G PROTEIN AND ANDROGEN SIGNALING IN OVARIAN FUNCTION

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Dedicated to my family for their unyielding love and support.

G PROTEIN AND ANDROGEN SIGNALING IN OVARIAN FUNCTION

by

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Oocytes are held in meiotic arrest in prophase I until ovulation when gonadotropins trigger a subpopulation of oocytes to resume meiosis in a process termed “maturation.” Meiotic arrest is maintained through a mechanism whereby constitutive cAMP production exceeds phosphodiesterase-mediated degradation, leading to elevated intracellular cAMP. Studies have implicated a constitutively activated Gαs-coupled receptor, G protein-coupled receptor 3 (GPR3), as one of the molecules responsible for maintaining meiotic arrest in mouse oocytes.

Here, we characterized the signaling and functional properties of GPR3 by using the more amenable model system of *Xenopus laevis* oocytes. We cloned the *Xenopus laevis* isoform of GPR3 (XGPR3) from oocytes and showed that overexpressed XGPR3 elevated intra-oocyte cAMP, in large part due to $G_{\beta\gamma}$ signaling. Overexpressed XGPR3 suppressed steroid-triggered kinase activation and maturation of isolated oocytes, as well as gonadotropin-induced maturation of follicle-enclosed oocytes. In contrast, depletion of XGPR3 using antisense oligodeoxynucleotides enhanced steroid- and gonadotropin-mediated oocyte maturation. Interestingly, collagenase treatment of Xenopus oocytes cleaved and inactivated cell surface XGPR3, which enhanced steroid-triggered oocyte maturation and activation of MAPK. In addition, hCG treatment of follicle-enclosed oocytes triggered matrix metalloproteinase-mediated cleavage of XGPR3 at the oocyte cell surface.

Collectively, these results suggest that GPR3 moderates the oocyte response to maturation-promoting signals, and that gonadotropin-mediated activation of metalloproteinases may sensitize oocytes for maturation by inactivating constitutive GPR3 signaling.

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Carbajal, L., **Deng, J**., Dressing, G.E., Hagan, C.R., Lange, C.A., and Hammes, S.R. (2009). Meeting review: Extra-nuclear steroid receptors-Integration with multiple signaling pathways. Steroids 74, 551-554.

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

Deng, J., Lang, S., Wylie, C., and Hammes, S.R. (2008). The *Xenopus laevis* isoform of G protein-coupled receptor 3 (GPR3) is a constitutively active cell surface receptor that participates in maintaining meiotic arrest in X. laevis oocytes. Mol Endocrinol 22, 1853-1865.

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LIST OF ABREVIATIONS

AC – adenylyl cylcase

AKAP – A-kinase anchoring protein

AR – androgen receptor

cAMP – cyclic adenosine monophosphate

cdc2 – also referred to as CDK1

CDK – cyclin-dependent kinase

cRNA – derived from cDNA through *in vitro* RNA synthesis

ERK2 – p42 MAPK

FSH – follicule-stimulating hormone

G protein - guanine nucleotide-binding proteins

GDP - Guanosine-5'-diphosphate

GnRH – gonadotropin-releasing hormone

GPCR – G protein-coupled receptor

GTP - Guanosine-5'-triphosphate

GVBD – germinal vesicle breakdown

hCG – human chorionic gonadotropin, is structurally similar to LH/FSH and serves as cheaper alternative

HPG axis – hypothalamic-pituitary-gonadal axis

LH – luteinizing hormone

MAPK – mitogen-activated protein kinase or ERK

MEK1 – MAP kinase kinase

MPF – maturation-promoting factor, complex of CDK1 and cyclin B1

mRNA – messenger RNA

PAR1 – protease-activated receptor 1, also known as thrombin receptor

PDE – phosphodiesterase

PKA – protein kinase A

PR – progesterone receptor

Chapter One

General Introduction

Oocyte maturation

Normal female fertility requires the precise regulation and initiation of meiosis in oocytes, a process also known as oocyte maturation. The term "oocyte maturation" simply means the progression of an immature oocyte into a mature oocyte or egg ready for fertilization by a sperm. Improper signaling during these events can result in a myriad of disorders, including infertility. Therefore, understanding the complex processes involved in fertility and meiosis is crucial to realizing how both normal and abnormal reproduction occur.

In nearly all vertebrates, females are born with their full sets of oocytes. These oocytes are held in meiotic arrest, and only at the time of sexual maturity will these oocytes re-enter the meiotic cycle. This arrested state is the default fate of the oocytes. The induced fate is for the oocyte to undergo maturation, which produces a ripe oocyte arrested in metaphase of meiosis II, ready for ovulation and fertilization.

Meiosis is a process that occurs only in gametes and involves the segregation of chromosomes along the mitotic spindle to generate haploid gametes from diploid gamete precursors (Whitaker, 1996). In most species,

there are two cell cycle arrests during meiosis. The first meiotic arrest, as described above, occurs at prophase of meiosis I, resulting in the accretion of immature oocytes within the ovary. When these immature oocytes progress through the meiotic cycle, they are again arrested at metaphase of meiosis II, in which, the oocytes are then deemed mature and become fertilizable eggs (Fig. 1-2).

Just prior to ovulation, meiotic progression through the first prophase arrest occurs through stimulation by the hypothalamic-pituitary-gonadal or HPG axis (Fig. 1-1). The hypothalamus releases gonadotropin-releasing hormone (GnRH), which stimulates the pituitary to release the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins travel to the ovary and stimulate the synthesis of the sex steroids: progesterone (P), testosterone (T), and estrogens (Fig. 1-1). When the immature oocytes held at prophase I of meiotic arrest receive these gonadotropin-induced steroid signals, the oocytes resume meiosis, as indicated by germinal vesicle breakdown (GVBD) and completion of the first meiotic division and extrusion of the first polar body. These oocytes then progress through meiosis, until they are again arrested at metaphase of meiosis II. Now deemed mature, (Fig. 1-1) the oocytes require fertilization by a sperm as the signal to complete meiosis (Albertini and Carabatsos, 1998; Maller, 1985; Maller and Krebs, 1980; Masui and Clarke, 1979; Shibuya and Masui, 1989).

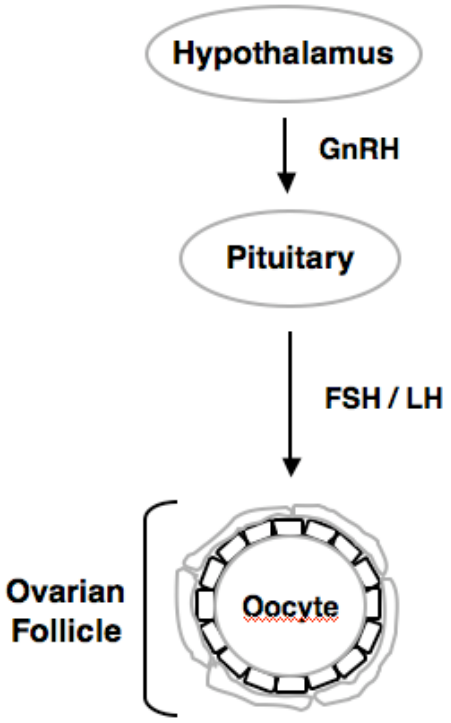

Figure 1-1: Overview of Hypothalamic-Pituitary-Gonadal (HPG) Axis.

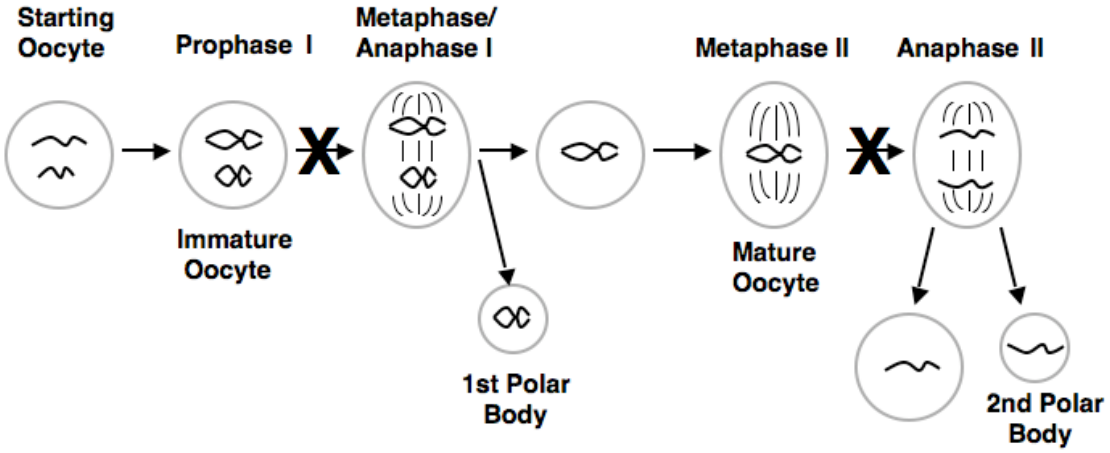

Figure 1-2: Overview of Meiosis.

Xenopus laevis as a Tool to Study Oocyte Maturation

Both the mouse and frog animal models have long been used to study fertility and reproduction. *Xenopus laevis*, in particular, has served as an excellent experimental model for studying maturation and cell cycle regulation. Unlike mouse oocytes, *X. laevis* produces hundreds to thousands of relatively large (Hammes, 2003) oocytes visible to the naked eye, thus allowing for easier manipulation and experimentation. This ease of isolating large numbers of oocytes in *Xenopus laevis* is especially useful for gene overexpression and knockdown studies, as well as for assaying signals associated with meiosis (Hammes, 2003, 2004; Maller and Krebs, 1980; Rasar and Hammes, 2006).

Although significant progress has been made in showing that steroids trigger maturation in oocytes via classical steroid receptors in a transcription-independent, or nongenomic, manner in *X. laevis* (Hammes, 2004), relevance of steroids in regulating mammalian oocyte maturation has remained controversial. Thus, *Xenopus* oocytes are one of the few well-accepted, biologically relevant examples of nongenomic steroid-mediated signaling (Gill et al., 2004; Jamnongjit and Hammes, 2005; Schmitt and Nebreda, 2002).

Overview of the Oocyte Maturation Signaling Pathway

How does an immature oocyte become a fertilizable egg? Most studies suggest a "release of inhibition" model for oocyte maturation, whereby oocytes are held in meiotic arrest by constitutive G protein signals that stimulate adenylyl cyclase (AC) to keep intracellular cyclic AMP (cAMP) levels elevated (Gallo et al., 1995; Lutz et al., 2000; Sheng et al., 2001). In *Xenopus laevis*, during ovulation, gonadotropins act to stimulate ovarian steroid production, which overcome or inhibit these signals. In turn, intracellular cAMP levels are reduced, and thus, meiotic progression can occur (Fig. 1-3). When cAMP levels are decreased, downstream kinases are then activated, leading to germinal vesicle breakdown (GVBD), an indicator of meiotic progression (Ferrell, 1999b; Maller and Krebs, 1980). The rapid decrease in cAMP levels is followed by a "lag period" during which protein synthesis occurs, followed by activation of the MAPK cascade, including MEK1 and p42 MAPK. Finally, after several hours, the activation of the MAPK cascade results in the activation of the maturation promotion factor (MPF) and the resumption of meiosis and germinal vesicle breakdown (Fig. 1-3). This germinal vesicle breakdown and resumption of meiosis is easy to score for in *Xenopus* oocytes, because a white spot, resulting from rearrangement of cortical pigment granules, appears at the oocyte's animal pole (Fig. 1-4).

To fully understand oocyte maturation, we must discuss in detail the mechanisms that hold the oocytes at meiotic arrest, the signals that activate

meiotic progression, and finally, the signaling pathways that lead to the ultimate activation of MPF for meiotic progression.

The "Release of Inhibition" Model

How does steroid signaling through classical steroid receptors regulate oocyte maturation? As previously described, most studies suggest a "release of inhibition" model (Fig. 1-3) for *Xenopus* oocyte maturation in which promoters of maturation, such as steroids, overcome inhibitory signals, causing a decline in intracellular cAMP, therefore triggering activation of the maturation signaling cascade (Gill et al., 2004; Lutz et al., 2000).

To date, it is still unclear how steroid receptors can communicate with G protein signaling pathways to mediate such rapid changes in signaling. Interestingly, it has been shown that a scaffolding molecule termed proline-, gluatmic acid-, and leucine-rich protein 1 (PELP1), also known as the modulator of nongenomic actions of the androgen receptor (MNAR), binds to both the AR and G_{β}, and its expression appears to be critical for maintaining basal levels of $G_{\beta\gamma}$ signaling (Haas et al., 2005), thus suggesting a possible involvement of PELP1 in facilitating this communication between steroid receptors and G protein signaling.

It was further proposed that ligand binding might induce a change within the complex resulting in rapid attenuation of cAMP levels by altering βγ-signaling. This hypothesis has been supported by recent work using a βγ-

sensitive potassium channel as a real-time reporter for rapid changes in βγ-signaling that showed androgens can significantly decrease βγ-signals in as little as ten minutes. This effect was confirmed to occur through the AR since the competitive antagonist, flutamide, or reduction of AR levels by RNA interference blocked the androgen-induced changes in βγ-signaling. Because βγ is known to activate AC activity, this experiment indicates one way in which steroids could affect cAMP levels rapidly (Evaul et al., 2007). This is likely only one of many ways cAMP levels decline, and most probably, other signals within the oocyte are altered upon steroid treatment, including altered $G\alpha_s$ and phosphodiesterase activity, both of which could adjust cAMP levels (Masciarelli et al., 2004; Sadler and Maller, 1987).

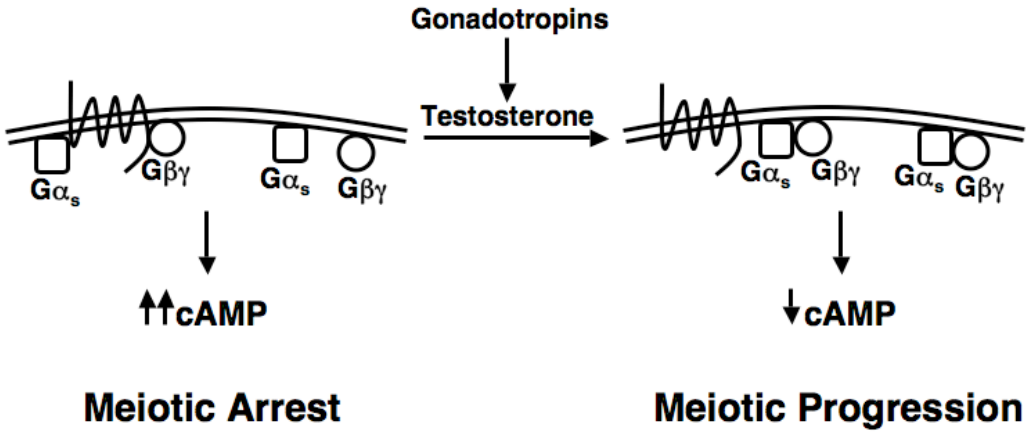

Figure 1-3: Overview of "Release of Inhibition" Model. Oocytes are held in meiotic arrest due to constitutive G protein signaling (left). This G protein signaling results in elevated levels of cAMP, a key factor thought to hold oocytes in arrest. During ovulation, gonadotropins trigger the release of steroids, such as testosterone. The steroids bind to their respective receptors and disrupt the G protein signaling, which causes cAMP levels to decrease and allows meiosis to progress.

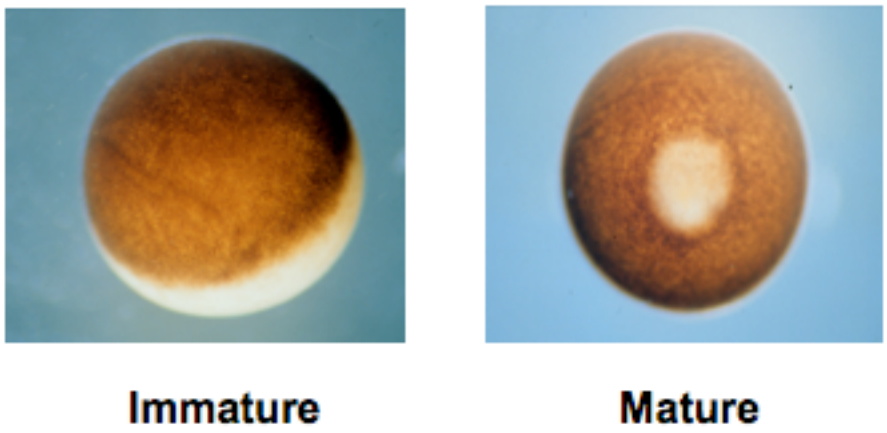

Figure 1-4: Immature versus Mature *Xenopus* Oocytes. The presence of the "white spot" on the animal pole indicates GVBD has occurred; thus, meiosis has resumed.

G Protein Signaling

To understand how constitutive G protein signaling functions to keep oocytes in meiotic arrest, general principles of G protein signaling must be discussed.

Guanine nucleotide-binding proteins (G proteins) are a family of proteins involved in second messenger cascades and are important signal transducing molecules in cells. Two main families of G proteins exist: heterotrimeric G proteins that are activated by G protein-coupled receptors (GPCRs) and monomeric "small" G proteins that belong to the Ras superfamily (Casey et al., 1988; Casey et al., 1989). GPCRs are a large family of seven-transmembrane receptors that mediate the responses to an enormous range of signal molecules, including hormones, neurotransmitters, and local mediators. Abnormalities involving GPCRs or G protein signaling can result in disease, and because of their diverse roles in many physiological functions, there are also a variety of human diseases associated with GPCRs. Currently, GPCRs serve as the target for over fifty percent of drugs.

Structurally, all GPCRs are similar. A GPCR consist of a single polypeptide chain that threads back and forth across the lipid bilayer seven times and are therefore sometimes called serpentine receptors.

Typically, when extracellular signaling molecules bind to these GPCRs, the receptors undergo a conformational change that enables them to activate

their associated heterotrimeric (G proteins). These G proteins are attached to the cytoplasmic face of the plasma membrane, where they serve as relay molecules to transduce the signal to other parts of a pathway. There are various types of G proteins, each specific for a particular set of GPCRs and for a particular set of downstream target proteins in the plasma membrane. All G proteins, however, share similar structures, and they operate in a similar ways (Casey and Gilman, 1988; Casey et al., 1988; Freissmuth et al., 1989). The trimeric family of G proteins consists of three subunits: α, β, γ. There are also four main classes of G proteins: G_s, which activates adenylyl cyclase; G_i, which inhibits adenylyl cyclase; G_q, which activates phospholipase C, and G_{12} and G_{13}, of unknown functions.

In the inactive state, the α subunit has GDP bound and the G protein is inactive. When a signal stimulates the associated receptor, the altered receptor stimulates a change in the G protein: GDP dissociates from the α subunit, and GTP takes its place. This causes the trimer to dissociate into two activated components—an α subunit and a βγ complex (Bourne and Stryer, 1992; Dohlman and Thorner, 1997; Dohlman and Thorner, 2001; Kjeldgaard et al., 1996; Nathans and Hogness, 1983; Sondek et al., 1994).

Both G_α-GTP and $G_{\beta\gamma}$ can activate different downstream signaling pathways and effectors, and activated trimeric G proteins can affect ion pores and enzymes such as adenylyl cyclases and phospholipases. These enzymes will then stimulate the production of second messengers such as cyclic

adenosine monophosphate (cAMP), inositol triphosphate (IP_3), and diacyclglycerol (DG) (Stryer and Bourne, 1986).

cAMP was first identified as a small intracellular mediator in the 1950's. cAMP is synthesized from ATP by the plasma-membrane-bound enzyme, adenylyl cyclase (AC) and is degraded by cAMP phosphodiesterases (PDEs) that hydrolyze cAMP to adenosine 5'-monophosphate (5'-AMP). cAMP serves to activate downstream targets such as cyclic-AMP-dependent protein kinase (PKA). This kinase can catalyze the transfer of the terminal phosphate group from ATP to specific serines or threonines of its targets, thereby regulating their activity.

PKA is found in all animal cells, but the substrates for PKA differ in different cell types, which explains how cAMP can have different effects in different cells. PKA consists of two catalytic subunits and two regulatory subunits in its inactive state. The regulatory subunits are also important for localizing the kinase inside the cell. Special PKA anchoring proteins (AKAPs) bind both to the regulatory subunits of PKA and tether the enzyme complex to a particular subcellular compartment. Some AKAPs also bind other kinases and phosphatases, creating a signal complex (Dekel, 2005).

The binding of cAMP to the regulatory subunits of PKA alters their conformation and thus causing them to dissociate from the complex. PKA is now active, and its catalytic subunits will phosphorylate downstream targets.

These concepts are important to grasping the importance of high cAMP levels in meiotically arrested oocytes.

Oocytes Are Held in Meiotic Arrest

Meiotic arrest in oocytes seems to be an active process involving inhibitory signals responsible for holding these oocytes in arrest. In mammals, 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, b). In contrast, the signals maintaining meiotic arrest appear to be endogenous to the oocytes themselves in lower vertebrates such as frogs. Isolated amphibian and fish oocytes remain 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). A meiosis-related decrease in cAMP levels has long been observed in animals ranging from fish to frog to mouse (Conti et al., 2002; Morrill et al., 1977; Sadler and Maller, 1981, 1985).

cAMP has been extensively shown to act as an inhibitor of oocyte maturation, perhaps through activation of PKA. Typically denuded (not encased in follicles) mouse oocytes spontaneous mature in media. However,

elevating intracellular cAMP levels through cAMP analogs such as dibutyryl cAMP or PDE antagonists such as isobutylmethylxanthine (IBMX) or milrinone in these denuded mouse oocytes can prevent spontaneous maturation (Conti et al., 2002; Conti et al., 1998). Also, oocyte maturation can be blocked *in vivo* by feeding mice PDE inhibitors prior to and during ovulation (Wiersma et al., 1998).

In contrast, mouse oocytes still encased in follicles are held in meiotic arrest. Release of meiotic arrest in these follicle-enclosed oocytes can be accomplished through injection of antibodies targeted against $G\alpha_s$ into oocytes (Mehlmann et al., 2002). Intracellular cAMP also appears to drop rapidly under some conditions at the start of mammalian oocyte maturation (Conti et al., 2002).

Interestingly, 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 PDE 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).

Finally, similar studies where cAMP levels in frog oocytes were artificially elevated resulted in the inhibition of steroid-induced maturation,

and likewise a drop in cAMP upon activation of meiosis has been examined (Morrill et al., 1977; Sadler and Maller, 1981, 1985).

Taken together, these findings show that 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 regulate PDE activity are less well characterized, but may involve short-term activation in response to PKA-mediated phosphorylation, as well as long-term regulation that entails changes in mRNA and protein expression (Mehats et al., 2002).

In *Xenopus* oocytes, cAMP levels appear to be elevated, at least in part, due to constitutive $G\alpha_s$ and $G_{\beta\gamma}$ signaling, which either or both can activate adenylyl cyclase (Gallo et al., 1995; Lutz et al., 2000; Sheng et al., 2001). Triggers of maturation in *X. laevis*, such as steroids, appear to overcome this inhibitory signal, releasing meiotic arrest and allowing maturation to occur (Hammes, 2003, 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. This also appears to be physiologically significant since inhibition of $G_{\beta\gamma}$ and/or $G\alpha s$

signaling results in induction of spontaneous maturation, presumably due to a reduction in intracellular cAMP (Gallo et al., 1995; Lutz et al., 2001; Sheng et al., 2001).

How does elevated cAMP levels hold oocytes at meiotic arrest and inhibit maturation? Much still remains unclear, but the theory is that cAMP regulates kinase signaling cascades, such as the PKA pathway, which are critical for meiotic arrest (Conti et al., 2002; Morrill et al., 1977; Sadler and Maller, 1981). 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).

A large amount of attention has been given to how steroids might decrease cAMP levels, but it is important to recognize that this is not required in all species. In some animals, such as sheep, rabbit, and pig, cAMP may not change or may actually increase at the onset of maturation (Crosby et al., 1985; Mattioli et al., 1994; Yoshimura et al., 1992). In an attempt to reconcile this difference to mouse and frog maturation, some argue that global cAMP changes may not be required, but instead local changes in cAMP concentration at key signaling sites may be important. In theory, this could be regulated by proteins that target cAMP signaling, such as AKAPs (Dekel, 2005). This theory is maintained partially by the observation that AKAP1 knockout mice demonstrate reduced fertility due to a defect in meiotic

maturation, likely resulting from mislocalization of PKA activity (Newhall et al., 2006). Nonetheless, there are possibly many signaling changes that occur to release meiotic arrest, some more important in some species than in others, and future research will unveil what these signals might be.

Steroids Can Trigger Meiotic Progression in *Xenopus laevis*

The study of steroid-induced *Xenopus laevis* oocyte maturation is one of the best-studied animal models of maturation available. The use of this model has allowed researchers to identify some of the key signals that trigger meiotic progression in a meiotically arrested oocyte.

Almost sixty years ago, female frogs that were treated with pituitary extracts were shown to trigger ovulation (Rugh, 1935). From those experiments, it was hypothesized that the pituitary released a hormone which caused the release of a factor within the ovary that was responsible for inducing maturation and ovulation (Heilbrunn, 1939; Ryan and Grant, 1940; Wright, 1945). From the 1960's until recently, it was thought that this factor was the hormone progesterone (Smith et al., 1968). Thus, most studies involving *Xenopus* oocyte maturation use progesterone as the *in vitro* promoter of *X. laevis* oocyte maturation. Because it works well *in vitro*, progesterone was assumed to be the *in vivo* mediator as well; however, significant evidence suggested otherwise.

First, mifepristone (RU486), a potent inhibitor of *Xenopus* progesterone receptor (PR)-mediated transcription, did not block progesterone-mediated maturation (Bagowski et al., 2001; Lutz et al., 2001). Second, reduction of endogenous PR levels or overexpression of exogenous PR in *Xenopus* oocytes only partially altered progesterone-induced maturation (Bayaa et al., 2000). Finally, *in vitro* stimulation of *Xenopus* ovarian fragments or follicles with gonadotropin revealed that other steroids, such as testosterone (Lutz et al., 2001; Smith and Ecker, 1971) were secreted at significantly higher levels than progesterone.

In an attempt to determine the true physiologic mediator of *X. laevis* oocyte maturation, female frogs were injected with human chorionic gonadotropin (hCG), which is structurally similar to LH/FSH and is used as a less expensive alternative to those particular gonadotropins. A measurement of serum and ovarian steroid levels were then taken (Lutz et al., 2001). At every time point, progesterone was nearly undetectable, while concentrations of the androgens androstenedione and testosterone were more than 10-fold that of progesterone. Furthermore, *in vivo* inhibition of androgen production using a CYP17 (the enzyme that converts progestins to androgens) inhibitor markedly reduced hCG-induced oocyte maturation and significantly delayed ovulation (White et al., 2005). Together with the aforementioned *in vitro* studies, these observations indicate that androgens rather than progesterone are the true primary physiologic mediators of oocyte maturation.

Xenopus oocytes also express high levels of CYP17 (Lutz et al., 2001; Mulner et al., 1978; Thibier-Fouchet et al., 1976; Yang et al., 2003). In fact, nearly all CYP17 in the frog ovary is localized to the oocytes rather than follicular cells (Yang et al., 2003), suggesting an unusual paradigm whereby oocytes are regulating production of the steroid that then promotes its own maturation. Furthermore, expression of CYP17 in oocytes means that addition of progesterone to oocytes *in vitro* actually results in the presence of two equally potent promoters of oocyte maturation: progesterone and androstenedione.

Interestingly, no matter which steroid hormone is used, steroid-induced maturation occurs independent of transcription in *Xenopus laevis* because: (1) very little transcription occurs during the maturation process; (2) addition of transcriptional inhibitors, such as actinomycin D, has no effect on steroid-mediated maturation *in vitro*; and (3) removal of nuclei from oocytes has no effect on steroid-triggered cytoplasmic signals associated with maturation. In the past, this fact may have prompted many researchers to rule out the possibility that steroids were working through the classical steroid receptors. However, the relatively recent discovery of nongenomic steroid-induced signals of the classical nuclear/cytoplasmic steroid receptors, as indicated by estrogen-induced activation of MAPK and endothelial nitric oxide synthase in breast and endothelial cells (Razandi et al., 2002; Shaul, 2002; Simoncini et al., 2002) and estrogen and androgen-mediated anti-

apoptotic signals in bone cells (Kousteni et al., 2001; Kousteni et al., 2002) have reinforced the possibility that steroids could promote oocyte maturation through classical receptors.

Over the last decade, much research has been devoted to identifying the receptor(s) responsible for mediating oocyte maturation. Classical *Xenopus* androgen receptor (AR) and PR have been cloned from oocytes and overexpression and antisense experiments evaluating the result of receptor level modulation have been conducted (Lutz et al., 2001; Maller, 2001). Biochemical studies of the classical *Xenopus* AR indicated the androgen receptor mediates androgen-induced maturation in the frog. Elimination of endogenous AR by RNA interference specifically reduced androstenedione-mediated maturation *in vitro*. Also, knockdown of AR expression or treatment with the AR antagonist flutamide markedly reduced testosterone-mediated maturation (Lutz et al., 2001; Lutz et al., 2003).

Studies in which the levels of PR were manipulated have also been conducted, but these studies demonstrated only small changes in the ability of progesterone to induce maturation. Such modest changes could be explained by the fact that AR may have actually mediated the progesterone signals due to the fact that progesterone can be metabolized to androgens by the oocyte, and progesterone is capable of binding the *Xenopus* AR in oocytes (Evaul et al., 2007; Yang et al., 2003).

Taken together, these data again show the excellent role *Xenopus laevis* plays as an animal model: since transcription plays no role in the meiotic process, steroid-triggered *Xenopus* oocyte maturation provides an ideal physiologic model for studying transcription-independent, or nongenomic, steroid signaling (Maller and Krebs, 1980).

In sum, when androgens bind to their respective androgen receptors, they disrupt the inhibitory G protein signaling present in the oocyte, leading eventually to meiotic progression.

The Nongenomic Signaling Pathway

How does a steroid-triggered decrease in cAMP levels correspond to oocyte maturation? The answer lies within the complex signal transduction network following the disruption of the constitutive G protein signaling and subsequent activation of a kinase cascade.

Briefly, once cAMP is reduced, the downstream kinases are activated (Fig. 1-5), leading to GVBD (Ferrell, 1999a; Maller and Krebs, 1980). At the top of this cascade is MOS, an oocyte-specific mitogen-activated protein kinase (MEK) similar to Raf. Increased polyadenylyation of *Mos* mRNA leads to increased MOS protein expression. MOS then activates MEK1 (Resing et al., 1995), which in turn activates extracellular signaling-regulated kinase 2 (ERK2, or p42 MAPK), and finally cyclin-dependent kinase 1 (CDK1), and

thus activating the MPF and meiotic resumption (Castro et al., 2001; Howard et al., 1999; Nebreda et al., 1995).

Although cAMP levels decline rapidly after triggering maturation, there is a significant delay, on the order of hours, before maturation is complete suggesting a significant number of signals are activated downstream of cAMP changes.

Some studies have proposed that a decrease in cAMP levels may lead to reduced activity of a cAMP-dependent protein kinase, PKA. Previous work has suggested that a decrease in PKA activity may be necessary for steroid-induced maturation since injection of active catalytic subunits of PKA blocks steroid-induced maturation and PKA inhibitors can induce spontaneous maturation (Maller and Krebs, 1977).

Interestingly, molecules that anchor PKA regulatory subunits to specific locations within the oocyte, such as AKAPs, seem to be crucial for maintenance of meiotic arrest (Brown et al., 2002; Kovo et al., 2006; Kovo et al., 2002; Newhall et al., 2006). AKAPs are temporally and spatially regulated during meiosis and may be important for maturation since they could potentially target cAMP responsive signals within the oocyte.

These AKAP-bound PKA molecules then activate further downstream kinases leading eventually to the activation of MPF, and subsequent resumption of meiosis.

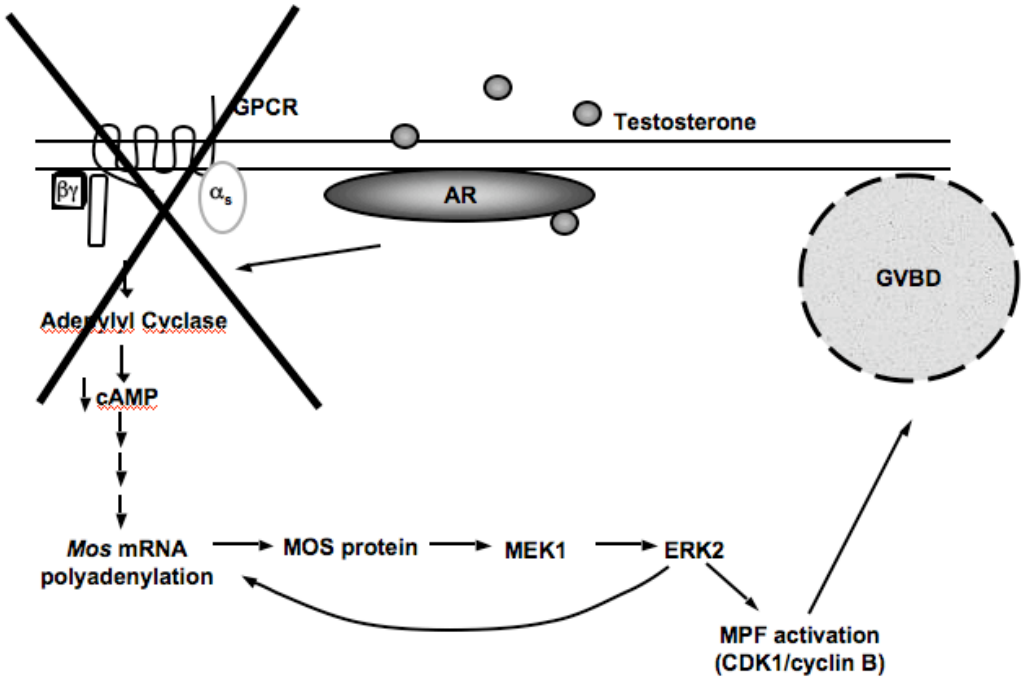

Figure 1-5: Overview of the Oocyte Maturation Pathway. The mechanisms that maintain meiotic arrest and the signals thought to be activated upon release of these signals are listed. When the constitutive G protein signaling is disrupted upon binding of steroids, cAMP levels are reduced which eventually leads to *Mos* mRNA polyadenylation. This polyadenylation serves as a translational regulation to generate MOS protein, which acts upon MEK1, which activates p42 MAPK/ERK2. ERK2 then activates the MPF, thus causing germinal vesicle breakdown, and now oocyte maturation has occurred.

Conclusions

Understanding the complex processes that regulate oocyte maturation allows for further insight into normal and abnormal female fertility. There are still many unanswered questions in this field, and further ongoing research is necessary to understand the processes that are occurring, both at a physiological and molecular level. Therefore, ongoing research will likely be focused on understanding what other proteins may be required for meiotic arrest and the subsequent progression of meiosis after stimulation by gonadotropins-induced signals to better understand the larger picture of signal that are activated during maturation.

Chapter 2

GPR3 is a Novel Constitutively Active G-Protein Coupled Receptor

Introduction

Oocytes are held in meiotic arrest in prophase I until ovulation when gonadotropins trigger a subpopulation of oocytes to resume meiosis in a process termed "maturation." Meiotic arrest is maintained through a mechanism whereby constitutive cAMP production exceeds phosphodiesterase-mediated degradation, leading to elevated intracellular cAMP. A major goal in the field of oocyte maturation has been to identify and characterize potential G protein-coupled receptors (GPCRs) that could be stimulating this inhibitory G protein signaling. The question as to what proteins promote this constitutive $G\alpha_s$ and $G_{\beta\gamma}$ to maintain meiotic arrest has recently been addressed, and has implicated a constitutively activated G protein-coupled receptor, GPR3 (Deng et al., 2008; Mehlmann, 2005; Mehlmann et al., 2004; Rios-Cardona et al., 2008).

G-protein coupled receptor 3, or GPR3, was found in an expressed sequenced tag (EST) library derived from a cDNA library of prophase-arrested mouse oocytes. There are both human and *Xenopus* orthologs of GPR3. *Gpr3* RNA was found predominantly in the ovary, testis, and brain, and at very low levels in the kidney and lung. It was shown to stimulate elevated levels of cAMP in a variety of somatic cells. Data from a GPR3

knockout mouse showed the presence of spontaneous oocyte maturation in *Gpr3-/-* antral follicles. In addition, female *Gpr3-/-* null mice had smaller than normal litter sizes, with premature ovarian failure. However, these mice were still fertile, and anywhere from 10% to 30% of the antral oocytes in *Gpr3-/-* null mice still remain in meiotic arrest. Furthermore, similar numbers of oocytes in wild-type mice remained in meiotic arrest after being injected with short interfering RNAs (siRNAs) directed against GPR3 mRNA (Mehlmann et al., 2004).

Taken together, these observations confirm that although GPR3 is important, it is neither essential nor is it the only player in the complex signaling processes that regulate oocyte maturation.

In fact, a novel family of $G\alpha_s$-coupled receptors that includes both GPRs 3 and 12 has been shown to participate in maintaining meiotic arrest in mammalian oocytes (Eggerickx et al., 1995; Freudzon et al., 2005; Hinckley et al., 2005; Mehlmann et al., 2004). These proteins have been shown as orphan receptors that appear to constitutively increase cAMP levels when overexpressed in a variety of cells. Unfortunately, direct evaluation of the effect of GPR3 on intracellular signals that accompany maturation, including changes in cAMP levels, activation of protein kinases, and detection of receptor on the cell surface, have been difficult due to the limited numbers of mouse oocytes that can be cleanly isolated and examined.

To study the signaling and biological properties of GPR3 in a more amenable model of meiosis, we examined the role of GPR3 in regulating *X. laevis* oocyte maturation. We generated a *Xenopus laevis* GPR3 clone that was similar in homology to the murine GPR3. Next, we wanted to determine whether functionally our XGPR3 protein behaved as we would expect: would XPGR3 increase levels of cAMP regardless of what cell type it was introduced into? Would XGPR3 be the correct size? Would the FLAG-tag that we engineered into the amino terminus of XGPR3 affect its function?

The aim of these studies was to determine the answers to these questions. We overexpressed our XGPR3 in both COS cells and oocytes, and then looked for several indicators that XGPR3 was functioning properly: cAMP levels, maturation assay results, and MAPK signaling activation levels.

For all experiments involving *Xenopus* oocytes, it is important to note that denuded oocytes refers to oocytes that have been isolated using collagenase methods and essentially have no follicular cells surrounding the oocyte, and manually defolliculated (follicle-enclosed) oocytes are defined as oocytes that were separated into singular follicle-oocyte units via manual forcep methods, and still are encased with a layer of follicular cells.

Both denuded and manually defolliculated oocytes are naturally held at meiotic arrest and require steroids to trigger maturation. Denuded oocytes no longer respond to hCG treatment, because hCG acts upon the follicular cells surrounding the oocyte, and thus only mature in response to

testosterone treatment. Meiotic resumption is triggered in response to hCG treatment in manually defolliculated oocytes due to the presence of follicular cells surrounding the oocyte (Rasar and Hammes, 2006; White et al., 2005).

Materials and Methods

Cloning of the Xenopus GPR3

The XGPR3 coding sequence was isolated by nested PCR techniques using the following primers: forward (outside): GACAGAGCTGGAGACGGAGGA; forward (inside), CGGATCCAGCCATGCTTCACCAGCCCTGCAGTC; reverse (outside), CTCCAAACATACAGTCCCGGA; reverse (inside), GGCGGCCGCGAATTCTTATACGTCACTGGAAGTTCT. The cDNA encoding XGPR3 was ligated into the mammalian expression vector pEF2-FlmugR-DNM, which contains the sequence encoding an amino-terminal FLAG tag (a generous gift from Mark Kahn, University of Pennsylvania, Philadelphia, PA). We then used the forward primer CGCCCGGGCCAGCCATGCGACCGACGCTGCTGTGGTCG to insert the complete N-terminal FLAG-tagged XGPR3 cDNA clone into the pGEM-HE vector (from L. Jan, University of California, San Francisco, CA). During the course of these studies, a clone matching ours, designated ABS19626, was submitted by others to the GenBank database and is designated GPRx.

Oocyte Preparation

All frogs were treated in accordance with accepted NIH and University of Texas standards of humane animal care. Oocytes were prepared using two different methods as indicated in the figure legends. To isolate denuded oocytes, ovaries were harvested from female *X. laevis* (Nasco, Fort Atkinson, WI) and treated as described elsewhere (Lutz et al., 2000). Briefly, follicle cells were removed by incubation of ovaries in 0.8 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN) in modified Barth's solution (MBSH) without Ca^{2+} for 3-4 h. Oocytes were then washed and incubated overnight at 16 °C in MBSH containing 1 mg/mL Ficoll, 1 mg/mL BSA, 100 U/mL penicillin, and 0.1 U/mL streptomycin. Testosterone (Steraloids, Newport, RI)-induced maturation assays were performed on stage V/VI oocytes from each preparation to determine sensitivity to steroid, because this varies considerably with each batch of oocytes. Maturation was scored as germinal vesicle breakdown, which was visualized as a white spot on the animal pole of the oocyte. Twenty oocytes were used for each data point in all experiments.

In the second method, stage V/VI oocytes were manually defolliculated from female *X. laevis*, injected with oligodeoxynucleotides or cRNA, as discussed in the appropriate materials and methods section and in the figure legends, and incubated in oocyte culture medium (OCM) as described

elsewhere (Zuck et al., 1998). Oocytes were then stimulated for maturation using either testosterone or human chorionic gonadotropin (hCG) (Intervet, Millsboro, DE). Maturation was scored as GVBD.

RNA Synthesis and Injections

The pGEM-HE plasmid containing the FLAG-tagged XGPR3 cDNA sequence was linearized with AatII or SphI. Capped cRNA was transcribed *in vitro* with T7 RNA polymerase according to the manufacturer's protocol (Ambion, Inc., Austin, TX). RNA was suspended in injection buffer (10 mM HEPES, pH 7.4) and Stage V/VI oocytes were injected with the amounts of cRNA indicated in the figure legends using a Drummond or Harvard Apparatus automatic injector.

After all injections, oocytes were incubated at least 36-48 h before any assay was begun.

Testosterone-Mediated Maturation Assays

Maturation assays were conducted by incubating 20 oocytes per condition with the indicated concentration of testosterone (Steraloids) or ethanol in MBSH or OCM for 12-16 h, at which time oocytes were scored for germinal vesicle breakdown. Dilutions were performed such that ethanol concentration remained at 0.1%.

Western Blots

Oocytes were incubated with steroid or vehicle, permeabilized in 20 μL/oocyte lysis buffer (1% Triton X-100; 50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 2 mM EDTA; 2 mM NaF; 0.5 mM sodium vanadate; 100 μg/mL phenylmethylsulphonyl fluoride), and microcentrifuged at 14,000 x g for 10 min to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2X sodium dodecyl sulfate sample buffer (Lutz et al., 2000). The equivalent of 0.5 oocytes was loaded in each lane for SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA), blocked in 5% Tris-buffered saline-Tween 20-milk for 1 h, and then incubated with primary antibody overnight at 4 °C (1:5000 for anti-FLAG M1, 1:5000 for anti-FLAG M2, 1:2000 for anti-phospho-p42 and anti-total p42, 1:2000 for anti-phospho- and anti-total CDK1). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and signal was detected by ECL Plus (Amersham Biosciences, Piscataway, NJ). Anti-FLAG M1 and M2 were obtained from Sigma-Aldrich (St. Louis, MO), anti-phospho-CDC2, and anti-total-CDC2, anti-phospho p42/p44 MAPK, and anti-total p42/p44 MAPK were from Cell Signaling Technology (Beverly, MA).

Membrane Preparations

Crude oocyte membrane and cytoplasmic fractions were prepared as described elsewhere (Lutz et al., 2000). For the Western blots using these fractions, approximately 0.5 oocyte equivalent was added to each lane.

Cell Culture and Transfection

COS-7 cells (American Type Culture Collection, Manassas, VA) were maintained at 37 °C in DMEM (Fisher Scientific, Pittsburgh, PA) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA). Transfections were performed in six-well plates using Lipofectamine reagent (Invitrogen). Each well was transfected with 1 μg of total DNA as indicated. After 48 h incubation in 10% serum, cells were washed two times with ice-cold PBS (pH 7.4), and permeabilized in 300 μL of oocyte lysis buffer. Wells were scraped, cell debris was removed by centrifugation, and the cleared lysates were mixed 1:1 with 2X sodium dodecyl sulfate sample buffer and immunoblotted as described.

cAMP Competitive ELISA

COS-7 cells were transfected with XGPR3 plasmid or vehicle, and oocytes were injected with XGPR3 cRNA or vehicle as described previously. The cells and oocytes were treated with 0.1 M HCl 48 h after transfection/injection to prevent endogenous phosphodiesterase activity and microcentrifuged at 0.6 rcf to remove debris. The samples were then added to

a 96-well plate, and cAMP levels were measured using a colorimetric assay per manufacturer's instructions (Endogen, Inc., Woburn, MA).

Cell Surface Expression Assay

COS-7 cells were transfected with XGPR3 plasmid, and oocytes were injected with XGPR3 cRNA as described. Cells were washed 48 h after transfection/injection and then incubated for 1 h with the M2 antibody at 1:1000 dilution in DMEM (cells) or the M1 antibody at 1:1000 dilution in modified Barth's solution (oocytes). After several washes with PBS, the cells were incubated with an anti-mouse secondary antibody (Bio-Rad Laboratories, Inc., Hercules, CA) at 1:2000. To assess the relative values of GPR3 cell surface expression levels, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), also known as ABTS solution (Pierce Chemical Co., Rockford, IL) was added, and absorption was measured by a spectrophotometer (BioTek Instruments, Inc. Winooski, VT) at 405 nm.

^{45}Ca Efflux Assay

Oocytes were injected with either mock cRNA or cRNA encoding PAR1 (20 ng) or XGPR3 (20 ng). ^{45}Ca efflux assays were performed after 48 h as described previously (Nanevicz et al., 1996). Oocytes were stimulated for the indicated times with 10.6 nM α-thrombin.

Results

Cloning of *Xenopus* GPR3

A cDNA encoding a 340-amino acid isoform of *X. laevis* GPR3 protein was cloned from oocyte RNA using reverse-transcription and nested PCR techniques. A FLAG epitope tag at the amino terminus of XGPR3 was added to allow for detection of the protein (Fig. 2-1). XGPR3 (GenBank accession no. ABS19626; also known as GPRx) shares approximately 42% overall identity and 65% homology with murine GPR3 and GPR12 (Fig. 2-1). Most of the differences in sequence are located in the 50-amino acid extracellular amino-terminal portion of the protein. A thorough search of all GenBank and *Xenopus* (accessed via Xenbase) databases revealed no other similar *X. laevis* G protein-couple receptors. Furthermore, the first 50 matches on GenBank homology searches are either GPR3 or GPR12. These results indicate that the clone identified encodes the only currently known member of the GPR3/12 family in *X. laevis*.

Overexpression of XGPR3

The effects of XGPR3 on steroid-triggered signaling in *X. laevis* oocytes were explored by first ensuring that overexpression of FLAG-XGPR3 allows for detection via Western blots and cell surface expression assays. Injection of cRNA encoding FLAG-tagged XGPR3 into *Xenopus* oocytes

resulted in substantial cell surface expression of protein, as detected using an anti-FLAG antibody in a cell surface ELISA (Fig. 2-2A). Membrane expression of XGPR3 was confirmed by Western blot using the anti-FLAG antibody where protein was present in total membrane, but not cytosolic, oocyte fractions (Fig. 2-2B).

Overexpressed murine GPR3 has been shown to increase cAMP levels in somatic cells and oocytes, and to block oocyte maturation (Freudzon et al., 2005; Hinckley et al., 2005; Mehlmann et al., 2004). Transfection of a cDNA encoding XGPR3 into COS-7 cells resulted in significant cell surface expression of XGPR3 (data not shown), as well as detection of an approximately 39 kD band on Western blots (Fig. 2-3A). To further confirm similar results in *Xenopus laevis*, we also injected XGPR3 cRNA *Xenopus* oocytes, and the presence of a XGPR3 band only in XGPR3-injected oocytes was detected (Fig. 2-3B) via immunoblot.

To confirm that XGPR3 would similarly increase intracellular cAMP, we also measured cAMP levels in both COS cells and Xenopus oocytes transfected/injected with buffer or cDNA/cRNA encoding XGPR3. Cells/oocytes overexpressing XGPR3 contained significantly more intracellular cAMP relative to mock-injected oocytes (Fig. 2-3 A & B).

Overexpressed XGPR3 Inhibits Steroid-Mediated Maturation

Because overexpressed XGPR3 increased intracellular cAMP, it would be predicted to inhibit steroid-triggered oocyte maturation. Indeed, overexpression of XGPR3 in denuded frog oocytes by cRNA injection almost completely abrogated testosterone-induced maturation as compared with mock-injected control oocytes (Fig. 2-4A). XGPR3 also completely abrogated steroid-triggered activation of MAPK, an important signal associated with oocyte maturation, as evidenced by the loss of testosterone-induced phosphorylation of p42 protein (Fig. 2-4B). XGPR3 also suppressed steroid-mediated activation of CDK1/cdc2, an important cyclin that promotes meiotic progression and is dephosphorylated during activation. As shown in Fig. 2-4B, overexpression of XGPR3 blocked testosterone-induced dephosphorylation of the CDK1 protein. Finally, overexpression of XGPR3 in follicle-enclosed, manually defolliculated oocytes similarly inhibited both testosterone- and hCG-mediated oocyte maturation (Fig. 2-4C).

Together, these data suggest that XGPR3-mediated increases in intracellular cAMP under overexpression conditions are sufficient to maintain Xenopus oocytes in meiotic arrest, even in the presence of amounts of steroid or hCG that would normally initiate maturation.

Notably, overexpressed XGPR3 lacking the FLAG tag still stimulated cAMP production in both tissue culture samples and oocytes and almost completely suppressed testosterone-induced activation of MAPK, CDK1, and

maturation (data not shown). This confirms that the FLAG tag was not significantly contributing to the constitutive activity of the exogenous XGPR3.

XGPR3 Signals Through $G_{\beta\gamma}$

Based on the fact that overexpression of XGPR3 increased cAMP levels in both COS cells and oocytes suggests that, as described in somatic cells and mouse oocytes, overexpressed XGPR3 might be stimulating $G\alpha_s$ to promote adenylyl cyclase activity.

However, substantial evidence demonstrates that, in *Xenopus* oocytes, both $G\alpha_s$ and $G_{\beta\gamma}$ act in concert to elevate intracellular cAMP levels and maintain meiotic arrest (Lutz et al., 2001; Sheng et al., 2005; Sheng et al., 2001). Furthermore, overexpression of just the carboxyl tail of G protein coupled kinase (GRK1) peptide in frog oocytes is known to enhance steroid-triggered oocyte maturation by suppressing the constitutive $G_{\beta\gamma}$ signaling that holds oocytes in meiotic arrest (Lutz et al., 2000; Sheng et al., 2001). Therefore, to determine whether $G_{\beta\gamma}$ might mediate part of the effects of XGPR3 on intracellular cAMP, oocytes were injected with cRNAs encoding both XGPR3 and the $G_{\beta\gamma}$ scavenger carboxyl-GRK1 peptide. Interestingly, the carboxyl-GRK1 peptide had minimal effect on cAMP levels in mock-injected oocytes but markedly attenuated the XGPR3-mediated rise in intracellular cAMP (Fig. 2-5A). Furthermore, the carboxyl-GRK1 peptide significantly enhanced testosterone-mediated oocyte maturation in both mock-injected

and XGPR3-expressing cells (Fig. 2-5B). XGPR3 expression was not affected by co-expression of the GRK peptide (Fig. 2-5A, *inset*). These results suggest that, unlike in mouse oocytes (Gill and Hammes, 2007), XGPR3 might, in fact, require $G_{\beta\gamma}$ signaling to fully stimulate adenylyl cyclase and elevate cAMP levels in Xenopus oocytes.

After showing that XGPR3 signals through $G_{\beta\gamma}$ in G_s-linked pathways by stimulating the activity of adenylyl cyclase to elevate high levels of cAMP, we wondered if XGPR3 also stimulated other G protein pathways. Other G protein-coupled receptors have been capable of activating multiple second messengers. For example, the M2 muscarinic receptor is coupled to both adenylyl cyclase and phosphoinositide turnover. We performed a calcium efflux assay to assess whether XGPR3 operated via a phospholipase C pathway (PLC). We compared our levels of activation to a known G_q receptor, protease activated receptor 1 (PAR1) (Hammes and Coughlin, 1999). Based on the results we saw (Fig. 2-5), it does not seem that XGPR3 is signaling via a G_q mechanism, but further studies need to be performed to confirm this.

Discussion

As discussed previously, *Xenopus laevis* serves as a superb physiologic model for studying transcription-independent steroid signaling. Therefore we decided to use this more amenable animal model in order to better study this

novel G-protein coupled receptor implicated in oocyte maturation. We cloned a *Xenopus* isoform of GPR3 using *X. laevis* oocyte mRNA, demonstrating the conservation of GPR3 expression in lower vertebrate oocytes.

Notably, the cloned X. laevis GPR3 bears similar homology to both mouse GPR3 and GPR12, both of which appear to promote adenylyl cyclase activity in the absence of ligand. In fact, these receptors seems to have differential effects in mice versus rats, because injection of morpholino oligonucleotides directed against GPR3, but not GPR12, into isolated mouse oocytes held in meiotic arrest with hypoxanthine resulted in increased maturation, whereas injection of morpholino oligonucleotides directed against GPR12, but not GPR3, into isolated rat oocytes held in meiotic arrest with hypoxanthine enhanced maturation.

For the studies described here, the *X. laevis* clone was arbitrarily designated XGPR3; however, although none to date have been found in existing sequence databases, *Xenopus* oocytes may also express other still unknown members of this constitutively active G protein-coupled receptor family.

We demonstrated that overexpressed XGPR3 localized to the cell surface and inhibited maturation induced by both gonadotropin and the direct physiological trigger, testosterone. Furthermore, we showed that XGPR3 increased intra-oocyte cAMP in isolated oocytes and inhibited kinase signals associated with maturation, including MAPK and CDK1. Importantly,

overexpressed XGPR3 increased intracellular oocyte cAMP in the absence of any known ligand, confirming that XGPR3 increased intra-oocyte cAMP in the absence of any known ligand, confirming that XGPR3 likely inhibits steroid-triggered maturation by constitutively increasing intracellular cAMP and operating via an adenylyl cyclase-mediated pathway.

Previous research has shown that frog oocyte maturation seems to involve $G\alpha_s$ pathways (Gallo et al., 1995) and does not utilize $G\alpha_i$ signaling pathways (Lutz et al., 2003). We show here that XGPR3 signals through $G_{\beta\gamma}$ to mediate its increase of cAMP levels within the oocytes. Overexpression of XGPR3 constitutively elevates cAMP levels suggests that XGPR3 is potentially also signaling via $G\alpha_s$ to activate adenylyl cyclase. However, further work needs to be performed to confirm whether $G\alpha_s$ is indeed directly involved XGPR3's ability in stimulating adenylyl cyclase activity in *Xenopus* oocytes.

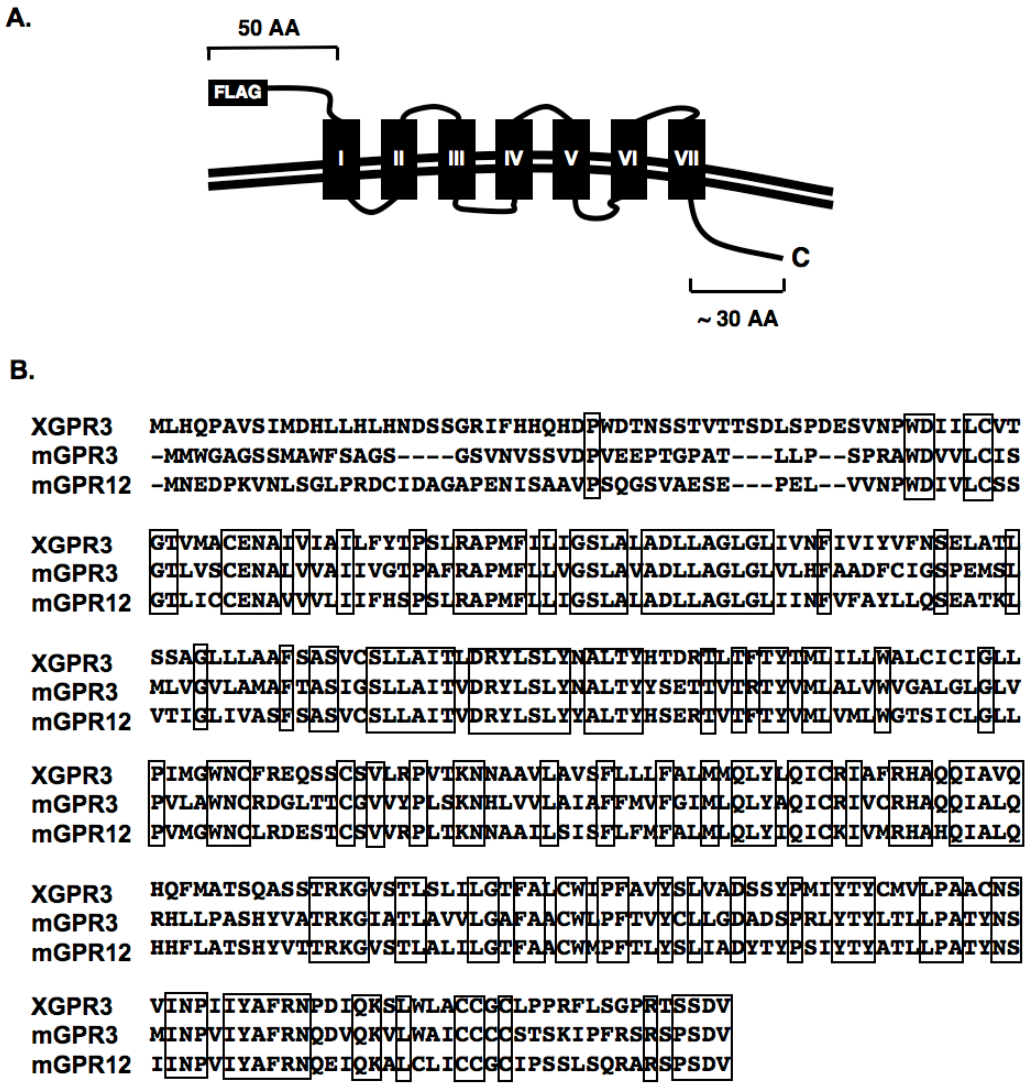

Fig. 2-1: Schematic and sequence of the *Xenopus* GPR3 (XGPR3). **A)** XGPR3 is a seven-transmembrane domain protein and approximately 340 amino acids (AAs) in length with a predicted 30 AA carboxyl-terminal intracellular tail and 50 AA amino-terminal extracellular domain. For these studies, an amino-terminal FLAG sequence has been engineered. **B)** Sequence comparison of XGPR3 with the mouse GPR3 and GPR12 (mGPR3 and mGPR12). Amino acids that are identical between all three proteins (42%) are in boxes. XGPR3 shares 49% and 54% identity with mGPR3 and mGPR12, respectively. XGPR3 shares approximately 70% sequence similarity with both mouse receptors.

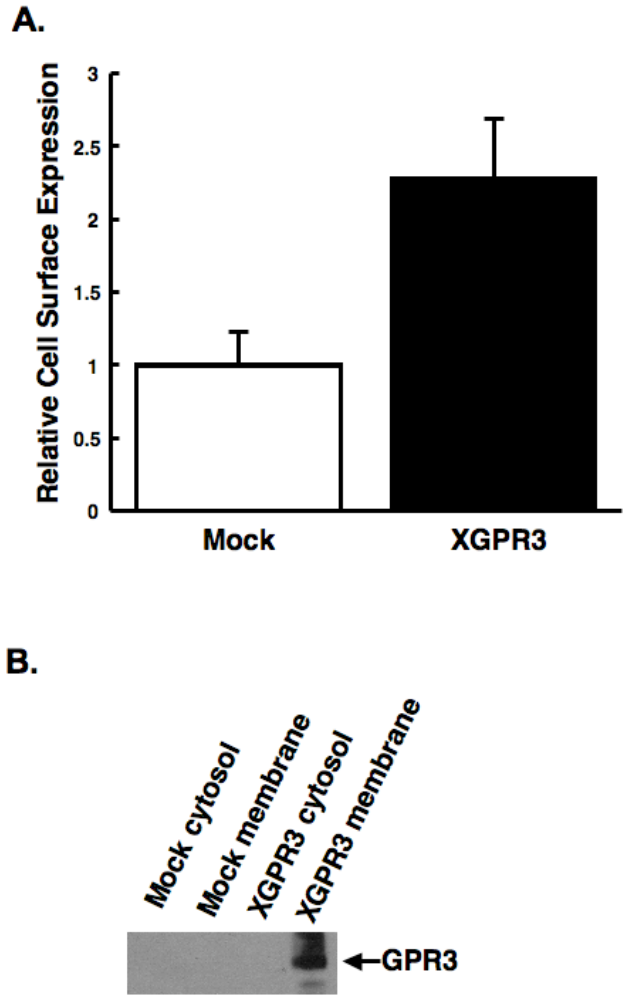

Fig. 2-2: Overexpression of XGPR3 in Oocytes. A) Oocytes injected with cRNA encoding XGPR3 expressed high levels of XGPR3 on the cell surface relative to mock-injected oocytes as determined by an anti-FLAG ELISA assay on fixed, intact oocytes. Values are the average ± S.D. (n=3). **B)** XGPR3 was expressed in membrane, but not cytoplasmic, oocyte fractions, as detected by Western blot using an anti-FLAG antibody. All studies were performed at least three times with similar results.

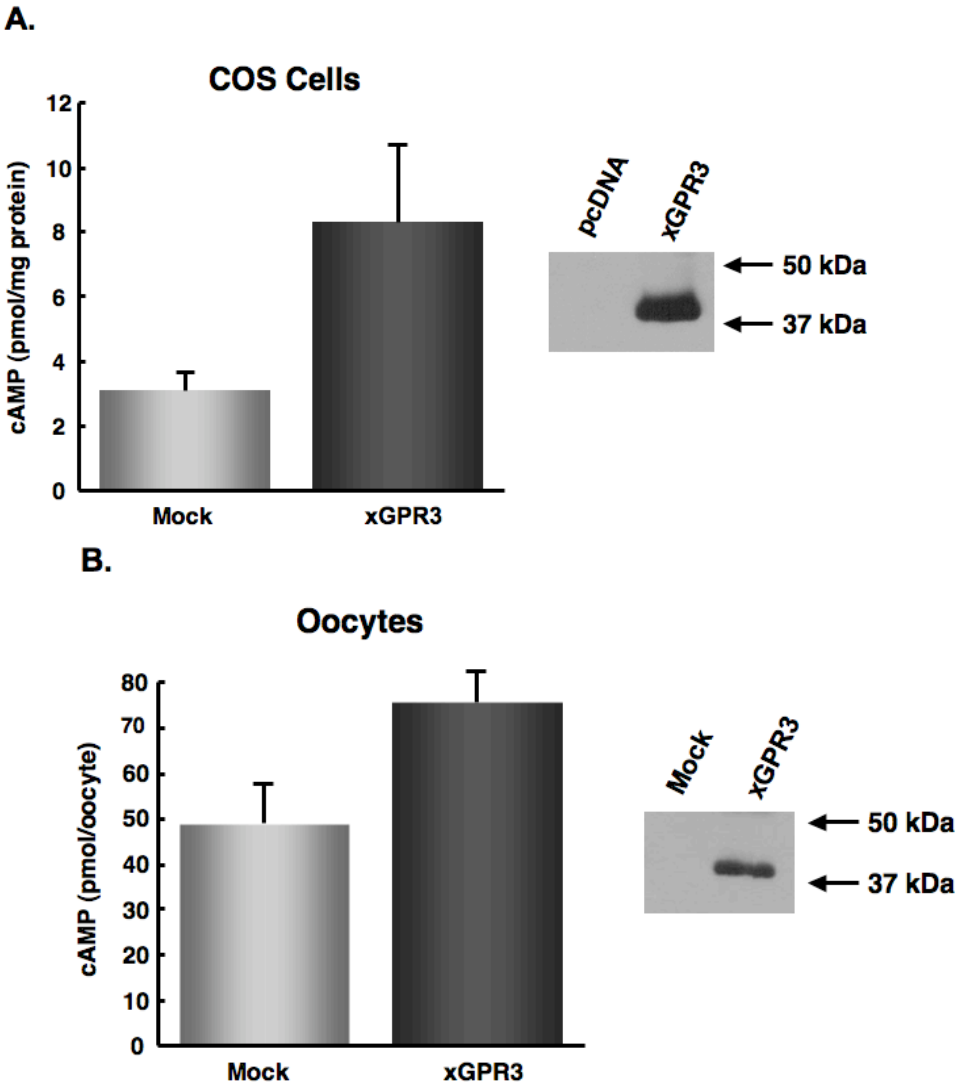

Figure 2-3: XGPR3 Increases cAMP Levels. A) Transfections were carried out in COS-7 cells as described, followed by using the cell lysates for Western blot analysis and a cAMP competitive ELISA. XGPR3 overexpressing cells had substantial elevated levels of cAMP compared to mock-transfected cells. **B)** Oocytes were injected as described, and oocyte lysates were used to confirm expression of XGPR3 via Western blot. A cAMP assay was also performed 48 h after the injection. XGPR3 again increased cAMP levels compared to mock-injected oocytes. All studies were performed at least three times with similar results.

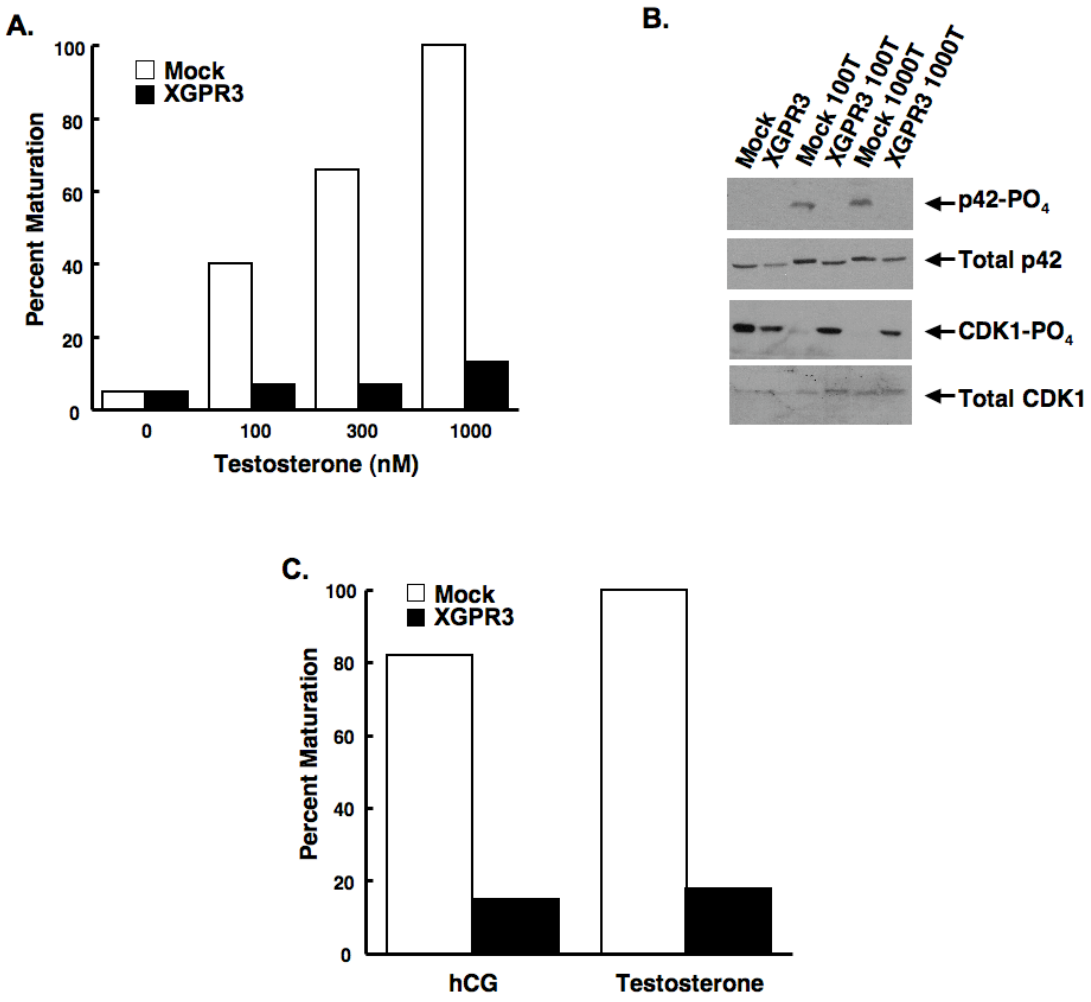

Figure 2-4: Overexpression of XGPR3 in *Xenopus* Oocytes Inhibits Maturation. A & B) Overexpression of XGPR3 markedly inhibited testosterone-mediated oocyte maturation **(A)** and activation of MAPK and CDK1 **(B)** in denuded oocytes. Maturation was determined by examining oocytes for Germinal Vesicle Breakdown (GVBD) after overnight incubation with testosterone. After six hours with testosterone, MAPK activation was detected by Western blot for increased phosphorylation of p42, while CDK1 activation was determined by Western blot for decreased phosphorylation of CDK1. Total p42 and MAPK expression in the same samples are also shown. **C)** Overexpression of XGPR3 markedly inhibited hCG (150 units/mL)- and testosterone (500 nM)-induced oocyte maturation in follicle-enclosed oocytes. All studies were performed at least three times with similar results.

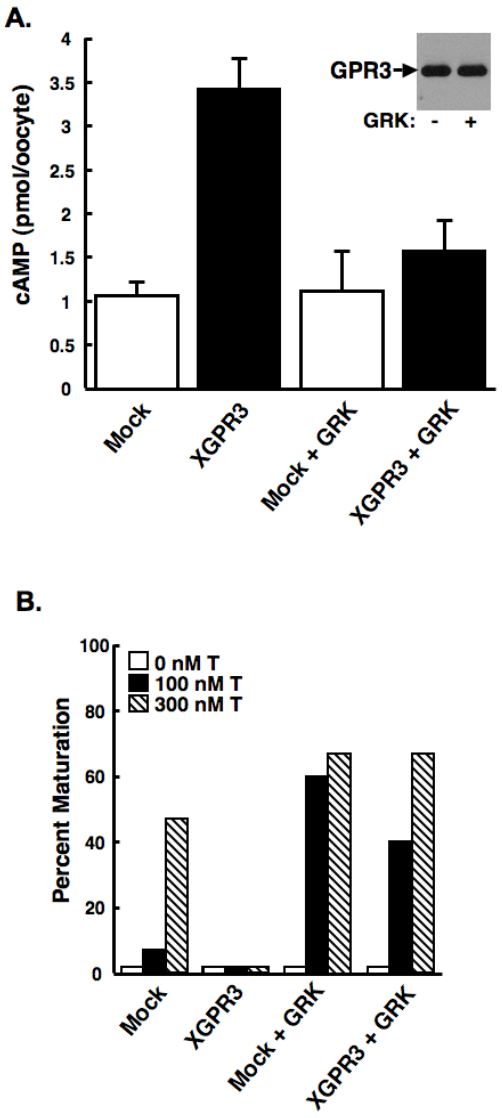

Figure 2-5: XGPR3 Signals Through $G_{\beta\gamma}$. A) Co-expression of the carboxyl tail of GRK1 (GRK) abrogated this XGPR3-mediated increase in cAMP, suggesting that $G_{\beta\gamma}$ at least partially mediates XGPR3 signaling. Values are the average ± S.D. (n=5). Equal amounts of XGPR3 were expressed in both with and without GRK co-expression (inset). **B)** Overexpression of the GRK peptide enhanced testosterone-mediated oocyte maturation in both mock-injected oocytes and oocytes over-expressing XGPR3. T = testosterone

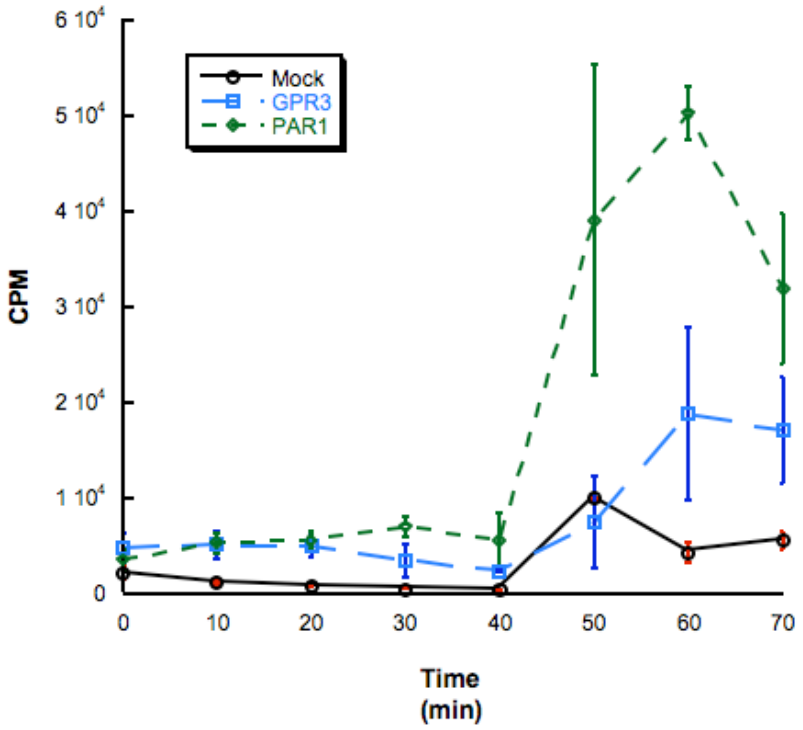

Figure 2-6: GPR3 Does Not Constitutively Signal Via Phospholipase C Mechanisms. Oocytes were injected with cRNA encoding mock, XGPR3, or PAR1 constructs. Oocytes were incubated with ^{45}Ca followed by treatment for the indicated times with 10.6 nM α-thrombin as agonist for PAR1- and mock-injected oocytes, and with 0.8 mg/mL collagenase A for XGPR3-injected oocytes and measured as counts per min (CPM). Initial treatment started at 40 min. As confirmed, PAR1 receptor is known to activate G_q pathways, however XGPR3 does not seem to functioning via these G_q pathways in comparison to the levels seen when PAR1 is stimulated with its agonist.

Chapter Three

Knockdown of Endogenous XGPR3 Expression Enhances Steroid- and hCG-Mediated Signaling and Maturation

Introduction

If XGPR3 plays a physiological role in repressing maturation in response to endogenous signals, then its removal would be predicted to enhance the maturation response to these triggers. The lack of anti-GPR3 antibodies precluded demonstrating the loss of endogenous XGPR3 protein expression. However, we utilized antisense phosphothiorated oligodeoxynucleotides in conjunction with quantitative real-time PCR to determine the importance of endogenous XGPR3 in *Xenopus* oocytes.

Materials and Methods

Oocyte Preparation

All frogs were treated in accordance with accepted NIH and University of Texas standards of humane animal care. Oocytes were prepared using two different methods as indicated in the figure legends. To isolate denuded oocytes, ovaries were harvested from female *X. laevis* (Nasco, Fort Atkinson, WI) and treated as described elsewhere (Lutz et al., 2000). Briefly, follicle

cells were removed by incubation of ovaries in 0.8 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN) in modified Barth's solution (MBSH) without Ca^{2+} for 3-4 h. Oocytes were then washed and incubated overnight at 16 °C in MBSH containing 1 mg/mL Ficoll, 1 mg/mL BSA, 100 U/mL penicillin, and 0.1 U/mL streptomycin. Testosterone (Steraloids, Newport, RI)-induced maturation assays were performed on stage V/VI oocytes from each preparation to determine sensitivity to steroid, because this varies considerably with each batch of oocytes. Maturation was scored as germinal vesicle breakdown, which was visualized as a white spot on the animal pole of the oocyte. Twenty oocytes were used for each data point in all experiments.

In the second method, stage V/VI oocytes were manually defolliculated from female *X. laevis*, injected with oligodeoxynucleotides or cRNA, as discussed in the appropriate materials and methods section and in the figure legends, and incubated in oocyte culture medium (OCM) as described elsewhere (Zuck et al., 1998). Oocytes were then stimulated for maturation using either testosterone or human chorionic gonadotropin (hCG) (Intervet, Millsboro, DE). Maturation was scored as GVBD.

RNA Synthesis and Injections

The pGEM-HE plasmid containing the FLAG-tagged XGPR3 cDNA sequence was linearized with AatII or SphI. Capped cRNA was transcribed *in*

vitro with T7 RNA polymerase according to the manufacturer's protocol (Ambion, Inc., Austin, TX). RNA was suspended in injection buffer (10 mM HEPES, pH 7.4) or water, and Stage V/VI oocytes were injected using a Drummond or Harvard Apparatus automatic injector with the amounts of cRNA indicated in the figure legends. The sequences of the sense and antisense XGPR3 HPLC purified oligodeoxynucleotides were G*C*A*TATAGCAATGCTTCA*C*C*A and T*A*G*GGTGGCCAGTTC*A*C*T, respectively, with phosphothiorated bonds indicated by asterisks.

Oocytes were injected with oligodeoxynucleotides as indicated in the figure legends. For the rescue studies, XGPR3 cRNA was injected with the oligodeoxynucleotides as indicated. After all injections, oocytes were incubated at least 36-48 h before any assay was begun.

For injection of intraovarian oocytes, ovaries were harvested from adult, non-virgin female *X. laevis* that had previously laid eggs and then cut into pieces containing approximately 30-50 large oocytes. Intraovarian stage V/VI oocytes were injected with a mixture of either XGPR3 antisense oligodeoxynucleotide and the lineage marker fluorescein-lysine-dextran or water and fluorescein-lysine-dextran. The ovarian pieces were incubated at 18 °C in OCM for 4 d. After incubation, the lineage-labeled oocytes were manually defolliculated under a fluorescent microscope (Nikon SMZ1500; Nikon, Melville, NY). The oocytes were then stimulated with 150 units/mL hCG and scored for GBVD every 30 min at room temperature.

Testosterone-Mediated Maturation Assays

Maturation assays were conducted by incubating 20 oocytes per condition with the indicated concentration of testosterone (Steraloids) or ethanol in MBSH or OCM for 12-16 h, at which time oocytes were scored for germinal vesicle breakdown. Dilutions were performed such that ethanol concentration remained at 0.1%.

Western Blots

Oocytes were incubated with steroid or vehicle, permeabilized in 20 μL/oocyte lysis buffer (1% Triton X-100; 50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 2 mM EDTA; 2 mM NaF; 0.5 mM sodium vanadate; 100 μg/mL phenylmethylsulphonyl fluoride), and microcentrifuged at 14,000 x g for 10 min to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2X sodium dodecyl sulfate sample buffer (Lutz et al., 2000). The equivalent of 0.5 oocytes was loaded in each lane for SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA), blocked in 5% Tris-buffered saline-Tween 20-milk for 1 h, and then incubated with primary antibody overnight at 4 °C (1:5000 for anti-FLAG M1, 1:5000 for anti-FLAG M2, 1:2000 for anti-phospho-p42 and anti-total p42, 1:2000 for anti-phospho- and anti-total CDK1). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and signal was

detected by ECL Plus (Amersham Biosciences, Piscataway, NJ). Anti-FLAG M1 and M2 were obtained from Sigma-Aldrich (St. Louis, MO), anti-phospho-CDC2, and anti-total-CDC2, anti-phospho p42/p44 MAPK, and anti-total p42/p44 MAPK were from Cell Signaling Technology (Beverly, MA).

cAMP Competitive ELISA

COS-7 cells were transfected with XGPR3 plasmid or vehicle, and oocytes were injected with XGPR3 cRNA or vehicle as described previously. The cells and oocytes were treated with 0.1 M HCl 48 h after transfection/injection to prevent endogenous phosphodiesterase activity and microcentrifuged at 0.6 rcf to remove debris. The samples were then added to a 96-well plate, and cAMP levels were measured using a colorimetric assay per manufacturer's instructions (Endogen, Inc., Woburn, MA).

Analysis of Gene Expression using Real-time PCR

Real-time RT-PCR was performed using total RNA extraction of two oocytes as described elsewhere (Kofron et al., 2001). cDNA was synthesized using approximately one-sixth oocyte equivalent and oligo deoxythymidine primers. Quantitative real-time PCR and quantification were performed using the LightCycler System version 3.5 (Roche) as described previously (Standley et al., 2006). Relative quantitative expressive levels were calculated using a standard curve, generated by a dilution series of control oocyte cDNA.

Samples were normalized to a housekeeping gene, ornithine decarboxylase, to serve as a loading control.

The primer sequences for the XGPR3 gene are forward, 5'-CTGGGGCTCATTGTGAATTT-3' and reverse, 5'-GTGGTAGGTGAGGGCATTGT-3'. The ornithine decarboxylase primer sequences are forward, 5'-GCCATTGTGAAGACTCTCTCCATTC-3' and reverse, 5'-TTCGGGTGATTCCTTGCCAC-3' (Kofron et al., 2001).

Results

Reduction of Endogenous XGPR3 Expression in *X. laevis* Oocytes Enhanced Testosterone- and hCG-Mediated Intracellular Signaling and Maturation

Injection of antisense, but not sense, phosphothiorated oligodeoxynucleotides directed against mRNA encoding XGPR3 completely suppressed expression of exogenous FLAG-tagged XGPR3 protein in denuded oocytes (Fig. 3-1A). This result indicates that the antisense oligodeoxynucleotides were capable of specifically abrogating XGPR3 protein expression.

Injection of oocytes with the antisense oligodeoxynucleotides directed against mRNA encoding XGPR3 also reduced intracellular cAMP relative to oocytes injected with the sense oligodeoxynucleotide (Fig. 3-1B), confirming

that endogenous XGPR3 plays a role in stimulating cAMP production. Interestingly, although cAMP levels dropped significantly, these oocytes did not spontaneously mature.

Although these denuded oocytes injected with the antisense oligodeoxynucleotide did not spontaneously mature (even up to 72 h after injection), they became significantly more sensitive to testosterone-triggered maturation (Fig. 3-2A). Similarly, follicle-enclosed oocytes injected with the antisense oligodeoxynucleotide became more sensitive to testosterone-mediated maturation (Fig. 3-3A). Quantitative PCR confirmed that XGPR3 mRNA levels were significantly decreased in response to the injected antisense, but not sense, oligodeoxynucleotides (Figs. 3-2B & 3-3B). The ability of overexpressed XGPR3 to rescue the effects of the antisense oligodeoxynucleotides in both oocyte preparations (Fig. 3-2A & 3-3A), as well as the inability of a nonspecific sense oligodeoxynucleotide to enhance maturation, confirmed the specificity of the antisense-mediated depletion.

Finally, depletion of XGPR3 mRNA by injection of the antisense oligodeoxynucleotide into oocytes that were still enclosed in their follicles within pieces of cultured ovary (Fig. 3-4A) enhanced the rate of subsequent oocyte maturation mediated by hCG, the initial *in vivo* signal for maturation.

Together, these data suggest that endogenous XGPR3 plays at least a partial role in maintaining meiotic arrest in *Xenopus* oocytes.

Discussion

The introduction of antisense RNA into eukaryotic cells by microinjection or expression from antisense DNA constructs has been successfully tested as a strategy to specifically inhibit the expression of target genes in several model systems (Crowley et al., 1985; Rosenberg et al., 1985; Wormington, 1986). However, the presence of an RNA duplex unwinding activity in *Xenopus* early embryos have precluded the use of antisense RNA techniques in *Xenopus* (Bass and Weintraub, 1987). An alternative strategy employing antisense oligodeoxynucleotides has shown an extensive ability to inhibit gene expression in both eukaryotic cells and microinjected *Xenopus* oocytes. The studies show that the oligonucleotides operate primarily by forming a DNA-RNA duplex that directs RNase H cleavage of the mRNA (Minshull and Hunt, 1986). In our knockdown studies, we specifically utilized phosphothiorated sense and antisense oligodeoxynucleotides, an excellent means to knock down expression via RNase H mechanisms.

We demonstrated that reduction of endogenous XGPR3 expression using antisense oligodeoxynucleotides lowered intracellular cAMP and enhanced both gonadotropin- and testosterone-triggered oocyte maturation. These findings confirm the studies in GPR3 null mice (Ledent et al., 2005; Mehlmann et al., 2004) and demonstrate that, unlike in mice, endogenous XGPR3-mediated signaling plays a role in maintaining meiotic arrest in *Xenopus* oocytes even after they have been removed from the ovary.

Notably, despite significant reductions in XGPR3 mRNA using antisense oligodeoxynucleotides, *Xenopus* oocytes did not spontaneously mature, even 72 h after injection. These oocytes also have significantly reduced cAMP levels. The only way to trigger maturation in these oocytes is to introduce steroids to them. This possibly suggests that although lowered cAMP levels are necessary for meiotic resumption, these decreased levels alone are not sufficient in causing meiotic progression. This yields speculation that meiotically arrested oocytes may require both an anti-inhibitory signal to overcome the inhibitory, elevated levels of cAMP within the oocyte, as well as a positive signal, such as steroids, to trigger meiotic progression.

Also, it is still unknown what threshold exists for the amount that cAMP levels need to decrease in order for meiotic progression to occur;perhaps even the significantly reduced cAMP levels we see in the XGPR3 knockdown oocytes are still adequate to maintain meiotic arrest.

A further option is that as previously discussed, it may not be the total levels of intracellular cAMP that maintains meiotic arrest of oocytes, but the localization of the intracellular cAMP coupled with their effector molecules, AKAP-PKAs.

Another possibility is that XGPR3 may not be the only signal maintaining meiotic arrest. For example, other members of the GPR3/GPR12 family of constitutively activated G protein-coupled receptors, novel G protein receptors such as the membrane progesterone receptor (mPR) family of

steroid receptors, or receptor-independent G protein signaling, may be stimulating adenylyl cyclase to elevate intracellular cAMP and prevent oocyte maturation. Further studies need to be performed to address these questions to better understand the complex signaling occurring through cAMP.

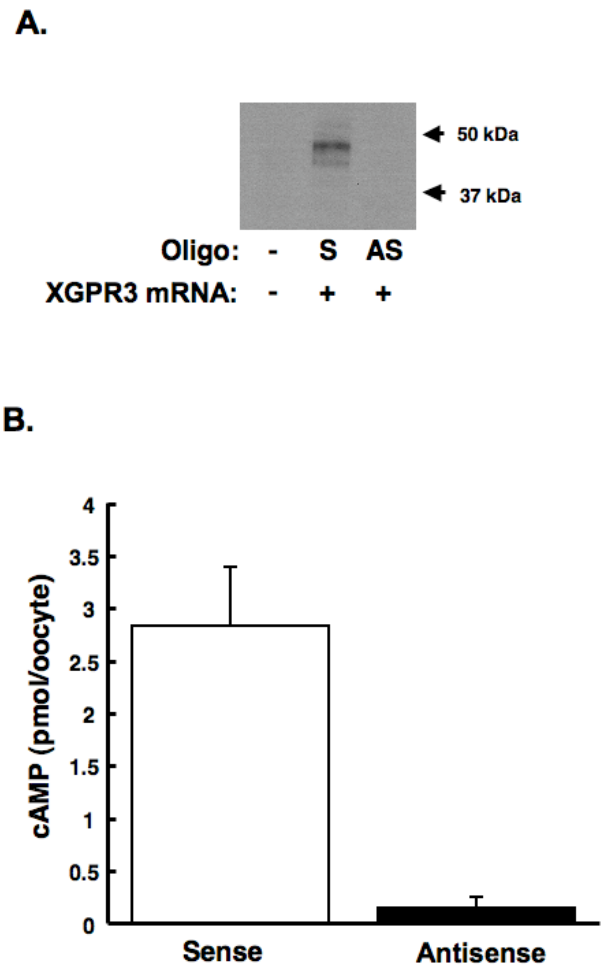

Figure 3-1: Knockdown of Endogenous XGPR3 Expression Reduced cAMP Levels. A) Injection of antisense, but not sense, oligonucleotides prevented the expression of exogenous XGPR3. Denuded oocytes were injected with buffer (-), sense (S), or antisense (AS) oligodeoxynucleotides, as well as mRNA encoding FLAG-tagged XGPR3. After 48 h, oocytes were lysed, and equal amounts of extracts were loaded in each lane. FLAG-tagged XGPR3 was then detected by Western blot. **B)** Injection of denuded oocytes (without attached follicle cells) with an antisense, but not sense, oligos directed against XGPR3 mRNA reduced intracellular cAMP. All studies were performed at least three times with similar results.

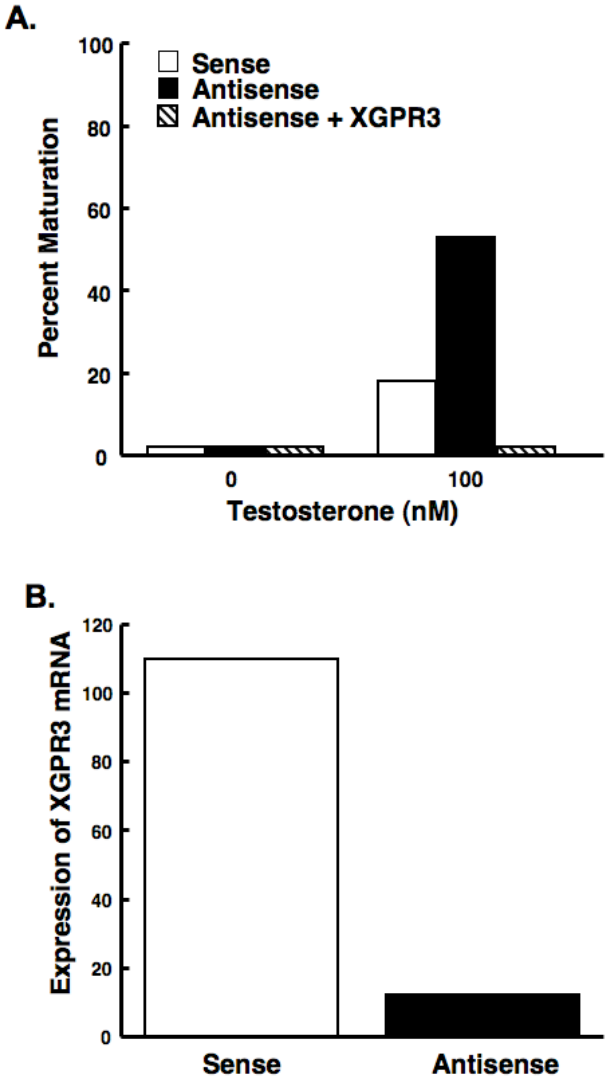

Figure 3-2: Knockdown of Endogenous XGPR3 Expression in Denuded Oocytes Enhanced Oocyte Maturation. A) Injection of antisense, but not sense, oligonucleotides into denuded oocytes also enhanced testosterone-mediated oocyte maturation. Importantly, co-injection of cRNA encoding FLAG-XGPR3 rescued this enhancement. **B)** Expression of *XGPR3* mRNA was reduced by approximately 90%, as determined by real-time PCR. All studies were performed at least three times with similar results.

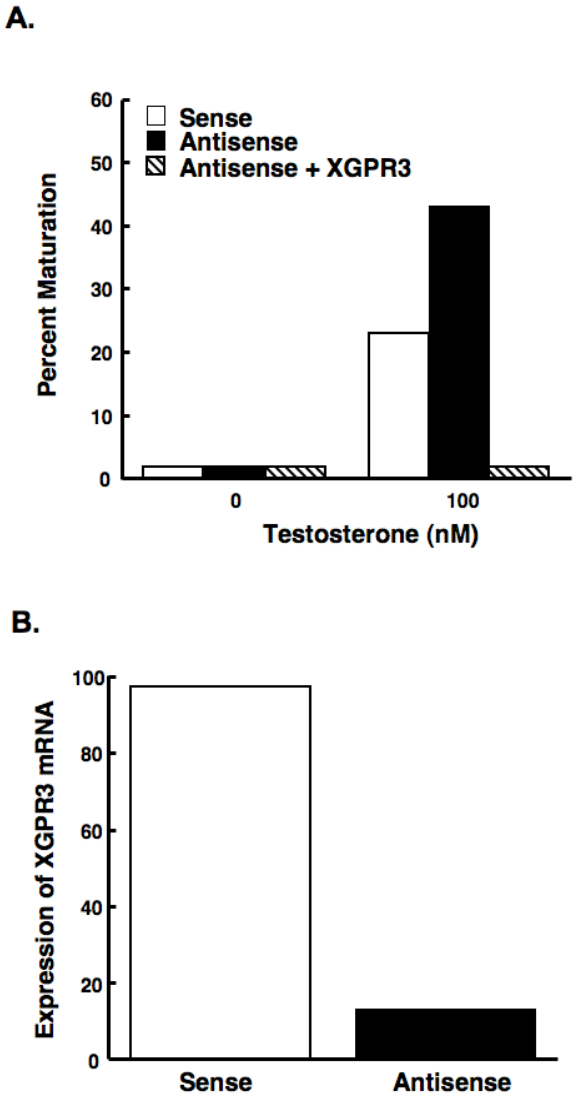

Figure 3-3: Knockdown of Endogenous XGPR3 Expression in Manually Defolliculated Oocytes Enhanced Oocyte Maturation. A) Similarly, injection of manually defolliculated oocytes (still has follicle cells) with antisense, but not sense, oligonucleotides directed against *XGPR3* mRNA enhanced testosterone-mediated oocyte maturation. Importantly, co-injection of cRNA encoding FLAG-XGPR3 rescued this enhancement. **B)** Expression of *XGPR3* mRNA was reduced by approximately 90%, as determined by real-time PCR. All studies were performed at least three times with similar results.

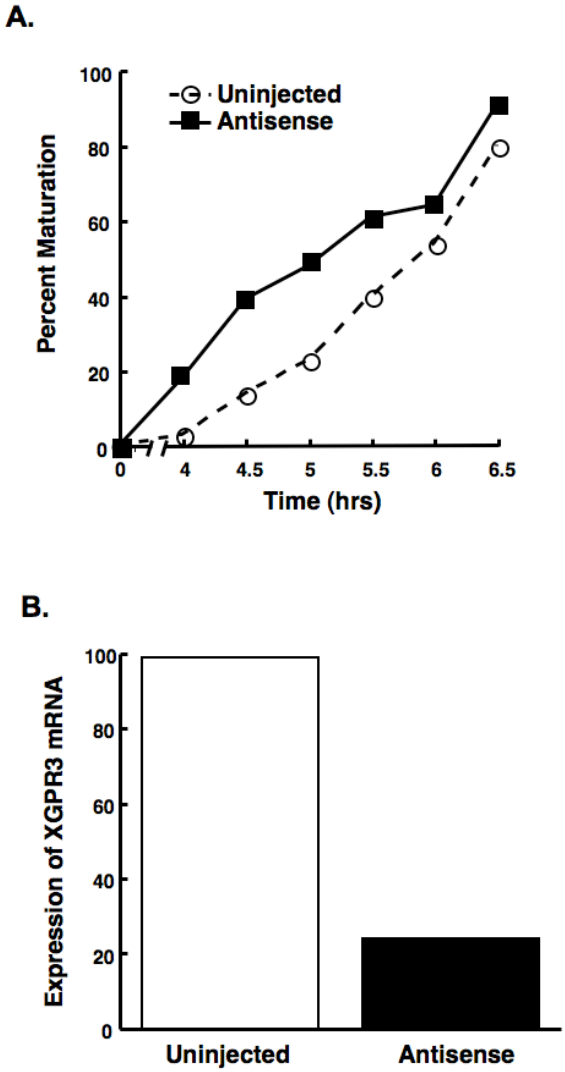

Figure 3-4: Knockdown of Endogenous XGPR3 Expression in Manually Defolliculated Oocytes in Ovaries Enhanced Oocyte Maturation. A) Manually defolliculated oocytes (still with attached follicle cells) that were injected with the antisense oligodeoxynucleotide directed against XGPR3 while still in ovarian fragments matured more quickly in response to hCG (150 units/mL), relative to uninjected oocytes. Expression of *XGPR3* mRNA was reduced by approximately 75% in these oocytes, as determined by real-time PCR **(B)**. All studies were performed at least three times with similar results.

Chapter Four

The Metalloproteinase Collagenase Can Cleave and Inactivate Cell Surface GPR3

Introduction

The main question left unanswered with GPR3 was a mechanism for how it was regulated during oocyte maturation. Because GPR3 appears to be a constitutively activated G protein-coupled receptor, we postulated that the inactivation of GPR3 might play a role in permitting meiosis to progress in follicle-enclosed oocytes. Specifically, we hypothesized that proteases may be capable of inactivating GPR3 at the cell surface because: (1) it is known that matrix metalloproteinases (MMPs) are activated and play an important role in ovulation. (2) It is also known that some GPCRs are activated due to cleavage (Hammes and Coughlin, 1999; Hammes et al., 1999), and we wondered whether GPR3 could instead be inactivated due to cleavage. (3) We have observed in previous experiments that collagenase, a matrix metalloproteinase used in oocyte isolation, seemed to sensitize oocytes to steroids. As we explored this hypothesis further, we questioned if collagenase could somehow affect GPR3's function and signaling.

Materials and Methods

Cloning of the Xenopus GPR3 Amino-terminal Deletion Mutants

The following primers were used to clone the amino-terminal deletion mutants from the full-length XGPR3 as template: forward (PDE mutant): GGGGATCCTATGCCTGATGAGTCCGTCAAT, forward (DPW mutant): GGGGATCCTATGGACCCTTGGGACACAAAC, forward (DHL mutant): GGGGATCCTATGGATCACCTGCTGCATCTC, and all mutants used the following reverse primer: GGCGGCCGCGAATTCTTATACGTCACTGGAAGTTCT. The cDNA encoding XGPR3 mutants were ligated into the mammalian expression vector pEF2-FlmugR-DNM, which contains the sequence encoding an amino-terminal FLAG tag (a generous gift from Mark Kahn, University of Pennsylvania, Philadelphia, PA).

Oocyte Preparation

All frogs were treated in accordance with accepted NIH and University of Texas standards of humane animal care. Oocytes were prepared using two different methods as indicated in the figure legends. To isolate denuded oocytes, ovaries were harvested from female *X. laevis* (Nasco, Fort Atkinson, WI) and treated as described elsewhere (Lutz et al., 2000). Briefly, follicle cells were removed by incubation of ovaries in 0.8 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN) in modified Barth's solution

(MBSH) without Ca^{2+} for 3-4 h. Oocytes were then washed and incubated overnight at 16 °C in MBSH containing 1 mg/mL Ficoll, 1 mg/mL BSA, 100 U/mL penicillin, and 0.1 U/mL streptomycin. Testosterone (Steraloids, Newport, RI)-induced maturation assays were performed on stage V/VI oocytes from each preparation to determine sensitivity to steroid as this varies considerably with each batch of oocytes. Maturation was scored as germinal vesicle breakdown, which was visualized as a white spot on the animal pole of the oocyte. Twenty oocytes were used for each data point in all experiments.

In the second method, stage V/VI oocytes were manually defolliculated from female *X. laevis*, injected with oligodeoxynucleotides or cRNA, as discussed in the appropriate materials and methods section and in the figure legends, and incubated in oocyte culture medium (OCM) as described elsewhere (Zuck et al., 1998). Oocytes were then stimulated for maturation using either testosterone or human chorionic gonadotropin (hCG) (Intervet, Millsboro, DE). Maturation was scored as GVBD.

RNA Synthesis and Injections

The pGEM-HE plasmid containing the FLAG-tagged XGPR3 cDNA sequence was linearized with AatII or SphI. Capped cRNA was transcribed *in vitro* with T7 RNA polymerase, according to the manufacturer's protocol (Ambion, Inc., Austin, TX). We suspended RNA in injection buffer (10 mM HEPES, pH 7.4), and using a Drummon or Harvard Apparatus automatic

injector, we injected Stage V/VI oocytes with the amounts of cRNA indicated in the figure legends.

After all injections, oocytes were incubated at least 36-48 h before any assay was begun.

Testosterone-Mediated Maturation Assays

Maturation assays were conducted by incubating 20 oocytes per condition with the indicated concentration of testosterone (Steraloids) or ethanol in MBSH or OCM for 12-16 h, at which time oocytes were scored for germinal vesicle breakdown. Dilutions were performed such that ethanol concentration remained at 0.1%.

Western Blots

Oocytes were incubated with steroid or vehicle, permeabilized in 20 μL/oocyte lysis buffer (1% Triton X-100; 50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 2 mM EDTA; 2 mM NaF; 0.5 mM sodium vanadate; 100 μg/mL phenylmethylsulphonyl fluoride), and microcentrifuged at 14,000 x g for 10 min to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2X sodium dodecyl sulfate sample buffer (Lutz et al., 2000). The equivalent of 0.5 oocytes was loaded in each lane for SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA), blocked in 5% Tris-buffered saline-Tween 20-milk for 1 h, and then incubated

with primary antibody overnight at 4 °C (1:5000 for anti-FLAG M1, 1:5000 for anti-FLAG M2, 1:2000 for anti-phospho-p42 and anti-total p42, 1:2000 for anti-phospho- and anti-total CDK1). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and a signal was detected by ECL Plus (Amersham Biosciences, Piscataway, NJ). Anti-FLAG M1 and M2 were obtained from Sigma-Aldrich (St. Louis, MO), anti-phospho-CDC2, and anti-total-CDC2, anti-phospho p42/p44 MAPK, and anti-total p42/p44 MAPK were from Cell Signaling Technology (Beverly, MA).

Cell Culture and Transfection

COS-7 cells (American Type Culture Collection, Manassas, VA) were maintained at 37 °C in DMEM (Fisher Scientific, Pittsburgh, PA) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA). Transfections were performed in six-well plates using Lipofectamine reagent (Invitrogen). Each well was transfected with 1 μg of total DNA as indicated. After 48 h incubation in 10% serum, cells were washed two times with ice-cold PBS (pH 7.4) and permeabilized in 300 μL of oocyte lysis buffer. Wells were scraped, cell debris was removed by centrifugation, and the cleared lysates were mixed 1:1 with 2X sodium dodecyl sulfate sample buffer and immunoblotted as described.

cAMP Competitive ELISA

COS-7 cells were transfected with XGPR3 plasmid or vehicle, and oocytes were injected with XGPR3 cRNA or vehicle as described previously. The cells and oocytes were treated with 0.1 M HCl 48 h after transfection/injection to prevent endogenous phosphodiesterase activity and then were microcentrifuged at 0.6 rcf to remove debris. Next, the samples were added to a 96-well plate, and cAMP levels were measured using a colorimetric assay per manufacturer's instructions (Endogen, Inc., Woburn, MA).

Cell Surface Expression Assay

COS-7 cells were transfected with XGPR3 plasmid, and oocytes were injected with XGPR3 cRNA as described. Cells were washed 48 h after transfection/injection and then incubated for 1 h with the M2 antibody at 1:1000 dilution in DMEM (cells) or the M1 antibody at 1:1000 dilution in modified Barth's solution (oocytes). After several washes with PBS, the cells were incubated with an anti-mouse secondary antibody (Bio-Rad Laboratories, Inc., Hercules, CA) at 1:2000. To assess the relative values of GPR3 cell surface expression levels, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), also known as ABTS solution (Pierce Chemical Co., Rockford, IL), was added, and absorption was measured by a spectrophotometer (BioTek Instruments, Inc. Winooski, VT) at 405 nm.

Collagenase Treatment

Cells or oocytes were transfected or injected with XGPR3 as described. For the cell surface assays, COS-7 cells were treated with either serum free media alone or serum free media containing 0.8 mg/mL collagenase A (Roche Applied Science) for 30 min. Injected oocytes were treated with either MBSH alone or with 0.8 mg/mL collagenase A for 30 min. Immediately after collagenase treatment, cell surface expression of FLAG-tagged XGPR3 was measured as described previously. For maturation and MAPK assays, isolated oocytes injected with cRNA encoding XGPR3 were incubated MBSH with the indicated concentration of testosterone or ethanol, ± 0.8 mg/mL collagenase A. To measure phosphorylation of p42-ERK, oocytes were incubated for 6 h followed by lysis and Western blot as described previously. To follow oocyte maturation, we treated oocytes for 12-16 h and scored oocytes for GVBD.

For Fig. 4-1A, manually defolliculated oocytes were treated with Collagenase Type IV (generous gift from Worthington Biochemical Corp., Freehold, NJ) at 1 mg/mL in 1X Mark's Modified Ringer (MMR) solution (1 M NaCl, 20 mM KCl, 20 mM $CaCl_2$, 10 mM $MgCl_2$, 150 mM HEPES). Before treatment with collagenase, the oocytes were washed five times in 1X MMR for 5 min. The oocytes were treated with 1 mg/mL collagenase solution for 1 h 45 min at room temperature. After treatment, oocytes were washed three times in 1X MMR for 5 min and then three times in OCM for 5 min.

Collagenase-treated oocytes were then incubated in OCM for 24 h, followed by treatment with testosterone as indicated in the figure legends. After 16 h incubation, oocytes were scored for GVBD.

Collagenase Treatment with Hoescht Staining

Oocytes were treated with MMR buffer alone (- Collagenase) or 1mg/mL Type IV collagenase (+ Collagenase) for 1 h and 45 min at room temperature. Cells were stained for 10 min with 10 micrograms/ml Hoescht dye and imaged on a Zeiss Axiovert 100M microscope using Axiovision Release version 4.6 software. Somatic cell nuclei are seen as individual green spots.

Immunohistochemistry

Oocytes were injected with either vehicle or 20 ng of XGPR3 cRNA. After 24 h, the oocytes were treated with either 5 IU hCG/mL in MBSH, 5 IU hCG with 50 μM Galardin (Calbiochem, La Jolla, CA) in MBSH, 0.8 mg/mL Collagenase A in MBSH, or MBSH alone for 4 h. The oocytes receiving Galardin were pre-treated with 50 uM Galardin in MBSH for 1 h. The oocytes were then fixed in paraffin, sectioned, and mounted on slides (Molecular Pathology Core Facility, University of Texas Southwestern). The immunohistochemistry was carried out as previously described (Rasar et al.,

2006) using 1:200 dilution of horse serum containing an anti-FLAG mouse monoclonal antibody.

Results

The Metalloproteinase Collagenase Can Cleave Cell Surface GPR3 in COS Cells

Interestingly, treatment of manually defolliculated oocytes with collagenase, a technique commonly used to remove follicular cells from *Xenopus* oocytes, markedly enhanced testosterone-mediated oocyte maturation (Fig. 4-1A). This maturation-enhancing effect was seen using every preparation of collagenase tested, including the highly purified collagenase batch shown in Fig. 4-1A. These results suggest that the collagenase itself, rather than a contamination, was mediating this enhancement.

To determine whether collagenase was enhancing maturation by cleaving and inactivating GPR3 on the cell surface, we first examined the effects of collagenase treatment for 30 min on GPR3 cell surface expression in COS cells. Compared to mock-transfected cells, cells transfected with cDNAs encoding XGPR3 and our positive control, M1R, both of which contain an amino-terminal FLAG tag, revealed significant cell surface expression. However, after 30 min of collagenase treatment, detectable levels of the

FLAG-epitope markedly decreased in the XGPR3-, but not the M1R-expressing cells, suggesting that collagenase cleaved the amino terminus of XGPR3 in a receptor-specific fashion (Fig. 4-1B). Importantly, mouse FLAG-GPR3 cell surface expression was similarly reduced by collagenase (Fig. 4-1B), demonstrating that collagenase-mediated cleavage of the amino terminus of GPR3 is not specific for only the frog isoform of GPR3.

The Metalloproteinase Collagenase Cleaves Cell Surface GPR3 in *Xenopus* Oocytes

Repeating the collagenase-cleavage experiment in *Xenopus* oocytes also showed a rapid collagenase-mediated reduction of overexpressed XGPR3 cell surface expression (Fig. 4-2A). Although the cell surface levels of overexpressed FLAG-tagged XGPR3 were decreased, total XGPR3 expression, as well as the size of the detected FLAG-tagged XGPR3, was unaffected, as determined by Western blot using an anti-FLAG antibody. This observation suggests that perhaps significant levels of intact, overexpressed GPR3 are present inside cells and, therefore, protected from the collagenase.

To test the effects of collagenase-mediated cleavage of GPR3 on cAMP levels, denuded oocytes were treated with collagenase both to remove follicle cells and to cleave endogenous XGPR3. As expected, subsequent overexpression of XGPR3 increased intracellular cAMP (Fig. 4-2B). Importantly, collagenase treatment of oocytes overexpressing XGPR3 reduced

intracellular cAMP (Fig. 4-2B). As seen in Fig. 4-3, it is clearly visible that collagenase can remove the follicular cell layer surrounding *Xenopus* oocytes.

Collagenase Partially Inactivates GPR3 Signaling

To further explore the idea of what collagenase could do to GPR3 signaling and oocyte maturation, denuded oocytes were again treated with collagenase both to remove follicle cells and to cleave endogenous XGPR3. As expected, subsequent overexpression of XGPR3 almost completely blocked testosterone-mediated maturation and activation of MAPK (Fig. 4-4A). Importantly, collagenase treatment of oocytes overexpressing XGPR3 partially restored steroid-triggered oocyte maturation and activation of MAPK (Fig. 4-4 A & B), indicating that, similar to reducing endogenous XGPR3 expression, collagenase-mediated cleavage and inactivation of XGPR3 sensitizes oocytes to testosterone. Notably, collagenase did not completely restore steroid sensitivity in oocytes overexpressing XGPR3 compared to that seen in mock-injected oocytes (Fig. 4-4 A & B). This lack of complete restoration is most likely caused by the large amounts of overexpressed intracellular full-length XGPR3 that are still detected by Western blot (data not shown) and that may be stimulating sufficient intracellular cAMP production to prevent maturation.

Treatment of Follicle-Enclosed Oocytes with hCG Triggers Matrix Metalloproteinase-Mediated Cleavage of XGPR3 at the Oocyte Cell Surface

To determine whether XGPR3 was being cleaved in follicles during gonadotropin-induced oocyte maturation and ovulation, follicle-enclosed oocytes were injected with cRNA encoding FLAG-tagged XGPR3, followed by treatment with hCG for 4 h. Individual oocytes enclosed in follicle cells were then fixed and mounted on slides for immunohistochemical analysis using an anti-FLAG antibody.

As expected, oocytes injected with the cRNA encoding XGPR3 expressed significant amounts of protein at the cell surface relative to mock-injected cells (Fig. 4-5 *top two panels*). Intriguingly, treatment of oocytes overexpressing XGPR3 with hCG reduced cell surface expression of the FLAG epitope (Fig. 4-5 *middle two panels*), suggesting that the amino terminus of XGPR3 was being cleaved in response to hCG. Furthermore, the metalloproteinase inhibitor Galardin partially abrogated the hCG-induced cleavage of XGPR3 at the cell surface (Fig. 4-5 *bottom two panels*), suggesting that hCG may be activating a matrix metalloproteinase to degrade XGPR3.

Interestingly, Galardin blocked hCG-mediated oocyte maturation in follicle-enclosed oocytes (data not shown). However, Galardin also reduced hCG-induced steroid production (data not shown), thus complicating the interpretation of this result; whether Galardin antagonizes oocyte maturation

by inhibiting an MMP or by somehow reducing steroid production so that oocytes do not have enough of the positive signal to mature requires future work.

Western blot analysis of oocyte lysates (data not shown) from all four conditions in Fig. 4-5 confirmed that FLAG-tagged XGPR3 was appropriately expressed in oocytes injected with cRNA encoding the receptor and that the total FLAG-xGPR3 levels were unchanged in response to hCG, despite the observed loss of expression at the cell surface. This observation again suggests that significant levels of intact overexpressed XGPR3 are present inside cells and are, therefore, protected from proteolysis at the cell surface.

Amino-terminal XGPR3 Mutants Show That Collagenase Is Not Acting Upon the Amino-terminus of XGPR3

We generated several amino-terminal deletion GPR3 mutants that lack a portion of the amino-terminal overhang before the transmembrane domain. Because of specific hydrophobic residues that could be target of MMPs contained in this region, we hypothesized that it was this overhang that was being cleaved by collagenase, and thus regulates GPR3 signaling. Therefore we presumed that mutants lacking in specific regions or all of this overhang, would no longer be cleaved by collagenase or other MMPs. Overexpression of the XGPR3 mutants (Fig. 4-6) shows that they express properly in both COS cells and *Xenopus* oocytes (data not shown), and they seem to stimulate

cAMP levels constitutively (Fig. 4-7). Thus, they seem to be functioning properly. Interestingly, collagenase-treatment of a mutant lacking almost the full amino-terminal head of XGPR3, PDE-XGPR3, still can be significantly cleaved by collagenase, although not to the levels seen in full-length XGPR3 (Fig. 4-8). This suggests that collagenase may be cleaving/inactivating XGPR3 at other sites, or another alternative, is that an unknown ligand may be stimulating GPR3 activity constitutively, and collagenase/MMPs are acting on this ligand rather than GPR3 itself.

Discussion

Our data shows that the metalloproteinase collagenase readily cleaves both *Xenopus* and mouse GPR3, potentially removing the amino terminus from the cell surface. The exact nature of this proteolysis is not known because collagenase cleaves proteins at several hydrophobic residues. It was thought that because the sequences ^{6}A^{7}V^{8}S and ^{5}A^{6}V^{7}G are conserved between *Xenopus* and mouse GPR3, respectively, these sites could serve as a potential target for the cleavage. In addition, the hydrophobic resides ^{13}L^{14}L in XGPR3 and ^{14}A^{15}G in mouse GPR3 may serve as collagenase targets.

However, based on preliminary cell surface expression assays using the FLAG-tagged XGPR3 mutants lacking the amino-terminal domain (Fig. 4-8), it seems that either the collagenase-mediated cleavage is not occurring only at the amino terminus or that there are other mechanisms and sites for

this cleavage event. Perhaps there is enough overexpressed XGPR3 that collagenase is only cleaving most cell surface XGPR3, but there is still a large amount of intracellular XGPR3 that has not localized to the cell surface, which is protected from this cleavage. Work using immunofluorescence indicates that overexpression of XGPR3 in COS cells can result in very high amounts of expression especially at the cell surface, but also intracellularly (data not shown). It is presumed this same situation can occur in *Xenopus* oocytes, but because of the thick, fatty membranes and layers of the oocytes, immunofluorescence work is difficult to accomplish in these oocytes. Further research is necessary to elucidate the exact nature of this collagenase-mediated cleavage of XGPR3.

Similar to the knockdown experiments using antisense oligodeoxynucleotides, treatment of manually defolliculated oocytes with collagenase enhanced testosterone-mediated maturation without triggering spontaneous maturation. This again supports the notion that two signals are required in oocyte maturation: an anti-inhibitory signal, such as collagenase/MMP or knockdown of GPR3, as well as a positive signal, such as steroids.

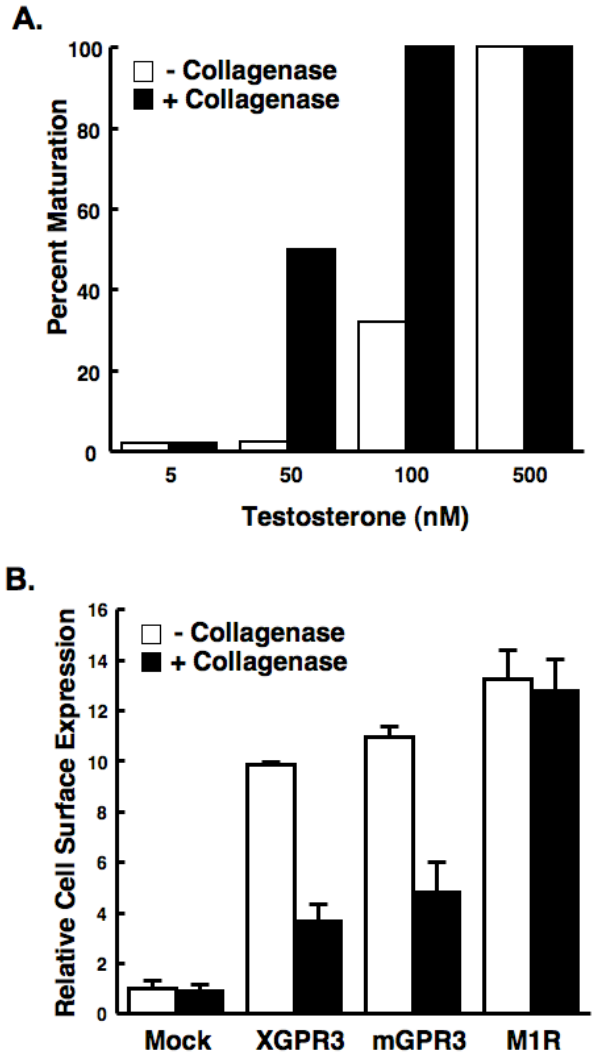

Figure 4-1: Collagenase Cleaves & Inhibits GPR3. A) Manually defolliculated oocytes (some follicle cells still attached) were more sensitive to testosterone after treatment with highly pure collagenase type IV (1 mg/mL). **B)** The amino-termini of both XGPR3 and mouse GPR3 (mGPR3) expressed in COS cells were cleaved by collagenase A (0.8 mg/mL), while the amino-terminus of the M1 muscarinic receptor (M1R) was unaffected. Cleavage was measured as the loss of cell surface FLAG expression by ELISA. Values are presented as cell surface expression relative to mock-transfected, untreated cells, and are the average ± S.D. (n=3). All experiments were repeated at least three times with similar results.

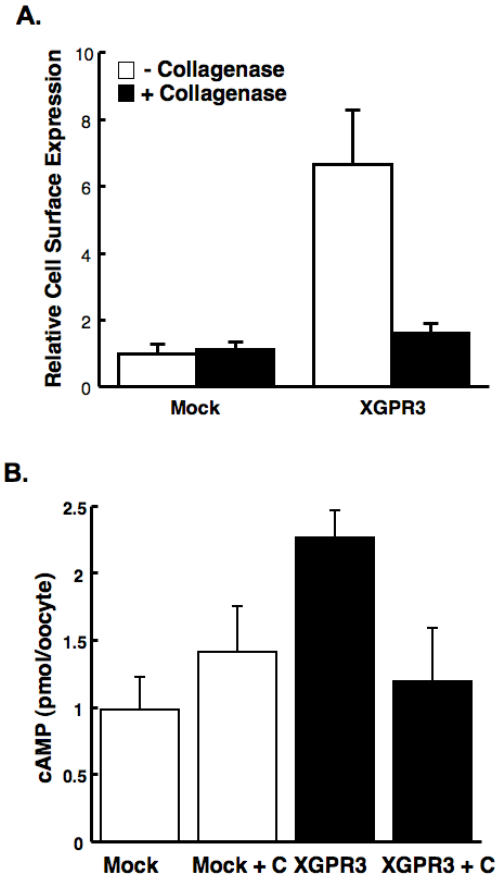

Figure 4-2: Collagenase Cleaves GPR3 & Inhibits Activity of GPR3 in Oocytes. A) The amino terminus of XGPR3 expressed in *Xenopus* oocytes was cleaved by collagenase A (0.8 mg/mL for 30 min). Cleavage was measured as the loss of cell surface FLAG expression by ELISA. Values are presented as cell surface expression relative to mock-transfected, untreated cells, and are the average ± S.D. (n=3). **B)** Collagenase treatment of oocytes injected with cRNA encoding XGPR3 abrogated the mRNA-dependent rise in intracellular cAMP, while having no significant effect on mock-injected oocytes. Values represent the mean ± S.D. (n=3). All experiments were repeated at least three times with similar results.

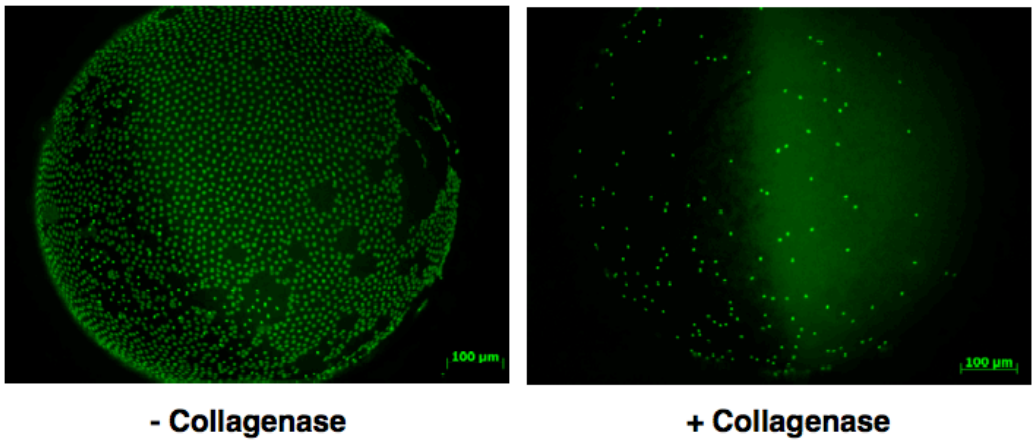

Figure 4-3: Collagenase Removes Somatic Follicle Cells from *Xenopus* oocytes. Oocytes were treated with MMR buffer alone (- Collagenase) or 1mg/mL Type IV collagenase (+ Collagenase) for 1 hour and 45 minutes at room temperature. Cells were stained for 10 minutes with 10 micrograms/ml Hoescht dye and imaged on a Zeiss Axiovert 100M microscope using Axiovision Release version 4.6 software. Somatic cell nuclei are seen as individual green spots.

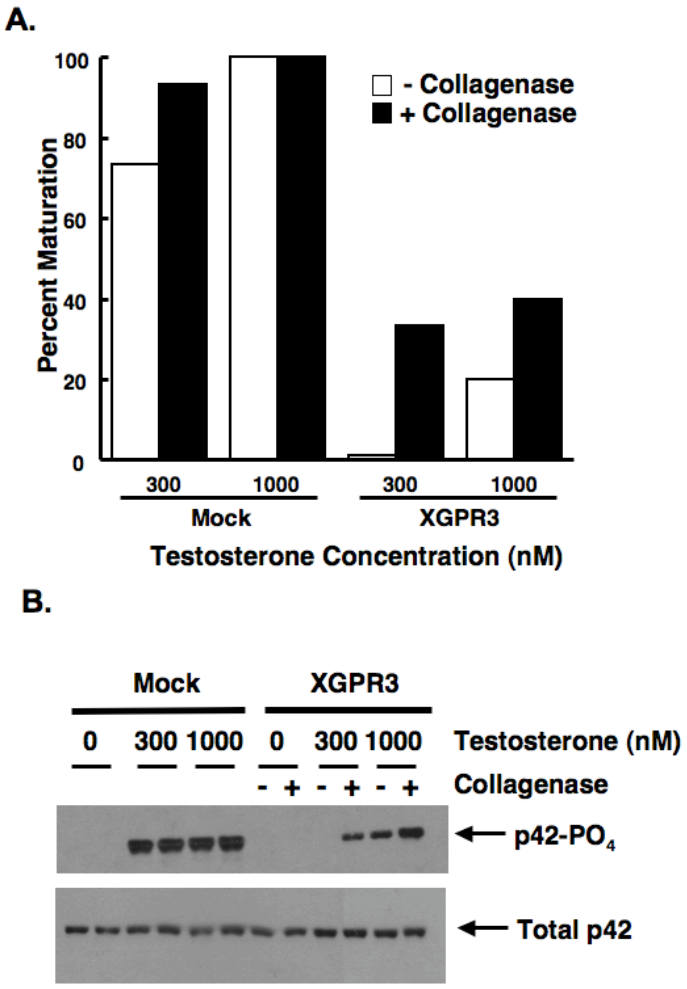

Figure 4-4: Collagenase Enhances Testosterone-Mediated Oocyte Maturation. A) Collagenase A (0.8 mg/mL) partially rescued the inhibitory effects of overexpressed XGPR3 on testosterone-mediated oocyte maturation (**A**, overnight treatment) and activation of MAPK (**B**, six hour treatment). All experiments were repeated at least three times with similar results.

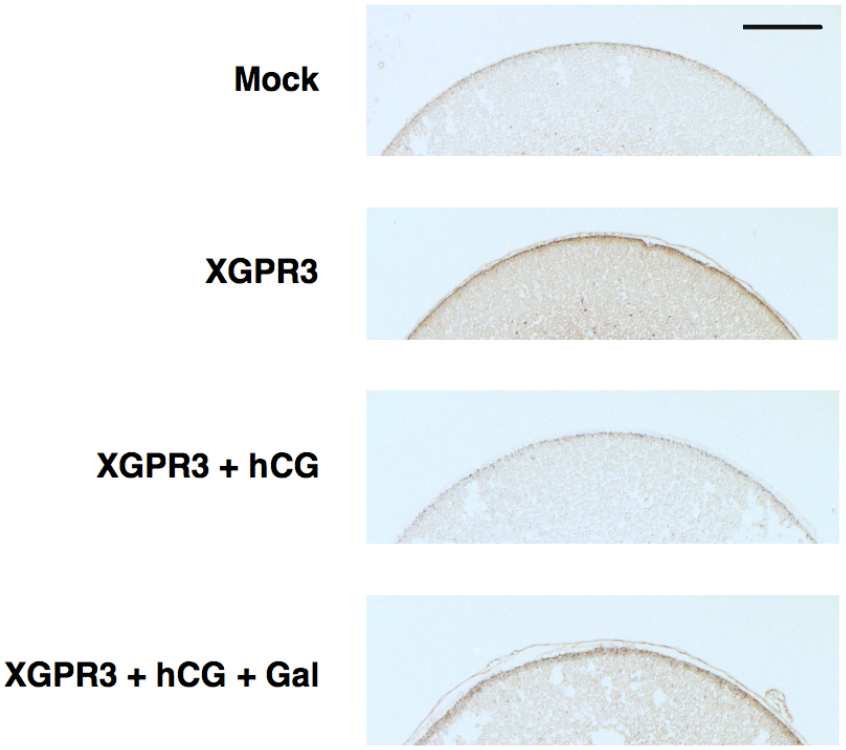

Figure 4-5: hCG Treatment of Follicle-Enclosed Oocytes Triggered Matrix Metalloproteinase-Mediated Cleavage of XGPR3 at the Oocyte Cell Surface. Oocytes were injected with either vehicle or 20 ng of XGPR3 cRNA. After 24 h, the oocytes were treated with either 5 IU hCG in MBSH, 5 IU hCG with 50 μM Galardin in MBSH, 0.8 mg/ml Collagenase A in MBSH, or MBSH alone for 4 h. The oocytes receiving the hCG and Galardin treatment were pretreated with 50 μM Galardin in MBSH for 1 h. The oocytes were then fixed in paraffin, sectioned, and mounted on slides (Molecular Pathology Core Facility, University of Texas Southwestern). The immunohistochemistry was carried out as previously described, (Rasar et al., 2006) using 1: 200 dilution of horse serum containing an anti-FLAG antibody. The slides were photographed using a Zeiss Axioskop 2 scope at 10X and digital camera using 36 ms exposure for all pictures. The black bar represents 100 μm. Note that the oocytes injected with the cRNA encoding FLAG-tagged XGPR3 demonstrated significant expression of XGPR3 at the cell surface relative to mock-injected oocytes. Treatment with hCG reduced all cell surface expression of FLAG-tagged XGPR3, but the addition of Galardin partially abrogated the effects of hCG. Photos are representative of three experiments with similar results. Gal, Galardin.

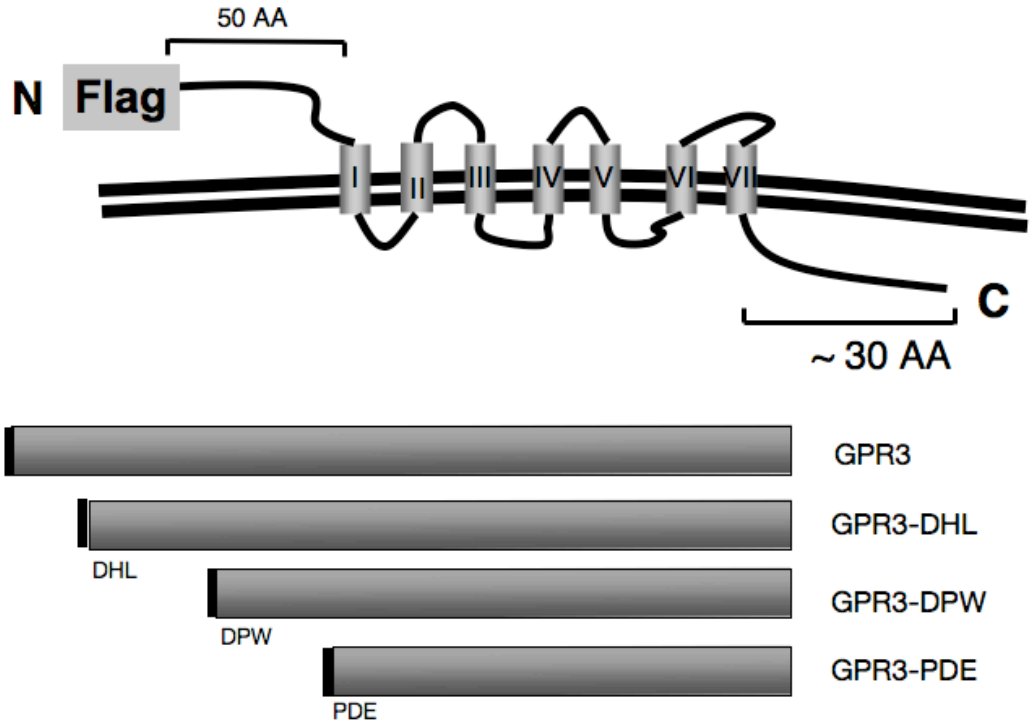

Figure 4-6: Schematic of Amino-terminal GPR3 Mutants. Several mutants were cloned and generated that still contain a FLAG-epitope tag at the amino-terminus. However, these mutants were designed with PCR techniques to have a certain portion of the 50 AA head before the 7-transmembrane domain of XGPR3 deleted. PDE-GPR3 is lacking all of the 50 AA head.

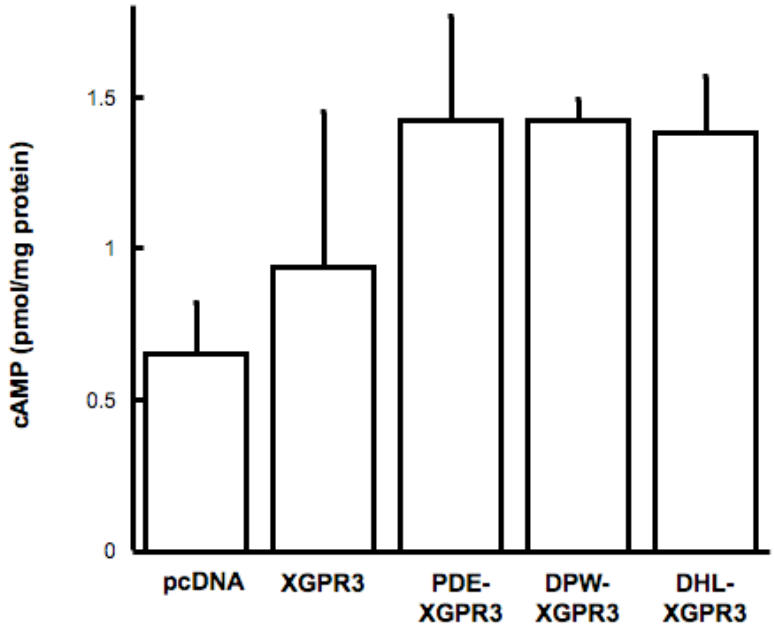

Figure 4-7: XGPR3 Mutants Still Can Increase cAMP Levels. Transfections were carried out in COS-7 cells as previously described, followed by using the cell lysates for Western blot analysis and a cAMP competitive ELISA. XGPR3 overexpressing cells had substantial elevated levels of cAMP compared to mock-transfected cells, and XGPR3 amino-terminal mutants could still stimulate cAMP levels, suggesting they are completely functional.

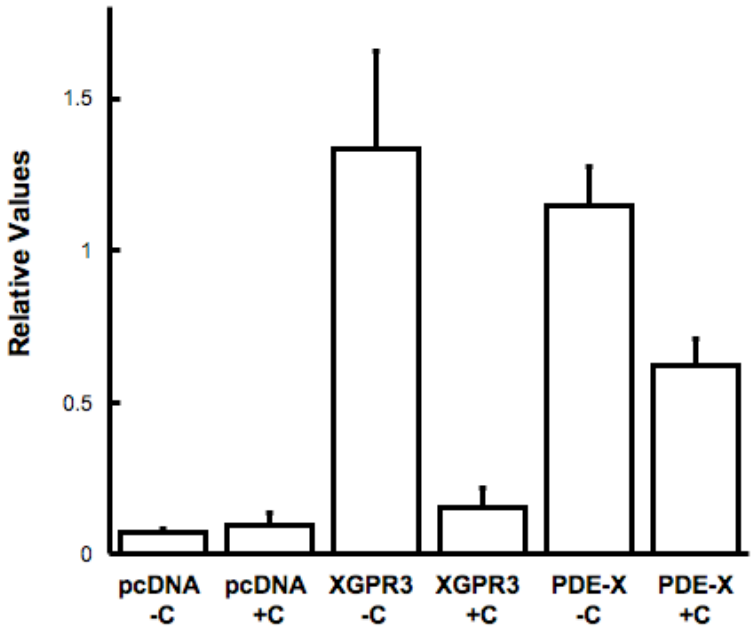

Figure 4-8: XGPR3 Mutants Are Still Cleaved by Collagenase. The amino terminus of XGPR3 mutants expressed in *Xenopus* oocytes was still cleaved by collagenase A (0.8 mg/mL for 30 min). Cleavage was measured as the loss of cell surface FLAG expression by ELISA. Values are presented as cell surface expression relative to mock-transfected, untreated cells, and are the average ± S.D. (n=3).

Chapter 5

Conclusions and Recommendations

The studies presented here were designed to carefully characterize the signaling properties of GPR3 in the *X. laevis* model system. Advantages of the Xenopus model over the mouse system are the relative ease in isolating large numbers of oocytes, overexpressing and depleting proteins, and measuring signals associated with maturation, including changes in cAMP and activation of MAPK and CDK1 (Hammes, 2003; Maller and Krebs, 1980; Rasar and Hammes, 2006).

In addition, the physiological trigger for mouse oocyte maturation is still unknown; mouse oocytes spontaneously mature upon removal from follicles via mechanisms that are also not well understood (Albertini and Carabatsos, 1998; Jamnongjit and Hammes, 2005). In contrast, *Xenopus* oocytes remain in meiotic arrest after removal from the ovary until stimulated by steroid (Maller and Krebs, 1980). Thus, timed responses to the physiological agonist testosterone can be readily followed with Xenopus oocytes (Hammes, 2004).

Finally, detection of overexpressed mouse GPR3 has been hampered by the lack of anti-GPR3 antibodies. By engineering the XGPR3 protein to contain a FLAG sequence at its amino terminus, overexpressed XGPR3 could

be detected by Western blot of oocyte extracts and cell surface XGPR3 expression could be measured with a whole-cell ELISA strategy.

Using the *Xenopus* model, we have confirmed and extended our knowledge regarding GPR3 signaling. First, we cloned a *Xenopus* isoform of GPR3 using *X. laevis* oocyte mRNA, demonstrating the conservation of GPR3 expression in lower vertebrate oocytes. Second, we demonstrated that overexpressed XGPR3 localized to the cell surface and inhibited maturation induced by both gonadotropin and the direct physiological trigger, testosterone. Third, we showed that reduction of endogenous XGPR3 expression using antisense oligodeoxynucleotides lowered intracellular cAMP and enhanced both gonadotropin- and testosterone-triggered oocyte maturation. Finally, we proposed a mechanism for the regulation of XGPR3 via matrix metalloproteinases that are activated during ovulation.

How is GPR3 stimulating adenylyl cyclase in *Xenopus* oocytes? As aforementioned, an unusual feature of *Xenopus* oocytes is that $G_{\beta\gamma}$ and $G\alpha_s$ appear to signal together to stimulate adenylyl cyclase, elevate intracellular cAMP, and hold oocytes in meiotic arrest (Gallo et al., 1995; Lutz et al., 2001; Sheng et al., 2005; Sheng et al., 2001). This differs from mouse oocytes, in which $G_{\beta\gamma}$ signaling may, in fact, inhibit adenylyl cyclase and promote oocyte maturation (Gill and Hammes, 2007). The difference between these species is likely due to the presence of adenylyl cyclase VII in *Xenopus* (Guzman et al., 2005; Sheng et al., 2005), but not mouse, oocytes. Adenylyl cyclase VII is

stimulated by both $G_{\beta\gamma}$ and $G\alpha_s$ (Federman et al., 1992); thus, the G protein signaling that maintains meiotic arrest in frog oocytes may be more powerful than that in mice.

In fact, the additional Gβγ-mediated stimulation of adenylyl cyclase in *Xenopus*, but not mouse, oocytes may partially explain why *Xenopus* oocytes uniquely remain in meiotic arrest after removal from the ovary. Interestingly, sequestration of G_{β} by overexpression of the carboxyl-terminal GRK1 markedly reduced XGPR3-mediated elevation of intracellular cAMP (Fig. 3-4A) and almost completely rescued GPR3-mediated inhibition of testosterone-induced maturation (Fig. 3-4B). These observations suggest that XGPR3 is likely activating both $G\alpha_s$ and $G_{\beta\gamma}$ in *Xenopus* oocytes to stimulate adenylyl cyclase and maintain meiotic arrest in *Xenopus* oocytes, with $G_{\beta\gamma}$ being the dominant signal that regulates these processes.

Further studies will be needed to confirm whether GPR3 stimulates $G_{\beta\gamma}$ in other cells or whether this $G_{\beta\gamma}$ stimulatory effect is specific only to cells containing adenylyl cyclase VII . However, recent findings that $G\alpha_s$ signaling may be relatively unchanged during LH-induced oocyte maturation in mouse oocytes suggest that other G protein-coupled signaling pathways mayindeed be involved (Norris et al., 2007).

It was hypothesized that GPR3 may be function via multiple G protein signaling pathways to carry out its effects. However, preliminary work utilizing a calcium efflux assay in oocytes to determine whether XGPR3 also

signals via other effectors such as phospholipase C has suggested that XGPR3 seems to only signal through stimulatory adenylyl cyclase means. Further studies, involving phosphoinositide turnover, in other cell lines are needed to determine whether GPR3 can signal via G_q mechanisms.

Moreover, recent work in GPR3 null mice has clearly demonstrated that GPR3 plays an important role in maintaining meiotic arrest in mouse oocytes because the majority of oocytes in *Gpr3-/-* antral follicles prematurely progresses through meiosis (Ledent et al., 2005; Mehlmann et al., 2004). However, 10-30% of oocytes in the *Gpr3-/-* preantral follicles remain in meiotic arrest, and female GPR3 null mice are still fertile. Furthermore, wild-type mouse oocytes removed from follicles spontaneously mature despite the continued presence of constitutively activated GPR3 (Albertini and Carabatsos, 1998; Freudzon et al., 2005). The results of the knockdown studies show that although GPR3 expression is significantly reduced, there is still no spontaneous maturation. In fact, maturation of these GPR3-knockdown oocytes only occurs with the addition of steroids. Moreover, in the knockdown experiments, cAMP levels are also substantially decreased, however there still is no spontaneous maturation. Maturation is only observed in these GPR3 knockdown, cAMP-reduced oocytes if steroids are added. As discussed previously, it is known that high levels of cAMP can inhibit oocyte maturation. In contrast though, there is still an unanswered question of whether a decrease in cAMP levels is necessary or sufficient. The

answer may actually involve the theory that localized cAMP changes are occurring that affect specific AKAP-PKA substrates. It is also unclear the exact amounts cAMP levels need to be reduced to activate a threshold, if any, to allow for meiotic progression, and further work needs to be performed to assay what this threshold of cAMP is in oocyte maturation.

Together, these observations indicate that, as we now confirm in *Xenopus* oocytes, GPR3 is very important but is neither necessary nor sufficient to maintain meiotic arrest in all oocytes. GPR3 plays a large role in the inhibitory arm of oocyte maturation. However, there also may be a stimulatory arm in oocyte maturation, involving steroids, that may involve separate signaling pathways and mechanisms from the inhibitory arm, as indicated by the knockdown experiments, that are necessary to promote the oocytes to resume meiosis.

Similar to the knockdown experiments using antisense oligos, treatment of manually defolliculated oocytes with collagenase enhanced testosterone-mediated maturation without triggering spontaneous maturation. In addition, collagenase partially rescued the inhibitory effects of overexpressed XGPR3. These data suggest that collagenase-mediated proteolysis may inactivate cell surface GPR3. Because LH is known to activate multiple metalloproteinases, including collagenase, during ovulation (Leung and Steele, 1992; Maller and Krebs, 1980), we postulated that LH-induced activation of ovarian metalloproteinases may prime oocytes for maturation by

inactivating GPR3 and eliminating one of the signals that holds cells in meiotic arrest. Accordingly, we demonstrated that hCG treatment of follicle-enclosed oocytes led to at least partial metalloproteinase-mediated (Galardin-sensitive) cleavage of cell surface XGPR3. Although we originally believed that collagenase and other MMPs were cleaving the amino-terminal portion of GPR3, preliminary data involving GPR3 amino-terminal deletion mutants suggest that the cleavage may be occurring at another site since the mutants still show some cleavage via cell surface expression assays. For example, perhaps GPR3 may actually have a ligand that has not been found yet. It has been suggested that sphingosine-1-phosphate and sphingosylphosphorylcholine may be potential ligands for GPR3 (Hinckley et al., 2005). Perhaps collagenase and other MMP3s are inactivating these or other ligands somehow, which explains how collagenase affects the activity of GPR3 in addition to the cleavage events occurring. Further studies will be needed to determine the physiological importance of these findings and to identify the specific metalloproteinases that may be inactivating GPR3 *in vivo* and the sites of action of these MMPs.

On a final note regarding collagenase and GPR3, collagenase is commonly used to isolate *Xenopus* oocytes for maturation studies. The studies presented in this dissertation highlight an important caveat against this technique: collagenase treatment will make oocytes more sensitive to steroid just after isolation; however, with time translation of new cell surface

GPR3 protein may lead to decreased sensitivity. This issue needs to be taken into consideration when comparing steroid sensitivities between different populations of oocytes.

To reconcile the results in the mouse and frog models, we propose that GPR3 indeed plays a partial role in elevating intracellular cAMP and maintaining meiotic arrest and that protease-mediated inactivation of GPR3 may be one of several signals which contribute to gonadotropin-induced oocyte maturation (Fig. 5-1). The existence of another highly similar, almost identical GPR12 implicates that GPR3 may only be one of multiple G protein signals that help hold oocytes at arrest.

Taken together, our findings show that in oocyte maturation, there exist both anti-inhibitory signals to turn off the constitutive G protein signaling and pro-stimulatory signals, such as steroids. Logically and physiologically, it makes sense for a mechanism that needs to be tightly regulated, such as oocyte maturation, to utilize multiple, complex signals to ensure that no aberrant signaling occurs, thus guaranteeing protection of a process so important to life: reproduction. It will be interesting to find what other receptors and interactors, in conjunction with GPR3, are involved in holding oocytes at meiotic arrest.

A final question remains: how do steroids inhibit G protein signaling in *Xenopus* oocytes? Evidence suggests that androgens bind to classical androgen receptors to attenuate G protein signaling, perhaps using the

scaffold molecule called proline-, glutamic acid-, and leucine-rich protein-1 (PELP1). PELP1 contains multiple leucine-rich domains that can interact with the AF2 domain of the androgen receptor (AR),as well as several proline-rich motifs that can interact with Src or other proteins containing SH3 domains. Thus, PELP1 may serve as a scaffold that links steroid receptors to Src and perhaps other signaling molecules. *In vitro,* PELP1 enhances estrogen receptor-mediated genomic and nongenomic signaling in response to estradiol, and both types of signaling appear to be regulated in large part through PELP1's nongenomic actions on Src (Barletta et al., 2004; Wong et al., 2002). In addition, MNAR interacts with other classical steroid receptors. For example, MNAR co-precipitates with the AR in LNCaP prostate cancer cells (Unni et al., 2004) and is, therefore, a potential regulator of nongenomic androgen-triggered signals in the prostate.

Furthermore, reducing PELP1 expression in *Xenopus* oocytes enhances testosterone-mediated maturation and reduces the ability of the $G_{\beta\gamma}$-coupled M2R receptor to trigger calcium mobilization (Haas et al., 2005). These observations indicate that PELP1 may be enhancing $G_{\beta\gamma}$ signaling in *Xenopus* oocytes, even in the absence of steroid and suggest the intriguing possibility that $G_{\beta\gamma}$ might enhance GPR3 signaling. Preliminary work overexpressing PELP1 and XGPR3 together did not seem to further enhance the levels of cAMP as compared to overexpressing XGPR3 alone (data not

shown). However, the cells and oocytes may already be saturated to the maximum levels of cAMP, causing no further visible enhancement by PELP1.

Based on this data, it is, therefore, more useful to determine the role of endogenous PELP1 in oocyte maturation. To do so, experiments in knocking down levels of PELP1 may prove important. Initial data is still inconclusive about the effects of knocking down PELP1. PELP1 is ubiquitously expressed in almost all cell types (Greger et al., 2006), and as an important scaffold protein for a variety of cellular functions, PELP1 may require a combination of several antisense oligos or siRNAs rather than a single oligo/siRNA in order to efficiently knock down the levels PELP1 RNA and protein. After knockdown of PELP1 is achieved, studies involving the characterization of whether PELP1 operates in conjunction with G protein signaling in ovarian functions? should be carried out. If PELP1 does seem to interact with G protein signaling in oocyte maturation, research into whether PELP1 utilizes cAMP pathways or the PI pathways to signal should be conducted. Initial studies with both cAMP and PI hydrolysis assays have proved inconclusive since PELP1 levels were not significantly knocked down.

Regardless of the mechanism that allows G protein signals to interact and communicate with androgen signals, these data and future observations will certainly provide important information about how this complex network can be integrated into allowing oocytes to meiotically progress. In general, *Xenopus* oocytes are a superb model system for exploring MAPK scaffolds, G

protein signaling, and nongenomic actions of steroid receptors. In the future, further insight into G protein and androgen signaling in these oocytes will prove invaluable for the field of reproduction to truly understanding the underlying mechanisms involved in normal fertility so that we can find treatments and cures for abnormal fertility.

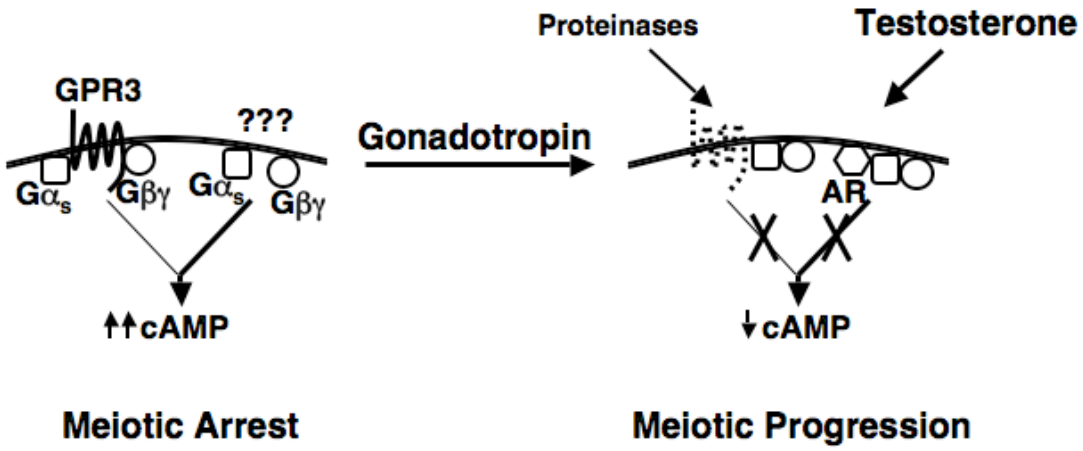

Figure 5-1: Model - GPR3 is Partially Responsible for Maintaining Meiotic Arrest in *Xenopus* Oocytes. XGPR3, as well as other unknown factors, stimulate both $G\alpha_s$ and $G_{\beta\gamma}$ to elevate cAMP and maintain meiotic arrest. Stimulation of follicles with gonadotropin leads to the activation of proteinases, which may cleave GPR3 and partially reduce the inhibitory signals maintaining meiotic arrest. Testosterone then suppresses the other sources of $G_{\beta\gamma}$ signaling, resulting in significant reduction of intracellular cAMP and meiotic progression.

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