from 21-day old mice. Follicles were incubated for 8 hours steroid and results show that null mice as compared to heterozygote littermates no longer matured in response to progestin but were still capable of responding to estradiol (Fig.2.5). The fact that 100% of the PRKO oocytes underwent GVBD in response to estradiol indicate that these oocytes were still capable of maturing; however, their inability to respond to progestin suggests that steroids are specifically mediating maturation through their respective classical steroid receptors.

**Ovarian steroid concentration increase prior to ovulation**

To further support the physiologic role of steroids in mouse oocytes maturation we examined ovarian steroid levels during ovulation. Previous studies have shown that serum steroid concentrations increase during the LH-surge; however, there were few studies examining ovarian steroid levels or androgen levels. To measure ovarian steroid content in cycling mice, the estrous cycles of eight week-old female mice were tracked by vaginal cytology through a minimum of two cycles, ovaries removed on the indicated days, and steroid content measured by RIA. Levels of progesterone, testosterone, and estradiol all increased at ovulation and remained high throughout diestrus II (Fig. 2.6). Of note, ovarian progesterone content (Fig. 2.6B) was approximately 100-fold higher than that of testosterone and estradiol. These results suggest that steroids play a role in mammalian oocyte maturation *in vivo*.

**Discussion**

In summary, these studies demonstrated that steroids were sufficient at promoting maturation and signaling in mammalian oocytes *in vitro*. Examining maturation of denuded mouse oocytes held in meiotic arrest with a phosphodiesterase (PDE) inhibitor demonstrated that mouse oocytes were triggered to mature by nanomolar amounts of steroid independent of transcription (Fig.2.1A). Importantly, maturation was accompanied by activation of MAPK and cdc2 (Fig.2.1C & D), signaling pathways previously shown to be important in steroid induced-maturation in frogs. Attempts to demonstrate steroid-mediated maturation of mammalian oocytes have been difficult to interpret (Eppig et al., 1987; Rice and McGaughey, 1981; Smith and Tenney, 1980), perhaps due in part to the background of spontaneous oocyte maturation upon removal from the ovary, as well as methods of oocyte removal that pre-expose oocytes to sex steroids (*e.g*. pretreatment of mice with gonadotropins or removal of oocytes from mice that are postpubertal).

While other receptors such as the membrane progesterone receptor family (Zhu et al., 2003) may also be involved in steroid-induced maturation, our data are consistent with classical receptors having important physiologic roles in mouse oocyte maturation. First, the presence of the classical PR, AR, ERβ was detected in oocytes (Fig.2.4). Secondly, the antagonists mifepristone, flutamide, and faslodex blocked progestin, androgen, and estradiol-mediated maturation, respectively (Table 1), indicating that steroids are mediating their effects through classical steroid receptors likely directly in oocytes. Third, oocytes from progesterone receptor null mice in contrast to heterozygote littermates did not mature in

response to progestin but were capable of maturing in response to an alternative steroid, estradiol (Fig. 2.5).

Although further work is needed to confirm the physiologic relevance of these steroid-mediated activities, several pieces of evidence support an *in vivo* role for steroid actions in the ovary. For example, estradiol-mediated signaling through the estrogen receptors ERα and ERβ is critical for follicle development (Couse and Korach, 1999; Krege et al., 1998; Schomberg et al., 1999), since mice with reduced expression of one or both or these receptors have abnormal follicle growth. In addition, local progesterone production and actions at the time of the LH surge are crucial for ovulation, as mice lacking progesterone receptors (PRs) do not ovulate in response to gonadotropin (Lydon et al., 1995). Finally, androgen signaling via the androgen receptor (AR) appears to be important for normal follicle development, as mice lacking the AR have reduced follicle growth and premature ovarian failure (Hillier and Tetsuka, 1997; Hu et al., 2004; Shiina et al., 2006).

Importantly, hyperandrogenemia regardless of source, promotes abnormal ovarian growth with development of polycystic ovaries, and the AR antagonist flutamide improves infertility in some of these individuals (Rittmaster, 1999). These observations in humans confirm that androgen actions through the AR may be mediating critical signals in the ovary. Perhaps nongenomic androgen signaling in oocytes may be contributing to the polycystic phenotype induced by excess androgens, perhaps by promoting the growth of multiple follicles while preventing the development of dominant ones. To accommodate our data with other studies of mouse oocyte maturation, we propose a model of *in vivo* mouse oocyte maturation whereby constitutive inhibitory signals within the ovary (I) hold oocytes in

prophase I of meiosis (Fig. 2.7). Although the precise intracellular mechanisms regulating this meiotic arrest are not known, they may involve G protein-induced elevations in cAMP levels. Before ovulation, gonadotropins promote follicular growth and production of sex steroids (P, progesterone, T, testosterone; E, estrogen). In frogs, many follicles are stimulated to grow, resulting in maturation and ovulation of hundreds of oocytes. In contrast, only a few dominant follicles in mice or other mammals might be sufficiently stimulated by gonadotropins, as well as by cross-talk between oocytes and surrounding follicle cells, to produce enough steroid to overcome the inhibitory signals and allow meiosis and subsequent ovulation to progress. In support for the role of steroids in the selection process is the finding that dominant follicles contain the highest steroid concentrations (Teissier et al., 2000). In individuals with androgen excess, one might speculate that multiple ovarian follicles would be equally stimulated with androgens, which might result in unregulated growth and the lack of dominant follicle production. This inability to produce dominant follicles may explain the anovulatory state of many individuals with androgen excess.

The pharmacology of androgen signaling using AR antagonists and SARMs was the same in mouse and frog oocytes. Notably R1881, a potent activator of AR-mediated transcription, was a poor promoter of oocyte maturation, and in fact, inhibited testosterone-induced activation of MAPK (Fig.2.3B). This suggests that selective AR modulators could be used to specifically promote genomic vs. nongenomic androgen-mediated effects in mammals. Given the nongenomic nature of androgen-induced oocyte maturation in mammals, R1881 might prove useful in regulating oocyte maturation and follicular development both *in vitro* and in women with reduced fertility.

The paracrine signaling that occurs between oocytes and surrounding cells allow the oocyte to govern their environment with the ultimate goal of completing meiosis and fertilization. This crosstalk plays an integral role in follicular development. For example, oocyte-derived factors such as, growth differentiation factor 9 (GDF9) and bone morphogenic protein 15 (BMP-15) are secreted from the oocyte and are essential for folliculogenesis as null animals contain no Graafian follicles ((Eppig, 2001; Matzuk, 2000). The varying exposure of follicular cell types to growth factors and sex steroids may be responsible for the observed shift in gonadotropin sensitivity in these cells. In addition to understanding nongenomic steroid signaling in the context of oocyte maturation, it is also important to examine steroid signaling and its role in modulating gonadotropin action for follicle growth.

Interestingly, androgens, progestins, and estrogen all promote oocyte maturation via their respective receptors in this *in vitro* model system and which likely represents redundancy in this important physiologic signaling pathway. However, progesterone is the most abundant ovarian steroid in ovulating mice (Fig. 2.6) and therefore is likely the primary steroid regulating meiosis *in vivo*. Interestingly, administration of progesterone to ovulating primates enhances oocyte maturation (Borman et al., 2004) confirming progesterone's potential physiologic role in regulating meiosis. Taken together, this study indicates that steroids were capable of triggering oocyte maturation in a fashion very similar to that seen in the well-characterized frog model, including the nongenomic nature of signaling, the similarities in steroid-mediated signaling pathways, and the selectivity of various AR ligands implying a conserved mechanism of oocyte maturation from amphibians to mammals.

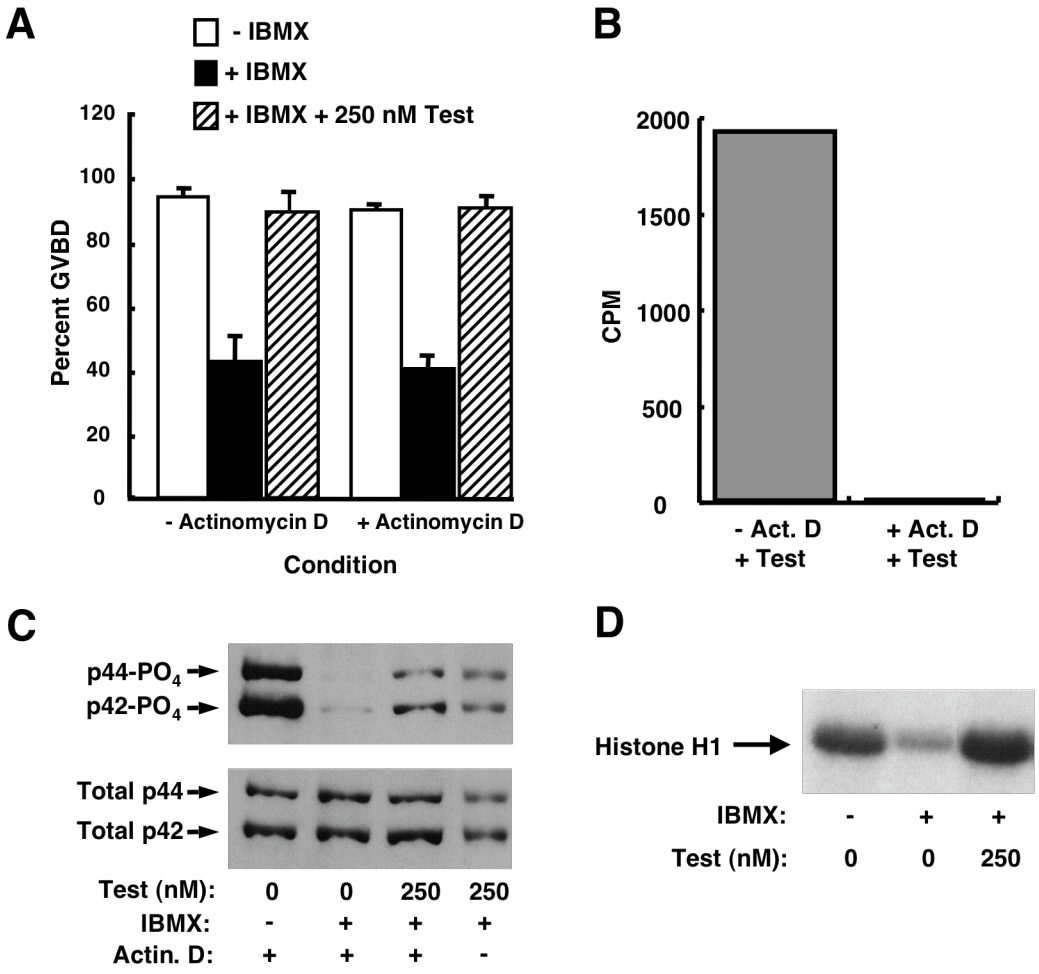


**Figure 2.1 Testosterone promotes maturation of mouse oocytes, as well as activation of MAPK and CDK1/cdc-2, in a transcription-independent manner.** A, Spontaneous maturation was inhibited *in vitro* by the addition of 200 μM IBMX. In the absence of IBMX, nearly 100% of oocytes underwent GVBD. In the presence of IBMX, maturation was inhibited by 60%. The addition of testosterone reversed the IBMX mediated inhibition. Bars represent an average of five experiments +/- SEM. Results were similar in the presence or absence of 10 μg/ml Actinomycin D. Bars represent an average of 2 experiments +/- SEM. (B) 10μM Actinomycin D inhibits transcription in the presence of 250 nM testosterone as measured by the incorporation of radiolabeled d-UTP into mouse oocytes. (C) IBMX significantly inhibited P42/P44 phosphorylation in mouse oocytes, while addition of testosterone in the presence of IBMX activated P42/P44 phosphorylation. Similar activation of MAPK was observed in the presence or absence of 10 μg/ml Actinomycin D. (D) CDK1/cdc-2 activity was elevated in untreated mouse oocytes, inhibited by IBMX, and increased by the addition of 250 nM testosterone. Experiments in (C) and (D) were performed four times with identical results.

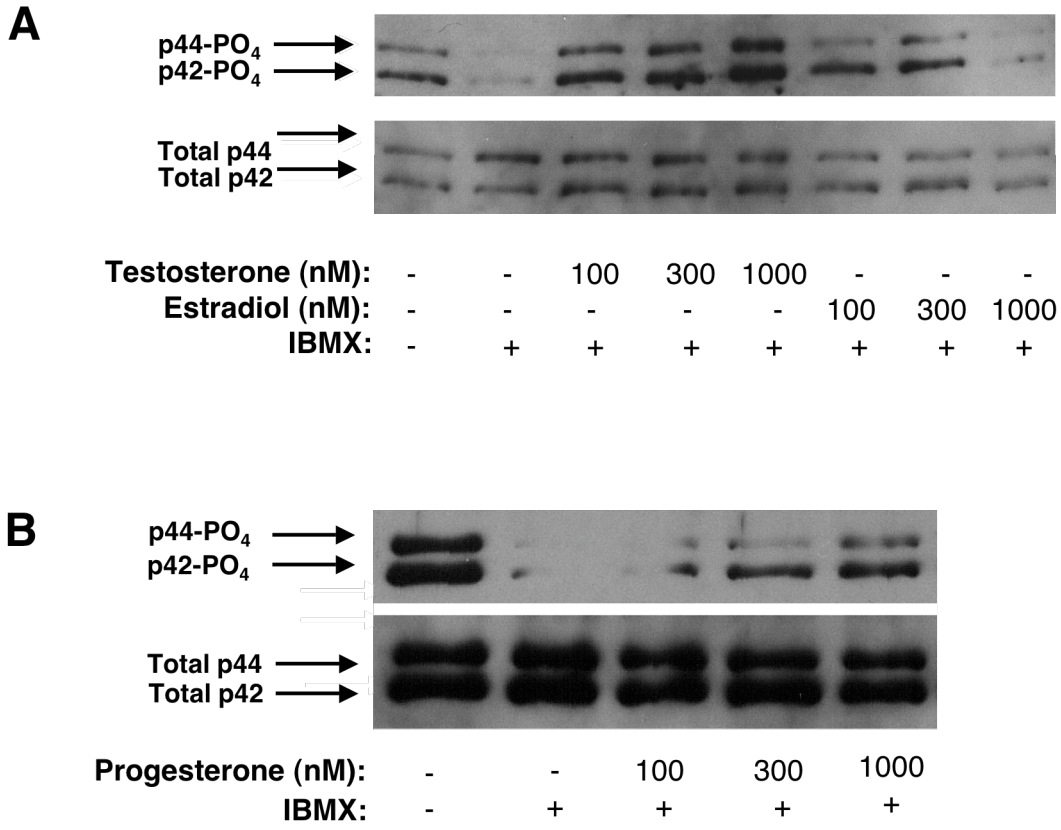


**Figure 2.2. Dose response of testosterone, estradiol (A) and progesterone (B)- mediated activation of MAP-kinase.** Denuded oocytes were incubated with the indicated steroid concentrations for 16 hrs, lysed, then extracts were analyzed by western blot using anti-phospho MAPK (top panels) or anti-total MAPK antibodies ( bottom panels). Experiments were performed a minimum of 3 times with nearly identical results.

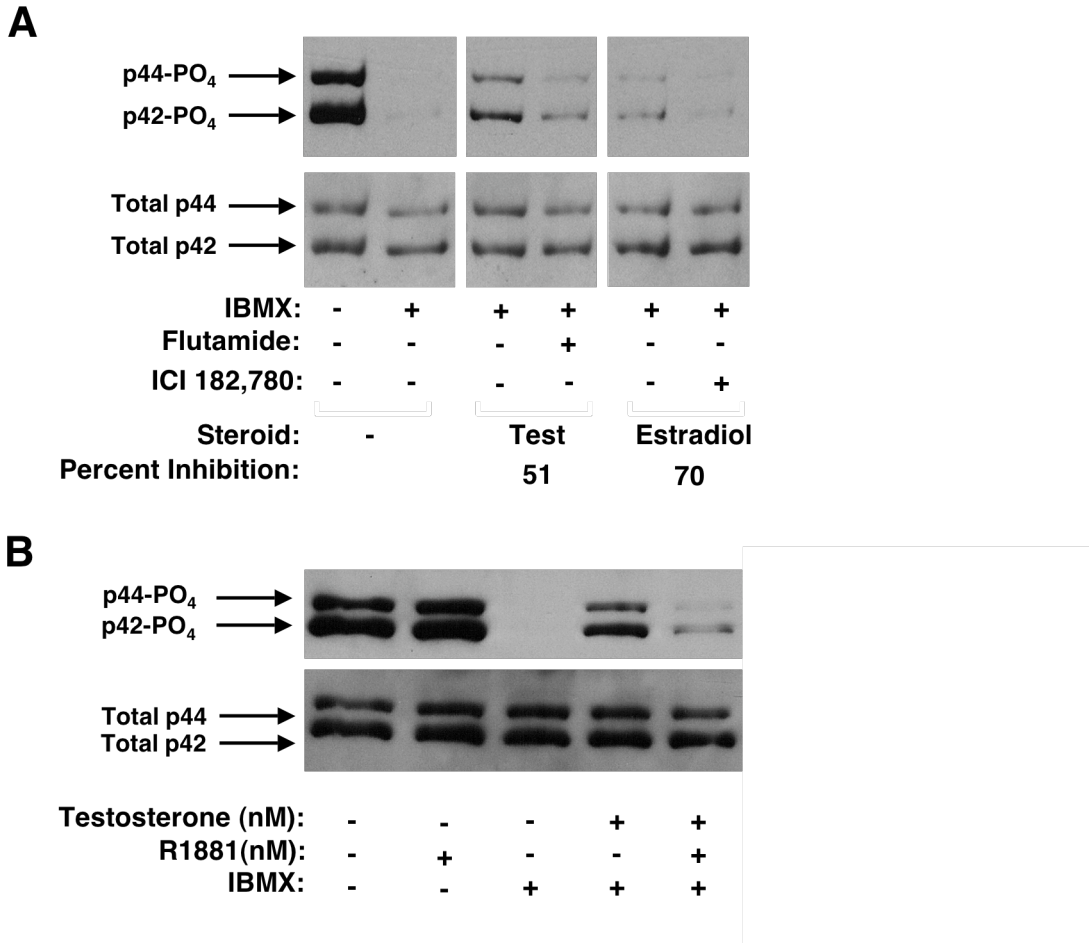


**Figure 2.3. The androgen receptor (AR) and estrogen receptor (ER) play a role in steroid-mediated signaling in oocytes**. (A) 10 μM flutamide reduced testosterone mediated phosphorylation of p42/p44 by 50% and 1 μM ICI 182, 780 inhibited estradiol mediated phosphorylation by 80%. (B) 1 μM R1881 inhibited testosterone phosphorylation of P42/P44. Oocytes pretreated for 1 hour with either ethanol or inhibitor (flutamide, ICI 182, 780, or R1881) were incubated with IBMX plus 250 nM steroid. Extracts were analyzed by western blot using anti-phospho MAPK (top panels) or anti-total MAPK antibodies (bottom panels). These experiments were repeated a minimum of 3 times with nearly identical results.

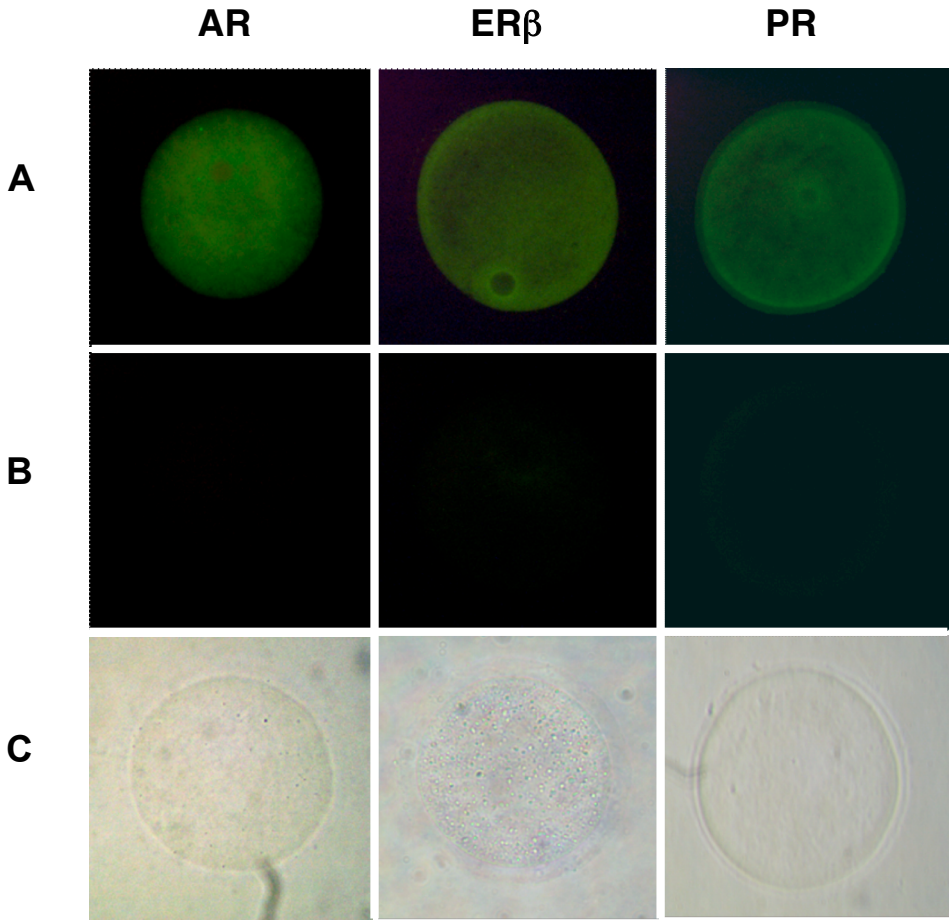


**Figure 2.4. Intracellular steroid receptors are expressed in mouse oocytes.** (A) Oocytes were incubated with antibodies against the androgen (AR), estrogen (ERβ), and progesterone (PR) receptors. Immunofluorescence demonstrates the expression of all three receptors throughout the oocytes. (B) Oocytes were incubated with primary antibodies pretreated with peptide to which they were directed (AR and PR) or no primary antibody (ERβ). (C) White light images of the oocytes in (B).

**Table 1. Classical steroid receptor antagonists inhibit steroid-triggered maturation in OCCs**

| Receptor | Control | Agonist | Inhibitor | Steroid Rescue |
|---|---|---|---|---|
| PR | ETOH 20% | Promegestone 90% | Mifepristone 20% | Estradiol 75% |
| ER | ETOH 15% | Estradiol 90% | Faslodex 25% | Promegestone 100% |
| AR | ETOH 19% | DHT 75% | Flutamide 19% | Estradiol 81% |

**Classical steroid receptor antagonists inhibit steroid-triggered maturation in oocytes.** The percentages represent percent of oocytes within OCCs that have undergone GVBD. OCCs were treated with agonist alone (Agonist), agonist plus a corresponding antagonist (Inhibitor), or agonist, antagonist, and an alternative steroid agonist (Rescue). Antagonists were added 30 minutes before and through the addition of agonists. Concentrations were 100 nM for agonists, 2.5 μM for antagonists, and 250 nM for rescuing steroids. GVBD was scored after 8 hrs. Each experiment was performed twice with similar trends.

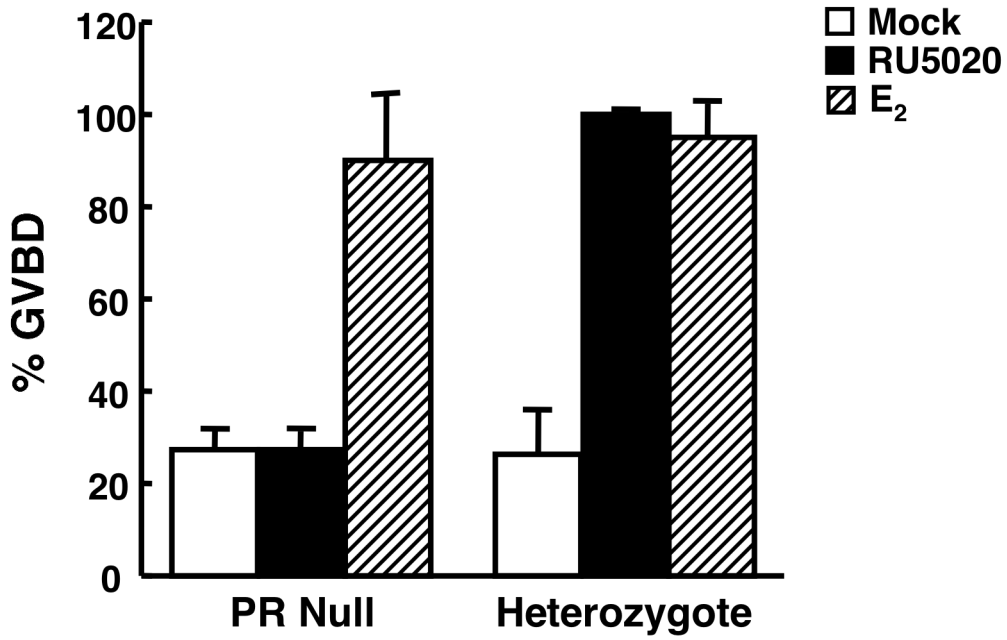


**Figure 2.5. Oocytes from PRKO mice do not mature in response to progestin.** These oocytes still respond to estradiol suggesting that the PR is necessary for progesterone-mediated maturation. OCCs were isolated from 3 week old PRKO heterozygote or null mice and incubated with 250 nM steroid or vehicle and scored for GVBD after 8 hrs. Bars represent an average of 2 experiments +/- SD.

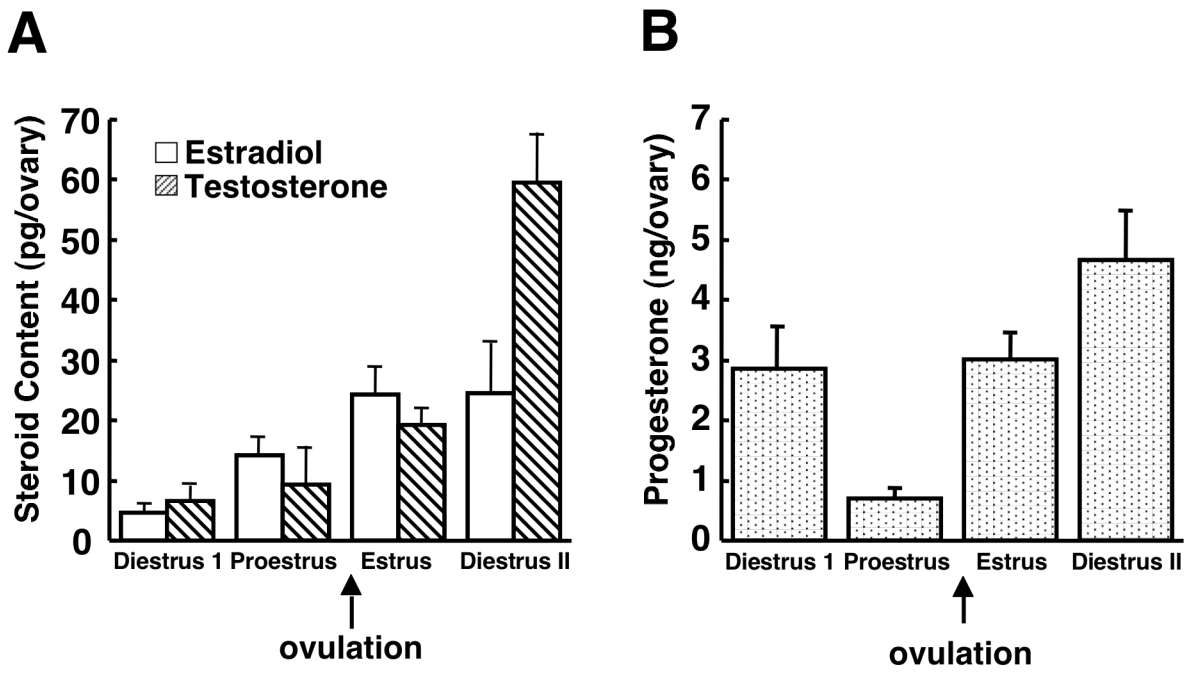


**Figure 2.6. Ovarian steroid levels in cycling mice.** On the indicated days, steroids were extracted from mouse ovaries and measured by RIA. Bars represent an average ± S.D. of 10-12 animals. A) Testosterone, estradiol, and B) progesterone ovarian levels.

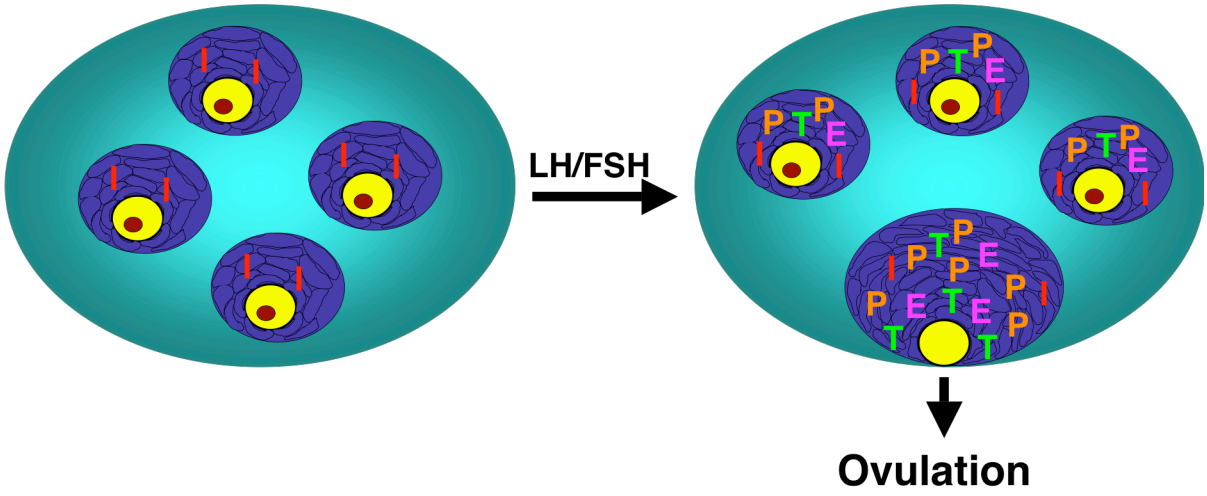


**Fig. 2.7. Model for *in vivo* mouse oocyte maturation**. Unknown ovarian inhibitory signals (I) maintain meiotic arrest of oocytes (yellow with brown nucleus). Prior to ovulation, gonadotropins promote follicle growth and production of sex steroids ( P = progesterone, T = testosterone, E = estrogen). Large, dominant follicles (right bottom) produce sufficient amounts of steroid to overcome and release the inhibitory signals, thus allowing GVBD (loss of defined brown nucleus), meiosis, and subsequent ovulation to progress.

# CHAPTER THREE

## EGF signaling in steroidogenesis and oocyte maturation

### Introduction

LH triggers multiple processes within the ovary that are all critical for normal ovulation, including steroidogenesis, cumulus cell expansion, and oocyte maturation. Understanding the signaling pathways induced by LH in the ovary is therefore essential for appreciating how these activities relate to one another. Several pieces of evidence have implicated epidermal growth factor receptor (EGFR) signaling as a potential central pathway coordinating these important LH-mediated events.

First, EGF triggers steroidogenesis in gonadal cells, including Leydig cell lines (Ascoli and Segaloff, 1989; Manna et al., 2002) and possibly granulosa cells (Jezova et al., 2001). While EGF promotes steroidogenesis in these models, an essential role for EGF and the EGFR in regulating gonadotropin-induced steroidogenesis has not been established.

Second, the EGFR plays a role in regulating oocyte maturation (Ashkenazi et al., 2005; Park et al., 2004). Oocyte maturation refers to the meiotic progression of oocytes from prophase I to metaphase II that is required for normal ovulation and fertilization. LH triggers secretion of EGF family members, which in turn signal in a paracrine fashion via the EGFR to stimulate cumulus cell expansion and oocyte maturation (Ashkenazi et al., 2005; Park et al., 2004). Notably, EGF cannot directly trigger maturation of denuded oocytes, suggesting that secondary messengers induced by EGFR-mediated signaling in the cumulus cells are required for meiotic progression.

Finally, in contrast to EGF, progesterone, testosterone and estradiol are capable of promoting maturation of denuded mouse oocytes held in meiotic arrest *in vitro* (Gill et al., 2004). This steroid-mediated process is transcription-independent and appears to be regulated by classical steroid receptors. While steroids are established physiologic mediators of maturation in frogs and fish (Hammes, 2004; Maller and Krebs, 1980; Smith and Tenney, 1980; Tian et al., 2000), their role in regulating mammalian oocyte maturation has remained controversial. Though steroids promote maturation in denuded mouse oocytes, the effects of steroids on more complex models whereby oocytes are held in meiotic arrest by surrounding follicular cells needs to be examined.

Together, these earlier studies raise the possibility that EGFR-mediated signaling may trigger steroidogenesis, which in turn plays a role in regulating oocyte maturation. In the present study, we utilized several models to examine EGF-mediated steroidogenesis and maturation, including: 1) cultures of oocyte-granulosa cell complexes (OGC), which are isolated from pre-antral follicles in 12 day old mice and grown to meiotic competence; 2) meiotically competent oocyte-cumulus cell complexes (OCCs), which are isolated from 21-24 day old mice and used immediately; and 3) intact pre-ovulatory follicles from mice primed with pregnant mare serum gonadotropin (PMSG). In each of the oocyte-granulosa cell models, EGF promoted steroid production. In addition, gonadotropin-induced steroidogenesis was regulated in ovarian follicles via EGFR-mediated signaling. Finally, EGFR-induced steroidogenesis produced sufficient steroid to promote oocyte maturation in oocyte-granulosa cell models.

## Materials and Methods

### Culturing of oocyte-granulosa cell complexes and measurement of steroid production and maturation.

Oocyte-granulosa cell complexes (OGCs) were isolated as previously described in (Eppig and Schroeder, 1989). Ovaries were dissected from 12 day old C57BL/6J x SJL/J $F_1$ mice purchased from The Jackson Laboratory (Bar Harbor, ME) and placed in 2 mls of syringe-filtered Waymouth's MB752/1 medium (Invitrogen, Carlsbad, CA) medium supplemented with 3 mg/ml BSA, 5 μg insulin/5 μg transferring/5 ng sodium selenite (ITS), 5% FBS, 0.02 mg/ml DNase, and 1 mg/ ml Type I Collagenase in a 35 mm diameter Petri dish. All reagents were purchased from Sigma unless otherwise indicated. Ovaries were incubated at 37°C for ten minutes. Dissociation occurs by pipetting with a P1000 (set at 700μl) 50 times every 5 minutes. The pipet is kept in the same position and the Petri dish tilted. Pipetting too hard will burst follicles. Ovaries are completely dissociated into individual oocyte-granulosa cell complexes in 30-40 minutes. One to three layers of granulosa cells should surround oocytes. The complexes are washed and cleaned of debris by sequential transfer through three dishes containing 2 mls of medium to remove undigested clumps. Complexes are picked up in 100μl of medium and transferred to membrane inserts supplied with 2 ml of Waymouth's medium + 5% FBS + BSA + ITS inside and outside the insert in a 6-well plate. Complexes are spread evenly on the membrane without touching and the empty wells filled with water to humidify.

The OGCs are grown for 10 days at 37° in a modular incubator chamber (Billups Rothenberg, Del Mar, CA) that has been perfused with 90%N - 5%O2 - 5% CO2. Cultures

are fed every other day by removing approximately 2 mls of media from outside the insert and then adding the same volume of fresh media. Care must be taken to avoid jolting the cultures, which dislodges the growing complexes. To determine EGF-mediated steroid production, medium was removed on day 10 of culture and 16 hrs after incubation 20 ng/ml EGF, steroids were extracted (Lutz et al., 2001), and radio-immune assays (RIAs) were performed to detect progesterone, testosterone, and estradiol levels (MP Biomedicals, Irvine, CA). For maturation assays, after 10 days of culture the insert is removed to a Petri dish containing 2 mls of Waymouth's 5% FBS + BSA and the complexes are dislodged by sharply tapping the edge of the insert with a fingertip. The dislodged complexes are collected, washed twice to remove ITS and allowed to mature for 16-17 hours in medium containing 10 ng/ml of EGF or steroid at the concentrations indicated in the figure legends. Maturation was scored by gently denuding OGCs with pulled glass pasteur pipettes and visually examining the oocytes for germinal vesicle breakdown (GVBD). Results were verified by blinding the individuals scoring for GVBD.

**Oocyte-cumulus cell complex harvest and measurement of steroid production and maturation.**

Ovaries from unprimed immature 21-to 24-day-old (C57BL/6J x SJL/J) $F_1$ or PRKO (a gift from Dr. O. Conneely, Baylor College of Medicine) were isolated and placed into M2 medium (Chemicon, Temecula, CA) containing 200 mM IBMX (Calbiochem, San Diego, CA). Large follicles were punctured with 30 gauge needles and oocyte-cumulus cell complexes (OCCs) were harvested by gentle aspiration with pulled-glass pasteur pipettes. OCCs were washed with M2 medium, allocated into various conditions in four-well plastic

culture dishes (Nunclon, Nunc, Copenhagen, Denmark) containing 500 µl of M16/IBMX, and incubated at 37 °C. OCCs were pretreated with EGFR kinase inhibitor AG1478 (Sigma, St. Louis, MO), classical steroid receptor antagonists, or equal amounts of vehicle for 30 minutes before and after the addition of stimulators. Concentrations of dimethysulfoxide (DMSO) or ethanol were 0.1% in all samples. Maturation, or GVBD, was scored after 8 hrs by gently denuding the oocytes. Medium containing oocytes were collected for steroid detection by RIA.

**Phospho-StAR, StAR and CYP11A1 immunoblotting**

Fifteen oocyte-cumulus cell complexes (OCCs) were placed in M-16 medium with or without 20 ng/ml EGF and incubated at 37 °C for 16 hours. OCCs were collected and placed in 2X sodium dodecyl sulfate (SDS) buffer plus 10% *beta*-mercaptoethanol. Lysates were separated by electrophoresis on 12% polyacrylamide gels and proteins transferred to Immobilon-P membranes. Membranes were probed with rabbit anti-phospho StAR (gift from Dr. Stephen King, Children's Hospital, Brooklyn, NY), rabbit anti-StAR (gift from Dr. D.M. Stocco, Texas Tech University Health Sciences Center, Lubbock, Texas) and rabbit anti-CYP11A1 (gift from Dr. B.C. Chung, Institute of Molecular Biology, Taipei, Taiwan) antibodies.

**StAR null mice follicle isolation and steroid production**

StAR null mice were rescued by daily injections of by dexamethasone 0.04 µg and fludrocortisone 0.05 µg s.c) as previously described (Caron, 1997). Follicles were isolated from 21-day old mice and placed in M16 media and incubated at 37 °C for 16 hours with 200

ng/ml EGF. Medium and follicles were collected and progesterone content measured by RIA.

**Overnight EGF-induced steroidogenesis in oocyte-cumulus cell complexes**

Approximately 150 OCCs were isolated as described above and placed in 2 mls of M16 medium containing, 200mM IBMX, and 20 ng/ml EGF. OCCs were incubated for 16 hrs at 37 °C in a modular incubation chamber infused with 5% $O_2$, 5% $CO_2$, and 90% $N_2$. Medium was collected and centrifuged at 0.8 relative centrifugal force to remove all cells. Medium was then added to newly harvested OCCs in four separate conditions; 1) Medium was unmodified; 2) Medium was treated with 20 mM AG1478; 3) Steroids were removed from the medium using a Sep-Pak (Waters, Milford, MA); 4) Steroids were removed and medium was treated with AG1478. Of note, the Sep-Pak removed 95% of steroid from the medium; however, one cannot rule out the possibility that the filter was removing other important hydrophobic molecules that may be affecting oocyte maturation. Maturation was scored after 8 hrs by gently denuding OCCs with pulled glass pasteur pipettes and visualizing the oocytes for GVBD.

**Pre-ovulatory follicle harvest and determination of steroid production and maturation**

Immature 21-28 day old C57BL/6J mice from The Jackson Laboratory were injected with 5 IU PMSG (Sigma, St. Louis, MO) into the peritoneum to stimulate follicular development. Follicles were isolated 44–48 hr post-PMSG injection by puncturing the ovary with 30 gauge needles. Intact follicles greater than 400 microns were selected, washed in M2 medium, then placed in M16 media and incubated at 37 °C for 6 hours with either 3 mg/ml LH, 200 ng/ml EGF, or 20 μM 22*R*-hydroxycholesterol (Steraloids, Newport, RI).

Maturation was determined in denuded oocytes by examining germinal vesicle breakdown (GVBD), and steroids measured by RIA following extraction from the medium and follicles. Follicles were pretreated with AG1478 , Galardin (Calbiochem, San Diego, CA), or equal amounts of vehicle for 30 minutes before and after the addition of stimulators. DMSO or ethanol concentrations were kept at 0.1% for all samples.

## Results

### Steroidogenesis and steroid effects in oocyte-granulosa cell complexes (OGCs).

Steroid-induced maturation was examined in cultured oocyte-granulosa cell complexes, whereby oocytes are naturally held in meiotic arrest by the surrounding granulosa cells. OGCs are isolated from pre-antral follicles of 12 day-old mice and cultured for ten days after which time they are competent to mature (Eppig and Schroeder, 1989). Interestingly progesterone, testosterone, and estradiol all promoted oocyte maturation as well as EGF (Fig 3.1A). Steroid production was then examined in this growing oocyte/follicle cell model system. To determine whether EGF could stimulate steroid production, ten-day old OGC cultures were incubated with 20 ng/ml EGF for 16 hrs. EGF increased progesterone, testosterone, and estradiol levels by 6-fold (absolute concentration ≈10 ng/ml), 3-fold (absolute concentration ≈6 ng/ml), and 2-fold (absolute concentration ≈4 ng/ml), respectively, relative to mock-treated cells (Fig. 3.1B).

### Steroids are potent and rapid activators of maturation in oocyte-cumulus cell complexes (OCCs).

Progesterone similarly triggered oocyte maturation in meiotically competent oocyte-cumulus cell complexes (OCCs), which are isolated from 21-24 day old mice. Furthermore, the $EC_{50}$ for progesterone-mediated oocyte maturation in OCCs was approximately 50 nM (Figure 3.2A), which is likely within the range of normal ovarian steroid concentrations during ovulation. In fact, examining the kinetics in more detail revealed that progesterone promoted maturation more rapidly than EGF, suggesting that steroids may act downstream of EGFR signaling (Fig. 3.2B). Finally, unlike EGF, progesterone did not promote cumulus cell

expansion in oocyte-cumulus cell complexes (Fig. 3.2C), suggesting that it may act directly on oocytes rather than on surrounding cumulus cells.

**Progesterone is sufficient to promote oocyte maturation in the absence of EGFR signaling.**

The EGFR kinase inhibitor AG1478 is known to attenuate EGF-induced maturation in oocyte-cumulus cell complexes (Ashkenazi et al., 2005). Since steroid production appears to be downstream of EGF receptor signaling, progesterone would be predicted to overcome this AG1478-mediated inhibition of meiosis. Freshly isolated oocyte-cumulus cell complexes were therefore treated with EGF, EGF plus AG1478, or EGF plus AG1478 and progesterone. Progesterone completely rescued AG1478-mediated inhibition of oocyte maturation (Fig. 3.3A), confirming that steroid-triggered maturation occurs independent, and likely downstream, of EGFR signaling. Corresponding steroid levels (Fig. 3.3A, below graph) verified that EGF-mediated steroid production required an active EGFR, as the specific EGFR kinase inhibitor AG1478, abrogated EGF-induced progesterone secretion in oocyte-cumulus cell complexes (Fig 1B). Notably, under these conditions AG1478 abrogated EGFR phosphorylation (Park et al., 2004).

**EGF-induced steroidogenesis in oocyte-cumulus cell complexes increases phosphorylated steroidogenic acute regulatory (StAR) protein.**

StAR is the primary regulator of cholesterol transport into the mitochondria, where the steroid precursor is then converted by CYP11A1 (side chain cleavage enzyme) to pregnenolone. In some cell types, EGF-induced steroidogenesis is regulated by increased steroidogenic acute regulatory (StAR) protein expression (Manna et al., 2002). To determine

whether EGF increased StAR or CYP11A1 protein expression, freshly isolated OCCs were incubated with EGF for 16 hours and cell lysates were analyzed for StAR and CYP11A1 expression by Western blot. Interestingly, neither total StAR (Fig. 3.3B *middle*) nor CYP11A1 (Fig. 3.3B *bottom*) expression levels were altered in EGF-treated OCCs relative to mock-treatment. Rather, EGF-induced steroidogenesis in OCCs relies on post-translational activation of StAR protein as suggested with the increase in phosphorylated-StAR protein levels (Fig.3.3B *top*).

Accordingly, we demonstrated the necessity of StAR in EGF-induced steroidogenesis using StAR null mice. Follicles were isolated from 21 day-old StAR null mice rescued by daily injections of dexamethasone/fludrocortisone and wild-type littermates. EGF did not promote steroidogenesis in follicles isolated from StAR null mice compared to wild-type littermates whereby EGF increased progesterone production by 30-fold (Fig. 3.4A). Surprisingly, steroidogenesis was not observed from follicles of StAR null mice incubated with 22*R*-hydroxycholesterol, a membrane-permeable analogue of cholesterol, suggesting that these mice are unable to produce steroid from cholesterol (Fig.3.4B).

**EGF-induced steroidogenesis is sufficient to promote maturation**.

To determine whether EGF-induced steroidogenesis was sufficient to promote maturation, oocyte-cumulus cell complexes were treated with EGF for 16 hours. The surrounding medium, which contained EGF and steroids, was then collected and added to newly isolated oocyte-cumulus cell complexes under four different conditions. First, the medium was left unmodified, resulting in 100% maturation (Fig 3.5). This maturation was likely triggered by both steroids and EGF in the medium. Second, medium was added to

OCCs in the presence of the EGFR kinase inhibitor AG1478, causing 94% maturation. Maturation here was likely regulated by steroids and/or other EGF-induced factors. Third, steroids, but not EGF, were extracted from the medium, resulting in 100% maturation. Fourth, steroids were extracted and AG1478 was added, thus inhibiting both steroid- and EGFR-mediated signaling. Under this condition, oocyte maturation was significantly impaired. Together, these results provide proof-in-principal that EGF-induced steroid production is sufficient to mediate oocyte maturation.

**LH-mediated steroidogenesis in ovarian follicles requires cleavage of membrane-bound EGF family members and activation of the EGFR.**

Pre-ovulatory follicles from PMSG-primed mice were isolated and stimulated for 6 hours with LH. As expected, LH triggered an approximately 30-fold increase in progesterone production (Fig. 3.6A). Inhibition of EGF signaling with the EGF receptor kinase inhibitor AG1478 prevented the LH-induced increase in progesterone production (Fig. 3.6A), suggesting that EGFR signaling is critical for normal gonadotropin-mediated steroidogenesis in ovarian follicles.

G protein-coupled receptors are known to activate EGFR signaling by several mechanisms (Herrlich et al., 1998; Pierce et al., 2001). For example, stimulation of the $G\alpha_s$-coupled β2-adrenergic receptor activates membrane-bound matrix metalloproteinases (MMPs) that cleave heparin-bound EGF (HB-EGF) from the cell surface to auto-activate the EGFR (Maudsley et al., 2000). MMPs may also activate other members of the membrane-bound EGF family, including amphiregulin (Thorne and Plowman, 1994). Since MMPs are critical for numerous ovarian functions (Curry and Osteen, 2003), and since the LH receptor

similarly signals via $G\alpha_s$, the role of membrane-bound EGF family members in regulating LH-induced follicular steroidogenesis was examined. Inhibition of MMP activity was achieved by co-incubating LH-treated follicles with the broad-spectrum MMP inhibitor, Galardin. As seen in figure 3.6B, Galardin blunted LH-induced progesterone production, implicating both MMPs and membrane-bound EGFs as important factors regulating gonadotropin-induced steroidogenesis. Galardin's inhibitory effects could be reversed by adding EGF (Fig. 3.6B), confirming that Galardin was acting upstream of the EGF receptor. Furthermore, Galardin had no visually toxic effects on follicles, and a commercially available, structurally similar negative control compound for Galardin did not change LH-induced follicular progesterone production (data not shown).

**Progesterone is sufficient to promote oocyte maturation in intact follicles.**

Steroid-mediated maturation was next examined in pre-ovulatory follicles from PMSG-primed mice. As seen with the oocyte-granulosa cell cultures and with the freshly isolated oocyte-cumulus cell complexes, both progesterone and testosterone promoted oocyte maturation in the pre-ovulatory follicles (Fig. 3.6C).

EGFR activation is known to be required for oocyte maturation (Park et al., 2004). Since EGFR signaling is also necessary for LH-induced steroidogenesis (Fig. 3.6A), and since steroids trigger maturation of denuded mouse oocytes (Gill et al., 2004), we postulate that the EGFR may be regulating oocyte maturation through steroid production. Accordingly we show that inhibiting release of membrane-bound EGFs with Galardin inhibits maturation and this effect is rescued by the addition of progesterone, the putative downstream effector

(Fig. 3.6D). The corresponding steroid content (Fig. 3.6D below) confirmed that Galardin did indeed knock-down progesterone levels.

**Discussion**

These studies examined the role of the EGFR in regulating LH-mediated processes in gonadal tissue. Previous work showed that EGF could stimulate steroidogenesis in gonadal cells (Ascoli and Segaloff, 1989; Jezova et al., 2001; Manna et al., 2002). We have confirmed and advanced these observations using several experimental models. Our studies demonstrate that EGF-mediated signaling via the EGFR is sufficient to promote steroid production in two models of mouse oocyte-granulosa cells complexes (Fig. 3.1B and 3.3A). Furthermore, we show that EGFR-mediated signaling is necessary for maximal gonadotropin-induced steroidogenesis in the ovary, as the specific EGFR kinase inhibitor AG1478 ablated LH-induced steroidogenesis in PMSG-primed ovarian follicles (Fig 3.6A). Notably, EGF is less potent than LH in promoting steroidogenesis in oocyte-cumulus cell complexes, which is consistent with similar studies performed in Leydig cells (Ascoli et al., 1987). These observations suggest that EGFR signaling is necessary but not sufficient for maximal gonadotropin-induced steroid production.

Our studies also address the mechanisms by which LH receptor signaling leads to EGFR activation. In ovarian follicles, LH-induced steroidogenesis required MMP-mediated cleavage of membrane-bound EGFs (Fig 3.6B). Given the similarities between this result and work in other systems (Herrlich et al., 1998; Maudsley et al., 2000; Pierce et al., 2001; Prenzel et al., 1999), we propose the following model (Fig 3.7): LH activates its receptor located on theca cells and granulosa cells (Peng et al., 1991). This results in the activation of MMPs and cleavage of membrane-bound EGFs, possibly including HB-EGF and amphiregulin. Soluble EGF molecules then bind to EGFRs on cumulus granulosa cells, as

well as on mural granulosa and theca cells, to enhance steroid production. However, progesterone, the most abundantly produced steroid, is likely coming primarily from the granulosa cells, as these cells lack CYP17 and cannot convert progesterone to androgens.

How does activation of the EGFR stimulate steroidogenesis? Studies utilizing follicles from StAR null mice revealed that StAR is necessary for EGF-induced steroidogenesis (Fig.3.4). Notably, total StAR protein expression was unchanged by EGF in oocyte-cumulus cell complexes (Fig 3.3B), but phosphorylated StAR protein expression increased indicating that activity is dependant upon post-translational modifications.

The consequences of EGFR-induced steroidogenesis are significant, as steroids mediate many important ovarian functions. The present study focused on one pre-ovulatory process: steroid-induced oocyte maturation. Steroids are the physiologic mediators of oocyte maturation in *Xenopus laevis*, and promote maturation of denuded mouse oocytes held in meiotic arrest by phosphodiesterase inhibitors (Gill et al., 2004). We confirm here that progesterone, testosterone, and estradiol trigger maturation in models where oocytes were held in meiotic arrest by surrounding granulosa cells (Fig.3.1A) and in whole follicles (Fig.3.6C).

As mentioned, the EGFR regulates oocyte maturation (Ashkenazi et al., 2005; Park et al., 2004) but EGF cannot trigger maturation of denuded oocytes, implying that secondary messengers in cumulus cells are essential for EGF-mediated maturation. Our studies confirm these results, and suggest that steroids may signal downstream of the EGFR. First, progesterone induced maturation more rapidly than EGF (Fig.3.2B). Second, progesterone rescued AG1478-mediated inhibition of EGF-induced oocyte maturation in oocyte-cumulus

cell complexes (Fig.3.3A), as well as AG1478 (data not shown) and Galardin-mediated inhibition of LH-induced maturation in intact follicles (Fig.3.6D). Finally, the media surrounding EGF-treated oocyte-cumulus cell complexes contained sufficient steroid to trigger oocyte maturation in newly harvested oocyte-cumulus cell complexes (Fig.3.5). These findings imply that LH-induced activation of EGFR increases steroid production, which in turn can promote oocyte maturation (Fig.7).

Blockade of follicular steroid production with inhibitors of several early steroidogenic enzymes does not completely block gonadotropin-induced oocyte maturation (Lieberman et al., 1976; Lu et al., 2000; Tsafriri et al., 1998; Yamashita et al., 2003), suggesting that EGFR signaling promotes oocyte maturation by more than one mechanism. Pathways other than steroidogenesis that might promote maturation include EGF-mediated cumulus expansion, which promotes breakdown of cumulus cell-oocyte gap junctions (Eppig, 1994; Kanemitsu and Lau, 1993), and attenuation of G protein-mediated signals holding oocytes in meiotic arrest (Mehlmann et al., 2002; Mehlmann et al., 2004). Each activation pathway may regulate different aspects of follicular and oocyte development. For example, steroid-mediated maturation might ensure that oocytes in dominant follicles mature first, as mature oocytes are most often found in the larger follicles that contain the highest steroid concentrations (Teissier et al., 2000).

Finally, steroid-mediated maturation may be significant in disorders of androgen excess such as polycystic ovarian syndrome (Dunaif, 1997; Ehrmann et al., 1995). This condition is characterized by anovulation due to unregulated follicle growth with the absence of dominant follicles (Nelson et al., 1999). The status of oocytes within these abnormal follicles is

uncertain; however, treating these patients with gonadotropins can lead to ovarian hyperstimulation (Balen, 2000), suggesting that their follicles and oocytes have been "primed" by excess androgen. Treatment with anti-androgens improves ovarian function in these patients, and sometimes leads to the resumption of ovulation (De Leo et al., 1998; Eagleson et al., 2000; Gambineri et al., 2004; Rittmaster, 1999). Perhaps regulating EGFR or MMP activity in the ovary may similarly prove beneficial for women with androgen excess, as well as for controlling fertility in both men and women.

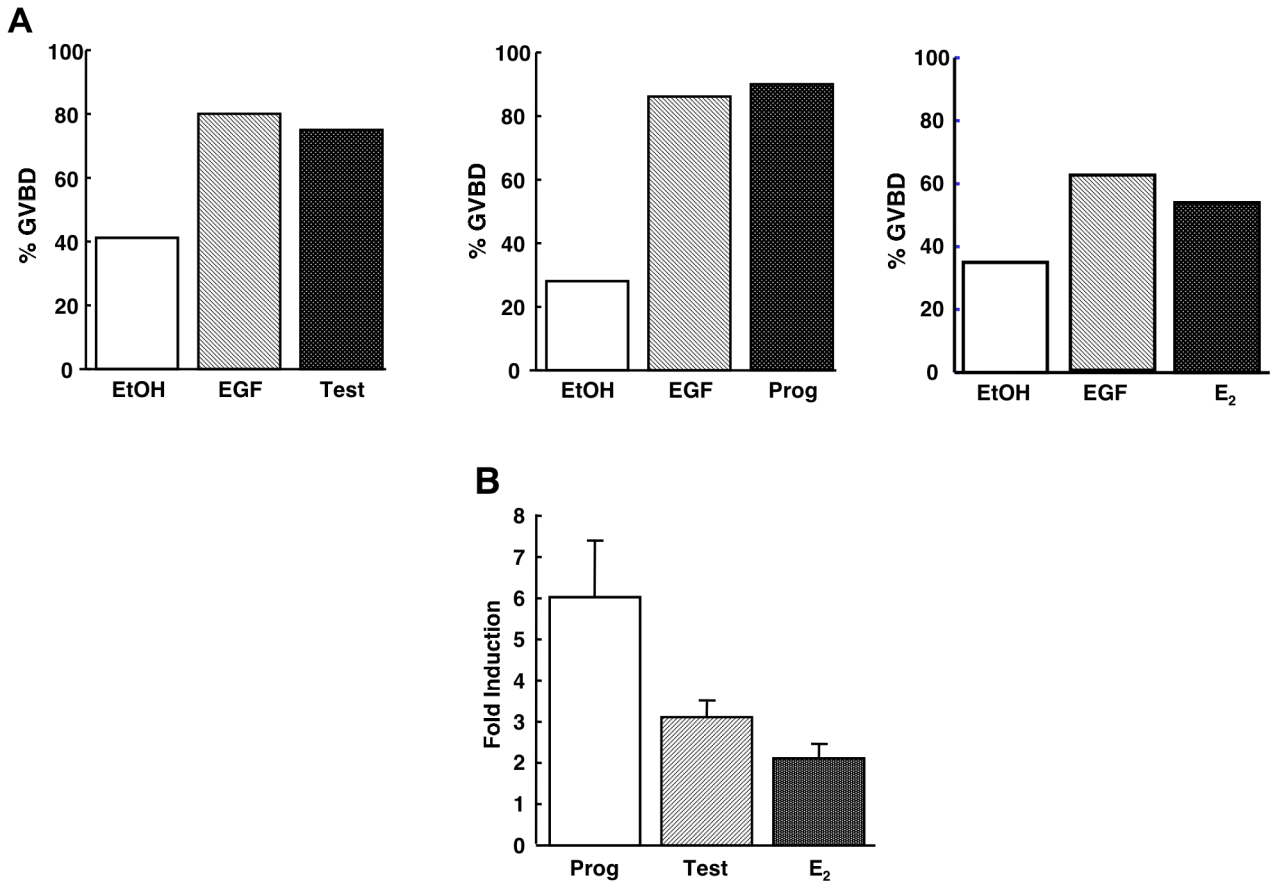


**Figure 3.1 (A) Progesterone, testosterone, and estradiol promote maturation in cultured oocyte-granulosa cell complexes (OGCs).** OGCs were grown for ten days and then incubated with 250 nM steroid or 20 ng/ml EGF for 16 hours. Maturation was assessed by denuding OGCs and scoring for the percent of oocytes that had undergone germinal vesicle breakdown (GVBD). Similar results were seen from three separate experiments. (B) **EGF stimulates steroid production in oocyte-granulosa cell complexes**. After 10 days of culture, approximately 120 OGCs were incubated for 16 hours at 37° C with 20 ng/ml EGF or 0.1% ethanol. Medium was removed and steroid content measured by RIA. Fold induction was normalized to OGCs treated with ethanol. Each bar represents the average $\pm$ S.D. (n=3).

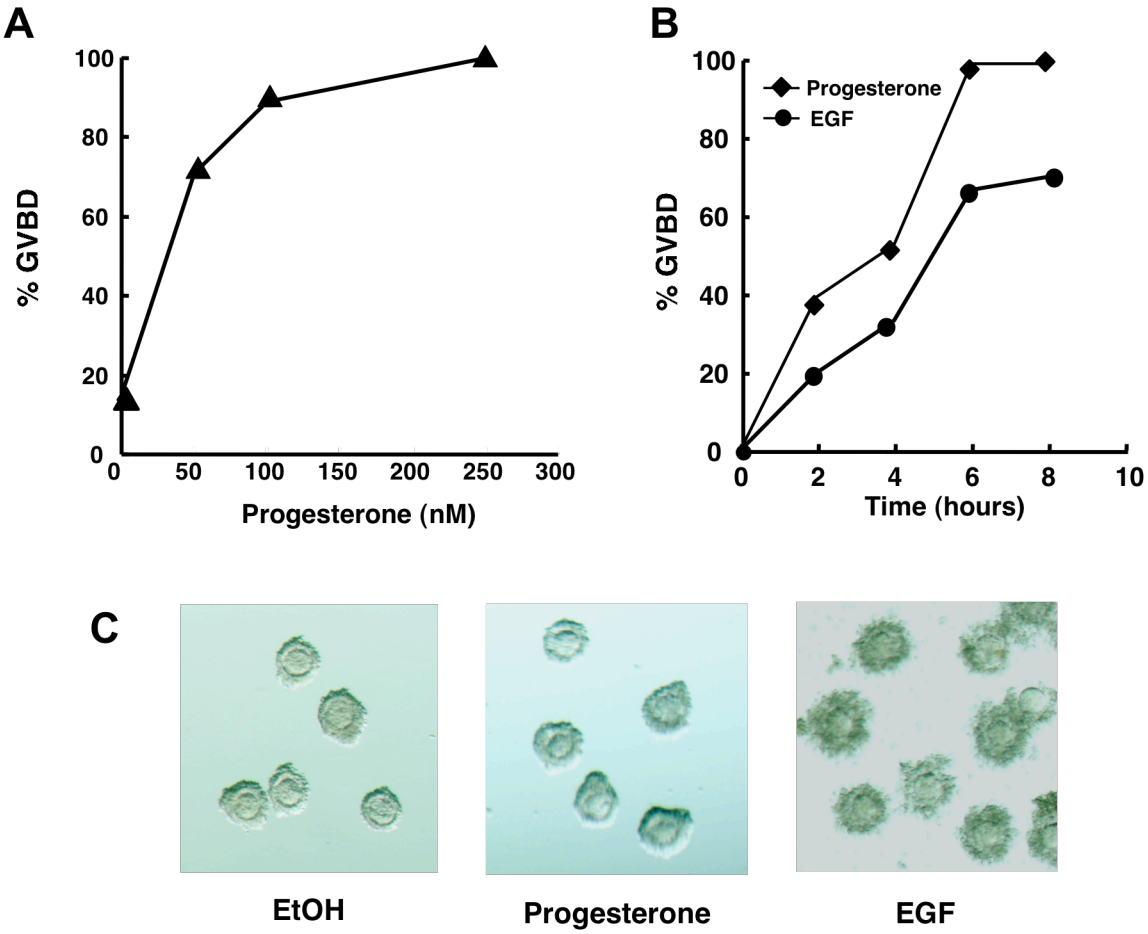


**Figure 3.2 Steroids are potent promoters of maturation acting directly on the oocyte.** (A) Dose response for steroid-induced maturation in isolated oocyte-cumulus cell complexes (OCCs). Similar results were seen in three separate experiments. (B) Time course for steroid-induced maturation. OCCs were incubated with 250 nM progesterone or 20 ng/ml EGF. Oocytes were denuded and scored for GVBD at the indicated time points. An identical trend was observed from two separate experiments. (C) Steroids do not promote cumulus cell expansion in OCCs. Cells were placed in M-16 media containing EtOH, 250 nM progesterone, or 20 ng/ml EGF. Images were taken after 16 hrs.

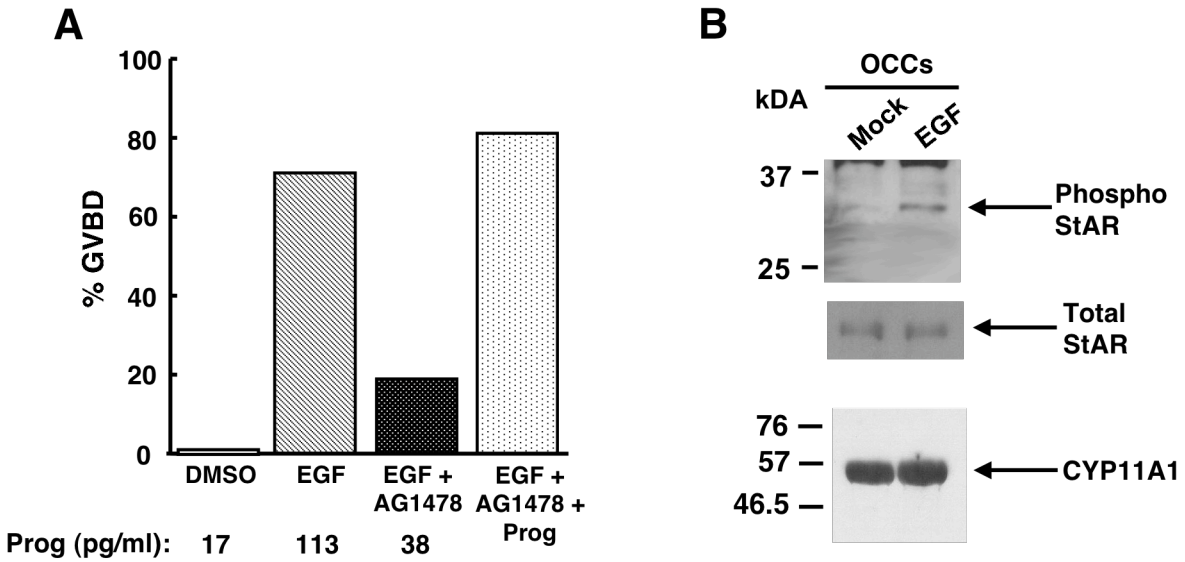


Figure 3.3 **EGFR signaling promotes steroidogenesis via activation of StAR in OCCs.** (A) Steroids rescue AG1478-mediated inhibition of maturation in OCCs. 20 OCCs were treated with AG1478 or DMSO for 30 minutes before and after the addition of 20 ng/ml EGF. 250 nM progesterone was added to rescue inhibition. Maturation was scored after 6 hrs by visualizing GVBD. Progesterone content is shown below the graph. The experiment was performed 3 times with similar results. (B) EGF increases phospho-StAR but not total StAR or CYP11A1 protein levels in oocyte-cumulus cell complexes. Approximately 25 OCCs were incubated in M-16 medium with or without 20 ng/ml EGF for 16 . Protein levels were analyzed by Western blot. Results were identical from three separate experiments.

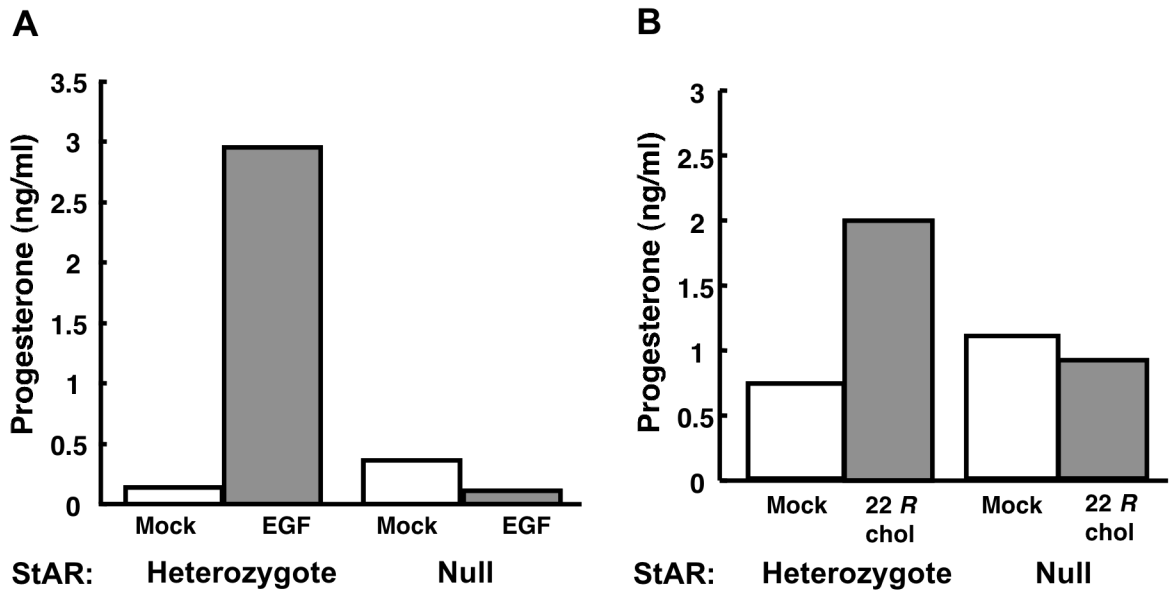


**Figure 3.4 StAR is necessary for EGF-induced steroidogenesis.** Follicles were collected from 21-day old StAR null mice (rescued by daily injection of dexamethasone 0.04 μg and fludrocortisone 0.05 μg s.c) or heterozygote littermates and incubated in the above conditions for 16 hours. Media and follicles were collected and progesterone content was measure by RIA. Note that 200 ng/ml EGF (A) and 20 μM 22*R*-hydroxycholesterol (B) did not promote progesterone production in the StAR null mice.

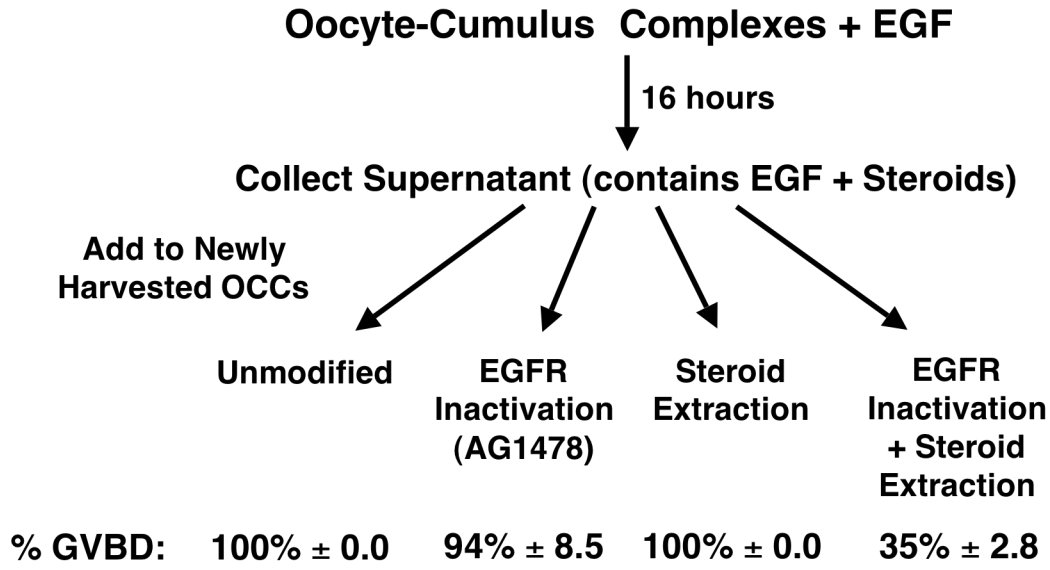


**Figure 3.5 EGF-mediated steroid production is sufficient to promote oocyte maturation.** 150 OCCs were incubated with 20 ng/ml +EGF for 16 hrs. Medium was collected and added to newly harvested OCCs in four conditions: (1) Unmodified (containing EGF and steroid); (2) With 20 mM AG1478 to block EGF receptor activity (steroids still present); (3) With steroids extracted (EGF signaling still intact); (4) With AG1478 and steroid extraction (no EGF signaling or steroids). Maturation (GVBD) was scored after 8 hrs. Results are the average of two separate experiments $\pm$ S.D.

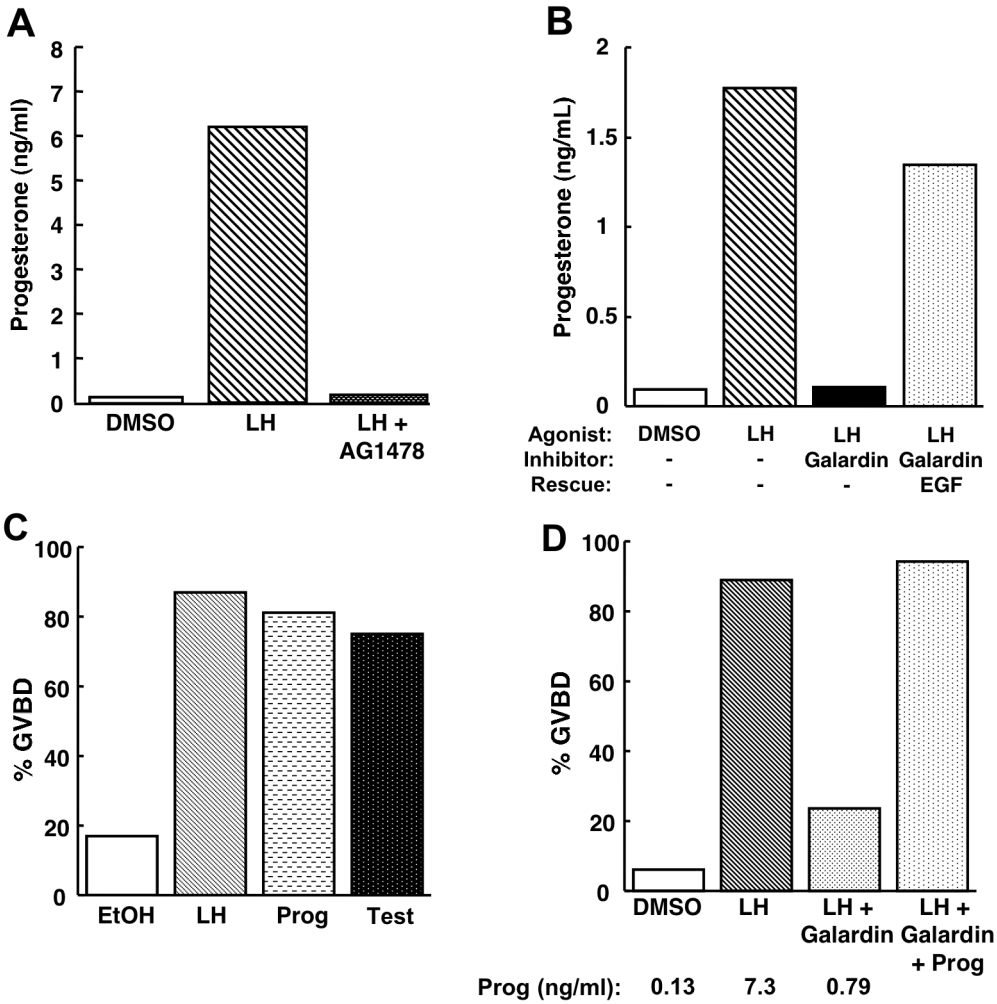


**Figure 3.6. EGFR signaling is necessary for LH-induced steroidogenesis and maturation.** LH-induced steroid production is inhibited in follicles by blocking EGFR signaling (A) and membrane-bound EGF release (B). Similar results were observed from six experiments. This latter inhibitory effect can be rescued by adding EGF. Pre-ovulatory ovarian follicles were isolated from PMSG-primed mice and incubated with DMSO, 3 mg/ml LH, or 200ng/ml EGF in the presence or absence of 20 μM AG1478 or 20 μM Galardin . Medium was collected after 6 hrs and progesterone content measured by RIA. Fifteen follicles were used for each condition. (C) Steroids and LH equally promote maturation in follicles. Intact follicles were incubated with 250 nM steroid or 3 mg/ml LH for 6 hours, then scored for GVBD. A representative graph is shown from 3 separate experiments with similar results. (D) Steroids rescue Galardin-mediated inhibition of maturation in follicles. Follicles were treated with Galardin or DMSO for 30 minutes before and after the addition of 3 mg/ml LH. 250 nM progesterone was used to rescue inhibition. Maturation was scored after 6 hours. Progesterone content was then measured by RIA and is shown below the graph. The experiment was performed twice with nearly identical results.

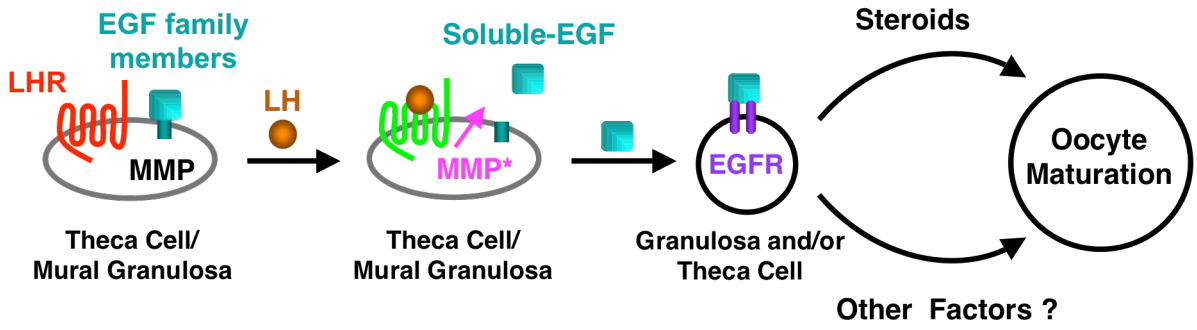


**Figure 3.7 Model for gonadotropin-induced oocyte maturation.** Before ovulation, LH (orange) binds to its receptors (red) on theca and mural granulosa cells. Activation of the LH receptor (green) results in MMP-mediated cleavage of EGF family members (blue). Upon release, these soluble growth factors then bind to EGFRs (violet) on cumulus cells (and likely mural granulosa and theca cells as well) to enhance the production of steroids and probably other unknown factor(s) that promote oocyte maturation.

# CHAPTER FOUR

## Matrix metalloproteinases: a potential target for regulating ovarian steroidogenesis

## Introduction

Unregulated LH stimulation of the ovary can lead to excess ovarian steroidogenesis, in particular androgen production, resulting in PCOS. Identifying novel signaling pathways that regulate LH-induced steroidogenesis in the ovary could aid in developing new means for attenuating excess androgen production in patients with PCOS.

We have previously found that signaling through the epidermal growth factor receptor (EGFR) is absolutely necessary for gonadotropin-induced steroidogenesis in ovarian follicles. Interestingly, activation of matrix metalloproteinases (MMP) was an essential upstream component for EGFR activation. In the ovary, we propose that MMPs are likely cleaving membrane-bound EGF moieties on the surface of LH expressing theca cells, which then activate EGFRs on granulosa cells, resulting in increased StAR activity and subsequent steroid production. Next, we began to examine the importance of this signaling pathway in other steroidogenic tissue. Surprisingly, we found that EGFR signaling is essential for LH and ACTH-induced steroidogenesis in Leydig and adrenal cell lines respectively, making the EGFR a universal regulator of steroidogenesis. In contrast to the ovary, activation of MMPs was not necessary for steroid production in Leydig and adrenal cells.

Given the ovarian specificity of MMP activation, we wanted to examine whether MMPs could be targeted *in vivo* to specifically reduce ovarian steroid production while

minimally affecting essential adrenal cortisol production. Using both a gonadotropin-primed as well as naturally cycling mouse model, we were able to show that Galardin, a broad-spectrum MMP inhibitor was able to reduce steroid production in the ovary without affecting corticosterone secretion from the adrenal glands. This study provides a proof-in-principle that MMP inhibitors can be given as treatment *in vivo* to specifically target ovarian steroidogenesis.

## Materials and Methods

### MA-10 and Y1 cell culture, measurement of steroid levels, and immunoblotting

The MA-10 mouse Leydig tumor cell line (a generous gift from Dr. M. Ascoli, University of Iowa College of Medicine, Iowa, City, Iowa) was grown in RPMI medium supplemented with 15% horse serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere under 5% CO2, 95% air. The Y1 cell line was grown in Hams-10 media with 15% horse serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere under 5% CO2, 95% air. All media and supplements were purchased from Invitrogen (Carlsbad, CA). Cells were plated in 12-well plates in complete media 48 hours preceding experiments. Media was then replaced with serum-free medium after 24 hours. On the day of the experiments, cells were washed with phosphate-buffered saline, and then placed in serum-free medium supplemented with 20 mM Hepes and 3 mg/ml BSA. Cells were pretreated with 20 μM AG1478, GM6001 (Galardin), or equal amounts of DMSO for 30 minutes before and through the addition of stimulators, 3 μg/ml LH (Sigma) or 20 μM 22R-hydroxycholesterol. Medium was collected 4 hours after the addition of stimulant and steroids extracted and measured by RIA. 200 μl of 2X sodium dodecyl sulfate (SDS) buffer plus 10% *beta*-mercaptoethanol was added and cells harvested with a cell scraper. Cells were sheared with 25-gauge needle and lysates separated by electrophoresis on 12% polyacrylamide gels and proteins transferred to Immobilon-P membranes. Membranes were probed with rabbit anti-phospho StAR (gift from Dr. Stephen King, Children's Hospital, Brooklyn, NY) and rabbit anti-StAR (gift from Dr. D.M. Stocco, Texas Tech University Health Sciences Center, Lubbock, Texas) antibodies.

**Pre-ovulatory follicle harvest and determination of steroid production**

Immature 21-28 day old C57BL/6J mice from The Jackson Laboratory were injected with 5 IU PMSG (Sigma, St. Louis, MO) into the peritoneum to stimulate follicular development. Follicles were isolated 44–48 hr post-PMSG injection by puncturing the ovary with 30 gauge needles. Intact follicles greater than 400 microns were selected, washed in M2 medium, then placed in M16 media and incubated at 37 °C for 6 hours with 3 mg/ml LH. Steroids measured by RIA following extraction from the medium and follicles. Follicles were pretreated with 20 μM Galardin or 20 μM TAP1-1 (Calbiochem, San Diego, CA), or equal amounts of vehicle for 30 minutes before and after the addition of stimulators. DMSO or ethanol concentrations were kept at 0.1% for all samples.

***In vivo* inhibition of matrix metalloproteinase (MMP)**

Three week-old prepubertal C57BL/6 females were primed with 2 IU PMSG plus 20 μM Galardin or AG1478, or vehicle into the peritoneum. After 40 hours animals were injected with 2 IU HCG plus previous the condition. Animals were sacrificed after 12 hours, ovaries removed, steroid extracted and measured by RIA. 2 animals were utilized in each condition.

Mature, 8 week-old mice were followed through two estrous cycles by vaginal cytology. Mice were injected with 20 μM Galardin or vehicle at the beginning of Diestrus 1 and Proestrus, 12 hours after ovulation serum was retrieved through retro-orbital sinus and ovaries removed. Steroid was extracted from ovary and serum and progesterone and corticosterone content measured by RIA. 5 animals were utilized in each condition.

## Results

### Normal LH-mediated steroidogenesis in MA-10 Leydig cells requires activation of the EGFR, but not membrane-bound EGFs.

To determine whether EGFR signaling may serve as a universal mechanism for regulating steroidogenesis in gonadal tissue, we examined LH-induced steroidogenesis in the MA-10 mouse Leydig cell line. As expected, LH triggered a 10-fold increase in progesterone production over the course of 4 hours (Fig. 4.1A). Similar to the results in ovarian follicles, LH-induced progesterone production was abrogated by the EGFR kinase inhibitor AG1478; however, unlike the follicles, gonadotropin-induced steroidogenesis in MA-10 cells was unaffected by the MMP inhibitor Galardin. This result suggests that LH-mediated steroidogenesis in MA-10 cells requires activation of the EGFR; however, release of membrane-bound EGFs is not required. Addition of 22*R*-hydroxycholesterol, a StAR-independent steroid precursor rescued the suppressive effects of AG1478 on LH-mediated progesterone production (Fig.4.1A), confirming that, as in the ovary, EGFR-mediated steroid production in Leydig cells is primarily regulated by increased StAR activity (Manna et al., 2002). Accordingly, upon incubation with EGF, an increase in both total and phosphorylated StAR protein levels are observed similar to induction with gonadotropin (Fig.4.1B). When cells were pretreated with AG1478, a slight inhibition in EGF-mediated protein induction is seen suggesting that an activated EGF receptor is necessary for full activation of StAR.

**ACTH-induced steroidogenesis in Y1 adrenal cells requires activation of the EGFR.**

We continued to examine the necessity of EGFR signaling for steroid production in an additional steroidogenic tissue using the Y1 mouse adrenal cell line. ACTH-mediated steroid production in the mouse adrenal Y1 cell line was abrogated when the EGFR was inactivated by the AG1478 compound (Fig. 4.1C), confirming that functional EGF receptor was necessary for normal steroid pituitary hormone-induced steroidogenesis. Notably, as in MA-10 cells, Galardin has a relatively small effect on steroidogenesis relative to AG1478 in the Y1 adrenal cells, suggesting that MMPs may be specific for regulating steroidogenesis in the ovary.

**TACE inhibition does not affect gonadotropin-induced steroidogenesis**.

Members of the adamalysins (ADAMs, a disintegrin and metalloproteinase domain) family of membrane metalloproteinases are known to shed ectodomains of membrane-bound EGF family members. For example, ADAM17, or TNF-a converting enzyme (TACE), cleaves amphiregulin, epiregulin, and HB-EGF (Sahin et al., 2004). Initial studies aimed to identify specific MMPs regulating gonadotropin-induced steroidogenesis utilized a potent TACE inhibitor, TAP1-1. Results indicate that follicles from primed mice were not affected by TACE inhibition (Fig.4.2A) as compared to Galardin, indicating that this MMP is likely not involved in regulating gonadotropin regulated steroid production.

**Inhibiting MMPs in vivo decreases ovarian steroidogenesis.**

To further explore the ovarian specificity of MMP-mediated steroidogenesis *in vivo,* we used two separate mouse models. Initially, ovarian steroidogenesis was examined in mice whose ovaries were hyper-stimulated with exogenous gonadotropins. This model

allowed for amplification of steroid production as well as precise timing of experimental procedures in relation to the gonadotropin surge. On day 1, the broad-spectrum MMP inhibitor Galardin, which significantly reduced gonadotropin-induced follicular steroid production *in vitro,* was injected into the peritonea of pre-pubertal 21-day old mice in addition to PMSG to promote follicle growth. Control mice were injected with PMSG and vehicle alone. On day 2, mice were again injected with Galardin or vehicle and finally on day 3, the mice received another injection of the MMP inhibitor or vehicle in conjunction with hCG. Mice were sacrificed 8 hours after hCG injection and ovarian steroid content measured. The same injection schedule was performed using AG1478, the EGF receptor kinase inhibitor. Results indicate that PMSG alone induced some background progesterone production of approximately 3 ng/ovary (Fig4.2B). hCG plus vehicle injection increased steroidogenesis by five fold and interestingly, both Galardin and AG1478 attenuated this effect by 90% similar to the results seen *in vitro*.

The effectiveness of Galardin was further examined in normally cycling mice. Vaginal smears were examined to track the estrus cycle of the mice through a minimum of two cycles. Once the cycling patterns were confirmed, intra-peritoneal injection of Galardin or vehicle alone were given on diestrus I. Animals were again injected at the beginning of proestrus, and sacrificed 8 hours after ovulation. Similar to the results obtained from superovulated mice, Galardin was effective at inhibiting ovarian steroid production by approximately 65% (Fig.4.2C). In addition, no changes in serum corticosterone (the physiologic corticosteroid in mice) levels were observed (Fig.4.2D) and confirmed that MMP inhibition did not significantly alter adrenal steroid secretion.

**Discussion**

In Leydig cells, EGF increases the expression and activity of StAR protein, thus increasing cholesterol transport into the mitochondria (Fig. 4.1B) (Manna et al., 2002). In contrast, MMP-mediated activation of membrane-bound EGFs is not required for gonadotropin-induced steroidogenesis in MA-10 Leydig cells (Fig. 4.1A), suggesting that alternative mechanisms activate the EGFR after LH receptor stimulation in these cells. Likewise ACTH-induced steroidogenesis is dependent upon activation of the EGFR but not cleavage of soluble EGF factors. These differences may reflect the greater role of paracrine signaling in the ovary, where multiple cell types are required for steroidogenesis. Notably, in primary granulosa cell cultures, LH-induced steroid production is no longer sensitive to to MMP inhibition (data not shown), indicating that the three dimensional structure of the ovary is necessary for this type of signaling mechanism to be preserved.

Taking lessons learned from *in vitro* studies and exploiting the specificity of MMP activation for ovarian steroidogenesis, we moved into an *in vivo* mouse model. In both gonadotropin-primed mice as well as naturally cycling mice, Galardin inhibited ovarian steroid production (Fig.4.2B &C) without affection adrenal corticosterone production (Fig. 4.2D.) These findings confirm that MMP inhibitors can be administered *in vivo* to specifically decrease ovarian steroid production in response to an artificial or natural gonadotropin surge in mice.

Although there is no mouse model for PCOS, mice still serve as a useful model for studying gonadotropin-induced steroidogenesis. Importantly, gonadotropin-mediated androgen production is a major contributor to hyperandrogenemia seen in PCOS (Adashi,

1988). Furthermore, PCOS patients often have increased sensitivity to gonadotropins during in vitro fertilization protocols (Balen, 2000; Coffler et al., 2003; Koivunen et al., 2001), suggesting that follicles and/or oocytes may be primed by the excess androgens. Clinical trials using GnRH agonists to suppress gonadotropin secretion demonstrate substantial lowering of androgen levels in PCOS patients (Steingold et al., 1987; Szilagyi et al., 2000). If elevated androgen signaling in the ovary is a major contributor to the ovarian pathology seen in PCOS, then patients could be treated with AR antagonists to improve fertility. Both flutamide and spironolactone have indeed been used successfully to treat the infertility associated with PCOS (Rittmaster, 1999; Eagleson et al., 2000; De Leo et al., 2000); however, the efficacy of these drugs is limited due to their low potency. The observations regarding the specificity or MMPs to decrease gonadotropin-induced steroid production may be beneficial to reduce the virulizing effects of hyperandrogenemia and thus improve fertility.

We use progesterone as a marker for gonadotropin-induced steroidogenesis because it is the most abundant LH-induced steroid in normal mice, similar to humans. Given that MMP activation is an early signal in the LH-induced steroidogenic pathway, any gonadotropin-induced steroid will likely be similarly regulated. Preliminary studies demonstrate that LH-induced testosterone production in follicles is EGF receptor and MMP sensitive. There are MMP inhibitors in human clinical trials for the treatment of lung tumors and refractory solid tumors. These studies are a step towards determining whether a similar approach in humans can be used to safely decrease ovarian steroid production to treat the pervasive disease of PCOS.

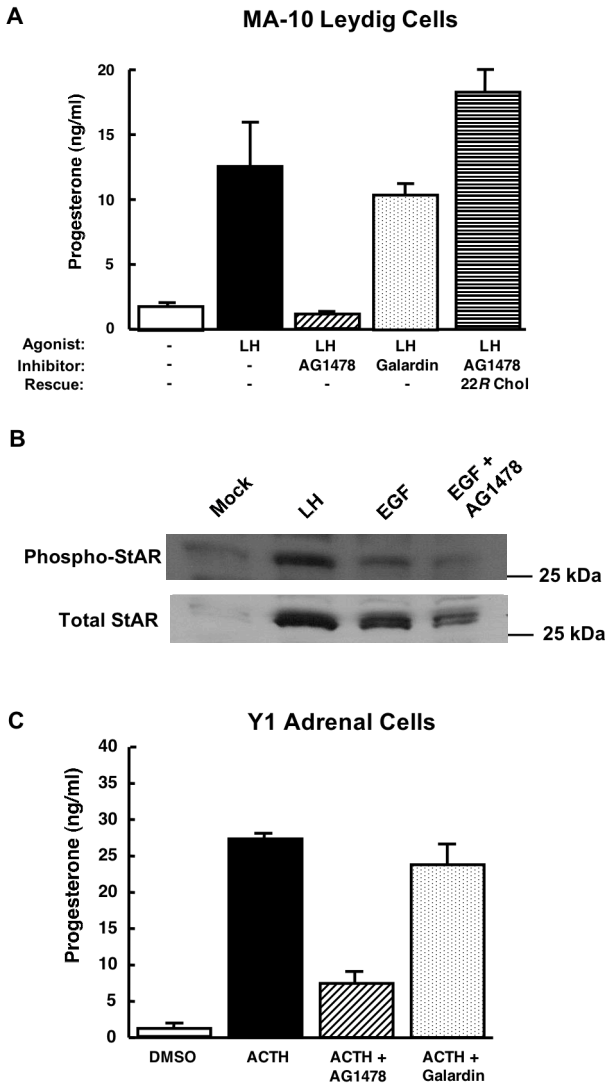


**Figure 4.1. EGFR signaling is necessary for gonadotropin and ACTH-induced steroidogenesis**. MA-10 mouse leydig cells and Y1 mouse adrenal cells were plated in 12-well plates with serum free media 48 h before the experiments. On the day of the study, cells were washed with PBS then pretreated with 20 μM AG1478, Galardin, or DMSO for 30 min before and throughout the addition of 3 μg/ml LH, 20 μM 22R-hydroxycholesterol or 300 nM ACTH. Medium was collected after 4 hours, and steroids were extracted and measured by RIA. LH-induced steroid production in MA-10 Leydig cells (A) and ACTH-induced steroid production in Y1 adrenal cells (C) were sensitive to EGFR inhibition but not MMP inhibition. This affect was rescued by addition of the StAR independent steroid precursor 22R-hydroxycholesterol in Leydig cells, again suggesting that EGF increases steroid production by activating StAR. Bars are an average ± S.D. (n=3), independent experiments were performed three times. (B) Total StAR and phosphorylated StAR levels increase upon incubation with LH and EGF. MA-10 cells were treated for 30 min with 20 μM AG1478 or vehicle before and throughout the addition of 20 ng/ml EGF or LH alone for 4 hours. Protein levels were analyzed by Western blot. A similar trend was observed from 2 separate experiments.

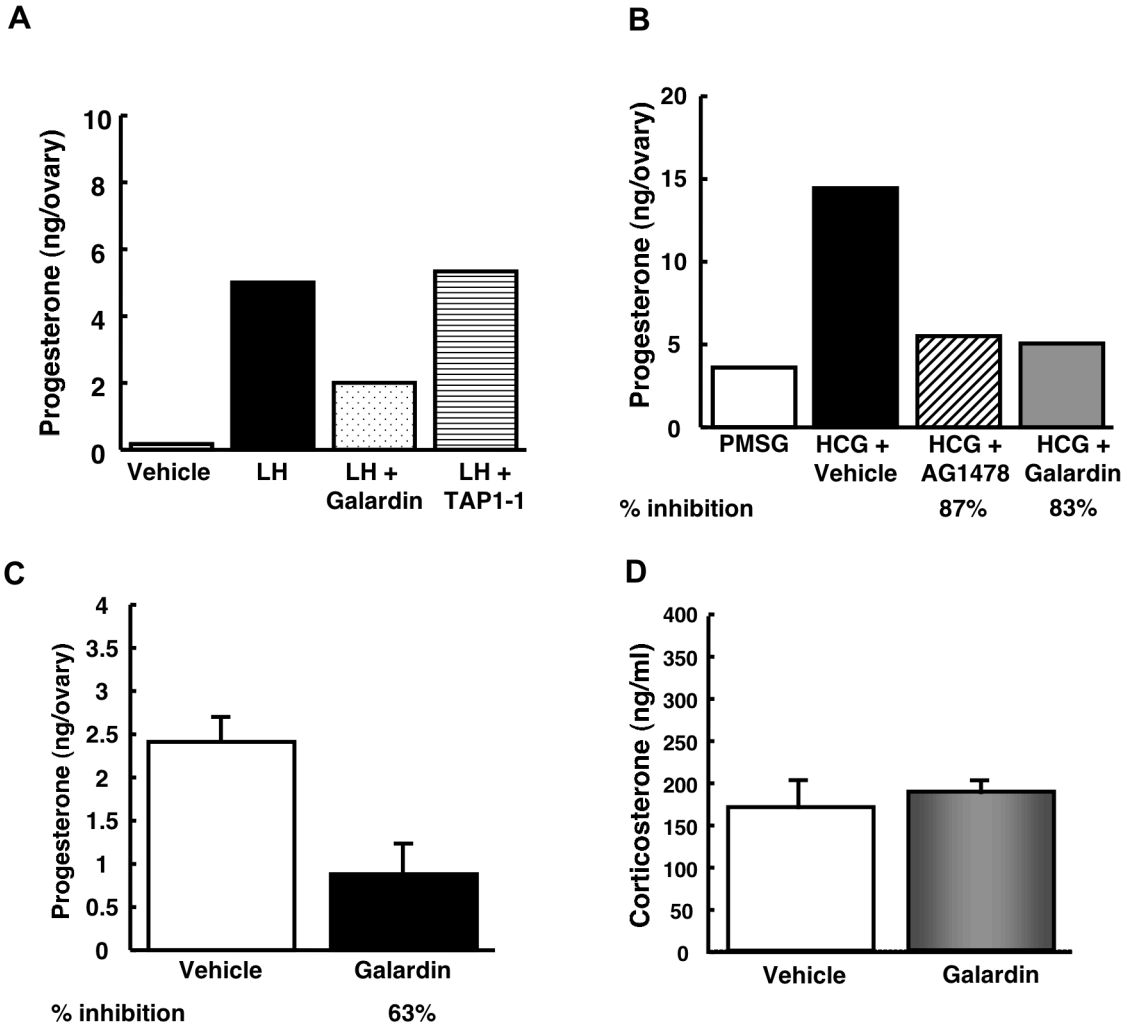


**Figure 4.2. Inhibiting matrix metalloproteinases specifically reduces LH-induced ovarian steroid production in mice.** (A) Gonadotropin-induced steroid production from follicles of 3 week-old primed mice is inhibited with 20μM galardin but not 20μM TAP1-1. (B) 3 week-old prepubertal mice were primed with 2 IU PMSG plus 20 μM Galardin or AG1478, or vehicle. 40 hours later animals were injected with 2 IU HCG plus previous condition. Animals were sacrificed after 12 hours. Identical trends were seen from two independent experiments. Each bar represents steroid from 4 ovaries. (C) 8 week-old mice were followed through two estrous cycles by vaginal cytology. Mice were injected with 20 μM Galardin or vehicle on Diestrus 1 and Proestrus, ovaries were removed 12 hours after ovulation and serum retrieved through retro-orbital sinus. Steroid was extracted from ovary and serum and steroid content measured by RIA. Similar results were seen from 2 separate experiments. Each bar represents average steroid production ± S.D from 10 ovaries. Galardin treatment inhibited progesterone content by 63%. (D) Serum corticosterone levels were unaffected by treatment, confirming that Galardin does not affect steroidogenesis in the adrenal gland. Bars represent average corticosterone content ± S.D. from the serum of 5 animals.

# CHAPTER FIVE

# Conclusions and Recommendations

## Activation of MMPs

Within our model for gonadotropin-induced steroidogenesis, questions still remain as to how stimulation of the LH receptor leads to activation of MMPs. The LH receptor is a $G\alpha_s$ coupled receptor and although the classical function of GPCR's is to generate second messengers, there is a large body of work showing how they can transactivate the EGFR. The crosstalk between these two types of receptors is necessary to perhaps amplify or diversify the type of signaling that can occur in response to the cellular environment. There are several examples showing agonist stimulation of the β2-adrenergic receptor, M1-muscarinic receptor, and Gq coupled receptors, leading to a very rapid and transient tyrosine phosphorylation and dimerization of the EGF receptor (Herrlich et al., 1998; Keely et al., 2000; Maudsley et al., 2000). The kinetics of these models has supported a ligand-independent mechanism for EGFR activation. Though the mechanism is still largely unknown, several studies have shown that Src kinase activation (Keely et al., 2000; Luttrell et al., 1997; Maudsley et al., 2000) and Pyk2 activation (Andreev et al., 2001; Keely et al., 2000) precede EGFR transactivation.

Ligands for the EGF receptor are synthesized as transmembrane precursors and are prototypically cleaved by matrix metalloproteinases to yield the mature growth factor. An alternative mechanism for EGFR transactivation that is ligand-dependent was first described by (Prenzel et al., 1999). They showed that GPCR activation rapidly induced MMP activity

and inhibition of proHB-EGF processing via MMP inhibition attenuated EGFR signaling in prostate carcinoma cells. Since ovarian steroid production is dependent upon release of soluble EGFs, characterizing the LH-triggered signals that regulate MMP activation is crucial for understanding steroidogenesis in the ovary. In particular, the involvement of cAMP, phospholipase C (PLC), Src, or mitogen-activated protein kinase kinase (MEK) in regulating LH-mediated activation of MMPs and subsequent EGF receptor signaling in follicles should be determined. For example, cAMP levels could be artificially upregulated with forskolin or dbcAMP in follicles and various downstream effectors such as EGFR phosphorylation and steroidogenesis can be measured to determine the role or cAMP in MMP activation. Similarly, inhibiting Src with PP2, or MEK with PD98059 then measuring downstream effects will verify the involvement of these pathways in LH-induced cleavage of membrane-bound EGFs. To date, experiments designed to detect shedding of HB-EGF into the media have been unsuccessful (Tsai et al., 1997). In order to further support our model, an assay to detect and measure soluble EGFs upon stimulation by gonadotropin should be developed.

**MMPs that regulate ovarian steroidogenesis**

The MMP inhibitor used for our studies was Galardin, a broad-spectrum inhibitor. Identifying the specific MMPs that are activated in response to gonadotropin in the ovary would be useful for determining the MMPs to target in order to precisely regulate ovarian steroid production *in vivo*. Several approaches can be used towards initially identifying these specific enzymes including reverse-transcription polymerase chain reaction (RT-PCR), gelatin zymography, and western blotting. After the expression of specific MMPs in response to gonadotropin are confirmed, their effects on steroid production in isolated

follicles then cycling mice should be examined. In addition, a murine model that closely mimics the excess androgen levels seen in PCOS should be developed. This could be accomplished by titrating the amount of gonadotropin stimulation to achieve a three to five fold increase in ovarian androgen levels. The effects of inhibiting ovarian specific MMPs in this PCOS model should be examined by measuring ovarian steroid concentrations as well as serum corticosteroid levels. In addition, studies that examine the long-term effects of chronic MMP inhibition and potential side effects should be determined.

**Activation of StAR**

StAR protein mediates the rate-limiting step in steroid hormone production, the transfer of cholesterol across mitochondrial membranes, though the mechanism by which StAR promotes cholesterol movement is still unknown. StAR contains two phosphorylation sites, Ser 56 and Ser 194 (Arakane et al., 1997). Though no function has been attributed to Ser 56, it has been shown that phosphorylation of Ser194 permits maximal cholesterol transfer by StAR (Jo et al., 2005) as well as affects protein stability (Clark et al., 2001). Some studies have shown that de novo steroid synthesis requires ongoing StAR production, similar to what we and others observe in Leydig cells (Fig 4.1B) (Manna et al., 2002); however, other studies have shown that it is an increase in phosphorylated StAR protein that is necessary for steroid production. For example, angiotensin II- induced steroid production correlated with an increase in phosphorylated but not total StAR protein levels. (King, 2005). They found in adrenocortical cells that both steroid production and StAR phosphorylation was abrogated upon incubation with a PKA inhibitor indicating that angiotensin II is mediating its effects through PKA, though another group has implicated the PKC signaling

pathway (Betancourt-Calle et al., 2001). Similarly, it is important to determine which kinases and molecular events are being activated downstream of EGF signaling to activate StAR phosphorylation.

**Signaling pathways activated by steroid-mediated maturation**

Our data suggests that steroid-induced maturation in the frog and mouse utilize similar signaling pathways. In light of this, it would be interesting to further explore the role of G proteins in mouse oocyte maturation as constitutive Gβγ, and possibly $G\alpha_s$, signaling appears to maintain meiotic arrest in frog (Lutz et al., ; Sheng et al., 2001). Notably, it has been shown in mice that a constitutively active $G_s$-linked orphan receptor GPR3, is crucial for maining meiotic arrest (Mehlmann et al., 2004). By doing studies to modulate Gβγ or $G\alpha_s$, the role of G proteins in regulating steroid-induced meiotic resumption can be further examined. In addition, changes in intracellular cAMP levels or adenylyl cyclase activity in response to steroid stimulation should also be determined.

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# VITAE

Michelle Jamnongjit was born in Garland, Texas on April 13, 1982, the daughter of Thuc Thi Dinh and Vichai Jamnongjit, US immigrants from Vietnam and Thailand. After completing her sophomore year at Garland High School, she was admitted to the Texas Academy of Mathematics and Science (TAMS) in Denton, Texas a program that allowed her to finish high school while concurrently taking college courses at the University of North Texas. She graduated from the program in May 2000 and attended the University of Texas at Austin for her junior and senior year of college. In May 2002, she received the degree of Bachelor of Arts with a major in psychology, minor in biology graduating with high honors. In June 2002 she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. She was awarded the degree of Doctor of Philosophy in December 2006.

Permanent Address: 1119 Creekwood Dr.
Garland, Texas 75044-2413