# GATA LIKE PROTEIN-1: A SOMATIC CELL FACTOR REQUIRED FOR NORMAL OVARIAN DEVELOPMENT AND FUNCTION

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## DEDICATION & ACKNOWLEDGEMENT

To Nick and Carter:

**"**If any of you lacks wisdom, he should ask God, who gives generously to all without finding fault, and it will be given to him. But when he asks, he must believe and not doubt, because he who doubts is like a wave of the sea, blown and tossed by the wind." **James 1:5-6**

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by

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GLP-1: A SOMATIC CELL FACTOR REQUIRED FOR NORMAL OVARIAN DEVELOPMENT AND FUNCTION

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Oogenesis and follicular maturation are processes that require organized and precisely timed communication through paracrine and endocrine signals of neighboring tissues. Deviations in the cross talk between ovarian cells, or aberrant gene expression within one of the cell populations, can lead to germ cell loss and infertility in the adult female. Expression of Glp-1

in the somatic cells of the ovary is required for normal fertility in female mice, as a deficiency for Glp-1 leads to the absence of oocytes at birth and ovarian tubular formation in the adult. However, the nature of germ cell loss and tubular adenoma formation, in the setting of a somatic cell protein deficiency, is not well understood. In this report, I characterize the embryonic germ cell loss phenotype in *Glp-1*$^{LacZ}$ null mice. Immunohistochemical analyses of *Glp-1*$^{LacZ}$ null mouse ovaries show that germ cells are appropriately specified and migrate to the nascent gonad similarly to wild type. After their arrival at the gonad, precocious loss of the germ cells begins at or around E13.5. This loss is completed by birth and is accompanied by defects in the expression of oocyte-specific genes associated with meiotic entry. Interestingly, somatic pregranulosa cells retain their ability to secrete paracrine signaling molecules to the oocyte and are still able to form the basement membrane surrounding the germline cysts. In the adult, the structure of the germline cyst persists, albeit without germ cells, and there is loss of HPG axis communication. The loss in HPG communication in *Glp-1*$^{LacZ}$ null mice can be accounted for by loss of regulated steroidogenesis through the GATA4-dependent transcriptional activation of *StAR*. These data imply that the somatic cell protein Glp-1 regulates 1) germ cell survival early in embryogenesis and 2) steroidogenesis through *StAR* promoter activation.

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## LIST OF ABREVIATIONS

Alk5 – Activin receptor-like kinase 5

AP – Alkaline-phosphatase

Bax – Bcl-2 associated X

Blimp1 – B-lymphocyte-induced maturation protein-1

Bmp – Bone morphogenic protein

bt – Bos taurus

cAMP – cyclic AMP

C/EBPβ – CCAAT-enhancer-binding protein β

c-Kit – KitL receptor

Cx43 – Connexin 43

Cxcr4 – Chemokine receptor 4

Dazl – Deleted in azoospermia-like

Dmc1 – Disruption of meiotic control

Dmrt1 – Dsx- and mab-3- related transcription factor 1

Dnd1 – Dead end 1

E – embryonic day

ECM – extra-cellular matrix

Ereg – Epiregulin

Erk2 – Extracellular signal-regulated kinase 2

Figlα – Factor in the germline α

Fog-2 – Friend of GATA-2

FSH – Follicle stimulating hormone

Fshr – FSH receptor

Gcd – Germ cell deficient

Gcnf – Germ cell nuclear factor

Gdnf – Glial cell-line derived neurotrophic factor

Glp-1 – GATA like protein-1

GnRH – Gonadotropin-releasing hormone

Gsk3β – Glycogen synthase kinase 3β

H3K9 – histone H3 lysine 9

H3K27 – histone H3 lysine 27

Hsd-3β - Hydroxysteroid dehydrogenase-3β

HPG – hypothalamic-pituitary-gonadal

KitL – Kit ligand

LH – Luteinizing hormone

Lhr – LH receptor

Lrh-1 – Liver receptor homolog-1

Mapk – Mitogen-activated protein kinase

me2 – dimethylation

me3 – trimethylation

Mis – Müllerian-inhibiting substance

Mvh – Mouse VASA homolog

Oct4 – Octamer-4

OX-Glp-1 – Glp-1 over-expressing MLTC1 cell line

P450ssc – cholesterol side chain cleavage cytochrome P450

PGC – primordial germ cell

PI3K – Phosphatidylinositol 3-kinase

PKA – Protein kinase A

PND/PD – post natal day

POF – premature ovarian failure

PR – Progesterone receptor

Prdm – PRDI-IBF1 and RIZ homologous domain

RA – retinoic acid

Prmt5 – Protein arginine methlytransferase 5

Sdf-1 – Stromal derived factor –1

SF-1 – Steroidogenic factor-1

Sox-2 – SRY-box-2

SP-A – Surfactant protein A

Spo11 – Sporulation protein 11

Stra8 – Stimulated by retinoic acid gene-8

StAR – Steroidogenic acute regulatory protein

Sycp3 – Synaptonemal complex protein 3

Tgfβ – Transforming growth factor β

Tnap – Tissue nonspecific alkaline phosphatase

ZP – Zona pellucida

# CHAPTER I: FORMATION OF A FOLLICLE AND THE ROLE OF GATAS AND GLP-1 IN THE ADULT OVARY

## I. Introduction

Perturbation of gonadal development and function resulting in infertility is devastating for families wanting to experience parenthood. Discoveries made through reproductive biology and endocrinology research provide clues with which the medical profession can better treat patients with decreased fertility. Specification of germ cells, which go on to become part of a follicular structure that can ultimately undergo ovulation, is essential to determining the reproductive potential of a female. Establishment of the follicular pool requires the coordinated interactions of several cell types, all of which are regulated in their own independent fashion. Importantly, this process is conserved in humans and mice. This literature review focuses on mouse female reproduction from primordial germ cell (PGC) specification to primordial follicle formation and adult ovarian function with an emphasis on GATA4/6 and GATA like protein-1 (Glp-1).

## II. PGC specification through primordial follicle formation

The nature of follicular development requires numerous processes to occur along with dynamic cellular migration of the PGC to the gonad from their site of origin. The functional female reproductive unit begins by being specified

as a specialized cell type called a PGC. Upon specification, PGCs must become competent to undergo subsequent maturation. The early processes of oogenesis, from embryonic day (E) 7.25 to 13.5, including acquiring pluripotent competency accompanied by loss of somatic gene expression, epigenetic reprogramming, migration, limited proliferation, and meiotic entry, are illustrated in Figure 1.a along with the time line in which they occur (De Felici 2009). Figure 1.b delineates later processes, from E11 to postnatal day (PND) 6, slightly overlapping with Figure 1.a., including meiotic entry and arrest, germline cyst formation, and cyst breakdown into primordial follicle formation (Pepling 2006). These figures illustrate the major processes that are required for primordial follicle formation while the molecules involved in these processes are discussed in the text.

*a. The origin of PGC specification*

PGCs were shown to be specified very early in development by lineage analysis and transplantation experiments. The formation of PGCs requires inductive signals from neighboring tissue. Between E5.5 and 7.5, a subset of cells, which include the PGCs, are allocated in the posterior portion of the epiblast/primitive streak (also known as the embryonic ectoderm) (Snow 1981, Tam & Snow 1981, Ginsburg *et al.* 1990, Lawson & Hage 1994, Tam & Zhou 1996). PGC specification is induced by secreted growth factors belonging to the transforming growth factor β (Tgfβ) superfamily. Bone morphogenetic protein

(BMP) 4 and Bmp8b are secreted from the neighboring extra-embryonic ectoderm and Bmp2 from the visceral endoderm. Mice deficient for any of these genes have fewer PGCs than wild type controls (Lawson *et al.* 1999, Ying *et al.* 2000, Ying & Zhao 2001, de Sousa Lopes *et al.* 2007). A subset of Smad family members, Smad1, Smad5, and Smad8, are the effector molecules for Bmp signaling which bind a common co-factor, Smad4. To test their role in PGC specification through Bmp signaling, Smad mutants were generated. *Smad4* mutants have an early embryonic lethal phenotype and die prior to E8.5 (Dunker & Krieglstein 2000). Thus, the precise role of Smad4 in PGC specification has not yet been studied. *Smad5* mutants showed significant reduction in PGC number, in a dose dependent manner during specification, as early as E8.0 (Chang & Matzuk 2001). Similar to Smad5, loss of Smad1 prevents specification of PGCs as demonstrated by the lack of tissue nonspecific alkaline phosphatase (Tnap) (an early marker of PGCs) staining at E7.5 (Hayashi *et al.* 2002). Mutant alleles for Smad8 were generated and these animals were fertile; thus, Smad8 may function redundantly with Smad1 and Smad5 (Arnold *et al.* 2006). These data suggest that Bmp signaling occurs through the canonical effector proteins, Smad5 and Smad1. Genetic ablation studies of these Bmp family members and the downstream effector molecules support more historical fate-mapping data obtained from lineage analysis and transplantation experiments; thus deducing the mechanism of PGC specification (Tam & Snow 1981, Lawson & Hage 1994, Tam & Zhou 1996).

*b. Tracking PGC specification with markers*

The Bmp inductive signals secreted from the extra-embryonic ectoderm and the visceral endoderm not only give rise to PGCs but somatic tissue as well. This prompted the investigation of germ cell-specific genes that are turned on during the process of PGC specification. *Fragilis/Ifitm*, *Stella/Dppa3/Pgc7*, and *B-lymphocyte-induced maturation protein-1* (*Blimp1*)*/Prdm1* are the most upstream genes expressed in tissue where the PGCs are restricted to form. They were identified in a cDNA library screen of single PGCs isolated from the posterior portion of the epiblast from E7.0 to 7.5 embryos. *Fragilis* expression, starting at E6.0, follows the movement of cells previously described by clonal analysis to include the PGCs (Lawson & Hage 1994, Saitou *et al.* 2002). Unfortunately, Fragilis functions only as a marker of PGC specification as *Fragilis* null mice show no defects in fertility (Lange *et al.* 2008). *Stella* expression begins at E7.0 and co-stains a subset of Fragilis positive cells (Saitou *et al.* 2002). Once again, Stella, an early marker for PGCs, was shown to be nonessential for PGC specification (Bortvin *et al.* 2004). Previous to the identification of these additional PGC markers, Tnap staining was the only marker of PGCs. Inactivation of the *Tnap* gene, as is the case with *Stella* and *Fragilis*, has no biological consequence on PGC formation and thus only functions as a marker for PGCs (MacGregor *et al.* 1995). Unlike Fragilis, Stella and Tnap, Blimp1 plays a crucial role in PGC formation and is discussed in the next section.

*c. Loss of Somatic Gene Expression and Acquisition of Pluripotency in PGCs*

Concomitant with upregulation of pluripotency genes that specify PGCs, down regulation of somatic program genes, including homeobox genes, and epigenetic reprogramming need to occur. Identification of Blimp1, a SET-PR containing protein, and PRDI-BF1 and RIZ homologous (PR) domain protein (Prdm) 14 demonstrates the importance of these three processes during PGC specification. Blimp1, identified as a repressor of homeobox genes, co-localizes with Fragilis expression even earlier than Stella expression in the epiblast at E6.25 (Saitou *et al.* 2002, Ohinata *et al.* 2005). Loss of Blimp1 leads to a severe reduction in the number of PGCs as early as E7.5 with none present by E12.5 and sustained expression of the homeobox genes, *Hoxa1* and *Hoxb1* (Ohinata *et al.* 2005). PGCs also showed decreased expression of the pluripotency genes, *SRY-box 2* (*Sox2*) and *Nanos3* (Ohinata *et al.* 2005). Prdm14 was identified in a quantitative single-cell cDNA amplification screen followed by qPCR looking for genes upregulated during PGC specification that were also Prdm1 positive (Yabuta *et al.* 2006). Prdm14 is required for proper PGC specification and epigenetic reprogramming. *Prdm14* null mice have reduced numbers of germ cells at E7.25, suggesting a role for Prdm14 in PGC specification as determined by expression of *Sox2*, a pluripotency gene (Yamaji *et al.* 2008). Octamer-4 (Oct4), a POU-domain transcription factor, also marks pluripotent cells. Oct4 is expressed as early as E3.5 in the developing embryo, but is then restricted to PGCs by E8.75 (Yeom *et al.* 1996). This restricted expression is due to

repression of Oct4 in neighboring tissues by germ cell nuclear factor (Gcnf) (Fuhrmann *et al.* 2001). Nanog, another marker for pluripotency, is expressed in PGCs until E13.5 (Yamaguchi *et al.* 2005). Its expression is dependent on transcriptional activation by the Sox2/Oct4 co-activators (Kuroda *et al.* 2005). Many of these genes continue to be expressed until differentiation of PGCs by entering into meiosis, at which point meiotic entry markers start to be expressed.

*d. Epigenetic reprogramming of PGCs*

During PGC migration changes in DNA epigenetic status occur that lead to the loss of somatic gene expression and acquisition of pluripotent gene expression. Using imunofluorescent techniques, it was shown that histone H3 lysine 9 (H3K9) dimethylation (me2) is lost beginning at E8.0. At E8.0, somatic cells and PGCs have similar levels of DNA methylation, which is lost in the PGCs during the migratory period (E9.5). Subsequent to the loss of H3K9me2 and DNA demethylation, there is an increase in histone H3 lysine 27 (H3K27) trimethylation (me3) (Seki *et al.* 2005). Prdm14 has been shown to be important for these changes. *Prdm14* null germ cells fail to reduce H3K9me2 at E8.5 and subsequently upregulate H3K27me3 at E9.5. Blimp1, as described above, interacts with chromatin modifiers, thus demonstrating a role in establishment of the epigenetic status of PGCs, which results in changes of gene expression. Blimp1 physically interacts with protein arginine methlytransferase 5 (Prmt5), an arginine-specific histone methyltransferase that functions on arginine 3 of histone

H2A and/or H4 tails (Ancelin *et al.* 2006). Loss of Blimp1, as described above, leads to PGC death (Ohinata *et al.* 2005). By E12.5, all genomic imprinting is erased (Lee *et al.* 2002, Seki *et al.* 2005). These changes allow for maintained gene expression and increased DNA plasticity. These epigenetic changes to the DNA and histones are essential for changes in gene expression leading to the pluripotent status of the PGCs.

*e. PGC migration to the genital ridge*.

PGC specification is completed around E7.5. Once this process is over, the PGCs begin their migration to the nascent gonad. PGCs labeled by alkaline-phosphatase (AP) migrate from the base of the epiblast into the primary endoderm, then into the hindgut, finally entering the genital ridge by E12 (Tam & Snow 1981). Time lapse movies using a *Oct4ΔPE:GFP* transgenic reporter and β-galactosidase staining of the germ cells using a second *Oct4/LacZ* transgenic reporter confirm these results (Yeom *et al.* 1996, Molyneaux *et al.* 2001).

Migration of PGCs has been shown to occur through chemo-attractant signaling. Secreted molecules expressed from the neighboring somatic tissue signal through their respective receptors expressed on the germ cell, thus directing PGC migration to the gonad. Kit ligand (KitL)/ kit ligand receptor (c-Kit), stromal-derived factor-1(Sdf-1)/chemokine receptor 4 (Cxcr4), and Tgfβ signaling pathways have been shown to be important for proper PGC migration.

Both KitL and c-Kit are required for migration. PGC migration is disrupted in mice deficient for KitL (Murphy 1972). KitL is secreted from the developing gonad while the germ cells express the receptor, c-Kit (Manova *et al.* 1993). Supporting a role for KitL/c-Kit in PGC migration, cultured PGCs are stimulated to migrate with the addition of KitL. This occurs through activation of phosphatidylinositol 3-kinase (PI3K). Addition of a PI3K inhibitor to the medium of PGC cultures abolished KitL dependent migration (Farini *et al.* 2007).

Similar to KitL/c-Kit, the SDF-1 receptor, Cxcr4, is expressed on the germ cells, while the neighboring tissues secrete SDF-1 (Molyneaux *et al.* 2003). Mice deficient for Cxcr4 lose PGCs during migration and thus have a reduced number of PGCs that colonize the gonad. PGCs migrating through the hindgut at E9.5 were treated in culture with or without exogenous SDF-1. Treated PGCs showed aberrant migration while untreated PGCs migrated out of the hindgut similar to their migration projectory *in vivo*. These data suggest a role for SDF-1/Cxcr4 in PGC survival during migration (Molyneaux *et al.* 2003). The role for SDF-1/Cxcr4 in PGC migration is conserved in zebrafish and in the chick (Doitsidou *et al.* 2002, Knaut *et al.* 2003, Stebler *et al.* 2004).

Before these instructive signals are processed by the PGCs, they require a stationary signal to prevent precocious migration. This stationary requirement

was shown to be performed by Tgfβ signaling to the PGCs through its receptor, activin receptor-like kinase 5 (Alk5). To demonstrate this, *Alk5* null mice were generated and the PGCs were observed for migration defects. Interestingly, there was no PGC loss during migration and no obvious defect in migration to the gonad. Instead, *Alk5* null PGCs precociously migrated to the gonad. Alk5 was shown to promote deposition of collagen type I in the extra-cellular matrix (ECM) which strongly adheres to PGCs. Thus down regulation of collagen type I leads to early release of the PGCs from the hindgut, allowing for their precocious migration (Chuva de Sousa Lopes *et al.* 2005).

Migration requires cell to ECM and cell to cell interactions. One of the most important factors for this interaction is integrin β1. Integrin β1 is expressed on the PGCs and is required for PGC migration to the gonad. Loss of Integrin β1 on the PGCs prevents the majority of PGCs from migrating to the gonad (Anderson *et al.* 1999). The gap junction protein, Connexin 43 (Cx43), was also found to be important for cell-cell interactions and is expressed on the PGCs as they begin their migration. Cx43 deficient PGCs are slower to migrate while undergoing increased apoptosis as a result of loss of adhesion through Integrin β1 function (Francis & Lo 2006). These data demonstrate the importance of cell to cell and cell to ECM interactions of the PGCs for PGC survival during migration.

*f. Survival of proliferating PGCs during migration*

It has been shown that as the PGCs are migrating, they are also proliferating (Tam & Snow 1981). They undergo several rounds of mitosis with incomplete cytokinesis forming interconnected cysts surrounded by somatic cells before finally, at E13.5, arresting in meiosis (Pepling & Spradling 1998). Several oocyte specific genes are required for survival of the germ cells as they migrate. *Nanos3*, a PGC specific gene, is expressed from E9.5 through 14.5 in the germ cells. The importance of Nanos3 during migration has been shown through genetic ablation. PGCs deficient for Nanos3 have normal numbers of germ cells before migration begins, but they rapidly disappear during migration (Tsuda *et al.* 2003). PGC survival requires Nanos3 expression to protect the PGCs from bcl-2 associated X (Bax)-dependent and –independent apoptosis (Suzuki *et al.* 2008). Nanog, a pluripotency marker, is also expressed in migrating PGCs. To address the function of Nanog, *Nanog* short-hairpin (sh)RNA transgenic mice were generated. *Nanog* mRNA levels were reduced by Cre/loxP-mediated conditional knockdown using either estrogen receptor-Cre or Tnap-Cre. These mice show a 50% decrease in the number of PGCs at E12.5 (Yamaguchi *et al.* 2009). Bax is a proapoptotic protein shown to be involved in proper establishment of PGC numbers during migration. Bax deficient PGCs are increased in numbers in neonates and adults, resulting in prolonged reproductive longevity in females (Perez *et al.* 1999, Greenfeld *et al.* 2007). These data show that Nanos3 and

Nanog are important for PGC survival during migration and that BAX regulates the number of PGCs allowed to occupy the embryonic gonad.

*g. Mitotic arrest of PGCs*

PGCs undergo limited rounds of proliferation upon entry into the gonad, at which point they lose expression of pluripotency genes followed by entry into meiosis at E13.5 (Figure 1.a and 1.b). Expression of Nanog, for example, is lost at the time of meiotic entry (Yamaguchi *et al.* 2009). Dsx- and mab-3 related transcription factor 1 (Dmrt1) has been shown to be important in shutting off pluripotency gene expression in germ cells. *Dmrt1* mutant germ cells have ectopic expression of the pluripotency markers, *Sox2*, *Oct4*, and *Nanog*. *Dmrt1* mutant germ cells also fail to undergo mitotic arrest as shown by the increased numbers of Ki67 (a marker for proliferation)-positive germ cells compared to controls (Krentz *et al.* 2009). Similarly, a mutation in the *dead end* (*Dnd1*) locus of the mouse genome results in germ cells that fail to arrest mitosis (Youngren *et al.* 2005). Thus, mitotic arrest and simultaneous loss of pluripotent gene expression are required for PGCs to differentiate into meiotically active germ cells.

*h. Meiotic entry of PGCs*

PGC specification and migration occurs similarly in both male and female embryos before they reach the gonad. Once the germ cells arrive, they are

exposed to male (XY) and female (XX) specific factors within the respective gonadal tissue. At E13.5 differences in gene expression lead to germ cells in an XX gonad undergoing meiotic entry and subsequent arrest in dictyate of meiotic prophase I, while germ cells in the XY gonad arrest in G0 or G1 of mitosis (Bowles & Koopman 2007).

Several laboratories, including the Page and Koopman groups, have made significant progress in the field of germ cell meiotic entry. In the late 1990s it was suggested that PGCs of both sexes must be exposed to a meiotic-inducing substance for them to enter into meiosis (McLaren & Southee 1997). It was also postulated that a factor in the males was required to prevent them from precocious meiotic entry (McLaren & Southee 1997). In the early 2000s, the Page and Koopman groups independently determined that meiotic entry occurs in an anterior-to-posterior wave in the female gonad. Koopman's group demonstrated loss of expression of *Oct4*, a marker of pluripotency, and the gain of expression of *synaptonemal complex protein 3 (Sycp3)*, a marker of meiotic entry, in an anterior to posterior fashion (Bullejos & Koopman 2004). Page's group analyzed expression of Stimulated by retinoic acid gene-8 (Stra8). The Stra8 protein is expressed in the female but not the male at E13.5 and was identified in a screen looking for gene expression stimulated by retinoic acid to detect meiotic entry (Bouillet *et al.* 1995, Menke & Page 2002). They confirmed these results showing expression of disruption of meiotic control (Dmc1)/LIM15,

a marker of meiosis, and loss of Oct4 in an anterior to posterior fashion (Menke *et al.* 2003). These data demonstrate the importance of extinction of pluripotent gene expression with the switch from mitosis to meiosis.

As a result of the Stra8 expression pattern coinciding with meiotic entry, studies assessed the ability of retinoic acid (RA) to stimulate meiotic entry in PGCs. RA, secreted from the neighboring mesenchyme, was shown to stimulate meiotic entry through expression of Stra8 (Baltus *et al.* 2006, Bowles *et al.* 2006, Anderson *et al.* 2008). Stra8 activation by RA also requires the loss of *Cyp26b1* gene expression. Cyp26b1, a P450 enzyme, oxidizes RA into an inactive form and is expressed in both male and female gonads prior to meiotic entry (Bowles *et al.* 2006). Mice deficient for Stra8 undergo normal mitosis but fail to enter into meiosis (Baltus *et al.* 2006, Anderson *et al.* 2008).

Deleted in azoospermia-like (Dazl), an RNA-binding protein, is required not only for germ cell maintenance but also for expression of meiotic genes. *Dazl* knock-out female mice lose their germ cells after E12.5 (Haston *et al.* 2009). Dazl is required for the mRNA expression of *Stra8*, *Dmc1*, *sporulation protein 11* (*Spo11*), *Sycp3*, and *Rec8*, all of which are involved in meiosis (Lin *et al.* 2008). Dmc1 and Spo11 are responsible for proper recombination. Spo11 produces DNA double-stranded breaks while Dmc1 repairs the breaks (Cohen & Pollard 2001, Hunt & Hassold 2002). Stra8 expression is required for the proper DNA

loading of Rec8 and Sycp3, a cohesion molecule and a component of the synaptonemal complex, respectively, during meiotic prophase at E15.5 (Baltus *et al.* 2006). These results show that meiotic entry occurs due to stimulation by RA and subsequent expression of genes involved in meiosis.

*i. Germ cell-cyst encapsulation and primordial follicle formation*

A primordial follicle is morphologically characterized as a single layer of squamoid (i.e. flattened) epithelial cells (pregranulosa cells) surrounding a single, meiotically arrested oocyte (Chapter I, Figure 1.b). At birth, a finite follicular pool of oocytes is present and there are no post-natal mechanisms for oocyte renewal. Therefore, tight regulation of primordial follicle formation is critical to fertility.

As the germ cells are entering into meiosis, germline cyst formation occurs, followed closely by germline cyst breakdown. Before PGCs enter into meiosis, they have undergone 7-8 rounds of proliferation, increasing in number from 50 PGCs to 25,000 (Tam & Snow 1981). These mitotic divisions occur with incomplete cytokinesis thus forming interconnected germline cysts through E13.5 (Chapter I, Figure 1.b) (Pepling & Spradling 1998). The PGCs migrate via an ECM containing laminin, fibronectin, and collagen type IV. As PGCs approach the developing gonad, laminin expression increases, thus forming a ribbon-like structure onto which the PGCs adhere and migrate up to the gonad. Laminin

staining illustrates the encapsulation of germ cells by the pregranulosa cells, which deposit laminin into the basement membrane after PGC arrival to the gonad (Frojdman *et al.* 1995, Garcia-Castro *et al.* 1997).

Around birth, programmed cyst breakdown begins by somatic pregranulosa cells intercalating between germ cells of the cyst, thus disrupting gap junctions and forming primordial follicles (Chapter I, Figure 1.b) (Pepling & Spradling 2001). Ultrastructural studies utilizing the developing rat ovary beautifully describe the process of cyst breakdown. It demonstrates modifications in basement membrane deposition by pregranulosa cells and their interactions with future theca cells of mesenchymal origin. These studies also demonstrate the function of metalloproteinases in breaking apart the cysts to form primordial follicles (Mazaud *et al.* 2005).

Regulated germ cell death occurs along with germline cyst breakdown, as the total number of PGCs decreases by two-thirds during this process (Figure 1.b) (Pepling & Spradling 2001, McClellan *et al.* 2003). As described above, BAX, a proapoptotic gene, plays a critical role in apoptosis during migration and is thought to be essential for proper follicular endowment of primordial follicles in the adult (Perez *et al.* 1999, Greenfeld *et al.* 2007).

Paracrine and autocrine signaling between and within the oocyte and the pregranulosa cells is required for the survival of the germ cells during primordial follicle formation. The role for Notch signaling in this pathway is supported by localization studies of the receptor and ligand. Notch2, a receptor for Jagged1, is expressed in pregranulosa cells, while Jagged1 is expressed in the germ cells during the time of primordial follicle formation (Trombly *et al.* 2009). By culturing newborn mouse ovaries in the presence of the Notch pathway inhibitor, gamma-secretase, few primordial follicles formed and most oocytes remained within the germline cyst (Trombly *et al.* 2009). Thus, Notch signaling appears to be required for promoting primordial follicle formation.

In contrast to Notch signaling, which promotes primordial follicle formation, estradiol and progesterone are thought to inhibit primordial follicle formation, although discrepancies exist in the literature. Recent studies have shown that germline cysts undergo programmed cyst breakdown in response to withdrawal of estradiol and progesterone treatment. Likewise, primordial follicle formation can be rescued by addition of estradiol or progesterone to medium of ovarian cultures (Kezele & Skinner 2003, Chen *et al.* 2007). The idea behind performing these studies is that at birth when primordial follicles form, neonatal mice are withdrawn from exposure to the pregnancy hormones, estradiol and progesterone, from the mother, therefore causing germline cyst breakdown.

The discrepancies in the literature are found in studies using mice deficient for the progesterone receptor (PR) and aromatase. Although defects are seen in all steroidogenic tissues of *PR* null mice, it is not until adulthood that the ovaries exhibit a phenotype, long after fetal progesterone withdrawal. The ovary develops normally but adult female mice are sterile due to the inability to ovulate (Lydon *et al.* 1995). Aromatase, transcribed from the *cyp19* gene, catalyzes the formation of estrogen from C19 steroids. The ovaries of *aromatase* null adult female mice contain follicles that have also failed to ovulate. This suggests that exposure of the ovary to estrogen and progesterone is required for the late stages of follicular maturation, but not primordial follicle formation (Fisher *et al.* 1998, Britt *et al.* 2004). Therefore, although progesterone and estradiol withdrawal may promote primordial follicle formation in culture, the biological function during germline cyst breakdown in the mouse is unknown.

Figl$\alpha$ (factor in the germline $\alpha$) is a germ cell-specific transcription factor required for primordial follicle formation. In mice lacking Figl$\alpha$, normal oocyte numbers are found at E18.5 but oocytes are completely absent in the ovary by post natal day 7 (PD7). Rapid oocyte loss in these mice occurs as a result of a defect in primordial follicle formation due to the absence of expression of Figl$\alpha$-dependent transcriptional targets, *zona pellucida 1*, *2*, and *3* (*ZP1*, *ZP2*, and *ZP3*) (Soyal *et al.* 2000).

**III. The Adult Ovary: GATA2/4/6, Glp-1 and the HGP Axis**

*a. The GATA transcription factor family*

GATA transcription factor family members (1-6) bind to co-factors and thus regulate tissue-specific gene expression. GATA2, GATA4, and GATA6 are differentially expressed within the mammalian ovary. Sexually dimorphic gene expression of *GATA2* occurs such that its expression is limited to the germ cells of female embryos from E11.5 to E14.5. The role GATA2 plays in the germ cells during this time is unknown (Siggers *et al.* 2002). GATA4 expression is detected in the somatic cells of the XX gonad as early as E10.5. During this time GATA4 promotes formation of female reproductive structures derived from the Mullerian duct by interacting with its co-factor, Fog (Friend of GATA-2) and inhibiting *MÜllerian-inhibiting substance* (*Mis*) expression (Viger *et al.* 1998, Anttonen *et al.* 2003). In the adult ovary, GATA4 is highly expressed in granulosa cells of primary and antral follicles, its expression is limited in theca and interstitial cells, and it is not expressed in luteinized granulosa cells (Heikinheimo *et al.* 1997, Viger *et al.* 1998, Laitinen *et al.* 2000, Vaskivuo *et al.* 2001, Lavoie *et al.* 2004). GATA4 may play a role in the survival of mouse and human pre-ovulatory follicles as demonstrated by increased mRNA expression and decreased expression in atretic follicles upon stimulation with follicle-stimulating hormone (FSH) (Heikinheimo *et al.* 1997, Vaskivuo *et al.* 2001). GATA6 is expressed at all stages of the developing follicle including the corpus luteum (Heikinheimo *et al.* 1997). In cells, including granulosa cells, where both GATA4 and GATA6 are

present, studies have shown that GATA4 has preferential binding affinity to promoters over GATA6 (Gillio-Meina *et al.* 2003).

The GATA transcription factor family members contain two zinc finger domains each made up of a Cys-$X_2$-Cys-$X_{17}$-Cys-$X_2$-Cys motif flanked by a basic region. Structural analysis of GATA1 demonstrated that the carboxy-terminal zinc finger physically interacts with DNA at the consensus site: (A/T)GATA(A/G) (Ko & Engel 1993, Omichinski *et al.* 1993). The amino-terminal zinc finger of GATA transcription factors, specifically GATA4, GATA5, and GATA6, is bi-functional in that it binds co-factors thus leading to regulated expression of target genes and it is responsible for localizing the protein to the nucleus (Morrisey *et al.* 1997). The importance of the amino-terminal was first established by the interaction between GATA1 and Fog in erythrocytes (Fox *et al.* 1998). Since the initial characterization of the interaction between GATA1 and Fog-1, multiple other cofactors specific to ovarian function have been identified including Fog-2, CCAAT-enhancer-binding protein (C/EBP$\beta$), Smad3, liver receptor homolog-1 (Lrh-1), and steroidogenic factor-1 (SF-1) (Tremblay & Viger 2001, Tremblay *et al.* 2002, Martin *et al.* 2005, Anttonen *et al.* 2006, Robert *et al.* 2006).

Post-transcriptional modifications of GATA4 are critical to its function as a transcription factor. GATA4 was found to have increased binding affinity for the *StAR* promoter by phosphorylation at serine 261 by protein kinase A (PKA)

through activation of the cyclic (c) AMP/PKA signal transduction (Tremblay *et al.* 2002). Activation of the cAMP/PKA pathway occurs through luteinizing hormone (LH) and and/or FSH paracrine signaling, which is required for follicular maturation in the ovary (Clark & Stocco 1995, Silverman *et al.* 1999, Tremblay & Viger 2003). In cardiomyocytes, negative regulation of GATA4 activity was shown to occur through phosphorylation of the amino-terminus by glycogen synthase kinase 3β (Gsk3β) which retains GATA4 in the cytoplasm; thus it physically prevents the transcriptional activity of GATA4 (Morisco *et al.* 2001). In cardiomyocytes, GATA4 was shown to have increased binding affinity due to phosphorylation of serine 105 by extracellular signal-regulated kinase 2 (ERK2) of the mitogen-activated protein kinase (MAPK) pathway (Liang *et al.* 2001). The requirement of serine 105 phosphorylation has subsequently been shown to increase GATA4 binding affinity to the *cyp19* (Aromatase) promoter through FSH stimulation (Kwintkiewicz *et al.* 2007). The effects of GSK3 signaling on GATA4 in granulosa cells have yet to be investigated.

The role of GATA4 in granulosa cells of the adult ovary is limited to the control of expression of genes involved in steroidogenesis and ovarian function. This is demonstrated by the identification of GATA4 transcriptional targets, including *StAR* (*Steroidogenic Acute Regulatory protein*), *cyp19*, and *Inhibin-α* (Tremblay & Viger 2001).

StAR is the rate limiting protein vital to steroidogenesis in that it transports cholesterol from the outer mitochondrial membrane to the inner membrane where cholesterol side chain cleavage cytochrome P450 (P450scc), the first enzyme in the steroidogenic pathway, resides (Christenson & Strauss 2000). In the ovary, P450scc and StAR are primarily expressed within the interstitial and theca cells, but also in granulosa cells, and therefore co-localize with GATA4 (Thompson *et al.* 1997, Hiroi *et al.* 2004, Lavoie *et al.* 2004). *StAR* expression is activated by FSH stimulation in granulosa cells, through a cAMP-dependent process whereby phosphorylation of GATA4 by PKA increases the binding affinity of GATA4 to the *StAR* promoter (Tremblay & Viger 2003, Hiroi *et al.* 2004). Subsequent studies showed that in response to FSH, GATA4 bound within the *StAR* promoter at position –66/-61 to activate its transcription (Silverman *et al.* 1999, Silverman *et al.* 2006).

Aromatase is the steroidogenic enzyme required for conversion of androstenedione to estrone in granulosa cells. It uses a distinct ovary-specific promoter allowing for tight regulation whereby aromatase it is only expressed in growing follicles during proestrus and estrus of the menstrual cycle (Mendelson *et al.* 2005). The up regulation of aromatase is due to an increase in GATA4 mRNA, protein, and DNA binding affinity in response to FSH (Kwintkiewicz *et al.* 2007). The ovary-specific *cyp19* promoter contains two GATA binding sites, -113/-118 (site A) and –123/-128 (site B), of which only site B is essential for its

transcriptional activation (Kwintkiewicz *et al.* 2007). As mentioned earlier, mice with mutations in *Cyp19* fail to ovulate and are thus sterile (Fisher *et al.* 1998).

*Inhibin-*$\alpha$, a member of the Tgf-$\beta$ superfamily, is transcriptionally activated by GATA4 in response to FSH, similarly to *cyp19* and *StAR* in granulosa cells (de Kretser & Robertson 1989, Anttonen *et al.* 2006). Maximum activation of *Inhibin-*$\alpha$ expression is dependent on simultaneous binding of multiple transcription factors (Anttonen *et al.* 2006, Robert *et al.* 2006). Inhibin-$\alpha$ is the common subunit of two heterodimeric proteins; Inhibin A and Inhibin B (de Kretser & Robertson 1989). Inhibin A exhibits both autocrine and endocrine functions. It was recently shown to inhibit transcriptional activation of the FSH receptor (Fshr) on the granulosa cells in an autocrine manner, thereby blunting the effects of FSH stimulation. This in turn inhibits steroidogenesis due to decreased expression of *P450scc* and *cyp19* (Lu *et al.* 2009). Both Inhibin A and Inhibin B function as endocrine signaling molecules to inhibit FSH secretion from the pituitary (Gregory & Kaiser 2004).

*b. Characterization of the novel GATA-like protein: Glp-1*

Glp-1 was first identified by Dr. Morrisey's laboratory at the University of Pennsylvania by screening the GenBank EST database for novel GATA transcription factors using the C-terminal zinc finger of GATA6. Glp-1 was characterized as a GATA-like protein in that it contains two zinc fingers in the C-

terminus of the protein, although the second zinc finger only contains 13 of the 17 amino acids within the linker region. It is thought that this lack in conservation of the linker region results in the inability of Glp-1 to bind the GATA binding consensus sequence. Despite its inability to bind DNA, Glp-1 localizes to the nucleus and is a strong repressor of GATA4/6-dependent transcriptional activation of *surfactant protein A* (*SP-A*), a known GATA6 target expressed in the lung epithelium (Bruno *et al.* 2000). Deletion analysis revealed that the repressive function of Glp-1 resides within the C-terminus of the protein, which contains the two zinc finger motifs (Li *et al.* 2007).

To further study the function of Glp-1, a *Glp-1*$^{LacZ}$ knock-in mouse line was generated. The entire locus of *Glp-1* was replaced with the bacterial *LacZ* gene. By utilizing β-galactosidase enzymatic activity through LacZ staining, *Glp-1* was shown to be expressed within the somatic cells of the gonads, specifically the granulosa cells of an ovarian follicle and the Leydig cells of the testes, but not the germ cells. Characterization of these mice revealed no abnormalities in the heterozygotes or nulls except that male and female null mice were sterile. When either heterozygote or wild type male or female mice were mated with a null animal, no progeny were produced over a two year period (Li *et al.* 2007).

Due to the defect in fertility, the morphology of gonads of both sexes was assessed. Female gonads at E17.5, birth, one month, and six months were

analyzed. *Glp-1*$^{LacZ}$ null ovaries show a slight reduction of oocytes at E17.5 and by birth, all oocytes are gone. Ovaries at one month of age were shrunken, due to the loss of germ cells, but at six months of age, the ovaries were large, likely due to somatic cell proliferation. The composition of the six month old adult ovary lacking Glp-1 was suggested to be due to proliferation of both theca and granulosa cells. This was determined by diffuse staining throughout the ovary of theca and granulosa cell-specific markers, p450ssc and SF-1, and Wnt4 and follicle stimulating hormone receptor (Fshr), respectively.

The presence of both theca and granulosa cells lead the authors to question the ability of the ovary to participate in hypothalamic-pituitary-gonadal (HPG) signaling. Briefly, gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates production of the gonadotropins, LH and FSH from the anterior pituitary. LH and FSH function at the level of the ovary to promote follicular growth and ovulation. This in turn activates a negative feedback loop back to the hypothalamus and pituitary that inhibits LH by progesterone and estrogen and FSH by Inhibin B. Estrogen, progesterone, and Inhibin B are all produced in the ovary in response to LH and/or FSH through GATA4-dependent transcriptional activation (Thackray *et al.* 2010). Analysis of estradiol, LH, and FSH levels were performed. Estradiol levels were similar between *Glp-1*$^{LacZ}$ null and wild type, while both LH and FSH were increased (Li *et al.* 2007). These data suggest that there is misregulation of the HGP axis in spite of normal levels of

estradiol (Li *et al.* 2007).

The male gonads were also analyzed for defects. It was observed that adult testes had very few mature sperm and were smaller as early as 5 weeks of age. This was due to increased apoptosis of spermatocytes. Germ cell numbers were decreased in the *Glp-1*$^{LacZ}$ null while the somatic cell (Sertoli and Leydig cell) numbers remain similar to wild type. The authors concluded that expression of Glp-1 in Leydig cells is essential for spermatocyte survival and maturation. Importantly, transcriptional targets of GATA4/6 in the testes are misregulated, showing both increased and decreased expression. *Glp-1*$^{LacZ}$ null testes have increased expression of *SF-1* and *Sox9* but decreased expression of *cyp19* and *StAR*. These data suggest that like FOG-2, a co-factor for GATA4 during development Glp-1 can act as an inhibitor or a repressor of transcription depending on the cellular context of the promoter (Li *et al.* 2007).

*c. Infertility and ovarian pathology with respect to the HPG axis*

Primary amenorrhea, a type of premature ovarian failure (POF) is defined as the precocious loss of germ cells (sometimes associated with chromosomal abnormalities) leading to the absence of secondary sexual characteristics (Anasti 1998; Davis 1996). It is characterized by the absence of menstruation in girls fifteen years of age or older. There are many causes for primary amenorrhea in

humans and rodents including disruptions in HPG axis signaling and loss of germ cells (similar to the Glp-1 phenotype), both of which result in sterility (Anasti 1998, Chand *et al.* 2010).

Mutant mouse models where germ cell loss has occurred, commonly results in tubular adenoma formation (Vanderhyden *et al.* 2003). In the early 1970s tubular adenoma formation was thought to occur as a result of loss of normal follicular tissue, interstitial cell hyperplasia, and ingrowth of germinal epithelial tubules and interstitial cells (Murphy 1972). The current belief is that tubular adenomas are derived from granulosa or pregranulosa (Personal communication with Dr. Diego Castrillon, UTSW). Tubular adenomas have been identified in *KitL*, *c-Kit*, *germ cell-deficient* (*Gcd*), *Glp-1*, and in *Dazl* mutants (Murphy & Beamer 1973, Ishimura *et al.* 1986, Duncan & Chada 1993, McNeilly *et al.* 2000, Li *et al.* 2007). *c-Kit* null females develop tubular adenomas (95% incidence) by five months of age and 56% of *Gcd* mutants do so by one year of age (Murphy 1972, Duncan & Chada 1993). The occurrence increases over time in these models. The percentage of occurrence has not been characterized in *Dazl*, *KitL*, or *Glp-1* mutants.

Cross-talk within the HGP axis is critical in the sexually mature female for proper estrous cycling and subsequent ovulation. Additional ovarian features of germ cell-less mutants include high circulating gonadotropin levels and the ability

to make steroids. *c-Kit*, *Dazl,* and *Glp-1* mutants have high levels of circulating LH and FSH compared to control animals (Murphy & Beamer 1973, McNeilly *et al.* 2000, Li *et al.* 2007). This also occurs in ovariectomized wild type sexually mature females, which eliminates ovarian feedback to the pituitary and hypothalamus (Murphy & Beamer 1973). This prolonged ovarian exposure to LH and FSH has been shown to directly cause tubular adenoma formation (Murphy & Beamer 1973). Loss of controlled LH and FSH production, as shown in these mouse models, suggests a signaling defect in the HPG axis. This is partially due to the loss of feedback of Inhibin B from the ovary, which normally suppresses FSH secretion (Gregory & Kaiser 2004).

It was previously reported that the random groups of cells within a tubular adenoma express enzymes required for the production of progesterone and estrogen (McNeilly *et al.* 2000). This differs from wild type ovaries in which layered cellular compartments, theca and granulosa cells, are required for steroid synthesis. Similar to other germ cell-less mouse models, *Glp-1*$^{LacZ}$ null adult ovaries were able to make steroids as shown by the similar levels of estradiol compared to controls (Terada *et al.* 1984, McNeilly *et al.* 2000, Li *et al.* 2007).

**IV. The importance of understanding the embryonic and adult function of Glp-1**

The known mechanisms by which oogenesis occurs is currently limited.

Few animal models that demonstrate embryonic germ cell loss and subsequent tubular adenoma formation exist in the literature. These include *KitL*, *cKit*, *Dazl*, *Figl*$\alpha$, *Gcd*, and *Glp-1*. Data from *KitL/cKit* mutants emphasize the importance of cross-talk between germ cells and the neighboring somatic cells for survival of germ cells during migration. Loss of somatic, pregranulosa cell gene expression resulting in prenatal germ cell loss is a novel phenotype and only seen in *Glp-1*$^{LacZ}$ mutants, thus loss of Glp-1 in the somatic, pregranulosa cells is required for germ cell survival. Understanding when the germ cells are lost in *Glp-1*$^{LacZ}$ mutants will provide insight into which know biological process or identify a novel process by which Glp-1 functions to promote germ cell survival. Similarly, in the adult, understanding the function of Glp-1 will provide insight into adult ovarian function. The research presented here in the embryos and in an adult gonadal *in vitro* system will provides insight into normal ovarian function and development.

**IV. Figures**

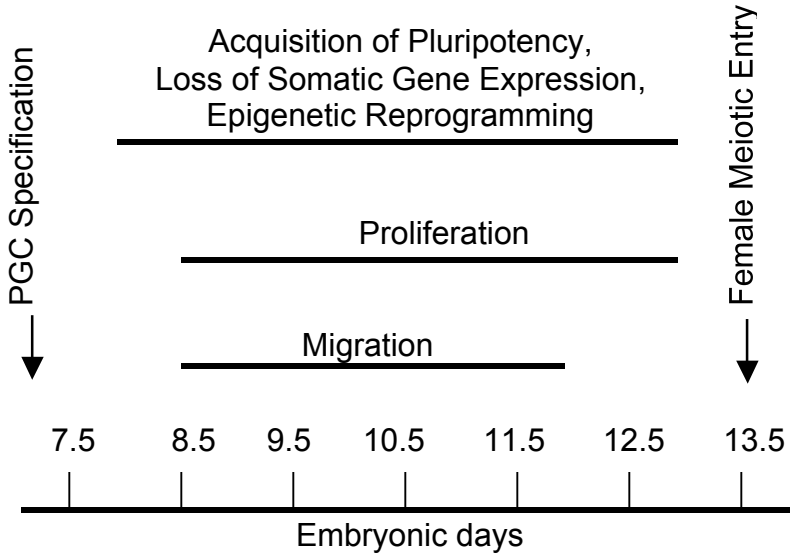



Figure 1.a. Timeline of mouse early oocyte development: From specification through meiotic entry. PGCs are specified before E7.5. At E8.5, they begin migrating while undergoing multiple rounds of proliferation. Before and during migration, they undergo acquisition of pluripotency accompanied by loss of somatic gene expression due to epigenetic reprogramming which occur before entering into meiosis at E13.5. These processes are explained in more detail in the text. Adapted from De Felici 2009.

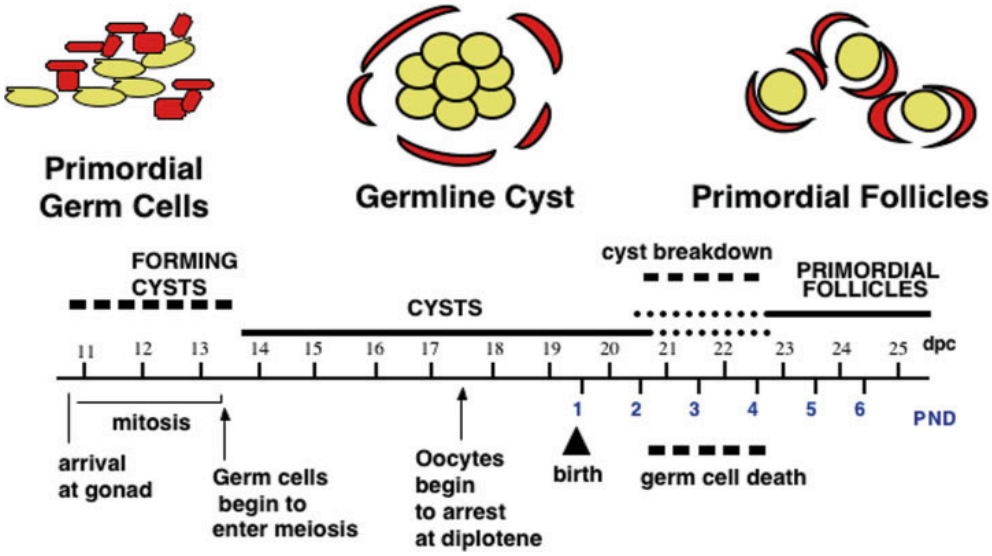



Figure 1.b. Timeline of oogenesis from PGC arrival at the gonad to primordial follicle formation in the mouse. As the germ cells (yellow) are arriving at the gonad, they are undergoing several rounds of mitosis with incomplete cytokinesis forming interconnected cysts surrounded by somatic cells (red). At E13.5, they stop proliferating and enter into meiosis. As the germ cells enter into meiosis, germline cyst formation is also occurring. Pregranulosa cells first surround the germline cyst. After birth, pregranulosa cells intercalate between the germ cells of the cyst thus breaking them apart and forming primordial follicles. This occurs along with regulated atresia of some germ cells. Notice the squamoid pre-granulosa cells in the primordial follicle. (PND)=post-natal day. Adapted from Pepling 2006.

# CHAPTER II: MATERIAL AND METHODS

## I. Mice

The *Glp-1*$^{LacZ}$ mouse line was generously provided by Edward Morrissey, Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA. Generation of these mice was previously described (Li *et al.* 2007). The WBB6F1/J-*Kit*$^{W}$/*Kit*$^{W\text{-}v}$ mouse in was obtained from Jackson Laboratories. *Glp-1*$^{LacZ}$ mice were maintained by heterozygous crosses and assayed by PCR as previously described (Li et al., 2006). Experimental protocols involving mice were approved by the University of Texas Southwestern Medical Center Animal Care and Use Committee.

## II. Gonad collection and sexing

Timed matings were established by housing female mice with male mice overnight. The presence of a vaginal plug seen before noon of the next day was considered E0.5. Gonads were dissected in PBS and the sex determined by the presence or absence of testicular cords.

## III. RNA isolation and qPCR

C57BL/6J and WBB6F1/J-*Kit*$^{W}$/*Kit*$^{W\text{-}v}$ (Jackson Laboratories) adult tissues (n=2) were processed in Trizol (Invitrogen) or TriPure (Roche). Ovaries from *Glp-*

*1*$^{LacZ}$ experimental animals (n=2) were taken and RNA isolated using TriPure. Embryonic gonads were placed into Trizol (Invitrogen) or TriPure (Roche). RNA was isolated for temporal expression data for *Glp-1* by pooling same sex C57BL/J6 littermate tissues at various times during development. Experiments were performed on pooled littermate samples from two independent mothers. The gonads from *Glp-1*$^{LacZ}$ experimental or *Glp-1*$^{LacZ/+}$ heterozygous littermate animals (n=3-5) were collected for gene expression experiments. RNA was isolated for all cell culture experiments using Trizol. Total RNA was isolated from Bos taurus (bt) granulosa cells using Trizol. Total RNA collected using either Trizol or TriPure was done according to the manufacturer's guidelines.

Gene expression analysis was performed for the following genes: *mGlp-1, mFoxL2*, *mMVH*, *mCyp26b1*, *mDazl*, *mStra8*, *mRec8*, *mSycp3*, *mSpo11*, *mDmc1*, *mBmp-2*, *mGdnf*, *mEreg*, *mKitL*, *mGAPDH*, *btGlp-1*, and *btGAPDH*. FAM-labeled TaqMan probes were generated using the Custom TaqMan Assay Design Tool from ABI or were ordered from the ABI pre-designed TaqMan® primer/probe pairs. Probe sequences are listed in Table 1-1 and Table 1-2. Equal amounts of RNA were used in each experiment. SuperScript® III Platinum®One-Step qRT-PCR Kit (Invitrogen) was used to run the qPCR reaction on the 7300 Real time PCR Instrument System. All experiments were analyzed using the ddCT method to obtain relative fold change when normalized to *GAPDH*.

**IV. Northern blot analysis**

20ug of total RNA was size separated on a formaldehyde gel and then transferred to a nylon membrane in 20x SSC overnight. Hybridization was performed in Church-Gilbert hybridization buffer for two days with $1x10^6$ CPM/ml, alpha-32P dCTP-labeled (Amersham) full length Glp-1 cDNA probes made with the Radprime DNA labeling system (Invitrogen). Following hybridization, membrane was washed two times with 0.1x SSC / 0.1%SDS at 65$^{o}$C for 30 minutes. The membrane was then exposed to film at –80$^{o}$C for nine days.

**V. *In situ* hybridization**

Whole-mount in situ hybridization was performed as previously described (Carroll *et al.* 2005). Briefly, E14.5 gonads were harvested and fixed in 4% paraformaldehyde in PBS at 4°C overnight. Gonads were treated with 10$\mu$g/ml proteinase K in PBST for 20 minutes at room temperature and hybridized overnight at 72°C with an antisense digoxigenin-UTP labeled *Glp-1* (DQ286956.1) probe that was linearized with EcoRI and transcribed with T3 polymerase. Embryos were then incubated overnight at 4°C with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche Applied Science). Color reaction was developed using BM Purple (Roche). Pictures were taken with the Olympus SZX7 or Leica MZ6 microscope. Images were taken and the background was changed to black to highlight the tissues.

Radioactive *in situ* hybridization experiments were performed by the Molecular Pathology Core Facility as previously described (Hinshelwood *et al.* 2003). Briefly, ovaries and pituitaries obtained from perfused animals were fixed overnight in 4% PFA followed by paraffin embedding and sectioning. [35S]-UTP-labeled cRNA probes were made against *Lrh-1*, an N-terminal fragment of *Glp-1* and full-length *Glp-1* (pCMVGlp-1) using the T3/T7 MaxiScript kit (Ambion). A construct containing a fragment of *Lrh-1* was provided by Dr. Carole Mendelson, UT Southwestern Medical Center (Hinshelwood *et al.* 2003). The N-terminal fragment of *Glp-1* was cloned into pCMV and transcribed using the T3 promoter.

**VI. Germ cell counts**

Gonads from *Glp-1*$^{LacZ}$ experimental or *Glp-1*$^{LacZ/+}$ heterozygous littermates (n=2) were fixed in 4% paraformaldehyde overnight at 4$^{o}$C. The tissue was then paraffin embedded and five micron sections were taken. Samples were stained with hematoxylin and eosin (H&E). All germ cells were counted within a fixed area from three adjacent sections at least 20 microns apart in the ovary. The values were averaged and then the percent remaining in the *Glp-1*$^{LacZ}$ null ovary was calculated.

**VII. Immunohistochemistry**

Gonads were collected in PBS and then fixed in 4% paraformaldehyde overnight at 4°C. The tissue was then paraffin embedded and five micron sections were taken. Immunohistochemistry experiments were performed as follows for Laminin (Sigma), Mvh (Abcam), β-galactosidase (Abcam), and Glp-1. Slides were rehydrated and washed in PBST (PBS plus 0.1% Triton-X). Antigen retrieval was performed by boiling the samples in 1mM Tris/5mM EDTA pH 7.5-8.0 for 5 minutes. Endogenous peroxidase activity was quenched by incubating samples in 3% $H_2O_2$ for 30 minutes. Samples were then blocked in 5% FBS/PBST for 30 minutes at room temperature. Primary antibodies, laminin (1:100), Mvh (1:500), β-galactosidase (1:100), and Glp-1 (1:500) in 2% FBS, were added and incubated at 4°C overnight. The next day, samples were washed in PBST. From here onward, the Vectastain Elite ABC Kit (Vector Laboratories) was used according to manufacturer's specifications. Immunostaining for pH2AX (phosphorylated at serine 319) (1:1000) (Biolegend) was performed as previously described (Akbay *et al.* 2008).

FoxL2 (Santa Cruz Biotechnology) immunohistochemistry was performed as follows. Slides were rehydrated and washed in water. Antigen retrieval was performed by gentle boiling in 1x sodium citrate pH 6.0 for 15 minutes. Endogenous peroxidase activity was quenched by incubating samples in 3% $H_2O_2$ for 30 minutes. Samples were then blocked in 1% BSA/PBS for 30 minutes

at room temperature. Samples were incubated with a FoxL2 antibody at 1:100 in 2% BSA at 4°C overnight and then washed with TBST (0.1% Tween-20). A rabbit HRP polymer secondary conjugated antibody (ZYMEB Superpicture) was added to the samples for 30 minutes at room temperature.

All samples were incubated with DAB (Vector Laboratories) until desired staining intensity appeared. Samples stained for FoxL2 and MVH were counterstained with H&E. All samples were mounted in Permount Mounting Media.

**VIII. Transmission electron microscopy**

Gonads from *Glp-1*$^{LacZ}$ experimental or *Glp-1*$^{LacZ/+}$ heterozygous littermates were harvested at E14.5 and fixed in 2% glutaraldehyde/0.1M cacodylate overnight at 4°C. Tissue was Epon embedded. Thick sections were taken for toluidine blue staining or thin sections were taken for electron microscopy (EM). EM was performed on the FEI Tecnai G2 Spirit BioTWIN microscope and images were taken using the Gatan CCD camera.

**IX. Antibody production and peptide purification**

Bio-Synthesis generated two antibodies against mouse Glp-1 for each of the following peptide sequences: PASLQRRPRKQLNPRMGI and MEAAGAGDLTRRQELLAPP. Two rabbits were inoculated for each peptide.

Both antibodies were confirmed to recognize Glp-1 before the final bleeds were taken. The bleed with the most reactivity against Glp-1 was purified using a peptide column (Bio-Synthesis). Briefly, crude serum was incubated with peptide-conjugated beads for 2hrs, washed with PBS, and eluted with 0.1M Citric Acid, pH 3.0. Purified antibody was neutralized with 1M Tris, pH 8.8, dialyzed overnight in PBS and then concentrated.

**X. Cell culture**

All cell lines were maintained at 37$^{o}$C with 5% carbon dioxide. COS-7 cells and L$\beta$T2 cells were cultured in DMEM media (Fisher) supplemented with 10% fetal bovine serum (Fisher), 5% penicillin/streptomycin (Invitrogen). MLTC1 cells were cultured in RPMI 1640 medium (Fisher) supplemented with 10 mM Hepes, 5% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS) (Invitrogen).

**XI. Western blot analysis**

Cells in culture dishes were lysed in RIPA buffer (0.1% SDS, 0.1% deoxycholic acid, 1.0% Triton X-100, 150mM NaCl, 50mM Tris-HCl, pH 7.6) plus protease inhibitors (10 ug/ml pepstatin A (Sigma), 10 ug/ml leupeptin (Sigma), 10 ug/ml aprotinin (Sigma), and 100 ug/ml phenylmethylsulfonyl fluoride) and incubated at 4$^{o}$C for 15 minutes. Wells were scraped, lysates sheared with a 21 gauge, followed by a 25 gauge needle, and then cellular debris precipitated.

Equal amounts of protein were loaded and SDS-PAGE performed. Protein was transferred to Immobilon-P membranes (MilliporeCorp.), blocked in 5% milk in TBS-Tween 20 for 1 h, and then incubated with primary antibody overnight at 4$^{o}$C (1:50-1:5000 for anti-Glp-1, 1:5000 for anti-GATA4 (Santa Cruz), 1:2000 for anti-cMyc (9E10) (Santa-Cruz), 1:4000 anti-beta-actin (Sigma), 1:20,000 for anti-SF-1 (a gift from Keith Parker at UT Southwestern Medical Center). Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h, and signal was detected by ECL Plus (GE Healthcare).

**XII. siRNA knockdown**

siRNAs were designed using the siDESIGN Center and purchased from Dharmacon to target *Glp-1*, *Gata4*, and *cyclophilin B*, or pre-made siCONTROL, non-targeting siRNA #3. 12-well plates were seeded with MLTC1 cells or L$\beta$T2 cells overnight in RPMI or DMEM media, respectively, both supplemented with 10% FBS and without antibiotics. 2uM siRNAs, unless otherwise specified, were transfected with DharmaFECT 1 according to the manufacturer's guidelines and harvested at 48hrs, for either mRNA analysis, or 96hrs, for protein analysis. SiRNA sequences generated by Dharmacon for the listed genes are as follows: Glp-1: GCAUCAGGUACAAGAAAUA, GCGAAUGGGUAUUGAGAAG, GGAAAAGCAUCCAGCCCAA, GGCAUCAGGUACAAGAAAU, CCAGAGAAGACCACGGAAG, ACACCGAGUCGCUCCGGAA; GATA4: GGACAUAAUCACCGCGUAA, CCCAAGAACCUGAAUAAAU; Cyclophilin B:

ON-TARGETplus Cyclophilin B Control Pool (Catalog #: D-001820-20)

**XIII. Serum collection and analysis**

Female *Glp-1*$^{LacZ}$ null and wild type sibling mice at 13wks, 5mo, and 6mo of age were anesthetized by IP injection of 275uL per 10g of total mouse body weight with 2.5% 2,2,2-tribromoethanol (Avertin) (Sigma). Whole blood was obtained by lethal cardiac heart punctures. Serum was collected using BD Microtainer Plastic Capillary Blood Collectors (Fisher Scientific). Serum was sent to the University of Virginia Core Ligand and Assay Laboratory to be analyzed for Inhibin-B, LH, FSH, and estrogen circulating protein levels by Elisa.

**XIV. Steroid radioimmunoassays**

MLTC1 cells, where Glp-1 has been knocked-down or a MLTC1 cell line stably over-expressing Glp-1 were serum starved for 4hrs and then stimulated with 0.1U/ml hCG for 30 minutes. The media was removed from culture wells and used to measure steroid levels according to the manufacturer's guidelines (MP Biomedicals).

**XV. Transient transfection**

Cells were plated and LipofectAMINE PLUS transfections (Life Technologies) were performed the following day according to the manufacturer's guidelines. The DNA-PLUS-LipofectAMINE solution was added to cells and

incubated for 4hrs. 6-well plates were transfected with 1ug DNA. 12-well plates were transfected with 0.7ug DNA. Plasmids transfected for all experiments include pSP-A.luc, p-966StAR/luc, p-254StAR/luc, β-galactosidase, pCMVGlp-1, pCDNA3.1Glp-1, pCMVGATA6, and/or pCDNA3.1GATA4.

**XVI. Nuclear vs. cytoplasmic fractionation**

MLTC1 cells or transiently transfected MLTC1 cells were grown in a 10mm plate to confluency. Cells were removed from the plate by scraping, washed in PBS, and resuspended in hypotonic buffer (10mM HEPES, pH 7.9, 1.5mM MgCl2, 10mM KCl, 0.5mM DTT, 0.2mM PMSF). Cells were allowed to swell on ice for 10 minutes before being homogenized. Nuclei were then pelleted and the supernatant kept which contains the cytoplasmic extract. Nuclei resuspended in low salt buffer (20mM HEPES, ph 7.9, 25% glycerol, 1.5mM MgCl2, 0.02M KCl, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF) were lysed by the addition of high salt buffer (20mM HEPES, pH 7.9, 25% glycerol, 1.5mM MgCl2, 1.2M KCl, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF). Nuclear debris was pelleted and the supernatant kept, which contains the nuclear extract. Lysates were resuspended in SDS-loading buffer with BME. Western blots were performed and protein detected with the appropriate antibody.

**XVII. Immunofluorescence of endogenous Glp-1**

MLTC1 cells were grown in 12 well plates on sterilized, round coverslips.

Once the cells reached confluency, the serum was removed and they were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed in PBS and then permeabilized in 0.25% Triton X-100 in PBS for 5 minutes at room temperature. Cells were washed again and blocked in 10% BSA in PBS for 30 minutes at 37$^{o}$C. A dilution series of primary antibody (1:10-1:5000), diluted in 3% BSA in PBS, was incubated for 2hrs at 37$^{o}$C. Primary antibody was removed by washes in PBS. Coverslips where then incubated with the Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (Invitrogen) at 1:500. Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories).

**XVIII. Estrous dating**

Vaginal smears from adult wild type and *Glp-1*$^{LacZ}$ mutant sibling females were taken everyday through three cycles. The vaginal orifice was flushed with 30ul of PBS. Samples were placed on a microscope slide and analyzed by light microscopy. Vaginal cytology was recorded each day based on published descriptions of each phase (Goldman *et al.* 2007).

**XIX. Reverse transcription reactions**

Total RNA from cell lines was harvested from 6-well or a 10cm plate. Total RNA extraction from ovarian tissue was described above. SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen) was used to generate cDNA followed by PCR amplification. PCR products were run on an agarose gel. The

following primers were used to detect *mGlp-1*: forward 5' CGAATGGGTATTGAGAAGGTGGAC 3' and reverse 5' TTTGGGCTGGATGCTTTTCC 3'.

Total RNA was harvested from bovine ovaries as described above. The following primers were used to perform RT-PCR of *btGlp-1*: forward 5' GAGACCCTGGGACCTCTACC 3' and reverse 5' GGTCCTACAGGAAGCACAGC 3'.

**XX. Cloning**

The p-254StAR minimal promoter (p-254StAR/luc) was cloned into pGL2 from p-966StAR/luc in pGL2 (Caron *et al.* 1997) using PCR followed by restriction cloning using the following primers: forward 5' CCCTCGAGTTTACAACTTTAGA 3', reverse 5' CCAAGCTTACTTAGATCCTCTAGAG 3.' PCR was performed, products and vector digested, gel purified and ligated together. The pCMVGlp-1 construct was previously described (Li *et al.* 2007). An untagged version of Glp-1, pCDNA3.1Glp-1 was generated by restriction cloning the Glp-1 cDNA into pcDNA3.1+ (Invitrogen) using EcoRI and XhoI restriction enzymes. Glp-1 cDNA was cloned into pBKS+ (Stratagene) for *in situ* hybridization and Northern blot experiments by restriction cloning using EcoRI and XhoI restriction enzymes. A construct containing the N-terminal portion of Glp-1 (pCMVN-Glp-1) was cloned

into pCMV using the restriction sites EcoRI and HindIII for *in situ* hybridization experiments.

**XXI. Stimulation with hCG**

In all cell culture experiments where cells were stimulated with hCG, 0.1U/ml of hCG was used. Cells were serum starved from 4hrs to overnight before stimulation with hCG for 4hrs.

**XXII. Luciferase reporter assay**

Cells were transfected with β-galactosidase alone or with both a luciferase reporter (pSP-A.luc, p-966StAR/luc, p-254StAR/luc) and pCMVGlp-1, pCMVGATA6, and/or pCDNA3.1GATA4. β-galactosidase was used as a control for transfection efficiency. Cells were lysed in 300ul Reporter Lysis Buffer (Promega) for 20 minutes at room temperature. Plates were placed at –80$^{o}$C for 30 minutes and then allowed to thaw completely. Lysis buffer in wells was pipetted up and down repeatedly to remove lysed cells from the bottom of the plate. Lysates were transferred to a microfuge tube, vortexed, and cellular debris precipitated. Twenty ul of lysate was loaded in triplicate into two separate FluoroNuc 96-well black plates (Fisher). One plate was incubated for 1hr with the Galacto-Star System (Applied Biosystems) to detected β-galactosidase activity. Luciferin (Promega) was added to the second plate immediately before detection. Both β-galactosidase and luciferase activity was detected using the

TopCount 5.0 Scintillation and Luminescence counter (Packard). Luciferase data was normalized by β-galactosidase activity for each well. Additional wells were transfected for Western blot analysis to ensure protein expression.

**XXIII. Isolation of bovine granulosa cells**

Fresh adult bovine ovaries at random stages in the estrous cycle were purchased from the Dallas City Packing slaughterhouse. Granulosa cells were isolated by puncturing large (3- to 5-mm) antral follicles with a 25 gauge needle attached to a 1mL syringe and aspirating out the follicular fluid containing granulosa cells. Five ovaries were used to isolate granulosa cells for RT-PCR and qPCR. The granulosa cells were pelleted by centrifugation at 5,000 rpm for 6 minutes at 4$^{o}$C. Fifteen ovaries were used to collect granulosa cells for cell culture/transfection experiments. Granulosa cells were pelleted and resuspended in 3mLs DMEM media with L-Glutamine, 4.5g/L Glucose and sodium pyruvate supplemented with 5% penicillin/streptomycin (Invitrogen), 0.25 ug/mL fungizone (Invitrogen), and 10% FBS (Fisher) (modified from (Hu *et al.* 2004). 0.5 mLs was aliquoted into each well of a 6-well plate. A single well was transfected as described above with β-galactosidase and the pSP-A.Luc reporter construct alone and mGlp-1 with and without mGATA4 for the luciferase reporter assay after the cells were allowed to settle overnight. Additional wells were transfected with β-galactosidase and the pSP-A.luc reporter construct alone and with mGlp-1 plus mGATA4 for qPCR experiments to determine expression.

**XXIV. LacZ staining**

Adult ovaries and testes were harvested from *Glp-1*$^{LacZ/+}$ and wild-type animals. Tissues were fixed in 2% PFA on ice for 1hr then rinsed multiple times for 2hrs in PBS. Adult ovaries were incubated in high temperature buffer, pH 7.5 (100mM HEPES, 5mM DTT, 1mM MgSO4, 2% Triton X-100) to quench endogenous β-galactosidase-like activity for 1hr at 50°C before adding staining solution. Staining solution (2mM MgCl, 5mM K+ferricyanide/K+ferrocyanide, 0.01% NP-40, 0.1% deoxycholate) plus 4% X-gal was added to the tissues and incubated at 37°C until the desired color was observed. Tissues were post-fixed in 2% PFA overnight at 4°C, processed for paraffin embedding and sectioned.

**XXV. Stable Glp-1 over-expressing MLTC1 cell line**

Briefly, MLTC1 cells were transiently transfected with pCMVGlp-1 and clones were selected for using media supplemented with G418. Clones containing Glp-1 were chosen based on the level of Glp-1 expression as determined by western blot analysis.

## XXVI. Tables

Table 1-1. Custom made TaqMan qRT-PCR probes

| Gene | Forward | Reverse | 5' FAM-labeled Probe |
|---|---|---|---|
| MGdnf | TGTCTGCCTGGTGTTGCT | CGCTTCGAGAAGCCTCTTACC | CCGCGTCTGCCTTC |
| mGlp-1 | CCTGAGTATGGCCAGGAATCC | AGTGAGGCAGGAGAAGTAGCA | CTAACCTGGAAATTTC |
| mTgfbi | GAAATACTTCACCAACTGCAAGCA | CCAGGACAGCACTCATAACTGATG | TTGCCGCAGATCTT |
| mkitL | ACCTCGTGTTATGCATGGAAGAA | AGTAAAGGATCTAGTTTCTGGCCTCTT | ACGCACCGAAGAATAT |
| mStra8 | CTCTGTTGCCGGACCTCAT | CCATGGTCTGCTTGTAAAAGTTGAG | ACCGTTCAAATTCC |
| MEreg | TCTACAGGCAGTTATCAGCACAAC | CATCTGAACTAAGGCGGTACAGT | ATCCCAGGAGAATCC |
| mBmp2 | CACCAGGTTAGTGAATCAGAACACA | GTGGTCCACCGCATCACA | ACGTCGAAGCTCTCC |
| btGlp-1 | GGCCTGCACACCAAGACT | CAGCTCCTCTGCTGTCTCTT | CCTGCGCTTCCTCC |
| btGapdh | CATGACCACTTTGGCATCGTG | GGGCCATCCACAGTCTTCTG | TCCACGCCATCACTGC |
| MGATA4 | GGTAACTCCAGCAATGCCACTA | CCCGGGCTCTGTCTTGATG | CTGCTGCTGCTGCTGC |
| mFoxL2 | GCAGCCCCGGTGCA | GCACGCGCGAGTACG | CCGCCGCCTCCTACG |

Table 1-2. Pre-made TaqMan qRT-PCR probes

| Gene | Assay ID |
|---|---|
| mRec8 | Mm00490939_m1 |
| mCyp26b1 | Mm00558507_m1 |
| mDmc1 | Mm00494485_m1 |
| mSycp3 | Mm00488519_m1 |
| mSpo11 | Mm00488871_m1 |
| mGapdh | Mm99999915_g1 |

# CHAPTER III: CHARACTERIZATION OF THE *Glp-1*$^{LACZ}$ NULL EMBRYONIC PHENOTYPE

## I. Objective

The objective of this study is to better understand the biology behind oogenesis. Here I use a genetically modified mouse line in which the entire locus of *Glp-1* has been replaced with the bacterial *LacZ* gene. *Glp-1*$^{LacZ}$ null mice develop as healthy adults but are unable to reproduce. Characterization of the female mice showed that at birth all their germ cells were missing (Li *et al.* 2007). My goal is to understand where Glp-1 is normally expressed in the developing ovary and during which embryonic process Glp-1 functions. I analyze the specification, proliferation, migration, and meiotic entry of PGCs and the function of the somatic cells in Glp-1 deficient embryos.

## II. Introduction

Establishment of the primordial follicular pool requires the coordinated interactions of several cell types, all of which are regulated in their own independent fashion. Three major processes in the mouse are known to be involved in oogenesis around the time of their arrival to the gonad (Summarized in Figure 1.a and 1.b). First, specified primordial germ cells migrate to the nascent gonad while undergoing mitosis with incomplete cytokinesis through

E11.5 (Buehr *et al.* 1993, Pepling & Spradling 1998, Lawson *et al.* 1999, Ying & Zhao 2001, Agoulnik *et al.* 2002, Tsuda *et al.* 2003, Mahakali Zama *et al.* 2005, Youngren *et al.* 2005, Farini *et al.* 2007). Second, after arriving in the gonad, meiotic entry occurs at E13.5, followed by arrest at the diplotene phase of prophase I, beginning at approximately E17.5. Meiotic entry is in part due to retinoic acid exposure (Menke *et al.* 2003, Baltus *et al.* 2006, Bowles *et al.* 2006, Koubova *et al.* 2006, Anderson *et al.* 2008, Lin *et al.* 2008), which occurs in a wave, starting at the anterior region of the ovary and moving toward the posterior end (Menke *et al.* 2003, Bullejos & Koopman 2004). Third, as the germline cyst enters the gonad, it is surrounded by pregranulosa cells which are responsible for primordial follicle formation just after birth in the mouse (Pepling & Spradling 2001, Uda *et al.* 2004). Germline cyst breakdown occurs along with regulated apoptosis of some germ cells (Ratts *et al.* 1995, Pepling & Spradling 2001). At the onset of puberty, primordial follicles are reactivated by a surge in LH and FSH and proceed into Meiosis II, at which point they arrest again and are not reactivated until fertilization (Jamnongjit & Hammes 2005, Mehlmann 2005).

As mentioned, all of the aforementioned processes require cross talk between somatic and germ cells within the ovary (Matzuk *et al.* 2002, Molyneaux *et al.* 2003, Mahakali Zama *et al.* 2005, Chen *et al.* 2007, Hsieh *et al.* 2009). As such, perturbations in normal function of either the germ cells or the surrounding somatic cells can have profound effects on normal ovarian development. One

such example is Glp-1. Glp-1 is a relative of the GATA transcription factor family in that it contains two zinc fingers in the C-terminus of the protein. However, Glp-1 does not appear to bind the GATA DNA sequences due to differences in sequence at and near the second zinc finger (Li *et al.* 2007). Glp-1 is expressed primarily in granulosa cells within the ovary; interestingly, knockout of the *Glp-1* gene by replacement with the bacterial *LacZ* gene leads to sterility in both males and females, with a complete lack of oocytes detected in the ovaries of the females by birth (Li *et al.* 2007).

To investigate when and how *Glp-1* expression in pregranulosa cells regulates oocyte survival, I performed detailed studies on ovarian embryonic development in *Glp-1*$^{LacZ}$ null mice focusing on the processes that occur around E13.5 to birth, including proliferation and migration, germline cyst formation, and meiotic entry (Reviewed in Chapter I). I found that germ cells proliferate and colonize the ovary normally; however, at or around E13.5, oocytes began to disappear at an abnormally rapid rate, and are completely lost by birth. Accompanying germ cell loss, I saw a reduction in mRNA expression of genes responsible for meiotic entry of germ cells. In contrast, depletion of Glp-1 in the somatic pregranulosa cells did not have an obvious effect on pregranulosa cell appearance or numbers during embryogenesis, nor did it affect the secretion of laminin or the expression of a select array of pregranulosa cell mRNAs. Together, these data suggest that, through a still unknown alteration in somatic-

germ cell crosstalk, Glp-1 regulates meiotic entry and subsequent germ cell survival during ovarian embryogenesis.

**II. Results**

*a. Glp-1 mRNA expression peaks in early ovarian development and again at birth*

Since previous work demonstrated embryonic loss of germ cells in *Glp-1*$^{LacZ}$ null female mice, I quantified *Glp-1* mRNA levels throughout oogenesis to confirm whether *Glp-1* was indeed present and therefore able to contribute to germ cell survival during embryogenesis. *Glp-1* mRNA expression was detected as early as E13.5 and was maintained at the same level until E15.5. Its expression then dropped between E16.5 and E17.5, followed by another increase in expression at E18.5 through birth, at which point its expression remained detectable but low into adulthood (Figure 3.a). Interestingly, the *Glp-1* expression data mimicked microarray data uploaded to the GEO repository by another group using multiple time points of embryonic gonads (Data not shown) (Small *et al.* 2005). Finally, *in situ* hybridization experiments using DIG-labeled riboprobes against *Glp-1* in wild type animals at E14.5, when *Glp-1* expression is high, demonstrated the *Glp-1* expression pattern in the ovaries and testes. *Glp-1* was expressed within the cords of the testis (arrow), while its expression in the ovary was present in an anterior to posterior gradient fashion (Figure 3.b). The arrowhead in the ovary denotes the anterior portion where *Glp-1* expression was

reduced and the arrow points to the posterior portion of the ovary where *Glp-1* expression was higher.

*b. Germ cell loss in Glp-1$^{LacZ}$ null embryos occurs after migration and before birth*

To determine the commencement and progression of germ cell loss in *Glp-1$^{LacZ}$* null mice, germ cell counts were performed on H&E sections by calculating the percentage of germ cells in *Glp-1$^{LacZ}$* null ovaries relative to their wild type siblings during oogenesis. At E13.5, *Glp-1$^{LacZ}$* null ovaries contained equal numbers of germ cells as compared to their sibling controls, suggesting that germ cell migration and proliferation occurred appropriately in the *Glp-1$^{LacZ}$* null mice. One day later, at E14.5, germ cell numbers were already reduced by nearly 40% relative to wild type littermates. This loss continued throughout embryogenesis until birth, at which point the *Glp-1$^{LacZ}$* null ovary was devoid of germ cells (Figure 3.c). Notably, *Mouse VASA homolog* (*Mvh*), a germ cell specific marker, expression was also similar between wild type and *Glp-1$^{LacZ}$* null mice at E13.5 (Figure 3.d) and disappeared at a similar rate to that observed in Figure 3.c. Germ cell loss can also be visualized in the FoxL2 stained ovaries, which are marked with asterisks (Figure 3.f.A).

Morphologically, germ cells in *Glp-1$^{LacZ}$* nulls tend to have an irregular cell shape and size as compared to their wild type littermates (Figure 3.i.A) but they have similar chromatin patterns at E14.5 (Figure 3.i.B). These data, compared

with the temporal mRNA expression pattern of *Glp-1* (Figure 3.a), suggest that a loss in Glp-1 expression could affect germ cell survival beginning as early as E13.5, relatively soon after they have arrived at the gonad.

*c. Germ cell loss in Glp-1$^{LacZ}$ null embryos occurs without defects in somatic pregranulosa cell numbers or known functions*

Since Glp-1 is expressed in somatic cells within the ovary, I examined embryonic pregranulosa cell numbers and known functions in *Glp-1$^{LacZ}$* null mice. Interestingly, despite the rapid loss of germ cells from E14.5 to birth, pregranulosa cell pattern and function remained similar to that of wild type mouse ovaries. The expression of FoxL2, the earliest known pregranulosa cell marker (Schmidt *et al.* 2004), was similar from E14.5 through birth (Figure 3.e). These stains demonstrated that pregranulosa cell numbers, size, and shape were relatively normal in the *Glp-1$^{LacZ}$* null mice. Importantly, in FoxL2 deficient mice, pregranulosa cells are unable to differentiate and secrete factors such as laminin, which are required for proper formation of the basement membrane (Schmidt *et al.* 2004, Uda *et al.* 2004). Here I find that laminin production is completely normal in *Glp-1$^{LacZ}$* null mice relative to wild type mice (Figure 3.f), confirming that the function of pregranulosa cell encasement of germline cysts is also spared.

To investigate other markers of pregranulosa cell function, I examined expression of mRNAs encoding proteins known to be important for follicular

development and pregranulosa cell-oocyte cross-talk during embryogenesis. The molecules chosen to investigate by qPCR were identified from a screen examining sexually dimorphic gene expression of the somatic, SF-1 positive, cells during early gonadogenesis. From this list, known and novel candidate signaling molecules were chosen that were up-regulated in the developing ovary at E13.5 (Nef *et al.* 2005). These include *Bmp-2, glial cell-line derived neurotrophic factor (Gdnf)*, *epiregulin (Ereg)*, and *KitL*. Similar levels of these mRNAs were found to be expressed in both *Glp-1*$^{LacZ}$ null and wild type littermates at E13.5 (Figure 3.g). Together, the pregranulosa cell data indicates that markers of pregranulosa cell expression and function, both as a mediator of basement membrane deposition by laminin staining (Figure 3.e) and possibly pregranulosa-germ cell cross talk, are relatively normal in Glp-1 null ovaries, despite the rapid loss of germ cells.

*d. Expression of meiotic entry markers is altered due to the loss of Glp-1 and in the absence of structural evidence of precocious or delayed entry into meiosis*

As described above, three major processes are known to be involved in oogenesis, including germ cell migration and mitosis (through E10.5), meiotic entry (E13.5) and arrest (E17.5), and cyst formation (E11) and subsequent breakdown (PD3) (Chapter I, Figures 1.a and 1.b). My data suggest that germ cell migration and proliferation occur appropriately (Figure 3.c) and that the pregranulosa cells are specified and are able to encompass the developing

germline cyst (Figure 3.e and 3.f). The last process that I investigated was meiotic entry. I therefore analyzed meiotic entry by looking at the mRNA levels of several, well characterized meiotic entry markers.

I focused on Stra8, which mediates meiotic entry in response to RA (Baltus *et al.* 2006, Bowles *et al.* 2006), Cyp26b1, a cytochrome p450 enzyme that cleaves RA into an inactive form (Bowles *et al.* 2006), Dazl, an RNA-binding protein required for germ cell maintenance (Lin *et al.* 2008, Haston *et al.* 2009), and Dmc1 and Spo11, proteins that mediate DNA recombination (Cohen & Pollard 2001, Hunt & Hassold 2002). All these markers of meiotic entry are specifically expressed in the germ cells with the exception of *Cyp26b1*, which is somatic in origin. QPCR of these markers demonstrated a significant decrease in *Stra8* and *Dmc1* mRNA expression, and a trend toward reduction of *Cyp26b1*, *Rec8*, and *Sycp3* mRNA levels. Both *Dazl* and *Spo11* expression levels remained unchanged (Figure 3.h).

Despite the change in expression of the meiotic entry markers, ultrastructural analyses of E14.5 ovaries from Glp-1 null mice demonstrate normal nuclear condensation and meiotic progression relative to wild type littermates (Figure 3.h and 3i).

**IV. Discussion**

Glp-1 was recently identified as a novel GATA-like transcription factor specific to the somatic cells of the gonad and important for germ cell development. The entire open reading frame of *Glp-1* was replaced with the bacterial *LacZ* gene to generate a Glp-1 deficient mouse line. Preliminary characterization of these mice showed defects in the gonads of both male and female Glp-1 null mice. In particular, ovaries were devoid of germ cells at birth (Li *et al.* 2007). Further characterization of ovarian development is necessary to determine what role Glp-1 may be playing in the developing ovary. Here I show that Glp-1 is a somatic cell factor important during ovarian development for germ cell survival, affecting expression of meiotic entry markers.

The previously reported *Glp-1*$^{LacZ}$ null ovarian phenotype is a novel ovarian phenotype in that no published reports have shown germ cell loss due to loss of a somatic cell factor occurring before birth. Therefore it was critical to define the expression pattern of Glp-1 during embryogenesis. I showed that *Glp-1* is expressed at E13.5 in the developing ovary at high levels and again around the time of birth and that this expression pattern occurs in a gradient fashion with expression moving from the anterior region to the posterior end. I was able to show that when Glp-1 expression would normally be high at in wild type ovaries, germ cell loss was beginning to occur in the *Glp-1*$^{LacZ}$ mutants. At E14.5 the percentage of germ cells remaining in the *Glp-1*$^{LacZ}$ mutant ovary is significantly

reduced and there are suggestive defects in cell survival as indicated by reductions in mRNA expression at E13.5. Interestingly, the timing and pattern of *Glp-1* expression and loss of germ cells beginning around E13.5, correlates with meiotic entry of the germ cells. All known processes that occur around E13.5 were investigated, including germline cyst encasement by pregranulosa cells, its subsequent dissemination into primordial follicles, and meiotic entry.

Analysis of germline cyst encasement of *Glp-1*$^{LacZ}$ null germline cysts shows no abnormalities. This suggests that ovaries deficient for Glp-1 still undergo proper secretion of glycoproteins by pregranulosa cells to make up the basement membrane, a process known to be important for germ cell survival (Frojdman *et al.* 1995, Uda *et al.* 2004). The laminin staining data suggest that, from E14.5 through birth, germline cysts are surrounded normally by pregranulosa cells. These data suggest that Glp-1 is not playing a role in cyst formation even though it is expressed in the somatic pregranulosa cells at this time. Unfortunately, I am unable to speculate about a role for Glp-1 during cyst breakdown and primordial formation due to the absence of germ cells, even though I know that Glp-1 is normally re-expressed at that time.

The idea that extrinsic and intrinsic factors are required for germ cell survival has recently been emphasized in the literature. Characterization of intrinsic germ cell-specific markers of meiosis is discussed below. First, I

analyzed extrinsic factors that may function as paracrine signaling molecules secreted from the somatic cells to the oocytes leading to their survival. These molecules were identified through a screen looking for sexually dimorphic gene expression at E13.5. These molecules include *KitL*, a known secreted molecule important for germ cell survival and migration, and novel factors functioning at E13.5 including *Gdnf*, *Tgfβi*, and *Bmp2* (Mahakali Zama *et al.* 2005). Normal expression of these molecules would suggest that the pregranulosa cells are able to support survival of the germ cells in the absence of Glp-1. Our data shows similar levels of these mRNAs expressed at E13.5, providing evidence that pregranulosa cells deficient for Glp-1 are able to secrete appropriate signals to the germ cells and thus support their survival through these pathways (Figure 3.g). The list of paracrine signals that I investigated was limited and therefore underscoring the importance of further identification of other signaling pathways during early oogenesis.

I showed that germ cell loss in the *Glp-1*$^{LacZ}$ mutant ovaries begins around E13.5 and that meiotic entry in the wild type animal commences at this time. The gradient expression of *Glp-1* is reminiscent of expression of many meiotic entry markers, although meiotic entry markers are expressed in the opposite manner beginning in the anterior at E14.5, and then expression moves in a wave to the posterior (Menke *et al.* 2003, Bullejos & Koopman 2004). These data, taken

together with the expression of *Glp-1* at E13.5 and E14.5, led me to examine if Glp-1 is playing a role in meiotic entry.

The process of meiosis involves several molecules. First, RA stimulates meiotic entry by allowing protein expression of Stra8 (Baltus *et al.* 2006, Bowles *et al.* 2006). Stra8 activation and function requires the loss of Cyp26b1, a p450 enzyme that cleaves RA into an inactive form (Bowles *et al.* 2006). Dazl, an RNA-binding protein, is required for germ cell maintenance as animals deficient for Dazl lose their germ cells after E12.5 (Haston *et al.* 2009). Dazl is then required for the mRNA expression of *Stra8*, *Dmc1*, *Spo11*, *Sycp3*, and *Rec8* (Lin *et al.* 2008). Finally, Dmc1 and Spo11 are responsible for recombination. Spo11 forms DNA double-stranded breaks while Dmc1 repairs the breaks (Cohen & Pollard 2001, Hunt & Hassold 2002). Stra8 expression was shown to be required for the proper DNA loading of Rec8 and Sycp3, a cohesion molecule and a component of the synaptonemal complex, respectively, during meiotic prophase at E15.5 (Baltus *et al.* 2006). QPCR of these markers demonstrated a significant decrease in *Stra8* and *Dmc1* mRNA expression, and a trend toward reduction of *Cyp26b1*, *Rec8*, and *Sycp3* mRNA levels. Both *Dazl* and *Spo11* expression levels remained unchanged. By looking at gene expression of these meiotic entry, germ cell-specific markers (except *Cyp2b1* which is somatic), I conclude that the germ cells are beginning to show signs of distress at E13.5. The significant loss of germ cells seen at E14.5 is not surprising. What is noteworthy

is the drastic effect loss of Glp-1, a somatic cell factor, is having on germ cell survival. Thus, despite the change in expression of the meiotic entry markers at E13.5, E14.5 ovaries from *Glp-1*$^{LacZ}$ null mice demonstrated normal nuclear condensation and meiotic progression relative to wild type littermates.

From these data, I conclude that in the *Glp-1*$^{LacZ}$ mutant ovaries, germ cells colonize the ovary and proliferate normally as seen by the presence of numerous germ cells in the null at E13.5 by germ cell counts and by the insignificant difference in *Mvh* mRNA levels at E13.5 when compared to wild type. Also based on the morphology of the somatic cells and FoxL2 expression, I can conclude that only the germ cells are being lost. Finally, loss of germ cells begins after E13.5 and is completed by birth. These data suggest that in the absence of Glp-1 there are severe deficiencies in oocyte development and/or survival beginning by E13.5. Therefore, *Glp-1*$^{LacZ}$ mutant ovaries are most likely having a problem with pregranulosa cell-oocyte cross-talk. The data do not conclusively demonstrate in which biological process the defect resides.

**V. Figures**

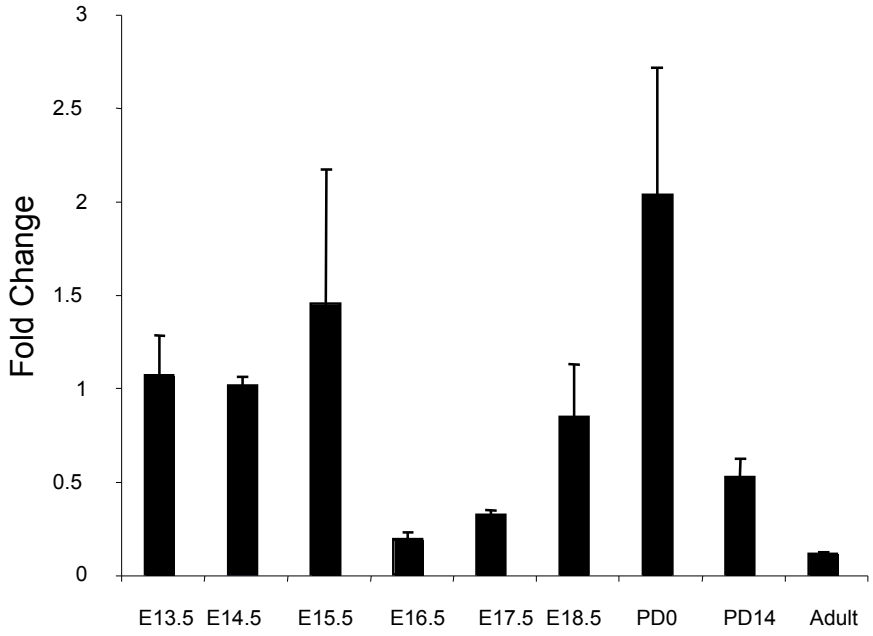



Figure 3.a. *Glp-1* is expressed during oogenesis. qPCR assay showing *Glp-1* mRNA levels in ovaries from E13.5 through adulthood. *Glp-1* is expressed from E13.5 through E15.5 at which time *Glp-1* levels drop. *Glp-1* is then re-expressed at E18.5, peaks at PD0 and then decreases. The Y axis displays fold change of *Glp-1* compared to *Gapdh* mRNA levels. n=2 for each sample. All female embryos from one litter were combined. Data was run in duplicate. All data points were averaged together and plotted. Error bars represent the S.E.M. of the two experiments.

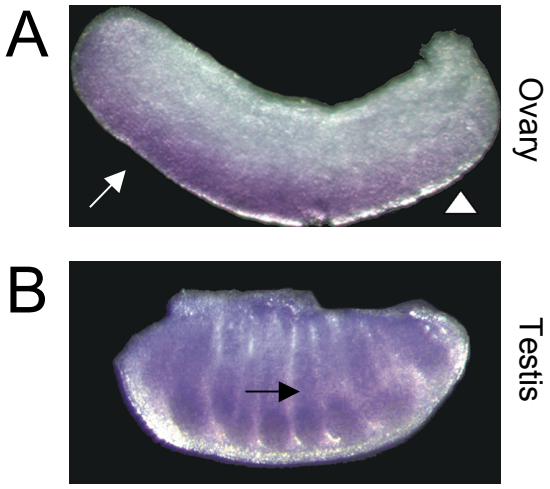



Figure 3.b. *In situ* hybridization at E14.5. A) Glp-1 is expressed throughout the ovary in a gradient fashion with the highest expression in the posterior part (white arrow) of the ovary and B) *Glp-1* is expressed within the cords of the testes (black arrow). White arrowhead indicates the anterior portion of the ovary. Positive staining is purple. Courtesy of Courtney Karner.

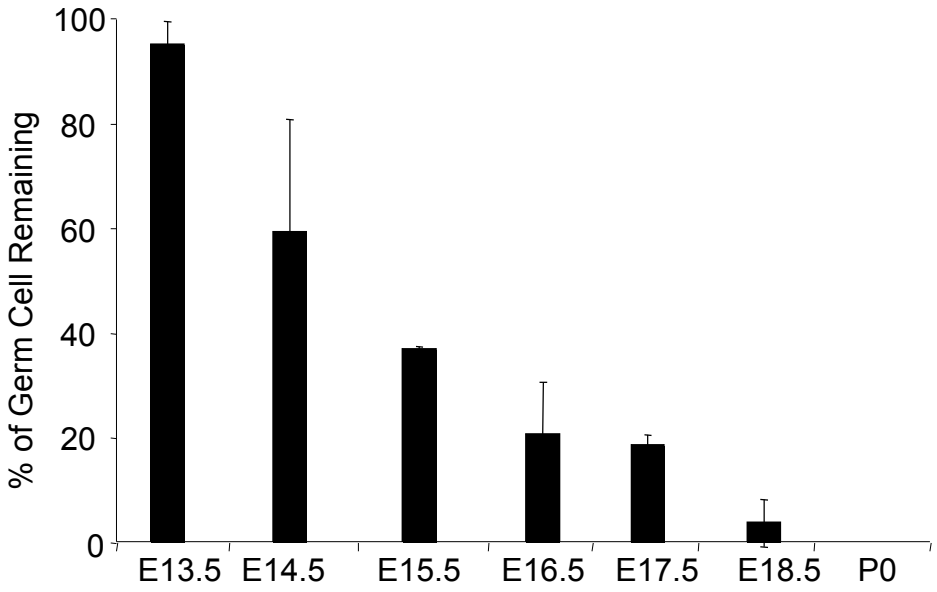



Figure 3.c. Glp-1 is required for germ cell survival during embryogenesis. The total number of germ cells from three fixed areas from different parts of the ovary from each genotype, heterozygotes and nulls, were counted. The average number of germ cells from the null animal was then divided by its heterozygous sibling and then the percent of germ cells remaining in the null was plotted. This was done for two separate animals for each genotype from different litters and the average was taken of the two experiments for each time point. Error bars represent the S.E.M. of the two experiments.

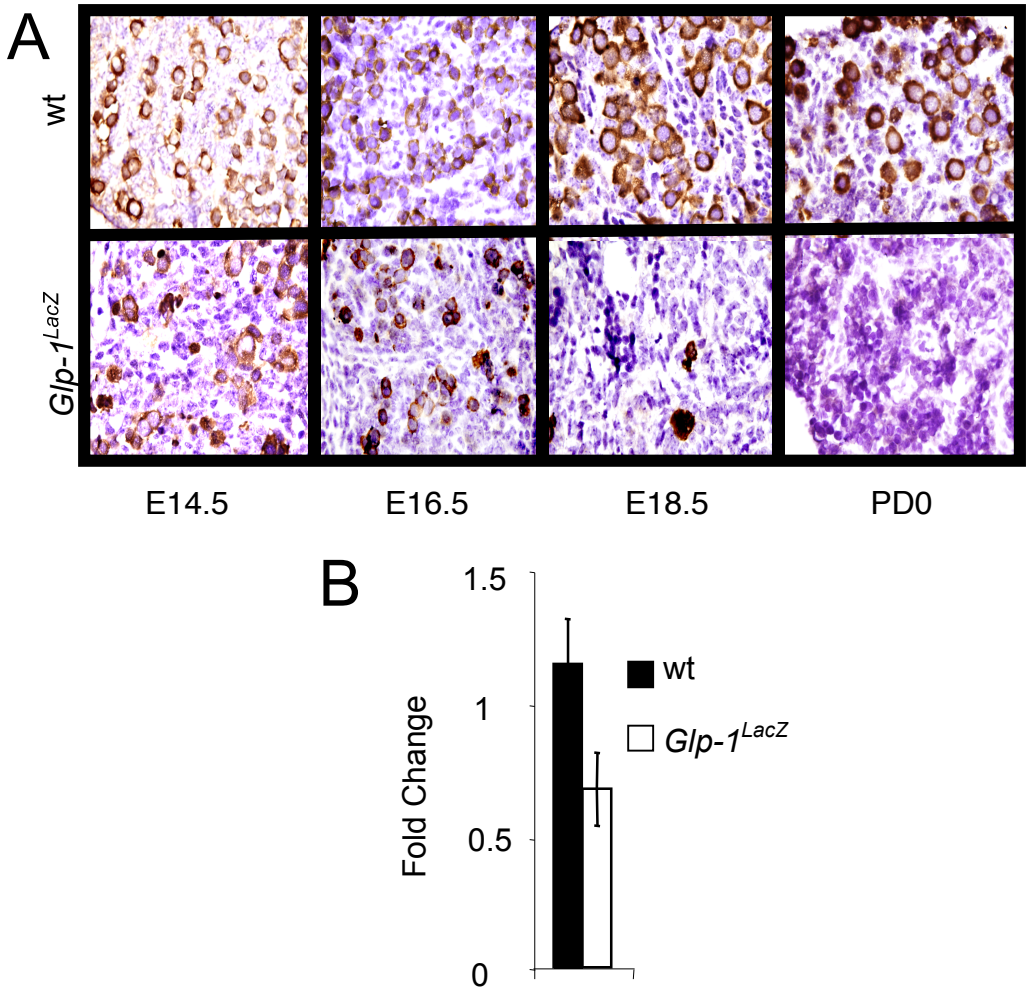



Figure 3.d. Confirmation of germ cell loss while not effecting pregranulosa cell specification in the absence of Glp-1. A) Mvh immunostaining of germ cells in *Glp-1*$^{LacZ}$ mutant and wild type ovaries. Germ cell loss has begun at E14.5 and is completed by birth. B) qPCR assay showing *Mvh* mRNA levels in *Glp-1*$^{LacZ}$ mutant and wild type ovaries at E13.5. The difference in *Mvh* mRNA levels is not significantly different between wild type and *Glp-1*$^{LacZ}$ mutants. The Y axis displays fold change of *Glp-1* compared to *Gapdh* mRNA levels. n=5 for each sample. Data was run in duplicate. All data points were averaged together and plotted. Error bars represent the S.E.M. of the five experiments.

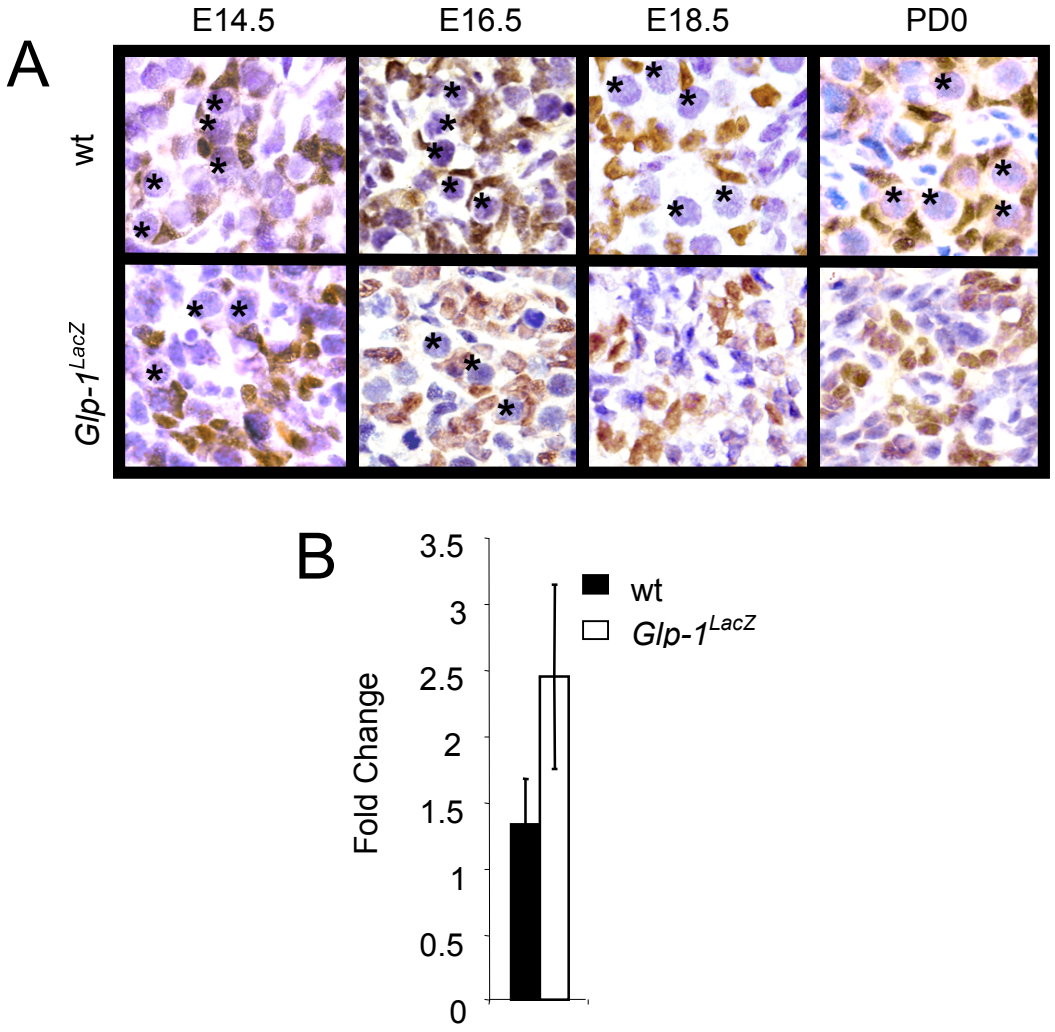



Figure 3.e. Pregranulosa cell specification in the absence of Glp-1. A) FoxL2 immunostaining of pregranulosa cells in *Glp-1*$^{LacZ}$ mutant and wild type ovaries at E14.5, E16.5, E18.5 and PD0. The asterisks indicate healthy looking germ cells in each genotype. B) qPCR assay showing *FoxL2* mRNA levels in *Glp-1*$^{LacZ}$ mutant and wild type ovaries at E13.5. The difference in *FoxL2* mRNA levels is not significantly different between wild type and *Glp-1*$^{LacZ}$ mutants. The Y axis displays fold change of *Glp-1* compared to *Gapdh* mRNA levels. n=5 for each sample. Data was run in duplicate. All data points were averaged together and plotted. Error bars represent the S.E.M. of the five experiments. This was done for separate animals for each genotype from different litters.

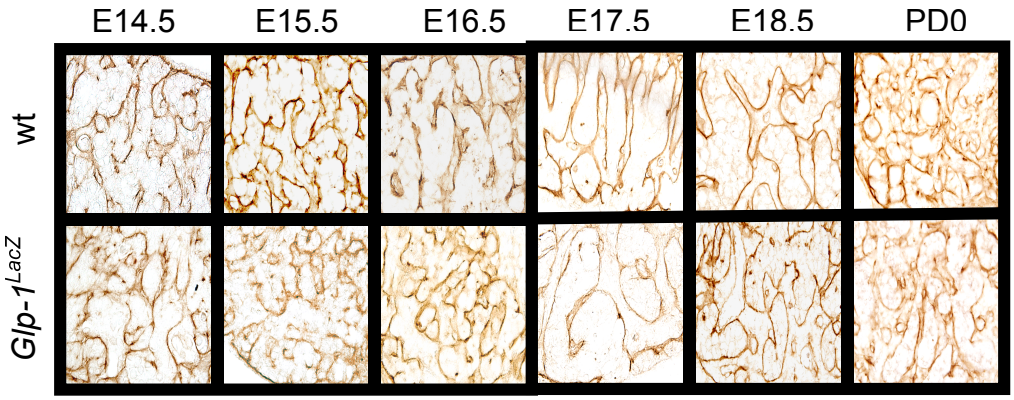



Figure 3.f. Loss of Glp-1 does not affect formation of a basement membrane. Laminin staining of *Glp-1*$^{LacZ}$ mutant and wild type ovaries is similar from E14.5 through PD0 even as germ cells are being lost.

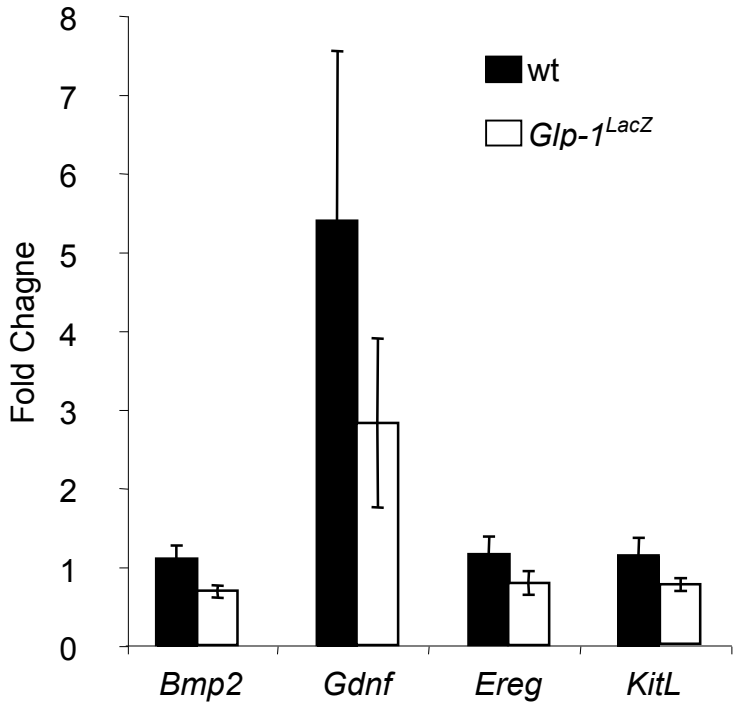



Figure 3.g. Normal somatic cell secretion of both novel and known molecules required during oogenesis. qPCR assay showing *Bmp2*, *Gdnf*, *Ereg* and *KitL* mRNA levels in *Glp-1*$^{LacZ}$ mutant and wild type ovaries at E13.5. The difference in *FoxL2* and *Mvh* mRNA levels is not significantly different between wild type and *Glp-1*$^{LacZ}$ mutants. *Bmp2*, *Gdnf*, *Ereg* and *KitL*, secreted somatic cells factors that remain unchanged in the absence of Glp-1. The Y axis displays the fold change of gene expression compared to *Gapdh* mRNA expression. Experiments were carried out two times and each experiment consisted of two replicates. All data points were averaged together and plotted. Error bars represent the S.E.M. of the two experiments. This was done for separate animals for each genotype from different litters.

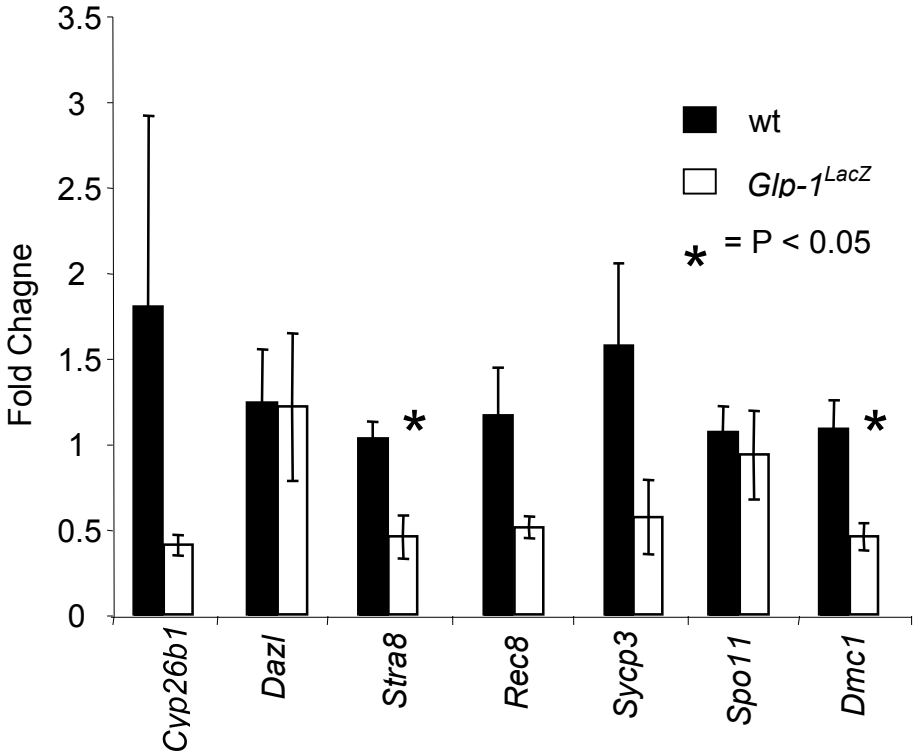



Figure 3.h. Loss of Glp-1 leads to aberrant expression of meiotic entry markers. qPCR assay showing *Cyp26b1*, *Dazl*, *Stra8*, *Rec8*, *Sycp3*, *Spo11* and *Dmc1* mRNA levels in E13.5 ovaries. Only *Stra8* and *Dmc1* mRNA levels are significantly different between *Glp-1*$^{LacZ}$ mutants compared to wild type. The Y axis displays the fold change of gene expression relative to *Gapdh* mRNA expression. The Y axis displays fold change of gene expression compared to *Gapdh* mRNA levels. n=3 for each sample. Data was run in duplicate. All data points were averaged together and plotted. Error bars represent the S.E.M. of the three experiments. All data points were averaged together and plotted. * $P < 0.05$. This was done for separate animals for each genotype from different litters.

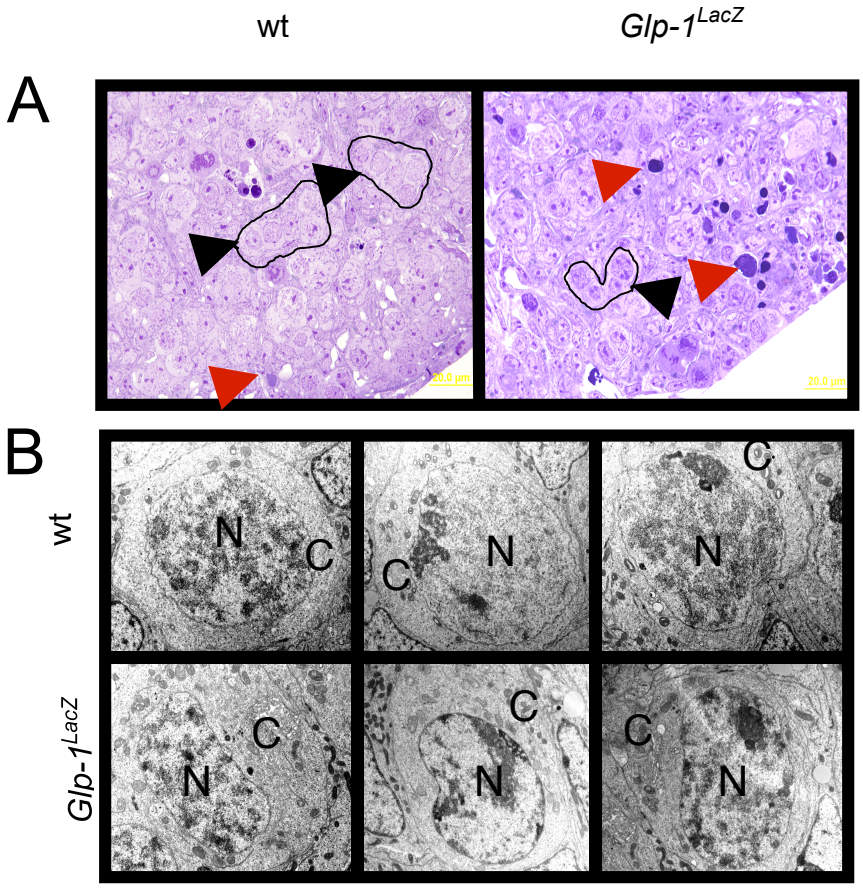



Figure 3.i. Embryonic ovaries show normal cyst formation and chromatin pattern with increased apoptosis of germ cells. A) Thick sections of E14.5 ovaries stained with toluidine blue to compare the morphology of the *Glp-1*$^{LacZ}$ and wild type ovaries. Black arrow heads point to germ cells enclosed in cyst. Red arrow heads indicate apoptotic cells. B) Transmission electron microscopy images of *Glp-1*$^{LacZ}$ and wild type germ cells in different steps in meiotic entry in E14.5. (N) germ cell nucleus (C) germ cell cytoplasm.

# CHAPTER IV: CHARACTERIZATION OF THE ADULT *GLP-1*$^{LACZ}$ NULL OVARIAN PHENOTYPE

## I. Objective

The objective of this study is to understand adult ovarian physiology in the absence of Glp-1. Ovaries deficient for Glp-1 were reported to form tubular adenomas containing theca and granulosa cells with the ability to make steroids (Li *et al.* 2007). First, I wanted to confirm the somatic cell expression of Glp-1. I also wanted to address the frequency of tubular adenoma formation in the absence of germ cells. Finally, I wanted to determine if *Glp-1*$^{LacZ}$ null animals have recognizable structures in ovaries that might allow them to make steroids. The research presented here addresses these issues.

## II. Introduction

*Glp-1* is a newly characterized gene shown to be important for ovarian development. Glp-1 is expressed in the granulosa cells of the ovary and the Leydig cells of the testis and acts as a transcription factor that represses GATA6/4-dependent transcription. In order to investigate the biological role of Glp-1, a *Glp-1*$^{LacZ}$ knock-in mouse line was generated in which the entire *Glp-1* locus has been replaced with the bacterial *LacZ* gene. Characterization of these mice revealed no abnormalities except that the null male and female mice were

sterile (Li *et al.* 2007). Due to the sterility phenotype in the *Glp-1*$^{LacZ}$ null mice, the testes and ovaries were analyzed for defects. Analyses of the gonads determined that in the adult *Glp-1*$^{LacZ}$ mouse testes failed to make sperm and that the ovaries were devoid of follicles. In the ovary, there was a complete loss of germ cells by birth. It was also reported that *Glp-1*$^{LacZ}$ null ovaries undergo excessive cellular proliferation resembling tubular adenomas and that these ovaries lose their structural integrity (Li *et al.* 2007).

Tubular adenoma formation is a common occurrence that arises secondarily to early germ cell loss in mouse models such as mutations in *KitL*, *c-Kit*, *Gcd*, *Dazl, and Glp-1* (Murphy & Beamer 1973, Duncan & Chada 1993, McNeilly *et al.* 2000, Li *et al.* 2007). Mutations in *c-Kit*, *KitL, Dazl, Glp-1* and ovariectomized wild type sexually mature females were shown to have high levels of circulating LH and FSH compared to control animals, and thus aberrant signaling occurs within the HPG axis (Murphy & Beamer 1973, McNeilly *et al.* 2000, Li *et al.* 2007). These high circulating levels of the gonadotropins, LH and FSH, result in prolonged stimulation of the granulosa cells which respond by proliferating thus leading to tubular adenomas (Personal communication with Dr. Diego Castrillon, UTSW).

Tubular adenomas are also able to make steroids. It has been suggested that the residing ovarian, somatic cell population has acquired the ability to

express all the enzymes required for steroidogenesis (Terada *et al.* 1984, McNeilly *et al.* 2000, Li *et al.* 2007). This differs from wild type ovary in that each individual cellular compartment of the follicle, theca and granulosa cells, is required for steroid synthesis. This suggests that either each cell contains the required enzymes to make steroids or that the ovarian structure is lost and the cells are no longer compartmentalized.

In addition, in *Glp-1*$^{LacZ}$ null ovaries, mRNA for the theca and granulosa cell markers, *p450ssc* and *SF-1, and Wnt4* and *Fshr,* respectively, were expressed in a diffuse manner throughout the ovary. The authors concluded that the ovaries of *Glp-1*$^{LacZ}$ null females have lost their cellular organization and that theca and granulosa cells are both present in an unorganized manner (Li *et al.* 2007). These opposing ideas regarding ovarian cellular composition demonstrate the need for further research to determine the exact changes that occur to the ovarian cells of a tubular adenoma. This suggests that in a tubular adenoma either theca and granulosa cells are present or that ovarian cells have undergone differentiation, thus becoming capable of expressing all the appropriate steroidogenic enzymes.

**III. Results**

*a. Glp-1 Expression in the adult mouse*

It has been reported that Glp-1 is expressed specifically in the granulosa cells of the ovary and the Leydig cells of the testis and not in any other tissue (Li et al. 2007). To investigate the possible function of Glp-1 in the adult ovary several techniques were employed. Northern blot analysis and qPCR were performed on wild type adult mouse tissues to determine the tissue mRNA expression pattern of *Glp-1*. The qPCR data show that *Glp-1* is expressed at limited levels (undetectable by Northern blot) in the ovary, lung, and brain and is most highly expressed in the testes (Figure 4.a and 4.b).

In parallel with qPCR and northern blot analysis, radio-labeled *in situ* hybridization experiments were performed without positive results. The *in situ* hybridizations were performed twice, each time using a different *Glp-1* riboprobe. The first experiment used a truncated *asGlp-1* cRNA probe, which contained only the N-terminal region of Glp-1, thus removing the C-terminal zinc fingers (pCMVNGlp-1) (data not shown). This eliminated cross hybridization with other GATA transcription factor family members already known to be expressed in the adult ovary. The second experiment used a full-length probe made as previously described (Li *et al.* 2007). *Lrh-1*, a granulosa cell marker, is expressed at high levels in the wild type adult ovary (Hinshelwood et al. 2003). A cRNA probe targeted to *asLrh-1* was generated and used as a positive control. In wild type

ovaries, *Lrh-1* is expressed within the follicles but is undetectable in the *Glp-1*$^{LacZ}$ mutant ovaries (Figure 4.c.A and B). *asGlp-1*, on the other hand is detected at similar levels using the *asGlp-1* and *sGlp-1* probes (Figure 4.c..E). *Glp-1*$^{LacZ}$ null ovaries were negative for *asGlp-1* (Figure 4.c.D).

Another experimental approach to determine the cellular localization of Glp-1 utilized adult heterozygotic *Glp-1*$^{LacZ/+}$ ovaries. Genetic inactivation of *Glp-1* by *β-LacZ* gene replacement can provide expression data due to the ability of the β-galactosidase enzyme to metabolize the X-gal substrate, thus leaving a blue precipitate easily visualized by microscopy. Unfortunately, the data obtained using this technique were inconsistent. This was possibly due to high levels of endogenous β-galactosidase-like activity known to be present in adult ovaries. Therefore, troubleshooting experiments were performed to minimize the background activity by comparing samples prepared with and without a high temperature (50$^{o}$C) incubation. In principle, the high temperature incubation step inactivates the endogenous enzyme while still allowing the bacterial β-galactosidase enzyme to function (Mercer 1995). This should lower background staining (Figure 4.d). Unfortunately, the *Glp-1*$^{LacZ/+}$ heterozygotic ovaries showed no positive β-galactosidase staining. Similarly, wild type ovaries had a similar staining pattern to *Glp-1*$^{LacZ/+}$ heterozygotic ovaries treated with and without the high temperature incubation step (data not shown).

Finally, two rabbit polyclonal Glp-1 antibodies were used on adult ovaries for immunofluorescence, immunohistochemistry, and western blot experiments. Unfortunately, due to the high level of non-specificity of the antibodies when used for immunostaining or western blot analysis, Glp-1 expression was undetectable even after several attempts to optimize the antibody (data not shown). Immunostaining experiments using an antibody against β-galactosidase in *Glp-1*$^{LacZ/+}$ heterozygous ovaries also failed to demonstrate the location of *Glp-1* promoter activity (data not shown).

Although several experimental techniques were not sensitive enough to detect *Glp-1* in the adult ovary, *Glp-1* mRNA levels are detectable by qPCR and RT-PCR. Confirmation that *Glp-1* is indeed expressed in the adult ovary is demonstrated by qPCR analysis. Comparisons between *Glp-1*$^{LacZ/LacZ}$, *Glp-1*$^{LacZ/+}$, and *Glp-1*$^{+/+}$ ovaries demonstrate that *Glp-1*$^{LacZ/+}$ ovaries contain 60% the amount of *Glp-1* mRNA that *Glp-1*$^{+/+}$ ovaries contain, and *Glp-1*$^{LacZ/lacZ}$ animals show negligible expression of *Glp-1* (Figure 4.e). These data demonstrate that *Glp-1* is present in the adult ovary and that the *Glp-1*$^{LacZ}$ null ovaries are true nulls.

*b. Glp-1 is expressed in the somatic cells of the ovary*

To prove that *Glp-1* is expressed within the somatic cells, specifically the granulosa cells of the ovarian follicle, *Glp-1* expression levels were analyzed in *c-Kit* (*Kit*$^{W}$/*Kit*$^{W-V}$) mutant adult gonads. *Kit*$^{W}$/*Kit*$^{W-V}$ mutant ovaries and testes are

devoid of germ cells due to the loss of appropriate germ cell migration and proliferation during embryogenesis (Mahakali Zama et al. 2005). QPCR experiments on adult $Kit^{W}/Kit^{W-V}$ mutant ovaries demonstrate that not only is *Glp-1* mRNA present in an ovary without germ cells but the levels are actually up-regulated (Figure 4.f.A). mRNA levels of *FoxL2*, a granulosa cell specific marker, in the $Glp\text{-}1^{LacZ}$ mutants are similar to wild type ovaries (Figure 4.f.B). This increase in *Glp-1* expression is not due to just an increase in granulosa cell number in the mutant ovaries compared to the wild type ovary, but is actually over-expression. Northern blot analysis comparing adult wild type and $Kit^{W}/Kit^{W-V}$ mutant testes confirms somatic cell expression (Figure 4.b). These data strongly suggest that Glp-1 is a somatic cell factor and not expressed in the germ cells.

*c. $Glp\text{-}1^{LacZ}$ null adult ovaries undergo aberrant proliferation but retain their structural integrity*

Two questions have arisen regarding the adult ovarian phenotype. First, what physiological differences exist between wild type and $Glp\text{-}1^{LacZ}$ null ovaries? Li *et al.*, 2007 reported that adult $Glp\text{-}1^{LacZ}$ null ovaries undergo cellular proliferation resulting in disorganized theca and granulosa cells and resemble tubular adenomas. To address the frequency of adenoma formation, ten six month old $Glp\text{-}1^{LacZ}$ mutant females ovaries were assessed for their size (n=5). Of these ten ovaries, only three (30%) were of similar size (large type) to their littermate controls, demonstrating that aberrant somatic cell growth does not

occur in all ovaries, nor was it observed to happen to both ovaries of the same female. The other 70% were shrunken (small type) and showed signs of dysgenesis (Figure 4.g). These data suggest that aberrant somatic cell growth at six months is not fully penetrant and thus does not always occur as suggested.

Second, I wanted to understand the structural organization of the *Glp-1*$^{LacZ}$ mutant ovary. The previous study concluded that cell populations in the adult *Glp-1*$^{LacZ}$ mutant ovaries were unorganized, as markers for theca and granulosa cells were diffusely expressed throughout the ovary. Interestingly, the ovaries retained their ability to make steroids, suggesting the presence of both theca and granulosa cells (Li et al. 2007). To investigate the gross morphology of the *Glp-1*$^{lacZ}$ null ovaries, immunostaining for laminin was performed. Laminin is a major component of the basement membrane surrounding each follicle and is produced by granulosa cells. The basement membrane is important for physically separating the theca cells from the granulosa cells of a follicle. Interestingly, it seems that a basement membrane is present in both the large and small type *Glp-1*$^{LacZ}$ null ovaries (Figure 4.h). This is an unexpected observation since it was previously concluded that the ovaries have lost their structural integrity (Li *et al.* 2007).

*d. Glp-1*$^{LacZ}$ *null females have abnormal levels of circulating blood factors required for proper estrous cycling*

*Glp-1*$^{LacZ}$ null females are sterile due to the loss of germ cells by birth. To ask how sterility effects the communication within the HPG axis, I followed adult *Glp-1*$^{LacZ}$ null females through the estrous cycle. After three cycling rounds, the *Glp-1*$^{LacZ}$ null females failed to cycle and remained in diestrus as compared to their cycling wild type littermates (Figure 4.i). I collected serum from three experimental and heterozygous females in diestrus to evaluate the levels of estradiol, the gonadotropins, LH and FSH, and Inhibin B. The LH data supports the previous data, thus confirming an increase in LH levels in circulating serum (Table 3.B). Inhibin B levels were also analyzed due to its importance in the negative regulation of FSH. These data show that Inhibin B is undetectable in circulating blood serum of *Glp-1*$^{LacZ}$ nulls compared to their sibling controls (Table 3.A). I have not yet received data for FSH or estradiol but suspect they will be similar to the previously reported data, like LH.

**IV. Discussion**

The data presented here provide additional insight regarding the adult *Glp-1*$^{LacZ}$ mutant phenotype in more detail that what was previously described. I showed that *Glp-1* is a somatic cell factor expressed at high levels in adult testes and at limited levels in the ovaries. I also showed that *Glp-1*$^{LacZ}$ null ovaries contain structures that are similar to embryonic germline cysts and that the residing cell population fails to produce Inhibin-$\alpha$.

My first undertaking was to repeat the previously reported data (Li *et al.* 2007). When I was unable to repeat the β-galactosidase staining, I tried alternative approaches to determine the cellular expression pattern of Glp-1 in the ovaries. My data show that Glp-1 is expressed in the somatic cells of the ovary but at very low levels. Unfortunately, complete characterization of Glp-1 function in the wild type ovary is hindered by low levels of endogenous expression. Therefore, future experiments will need to use more sensitive techniques than what currently exist.

Ovarian tubular adenomas derived from ovaries with germ cell loss were previously characterized in the early 1970s using variations of genetically altered mouse lines (Murphy 1972, Duncan & Chada 1993). It was concluded that, in fact, the tumors actually arise due to prolonged exposure to the gonadotropins, LH and FSH, released from the pituitary upon GnRH stimulation (Murphy 1972, Murphy & Beamer 1973, Blaakaer *et al.* 1995). High levels of LH and FSH detected in the serum of *Glp-1*$^{LacZ}$ mutant females are indicative of ovarian dysgenesis (Li *et al.* 2007). Adult *Glp-1*$^{LacZ}$ mutant ovaries lack the expression of Inhibin B (Table 3A), thus withdrawing the negative feedback signal required to decrease FSH levels. As a result of the sustained LH and FSH exposure, formation of a tubular adenoma occurs in *Glp-1*$^{LacZ}$ mutant ovaries that have lost their germ cells.

The frequency by which *Glp-1*$^{LacZ}$ mutant ovaries harbor tubular adenomas at six months is 30% (Figure 4.g). Based on research by others, it is likely that significantly more ovaries from *Glp-1*$^{LacZ}$ mutant females would eventually develop proliferative lesions, thus forming a large type tubular adenoma (Vanderhyden *et al.* 2003).

*Glp-1*$^{LacZ}$ mutant ovaries retain their ability to make steroids, which was suggested to occur due to the disorganized presence of both theca and granulosa cells (Li *et al.* 2007). My data supplemented with previous data of germ cell-less ovaries suggest that in a tubular adenoma normal ovarian tissues, such as theca and granulosa cells, are no longer present (Terada *et al.* 1984). Therefore, the ability of *Glp-1*$^{LacZ}$ null ovaries to makes steroids is not necessarily due to the presence of theca and granulosa cells but may be due to aberrant gene expression in the cells of the ovary. Interestingly, steroidogenesis, producing both androgens and estrogens, has been described as an inherent property of tubular adenomas and not due to the presence of theca and granulosa cells. (Terada *et al.* 1984, McNeilly *et al.* 2000).

To determine if the ovaries retained any cellular organization they were stained for laminin (Figure 4.h). Theca cells are physically separated from granulosa cells by a basement membrane, detectable by laminin. Laminin

staining suggests that the ovary is structured. Interestingly, it was reported in the early 1970s that ovaries that develop without germ cells consist of cells derived from the germinal epithelium and consist of tubular structures. An alternative model can be proposed whereby the basement membrane of a germline cyst remains in the adult, and which is surrounded by granulosa-like cells. The expression of SF-1, p450ssc, Wnt4, and FSHR in a diffuse manner in *Glp-1*$^{LacZ}$ mutant adult ovaries could alternatively be suggestive of the granulosa cells changing their genetic program, thus allowing them to express these factors. It was demonstrated that the interstitial cells express hydroxysteroid dehydrogenase-3β (Hsd-3β) only in the context of a tubular adenoma, thus giving the ovary the ability to make steroids (McNeilly *et al.* 2000). It is possible, and probably more likely, that the laminin staining observed is actually staining of a basement membrane, which surrounded a now reminiscent, embryonic germline cyst and not a newly formed tubular structure.

## V. Figures

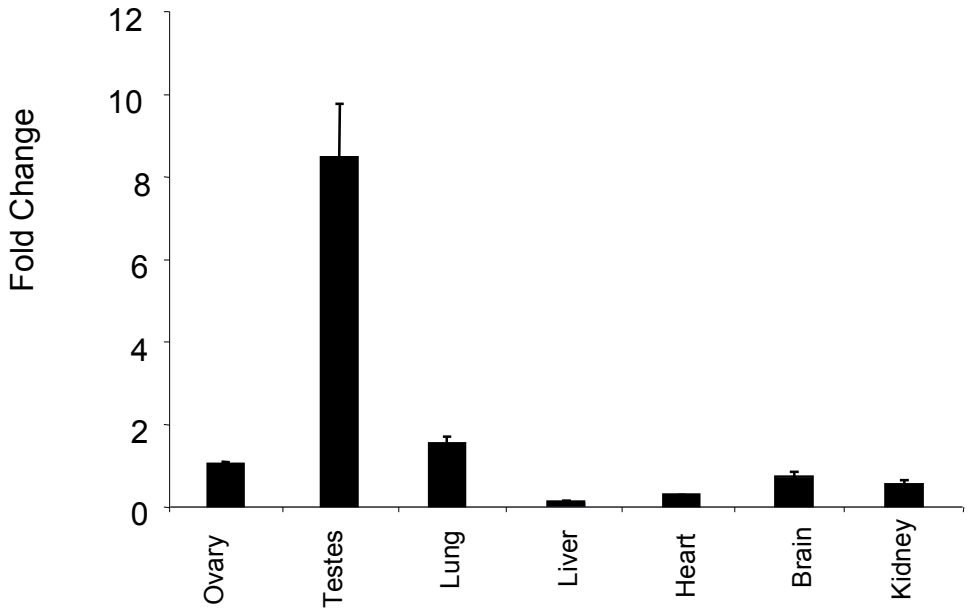



Figure 4.a. *Glp-1* expression in adult mouse tissues. Detected by qPCR *Glp-1* mRNA levels in various adult tissues. *Glp-1* is expressed highest in the testes with limited expression in the ovary, lung, and brain. The Y axis displays fold change of gene expression normalized to *Gapdh* mRNA levels. n=2 for each tissue. Samples were carried out in triplicate. All data points were averaged together and plotted. Error bars represent the S.E.M. of the experiments.

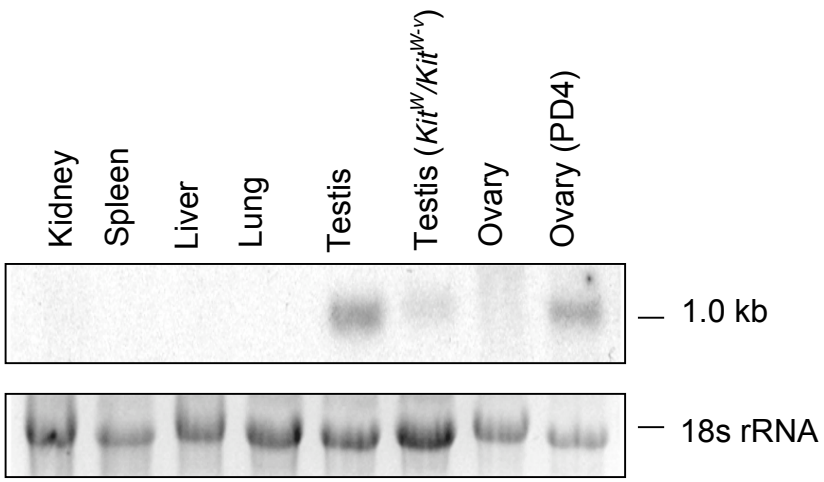



Figure 4.b. Glp-1 is a somatic cell factor expressed in gonads. Northern blot analysis shows detectable *Glp-1* mRNA levels in various adult tissues. *Glp-1* is expressed in wild type testes, germ cell-less, kit receptor mutant ($Kit^{W}/Kit^{W\text{-}v}$) testis, and wild type adult and PD4 ovaries.

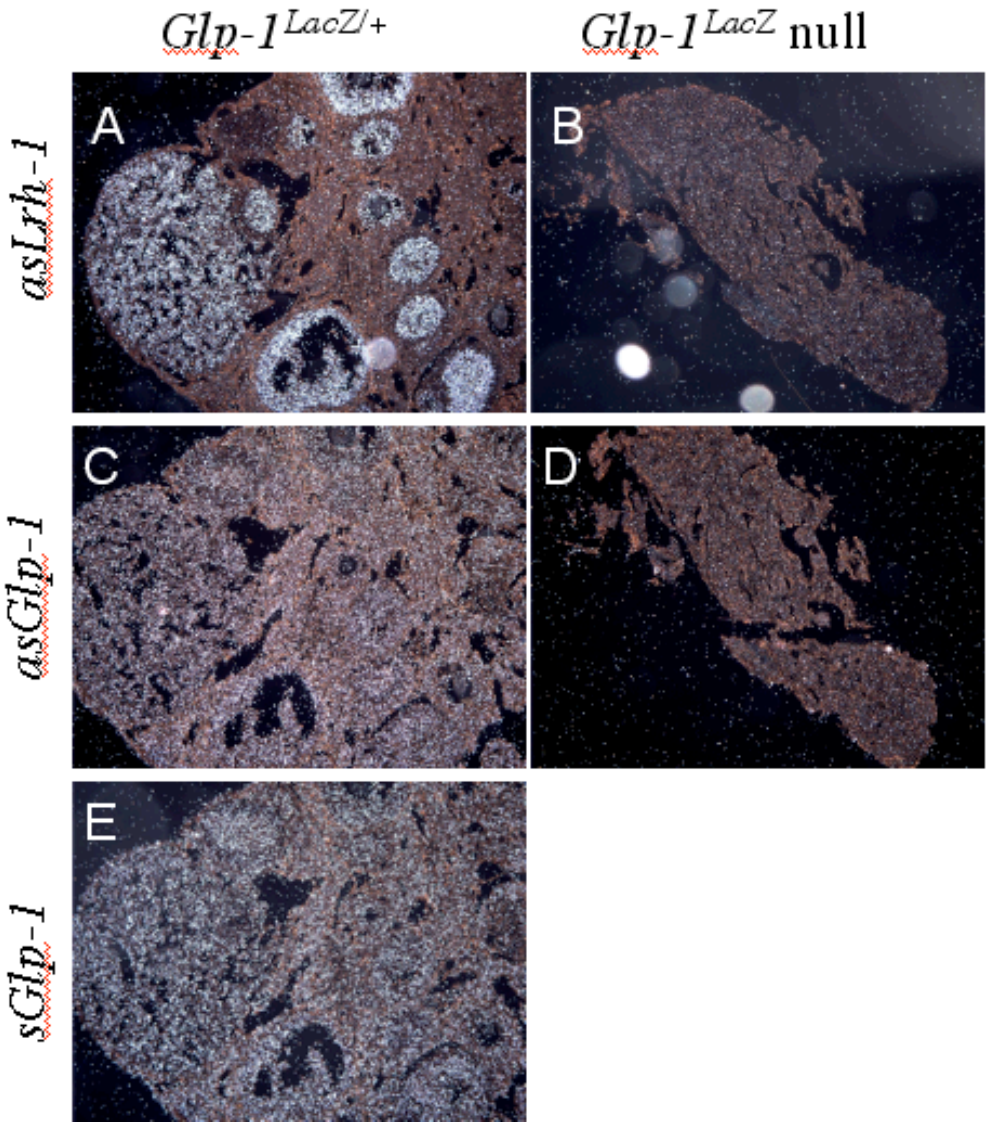



Figure 4.c. *Glp-1* mRNA is undetectable by *in situ* hybridization. *Glp-1*$^{LacZ}$ heterozygous (A, C, and E) and *Glp-1*$^{LacZ}$ null (B and D) ovaries were taken from 6 mo. old siblings females both in diestrus. Serial sections were incubated with an antisense probe targeted to *Lrh-1* or the N-terminus of *Glp-1*. A) *asLrh-1* is expressed in all granulosa cells of all follicular stages in the *Glp-1*$^{LacZ}$ heterozygous ovaries. B) *asLrh-1* is not expressed in *Glp-1*$^{LacZ}$ null ovary. C) *asGlp-1* mRNA is not specific in the *Glp-1*$^{LacZ}$ heterozygote as a similar staining pattern is seen using the *Glp-1* sense probe (*sGlp-1*) D) *Glp-1* staining is not seen in the negative control *Glp-1*$^{LacZ}$ null ovaries.

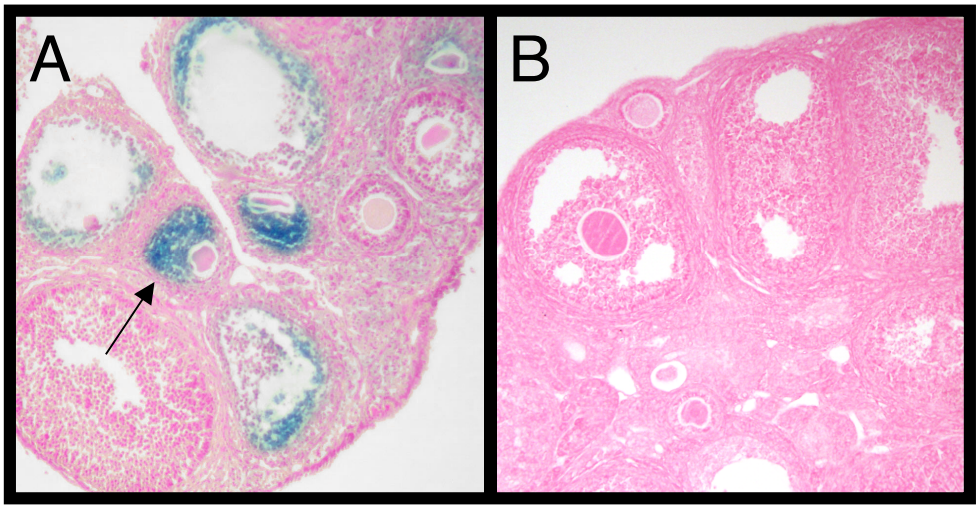



Figure 4.d. *Glp-1* promoter activity is undetectable using β-galactosidase staining in adult ovaries. A) An adult *Glp-1*$^{LacZ}$ heterozygotic ovary stained with the conventional β-galactosidase staining protocol. There is uneven β-galactosidase staining in the follicles of these ovaries. Arrow marks background staining. B) An adult *Glp-1*$^{LacZ}$ heterozygous ovary stained with a conventional β-galactosidase staining protocol with the high temperature incubation step at 50°C for 1hr prior to the addition of substrate. β-galactosidase staining is lost suggesting that endogenous β-galactosidase activity is responsible for the staining and not the bacterial *LacZ* gene inserted into the *Glp-1* locus.

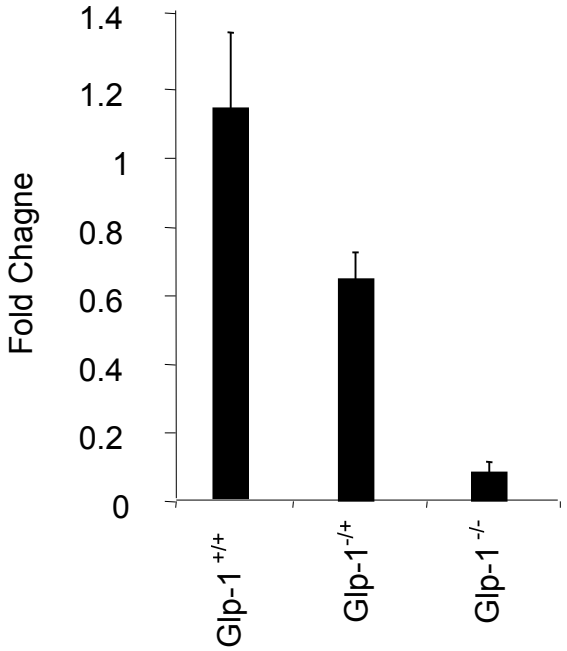



Figure 4.e. The *Glp-1*$^{LacZ}$ mouse line is a true null as determined by qPCR. *Glp-1* mRNA levels in adult ovaries of *Glp-1*$^{LacZ}$ wild type (+/+), heterozygotes (-/+), and null (-/-) animals. The Y axis displays the fold change of gene expression normalized to *Gapdh* mRNA levels. n=2 for each tissue. Samples were carried out in triplicate. All data points were averaged together and plotted. Error bars represent the S.E.M. of the experiments.

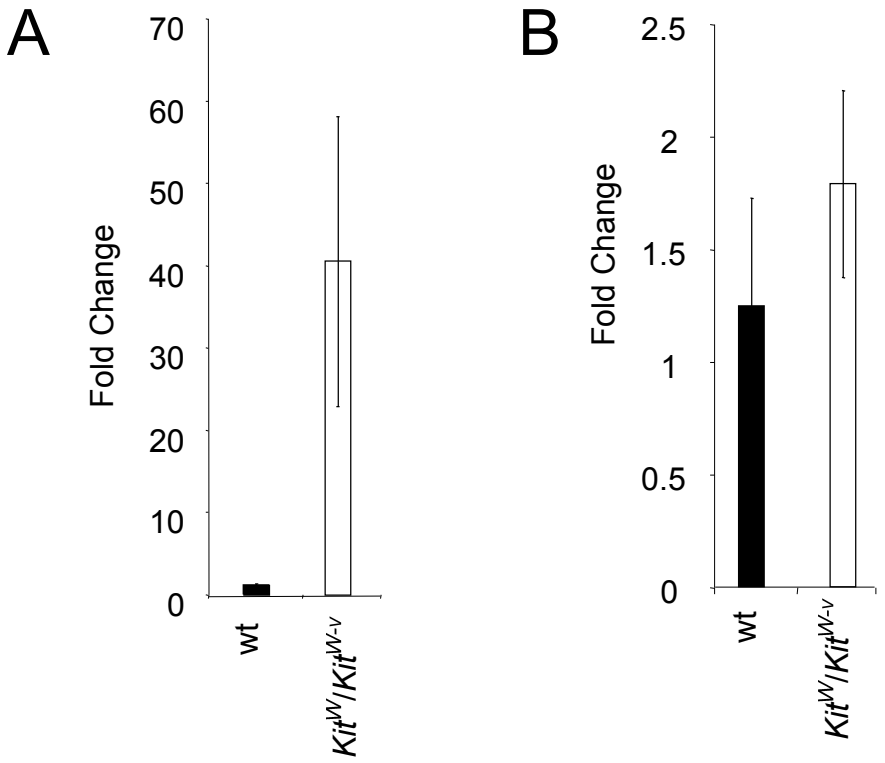



Figure 4.f. Glp-1 is a somatic cell factor expressed in female gonads as determined by qPCR. A) *Glp-1* mRNA is expressed in the granulosa cells of the ovary. *Glp-1* mRNA is detected at higher levels in Kit receptor transheterozygotic ($Kit^{W}/Kit^{W-v}$) ovaries that contain only somatic cells compared to wild type ovaries. B) *FoxL2* mRNA level in $Kit^{W}/Kit^{W-v}$ ovaries is not significantly different between $Kit^{W}/Kit^{W-v}$ and wild type adult ovaries. This suggests that the up-regulation of *Glp-1* mRNA is not due to a significant increase in the number of granulosa cells represented in the RNA samples. The Y axis displays the fold change of gene expression normalized to *Gapdh* mRNA levels. n=2 for each tissue. Samples were carried out in duplicate. All data points were averaged together and plotted. Error bars represent the S.E.M. of the experiments.

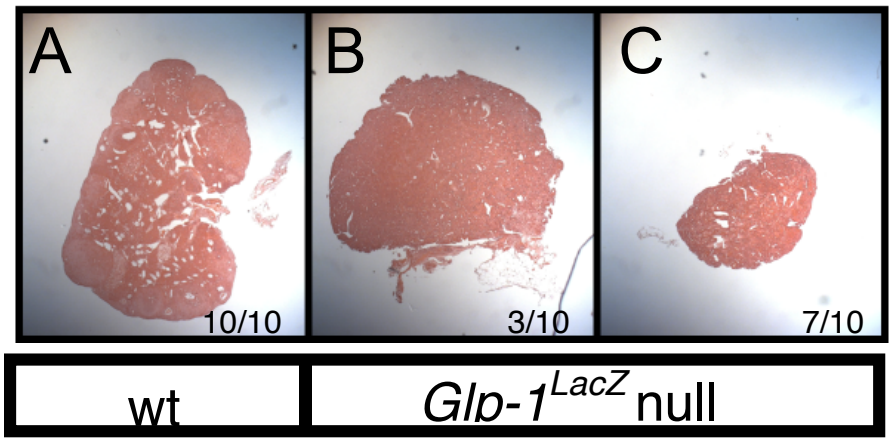



Figure 4.g. Large ovaries are seen 30% of the time in 6 month old *Glp-1*$^{LacZ}$ null females. A-C) Ten ovaries from five females were dissected and evaluated for their size. Ovary sections were stained with H&E. A) A normal wild type 6 mo old adult ovary. B) A typical large type ovary seen in 30% of 6mo old adult *Glp-1*$^{LacZ}$ null females that resembles a tubular adenoma. C) A typical small type ovary, most likely due to germ cell loss, seen in 70% of 6mo old adult *Glp-1*$^{LacZ}$ null females.

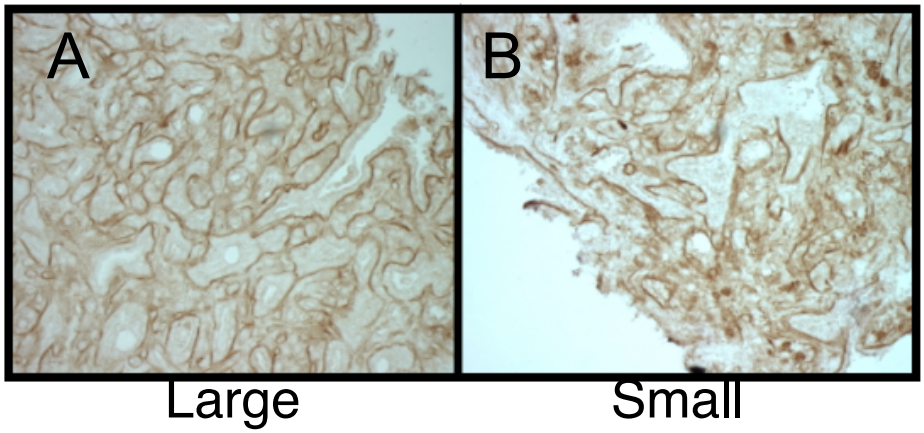



Figure 4.h. *Glp-1*$^{LacZ}$ null follicles retain their structural integrity into adulthood. A-B) Sections from 6mo old *Glp-1*$^{LacZ}$ null ovaries were stained for laminin. A) Tissue from a large type *Glp-1*$^{LacZ}$ null ovary. B) Tissue from a small type *Glp-1*$^{LacZ}$ null ovary.

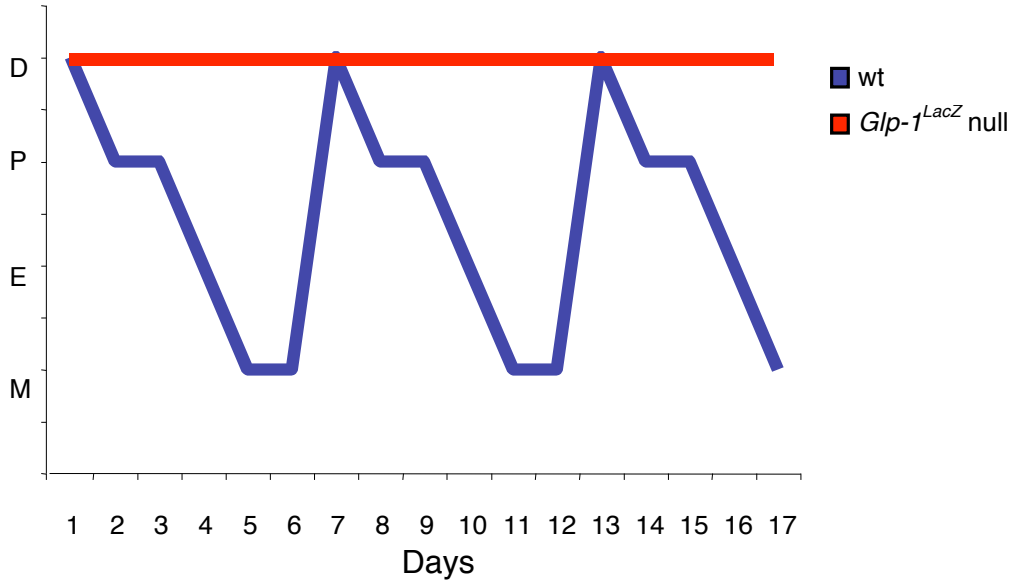



Figure 4.i. *Glp-1*$^{LacZ}$ null females do not progress through the estrous cycle and remain in diestrus. *Glp-1*$^{LacZ}$ null 8wk old females were housed with wild type littermates and vaginal smears were analyzed each day through three cycles. The data represent one of six sibling pairs analyzed. (D) diestrus, (P) proestrus, (E) estrous, (M) metestrus.

A Inhibin B pg/mL

| Age | wt | GLP-1LacZ null |
|---|---|---|
| 13wk | ud | ud |
| 5mo | 66.9 | ud |
| 6mo | 47.6 | ud |

B LH ng/mL

| Age | wt | GLP-1LacZ null |
|---|---|---|
| 13wk | 0.075 | 4.003 |
| 5mo | 0.055 | 2.576 |
| 6mo | 0.035 | 3.869 |

Table 4. Loss of Glp-1 leads to misregulation of molecules important for proper HPG signaling. Decreased levels of (A) Inhibin B and (B) increased levels of LH were found in circulating blood from *Glp-1*$^{LacZ}$ null females compared to wild type sibling controls. Serum was collected from one female animal at 13wk, 5mo, and 6mo old and sent to the University of Virginia Core Ligand and Assay Laboratory where an Elisa was performed according to their experimental protocol. (ud) undetectable.

# CHAPTER V: GLP-1 FUNCTIONS AS A REPRESSOR OF STEROIDOGENESIS THROUGH STAR PROMOTER ACTIVITY

## I. Objective

The objective of this study was to determine if Glp-1 regulates GATA4/6-dependent transcription of *StAR* and thus influence steroidogenesis. To perform these studies, I attempted to identify a cell line where I can perform transcriptional assays, radioimmunoassay, and manipulate endogenous *Glp-1* mRNA levels by either siRNA knock down or over-expression of *Glp-1*. I also addressed the subcellular localization of the endogenous Glp-1.

## II. Introduction

Recently, Glp-1 was characterized as GATA-like in that it contains two zinc fingers in the C-terminus of the protein. Glp-1 localizes to the nucleus and is a strong repressor of GATA4/6-dependent transcriptional activation of the surfactant protein-A luciferase (SP-A.luc) reporter vector. Interestingly, Glp-1 is unable to bind the GATA DNA consensus binding site (Li et al. 2007). This is thought to be due to the lack of homology in the length of the linker region in the second zinc finger which is essential for DNA binding and transcriptional activation in other GATA transcription factors (Li *et al.* 2007).

GATA transcription factors are known to have important functions in regulating promoters within steroidogenic tissues, including the ovary, at the level of transcription. Several ovarian GATA4-target genes have been identified including *StAR*, *cyp19*, and *Inhibin-*$\alpha$. Interestingly, each of these genes are transcriptionally activated through stimulation by FSH (Silverman *et al.* 1999, Anttonen *et al.* 2006, Kwintkiewicz *et al.* 2007). StAR is the rate limiting protein in the steroidogenic pathway, as it brings cholesterol from the outer mitochondrial membrane to the internal membrane (Thompson *et al.* 1997, Christenson & Strauss 2000). Expression of aromatase in the granulosa cells is responsible for the conversion of androstenedione to estrogen (Mendelson *et al.* 2005). The common subunit of Inhibin-A and Inhibin-B, *Inhibin-*$\alpha$, is also transcriptionally regulated by GATA4 activity (de Kretser & Robertson 1989).

Much research has been performed to elucidate the transcriptional mechanism by which the *StAR* promoter is activated. *StAR* expression is activated by FSH stimulation in granulosa cells, through a cAMP-dependent process whereby phosphorylation of GATA4 by PKA increases the binding affinity of GATA4 to the *StAR* promoter (Tremblay & Viger 2003, Hiroi *et al.* 2004). Subsequent studies showed that in response to FSH, GATA4 binds within the *StAR* promoter at position –66/-61 to activate its transcription (Silverman *et al.* 1999, Silverman *et al.* 2006). *StAR* expression is induced by LH/hCG with the greatest activation occurring within the first –254 nucleotides of the promoter

(Clark & Stocco 1995, Caron *et al.* 1997). The proximal promoter of *StAR* contains an unidentified repressor between nucleotides -966 and –254, which prevents maximum expression (Caron *et al.* 1997).

It is known that GATA4 binding within the *StAR* promoter is essential for its transcriptional activation in the ovary for proper steroidogenesis. To investigate if Glp-1 plays a role in steroid production, I tested its ability to regulate *StAR* promoter activation by GATA4. My data show, by transcriptional assays and by radioimmunoassay, that Glp-1 is able to repress GATA4-dependent transcriptional activation of *StAR*, which lead to a decrease in steroidogenesis.

**III. Results**

*a. Over-expressed Glp-1 localizes to the nucleus but endogenous is nuclear and cytoplasmic*

Over-expression studies of Glp-1 suggest that Glp-1 functions as a transcription factor in the nucleus (Li *et al.* 2007). These same studies were unable to show binding of the DNA by Glp-1, thus raising questions regarding the function of Glp-1 as a transcription factor. The previous localization study, which showed nuclear expression of Glp-1, over-expressed a myc-Glp-1 fusion protein (pCMV-Glp-1) and detected Glp-1 expression by immunofluorescence using an antibody direct toward myc (Li *et al.* 2007). Subsequent to these studies, working with Biosynthesis, I generated two rabbit polyclonal antibodies against different

peptide sequences within the coding region of Glp-1. These new reagents were used to determine endogenous Glp-1 localization by immunofluorescence and nuclear-cytoplasmic fractionation followed by western blot analysis. To demonstrate the specificity of the antibody, I collected nuclear and cytoplasmic fractions from COS7 cells transiently transfected with Glp-1. The nuclear and cytoplasmic fractions were analyzed for Glp-1 expression and compared to whole cell lysates in which cells were transiently transfected with either an empty vector control or Glp-1. Over-expressed Glp-1 is only seen in the nuclear fraction (Figure 5.a). This data supports the previously reported nuclear localization of over-expressed Glp-1.

Now that I have shown that the antibodies detect over-expressed Glp-1, I wanted to see if I could detect endogenous Glp-1 in a cell line naturally expressing Glp-1. Based on the previous gonadal-specific expression data, I analyzed *Glp-1* expression in the mouse Leydig tumor cell line, MLTC1s. *Glp-1* expression was analyzed by RT-PCR. *Glp-1* is expressed in the MLTC1 cell line as seen by a similar sized product detected from ovary RNA and by PCR of the pCMVGlp-1 plasmid (Figure 5.b). I have chosen to use the MLTC1 cell line for the following reasons. First, although a granulosa cell line would be optimal; one is not available. Second, I know that Glp-1 is expressed in the Leydig cells of the testes (Li *et al.* 2007). Finally, and most importantly, MLTC1 cells express all the enzymes required for steroidogenesis (Manna *et al.* 1999).

Next, I performed both immunofluorescence staining and western blot analysis after nuclear-cytoplasmic fractionation of MLTC1 cells to determine endogenous localization of Glp-1. Immunofluorescent staining of Glp-1 shows nuclear and cytoplasmic expression compared to DAPI staining of the nucleus (Figure 5.c.A). The western blot shows a band at the same size as cells transiently transfected with Glp-1 in both the cytoplasmic and nuclear fraction from MLTC1 cell lysates (Figure 5.c.B). Detection of SF-1 by western blot was used as a control for the nuclear and cytoplasmic fractionation (Figure 5.c.B). SF-1, a known nuclear protein, is only detected in the nuclear fraction (Val *et al.* 2003). Both techniques show nuclear and cytoplasmic staining of Glp-1 in MLTC1 cells.

*b. Glp-1 regulates GATA-dependent promoter activation in MLTC1 cells*

To examine Glp-1's effect on transcription, I first investigated, in MLTC1 cells, its effects on the *Sp-A* promoter, a known GATA-dependent transcriptional target that was used in the previous report using NIH3T3 cells (Li *et al.* 2007). Therefore, I repeated similar experiments as those performed by the Morrisey group but instead used MLTC1 cells and the COS7 cell line, which lacks Glp-1, as a control. COS7 and MLTC1 cells were transiently transfected with the SP-A.luc reporter vector, and a luciferase assay was performed 48hrs after transfection. In both cell lines, GATA4/6 are both able to activate the reporter.

Co-expression of Glp-1 caused repression of GATA4/6-dependent transcriptional activation of the SP-A.luc reporter, with the greatest effect of Glp-1 on GATA4 (Figure 5.d.A and B). Additional wells of MLTC1 cells were transfected and analyzed for protein expression. All constructs were expressed as determined by western blot analysis (Figure 5.d.B). My results are similar to the previously published results, and therefore I am able to use the luciferase system in the MLTC1 cells to perform future studies.

*c. Glp-1 is able to repress StAR promoter activation by GATA4/6 in MLTC1 cells in response to hCG*

With the identification of a cell line responsive to the effects of Glp-1 detectable by luciferase assay, I addressed if Glp-1 is involved in the expression of GATA-dependent transcriptional targets, with an emphasis on key players in the regulation of steroidogenesis. Granulosa cell GATA-dependent genes include *StAR*, *cyp19* and *Inhibin-*$\alpha$ (de Kretser & Robertson 1989, Thompson *et al.* 1997, Christenson & Strauss 2000, Mendelson *et al.* 2005). I have chosen to focus on *StAR* promoter regulation, as the reagents were readily available from Dr. Keith Parker's laboratory here at UTSW and because the proximal promoter region contains a single GATA binding site.

Transcriptional regulation of *StAR* has been well characterized. The promoter region contains several protein binding sites, including a single GATA

binding site. To test whether Glp-1 is able to regulate GATA4/6-dependent *StAR* promoter activation, I obtained the proximal promoter fused to luciferase (p-966StAR.luc). MLTC1 cells were co-transfected with p-966StAR.luc and with Glp-1 and/or GATA4/6. Cells were harvested and luciferase assays performed. The *in vitro* data show that Glp-1 is able to significantly suppress GATA4 and GATA6-mediated activation of the p-966StAR/luciferase reporter (Figure 5.e.A). A high level of basal activity is seen due to the presence of endogenous GATA4/6 (Ketola *et al.* 1999). When transfected alone, GATA4/6 modestly increase activation of the reporter, but with co-transfection of Glp-1, there is robust repression of both basal and GATA-dependent activation (Figure 5.e.A). Additional cells were transfected to detect protein expression. Western blot analyses show that each of the genes transfected are expressed (Figure 5.e.B).

One characteristic feature of *StAR* is that its expression is induced by gonadotropins secreted from the pituitary, including LH/hCG (hCG is functionally similar to LH) (Clark & Stocco 1995). To determine if Glp-1 has an effect on steroidogenesis, I first tested the sensitivity of the *StAR* proximal promoter (-966 nucleotides) to hCG in MLTC1 cells. MLTC1 cells were transiently transfected with the p-966StAR.luc reporter. After 48hrs, cells were serum starved for 4 hours and then stimulated with or without 5U/mL hCG. Cells were harvested and a luciferase assay performed. In MLTC1 cells, transcriptional activation of the proximal *StAR* promoter is not stimulated by hCG (Figure 5.f).

It was previously reported in MA-10 Leydig cells, that the maximal inducible activation of the *StAR* promoter resides within –254 nucleotides in the 5'UTR due to a repressor that resides between nucleotides –966 and –254 in the proximal reporter (Caron *et al.* 1997). To obtain the maximal response to hCG, I subcloned the –254 nucleotides of the promoter back into a vector containing luciferase. MLTC1 cells were transiently transfected with the minimal promoter, p-254StAR.luc, with or without Glp-1. In contrast to the longer promoter (p-966StAR.luc), the minimal *StAR* promoter (p-254StAR.luc) leads to an approximate two fold increase in luciferase activity upon hCG stimulation (compare Figure 5.f to 5.g).

To ensure that the myc-tagged version of Glp-1 functioned similarly to wild type Glp-1, I cloned *Glp-1* into pCDNA3.1, a vector containing no tags. MLTC1 cells were transfected with either myc-tagged Glp-1 (pCMVGlp-1) or Glp-1 (pCDNAGlp-1), and the minimal p-254StAR.luc reporter. After 48 hrs a luciferase assay was performed. Fortunately, both versions of Glp-1 behave similarly and are able to repress basal transcriptional activation of *StAR* (Figure 5.h).

*d. Over-expression of Glp-1 decreases steroidogenesis*

After establishing that Glp-1 negatively regulates *StAR* promoter activation by GATA4/6, I sought to evaluate the effect of Glp-1 on steroidogenesis. MLTC1 cells were transiently transfected with an empty vector or the Glp-1 expression vector. 48 hours after transfection, cells were serum starved followed by incubation with hCG. Medium was then collected and a radioimmunoassay to determine progesterone levels was performed. Transient expression of Glp-1 does not alter levels of progesterone with or without hCG stimulation (Figure 5.i.A).

I then investigated if the lack of increased steroidogenesis is possibly due to the limited levels of *Glp-1* expressed by transient transfection. To circumvent this technical limitation, a MLTC1 stably over-expressing Glp-1 cell line (OX-Glp-1) was generated. Glp-1 transiently transfected MLTC1 cells or the OX-Glp-1 cell lines were stimulated with hCG for 4hrs to initiate steroidogenesis. A radioimmunoassay was used to determine the amount of steroidogenesis occurring by detecting progesterone levels. These data indicate that Glp-1 decreased the levels of progesterone production (Figure 5.i.B). When considering the previous data I presented, it is possible this is occurring through transcriptional regulation of *StAR*.

*e. Knockdown of endogenous Glp-1 increases StAR promoter activity but without detectable changes in the Glp-1 mRNA level*

Next, I wanted to see what effect reducing *Glp-1* mRNA levels had on steroid production. MLTC1 cells were transfected with the proximal *StAR* reporter and then the media was immediately replaced with media containing either a control siRNA, siControl, or a siRNA directed to *Glp-1*, siGlp-1. A luciferase assay was performed at either 48h or 72hrs after transfection. These data suggest that knockdown of endogenous *Glp-1* allows for increased activation of the *StAR* promoter when comparing the siControl to siGlp-1 samples (Figure 5.j).

Unfortunately, I am unable to see significant knockdown of Glp-1 expression by western blot analysis (data not shown) or qPCR. Four different siRNAs transfected into MLTC1 cells, separately (data not shown) or in various combinations, failed to reduce *Glp-1* mRNA levels (Figure 5.k.A). To control for siRNA transfection efficiently, siRNAs targeting GATA4 (siGATA4) were used. Western blot analysis was used to determine GATA4 protein levels. SiRNAs targeting *GATA4* lead to an almost complete abolishment of GATA4 as seen by the absence of protein detected in the lane transfected with siGATA4 compared to the lane transfected with the siControl (Figure 5.k.B).

*f. Glp-1 is expressed at higher levels in L$\beta$T2 cells, compared to MLTC1 cells but with undetectable changes in Glp-1 expression with knockdown*

The fact that I am unable to prove knockdown of *Glp-1* prevents me from saying that the effects seen on steroid production are directly due to loss of Glp-1. In an attempt to re-address my questions regarding Glp-1 function, I switched cells lines. Dr. Lisa Halvorson, also interested in Glp-1, determined that *Glp-1* is also expressed in the pituitary, specifically within the anterior pituitary where the gonadotrope cells reside (personal communication with Dr. Halvorson, UTSW). The pituitary is similar to the gonads in that it is a steroidogenic tissue. Dr. Halvorson provided me with the LβT2 cell line, derived from immortalized mouse gonadotrope cells. I first compared *Glp-1* expression in LβT2 cells to expression in MLTC1 cells. QPCR was performed using equal amounts of RNA from each cell line and the results for *Glp-1* were normalized to *Gapdh*. *Glp-1* is expressed in LβT2 cells six times more than that of MLTC1 cells (Figure 5.l.).

To readdress the role Glp-1 may be playing in steroidogenesis, Glp-1 knockdown experiments were performed in LβT2 cells. Cells were transfected with a total of five different siRNAs targeted to Glp-1. Two separate experiments using different siRNAs against *Glp-1* at various concentrations both showed unchanged levels of *Glp-1* mRNA. *Glp-1* mRNA levels were assessed by qPCR (Figure 5.m.A and B). These data are similar to the data seen for *Glp-1* knockdown in MLTC1s in that there is no change in expression between cells treated with siGlp-1 and untreated cell or cells treated with an siRNA control

(Figure 5.m.A and B and Figure 5.k). As a transfection efficiency control, LβT2 cells were transfected with an siRNA targeting *cyclophilin B*. siRNA treatment drastically reduced *cyclophilin* mRNA levels when compared to untreated cells as determined by qPCR (Figure 5.m.C).

*g. Glp-1 is expressed in bovine granulosa cells but over-expression of mouse Glp-1 only moderately decreases basal and mouse GATA4-induced reporter activation*

The results from siRNA knockdown experiments in LβT2 cells and MLTC1 cells prompted me to try a third approach to elucidate the function of Glp-1 in steroidogenesis. I chose to work with granulosa cells isolated from bovine ovaries because the ovaries are large compared to mouse ovaries and granulosa cells are easily isolated from them. First, I wanted to determine if bovine granulosa cells express *Glp-1*. *Glp-1* is expressed in the granulosa cells of the bovine ovary, as a band of the expected size is detected, compared to the RT-PCR reaction without enzyme (Figure 5.n). Finally, I performed a transcriptional assay using cultured granulosa cells in which cells were transiently transfected with β-galactosidase and the SP-A.luc reporter with and without mouse Glp-1 and/or mouse GATA4. A luciferase assay was performed 48 hours after transfection and the activation of the reporter determined. Using primary granulosa cells, Glp-1 is modestly able to repress basal or GATA-dependent activation of the SP-A.luc reporter (Figure 5.o).

**IV. Discussion**

I wanted to address the possibility that Glp-1 performs a similar function on an ovarian GATA-dependent transcriptional target similarly to the known epithelium GATA target, SP-A. The reasoning behind this is that Glp-1 is co-expressed with members of the GATA transcription factor family in granulosa cells. My data suggest that Glp-1 may be shuttled between the two cellular compartments although a reason for this has not been identified. I also showed that Glp-1 can regulate *StAR* expression, a granulosa cell GATA4-dependent target, in the MLTC1 cell line and therefore, Glp-1 can repress the *StAR* promoter in an appropriate biological context. Repression of *StAR* gene expression reduced steroid production. Western blot analysis validated over-expression of Glp-1 thus confirming a role of Glp-1 in steroidogenesis. Unfortunately, reciprical experiments of *Glp-1* mRNA knockdown were not verifiable, although the transcriptional assay was sensitive to treatment of MLTC1 cells with siRNAs targeted to *Glp-1* resulting in increased *StAR* promoter activation.

As discussed in this Chapter and Chapter IV, Glp-1 expression is very low in adult ovaries making it difficult to manipulate by conventional experimental techniques. One has to wonder why expression so low in the whole ovary. Is Glp-1 expression limited to a subpopulation of follicles or granulosa cells? Alternatively, is Glp-1 expressed transiently in all granulosa cells? It is possible

that Glp-1 is regulated by post-translational modifications similarly to GATA4 (Tremblay & Viger 2003). Its expression could be regulated by LH, FSH, or by other growth factor signaling pathways, which could then regulate steroid production. Furthermore, shuttling of Glp-1 due post-translational modifications would control its availability to regulate transcription of its targets, including *StAR*.

StAR activity and thus steroid production is essential for proper signaling from the granulosa cells to the oocyte and into the HPG axis thus regulating ovulation. In the adult, the *in vivo* role for Glp-1 in steroidogenesis is difficult to study due to the primary defect of embryonic germ cell loss before birth (discussed in Chapter III). Creating a conditional *Glp-1* knockout mouse model would circumvent the role Glp-1 plays in early embryogenesis allowing one to study the effects of Glp-1 on *StAR* promoter activity and steroid production.

The mechanism by which Glp-1 functions to repress GATA4-dependent activation of *StAR* is unknown. It was reported that the C-terminal portion of Glp-1 is required for its activity but it is unable to bind the consensus GATA binding site. It remains to be determined where in the C-terminus, the transcriptional activity resides. Does Glp-1 bind at an unidentified DNA binding site? Alternatively, does Glp-1 interact with other proteins to repress transcription. Discriminating between these

possibilities will provide insight into the mechanism by which Glp-1 functions.

## V. Figures

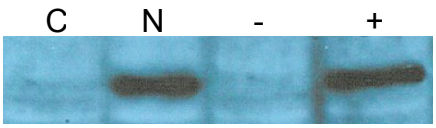



Figure 5.a. Over-expressed Glp-1 localizes to the nucleus. COS7 cells were transiently transfected with pCMVGlp-1. Cytoplasmic and nuclear lysates were collected and Glp-1 was detected by western blot analysis using an antibody against Glp-1. C) cytoplasmic fraction, N) nuclear fraction, -) whole cells lysate from cells transfected with an empty vector, +) whole cell lysate from cells transfected with pCMVGlp-1.

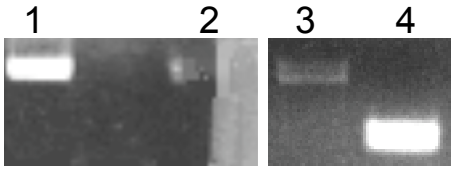
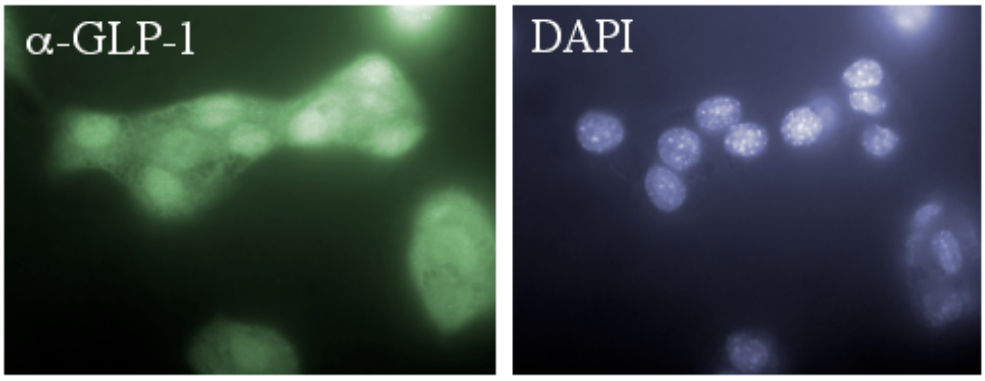



Figure 5.b. The MLTC1 cell line expresses *Glp-1*. *Glp-1* and *Gapdh* were detected by RT-PCR. Lanes 1) pCVMGlp-1 plasmid, 2) MLTC1 RNA, 3) adult mouse ovary RNA and 4) ratGAPDH.

A




B

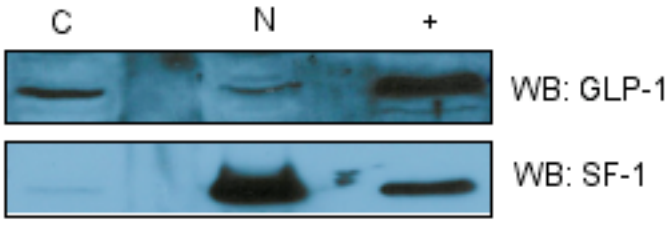



Figure 5.c. Nuclear and cytoplasmic detection of endogenous Glp-1 in MLTC1 cells by a Glp-1 antibody. A) Immunofluorescent staining of Glp-1 in MLTC1 cells shows both nuclear and cytoplasmic staining. DAPI staining denotes the nucleus. B) The top panel shows both nuclear and cytoplasmic localization of Glp-1 in MLTC1 cells compared to detection of over-expressed Glp-1 in MLTC1 whole cell extract. The bottom panel demonstrates SF-1 detection only in the nucleus compared to expression in the whole cell extract. (C) cytoplasmic fraction, (N) nuclear fraction, (+) over-expressed Glp-1 in MLTC1 whole cell extract.

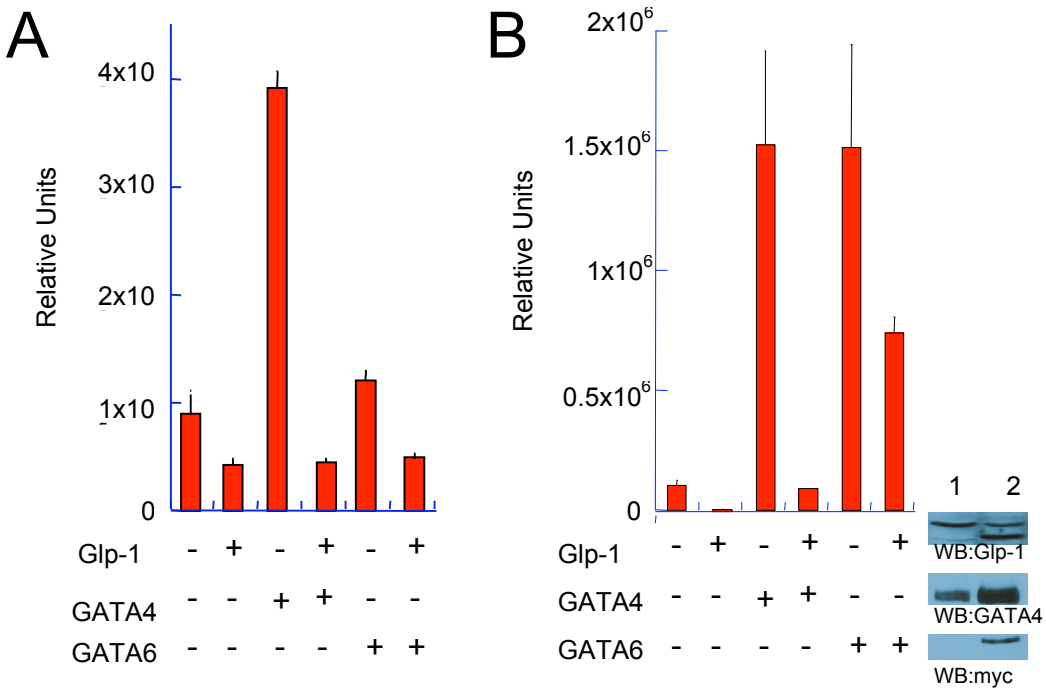



Figure 5.d. Glp-1 represses GATA4/6-dependent transcriptional activation of the SP-A luciferase reporter in MLTC1 cells. The greatest effect is seen with GATA4. A-B) Co-transfection experiment. All wells were transfected with β-galactosidase and the SP-A.luc reporter. A subset of wells were transfected with GATA4 or GATA6 with and without Glp-1 in A) COS7 cells or B) MLTC1 cells. Wells were transfected in triplicate and values were normalized against β-galactosidase activity. B) Western blot shows protein expression. Lane 1) transfection with β-gal and SP-A.luc only. Lane 2) transfection with either Glp-1, GATA4, or GATA6 (myc).

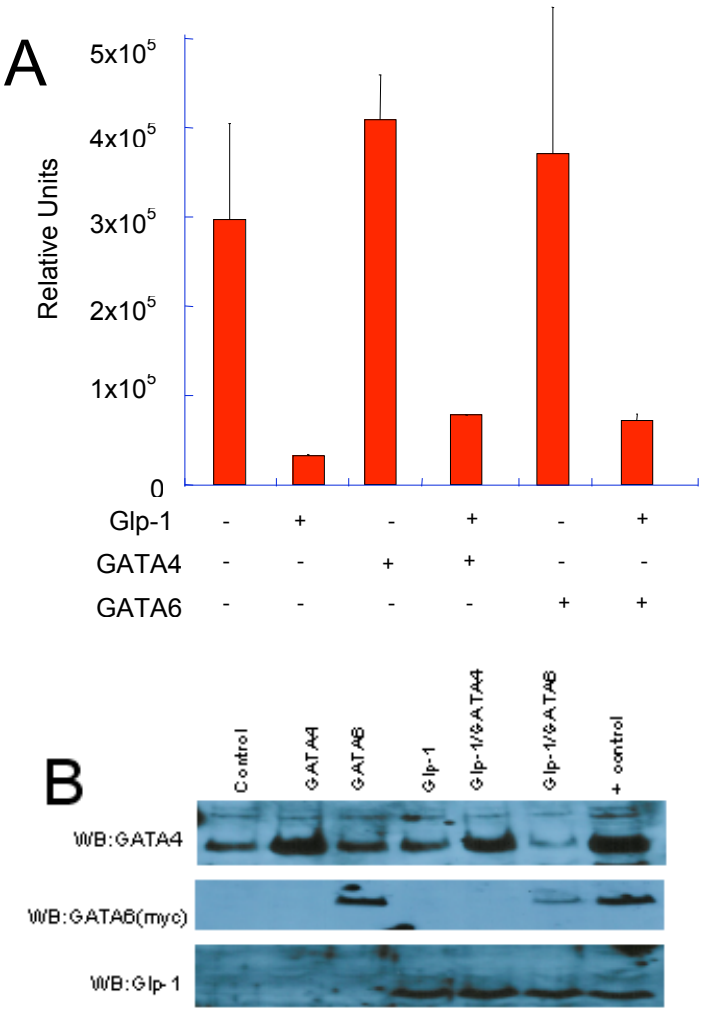



Figure 5.e. Co-transfection experiment showing repression of GATA4/6-dependent activation of *StAR* by Glp-1. A) All wells were transfected with β-galactosidase and the p-966StAR/luc. Wells were transfected in triplicate with GATA4 or GATA6 with and without Glp-1 MLTC1 cells. Values were normalized against β-galactosidase activity. B) Western blot shows protein expression for each representative well for each indicated antibody.

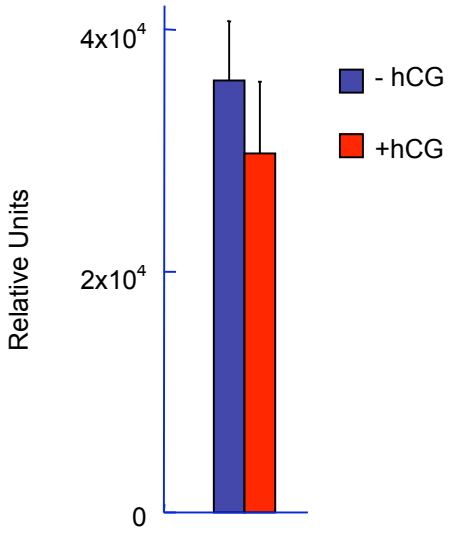



Figure 5.f. No significant effect of hCG on the proximal *StAR* promoter. MLTC1 cells were co-transfected with b-galactosidase and the p-966StAR.luc reporter, serum starved for 4hrs and then treated with 5U/mL hCG for 4hrs. There is no significant difference in the amount of relative units between cells not incubated with hCG and those that were. Values were normalized against β-galactosidase activity.

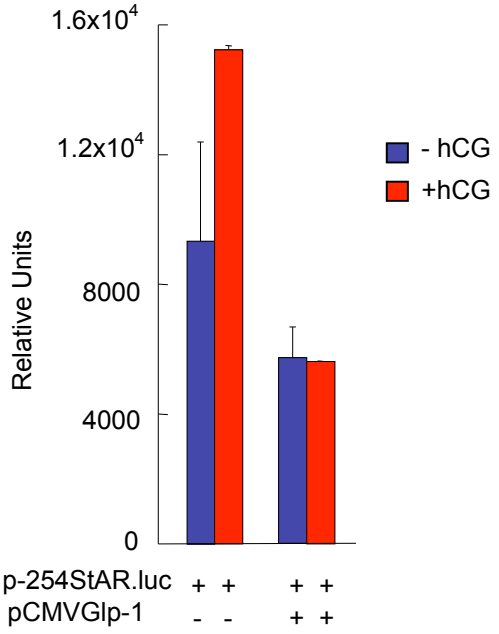



Figure 5.g. The minimal *StAR* promoter is stimulated by hCG. MLTC1 cells were co-transfected with β-galactosidase and the p-966StAR.luc reporter, serum starved for 4hrs and then treated with 5U hCG overnight. There is a trend toward significance in the amount of relative units between cells not incubated with hCG and those that were. Values were normalized against β-galactosidase activity.

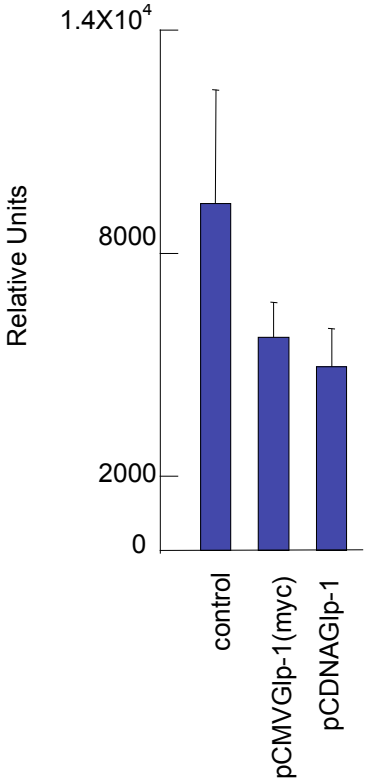



Figure 5.h. Myc-tagged and untagged versions of Glp-1 function similarly. MLTC1 cells were co-transfected with β-galactosidase and the p-254StAR.luc reporter with either pCMVGlp-1 (myc) or pCDNAGlp-1. Both the tagged and untagged versions of Glp-1 are able to repress endogenous activation of the p-254StAR.luc reporter. Values were normalized against β-galactosidase activity.

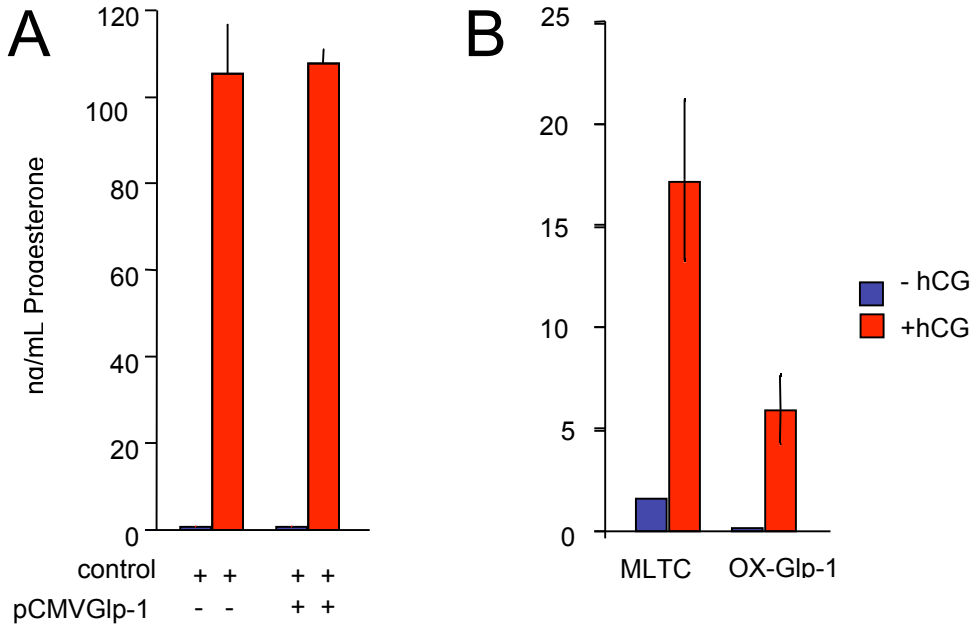



Figure 5.i. High levels of Glp-1 expression are required to repress steroidogenesis. A radioimmunoassay was performed in MLTC1 cells transiently transfected with Glp-1 (A) or stably expressing Glp-1 (B) treated with or without hCG. Glp-1 represses steroid production with and without hCG stimulation. OX-Glp-1 MLTC1 cells were compared to MLTC1 cells. Courtesy of Kristen Evaul and Aritro Sen.

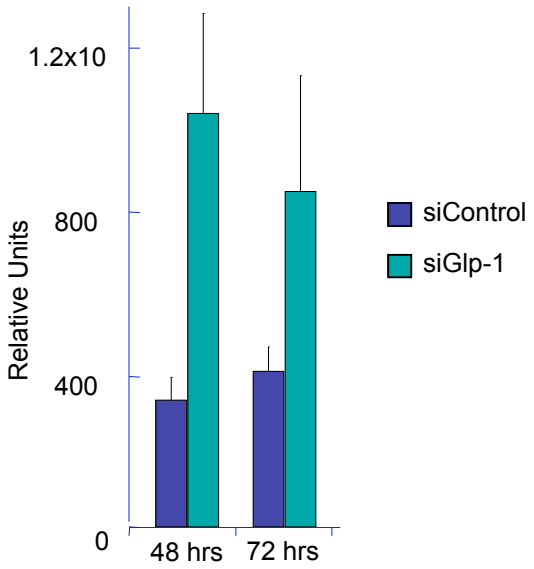



Figure 5.j. Knockdown of *Glp-1* allows for increased *StAR* promoter activity. siRNA experiments in MLTC1 cells transfected with β-galactosidase and the p-966StAR/luc reporter. A luciferase assay was performed 48 or 72hrs after introduction of a siRNA targeted to *Glp-1*. Values were normalized against β-galactosidase activity.

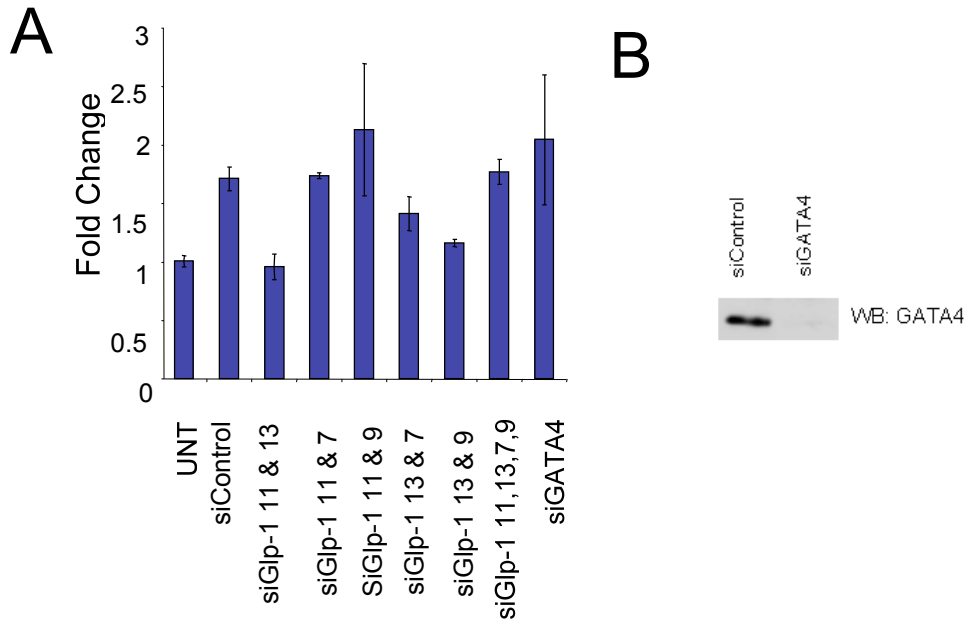



Figure 5.k. siRNAs targeted to multiple parts of *Glp-1* are unable to alter *Glp-1* mRNA levels in MLTC1 cells. A) qPCR assay showing *Glp-1* mRNA levels in MLTC1 cells transfected with a combination of siRNAs targeted to Glp-1 (7, 9, 11 and 13). *Glp-1* mRNA levels are not reproducibly different between untransfected or 2nM siControl transfected and siGlp-1 transfected cells. The Y axis displays the fold change of gene expression compared to *GAPDH*. Samples are plotted relative to the untransfected control. Data shown represents multiple experiments. Each experiment was performed in triplicate replicates. Error bars represent the S.E. of the experiment. UNT) untransfected B) 2nM siRNA targeting *GATA4* (siGATA4) almost completely abolishes expression as seen by the absence of protein detected by western blot analysis compared to the siControl.

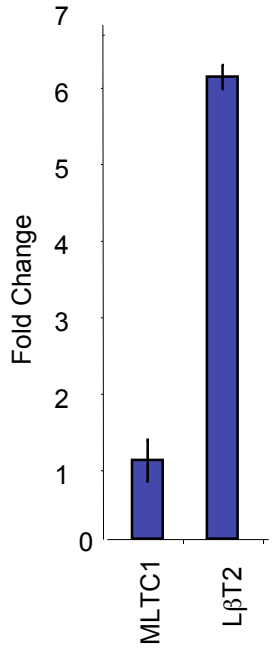
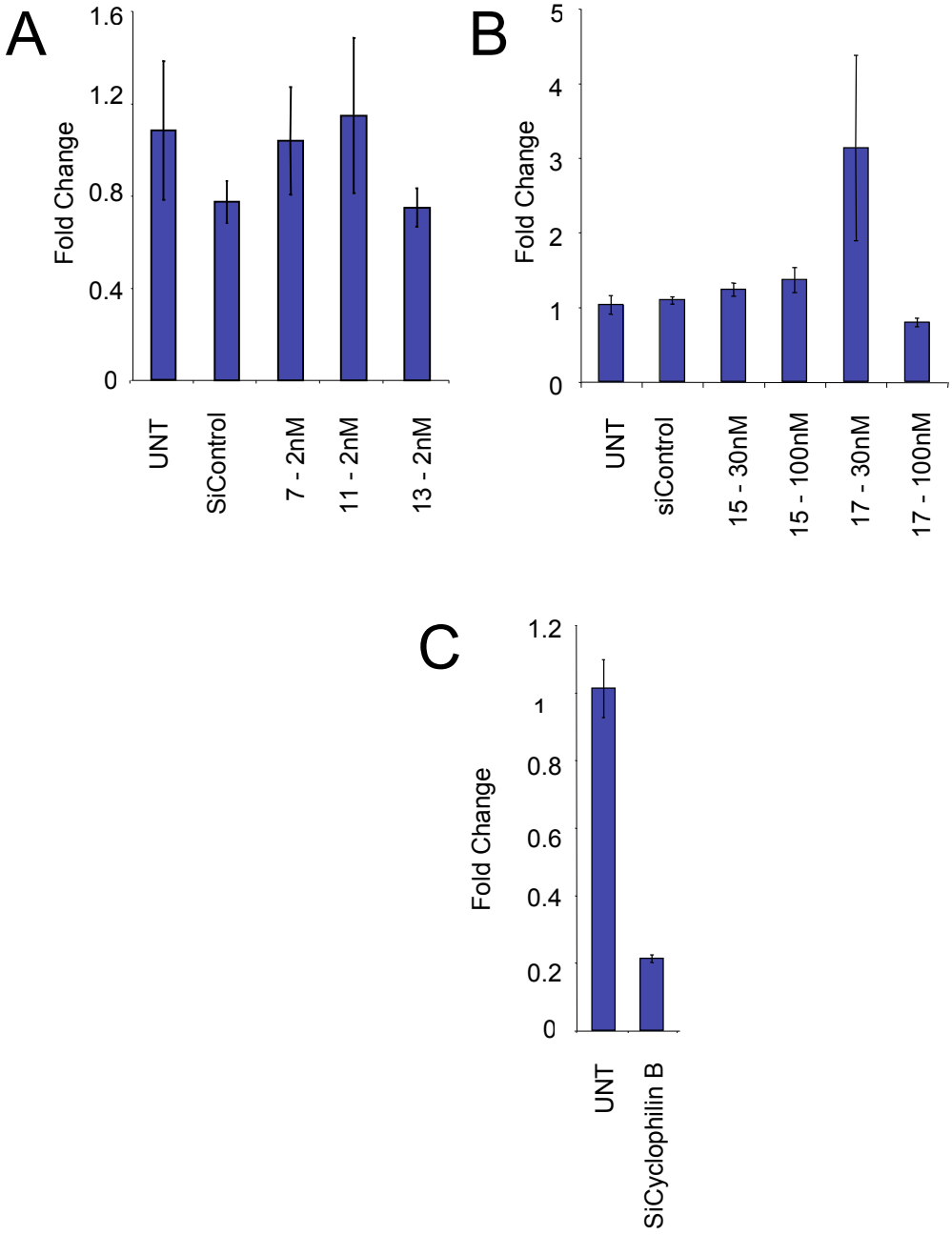



Figure 5.I. *Glp-1* is expressed over six times greater in LβT2 cells than MLTC1 cells. qPCR assay showing *Glp-1* mRNA levels in MLTC1 cells compared to LβT2 cells. The Y axis displays the fold change of gene expression compared to *GAPDH*. Data shown is representative two experiments. Each experiment was performed in triplicate replicates. Error bars represent the S.E. of the experiment.


A
1.6
1.2
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Fold Change
UNT
SiControl
7 - 2nM
11 - 2nM
13 - 2nM
B
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4
3
2
1
0
Fold Change
UNT
siControl
15 - 30nM
15 - 100nM
17 - 30nM
17 - 100nM
C
1.2
1
0.8
0.6
0.4
0.2
0
Fold Change
UNT
SiCyclophilin B

Figure 5.m. siRNAs targeted to multiple parts of *Glp-1* are unable to alter *Glp-1* at the mRNA level in LβT2 cells. A - B) qPCR assay showing *Glp-1* mRNA levels in cells transfected with a several siRNAs targeted to Glp-1 (7, 11, 13, 15, and 17). *Glp-1* mRNA levels are not reproducibly different between untransfected or siControl transfected and siGlp-1 transfected cells. C) qPCR assay showing *cyclophilin B* mRNA levels in cells transfected with a siRNAs targeted to *cyclophilin B*. *Cyclophilin B* mRNA levels are significantly reduced in siCyclophilin B transfected cells compared to untransfected cells. The Y axis displays the fold change of gene expression compared to *GAPDH*. Samples are plotted relative to the untransfected control. Data shown is representative of multiple experiments. Each experiment was performed in triplicate replicates. Error bars represent the S.E. of the experiment. UNT) untransfected


Figure 5.n. *Glp-1* is expressed in bovine granulosa cells. Granulosa cells were isolated from whole bovine ovaries and RNA was isolated. RT-PCR was performed with and without the enzyme reverse transcriptase.

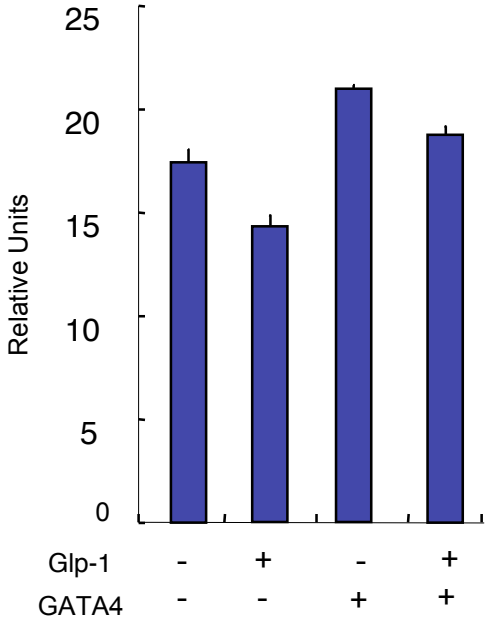


Figure 5.o. Glp-1 repression of GATA4-dependent transcription in cultured bovine granulosa cells. Bovine granulosa cells were isolated from whole ovaries and grown in culture. Granulosa cells were co-transfected with β-galactosidase and the SP-A.luc reporter with and without mGlp-1 and/or mGATA4. Glp-1 is modestly able to repress endogenous and GATA4-dependent activation of the reporter.

# CHAPTER VI: DISCUSSION AND FUTURE EXPERIMENTS

## I. Further Characterization of the *Glp-1*$^{LacZ}$ Embryonic Phenotype

From the data presented here, I conclude that in *Glp-1*$^{LacZ}$ mutant ovaries, germ cells colonize the ovary and initially proliferate normally as seen by similar germ cells numbers and *Mvh* mRNA levels in the null at E13.5 compared to wild type. The process of germ cell loss that begins after E13.5 and is completed by birth is specific to the germ cells as there is no evidence for loss of the somatic cells where Glp-1 is expressed. Ovarian expression of known and novel growth factors at E13.5 remain unchanged in *Glp-1*$^{LacZ}$ mutants compared to wild type controls. Finally, germ cells enter into meiosis appropriately as shown by the approximately normal chromatin patterns seen between experimental and control animals even though significant reductions in gene expression of meiotic entry markers exist. I propose that these reductions occur secondarily to defects already initiated at the time of investigation. These data suggest that in the absence of Glp-1 there are severe deficiencies in oocyte development and/or survival beginning by E13.5. My data suggest that Glp-1 is required upstream of germline cysts encasement by pregranulosa cells and meiotic entry and that *Glp-1*$^{LacZ}$ mutant ovaries are most likely having a problem with pregranulosa cell-oocyte cross-talk. Unfortunately, I am unable to say with which biological process

exactly the defect resides. Therefore additional analyses of the *Glp-1*$^{LacZ}$ phenotype during early oogenesis is warranted.

In order to further my preliminary studies on Glp-1 function in the embryonic ovary, initially, I would focus on the known processes that occur around E12.5. I would perform qPCR of *Glp-1*$^{LacZ}$ null ovaries to wild type ovaries at E12.5 looking for changes in gene expression of markers of pluripotency (*Oct4*, *Nos3*, and *Sox2*), epigenetic reprogramming (*Blimp1/Dmrt1)* and those involved in germ cell survival during migration (*Nanog* and *Crcx4*). Performing microarray analyses at E13.5 on tissues from *Glp-1*$^{LacZ}$ null ovaries and wild type ovaries would be particularly useful. Identification of candidate genes regulated by Glp-1 would provide insight not only into the mechanism by which Glp-1 functions but also into novel pathways that demonstrate the importance for oocyte-granulosa cell cross-talk.

**II. Adult ovarian physiology in the absence of Glp-1**

I would like to emphasize that it is possible Glp-1 has distinct functions in the embryo and the adult and that Glp-1 is not necessarily doing the same thing mechanistically in each developmental stage. I say this because in the adult, steroidogenesis is very important in follicular maturation but it does not seem to be an essential process during early ovarian development.

The function of the ovary is two-fold: 1) steroid production and 2) recruitment and maturation of an oocyte to be released from the ovary for subsequent ovulation. Glp-1, a recently identified novel GATA-like transcription factor, was shown to be specific to the somatic cells of the gonad, specifically the Leydig cells of the testis and the granulosa cells of the ovary (Li *et al.* 2007). Both of these cell types contain steroidogenic properties. The granulosa cells in particular, where Glp-1 is expressed, express all the steroidogenic enzymes to generate estrogen and progesterone. Both of these steroids feed into the HPG axis allowing for the cyclic secretion of LH. Granulosa cells also express Inhibin A and B which function in an endocrine fashion at the level of the brain by inhibiting FSH release and in an autocrine manner inhibiting expression of FSHR (Lu *et al.* 2009). Both LH and FSH release subsequently regulates the frequency of ovulation.

Initially, my research focused on understanding the physiology of an ovary that developed in the absence of Glp-1 using the bacterial *LacZ* knockin mouse line, *Glp-1*$^{LacZ}$. These mice were reported to develop tubular adenomas (Li *et al.* 2007). My studies determined that at six months of age, only 30% of *Glp-1*$^{LacZ}$ null mice formed these large tubular adenomas. It was also reported that the *Glp-1*$^{LacZ}$ ovaries contained both theca and granulosa cells, as circulating estradiol levels are similar between the null and wild type control and both theca cell-specific and granulosa cell-specific markers were expressed,

albeit in a diffuse manner (Li *et al.* 2007). Interestingly, formation of tubular adenomas with the ability to produce steroids is a common occurrence in mouse models where germ cell loss occurred during development (Terada *et al.* 1984, McNeilly *et al.* 2000).

Tubular adenoma formation occurs secondarily to a primary defect resulting in germ cells loss (communication with Diego Castrillon, UTSW). From my studies looking at laminin localization in the tubular adenoma ovaries compared to the small ovaries that have not proliferated, I conclude that the structures remaining in the ovary are germline cyst remnants due to the similar staining pattern between the two types of *Glp-1*$^{LacZ}$ mutant adult ovaries compared to the laminin staining pattern of the germ cell-less embryonic ovary at E18.5 (Figure 3.h and 5.e). To test this, future experiments looking at expression patterns of additional genes should be performed including markers for granulosa cells, LRH-1, Inhibin-$\alpha$, FSHR, and aromatase (cyp19), mural granulosa cells, LH receptor (Lhr), 17$\alpha$-hydroxylase (the protein production of the *cyp17* gene), and Fshr, theca cells, Lhr and 17$\alpha$-hydroxylase, and keratin. If individual cells do not co-express markers for both theca and granulosa cells, but are instead stain appropriately for each cell type, then the data would suggest that the cells of a tubular adenoma are unstructured. On the other hand, if cell-specific markers are coexpressed or diffuse, then I would be more likely to believe that the remaining cells have undergone aberrant gene expression, thus allowing for steroid

production. High levels of LH and FSH and the lack of a menstrual cycle are also features of tubular adenomas (Murphy & Beamer 1973, McNeilly *et al.* 2000). In the *Glp-1*$^{LacZ}$ null mice and other germ cell-less mouse models, this can be explained in part by the lack of inhibition of FSH secretion by Inhibin-$\alpha$ (Gregory & Kaiser 2004). Unfortunately, in humans, there are no reports of tubular adenoma formation and so the clinical significance of this phenomenon is unclear.

**III. A role for Glp-1 in ovarian development and function**

GATA4/6 are known transcription factors in the granulosa cells of the ovary that regulate expression of genes involved in embryonic development and steroidogenesis. Co-localization of Glp-1 and GATA4/6 and the ability of Glp-1 to regulate SP-A expression, a known target of GATA4/6 lead me to address the possibility that Glp-1 performs a similar function on the ovarian GATA-dependent transcriptional target, StAR (Anttonen *et al.* 2003, Li *et al.* 2007). My data show clearly, that Glp-1 is able to repress GATA4/6-dependent transcriptional activation of *StAR* by Glp-1 over-expression and knockdown studies in MLTC1 cells. Future experiments should be performed to address the mechanism by which this occurs.

One difficultly to overcome is detection of endogenous Glp-1 in the ovary. In the ovary, *Glp-1* is expressed at such low levels that changes at the mRNA

level are undetectable by qPCR. Changes in secondary outputs, such as progesterone production and transcriptional activity of target genes, are detectable but cannot be proven to be the direct cause of *Glp-1* expression differences. The antibodies I generated proved of limited utility. Immunostaining of Glp-1 in the ovary was hindered due to low expression and a high signal-to-noise ratio. Wild type ovaries and *Glp-1*$^{LacZ}$ null ovaries were indistinguishable with this method (data not shown).

One possible way to test the ability of ovaries to make steroids in response to hCG, is it to use an ovarian culture system. This could be done by generating an inducible CRE floxed-Glp-1 mouse line where *Glp-1* would be removed in the adult animal beginning at the time of sexual maturity. *Glp-1* mutant ovaries would be harvested, treated with hCG, and then a radioimmunoassay would be performed looking at progesterone production. This would circumvent *in vitro* knockdown experiments by siRNA because you can perform PCR on part of the ovary to determine efficient removal of *Glp-1*. To support these studies, an inducible *Glp-1* transgenic line would also be produced. Thus a CRE/lox system and a inducible *Glp-1* transgenic mouse line would allow for separation of the steroidogenic-independent processes that require Glp-1 function during embryonic development while allowing one to study the role of Glp-1 in the steroidogenic processes of the adult ovary.

Interestingly, *StAR* was recently identified as being transcriptionally activated by both cAMP-dependent and cAMP-independent mechanisms. It was reported that the cAMP-dependent pathway occurs in maturing follicles where GATA4 is expressed while the cAMP-independent pathway is activated only in the corpus luteum where GATA6 is expressed (Yivgi-Ohana *et al.* 2009). It has been shown that the activity of GATA4 is increased upon stimulation of FSH through cAMP to increase *StAR* expression and thus it would be interesting to see what role Glp-1 plays during the switch from cAMP-dependent to cAMP-independent *StAR* activation. Unfortunately, the low levels of Glp-1 expression make studying a role for Glp-1 *in vivo* in the adult ovary very challenging. If a better detection method for Glp-1 were developed, I would start by looking at Glp-1 expression in corpus lutea compared to growing follicles.

One of my first objectives was to confirm the nuclear localization of Glp-1 due to concerns arising from the inability of Glp-1 to bind the GATA DNA consensus sequence. My results using a Glp-1 antibody confirm previously reported nuclear detection of over-expressed Glp-1 (Figure 4.a). Interestingly, endogenous Glp-1 is detected in both the nucleus and cytoplasm of MLTC1 cells (Figure 4.c.A and B). This suggests that Glp-1 may be shuttled between the two cellular compartments; although a reason for this has not been identified.

In light of my current results that show nucleo-cytoplasmic shuttling of Glp-1 occurs, I would perform the following two experiments in MLTC1 cells to confirm these data. Over-expression of the nuclear exportin, Crm1/exportin 1, has been shown to decrease nuclear retention of GATA4 by increasing shuttling of molecules from the nucleus to the cytoplasm in cardiac myocytes (Morisco *et al.* 2001). I would test to see if Crm1 had a similar effect on Glp-1 thus increasing Glp-1 localization to the cytoplasm. Conversely, export of Glp-1 to the cytoplasm mediated by Crm1 would be tested by using the Crm-1-specific inhibitor, leptomycin B (Kudo *et al.* 1999). MLTC1 cells would be harvested after leptomycin B treatment followed by nuclear-cytoplasmic fractionation and western blot analysis. If my results prove to be true and Glp-1 is shuttled between the nucleus and the cytoplasm, these data could then be applied to understanding the mechanism of how Glp-1 regulates GATA-dependent transcriptional targets.

*In vitro* analyses of Glp-1 began by looking at the well characterized *StAR* promoter because it is known to be regulated by GATA4 through a single binding site and the reagents were accessible (Silverman *et al.* 1999). Further investigation of Glp-1 DNA binding activity of the *StAR* promoter and other GATA-dependent transcriptional targets, including *Inhibin-*$\alpha$, and *cyp19* can be addressed by performing luciferase assay, using the promoter of each of the listed genes fused to luciferase. These reporters will be transfected into MLTC1

cells with and without Glp-1 and GATA4. In light of the inability of Glp-1 to bind DNA, future experiments will be required to address if Glp-1 decreases the ability of GATA4 to bind its targets.

To further understand the mechanism by which Glp-1 functions both in the embryo and the adult, future experiments should include co-immunoprecipitation (co-IP) assay looking at GATA4 protein interaction in the granulosa cells of adult ovaries with the following known GATA4 cofactors; C/EBPβ, Smad3, Lrh-1, and SF-1 (Tremblay & Viger 2001, Tremblay *et al.* 2002, Martin *et al.* 2005, Anttonen *et al.* 2006, Robert *et al.* 2006). Possible results include: 1) expression of Glp-1 prevents the interaction between GATA4 and its cofactor on the promoter of the gene-of-interest, 2) Glp-1 physically binding to a complex of proteins including GATA4 could repress transcription of the gene-of-interest 3) Glp-1 may not bind another complex or the DNA itself to regulate gene transcription. Results obtained suggesting an interaction between a complex with GATA4 should be confirmed by electro-mobility shift assay. If Glp-1 is shown to not bind a complex containing GATA4 then promoter bashing of the gene-of-interest can be performed thus narrowing down the location of Glp-1 function.

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