Université d'Ottawa · University of Ottawa

Université d'Ottawa · University of Ottawa

FACULTÉ DE ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Xun LIU

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

M. Sc.(Biochemistry)

GRADE - DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Transcription-dependent and transcription-independent functions of the classical progesterone receptor in *Xenopus ovaries*

J. Liu

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

CO-DIRECTEUR DE LA THÈSE - THESIS CO-SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

H. McBride

J. Ngsee

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Transcription-dependent and transcription-independent functions of the classical progesterone receptor in *Xenopus* ovaries

Xun Liu

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology
in partial fulfillment of the requirements for the degree of Master of Science

University of Ottawa
Ottawa, Ontario, Canada
September 2004

Your file Votre référence
ISBN: 0-494-01535-7
Our file Notre référence
ISBN: 0-494-01535-7

ABSTRACT

The physiological functions of the classical progesterone receptor (XPR) in regulating oocyte maturation and ovulation in *Xenopus laevis* remain controversial. Using antibodies generated against cloned XPR, I demonstrated here that the somatic follicle cells expressed an 80 kDa XPR protein, termed XPR-1. XPR-1 underwent progesterone-induced, proteasome-mediated degradation. A smaller (~70 kDa) XPR protein, termed XPRo, was expressed in oocytes, but not in follicle cells. XPRo underwent progesterone-induced hyperphosphorylation, but not degradation. Treating isolated ovaries with progesterone caused oocyte maturation and the release of the mature oocytes from the ovaries (ovulation). Actinomycine D, a general transcription inhibitor, did not interfere with progesterone-induced oocyte maturation but blocked ovulation so that mature oocytes were trapped in the follicles. These results demonstrated that progesterone had dual functions in the process of ovulation: transcription-dependent follicle rupture and transcription-independent oocyte maturation. Furthermore, our results suggest that the dual functions of progesterone in *Xenopus* ovaries are mediated by the two forms of XPR proteins differentially expressed in the follicle cells and the oocytes respectively.

To my mother and father

Who give me the precious feeling of certainty in this chaotic world

ACKNOWLEDGEMENTS

I would like to thank Dr. Johné Liu for his guidance throughout the pursuit of my M.Sc. degree. My gratitude goes to him for motivating me to attain high standards. Thank you for teaching me the meaning of hard work.

All members of my lab and summer students for the pleasant memories, frequent help and kind companionship.

Yanwei Xi for his help in the lab, for his critical advice on my D.Phil. application, and for being such a great friend. His smart girlfriend Jing Zhang for her advice, disagreement and criticism.

I would like to thank the rest of the Ottawa Health Research Institute for making my stay a very enjoyable one.

My best friendsYan Zhong and Yang Li for being with me during those difficult times.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVATIONS

20β-S: 17, 20β, 21-trihydroxy-4-pregnen-3-one

AF: transactivation function domain

ATP: Adenosine 5'-triphosphate

BSA: bovine serum albumin

cAMP: Adenosine 3',5'-cyclic monophosphate

DBD: DNA binding domain

ER: estrogen receptor

ERα: estrogen receptor α

ERK: extracellular signal-regulated kinase

FPH: frog pituitary homogenate

GFP: green fluorescent protein

Gi: inhibitory G-protein

G_S: stimulatory G-protein

GpCR: G protein-coupled receptor

GpCR*: constitutively-activated GPCR

GST: glutathione S-transferase

GV: germinal vesicle

GVBD: germinal vesicle breakdown

HBD: hormone binding domain

hCG: human chorionic gonadotropin

hPR: human progesterone receptor

IGF-1R: insulin-like growth factor-1 receptor

MAPK: mitogen-activated protein kinase

MAPKK: MAPK kinase

MAPKKK: MAPK kinase kinase

MEK1: mitogen-activated or extracellular signal-regulated protein kinase 1 (a MAPK kinase)

MEKK1: MEK kinase 1 (a MAPK kinase kinase)

MI: meiosis I

MII: meiosis II

MPF: maturation promoting factor

MT: Myc tag

mPR: membrane progesterone receptor

NLS: nuclear localization signal

PI3-K: phosphatidylinositol-3 kinase

PKA: protein kinase A

PR: progesterone receptor

SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis

Shc: Src homology 2/α-collagen-related

SH2: Src homology 2

SH3: Src homology 3

siRNA: small interfering RNA

Src: steroid receptor coactivator

UTR: untranslated region

XPR: *Xenopus* progesterone receptor

XPRo: oocyte-specific XPR

INTRODUCTION

GONADOTROPIN-INDUCED FROG OOCYTE MATURATION AND OVULATION

A fully-grown frog oocyte is physiologically arrested at the G2-like stage (or prophase I) of the meiotic cell cycle with an intact nucleus named germinal vesicle (GV), and is enclosed in a follicle envelope before meiotic maturation and ovulation. Electron microscopy study of the follicular structure in Northern leopard frog (*Rana pipiens*) reveals that there are four tissue/cell layers outside the oocyte: the surface epithelium, the theca layer, the follicle cell layer, and the vitelline membrane. Both the follicle cell layer and the oocyte are anchored to the vitelline membrane by numerous processes (named macrovilli and microvilli, respectively) extending from their cell bodies (Smith et al., 1968).

After hormone stimulation, the GV migrates to the animal pole of the oocyte and undergoes germinal vesicle breakdown (GVBD) (Fig.1 D, E). The migrated GV disperses the pigment granules underneath the animal hemisphere and generates a "white spot" on the animal pole, which is routinely used as a marker of GVBD and initiation of oocyte maturation (Fig. 1C). After GVBD, the oocyte resumes meiosis I, extrudes the first polar body, and finally reaches metaphase II when it is arrested again. The transition from prophase I arrest to metaphase II arrest is called oocyte maturation, since this process transforms an immature oocyte to a mature egg that is able to fuse with a sperm and generate a zygote (Masui and Clarke, 1979 and Fig.1A).

Figure 1: Frog oocyte maturation

A) Schematic view of oocyte maturation in *X. laevis*. A fully-grown *Xenopus* oocyte is arrested at the G2-like stage (prophase I) of the meiotic cell cycle. After hormone stimulation, the oocyte undergoes GVBD, resumes meiosis I, produces the first polar body, and is arrested again at the metaphase of the meiosis II. Sperm binding will release this meiosis II arrest and the fertilized egg will then start mitosis and embryogenesis.

B) Light microscope picture of several fully-grwon oocytes before hormone stimulation. The darker side of the oocyte is the animal hemisphere, and the lighter side of the oocyte is the vegetal hemisphere.

C) Light microscope picture of several GVBD oocytes.

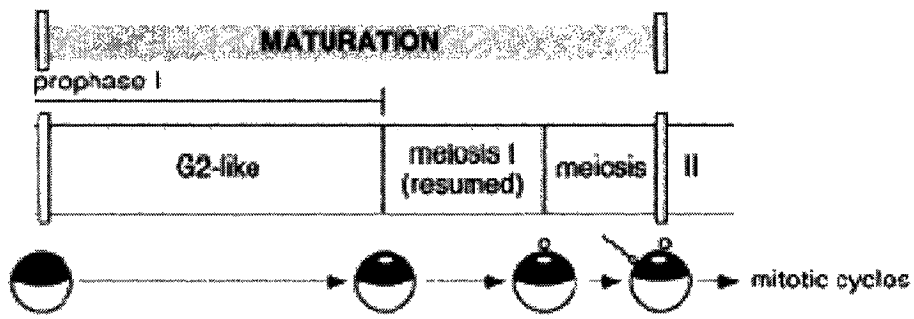
D) The cross-section of a GV oocyte through the animal-vegetal axis; the GV is indicated.

E) The cross-section of a GVBD oocyte through the animal-vegetal axis.

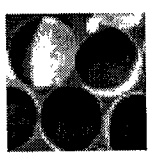
A) B) C) were modified from Ferrell, 1999. **D) E)** were modified from Hausen and Riebesell, 1991.

GVBD

A


MATURATION
prophase I
G2-like
meiosis I (resumed)
meiosis II
mitotic cycles

B

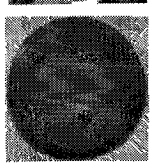


C

D


GV

E



Accompanying the oocyte maturation process, the three somatic tissue/cell layers (surface epithelium, theca, and follicle cell layer) will be separated from the maturing oocyte, and following a localized disintegration on a region of the follicle envelope (theca and peritoneal surface epithelium), the oocyte surrounded by the acellular vitelline membrane will be extruded into the body cavity. The follicle cell layer that is attached to the vitelline membrane in the preovulatory follicle will become detached during this process and remains within the empty ovulated follicle envelope (Schuetz and Lessman, 1982). This process by which a mature egg is released from ovary is called ovulation. It is known from early studies that amphibian ovulation is transcription-dependent and is blocked by transcription inhibitors such as actinomycin D (Yatvin and Pitot, 1969).

The physiological factors triggering oocyte maturation and ovulation are gonadotropins, which are heterodimeric glycoproteins secreted by the pituitary gland. Early studies using frog pituitary homogenate (FPH) demonstrated that frog gonadotropin could induce oocyte maturation and ovulation in frog ovary fragments *in vitro* (Heilburnn et al., 1939) and later studies found that human chorionic gonadotropin (hCG) had similar effect (Thibier-Fouchet et al., 1976).

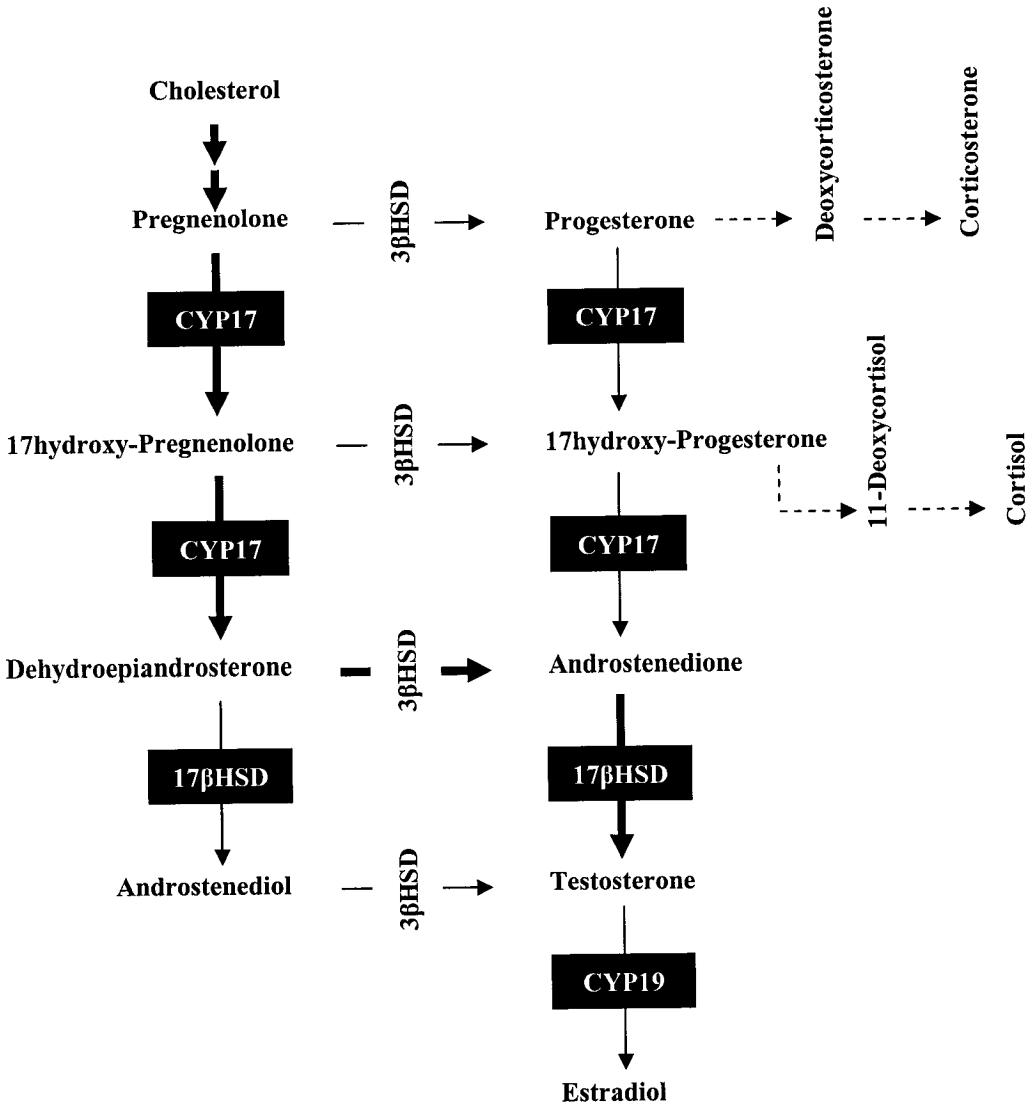
Amphibian follicles have basal steroidogenesis activity in the absence of gonadotropin stimulation, which is demonstrated by the facts that (1) basal secretion of steroids is detected in isolated follicles or perifused ovarian explants (Fortune,

1983; el-Zein et al., 1988) and (2) intact follicles or isolated follicle cells can convert exogenously-supplied pregnenolone and progesterone into their downstream metabolites (Schatz eand Ziegler, 1979). Gonadotropin stimulation causes an acute induction of steroidogenesis in frog ovary, whose dynamics and developmental pattern have been studied in detail. When continuous gonadotropin stimulation is given to perifused African clawed frog (*Xenopus laevis*) ovary explants, testosterone and androstenedione increase abruptly and then slowly decline, while progesterone and estradiol levels increase steadily (el-Zein et al., 1988). Study on follicles of different developmental stages, which coexist in the ovary, reveals that after gonadotropin stimulation, progesterone and testosterone are mainly secreted by stage V and VI follicles, while the secretion of 17β-estradiol peaks in medium-sized follicles (stage III and IV) (Fortune, 1983). Although most studies focus on progesterone, androgen, and estrogen, there is evidence to indicate that corticosterone and cortisol are also secreted by amphibian ovary (Gobbetti and Zerani, 1993). Fig. 2 is the summary of amphibian ovarian steroids biosynthesis pathways.

It was first thought that gonadotropin acted directly on the oocyte since manually "defolliculated" oocytes still responded to FPH and underwent GVBD, albeit with less efficacy compared to follicle-enclosed oocytes (Heilburnn et al., 1939; Dettlaff et al., 1964; Schuetz, 1967b). Later it was found that the follicle cell layer was still attached to manually-isolated oocytes (Masui, 1967; Smith et al., 1968), and the complete removal of this layer of somatic cells abolished the effect of gonadotropin

Figure 2: Ovarian steroid biosynthesis pathway

The sex steroid synthesis pathway, including several key enzymes, is shown. The thick arrows indicate the dominant steroidogenesis pathway in fully-grown amphibian follicles. The thin arrows indicate minor pathways. The dashed arrows indicate the putative synthesis of corticosterone. CYP17: 17 alpha-hydroxylase/17,20 lyase. 17βHSD: 17β-hydroxysteroid dehydrogenase. 3βHSD: 3β-hydroxy steroid dehydrogenase. CYP19: Aromatase. Modified from Yang et al., 2003; Gobbetti and Zerani, 1993.


Cholesterol
Pregnenolone
3βHSD
Progesterone
Deoxycorticosterone
Corticosterone
CYP17
CYP17
17hydroxy-Pregnenolone
3βHSD
17hydroxy-Progesterone
11-Deoxycortisol
Cortisol
CYP17
CYP17
Dehydroepiandrosterone
3βHSD
Androstenedione
17βHSD
17βHSD
Androstenediol
3βHSD
Testosterone
CYP19
Estradiol

on isolated oocytes (Masui, 1967). It was also found that gonadotropin was able to induce maturation of follicle cell-free oocytes packed together with empty follicular tissue, which strongly suggested that gonadotropin acted on follicular somatic cells and generated some intermediate factor, which then acted on the oocyte and induced meiotic maturation (Masui, 1967). Several lines of evidence suggest that this intermediate factor is steroid(s) secreted after gonadotropin stimulation. Progesterone, androgen (androstenedione, testosterone), and glucocorticoid (deoxycorticosterone, cortisol) - but not estrogen - are potent inducers of meiotic maturation in isolated oocytes (Schuetz, 1967a; Schorderet-Slatkine, 1972; Morrill and Bloch, 1977; Lin and Schuetz, 1983). In addition, steroidogenesis inhibitors block the gonadotrophic induction of maturation in follicle-enclosed oocytes in *R. pipiens* (Wright, 1971; Snyder and Schuetz, 1973) and *X. laevis* (Fortune et al., 1975).

In addition to their role in oocyte maturation, ovarian steroids are also suggested to be the principal mediator of the amphibian gonadotropin-induced ovulation. All the major ovarian steroids, with the exception of estrogen, are potent inducers of ovulation (Burgers and Li., 1960; Wright, 1961; Morrill and Bloch, 1977). Furthermore, steroidogenesis inhibitor also blocks the gonadotropin-induced amphibian ovulation (Synder and Schuetz, 1973).

STEROID-INDUCED FROG OOCYTE MATURATION

Overview

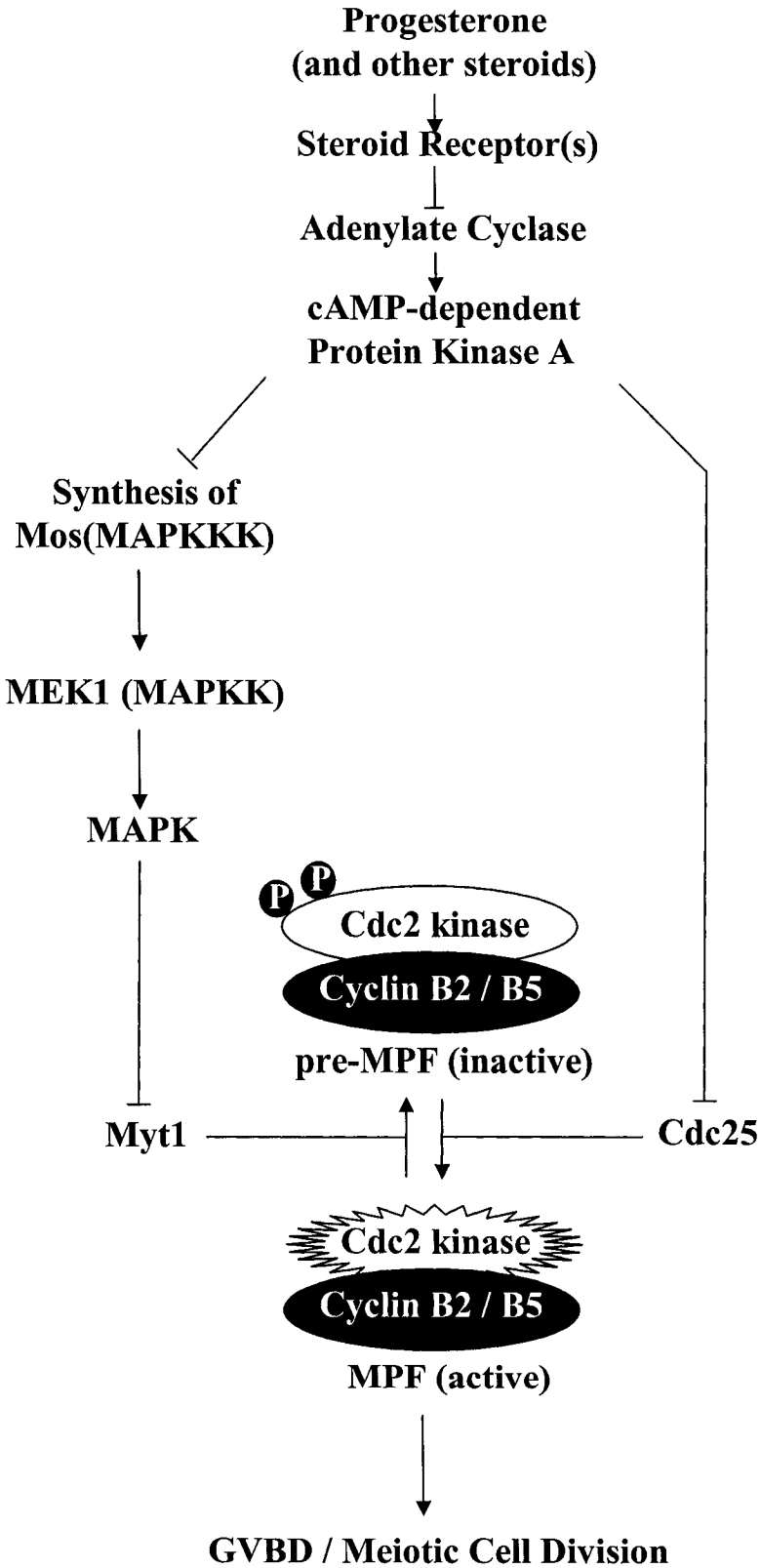
The classical theory of steroid hormone states that steroids exert their physiological effects by binding to their intracellular receptors, which then concentrate in the nucleus and modulate transcription of a subset of genes. Compared to the rapid cellular signaling initiated by ligands for surface receptors such as G protein-coupled receptors (GpCRs) or receptor tyrosine kinases, the typical effects of steroid hormones are characterized by a specific delay reflecting the time for the expression of target genes, and sensitivity to transcriptional inhibitors such as actinomycin D (Tsai and O'Malley, 1994). This simplistic model of steroid action, however, is giving way to a more dynamic paradigm in which another level of complexity is added to the original model, that is, steroids can induce rapid, transcription-independent cellular signaling events through their classical receptors, their novel membrane receptors, or other receptors whose natural ligands are not steroids (Norman et al., 2004).

Extensive studies have been done to understand the steroid-induced frog oocyte maturation, which is probably the most classical example of the non-genomic effects of steroids (Hammes, 2004). The non-genomic nature of this process was firmly established more than 30 years ago when Yoshio Masui showed in his seminal study, which led to the discovery of maturation-promoting factor (MPF), that after physical removal of GV, the remaining enucleated oocyte still responded to progesterone and generated MPF activity (Masui and Markert, 1971). Apart from this, it was also found

that (1) progesterone-induced oocyte maturation was not blocked by transcription inhibitors such as actinomycin D and α-amanitin (Schuetz, 1967b; Wassermann and Masui, 1974) and (2) progesterone stimulation triggered a series of cellular effects too rapid to involve transcription, such as inhibition of membrane-bound adneylate cyclase activities (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981), calcium influx (Wasserman et al., 1980), phospholipids methylation (Godeau et al., 1985) and 1,2-diacylglycerol / inositol 1,4,5-trisphosphate increase (Chien et al., 1991).

It is thought that progesterone induces a reduction of the protein kinase A (PKA) activity through the reduction of cAMP, which then activates two parallel pathways that converge on the activation of MPF, a heterodimer of B-type cyclin and Cdc2 kinase (Fig. 3). One branch induces activation of Aurora-A kinase and the translation of mRNA encoding Mos, a mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK). Mos induces activation of MEK1 (a MAPK kinase) and MAPK, which then leads to the inhibition of Myt1, the kinase responsible for the inhibitory phosphorylation of Cdc2 on threonine 14 and tyrosine 15. The other branch leads to the activation of Cdc25, which then dephosphorylates and activates Cdc2 (Duckworth et al., 2002; Schmitt and Nebreda, 2002; Maller, 2003). The activated MPF then initiates GVBD (Masui, 2001). It is helpful to note that two components in this signaling cascade, MAPK and cyclinB2, are phosphorylated just before GVBD and are routinely used as biochemical markers for oocyte maturation in our laboratory. The molecular identity of the steroid receptor(s) responsible for oocyte maturation

Figure 3: Steroid-induced oocyte maturation
A schematic representation of steroid signaling pathways involved in the steroid induction of meiotic cell division in *X. laevis*. Modified from Duckworth et al. 2002; Schmitt and Nebreda, 2002; and Maller, 2003.


Progesterone
(and other steroids)
Steroid Receptor(s)
Adenylate Cyclase
cAMP-dependent
Protein Kinase A
Synthesis of
Mos(MAPKKK)
MEK1 (MAPKK)
MAPK
P
P
Cdc2 kinase
Cyclin B2 / B5
pre-MPF (inactive)
Myt1
Cdc25
Cdc2 kinase
Cyclin B2 / B5
MPF (active)
GVBD / Meiotic Cell Division

remains controversial. Although, as mentioned before, a broad spectrum of ovarian steroids can trigger oocyte maturation in frog, progesterone is assumed to be the physiological steroid (Hammes, 2004) and hence most research efforts have been focused on the identification of the receptor for progesterone-induced oocyte maturation. It should be noted that a recent report suggested that androgen might be the primary maturation-inducing hormone *in vivo*. However, the same report also provided evidence to support an independent role of progesterone in oocyte maturation because progesterone induced GVBD even when its conversion to androgen was blocked by steroidogenesis inhibitor ketoconazole (Lutz et al., 2001).

The Surface Progesterone Receptor Hypothesis

For decades, progesterone was thought to act on some cell surface receptor to trigger oocyte maturation. This theory is supported by several lines of evidence. Firstly, progesterone inhibits adneylate cyclase activities in membrane-enriched high speed pellet from oocyte homogenate or in manually-dissected plasma membrane preparation (Finidori- Lepicard et al., 1981; Sadler and Maller, 1981), and this inhibition is suggested to be responsible for the reduction of cAMP level in intact oocytes after progesterone stimulation (Maller et al., 1979; Cicirelli and Smith, 1985; Cork et al., 1990). This is probably the best evidence to support the membrane action of progesterone in oocyte maturation because it is well established that high level of cAMP is responsible for the maintenance of the meiotic arrest in quiescent frog oocytes (Maller and Krebs, 1977; Bravo et al., 1978; Maller et al., 1979; Schorderet-

Slatkine and Baulieu, 1982; Cork et al., 1990; Daar et al., 1993; Gallo et al., 1995; Duckworth et al., 2002; Romo et al., 2002). In addition, cortisone and testosterone, which are also potent inducers of oocyte maturation, have similar inhibitory effect on the membrane-bound adenylate cyclase activities (Finidori- Lepicard et al., 1981).

Secondly, aqueous progesterone solution is ineffective in triggering oocyte maturation when microinjected into the oocyte, although it induces GVBD when applied extracelluarly (Smith and Ecker, 1969; Masui and Markert, 1971). In addition, steroid conjucated to agarose beads, polymer, or BSA induces oocyte maturation when applied extracellularly (Ishikawa et al., 1977; Godeau et al., 1978; Bandyopadhyay et al., 1998). All these results suggest that progesterone acts on the cell surface, but several factors need to be considered in the interpretation of this body of data. First of all, injection of progesterone dissolved in paraffin oil does induce oocyte maturation (Tso et al., 1982), and work in our lab shows that injection of progesterone dissolved in ethanol also induces 100% GVBD (Bayaa et al., 2000). Furthermore, steroid conjugates always have the problem of free steroid contamination; actually, a report shows that free estradiol in the BSA-estradiol conjucate is indeed responsible for its biological activity (Stevis et al., 1999).

Thirdly, several ligand binding studies suggest the existence of specific progestin-binding sites on frog oocyte plasma membrane (Sadler and Maller, 1982; Kostellow et al., 1982; Blondeau and Baulieu, 1984; Liu and Patino, 1993). The K_d values or the

molecular weights of the progesterone-binding proteins vary from study to study. However, binding studies can be misleading. For example, early studies found that progesterone bound specifically to the melanosomes located beneath the oocyte animal hemisphere, and a "melanosome receptor" was then proposed (Jacobelli et al., 1974; Drury and Ozon, 1975). However, later studies found that oocytes from albino mutant of *X. laevis*, which completely lacked melanosomes, still responded to progesterone and underwent GVBD (Smith, 1989), and the melanosome component that bound progesterone was eumelanin rather than a protein (Coffman et al., 1979).

A recent study in sea trout oocytes is believed to give new support to the oocyte surface receptor theory (Maller, 2003). Using a receptor capture assay to screen for protein that binds 17,20β, 21-trihydroxy-4-pregnen-3-one (20β-S), the natural inducer of fish oocyte maturation, Zhu et al. eventually obtained a cDNA from sea trout ovarian tissue encoding a putative GpCR, which was designated membrane progestin receptor (mPR). mPR transcript is restricted to the reproductive and neuroendocrine tissues, where most non-genomic effects of steroids are found. Notably, immmuocytochemical localization of mPR in oocyte and sperm is restricted to plasma membrane. mPR-transfected tissue culture cells respond to 20β-S and elicit intracellular signaling pathways, and the inhibition of the 20β-S signaling by pertussis toxin suggests that mPR is coupled to an inhibitory G protein (Gi). The mPR mRNA and protein level in ovarian tissue is up-regulated by 20β-S and gonadaotropin stimulation, and the mPR protein level in fish oocytes peaks in maturing oocytes and

then decline greatly in ovulated oocytes. Most importantly, antisense oligos against a zebrafish homolog of mPR significantly inhibit steroid-induced oocyte maturation in zebrafish (Zhu et al., 2003a). In addition, Zhu et al. reported the cloning of mPR homologs in other species including *X. laevis* (Zhu et al., 2003b). However, whether mPR plays any roles in *Xenopus* oocyte maturation remains unknown.

The Classical Progesterone Receptor Hypothesis

Alternatively, progesterone is thought to act through its classical receptor (PR) by a non-genomic mechanism (Liu and Ruderman, 2003). Results supporting this theory mainly come from two studies by our group and that of Dr. Ruderman, which independently reported the cloning of a classical progesterone receptor (designated XPR-2 and XPR-1, respectively) from *Xenopus* ovary and also presented multiple lines of evidence to support its role in mediating progesterone action in oocyte maturation (Bayaa et al., 2000; Tian et al., 2000; Liu and Ruderman, 2003). Subsequently another study suggests that XPR may exert its non-genomic effect by interacting with MAPK and phosphatidylinositol-3 kinase (PI3-K) (Bagowski et al., 2001). Since these three papers are immediately related to my research project, they will be further reviewed following brief introduction about (1) the structure, phosphorylation and subcellular localization of the classical progesterone receptor in other model systems and (2) the non-genomic effect mediated by classical estrogen and progesterone receptor in mammalian cells.

CLASSICAL PROGESTERONE RECEPTOR

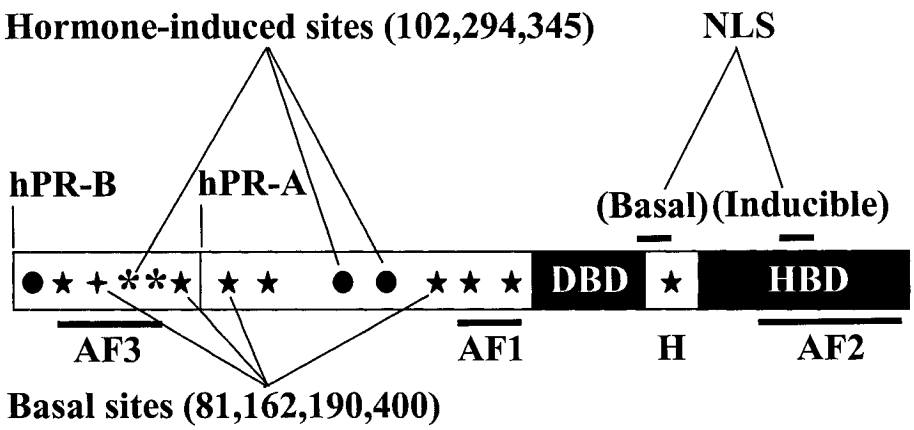
Overview

Classical progesterone receptor (PR) is a progesterone-regulated nuclear transcription factor. It is a member of the nuclear receptor superfamily, which also includes receptors for a wide range of ligands such as estrogen, androgen, vitamin D3 and bile acids, as well as a number of "orphan" receptors whose physiological ligands and functions are poorly understood (Tsai and O'Malley, 1994; Robinson-Rechavi et al., 2003). In chicken, rodent and human, two PR isoforms (termed PR-A and PR-B) are expressed from a single gene as the result of transcription from two alternative promoters, and translation from two alternative start codons. PR-A and PR-B differ only in that PR-B contains an additional sequence of 164 amino acids at its amino terminus which encodes a transactivation function domain (AF3) that is specific to PR-B (Conneely et al, 2003).

Domain Structure

The overall structural features of PR (full-length PR-B) are well defined (Fig. 4). The N terminal or A/B region of PR is the most hypervariable region which contains transactivation function domains (AF1 and AF3) as well as an inhibitory domain responsible for the recruitment of transcriptional inhibitory co-repressor proteins. AF1 and AF3 recruit co-activator proteins to the receptor to modulate the level and promoter specificity of target gene activation. In the middle is the DNA-binding domain (DBD) that is approximately 66–68 amino acids long and contains two type II

Figure 4: Domain structure and phosphorylation sites of human PR
Schematic representation of the human progesterone receptor. Shown here are the functional domains or motifs: AF, transactivation function; DBD,DNA binding domain; HBD, hormone binding domain; NLS, nuclear localization signal; H, hinge domain. The translation start points of A/B isoforms are indicated. 14 identified phosphorylation sites and their possible *in vivo* or *in vitro* kinases are also shown. Adapted from Ylikomi et al. 1992 and Lange 2004.



.....

● MAPK consensus sites (20,294,345)

★ CDK2 sites (25,162,190,213,400,430,554,676)

+ CK II site (81)

* Unknown kinases (102,130)

zinc finger structures. The zinc fingers facilitate binding of the receptor to specific *cis*-acting DNA sequences and are the hallmark of the nuclear receptor superfamily. DBD is followed by a variable hinge region, which contains a basal nuclear localization signal (NLS). A highly conserved hormone-binding domain (HBD) is located at the C terminal of PR. In addition to its ligand-binding function, HBD contains an additional transactivation function domain (AF-2) required for hormone-dependent co-activator recruitment, a hormone-inducible NLS, and sequences important for interaction of the inactive receptor with heat shock proteins and for receptor dimerization (Ylikomi et al., 1992; Tsai and O'Malley, 1994; Conneely et al., 2003)

Subcellular Localization

Immunocytochemical studies in chicken, rabbit, guinea pigs and human cells demonstrate that the predominant portion of PR is localized in the nucleus even in the absence of progesterone binding (Gasc et al., 1984; Perrot-Applanat et al., 1985 and 1987). Mechanistic studies reveal a dynamic situation of PR localization: Nuclear PR is continuously exported into the cytoplasm and then constantly and actively transported back into the nucleus; however, at any given time the majority of the protein is in the nucleus (Guiochon-Mantel et al., 1991). A basal NLS is identified within the hinge region; when this NLS is deleted, the receptor becomes cytoplasmic but is imported into the nucleus after the addition of progesterone (Guiochon-Mantel et al., 1989). This ligand-induced nuclear translocation is explained by the existence

of a second, hormone-inducible NLS within the DBD (Ylikomi et al., 1992). The import of PR is an energy-consuming process which can be blocked by ATP synthesis inhibitor, while the export is not (Guiochon-Mantel et al., 1991).

Phosphorylation

PR is extensively phosphorylated by multiple protein kinases; phosphorylation occurs primarily on serine residues and most of the phosphorylation sites (13 out of 14) are located within the N terminal region (Fig. 4). Ser81, 162, 190, and 400 are defined as "basal" sites, which are constitutively phosphorylated in the absence of hormone. Ser102, 294, and 345 are hormone-induced sites that are maximally phosphorylated 1～2 hours after progestin treatment. Specific kinases responsible for phosphorylation of selected sites have been identified. For example, Ser81 and 294 have been demonstrated to be phosphorylated by casein kinase II and MAPK, respectively. Eight of the total 14 sites (Ser25, 162, 190, 213, and 400; Thr430, 554, and 676) have been demonstrated to be phosphorylated by cyclin-dependent kinase 2 *in vitro* and five of these sites (Ser162, 190, 213, 400; Thr676) have been confirmed as authentic *in vivo* phosphorylation sites (Lange, 2004).

The functional consequence of PR phosphorylation is best understood in the case of Ser294. Mutation of Ser294 into alanine, treatment with MEK (MAPK kinase) inhibitors, or treatment with 26S proteasome inhibitor lactacystin, all block ligand-induced PR degradation in T4AD breast cancer cells, suggesting that

phosphoryaltion of Ser294 by MAPK is required for PR protein turnover (Lange et al., 2000). In addition, MEKK1 (a MAPKKK) overexpression leads to increased MAPK activity, phosphrylation of Ser294, and transcriptional hyperactivity of PR after ligand stimulation, and on the other hand, mutation of Ser294 and treatment with MEK inhibitor or lactacystin all abolish the hyperactivity of PR after MEKK1 overexpression and ligand stimulation, suggesting an intriguing coupling between transcriptional hyperactivity and degradation triggered by phosphorylation of Ser294 (Shen et al., 2001).

A recent study using T47D breast cancer cells suggests a relationship between Ser294 phosphorylation and receptor localization. EGF stimulation triggers phosphorylation of Ser294 and translocation of PR into the nucleus, which is blocked by Ser294 point mutation or MEK inhibitor. By contrast, ligand stimulation also causes fast phosphorylation of Ser294 and nuclear translocation of PR, but this phosphorylation is not blocked by MEK inhibitor, and the translocation is not blocked by Ser294 point mutation. In addition, MEK inhibitor alone causes nuclear accumulation of PR, and Ser294 mutant PR persists in the nucleus after ligand-induced translocation while wild-type PR gradually degrades. These results collectively suggest a role of Ser294 phosphorylation in PR nucleo- cytoplasmic shuttling, which is probably important for optimal receptor activity (Qiu et al., 2003).

NON-GENOMIC EFFECT OF ESTROGEN AND PROGESTERONE THROUGH THEIR CLASSICAL RECEPTORS

Classical Estrogen Receptor-mediated Non-genomic Effect of Estrogen

Extensive research has done to understand the non-genomic effect of estrogen in breast cancer cells. Estradiol induces a rapid and transient activation of the tyrosine kinase/Ras/MAPK pathway in human breast cancer MCF-7 cell line (Migliaccio et al., 1996), and the involvement of classical estrogen receptor α (ERα) is supported by substantial evidence. First of all, the effect of estradiol in MCF-7 cells is blocked by ER antagonist, and transfection of ERα in COS-7 cells reconstitutes the rapid response to estradiol (Migliaccio et al., 1996). It is also found that after estradiol stimulation, there is a physical association between ERα and Shc (Song et al., 2002), a protein required for the activation of Ras/MAPK pathway (Lennartsson et al., 1999). A recent study shows that in MCF-7 cells estradiol induces the formation of ERα/Shc/IGF-1R complex detected by immunoprecipitation assay, and the significance of this complex *in vivo* is demonstrated by three experiments using well-controlled siRNA against ERα, Shc, and IGF-1R: siRNA ablation of Shc abolishes the ERα/IGF-1R association and estradiol-induced ERα translocation to the membrane; siRNA ablation of IGF-1R also inhibits ERα translocation; and the siRNA ablation of ERα, Shc, or IGF-1R all block the estradiol-induced MAPK activation (Song et al., 2004). This study is consistent with an earlier report showing that in COS-7 or HEK 293 cells which express endogenous IGF-1R, estradiol stimulation after transfection of ERα induces rapid phosphorylation of IGF-1R and ERK 1/2, and

there is a rapid association of ERα and IGF-1R in both COS cells and IGF-1R-depleted cells transfected with ERα and IGF-1R (Kahlert et al., 2000).

Classical Progesterone Receptor-mediated Non-genomic Effect of Progesterone

Some studies provide evidence to indicate the involvement of classical PR in non-genomic signaling. Progestin stimulates human breast cancer T47D cells proliferation and rapidly activates the Src/Ras/ERK pathway, which is blocked by both anti-progestin and anti-estrogen. Furthermore, in COS-7 cells transfected with PR-B, progestin-induced MAPK activation depends on co-transfection of ER, and a PR-B mutant lacking transcription activities shows the same effect. In this study it is also found that PR-B does not interact directly with Src but with ER, which then binds to the SH2 domain of Src (Migliaccio et al., 1998). A recent study further supports the idea of ER-mediated PR action by identifying in PR two ER interaction domains (ERID-I and ERID-II) that are required for the interaction of PR with ER (Ballare et al., 2003).

An independent role of PR in Src activation is established by the identification of a proline-rich motif in PR which mediates the direct interaction of PR with the SH3 domain of Src and other SH3 containing proteins. Mutation of three critical prolines within this PR motif abolishes the association of PR with SH3 domain in GST- pull down assay. Similarly, a synthetic peptide containing this motif inhibits PR/SH3 interaction. Furthermore, PR becomes associated with Src within 5 minutes after

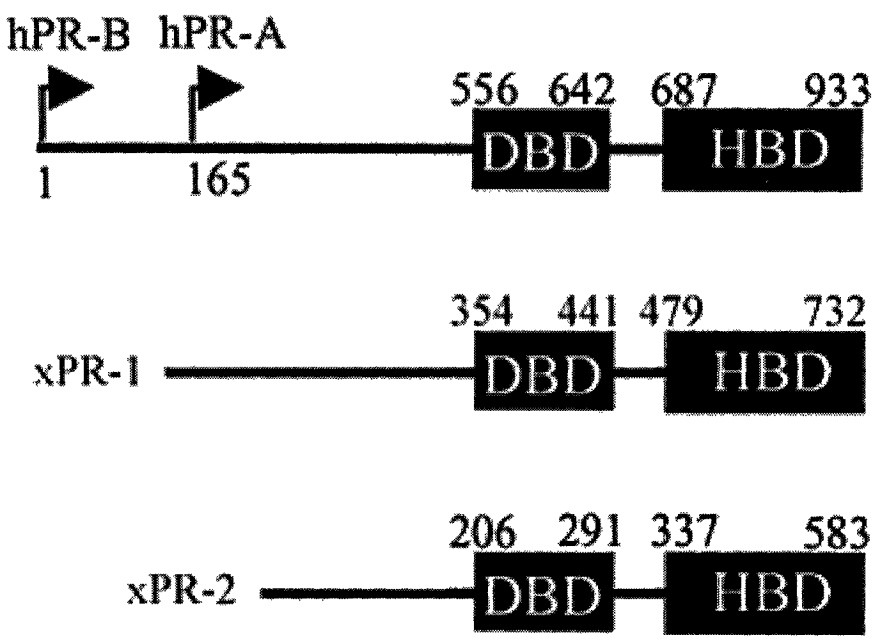
progesterone stimulation in T4AD cells. In MCF-12A cells which lack endogenous PR, transfection of the wild type PR reconstitutes progesterone-induced Src activation but transfection of the SH3-binding mutant PR does not (Boonyaratanakornkit et al., 2001).

XENOPUS CLASSICAL PROGESTERONE RECEPTOR

Bayaa et al. cloned the first *Xenopus* progesterone receptor, xPR (Bayaa et al., 2000). xPR encodes 583 amino acids with a predicated molecular weight of 66kDa (Fig.5). xPR is hereafter referred to as XPR-2, in keeping with the nomenclature of another distinct progesterone receptor, XPR-1 (Liu and Ruderman, 2003). A polyclonal antibody against N-terminus of XPR-2 recognizes the overexpressed Myc-tagged XPR-2, and it also recognizes a prominent endogenous band which is believed to represent the endogenous XPR-2 in oocyte. The mRNA of XPR-2 can be amplified from RNA preparation of oocytes from stage I to stage VI, and the endogenous XPR-2 band is also present in oocyte lysate from different stages. Furthermore, the endogenous XPR-2 is found to be extra-nuclear and is not associated with any membrane structure. Progesterone stimulation does not alter the subcelluar localization of endogenous XPR-2.

XPR-2 functions as a progesterone-regulated transcription factor in COS cells and has a normal nuclear localization in COS cells revealed by anti-Myc tag immunostaining. The transcriptional activity of XPR-2 in COS cells is suppressed by high concentration (50μM) of RU486, which is a common antagonist of human PR. Interestingly, RU486 is also able to induce (rather than suppress) oocyte maturation at similar concentrations. Overexpression of XPR-2 potentiates progesterone-induced oocyte maturation in a transcription-independent manner, and also accelerates progesterone-induced MAPK activation in enucleated oocytes. These data lead Bayaa

Figure 5: Domain structure of XPR-1 and XPR-2
Schematic comparison of the hPR (accession no. M151716), XPR-1 (accession no. AF279335) and XPR-2 (accession no. AY007198; originally named xPR). The DBD and HBD of the three PRs were indicated. Modified from Bayaa et al., 2000 and Tian et al., 2000.


hPR-B
hPR-A
556
642
687
933
1
165
DBD
HBD
354
441
479
732
xPR-1
DBD
HBD
206
291
337
583
xPR-2
DBD
HBD

et al. to conclude that XPR-2 is the long-sought after non-genomic progesterone receptor in frog oocytes (Bayaa et al., 2000).

Tian et al. independently cloned a *Xenopus* PR gene named XPR-1, which is 732 amino acids long and has a predicted molecular weight of 82 kDa (Fig.5). Similar to XPR-2, XPR-1 can function as a progesterone-regulated transcription factor, and potentiate progesterone-induced oocyte maturation. In addition, antisense oligos targeted to different regions of XPR-1 sequence block the translation of microinjected XPR-1 mRNA, and inhibit the progesterone-induced oocyte maturation. Subsequent injection of XPR-1 mRNA rescues progesterone response (Tian et al., 2000).

XPR-2 and XPR-1 are highly homologous in the DBD and HBD region but more variable in the N-terminal region. The difference between XPR-2 and XPR-1 indicates that they are two non-allelic copies of the *Xenopus* progesterone receptor gene (Liu and Ruderman, 2003). This phenomenon is common in many *X. laevis* genes due to its pseudotetraploid genetic makeup (Graf, 1991).

In support of the role of classical PR in frog oocytes, Bagowski et al. shows that antibodies against human PR (C-20) detect two immuno-reactive bands (82kDa and 110 kDa) in total *Xenopus* oocyte lysate, which they believe to represent the two *Xenopus* PR isoforms homologous to human PR-A (94kDa) and PR-B (116kDa), respectively. The "p110XPR" is exclusively localized in the cytoplasm while part of

the "p82XPR" is recovered in the membrane fraction. Progesterone (but not glucocorticoid or RU486) causes the appearance of PI3-K activity in C-20 immunoprecipitate of oocyte lysate; this activity increases modestly after 3.5hour and inhibition of this activity by wortmannin delays progeseterone-induced oocyte maturation. In addition, MAPK activity is also recovered in C-20 immunoprecipitate of GVBD oocytes, and activated MAPK phosphorylates an 110kDa protein *in vitro*, which is assumed to be "p110XPR" (Bagowski et al., 2001).

The objective of my M.Sc. research was to further investigate the functional roles of endogenous XPR proteins in frog oocyte maturation and ovulation. These studies revealed two forms of XPR proteins in the ovary. XPR-1, so called because it had identical apparent molecular mass (~82 KDa) as XPR-1 mRNA-derived protein, was found in the somatic follicle cells but not in the oocytes. In contrast, XPRo (oocyte-specific XPR) was found only in the oocytes and not in the follicle cells. When follicle cells were treated with progesterone, XPR-1 underwent proteasome-mediated degradation. In contrast, when oocytes were treated with progesterone, endogenous XPRo, over-expressed XPR-1, and over-expressed XPR-2 all underwent hyperphosphorylation. The differential biochemical changes of endogenous XPR-1 and XPRo correlated, respectively, with the genomic (transcription-dependent) action of progesterone in ovulation and the non-genomic (transcription-independent) action of progesterone in oocyte maturation.

MATEARIALS AND METHODS

Chemicals and Antibodies

α-manitin, lactacystin, and U0126 were purchased from Calbiochem. Sytox Green was purchased from Molecular Probes. Calcium ionophore A23187, cholera toxin, actinomycin D, collagenase type I, human chorionic gonadotropin (hCG), and pregnant mare serum gonadotropin (PMSG) were purchased from Sigma.

Rabbit antibody against XPR was raised by immunizing rabbits with a purified GST fusion protein containing XPR amino acids 1–215 by Mr. M. Bayaa in our laboratory. Rabbit anti-MAPK serum was produced in house with a coupled peptide kindly provided by J. A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, Washington). Sheep anti-cyclin B2 serum was kindly provided by J. L. Maller (University of Colorado School of Medicine, Denver, Colorado). Rabbit antibody against *Xenopus* nucleolin was a gift of P. J. DiMario (Louisiana State University, Baton Rouge) Mouse antibody against *Xenopus* β-integrin (8C8) was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Mouse antibody against β-tubulin was purchased from ICN Biomedicals. Mouse antibodies against human PR C-19 (sc538) was purchased from Santa Cruz Biotechologies. Mouse antibody against Myc tag and HA tag were produced in house.

Frog Manipulation and Oocyte Isolation

Sexually mature *Xenopus laevis* females were purchased from NASCO and

maintained in 18°C. 3-7 days after gonadotropin priming (PMSG, 50IU per frog), the frog was sacrificed and ovarian fragments were retrieved under hypothermia. Unless specified, stage V or VI oocytes used in this study were manually removed from follicles using Dumont No.5 forceps in OR2 medium (83mM NaCl, 2.5mM KCl, 1mM CaCl2, 1mM MgCl2, 1mMNa2HPO4, 5mM HEPES pH 7.8) minus calcium. Oocytes isolated from this method still contain a layer of follicle cells.

For the experiment dealing with XPRo detection in oocytes from different stages, collagenase method was used because it is too difficult to isolate the smaller (stage I-III) oocytes by manual defolliculation. This was achieved by treating pieces of isolated ovary tissue for 2-3 hours with collagenase solution (2mg/ml in calcium-free OR2 medium, with 1mg/ml soybean trypsin inhibitor) to release oocytes. The oocytes were then individually selected and grouped into various stages as detailed in Smith et al. 1991. Collagenase treatment was also used to obtain follicle-cell free oocytes for chromosome staining experiments.

The medium used for oocyte/ovarian tissue incubation experiments was OR2 medium supplemented with gentamicin (0.1mg/ml).

Expression Plasmids

All the expression plasmids used in this study were constructed by other researchers in the laboratory. The major expression vector used is pCS2+ vector, which contains a

SP6 promoter, a SV40 poly (A) sequence and a NotI restriction site for linearization. Another expression vector used is pSP64TM vector, which contains a SP6 promoter and an EcoRI restriction site for linearization. The information about these plasmids is summarized here:

1) pSP64TM-XPR-2: Encodes XPR-2 (1-583aa), the longest cDNA clone obtained in the cloning of *Xenopus* PR.

2) pCS2+-MT-XPR-2: Encodes five tandem copies of Myc tag followed by XPR-2 insert. The whole fusion protein (Myc-tagged XPR-2) has 565 amino acids and a calculated molecular weight of 72kDa.

3) pCS2+-XPR-1: Encodes the full length XPR-1 insert (1-732aa). The XPR-1 cDNA clone is a gift from J.V. Ruderman (Harvard Medical School, Boston).

4) pCS2+-MT-XPR-2-C: Encodes five tandem copies of Myc tag followed by XPR-2 C terminus (aa269-583). This truncated XPR contains the whole HBD and part of the DBD, including the basal NLS.

5) pCS2+-MT-XPR-2-N: Encodes five tandem copies of Myc tag followed by XPR-2 N terminus (aa1-215). This truncated XPR-2 contains the whole hypervariable N-terminal region and part of the DBD.

6) pCS2+-HA-14-3-3ζ: Encodes a single copy of HA tag followed by *Xenopus* full length 14-3-3 ζ (aa1-245).

7) pCS2+-HA-NLS-14-3-3ζ: Contains a single copy of the HA tag, two copies of the SV40 large T antigen NLS, and then the full length 14-3-3ζ.

8) pGFP-C1: Contains GFP2 insert after the CMV promoter.

***In vitro* Transcription**

The Plasmids were linearized with appropriate restriction enzyme (NotI for pCS2+ plamids and EcoRI for pSP64TM plasmid). *In vitro* transcription was carried out using mMESSAGE mMACHINE® SP6 Kit (Ambion, Austin, TX). The synthesized mRNA was dissolved in nuclease-free water, aliquoted, and stored in -80°C until injection.

Microinjection of mRNA or Other Reagents

Oocyte injection of mRNA or other reagents was conducted in calcium-free OR2 medium and usually 20nl of liquid was injected into each oocyte. For the experiment in which cholera toxin was injected into individual follicl-enclosed oocyte on ovary fragment, a minimum opening was made on the follicle envelope to enable passage of the glass needle. For the experiment in which GFP construct needed to be injected

into GV, oocytes were adjusted individually to make sure its animal pole appeared upmost, and DNA solution was injected after the tip of the glass needle pierced into the middle of the animal pole.

Oocyte Extract Preparation and Western Blotting

Oocytes were forced through a pipette tip and lysed in PBS lysis buffer (10mM sodium phosphate pH 7.5, 150mM NaCl, 0.1% Triton X-100, 10μg/ml of leupeptin, 1mM PMSF, 10μl lysis buffer per oocyte). Following centrifugation (13000g for 5 min), the clarified extract was removed and mixed with 2× reduced SDS sample buffer.

Boiled cell lysate samples were analyzed by SDS-PAGE on 15% polyacrylamide gel. Depending on the experiments being performed the amount of protein loaded on the gel varied as indicated. Usually 1 oocyte was loaded in xPR blot, 1/8 oocyte was loaded in MAPK blot and 1/2 oocyte was loaded in cyclinB2 blot. The immunoblots were developed using an ECL kit (Amersham, Arlington Heights, IL).

***In vitro* Ovulation Assay**

This protocol was modified from Morril and Bloch 1977. Ovary tissue was minced into piceces containing ~50 fully-grown follicles and these ovary fragments were incubated with hCG, steroids, and other reagents. After overnight incubation, the ovary fragment was shaken in the well to release all the ovulated eggs. The ovulated

oocytes, the GVBD oocytes, and the fully-grown oocytes remained on the ovary fragment were then counted and converted to ovulation or maturation ratio. Ovulation ratio is the number of ovulated oocytes divided by the total amount of oocytes on the fragment. Maturation ratio is the number of GVBD oocytes divided by the total amount of oocytes on the fragment.

Enucleation for Western Blotting

This protocol was modified from Heine et al. 1993. A small group of oocytes were put in a Petri dish cotaining Gall's medium plus Mg^{2+} (83mM KCl; 17mM NaCl; 1mM MgCl2; 10mM Tris pH7.2). An opening was made on the animal pole and the GV was gently squeezed out of the oocyte. The isolated GVs were transferred to an Eppendorf tube containing icy nucleus medium (100mM NaAc; pH5.2; 5mM EDTA). After brief centrifugation (2000 rpm, 4°C for 30 seconds) supernatant was carefully aspirated and the nucleus pellet was mixed with 2× reduced SDS sample buffer. The enucleated oocytes were also transferred to tubes and samples were made using the same method as intact oocytes.

Sytox Green Staining

The fluorescence DNA dye, Sytox Green, was used to reveal follicle clells attached to the oocyte surface. Briefly, oocytes were fixed in methanol overnight, rehydrated in 75%, 50%, 25% (v/v) methanol/TBS (Tris Buffer Saline: 10mM Tris, pH 7.5, 150mM NaCl), and washed with 50% TBS. Sytox Green (diluted at 1:10000) was added to

50% TBS followed by overnight incubation at room temperature. Oocytes were then dehydrated in 25%, 50%, 75% (v/v) methanol/TBS and were then transferred to methanol. The surface staining was viewed under the fluorescence microscope.

For chromosome staining a simplified protocol was used. Briefly oocytes were fixed in methanol for 30 minutes, rehydrated in 50% (v/v) methanol/TBS, and then transferred to TBS containing 1:10000 diluted Sytox Green. The meiotic spindle was right beneath the GVBD spot and was viewed under highest magnification of the fluorescence microscope.

Follicle Cell Isolation and Incubation

For experiments in which follicle cells were required, 120 manually "defolliculated" oocytes were stripped in sandpaper-coated Petri dishes containing 6ml OR2 medium for 2 hours. Oocytes were removed and the medium containing follicle cells were aliquoted into Eppendorf tubes, and the tubes were incubated in 18°C incubator after the addition of various reagents. At the end of the experiment, the tubes were centrifuged at 800g for 5 minute to pellet the follicle cells (Yang et al., 2003), the supernatant was carefully aspirated and the pellet was directly mixed with 2× reduced SDS sample buffer for Western blotting.

RESULTS

Mobility Shift of Overexpressed XPR-1 and XPR-2 during Oocyte Maturation

The mobility patterns of overexpressed XPR-1, Myc-tagged XPR-2, and XPR-2 in immature GV oocytes and in mature eggs (after overnight progesterone stimulation) were compared by Western blotting using polyclonal anti-XPR antibodies (Bayaa et al., 2000). Compared to the corresponding bands in GV oocytes, overexpressed XPR-1 (Fig. 6A, lane 3, 4), MT-XPR-2 (Fig. 6B, lane 7, 8), and XPR-2 (Fig. 6C, lane 3, 4) in mature eggs displayed a mobility shift, consistent with protein hyperphosphorylation.

To further characterize the mobility shift of XPR-2, two Myc-tagged XPR-2 truncated mutants, MT-XPR-2-C and MT-XPR-2-N, were overexpressed and their mobility patterns in GV oocytes and mature eggs were compared. Overexpressed MT-XPR-2-N displayed a mobility shift after oocyte maturation (Fig. 6D, lane 3, 4) while the mobility of MT-XPR-2-C did not change (Fig. 6D, lane 1, 2). This is consistent with the suggestion that the mobility shift of XPR-2 is due to phosphorylation because almost all of the reported mammalian PR phospohrylation sites (13 out of 14) are located in the N terminal region (Lange, 2004).

Characterization of XPRo: Mobility Shift, Nuclear Localization, and Expression Pattern

Three prominent bands, I, II and III, were detected in oocyte lysate, when probed with

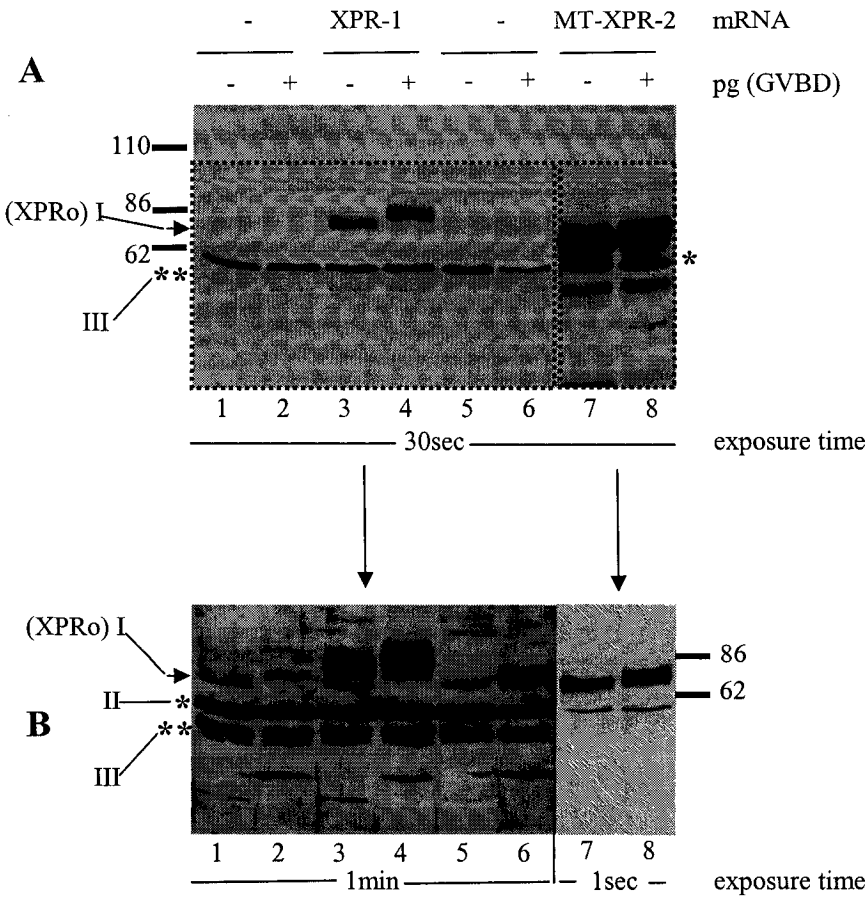
Figure 6: Mobility shift of overexpressed *Xenopus* PR constructs

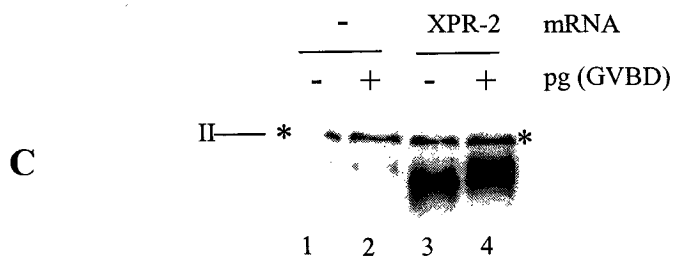
A) Oocytes injected with XPR-1 or MT-XPR-2 mRNA were incubated for 5 hours before addition of 1μM progesterone. After overnight incubation oocytes were lysed and subjected to Western blotting using anti-XPR antibodies.

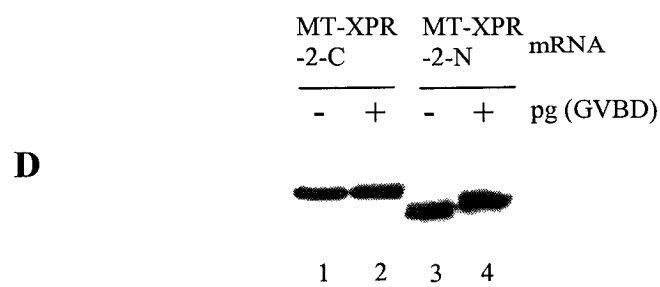
B) lane 1~lane 6 are longer exposure of the same lanes in **A)** and lane 7~lane 8 are shorter exposure of the same lanes in **A)**.

C) Oocytes injected with XPR-2 mRNA were incubated for 5 hours before addition of 1μM progesterone. After overnight incubation oocyte were lysed and subjected to Western blotting using anti-XPR antibodies.

D) Oocytes injected with MT-XPR-2-C or MT-XPR-2-N mRNA were incubated 5 hours before addition of 1μM progesterone. After overnight incubation oocyte were lysed and subjected to Western blotting using anti-Myc tag antibodies.


A
-
XPR-1
-
MT-XPR-2
mRNA
- + - + - + - +
pg (GVBD)
110
86
(XPRo) I
62
**
III
*
1 2 3 4 5 6 7 8
30sec
exposure time
(XPRo) I
II
*
**
III
B
86
62
1 2 3 4 5 6 7 8
1min
1sec
exposure time


-
XPR-2
mRNA
- + - +
pg (GVBD)
II
*
*
C
1 2 3 4


MT-XPR
-2-C
MT-XPR
-2-N
mRNA
- + - +
pg (GVBD)
D
1 2 3 4

anti-XPR antibodies (Fig. 6B, lane 1, indicated by "➔", "*", "**", respectively). The most prominent protein (II) was previously identified as endogenous XPR-2 (Bayaa et al., 2000). However, this protein did not display mobility changes following oocyte maturation. In contrast, band I shifted upwards in mature eggs (Fig. 6B, lane 1, 2 and lane 5, 6), similar to the shift exhibited by all XPR mRNA-derived proteins. These data prompted us to designate this protein as XPRo (oocyte-specific XPR).

The mobility shift of XPRo was characterized in more detail. Time course experiment revealed that the mobility shift of XPRo coincided with the phosphorylation of MAPK and cyclinB2, indicative of MAPK activation and MPF activation, respectively (Fig. 7A). The shifted XPRo was persistent through the meiotic cell cycle (Fig. 7A, 3 hour to 8.5 hour), and remained unchanged after overnight incubation (Fig. 7A, 17 hour). Similar mobility shift of XPRo was also observed in extracts derived from hCG-treated and *in vitro* "ovulated" eggs (Fig. 7B), suggesting that the same phosphorylation probably occurs in physiological oocyte maturation.

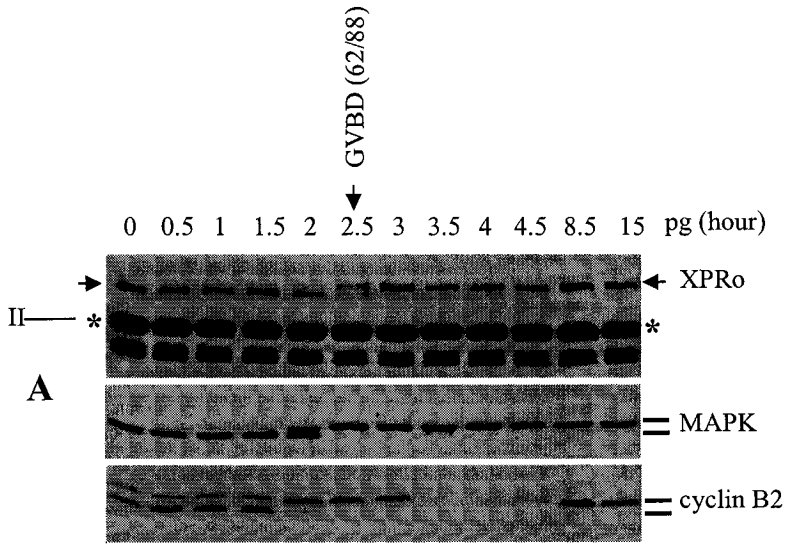
Calcium ionophore A23187 has been shown to trigger parthenogenetic activation of mature amphibian eggs, resulting in biochemical characteristics similar to those induced by sperm binding (Belanger and Schuetz, 1975; Masui et al., 1977). The fully-phosphorylated XPRo in mature egg underwent quick dephosphorylation after the addition of A23187 and completely shifted back at 50 minutes (Fig. 7C). The down-shift of XPRo happened in the same time frame of the dephosphorylation and

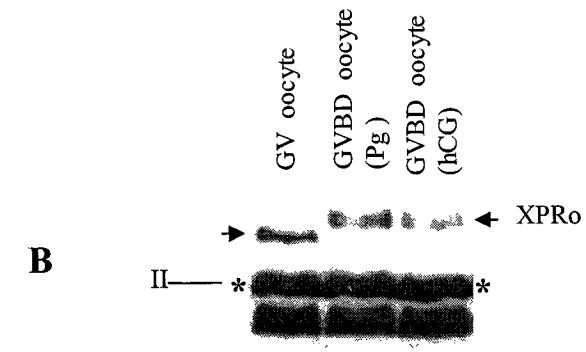
Figure 7: Mobility shift of XPRo

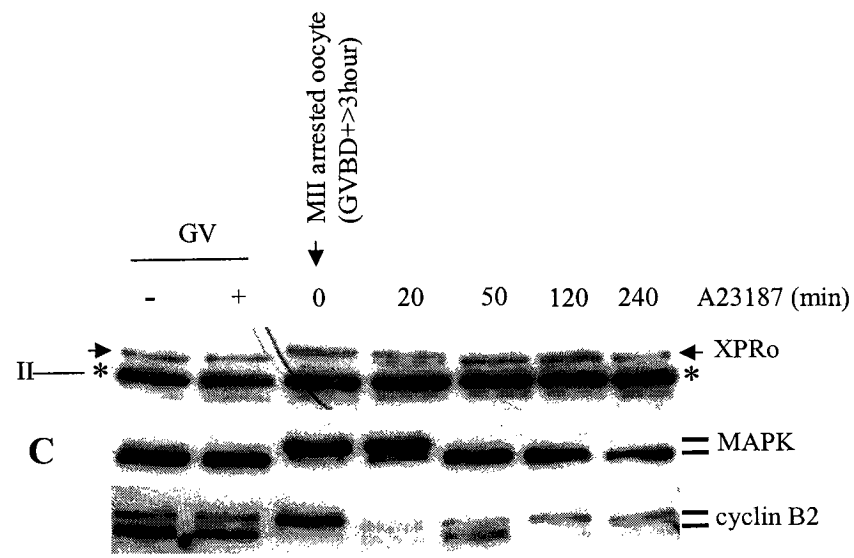
A) 88 oocytes were stimulated with 1μM progesterone (pg), and a group of 5 oocytes was randomly removed at each indicated time point, lysed and made into samples for Western blotting using anti-XPR, anti-MAPK, and anti-cyclinB2 antibodies. At 2.5 hour 62 out of 88 oocytes underwent GVBD and this time point was considered as "GVBD".

B) 20 manually defolliculated oocytes were stimulated with 1μM progesterone (pg) and incubated overnight with equal amount of unstimulated oocytes as control. A piece of ovarian fragment containing ~50 fully-grown follicles was stimulated with 50IU/ml hCG and also incubated overnight. At the second day 10 GV oocytes, 10 progesterone-induced mature eggs and 10 mature eggs ovulated from the ovary piece were lysed and subjected to Western blotting using anti-XPR antibodies.

C) After overnight stimulation with 1μM progesterone, 100 healthy-looking mature eggs were pooled and calcium ionophore A23187 (2μg/ml) was added to trigger mitotic response. A group of 10 oocytes was removed at each time point, lysed and made into sample for Western blotting using anti-XPR, anti-MAPK and anti-cyclinB2 antibodies.


GVBD (62/88)
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 8.5 15 pg (hour)
XPRo
II
*
*
A
MAPK
cyclin B2


GV oocyte
GVBD oocyte (Pg)
GVBD oocyte (hCG)
XPRo
B
II
*
*


MII arrested oocyte (GVBD+>3hour)
GV
- + 0 20 50 120 240 A23187 (min)
XPRo
II
*
*
C
MAPK
cyclin B2

inactivation of MAPK, a biochemical event critical for the progression from metaphase II arrest to the first mitotic cycle (Minshull et al., 1994; Abrieu et al., 1997; Sohaskey and Ferrell, 1999).

In an attempt to identify the protein kinase(s) responsible for XPRo phosphorylation we tested U0126, a potent inhibitor of MAPK kinase (MEK). U0126 blocks progesterone-induced MAPK activation, but not the activation of MPF or GVBD in *Xenopus* oocyte (Gross et al., 2000). Time course experiment showed that, when U0126-treated oocyte underwent GVBD 2 hours following the addition of progesterone (Fig. 8, lane 8), cyclinB2 was normally phosphorylated indicating normal MPF activation. At this point, MAPK remained in the unphosphorylated and inactive form, which correlated with partial "shift" of XPRo. Half an hour later (Fig. 8, lane 10), the level of cyclinB2 declined, while the status of MAPK and XPRo remained unchanged. These results suggested that inhibition of the MAPK pathway had an inhibitory effect on XPRo phosphorylation.

Enucleation experiment revealed that, while band II was located exclusively in the cytoplasm as previously reported (Bayaa et al., 2000), XPRo was predominantly localized in the GV. However, a small fraction was always recovered in enucleated oocytes (Fig. 9A). Quantification of the band intensity from five independent experiments showed that 22% (±4%) of XPRo was cytoplasmic and 74% (±2%) was nuclear. This ratio was in good accordance with that of PR in somatic cells (Gasc et

Figure 8: Effect of U0126 on XPRo phosphorylation

100 oocytes were pre-incubated with 100μM U0126 for 1 hour, and then stimulated with 1μM progesterone (pg) together with another group of control oocytes. The time when all control oocytes underwent GVBD was considered as time "0", and a group of 10 oocytes was removed at each time point after that, lysed and made into sample for Western blottting using anti-XPR, anti-MAPK and anti-cyclinB2 antibodies.

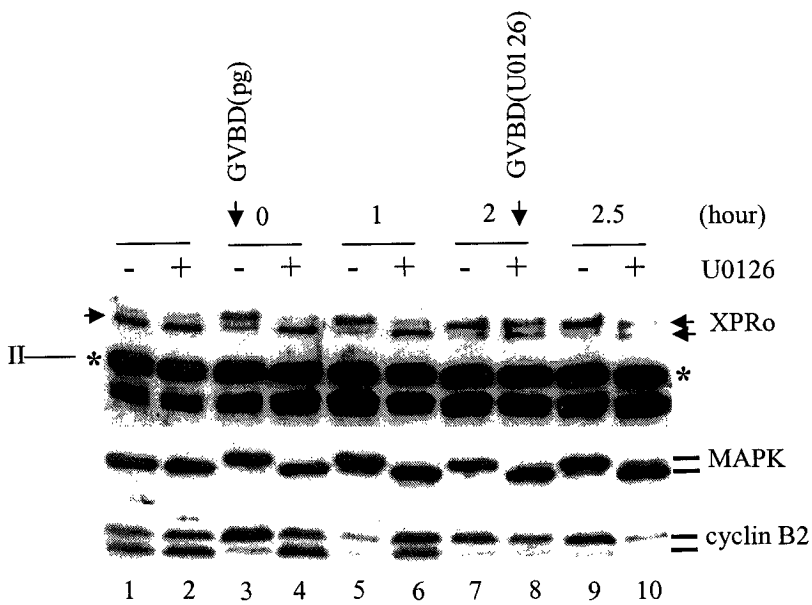
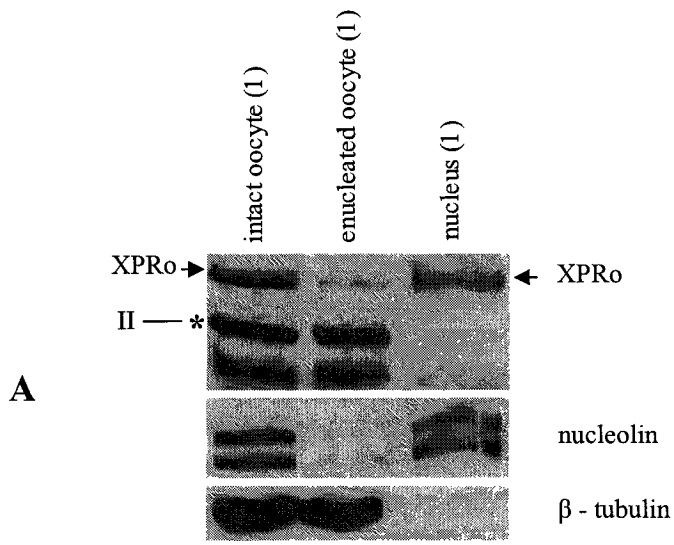
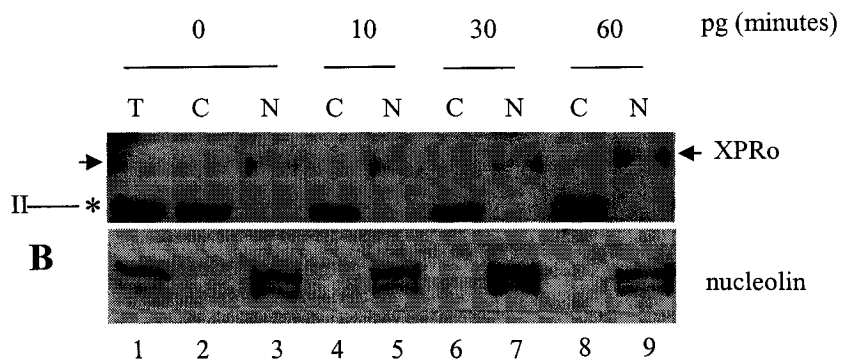

GVBD(pg)
GVBD(U0126)
0
1
2
2.5
(hour)
- + - + - + - + - +
U0126
XPRo
II
*
*
MAPK
cyclin B2
1 2 3 4 5 6 7 8 9 10

Figure 9: Subcellular localization of XPRo

A) A group of 5 oocytes were enucleated in Gall's medium. The nuclei and enuleated oocytes were lysed and subjected to Western analysis using anti-XPR, anti-nucleolin, and anti-β-tubulin antibodies. Nucleolin, a major nucleolar protein, was used as nuclear content control and β-tubulin, an abundant cytosolic protein, was used as cytosol control. 1 oocyte or nucleus was loaded in each lane. This is representative of five independent experiments.

B) 50 oocytes were stimulated with 1μM progesterone (pg), and 5 oocytes were removed at each time point and quickly enucleated. The nuclei and enucleated oocyte were lysed and made into sample for Western blotting using anti-XPR and anti-nucleolin antibodies. T: Total lysate. C: Cytoplasm. N: Nucleus.


intact oocyte (1)
enucleated oocyte (1)
nucleus (1)
XPRo
II
*
XPRo
A
nucleolin
β - tubulin


0
10
30
60
pg (minutes)
T C N C N C N C N
XPRo
II
*
B
nucleolin
1 2 3 4 5 6 7 8 9

al., 1984; Perrot-Applanat et al., 1985 and 1987). The effect of progesterone on the cytoplasmic/nuclear localization of XPRo was also examined. As shown in Fig. 8B, no substantial change of XPRo localization was found up to 60 minutes. Longer time points were not analyzed because oocytes began GVBD.

Previously it was found the XPR-2 mRNA and protein (represented by band II) was present in stage I – stage VI oocyte (Bayaa et al., 2000). In light of the identification of XPRo, Western blotting of oocyte in different stages was performed again and the XPRo was also present in all stages of oocyte with similar quantities (Fig. 10).

Functional Characterization of XPRo in Oocyte Maturation

In a previous yeast two hybrid screening, Dr. Chunqi Ma isolated 14-3-3ζ as a candidate XPR-2 binding protein. 14-3-3ζ is a member of a highly conserved multigene family of small acidic proteins that bind to a variety of proteins containing a phosphorylated peptide motif (Peng et al., 1997). We wished to explore the possible XPR/14-3-3ζ binding to change the subcellular localization of XPRo, and hence XPRo function. Two 14-3-3ζ expression plasmids were used: HA-14-3-3ζ (wild type) and HA-NLS-14-3-3ζ in which two copies of the SV40 large T antigen nuclear localization signal (NLS) were engineered between the HA tag and the 14-3-3ζ. Overexpressed HA-14-3-3ζ showed a predominant cytoplasmic localization (Fig. 11, lane 4~8), while overexpressed HA-NLS-14-3-3ζ was enriched in the nucleus (Fig. 11, lane 9~13). However, neither construct altered the cytoplasmic/nuclear distribution of

Figure 10: Expression pattern of XPRo during oogenesis
Oocytes of different stages were isolated and selected as described in the Materials and Methods section, lysed and made into sample for Western blotting using anti-XPR and anti-β-tubulin antibodies. β-tubulin blot was used as a protein quantity control.

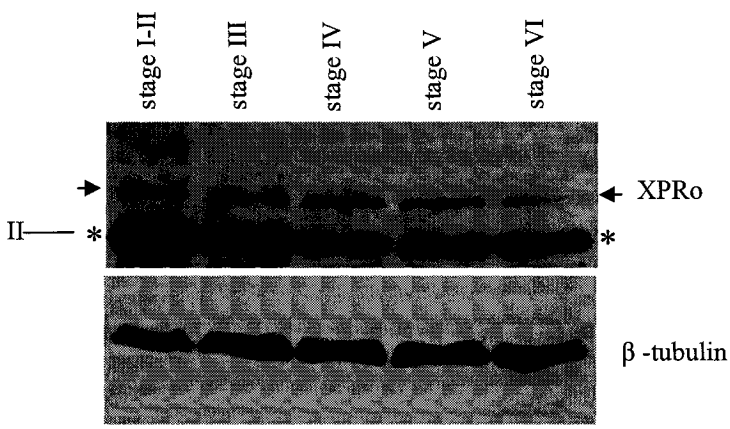
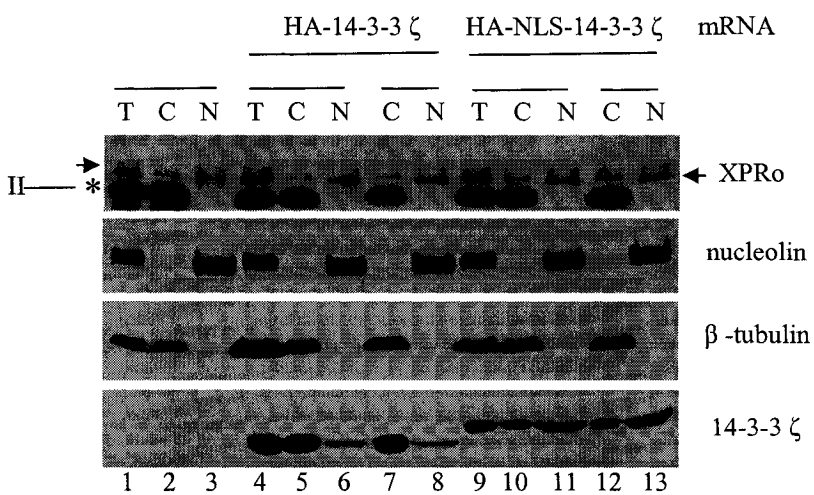

stage I-II
stage III
stage IV
stage V
stage VI
XPRo
II
*
*
β -tubulin

Figure 11: Effect of 14-3-3ζ overexpression on XPRo localization
Oocytes were injected with mRNAs of HA-tagged 14-3-3 ζ (HA-14-3-3 ζ) or HA tagged 14-3-3 ζ with double nuclear localization signals (HA-NLS-14-3-3 ζ), incubated overnight, and enucleated at the second day. 5 oocytes were enucleated as a group and duplicated enucleations were performed for mRNA-injected oocytes. The nuclei and enucleated oocytes were made into samples for Western blotting using anti-XPR, anti-nucleolin, anti-β-tubulin and anti-HA antibodies. Nucleolin blot was used as nuclear protein control. β-tubulin blot was used as cytoplasmic protein control. T: Total lysate. C: Cytoplasm. N: Nucleus.


HA-14-3-3 ζ
HA-NLS-14-3-3 ζ
mRNA
T C N T C N C N T C N C N
II
*
XPRo
nucleolin
β -tubulin
14-3-3 ζ
1 2 3 4 5 6 7 8 9 10 11 12 13

XPRo (Fig.11). Not surprisingly, neither 14-3-3ζ construct had any effect on progesterone- induced oocyte maturation when overexpressed (data not shown).

Although it is well established that progesterone can activate MPF in the absence of transcription, or even the nucleus, it remains possible that oocyte maturation may require transcription. For example, Iwashita et al. reported that, in enucleated oocyte, progesterone still activated MPF, but MPF activities soon diminished and remained inactivated, in contract to intact oocyte which exhibited biphasic pattern of MPF activation corresponding to entering metaphase I and metaphase II (Iwashita et al., 1998). To explore the possibility that transcription may be required for normal metaphase I to metaphase II transition, we tested the effect of transcription inhibitors on the biochemical profile and chromosome segregation during this period of the meiotic cell cycle.

During the MI - MII transition, the MAPK activity stays high, while the cyclinB2 is degraded after the beginning of the MI, and is re-synthesized in MII (Fig. 12A, control). Actinomycin D, a general transcription inhibitor, did not cause obvious alternation of the biochemical profile after GVBD as judged by MAPK and cyclinB2 immunoblots (Fig.12 A, +AMD). However, actinomycin D inhibits transcription by binding to chromosomal DNA and, in so doing, interferes with normal chromosome dynamics during MI-MII transition in frog and mouse oocyte (Ziegler and Masui, 1976; Jagiello 1969). This property of actinomycin D prevented it from being used to

Figure 12: Effect of transcription inhibitors on MI-MII transition

A) 50 oocytes were pre-incubated with 5μg/ml actinomycin D (AMD) for 1 hour, and then stimulated with 1μM progesterone. A group of 5 oocytes were removed at each time point, lysed, and made into sample for Western blotting using anti-MAPK and anti-cyclinB2 antibodies.

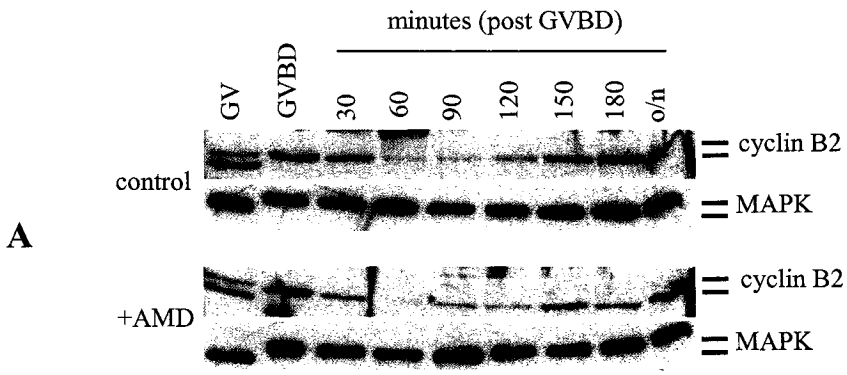
B) Oocytes were first injected with α-amanitin (10mg/ml, 20nl) and pre-incubated for 1 hour. The control oocytes and the α-amanitin-injected oocytes were then split into two groups, one group was stimulated with 1μM progesterone, and another group was nuclear-injected with GFP reporter plasmid (0.2μg/μl, 5nl). The morphology of meiotic spindle was observed at the next day by first staining the progesterone-stimulated oocytes using Sytox Green and then viewing them with animal pole upmost under the fluorescence microscope. The inhibitory effect of α-amanitin on transcription was monitored by comparing the GFP fluorescence of the GFP plasmid injected oocytes and the α-amanitin/ GFP plasmid double injected oocytes under fluorescence microscope.

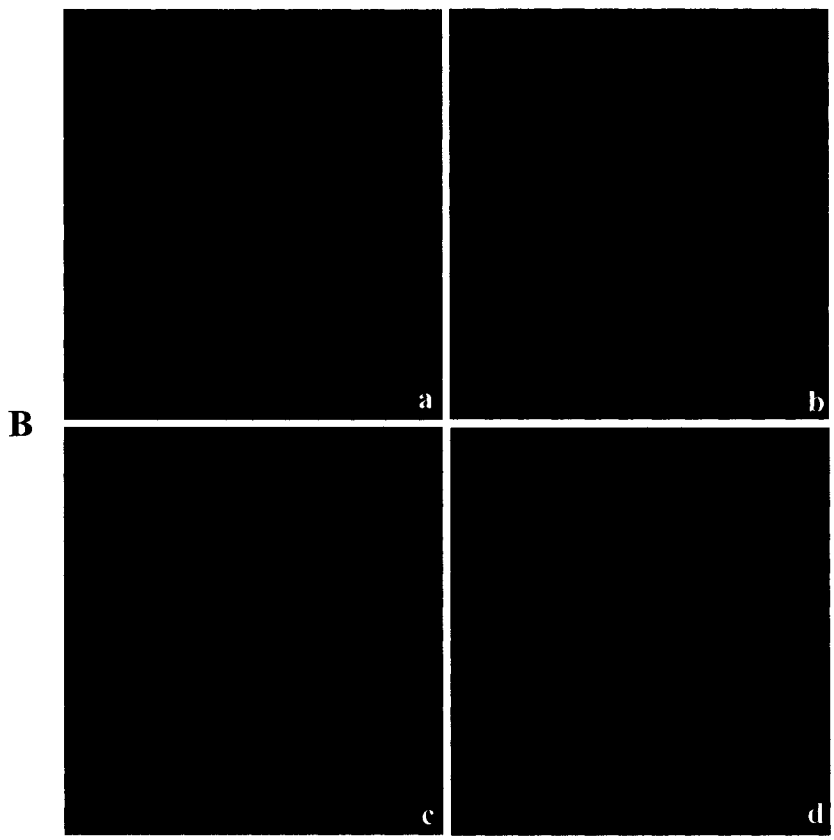
a) The chromosomes on MII spindle and polar body on control oocyte after overnight progesterone stimulation. The chromosomes on the MII spindle resemble a "flower" when viewed from the top of the animal pole, and the bright spot beside the spindle is the first polar body.

b) The chromosomes on MII spindle and polar body on α-amanitin injected oocyte after overnight progesterone stimulation.

c) The GFP fluorescence in GFP plasmid injected oocytes.

d) The GFP fluorescence in α-amanitin/GFP plasmid double injected oocytes.


A
minutes (post GVBD)
GV
GVBD
30
60
90
120
150
180
o/n
control
cyclin B2
MAPK
+AMD
cyclin B2
MAPK

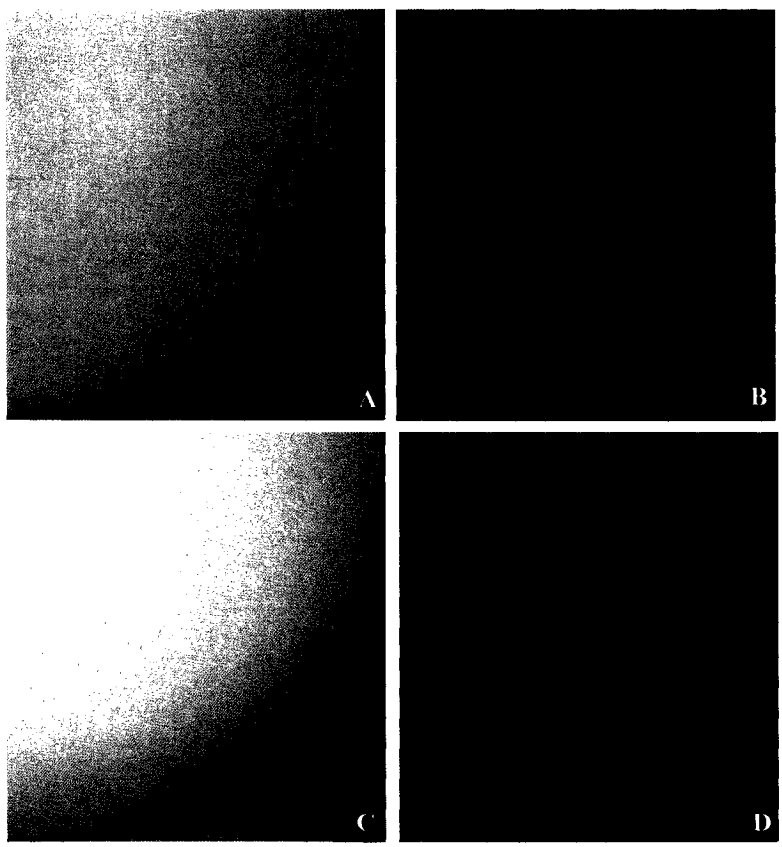

B
a
b
c
d

determine whether transcription *per se* was required for normal chromosome segregation during oocyte maturation. To circumvent this limitation, we employ α-amanitin, a specific inhibitor of RNA polymerase II, to inhibit transcription. Consistent with a previous report (Leonard and Patient, 1991), α-amanitin blocked mRNA transcription as indicated by the expression of GV-injected GFP plasmid in control oocytes but not in α-amanitin injected oocytes (Fig.12B, c and d). However, α-amanitin injected oocytes still displayed normal metaphase II spindle and the first polar body, indicating that inhibition of transcription did not affect chromosome dynamics during MI-MII transition period of the oocyte maturation (Fig.12B, a and b).

Progesterone-induced Degradation of XPR-1 in Follicle Cells

Follicle cells constitute the most inner layer of the follicle envelope, and they are connected to the oocyte surface via anchoring on the vitelline membrane. Sytox Green staining of manually "defolliculated" oocyte (Fig. 13B) revealed that this layer of somatic cells is still attached to the oocyte surface, which is consistent with an early report that described the surface of a manually "defolliculated" oocyte as "pebbled" under light microscope (Smith et al., 1968 and Fig. 13A). To remove this layer of follicle cells, we stripped the manually "defolliculated" oocytes with fine-grade sandpaper. Sandpaper-stripped oocytes had smooth surface and (Fig. 13C) lack follicle cells, as revealed by Sytox Green staining (Fig. 13D). To functionally test these oocyte preparations, we treated them with hCG and with progesterone. As

Figure 13: Follicle cell layer on manually "defolliculated" oocyte
The follicle cell layer on the manually "defolliculated" oocyte or sandpaper-stripped oocyte was viewed using light microscopy or fluorescence microscopy after Sytox Green staining.
A) B) Light and fluorescence microscopy pictures of the manually "defolliculated" oocyte.
C) D) Light and fluorescence microscopy pictures of the sandpaper-stripped oocyte.

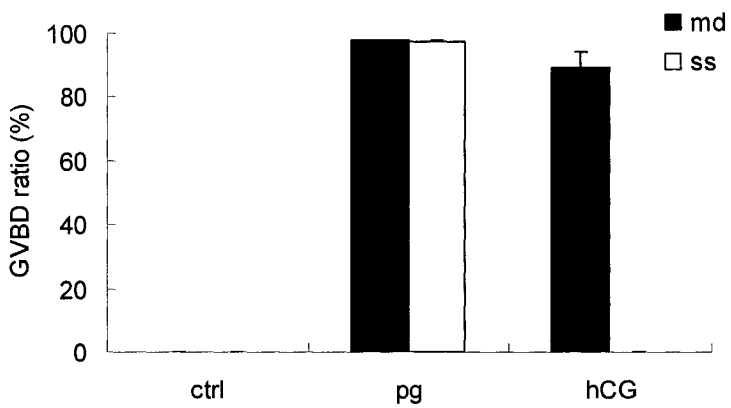

A
B
C
D

shown in Fig. 14, manually "defolliculated" oocytes responded to both hCG and progesterone but sandpaper-stripped oocytes only responded to progesterone, but not hCG. These results helped us establish new protocols for isolating frog oocytes (Liu and Liu, in press).

Our anti-XPR antibodies readily recognized overexpressed XPR-1 in oocytes (Fig. 6A, lane 3, 4), but it failed to detect any endogenous band at the same position. To examine whether XPR-1 was present in follicle cells, we recovered the follicle cells from the sandpaper stripping procedure (see above) for Western blotting with anti-XPR antibodies. Indeed, an immuno-reactive protein was detected which co-migrated with overexpressed XPR-1 from oocyte (Fig. 15). In contrast to XPRo which underwent progesterone-induced phosphorylation, XPR-1 in follicle cells was gradually degraded after progesterone stimulation; significant degradation was observed after 8 hours and complete degradation was observed after overnight incubation (Fig. 16A). This time course is consistent with the reported degradation of PR in human breast cancer cells (Lange et al., 2000). The mechanism of this degradation was studied by using MEK inhibitor U0126 and 26S proteasome inhibitor lactacystin, both of which have been shown to inhibit the progestin-induced PR degradation in tissue culture cells (Lange et al., 2000; Shen et al., 2001). Overnight treatment of progesterone caused complete degradation of XPR-1 in control follicle cells (Fig. 16B, lane 1~4), and this degradation was inhibited by U0126 (Fig. 16B, lane5) and lactacystin (Fig. 16B, lane6). Furthermore, this degradation was not

Figure 14: Response of the two oocyte preparation to progesterone and hCG
Manually "defolliculated" (md) oocyte or sandpaper-stripped (ss) oocyte was stimulated with 1μM progesterone (pg) and 50IU/ml hCG and at the second day the number of GVBD oocytes were counted. After GVBD scoring oocytes were lysed and made into sample for Western blotting using anti-MAPK and anti-cyclinB2 antibodies.
A) Summary of GVBD ratio from three independent experiments.
B) Western blotting from one experiment.

A


GVBD ratio (%)
100
80
60
40
20
0
ctrl
pg
hCG
md
ss

B

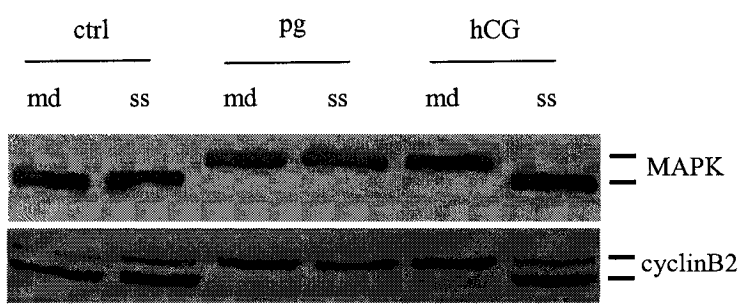

ctrl
pg
hCG
md
ss
md
ss
md
ss
MAPK
cyclinB2

Figure 15: XPR-1 in follicle cells

The follicle cells on the oocyte surface were isolated as described in the Materials and Methods section. An aliquot of follcle cells stripped from 20 oocytes was lysed and made into sample for Western blotting using anti-XPR antibodies. Also loaded is a sample from oocytes injected with XPR-1 mRNA (1 oocyte).

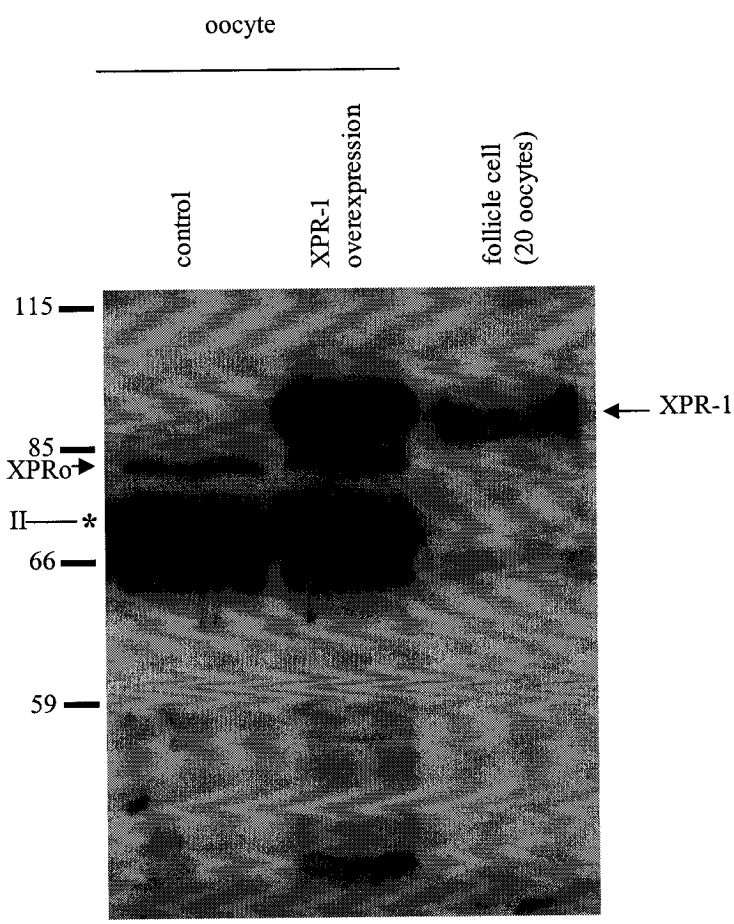
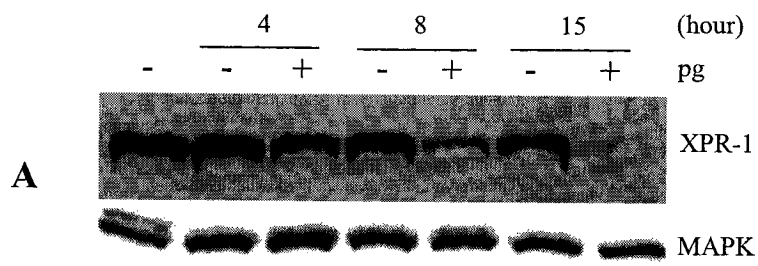

oocyte
control
XPR-1 overexpression
follicle cell (20 oocytes)
115
85
XPRo
II
*
66
59
XPR-1

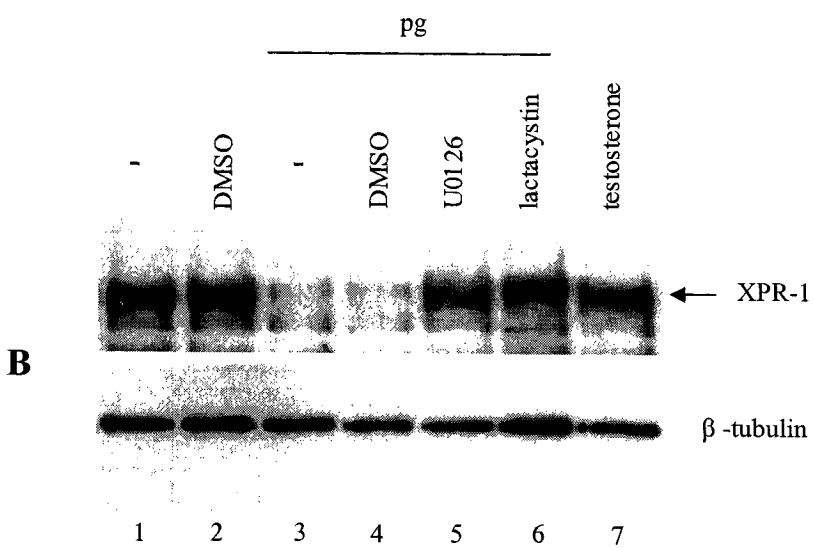
Figure 16: Degradation of XPR-1 after progesterone stimulation

A) Follicle cells were isolated, aliquoted and stimulated with 1μM progesterone (pg). Each aliquot contains follicle cells stripped from 20 oocytes. One aliquot was taken at each time point and made into sample for Western blotting using anti-XPR and anti-MAPK antibody. MAPK blot here was used as a protein quantity control.

B) Follicle cells were isolated, aliquoted and pre-incubated with vehicle (DMSO), 100μM U0126, 100μM lactacystin, and after 1 hour 1μM progesterone or testosterone was added. Each aliquot contains follicle cells stripped from 20 oocytes. After overnight incubation follicle cells were pelleted and made into sample for Western blotting for anti-XPR and anti-β-tubulin antibodies. β-tubulin blot was used as a protein quantity control.


4
8
15
(hour)
- - + - + - +
pg
XPR-1
MAPK

A


pg
-
DMSO
-
DMSO
U0126
lactacystin
testosterone
XPR-1
β -tubulin
1 2 3 4 5 6 7

B

induced by another ovulation-inducing steroid testosterone (Fig. 16B, lane 7), suggesting that the degradation is specific to progesterone. These results suggested that XPR-1 underwent progesterone-induced degradation with similar mechanism as mammalian PR.

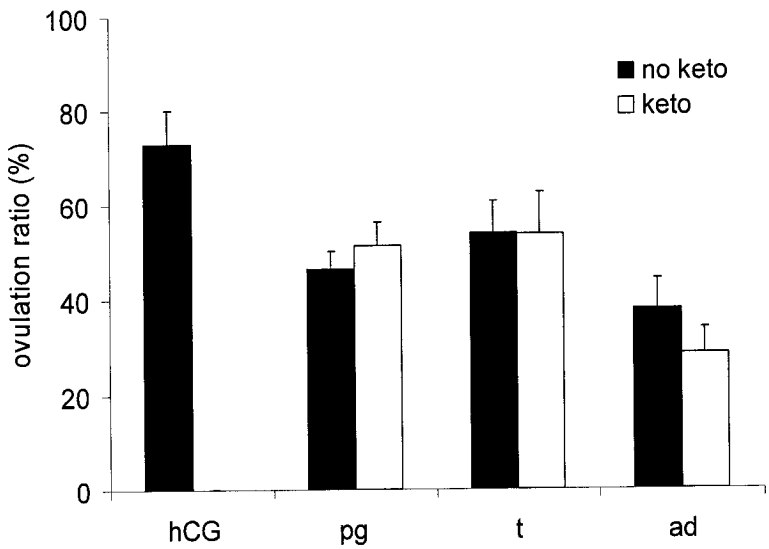
Genomic and Non-genomic Action of Progesterone in Frog Ovary

The presence of XPR proteins in both the oocyte and the follicle cells and their differential response to progesterone (hyperphosphorylation in oocytes and degradation in follicle cells) prompted us to examine the possible dual function of progesterone in frog ovary. As summarized in introduction, progesterone as well as its downstream metabolite androgen can induce "ovulation" (defined as the release of oocytes from ovarian tissues *in vitro*) in isolated ovarian tissues. The presence of 50μM ketoconazole did not inhibit progesterone-induced ovulation, suggesting that progesterone, not its metabolites, was responsible (Fig.17A). As expected, the ovulated oocytes were mature (Fig.17B). As a control, ketoconazole blocked hCG-induced ovulation, suggesting that steroidogenesis was inhibited (Fig.17A).

In the presence of the general transcription inhibitor actinomycin D, progesterone-induced ovulation was blocked. However, examining the oocytes trapped in follicles revealed that they were properly mature (Fig.18A). In contrast, actinomycin D blocked both ovulation and oocyte maturation when the stiumulus was hCG (Fig.18B). These results clearly indicated that progesterone-induced ovulation was dependent on

Figure 17: Effect of ketoconazole on steroid-induced ovulation and maturation
Isolated ovary fragments (each contain ~50 fully-grown follicles) were first pre-incubated with 50μM ketoconazole (keto) for 1 hour and then stimulated with 50IU/ml hCG, 10μM progesterone (pg), testosterone (t) and androstendione (ad). After overnight incubation, the number of ovulated eggs and GVBD oocytes in each well were counted.
A) The effect of ketoconazole on ovulation induced by hCG or steroids. Shown here are results from six independent experiments.
B) The effect of ketoconazole on oocyte maturation (GVBD) induced by hCG or steroids. Shown here are results from three independent experiments.

A


100
80
60
40
20
0
ovulation ratio (%)
no keto
keto
hCG
pg
t
ad

B

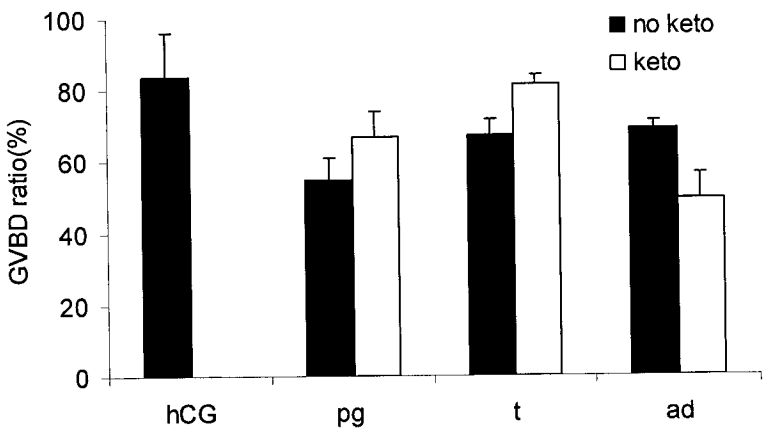
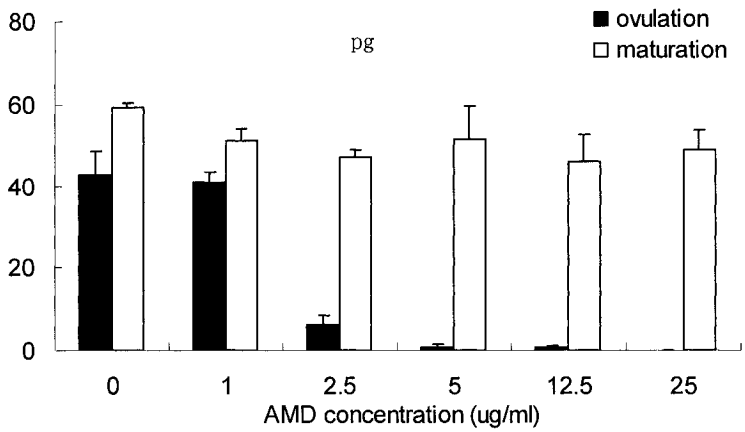
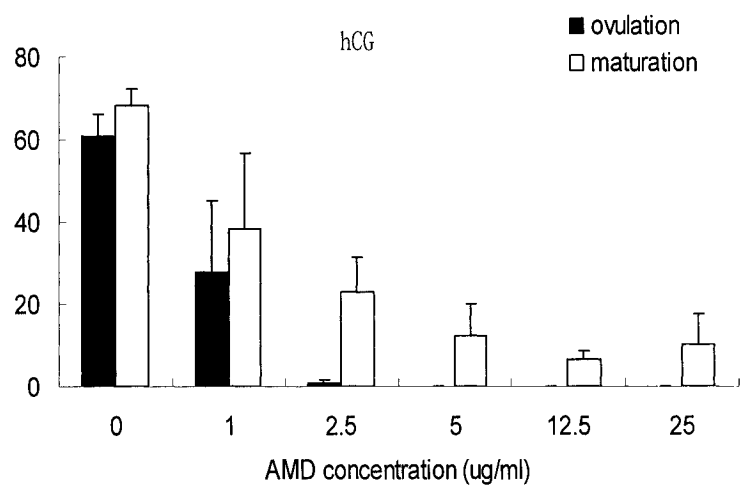

100
80
60
40
20
0
GVBD ratio(%)
no keto
keto
hCG
pg
t
ad

Figure 18: Effect of actinomycin D on the ovulation and oocyte maturation
After 1 hour pre-incubation with various concentrations of actinomycin D (AMD), ovary fragments (each contain ~50 fully grown follicles) were stimulated with 5μM progesterone (pg) or 50IU/ml hCG. After overnight incubation the number of ovulated eggs and GVBD oocytes in each well were counted.
A) The effect of actinomycin D on progesterone-induced ovulation/ maturation. Shown here are results from three independent experiments.
B) The effect of actinomycin D on hCG-induced ovulation/maturation. Shown here are results from three independent experiments.

A


pg
ovulation
maturation
80
60
40
20
0
0
1
2.5
5
12.5
25
AMD concentration (ug/ml)

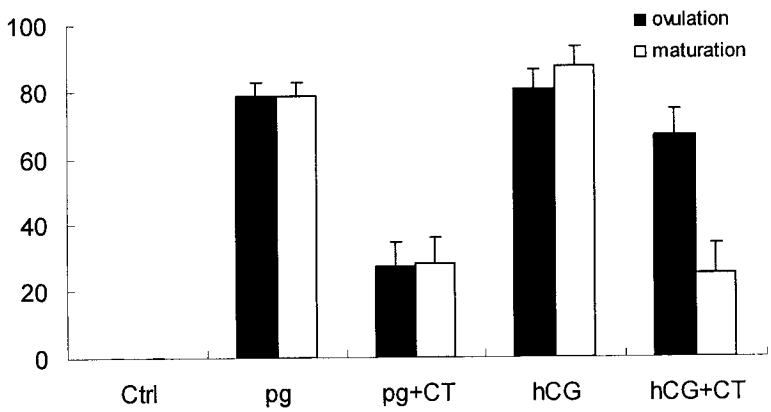
B


hCG
ovulation
maturation
80
60
40
20
0
0
1
2.5
5
12.5
25
AMD concentration (ug/ml)

gene transcription but progesterone-induced oocyte maturation was independent of transcription.

Cholera toxin activates the α subunit of Gs protein by ADP-ribosylation and, in doing so, was a potent inhibitor of oocyte maturation when injected directly into the oocytes (Schorderet-slatkine et al., 1978). To determine whether the inhibition of the non-genomic function of progesterone (oocyte maturation) would affect ovulation, we injected cholera toxin into oocytes enclosed in follicles prior to progesterone treatment. As shown in Fig. 19, cholera toxin injection strongly inhibited both progesterone- and gonadotropin-induced maturation of follicle-enclosed oocytes (the low percentage of maturation likely represented oocytes that had been buried inside the ovarian pieces and therefore not accessible during cholera toxin injection). Interestingly, progesterone-induced ovulation was inhibited to the same extent as maturation, which meant toxin-injected oocytes were also trapped in follicles. By contrast, cholera toxin injection did not interfere with hCG-induced ovulation.

Figure 19: Effect of cholera toxin on ovulation and oocyte maturation
Follicle-enclosed oocytes on ovary fragments were injected with cholera toxin (CT, 100μg/ml, 20nl). After 1hour incubation, ovary fragments were stimulated with 10μM progesterone (pg) or 50IU/ml hCG and incubated overnight. At the second day the ovulated eggs/oocytes and GVBD oocytes in each well were counted. Shown here are results from five independent experiments.


100
80
60
40
20
0
ovulation
maturation
Ctrl
pg
pg+CT
hCG
hCG+CT

DISCUSSION

Expression Pattern of XPR Proteins in Frog Ovary and its Functional Implication

The presence of two immuno-reactive XPR proteins (XPR-1 and XPRo) is reminiscent of two PR proteins (PR-B and PR-A) in other organisms. However, while both PR-A and PR-B are derived from the same gene through alternative transcription/translation, the origin of XPRo is not clear. Sequence analysis reveals that XPR-1 contains an internal ATG codon homologous to the start codon for the hPR-A isoform, and the putative protein derived from this alternative start codon has a molecular weight of 72.5 kDa. On the other hand, XPRo co-migrates with a 72kDa protein (MT-XPR-2) in Western blotting (Fig. 5B, lane 7, 8), so XPRo also has a molecular weight of ~72 kDa. Therefore it is tempting to conclude that XPRo represents an alternative translation product of *Xenopus* PR in frog oocytes. It should be pointed out that, despite much efforts in two laboratories, whether the cloned XPR-2 cDNAs represent the full length mRNA remain unclear, as no in-frame translation termination codons are found 5' to any of the ATG codons (Bayaa et al., 2000). Therefore, a definitive assignment of XPRo to either XPR-1 or XPR-2 remains an open question at the present time.

Regardless the origins and relationship between the two xPR proteins, their respective expression patterns (XPR-1 restricted in follicle cells *vs.* XPRo restricted in frog oocytes) and their differential biochemical behaviors (progesterone-induced XPR-1

degradation in follicle cells *vs.* progesterone- induced XPRo hyperphosphorylation in oocyte) would argue that they mediate different functions of progesterone in frog ovary, which includes the genomic action in ovulation and the non-genomic action in oocyte maturation.

Dual Functions of Progesterone in Frog Ovary I: Genomic Action in Ovulation

The function of progesterone as a mediator of gonadotropin in the induction of ovulation in mammals is well established. A specific and potent antagonist of PR, RU486, blocks gonadotropin-induced ovulation in mammals (Sanchez-Criado et al., 1990; Loutradis et al., 1991; Ledger et al., 1992). Furthermore, Progesterone receptor (PR) is transiently induced in granulosa cells of preovulatory mouse follicles in response to gonadotropin surge, and PR has been shown to be essential for ovulation, since PR knockout mice fail to ovulate and are infertile (Park and Mayo, 1991; Lydon et al., 1995). Several more recent studies have indicated that progesterone function in ovulation by activating gene expression of several metaloproteases in the follicle cells that help digest extracellular tissues in the follicle layer (Robker et al., 2000a, b).

In our *in vitro* assay using isolated *Xenopus* ovary fragment, progesterone was as effective as hCG in simulating ovulation (Fig.16A), and the effect of progesterone was blocked by actinomycin D (Yatvin and Pitot, 1969 and Fig. 17A), suggesting that progesterone induces ovulation in a genomic manner. Furthermore, progesterone-induced ovulation was not inhibited by ketoconazole, suggesting that progesterone

alone, and not its metabolites, was able to induce the whole ovulatory responses. Therefore, we propose that one function of progesterone in frog ovary is to activate its nuclear receptor (PR) in the follicle cells, which in turn activates gene expression of certain tissue-digesting metaloproteases just as its mammalian counterpart. The function of these metaloproteases is to carry out controlled tissue remodeling to allow the release of the eggs.

However, in the same assays, androgens (testosterone and androstenedione) were also effective in ovulation induction (Fig.16A). Furthermore, while progesterone-induced ovulation was inhibited when oocyte maturation was blocked (by injection of cholera toxin directly into the follicle-enclosed oocytes), hCG-induced ovulation was not affected by this maturation blockade. These results clearly indicate that gonadotropin can induce ovulation through multiple pathways. Similarly, androgens were as effective as progesterone in inducing frog oocyte maturation (Le Goascogne et al., 1985; Lutz et al., 2001). Why the redundant pathways? We propose that the presence of multiple gonadotropin-induced pathways to stimulate oocyte maturation and ovulation in frog ovary is to ensure that the two processes are highly coordinated. The need for this coordination is obvious because fertilization is impossible if mature eggs are trapped in follicles, or if immature oocytes are released from ovary.

Dual Functions of Progesterone in Frog Ovary II: Non-genomic Action in Oocyte Maturation

The legendary work by Dr. Masui in the late 60's to the early 70's clearly established the non-genomic nature of progesterone action in the induction of frog oocyte maturation (Masui, 1967; Masui and Markert, 1971). The identity of the receptor for progesterone in frog oocytes, however, remains disputed (Maller, 2001 and 2003). Following many years of disappointment in this field (Maller, 1998), work in our lab and Dr. Ruderman's lab independently concluded that the classical progesterone receptor (XPR) was responsible for progesterone-induced oocyte maturation.

However, the evidence supporting this notion is only circumstantial (see introduction). This situation reflects the unfortunate drawbacks of the *Xenopus* oocyte system as a biological model. First, it is not possible to generate XPR-null oocytes. Second, the traditional antisense approach (phosphorthioate or morpholino oligos) is not effective because the maternal XPR protein, like most other maternal proteins, is stable and apparently no new XPRo is synthesized during oocyte maturation (Fig. 6A). In addition, no neutralizing antibodies against XPR are available (in fact no neutralizing antibodies against PR of any species are available). Finally, classical PR antagonist RU486 does not inhibit progesterone-induced oocyte maturation. In fact, RU486 mimics progesterone in inducing oocyte maturation (Salder et al., 1985).

The recent cloning of a putative membrane progesterone receptor (mPR) in sea trout (Zhu et al., 2003a) suggested the possibility that a similar gene in the frog (Zhu et al., 2003b) may be the long sought-after progesterone receptor in frog ocoytes (Maller,

2003). At the moment, there is no evidence to support mPR as the non-genomic progesterone receptor in frog oocytes. In fact, the relevance of mPR in mediating fish oocyte maturation is not well established. The physiological inducer of fish oocyte maturation appears to be 20β-S and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-P), whereas progesterone, testosterone, or cortisol have only marginal effect on this process (Patino and Thomas, 1990). Oddly, mPR binds progesterone with high affinity but does not bind 20β-S and 17α, 20β-P (Zhu et al., 2003a). Furthermore, mPR appears to be a Gi-coupled GpCR and yet there are overwhelming evidence indicating that Gi is not involved in progesterone-induced oocyte maturation. First, progesterone-induced oocyte maturation is not sensitive to pertussis toxin (a classical Gi inhibitor) (Sadler et al., 1984; Goodhardt et al., 1984; Sheng et al., 2001). Second, activating endogenous Gi by overexpression of multiple Gi-coupled GpCRs does not induce frog oocyte maturation (Noh and Han, 1998; Romo et al., 2002; Kalinowski et al., 2003). Third, direct injection of activated Gi does not induce oocyte maturation (Kroll et al., 1991).

The main goal of my thesis work was to further examine the functional role of the classical progesterone receptor in mediating progesterone action in frog oocytes. To this end, I decided to further characterize the two cloned XPR cDNAs (XPR-1 and XPR-2) in frog oocytes. These efforts led to the previously unsuspected finding that XPR proteins derived from these cDNAs exhibited maturation-specific mobility shift in SDS-PAGE, indicative of hyperphosphorylation. These observations prompted us

to re-evaluate the previously-identified endogenous XPR protein (Bayaa et al., 2000), which does not exhibit similar shift. Indeed, a more minor anti-XPR-N immuno-reactive protein exhibited maturation-specific shift. Based on this criterion, we concluded that this newly identified protein more likely represented the endogenous XPR protein (designated as XPRo). In contrast to the previously identified XPR-2 (Bayaa et al., 2000) which resided outside of the nucleus, XPRo was predominantly nuclear.

The presence of the majority of XPRo in the nucleus was puzzling, as the signaling cascades leading to MPF activation clearly occurred outside of the nucleus (Masui and Markert, 1971). On the other hand, Tian et al. does not observed significant progesterone-activated transcription of a reporter construct containing progesterone response element (PRE) in frog oocytes, suggesting the lack of endogenous progesterone receptor capable of activating transcription (Tian et al., 2000).

It clearly remains possible that the non-genomic action of progesterone was mediated by the cytoplasmic portion of XPRo (~22%) whereas the nuclear XPRo remains silent. However, it is worth noting that XPRo was completely shifted to the hyperphosphorylated form following oocyte maturation, suggesting that both the cytoplasmic and nuclear XPRo undergoes similar hyperphosphorylation. An alternative interpretation of the complete "shift" of XPRo is that XPRo hyperphospho -rylation occurs following GVBD when the nuclear boundary disappears. The timing

of the XPRo hyperphosphorylation coincides with the "all-or-none" switch of MAPK activation (Ferrell and Machleder, 1998, Ohan et al., 1999) and GVBD, which is several hours following the initial addition of progesterone. This result suggests that XPRo hyperphosphorylation does not represent an initial signaling event. Therefore this XPRo hyperphosphorylation is either a feedback mechanism in progesterone signaling or an event unrelated to progesterone action.

How does the classical progesterone receptor (XPR) mediate cytoplasmic signaling in frog oocytes? Earlier work by others has suggested that endogenous XPR proteins may directly activate PI-3 kinase pathway (Bagowski et al., 2001). This was based on the observation that antibodies against human PR recognized two proteins of migration pattern similar to human PR-A and PR-B, respectively. Antibodies recognizing "p110XPR" and "p85XPR" (as they are called) were found to co-immunoprecipitate with PI-3 kinase. We employed the same anti-hPR antibodies and detected similar bands ("p85XPR" and "p110XPR") in frog oocyte extracts. However, these two bands didn't display mobility upshift in mature eggs (data not shown). Therefore the immuno-reactive "p85XPR" and "p110XPR" most likely represented non-specific proteins. Unfortunately, my thesis work did not provide further information regarding XPR signaling in frog oocytes.

As discussed above, we believe that there are redundant pathways (progesterone, androgens etc.) to trigger frog oocyte maturation. Recent work from Hammes'

laboratory suggested that the nuclear androgen receptor is responsible for androgen-induced oocyte maturation (Lutz et al., 2001 and 2003), although the evidence is, at best, circumstantial. Therefore, from a biological standpoint, it seems more relevant to ask the question of how gonadotropin induces oocyte maturation. To this end, accumulating evidence has indicated that, in frog oocytes and in mouse oocytes, a constitutively activated G-protein coupled receptor (GpCR*) is responsible for maintaining high levels of oocyte cAMP (Gallo et al., 1995; Romo et al., 2002; Wang and Liu, 2003; Kalinowski et al., 2004). The function of gonadotropins, and steroids, is to overcome this GpCR*. The molecular identity of this GpCR* remains unknown. One intriguing possibility is that this GpCR* may be related to mPR. Rrecent work suggests that mPR represents a multigene family (Zhu et al., 2003b). It is possible that other members of this family are coupled to Gs. In this case, progesterone, and perhaps androgens, may inhibit, instead of activate, its "receptor".

REFERENCE

Abrieu, A., Fisher, D., Simon, M.N., Doree, M., and Picard, A. (1997) MAPK inactivation is required for the G2 to M-phase transition of the first mitotic cell cycle *EMBO J.* 16:6407-13

Bagowski, C.P., Myers, J.W., and Ferrell, J.E. Jr. (2001) The classical progesterone receptor associates with p42 MAPK and is involved in phosphatidylinositol 3-kinase signaling in *Xenopus* oocytes *J. Biol. Chem.* 276:37708-14

Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., and Beato, M. (2003) Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells *Mol. Cell Biol.* 23:1994-2008

Bandyopadhyay, A., Bandyopadhyay, J., Choi, H.H., Choi, H.S., and Kwon, H.B. (1998) Plasma membrane mediated action of progesterone in amphibian (*Rana dybowskii*) oocyte maturation *Gen. Comp. Endocrinol.* 109:293-301

Bayaa, M., Booth, R.A., Sheng, Y., and Liu, X.J. (2000) The classical progesterone receptor mediates *Xenopus* oocyte maturation through a nongenomic mechanism *Proc. Natl. Acad. Sci. U. S. A.* 97:12607-12

Belanger, A.M., and Schuetz, A.W. (1975) Precocious induction of activation responses in amphibian oocytes by divalent ionophore A23187. *Dev. Biol.* 45:378-81

Blondeau, J.P., and Baulieu, E.E. (1984) Progesterone receptor characterized by photoaffinity labelling in the plasma membrane of *Xenopus laevis* oocytes *Biochem. J.* 219:785-92

Boonyaratanakornkit, V., Scott, M.P., Ribon, V., Sherman, L., Anderson, S.M., Maller, J.L., Miller, W.T., and Edwards, D.P. (2001) Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases *Mol. Cell* 8:269-80

Bravo, R., Otero, C., Allende, C.C., and Allende, J.E. (1978) Amphibian oocyte maturation and protein synthesis: related inhibition by cyclic AMP, theophylline, and papaverine *Proc. Natl. Acad. Sci. U. S. A.* 75:1242-6

Burgers, A.C.J., and Li., C.H. (1960) Amphibian ovulation *in vitro* induced by mammalian pituitary hormones and progesterone *Endocrinol.* 66: 255-259

Chien, E.J., Morrill, G.A., and Kostellow, A.B. (1991) Progesterone-induced second messengers at the onset of meiotic maturation in the amphibian oocyte:

interrelationships between phospholipid N-methylation, calcium and diacylglycerol release, and inositol phospholipid turnover *Mol. Cell Endocrinol.* 81:53-67

Cicirelli, M.F., and Smith, L.D. (1985) Cyclic AMP levels during the maturation of *Xenopus* oocytes *Dev. Biol.* 108:254-8

Coffman, G.K., Keem, K., and Smith, L.D. (1979) The progesterone receptor-like properties of *Xenopus laevis* oocyte melanosomes are probably due to eumelanin *J. Exp. Zool.* 207:375-382

Conneely, O.M., Mulac-Jericevic, B., and Lydon, J.P. (2003) Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms *Steroids.* 68:771-8

Cork, R.J., Taylor, M., Varnold, R.L., Smith, L.D., and Robinson, K.R. (1990) Microinjected GTP-gamma-S inhibits progesterone-induced maturation of *Xenopus* oocytes *Dev. Biol.* 141:447-50

Daar, I., Yew, N., and Vande Woude, G.F. (1993) Inhibition of mos-induced oocyte maturation by protein kinase A *J. Cell Biol.* 120:1197-202

Dettlaff, T.A., Nikttina, L.A., and Stroeva, O.G. (1964) The role of the germinal vesicle in oocyte maturation in anurans as revealed by the removal and transplantation of nuclei *J. Embryol. Exptl. Morphol.* 12: 851-873

Drury, K.C., and Ozon, R. (1975) *In vitro* progesterone binding to *Xenopus laevis* oocytes *Gen. Comp. Endocrinol.* 25:339-45

Duckworth, B.C., Weaver, J.S., and Ruderman, J.V. (2002) G2 arrest in *Xenopus* oocytes depends on phosphorylation of cdc25 by protein kinase A *Proc. Natl. Acad. Sci. U. S. A.* 99:16794-9

el-Zein, G., Boujard, D., Garnier, D.H., and Joly, J. (1988) The dynamics of the steroidogenic response of perifused *Xenopus* ovarian explants to gonadotropins *Gen. Comp. Endocrinol.* 71:132-40

Ferrell, J.E. Jr., and Machleder, E.M. (1998) The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes *Science* 280:895-8

Ferrell, J.E. Jr. (1999) *Xenopus* oocyte maturation: new lessons from a good egg *Bioessays.* 21:833-42

Finidori-Lepicard, J., Schorderet-Slatkine, S., Hanoune, J., and Baulieu, E.E. (1981) Progesterone inhibits membrane-bound adenylate cyclase in *Xenopus laevis* oocytes

Nature. 292:255-7

Fortune, J.E., Concannon, P.W., Hansel, W. (1975) Ovarian progesterone levels during *in vitro* oocyte maturation and ovulation in *Xenopus laevis Biol. Reprod.* 13:561-7

Fortune, J.E. (1983) Steroid production by *Xenopus* ovarian follicles at different developmental stages. *Dev Biol.* 99:502-9

Gallo, C.J., Hand, A.R., Jones, T.L., and Jaffe, L.A. (1995) Stimulation of *Xenopus* oocyte maturation by inhibition of the G-protein alpha S subunit, a component of the plasma membrane and yolk platelet membranes *J. Cell Biol.* 130:275-84

Gasc, J.M., Renoir, J.M., Radanyi, C., Joab, I., Tuohimaa, P., and Baulieu, E.E. (1984) Progesterone receptor in the chick oviduct: an immunohistochemical study with antibodies to distinct receptor components. *J. Cell Biol.* 99:1193-201.

Gobbetti, A., and Zerani, M. (1993) Prostaglandin E2 and prostaglandin F2 alpha involvement in the corticosterone and cortisol release by the female frog, *Rana esculenta*, during ovulation *J. Exp. Zool.* 267:164-70

Godeau, J.F., Schorderet-Slatkine, S., Hubert, P., and Baulieu, E.E. (1978) Induction of maturation in *Xenopus laevis* oocytes by a steroid linked to a polymer *Proc. Natl. Acad. Sci. U. S. A.* 75:2353-7

Godeau, F., Ishizaka, T., and Koide, S.S. (1985) Early stimulation of phospholipid methylation in *Xenopus* oocytes by progesterone *Cell Differ.* 16:35-41

Goodhardt, M., Ferry, N., Buscaglia, M., Baulieu, E.E., and Hanoune, J. (1984) Does the guanine nucleotide regulatory protein Ni mediate progesterone inhibition of *Xenopus* oocyte adenylate cyclase? *EMBO J.* 3:2653-7

Graf, J. (1991) Genetics of *Xenopus laevis* In Kay, B.K. and Peng, H.B. (eds.) *Xenopus laevis*: Practical Use in Cell and Molecular Biology. Academic Press, p19-34

Gross, S.D., Schwab, M.S., Taieb, F.E., Lewellyn, A.L., Qian, Y.W., and Maller, J.L. (2000) The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by $p90^{Rsk}$ *Curr. Biol.* 10:430-8

Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., and Milgrom, E. (1989) Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. *Cell* 57:1147-54.

Guiochon-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat,

M., and Milgrom, E. (1991) Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J.* 10:3851-9

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

Hausen, P. and Riebesell, M (eds.) (1991) The early development of *Xenopus laevis*: An Atlas of the histology, Springer-Verlag, plate 1 and plate 8

Heilburnn, L.V., Daugherty, K., and Wilbur, K.M. (1939) Initiation of maturation in the frog egg. *Physiol. Zool.* 12:97-100

Heine, M.A., Rankin, M.L., and DiMario, P.J. (1993) The Gly/Arg-rich (GAR) domain of *Xenopus* nucleolin facilitates in vitro nucleic acid binding and *in vivo* nucleolar localization *Mol. Biol. Cell.* 4:1189-204

Ishikawa, K., Hanaoka, Y., Kondo, Y., and Imai, K. (1977) Primary action of steroid hormone at the surface of amphibian oocyte in the induction of germinal vesicle breakdown. *Mol. Cell Endocrinol.* 9:91-100

Iwashita, J., Hayano, Y., and Sagata, N. (1998) Essential role of germinal vesicle material in the meiotic cell cycle of *Xenopus* oocytes. *Proc. Natl. Acad. Sci. U. S. A.* 95:4392-7

Jacobelli, S., Hanocq, J., Baltus, E., and Brachet, J. (1974) Hormone-induced maturation of *Xenopus laevis* oocytes: effects of different steroids and study of the properties of a progesterone receptor. *Differentiation.* 2:129-35

Jagiello, G.M. (1969) Meiosis and inhibition of ovulation in mouse eggs treated with actinomycin D *J. Cell Biol.* 42:571-4

Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R., and Grohe, C. (2000) Estrogen receptor α rapidly activates the IGF-1 receptor pathway *J. Biol. Chem.* 275:18447-53

Kalinowski, R.R., Jaffe, L.A., Foltz, K.R., and Giusti, A.F. (2003) A receptor linked to a Gi-family G-protein functions in initiating oocyte maturation in starfish but not frogs. *Dev. Biol.* 253:139-49

Kalinowski, R.R., Berlot, C.H., Jones, T.L., Ross, L.F., Jaffe, L.A., and Mehlmann, L.M. (2004) Maintenance of meiotic prophase arrest in vertebrate oocytes by a Gs protein - mediated pathway *Dev. Biol.* 267:1-13

Kostellow, A.B., Weinstein, S.P., and Morrill, G.A. (1982) Specific binding of

progesterone to the cell surface and its role in the meiotic divisions in *Rana* oocytes *Biochim. Biophys. Acta.* 720:356-63

Kroll, S.D., Omri, G., Landau, E.M., and Iyengar, R. (1991) Activated α subunit of Go protein induces oocyte maturation *Proc. Natl. Acad. Sci. U. S. A.* 88:5182-6.

Lange, C.A., Shen, T., and Horwitz, K.B. (2000) Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. *Proc. Natl. Acad. Sci. U. S. A.* 97:1032-7

Lange, C.A. (2004) Making sense of cross-talk between steroid hormone receptors and intracellular signaling pathways: who will have the last word? *Mol. Endocrinol.* 18:269-78

Ledger, W.L., Sweeting, V.M., Hillier, H., and Baird, D.T. (1992) Inhibition of ovulation by low-dose mifepristone (RU 486). *Hum. Reprod.* 7:945-50

Le Goascogne, C., Sananes, N., Gouezou, M., and Baulieu, E.E. (1985) Testosterone-induced meiotic maturation of *Xenopus laevis* oocytes: evidence for an early effect in the synergistic action of insulin *Dev. Biol.* 109:9-14

Lennartsson, J., Blume-Jensen, P., Hermanson, M., Ponten, E., Carlberg, M., and Ronnstrand, L. (1999) Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction *Oncogene.* 18:5546-53

Leonard, M.W., and Patient, R.K. (1991) Evidence for torsional stress in transcriptionally activated chromatin *Mol. Cell Biol.* 11:6128-38.

Lin, Y.W., and Schuetz, A.W. (1983) *In vitro* estrogen modulation of pituitary and progesterone- induced oocyte maturation in *Rana pipiens J. Exp. Zool.* 226:281-91

Liu, X and Liu, X.J. Oocyte isolation and enucleation In Liu, X.J. (ed.) *Xenopus* protocols: Cell biology and signal transduction, Humana Press (in press)

Liu, X.J. and Ruderman, J.V. (2003) The classical progesterone receptor mediates *Xenopus* oocyte maturation through a non-genomic mechanism. In Watson, C.S. (ed.) The identities of membrane steroid receptors, Kluwer Academic Publishers, p93-101

Liu, Z., and Patino, R. (1993) High-affinity binding of progesterone to the plasma membrane of *Xenopus* oocytes: characteristics of binding and hormonal and developmental control. *Biol. Reprod.* 49:980-8

Loutradis, D., Bletsa, R., Aravantinos, L., Kallianidis, K., Michalas, S., and

Psychoyos, A. (1991) Preovulatory effects of the progesterone antagonist mifepristone (RU486) in mice *Hum. Reprod.* 6:1238-40

Lutz, L.B., Cole, L.M., Gupta, M.K., Kwist, K.W., Auchus, R.J., and Hammes, S.R. (2001) Evidence that androgens are the primary steroids produced by *Xenopus laevis* ovaries and may signal through the classical androgen receptor to promote oocyte maturation *Proc. Natl. Acad. Sci. U. S. A.* 98:13728-33

Lutz, L.B., Jamnongjit, M., Yang, W.H., Jahani, D., Gill, A., and Hammes, S.R. (2003) Selective modulation of genomic and nongenomic androgen responses by androgen receptor ligands *Mol. Endocrinol.* 17:1106-16

Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, C.A. Jr, Shyamala, G., Conneely, O.M., and O'Malley, B.W. (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities *Genes Dev.* 9:2266-78.

Maller, J.L., and Krebs, E.G. (1977) Progesterone-stimulated meiotic cell division in *Xenopus* oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase *J. Biol. Chem.* 252:1712-8

Maller, J.L., Butcher, F.R., and Krebs, E.G. (1979) Early effect of progesterone on levels of cyclic adenosine 3':5'-monophosphate in *Xenopus* oocytes *J. Biol. Chem.* 254:579-82

Maller, J.L. (1998) Recurring themes in oocyte maturation *Biol. Cell* 90:453-60

Maller, J.L. (2001) The elusive progesterone receptor in *Xenopus* oocytes *Proc. Natl. Acad. Sci. U. S. A.* 98:8-10

Maller, J.L. (2003) Fishing at the surface *Science* 300: 594-595

Masui, Y. (1967) Relative roles of the pituitary, follicle cells, and progesterone in the induction of oocyte maturation in *Rana pipiens J. Exp. Zool.* 166:365-75

Masui, Y., and Markert, C.L. (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes *J. Exp. Zool.* 177:129-45

Masui, Y., Meyerhof, P.G., Miller, M.A., and Wasserman, W.J. (1977) Roles of divalent cations in maturation and activation of vertebrate oocytes *Differentiation.* 9:49-57

Masui, Y. and Clarke, H.J. (1979) Oocyte maturation *Int. Rev. Cytol.* 57:185-282

Masui, Y. (2001) From oocyte maturation to the in vitro cell cycle: the history of discoveries of Maturation-Promoting Factor (MPF) and Cytostatic Factor (CSF) *Differentiation.* 69:1-17

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol- receptor complex in MCF-7 cells *EMBO J.* 15:1292-300

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor *EMBO J.* 17:2008-18

Minshull, J., Sun, H., Tonks, N.K., and Murray, A.W. (1994) A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts *Cell.* 79:475-86

Morrill, G.A., and Bloch, E. (1977) Structure-function relationships of various steroids relative to induction of nuclear breakdown and ovulation in isolated amphibian oocytes *J. Steroid Biochem.* 8:133-9

Noh, S.J., and Han, J.K. (1998) Inhibition of the adenylyl cyclase and activation of the phosphatidylinositol pathway in oocytes through expression of serotonin receptors does not induce oocyte maturation. *J. Exp. Zool.* 280:45-56.

Norman, A.W., Mizwicki, M.T., and Norman, D.P. (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model *Nat. Rev. Drug Discov.* 3:27-41

Ohan, N., Agazie, Y., Cummings, C., Booth, R., Bayaa, M., and Liu, X.J. (1999) RHO-associated protein kinase alpha potentiates insulin-induced MAP kinase activation in *Xenopus* oocytes *J. Cell Sci.* 112:2177-84

Park, O.K., and Mayo, K.E. (1991) Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge *Mol. Endocrinol.* 5:967-78

Patino, R., and Thomas, P. (1990) Induction of maturation of Atlantic croaker oocytes by 17 alpha,20 beta,21-trihydroxy-4-pregnen-3-one *in vitro*: consideration of some biological and experimental variables. *J. Exp. Zool.* 255:97-109

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997) Mitotic and G_2 checkpoint control: regulation of 14-3-3 protein binding by

phosphorylation of Cdc25C on serine-216. *Science*, **277**, 1501-1505

Perrot-Applanat, M., Logeat, F., Groyer-Picard, M.T., and Milgrom, E. (1985) Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology*. 116:1473-84

Perrot-Applanat, M., Groyer-Picard, M.T., Lorenzo, F., Jolivet, A., Vu Hai, M.T., Pallud, C., Spyratos, F., and Milgrom, E. (1987) Immunocytochemical study with monoclonal antibodies to progesterone receptor in human breast tumors. *Cancer Res.* 1987 47:2652-61

Qiu, M., Olsen, A., Faivre, E., Horwitz, K.B., and Lange, C.A. (2003) Mitogen - activated protein kinase regulates nuclear association of human progesterone receptors *Mol. Endocrinol.* 17:628-42

Robinson-Rechavi, M., Escriva Garcia, H., and Laudet, V. (2003) The nuclear receptor superfamily *J. Cell Sci.* 116:585-6

Robker, R.L., Russell, D.L., Espey, L.L., Lydon, J.P., O'Malley, B.W., and Richards, J.S. (2000a) Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases *Proc. Natl. Acad. Sci. U. S. A.* 97:4689-94

Robker, R.L., Russell, D.L., Yoshioka, S., Sharma, S.C., Lydon, J.P., O'Malley, B.W., Espey, L.L., and Richards, J.S. (2000b) Ovulation: a multi-gene, multi-step process. *Steroids.* 65:559-70

Romo, X., Hinrichs, M.V., Guzman, L., and Olate, J. (2002) Gαs levels regulate *Xenopus laevis* oocyte maturation *Mol. Reprod. Dev.* 63:104-9

Sadler, S.E., and Maller, J.L. (1981) Progesterone inhibits adenylate cyclase in *Xenopus* oocytes. Action on the guanine nucleotide regulatory protein *J. Biol. Chem.* 256:6368-73

Sadler, S.E., and Maller, J.L. (1982) Identification of a steroid receptor on the surface of *Xenopus* oocytes by photoaffinity labeling *J. Biol. Chem.* 257:355-61.

Sadler, S.E., Maller, J.L., and Cooper, D.M. (1984) Progesterone inhibition of *Xenopus* oocyte adenylate cyclase is not mediated via the *Bordetella pertussis* toxin substrate. *Mol. Pharmacol.* 26:526-31

Sadler, S.E., Bower, M.A., and Maller, J.L. (1985) Studies of a plasma membrane steroid receptor in *Xenopus* oocytes using the synthetic progestin RU 486 *J. Steroid Biochem.* 22:419-26

Sanchez-Criado, J.E., Bellido, C., Galiot, F., Lopez, F.J., and Gaytan, F. (1990) A possible dual mechanism of the anovulatory action of antiprogesterone RU486 in the rat *Biol. Reprod.* 42:877-86

Schatz, F., and Ziegler, D. (1979) The role of follicle cells in *Rana pipiens* oocyte maturation induced by delta 5-pregnenolone *Dev. Biol.* 73:59-67

Schmitt, A., and Nebreda, A.R. (2002) Signalling pathways in oocyte meiotic maturation *J. Cell Sci.* 115:2457-9

Schorderet-Slatkine, S. (1972) Action of progesterone and related steroids on oocyte maturation in *Xenopus laevis*. An *in vitro* study *Cell Differ.* 1:179-89

Schorderet-Slatkine, S., Schorderet, M., Boquet, P., Godeau, F., and Baulieu, E.E. (1978) Progesterone-induced meiosis in *Xenopus laevis* oocytes: a role for cAMP at the "maturation- promoting factor" level *Cell.* 15:1269-75

Schorderet-Slatkine, S. and Baulieu, E.E. (1982) Forskolin increases cAMP and inhibits progesterone induced meiosis reinitiation in *Xenopus laevis* oocytes *Endocrinology.* 111:1385-7

Schuetz, A.W. (1967a) Effect of steroids on germinal vesicle of oocytes of the frog (*Rana pipiens*) *in vitro*. *Proc. Soc. Exp. Biol. Med.* 124:1307-10

Schuetz, A.W. (1967b) Mechanism of progesterone- and pituitary- induced germinal vesicle breakdown in oocytes of *Rana pipiens J. Cell. Biol.* 35,123

Schuetz, A.W. and Lessman, C. (1982) Evidence for follicle wall involvement in ovulation and progesterone production by frog (*Rana pipiens*) follicles *in vitro Differentiation.* 22:79-84

Shen, T., Horwitz, K.B., and Lange, C.A. (2001) Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. *Mol. Cell Biol.* 21:6122-31

Sheng, Y., Tiberi, M., Booth, R.A., Ma, C., and Liu, X.J. (2001) Regulation of *Xenopus* oocyte meiosis arrest by G protein βγ subunits. *Curr. Biol.* 11:405-16

Smith, L.D., Ecker, R.E., and Subtelny, S. (1968) *In vitro* induction of physiological maturation in *Rana pipiens* oocytes removed from their ovarian follicles *Dev. Biol.* 17:627-43

Smith, L.D., and Ecker, R.E. (1969) Role of the oocyte nucleus in physiological

maturation in *Rana pipiens Dev. Biol.* 19:281-309

Smith, L.D. (1989) The induction of oocyte maturation: transmembrane signaling events and regulation of the cell cycle *Development.* 107:685-99

Smith, L.D., Xu, W., and Varnold, R.L. (1991) Oogenesis and oocyte isolation In Kay, B.K. and Peng, H.B. (eds.) *Xenopus laevis*: Practical Use in Cell and Molecular Biology. Academic Press, p45-60

Snyder, B.W., and Schuetz, A.W. (1973) *In vitro* evidence of steroidogenesis in the amphibian (*Rana pipiens*) ovarian follicle and its relationship to meiotic maturation and ovulation *J. Exp. Zool.* 183:333-42

Sohaskey, M.L. and Ferrell, J.E. Jr. (1999) Distinct, constitutively active MAPK phosphatases function in *Xenopus* oocytes: implications for p42 MAPK regulation *in vivo Mol. Biol. Cell.* 10:3729-43

Song, R.X., McPherson, R.A., Adam, L., Bao, Y., Shupnik, M., Kumar, R., and Santen, R.J. (2002) Linkage of rapid estrogen action to MAPK activation by ERα-Shc association and Shc pathway activation *Mol. Endocrinol.* 16:116-27

Song, R.X., Barnes, C.J., Zhang, Z., Bao, Y., Kumar, R., and Santen, R.J. (2004) The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor α to the plasma membrane *Proc. Natl. Acad. Sci. U. S. A.* 101:2076-81

Stevis, P.E., Deecher, D.C., Suhadolnik, L., Mallis, L.M., and Frail, D.E. (1999) Differential effects of estradiol and estradiol-BSA conjugates *Endocrinology.* 140:5455-8

Thibier-Fouchet, C., Mulner, O., and Ozon, R. (1976) Progesterone biosynthesis and metabolism by ovarian follicles and isolated oocytes *Xenopus laevis Biol. Reprod.* 14:317-26

Tian, J., Kim, S., Heilig, E., and Ruderman, J.V. (2000) Identification of XPR-1, a progesterone receptor required for *Xenopus* oocyte activation *Proc. Natl. Acad. Sci. U. S. A.* 97:14358-63

Tsai, M.J. and O'Malley, B.W. (1994) Molecular mechanisms of action of steroid / thyroid receptor superfamily members *Annu. Rev. Biochem.* 63:451-86

Tso, J., Thibier, C., Mulner, O., and Ozon, R. (1982) Microinjected progesterone reinitiates meiotic maturation of *Xenopus laevis* oocytes *Proc. Natl. Acad. Sci. U. S. A.* 79:5552-6

Wang, J., and Liu, X.J. (2003) A G protein-coupled receptor kinase induces *Xenopus* oocyte maturation *J. Biol. Chem.* 278:15809-14

Wasserman, W.J., and Masui, Y. (1974) A study on gonadotropin action in the induction of oocyte maturation in *Xenopus laevis Biol. Reprod.* 11:133-44

Wasserman, W.J., Pinto, L.H., O'Connor, C.M., and Smith, L.D. (1980) Progesterone induces a rapid increase in $[Ca^{2+}]_{in}$ of *Xenopus laevis* oocytes *Proc. Natl. Acad. Sci. U. S. A.* 77:1534-6

Wright, P.A. (1961) Induction of ovulation *in vitro* in *Rana pipiens* with steroids *Gen. Comp. Endocrinol.* 1:20-3

Wright, P.A. (1971) 3-keto-delta-4 steroid: requirement for ovulation in *Rana pipiens Gen. Comp. Endocrinol.* 16:511-5

Yang, W.H., Lutz, L.B., and Hammes, S.R. (2003) *Xenopus laevis* ovarian CYP17 is a highly potent enzyme expressed exclusively in oocytes. Evidence that oocytes play a critical role in *Xenopus* ovarian androgen production *J. Biol. Chem.* 278:9552-9

Yatvin, M.B. and Pitot, H.C. (1969) Effect of the inhibition of RNA synthesis on *in vitro* ovulation by frog ovaries *Nature* 223:62-64

Ylikomi, T., Bocquel, M.T., Berry, M., Gronemeyer, H., and Chambon, P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors *EMBO J.* 11:3681-94

Zhu, Y., Rice, C.D., Pang, Y., Pace, M., and Thomas, P. (2003a) Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes *Proc. Natl. Acad. Sci. U. S. A.* 100:2231-6

Zhu, Y., Bond, J., and Thomas, P. (2003b) Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor *Proc. Natl. Acad. Sci. U. S. A.* 100:2237-42

Ziegler, D., and Masui, Y. (1976) Control of chromosome behavior in amphibian oocytes. II. The effect of inhibitors of RNA and protein synthesis on the induction of chromosome condensation in transplanted brain nuclei by oocyte cytoplasm. *J. Cell Biol.* 68:620-8

CURRICULUM VITAE

Education

September 2002 - Present	Master of Science in Biochemistry **UNIVERSITY OF OTTAWA**, Ottawa Ontario, Canada Dr. Johné Liu, Supervisor
September 1998 - 2002	Bachelor of Science, Major in Biochemistry **SUN YAT-SEN UNIVERSITY**, Guangzhou, Guangdong, China

Presentation

Xun Liu and X. Johné Liu (2004) "Differential localization and regulation of the classical progesterone receptor in *Xenopus* oocyte and follicle cells". Presented at the 10th International *Xenopus* Meeting, Woods Hole, Massachusetts

Publications

1: Yinglun Sheng, Ling Wang, **X. Shawn Liu**, Véronique Montplaisir, Mario Tiberi, Jay M. Baltz and X. Johné Liu (2004) A serotonin receptor antagonist induces oocyte maturation in both frogs and mice: evidence that the same G protein-coupled receptor is responsible for maintaining meiosis arrest in both species *Journal of Cellular Physiology* (in press)

2: **Xun Liu** and X. Johné Liu (2004) Oocyte isolation and enucleation In X. Johné Liu (ed.) *Xenopus* protocols: Cell biology and signal transduction, Humana Press (in press)

3: **Xun Liu**, Yan Zhong, and Qiong Shen (2002) Systematic molecular basis of beta-lactam antibiotic resistance in *Staphylococci* (Chinese) *Journal of Chinese Physician* 4: 553-555