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ROLE OF THE SHP-1 TYROSINE PHOSPHATASE IN THE REGULATION OF OOCYTE GROWTH AND FOLLICLE DEVELOPMENT

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Many thanks to my family and friends who have supported me throughout my studies. Your constant love and support have been wonderful and very encouraging. Mom, thank you for helping me strive for the best and for always believing in me, especially when I wasn't able to. And to my husband Tony, I thank you so much for your love, patience, and encouragement. Your support has been tremendously appreciated and your passion in life is an inspiration to me.

ABSTRACT

The tyrosine kinase Kit is expressed in oocytes and is involved in primordial germ cell (PGC) proliferation and oocyte growth. Our goal was to determine the involvement of the SHP-1 phosphatase in regulating PGC proliferation and oocyte growth by examining SHP-1 deficient *motheaten* mice. Ovaries from wild-type and *motheaten* mice were observed at 10-13 days of age and after transplantation under the kidney capsule of SCID mice. Ovaries were analyzed by histological and western analyses. SHP-1 was expressed in all ovarian cell types throughout follicle development. At 10-13 days, *motheaten* animals had smaller ovaries, increased numbers of PGCs and decreased granulosa cell proliferation compared to controls. After transplantation, both groups had formed large antral follicles in similar proportions. Interestingly, *motheaten* oocytes achieved larger sizes than controls. These results suggest that SHP-1 may interact with Kit to regulate PGC proliferation and oocyte growth, however, the loss of SHP-1 does not impair granulosa cell proliferation and follicle development.

LIST OF FIGURES & TABLES

LIST OF ABBREVIATIONS AND CHEMICAL FORMULAE

ANOVA	analysis of variance
°C	degrees Celcius
cAMP	cyclic adenosine monophosphate
CO_2	carbon dioxide
Csw	corkscrew
dH_2O	distilled water
d.o.	days old
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
FBS	fetal bovine serum
FSH	follicle stimulating hormone
GDF-9	growth differentiation factor-9
GST	glutathione S-transferase
GVBD	germinal vesicle breakdown
h	hour(s)
H&E	hematoxylin and eosin
hCG	human chorionic gonadotropin
HCl	hydrochloric acid
HGF	hepatocyte growth factor
H_2O	tap water
H_2O_2	hydrogen peroxide

IL-3	Interleukin 3
I.U.	international unit
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
kDa	kilodalton
KGF	keratinocyte growth factor
KL	kit ligand
LH	luteinizing hormone
LIF	leukemia inhibiting factor
μg	microgram
μl	microliter
μm	micrometers
min	minutes
NaCl	sodium chloride
NaF	sodium fluoride
NaPP	sodium pyrophosphate
Na_3VO_4	sodium orthovanadate
NK	natural killer
OCC	oocyte-cumulus cell complexes
PBS	phosphate buffered saline
PGC	primordial germ cell
PDGF	platelet-derived growth factor
PI-3K	phosphotidylinositol 3-kinase
PMSF	phenylmethylsulfonyl fluoride

PMSG	pregnant mares' serum gonadotropin
PTP	protein tyrosine phosphatase
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SCID	severe combined immune deficiency
sec	seconds
SH2	Src homology 2
SEM	standard error of the mean
SHP-1	SH2 homology Phosphatase
Sl	steel
STAT	signal transduction activators of transcription
TBS-T	tris-buffered saline supplemented with tween
TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
W	white spotting
WAY	Waymouth media

INTRODUCTION

1. Follicular Development

1.1 Overview

The mammalian ovary is the site of production of female germ cells, known as oocytes. Within the postnatal ovary, oocytes grow and mature in ovarian follicles that consist of oocytes, granulosa cells, and theca cells. Stromal cells fill the gaps between follicles while the surface epithelial cells form a single layer around the ovary. Communication between these different cell types, either by paracrine signaling or by gap junctional communication as well as their stimulation by endocrine factors are crucial in the development of mature and healthy follicles that are required for ovulation.

During follicular development, germ cells and somatic cells undergo dynamic changes to form the structures that we refer to as primordial, primary, preantral, antral, and preovulatory follicles (Fig. 1). These changes include the formation of the theca cell layer and of a fluid-filled antrum, and the differentiation of the granulosa cell compartment into cumulus and mural granulosa cell layers. Mouse oocytes can typically achieve sizes in the order of 70-80 μm within 2-3 weeks of birth. During their growth, oocytes must acquire the competence to complete meiosis and help prepare the follicle cells for ovulation. Similarly, the granulosa cells provide important signals and nutrients to support oocyte growth. Ultimately, the production of mature and healthy oocytes and follicles relies on a complex communication system, which maintains a tight balance of stimulatory and inhibitory functions (Driancourt and Thuel, 1998; McNatty et al., 2000).

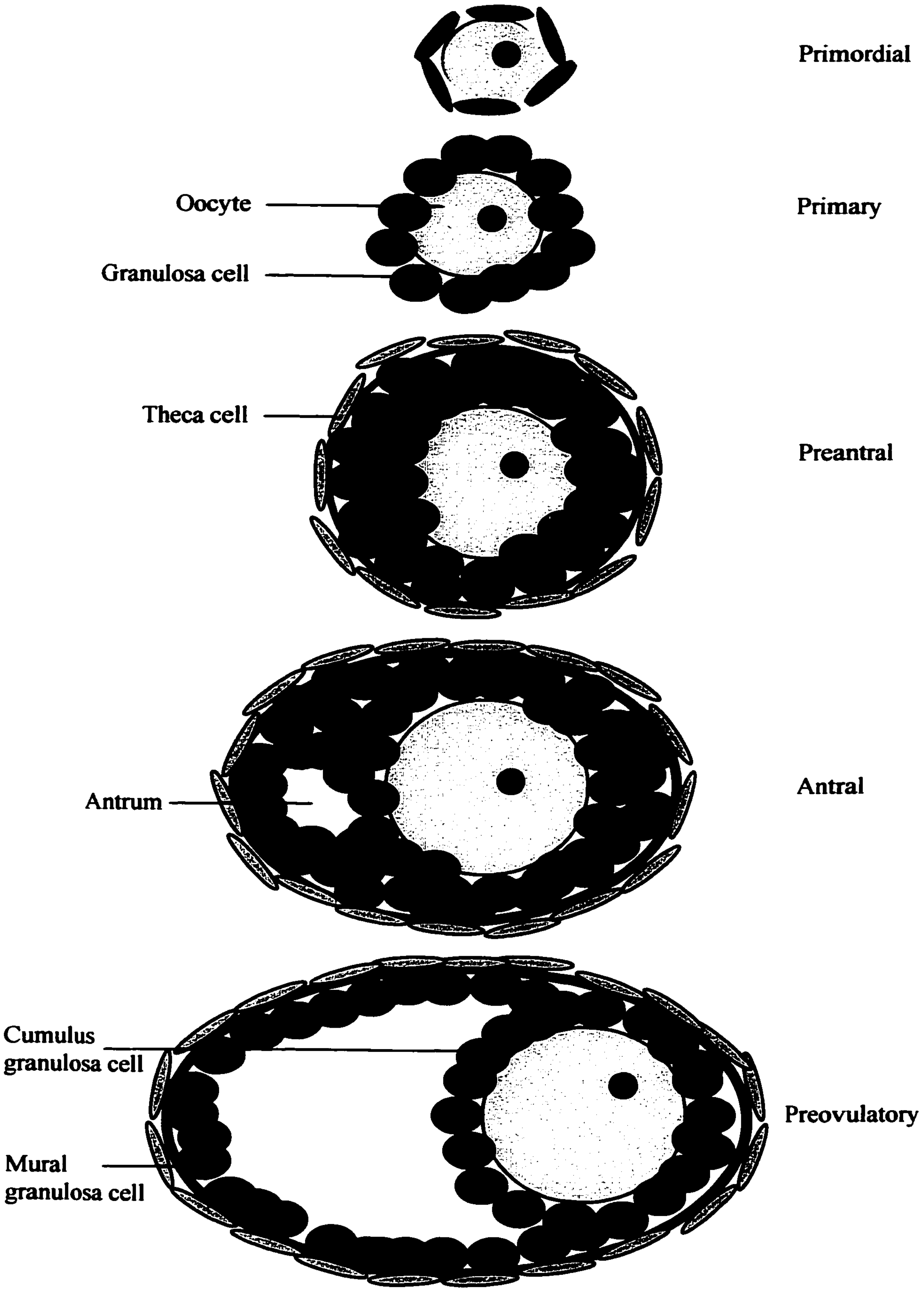
Although numerous factors are involved, primordial germ cell development, oocyte growth and follicle development are thought to be strongly regulated by two proteins known as Kit and Kit ligand (KL). Kit is a tyrosine kinase receptor whose gene, *Kit*, is encoded at the *W* locus while its ligand KL is a product of the *Kitl* gene located at the *Steel* (*Sl*) locus. Mutations in these loci are known to result in developmental problems associated with melanogenesis, hematopoiesis and gametogenesis (Russel, 1979). To date, the regulation of Kit activity in ovaries is not understood and was of primary interest to this research project.

Previously published evidence in other systems of the negative regulation of Kit activity by the SH2 homology phosphatase (SHP-1) provided a rational basis for this research project in which the role of SHP-1 in mouse ovaries was investigated with the goal of understanding the regulation of Kit activity in mouse oocytes.

1.2 Signals during embryogenesis

Primordial germ cell (PGC) development occurs during embryogenesis in a site other than where the gonads ultimately form. PGCs initially appear between embryonic days 7 and 9 in the yolk sac endoderm and in the region of the allantois that is derived from the primitive streak. The PGCs then migrate from this region to the genital ridge (Gomperts et al., 1994). They achieve this initially by passive transfer into the hindgut, and subsequently by ameboid movement to the genital ridges. Interestingly, PGCs are known to express the Kit receptor (Manova and Bachvarova, 1991) and their movement to the site of gonadal origin is thought to be stimulated by chemoattractants like KL

FIGURE 1. Schematic of follicular development in the mammalian ovary. Follicular stages from primordial through preovulatory are illustrated. Growing primary follicles emerge from the pool of primordial follicles present at the time of birth. The most striking feature associated with their entry into the growth phase is the transition of their granulosa cells from squamous to cuboidal. Primary follicles then undergo numerous changes in their growth and development including the formation of a theca cell layer and zona pellucida as well as granulosa cell proliferation to produce preantral follicles. The formation of a fluid-filled antrum defines the beginning of the antral phase which is associated with numerous changes including granulosa cell differentiation into mural and cumulus granulosa cells. Once oocyte growth is complete in antral follicles, the oocytes undergo nuclear maturation in preparation for ovulation. As well, the antrum enlarges and the granulosa cells secrete substances to facilitate extrusion of the oocyte at the time of ovulation


Primordial
Oocyte
Granulosa cell
Primary
Theca cell
Preantral
Antrum
Antral
Cumulus granulosa cell
Mural granulosa cell
Preovulatory

(Keshet et al., 1991; Matsui et al., 1991; Gomperts et al., 1994). These cells are also thought to be protected by survival factors like KL (Dolci et al., 1991; Godin et al., 1991) and leukemia inhibiting factor, and their proliferation has been shown to be stimulated by Kit activity, cyclic adenosine monophosphate (cAMP) and retinoic acid (reviewed by De Felici, 2000) .

PGCs undergo rapid proliferation during their migration to the genital ridge and produce more than 20,000 cells (in mice) which serve as the only pool of germ cells in female adults (Gomperts, 1994). Mice that are homozygous for the *W* or *Sl* mutations and thus lack complete expression of Kit or KL, show the presence of PGCs by embryonic day 8, however, these germ cells fail to proliferate and few, if any, reach the gonad (Bennett, 1956; Mintz and Russell, 1957). In addition, *in vitro* studies have shown that PGCs respond to KL stimulation with increased proliferation and survival (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991; Pesce et al., 1993). This evidence suggests that Kit and KL are essential for PGC migration and/or proliferation during embryogenesis. As well, migration of the PGCs is generally complete in day 13 embryos and changes occur at this time which convert mitotically active PGC into meiotic non-growing oocytes which arrest and remain at prophase I of meiosis until stimulated by the surge of luteinizing hormone (LH) in mature ovaries (Greenwald and Terranova, 1988; Wassarman and Kinloch, 1992) (Fig. 2).

FIGURE 2. Transition from mitotic cells to meiotically arrested cells.

During embryogenesis, once the primordial germ cells colonize the gonadal ridge, changes occur that convert the mitotically active germ cells to meiotically arrested oocytes. Oocytes become arrested at prophase I of meiosis and remain there until the LH surge which triggers the resumption of meiosis. Following the LH surge, oocytes undergo germinal vesicle breakdown followed by the extrusion of the first polar body at the time of ovulation. Upon fertilization, the oocyte undergoes the second meiotic division thus extruding the second polar body and retaining only 23 maternal chromosomes. Figure modified from Austin and Short (1982).

Developmental events

Multiplication by mitosis

Migration to genital ridge

Final interphase
DNA synthesis
Meiotic prophase begins

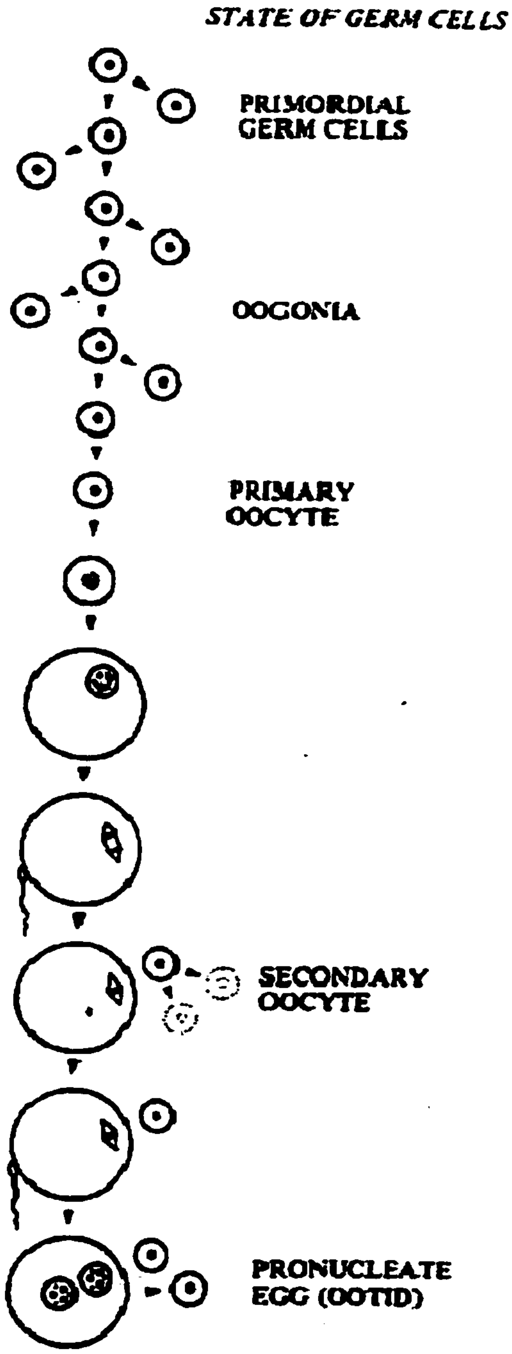
Birth

Growth of oocyte and follicle
Puberty

Follicular maturation

First polar body emitted

Ovulation



1.3 Oocyte and follicle growth and differentiation

In post-natal mouse ovaries, numerous primordial follicles populate predominantly the cortical region of the ovary. These follicles are generally in the range of 12-15 μm in diameter and are composed of a non-growing oocyte surrounded by a single layer of flattened pre-granulosa cells. Primordial follicles can remain dormant for varying lengths of time depending on the species. However, continual cohorts of these early follicles initiate growth until none are left in the pool and the animals undergo reproductive senescence. Although the trigger required for entry into the growth phase is poorly understood, the initiation of follicle and oocyte growth is marked by the transition of the granulosa cells from squamous to cuboidal (Lintern-Moore and Moore, 1979) . At this point, follicle growth is associated with numerous changes: 1) the rapid formation of a theca cell layer surrounding the granulosa cells, 2) the formation of the zona pellucida by the oocyte, as well as 3) granulosa cell proliferation, and 4) oocyte growth. During this period of oocyte growth, the germ cells are arrested at the dictyate stage of the first meiotic prophase and undergo numerous transcriptional and translational events as is evident by the active synthesis of ribonucleic acids (RNA) and proteins (Bachvarova et al., 1985).

In contrast to early follicular development which is mainly regulated by paracrine signaling, late preantral follicle development, antral follicle formation and the development of preovulatory follicles are highly dependent on gonadotropic stimulation. Follicle stimulating hormone (FSH) is an endocrine factor secreted from the anterior pituitary into the bloodstream in response to gonadotropin releasing hormone (GnRH) stimulation from the hypothalamus. In the ovary, FSH acts specifically on follicular granulosa cells where its receptors are located. Although FSH has stimulatory effects on granulosa cell proliferation in early preantral follicles it is not required for this function at the preantral stage of development nor is it required for the formation of the theca interna (Hirshfield, 1985). In fact, mice that have impaired gonadotropin levels show normal follicle development in small preantral follicles but these follicles rarely develop beyond the mid-preantral stage (follicles containing approximately 40 granulosa cells) (Wang and Greenwald, 1993).

The gonadotropin-dependent stage of follicle development involves the proliferation of granulosa cells in late preantral development, the formation of the antral cavity and subsequently the antral stages of follicle development including oocyte maturation, granulosa cell differentiation, and ovulation. The follicular changes that occur include the differentiation of the granulosa cells into the cumulus granulosa cells that surround the oocyte and the mural granulosa cells that line the follicle wall. The cumulus cells communicate with both the oocyte and other granulosa cells through gap junctions that provide important metabolic precursors for the growing and fully grown oocytes (Anderson and Albertini, 1976). Although oocyte growth, as measured by increases in diameter, is almost complete when the follicles reach the antral stage, oocytes gain considerable increases in volume during the final stages of development in preparation for ovulation (Gosden et al., 1997).

In mammalian ovaries, the preovulatory surge of gonadotropins is essential for ovulation. Estrogen is believed to be the trigger for the preovulatory surge since anti-estrogen antibodies inhibit this surge (Shirley et al., 1968) and the treatment of ovariectomized animals with estrogen reinstates a partial surge (Goldman et al., 1971). Also, progesterone is thought to play a synergistic effect in the magnitude of the gonadotropin surge (Chang and Jaffe, 1978; reviewed in Mahesh and Brann, 1998). Ovulation which occurs in response to the gonadotropin surge is preceded by important changes that prepare the oocyte for ovulation and fertilization. For instance, FSH and LH are known to be important for oocyte maturation. Studies have shown that the addition of FSH to culture media for the in vitro maturation of cumulus enclosed oocytes results in increased rates of germinal vesicle breakdown and extrusion of the first polar body (Eppig and Schroeder, 1989; Prins. 1987).

In response to the LH surge, granulosa cells secrete factors, like hyaluronic acid, that cause dispersion of the cumulus granulosa cells away from the oocyte in a process known as cumulus cell expansion (Eppig, 1979b; Salustri et al., 1989). In turn, removal of the oocyte from the cumulus cells *in vitro* results in an impaired ability of the granulosa cells to produce hyaluronic acid. Also, cumulus expansion not only requires the presence of an oocyte-derived cumulus enabling factor, but also needs gonadotropic stimulation (Buccione et al., 1990b; Salustri et al., 1990). Thus, follicle development from the late preantral stage to ovulation requires both paracrine and endocrine signals for the ovulation of mature oocytes.

1.4 Oocyte Maturation

The ability of the oocyte to resume meiosis occurs when the germ cells have reached a certain size (approximately 60 μm) and in response to the LH surge *in vivo*. Small oocytes are not competent to resume meiosis in contrast to the first cohort of large oocytes which achieve this competence approximately 15 days after birth (in mice). Although the competent oocytes have all the required factors for the resumption of meiosis, other factors produced by the granulosa cells maintain the oocytes in a state of meiotic arrest (Chesnel at al., 1994). Large oocytes that are removed from their follicular environment resume meiosis when released in an appropriate culture medium (Chesnel and Eppig, 1995). Indeed, it is known that the maintenance of meiotic arrest in oocytes is largely governed by the presence of meiosis-inhibiting substances like cyclic adenosine monophosphate (cAMP) (Cho et al., 1974; Dekel and Beers, 1978), FSH (Eppig et al, 1983; Vanderhyden and Armstrong, 1990), purines (Eppig and Downs, 1988), and müllerian inhibiting substance (Takahashi et al., 1986).

Meiotic maturation is characterized initially by the breakdown of the germinal vesicle and chromosome condensation. Oocytes progress from prophase I to metaphase II of meiosis with the extrusion of the first polar body (Sorensen and Wassarman, 1976). Upon fertilization, emission of the second polar body results in a diploid zygote that can undergo cleavage and further differentiation in preparation for implantation. Numerous factors have been shown to contribute to oocyte maturation. These include epidermal growth factor (EGF) (Merriman et al., 1998), transforming growth factor-β (TGF-β)

(Feng et al., 1988), insulin-like growth factor (IGF) (Feng et al., 1988), GnRH (Hillensjo et al., 1980), growth hormone (GH) (Apa et al., 1994) and KL (Ismail et al., 1996).

Ismail et al. (1996) demonstrated that animals treated with PMSG and hCG had a 2.3-fold increase in KL expression. They also showed that this increase in KL mRNA was not due to secondary effects mediated by estradiol production by the granulosa cells. Furthermore, when oocytes from PMSG-primed animals were cultured in the presence of KL the rates of germinal vesicle breakdown (GVBD) and polar body extrusion were decreased. Thus, the addition of KL to fully grown oocytes in culture resulted in a delay in the progression of meiotic maturation. Further studies by this same group (Ismail et al., 1997) demontrated that the inhibitory effects of KL on oocyte maturation could be inhibited by the addition of anti-Kit antibodies. They also showed that the resumption of meiosis in oocytes that were stimulated with human chorionic gonadotropin (hCG) was associated with a shift in the granulosa cell expression of KL from membrane-bound isoforms to the less active soluble isoforms, as well as with the loss of KL expression in the cumulus granulosa cells surrounding the oocyte. Together, these results suggest that Kit signaling is inhibitory to meiotic progression and that the resumption of meiosis due to gonadotropic stimulation may inhibit Kit activity due to decreased stimulation by its ligand (Ismail et al., 1997).

2. Communication between Somatic cells and Germ cells

2.1 Gap junctional communication

Gap junctions are intercellular membrane channels that allow for the direct transmission of inorganic ions, second messengers, and small metabolites from one cell to another adjacent cell. They are composed of two opposing hemi-channels called connexons, which in turn are comprised of six protein subunits known as connexins (Unger et al., 1999). There are 15 types of connexins, that differ in size and function, and can be paired together by type or in combinations creating a great deal of variety and specificity. In the follicle, gap junctions play an important role in facilitating the flow of low molecular weight molecules (<1000 kDa) to both granulosa cells and oocytes and are crucial to the development of a mature follicle (Eppig, 1977, 1979a; Buccione et al., 1990a; Eppig, 1991). They are thought to appear with the formation of primordial follicles at the time of birth and remain throughout folliculogenesis. Denuded oocytes cultured *in vitro* on monolayers of granulosa cells show minimal growth, if any. However, the culture of oocyte-granulosa cell complexes allows for oocyte growth, depending on the level of coupling that is maintained in culture (Eppig, 1977; Eppig, 1979). Furthermore, the rate of oocyte growth is directly correlated to the degree of gap junctional coupling between the oocyte and granulosa cells (Brower and Schultz, 1982). In addition to allowing the passage of nutrients to the oocytes and granulosa cells, gap junctions are important to mediate metabolic cooperativity between these two cell types, a function that is essential for oocyte growth and development.

In mice, the connexons that couple the oocytes with their surrounding granulosa cells are composed of connexin 37 (Simon et al., 1997). Female mice lacking connexin 37 are infertile. They are unable to ovulate even in response to pregnant mares' serum gonadotropin (PMSG) stimulation, lack the formation of large preovulatory follicles and only occasionally show the presence of antral follicles. As well, oocyte development is arrested before meiotic competence is achieved. Connexin 37 null mice have extensions from the granulosa cells across the zona pelucida and are capable of making adherens junctions with the oocyte as is the case in normal mice. However, the mutant animals do not express connexin 37 protein and gap junctions are not visible by electron microscopy (Simon et al., 1997). These results suggest that connexin 37 is required for oocyte maturation and follicle development and thus is essential for fertility.

Similarly, the connexons that couple the granulosa cells to each other are composed mainly of connexin 43 (Granot and Dekel, 1997). Studies of ovaries from connexin 43 null mice are difficult due to the neo-natal lethality of these animals as well as their severe depletion in germ cells thought to arise from impaired PGC migration (Reaume et al., 1995; Juneja et al., 1999). *In vitro* studies of post-natal folliculogenesis using follicles from connexin 43 null mice showed impaired development of the granulosa cell layers (Juneja et al., 1999). Further studies by Ackert et al. (2001) showed that neo-natal connexin 43 null ovaries transplanted under the kidney capsule of severe combined immuno-deficient (SCID) mice had severely impaired granulosa cell proliferation and compromised oocyte development (Ackert et al., 2001). Thus connexin

43 is required for PGC migration, granulosa cell proliferation, oocyte development and ultimately is essential for fertility.

Gap junctional communication is important to keep the oocyte in a state of meiotic arrest. The granulosa cells are known to transfer inhibitory signals like cAMP and purines via gap junctions to the oocyte in order to prevent nuclear maturation (Cho et al., 1974; Dekel and Beers, 1978; Eppig et al., 1985). Although GVBD normally occurs in the antral stage of development, oocytes in preantral follicles are capable of nuclear maturation if removed from their follicular context. Thus, gap junctional communication is not required for the acquisition of meiotic competence (Chesnel et al., 1994; Goodenough et al., 1999).

2.2 Paracrine signaling

Evidence has shown that the oocyte is an important factor contributing to normal granulosa cell proliferation and differentiation. For instance, oocytes release factors that contribute to cumulus expansion (Buccione et al., 1990; Salustri et al., 1990; Vanderhyden et al., 1990), the induction of granulosa cell proliferation (Vanderhyden et al., 1992), follicular structural integrity (Vanderhyden et al., 1990, 1992), and steroidogenesis by maintaining estradiol production and inhibiting progesterone production (Vanderhyden et al., 1993; Vanderhyden and Tonary, 1995). Moreover, oocytes can stimulate the secretion of hyaluronic acid which is known to enhance ovulation (Buccione et al., 1990b; Salustri et al., 1990).

During primordial follicle formation, oocytes are essential for follicle assembly, in contrast to gametogenesis in males, where the seminiferous tubules are capable of forming in the absence of spermatocytes. Exciting evidence on a novel protein known as growth differentiation factor-9 (GDF-9) has supported an important role for the oocyte in regulating early follicle development (Dong et al., 1996). The *Gdf-9* gene is a member of the TGF-β superfamily and is thought to be expressed mouse, cow, sheep, rat and human oocytes (McGrath et al., 1995; Laitinen et al., 1998; Aaltonen et al., 1999; Bodensteiner et al., 1999). In mice, *gdf-9* is expressed in oocytes of follicles beginning at the primary stage of development, although some species variability has been observed (Fitzpatrick et al., 1998; Bodensteiner et al., 1999; Hayashi et al., 1999). Interestingly, *gdf-9* null mice have follicles arrested at the primary stage with only a single layer of granulosa cells surrounding each oocyte, thus rendering the animals infertile. Initially, *gdf-9* null mice were found to have elevated KL levels suggesting that GDF-9 may regulate the expression of KL, another paracrine signal (detailed below) (Elvin et al., 1999). This finding was later confirmed by *in vitro* studies in which the addition of recombinant GDF-9 suppressed KL expression by the granulosa cells (Joyce et al., 2000). Interestingly, GDF-9 is known to stimulate the expansion of oocytectomized mouse complexes *in vitro* as well as stimulate the expression of hyaluronan synthase 2, an enzyme required for the synthesis of hyaluronic acid (Elvin et al., 1999).

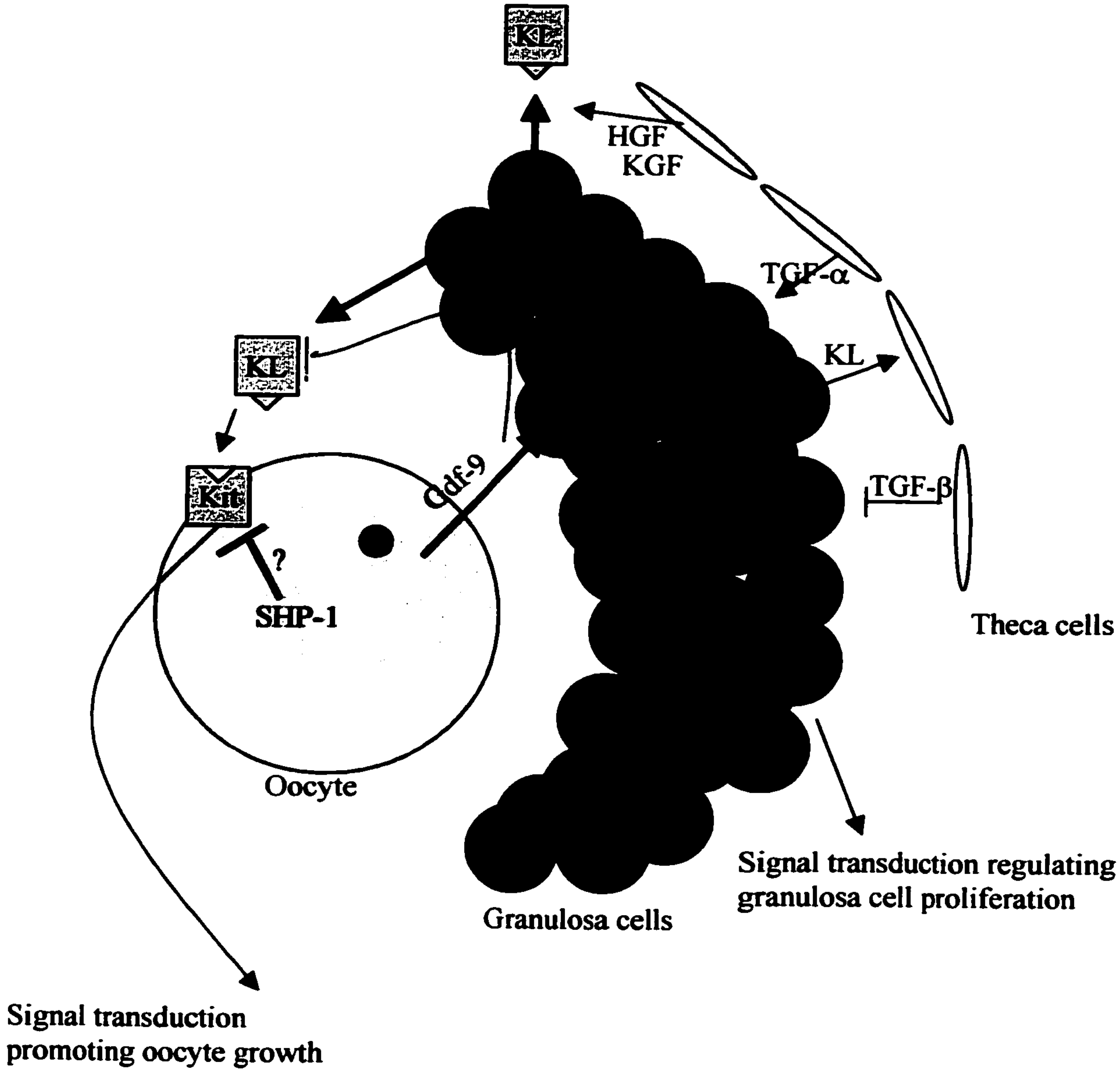
Studies have shown that in large antral follicles, fully grown oocytes can regulate the expression of gonadotropin-induced LH receptor. *In vitro* studies in granulosa cells have shown that fully grown oocytes can prevent FSH-induced increases in LH receptor mRNA, suggesting that oocytes can regulate LH receptor expression (Eppig, 1997). As well, oocytes also play an important role in ovulation. Fully grown oocytes are known to secrete a factor known as cumulus expansion-enabling factor (CEEF) which allows the granulosa cells to respond to FSH by producing hyaluronic acid. Mid-size oocytes from preantral follicles as well as ovulated eggs produce little or no CEEF suggesting that the active secretion of this factor is stage-specific (Buccione et al., 1990b; Salustri et al., 1990; Vanderhyden et al., 1990). Although no direct relationship has been proven, it is believed that GDF-9 may be the CEEF.

Interestingly, oocytes have recently been shown to regulate the expression of KL in mouse granulosa cells in a manner consistent with the growth and developmental patterns of the oocyte and dependent upon FSH stimulation (Joyce et al., 1999). Thus, when oocytes are growing, KL is highly expressed and predominantly in the form of KL-2, which is the most active isoform. However, they found that co-culture of fully grown oocytes with preantral granulosa cells showed a shift in KL isoforms from membrane-bound KL-2 to the less active soluble form KL-1. Thus factors secreted from the oocytes are capable of differentially regulating KL-1 and KL-2 mRNA expression (Joyce et al., 1999; Joyce et al., 2000). Therefore, there appears to be a feedback system whereby the following occurs: granulosa cells produce KL which in turn binds to the kit receptor and activates it. Then, Kit activity stimulates oocyte growth, which subsequently results in decreased KL expression and a change in isoform expression to the less active KL-1. Further biochemical experiments are necessary to better understand the exact signaling pathways that allow for this loop to occur.

Numerous other factors are also involved in the paracrine signaling between oocytes and granulosa cells. For instance, EGF and fibroblast growth factor (FGF) are expressed in the oocyte while their receptors are present on granulosa cells. Similarly, the oocyte bears receptors for EGF/TGF-α (Chia et al., 1995), IGF-1 and IGF-2 (Teissier et al., 1994), and platelet-derived growth factor (PDGF) (Watson et al., 1992) suggesting functional signaling pathways for these proteins within the oocyte itself as well as stimulatory or inhibitory effects on granulosa cell proliferation and/or differentiation (McNatty et al., 2000) (Fig. 3).

FIGURE 3. Model of paracrine signaling in the ovarian follicle.

Follicular development involves a coordinate interplay of paracrine signals between the oocyte, granulosa cells and theca cells. Although numerous other factors are involved, this diagram illustrates some of the important factors that contribute to granulosa cell proliferation, oocyte growth and follicle development. Oocyte growth is known to be regulated by Kit activity in oocytes as a result of the interaction between granulosa cell-derived KL and Kit-presenting oocytes. These germ cells are also known to regulate granulosa cell functions via growth differentiation factor -9 (GDF-9) which is required for both granulosa cell proliferation and the inhibition of KL expression. Theca cell-derived factors such as hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), transforming growth factor-alpha (TGF-α) and transforming growth factor-beta (TGF-β) can also influence granulosa cell proliferation. As well, granulosa cell-derived epidermal growth factor (EGF) is known to elicit a proliferative response in those cells. Our hypothesis is that SHP-1 can inhibit Kit signaling in oocytes and perhaps other signaling pathways in the granulosa cells and thus may be capable of altering oocyte growth and/or granulosa cell proliferation.


KL
HGF
KGF
TGF-α
KL
TGF-β
Theca cells
KL
Kit
?
SHP-1
Gdf-9
Oocyte
Granulosa cells
Signal transduction regulating
granulosa cell proliferation
Signal transduction
promoting oocyte growth

3. Regulation of Oocyte Growth

3.1 Background

Numerous attempts to examine oocyte growth *in vitro* have been complicated by the germ cells requirement for granulosa cell coupling (Buccione et al., 1987; Cecconi et al., 1996) (Buccione *et al.*, 1987; Cecconi *et al.*, 1996) and by the fact that oocytes are not mitogenic, thus do not proliferate in culture. However, using various techniques including *in vitro* culture systems and genetic manipulations, several studies have been able to address the following question: What signaling pathways are required for oocyte growth? To date, Kit and KL are the only known receptor-ligand system to be required for oocyte growth.

In 1994, Packer et al. exposed dissociated fetal ovaries to anti-Kit antibodies and demonstrated that blocking Kit activity resulted in reduced oocyte growth and provided evidence to suggest that KL can enhance the rate of oocyte growth *in vitro*. A few years later, Yoshida et al. (1997) investigated the role of Kit signaling in oocyte growth and follicle development by blocking Kit-KL interactions using anti-Kit antibodies that they administered to developing mice at various times after birth. Their study revealed several impaired functions: primordial follicle development, follicle growth, antrum formation, and preovulatory follicle maturation. They found that Kit signaling was especially important for oocyte growth during the first five days after birth prior to FSH receptor expression. And more recently, Kissell et al. (2000) demonstrated impaired follicle development at the cuboidal stages leading to infertility by mutating the binding site for the p85 subunit of phosphatidylinositol 3-kinase (PI-3K) using a knock-in strategy. Their study showed that impaired Kit signaling through the PI-3K pathway resulted in a reduction in the number of follicles entering the growth phase, smaller ovaries, as well as acentric and degenerating oocytes.

Others groups examining Kit activity failed to support a role for this pathway in oocyte growth. However, they were able to determine roles for Kit activity in follicle development and germ cell survival. In 1996, Cecconi et al. examined oocyte growth *in vitro* by culturing oocytes on granulosa cell monolayers. Their study showed that the addition of an antibody against mouse KL did not impair the growth of oocytes from 12 day old mice. Later, Reynaud et al. (2000) performed *in vitro* studies examining oocyte growth in culture systems with the addition of KL and anti-Kit antibodies. They reported that the addition of KL, regardless of concentration and culture conditions did not alter oocyte growth. However, they found that blocking Kit-KL interactions with anti-Kit antibodies resulted in decreased oocyte survival, impaired antrum formation and reduced aromatase activity suggesting that Kit activity is required for these functions *in vitro*.

3.2 Structure of the Kit tyrosine kinase

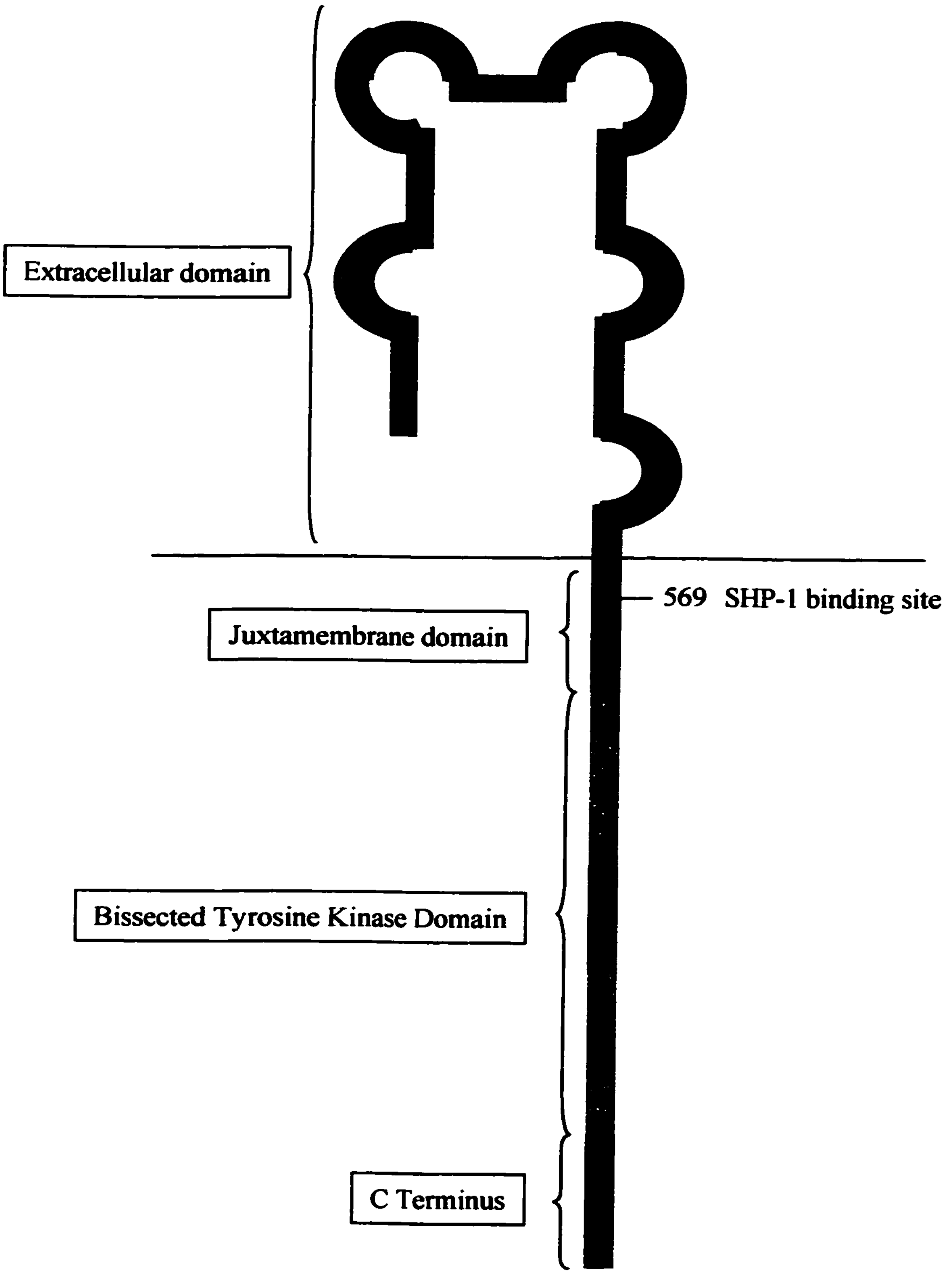
The protooncogene *c-Kit* encodes a receptor tyrosine kinase (RTK) and is homologous to the viral oncogene *v-kit* (Besmer et al., 1986). The *Kit* gene, located at the *W* locus on chromosome 5 in mice, encodes a transmembrane protein containing five immunoglobulin-like (Ig-like) repeats in the extracellular domain and bisected tyrosine kinase sequences in the cytoplasmic domain (Yarden et al., 1987; Qiu et al., 1988) (Fig. 4). The Kit receptor has a calculated molecular mass of 110 kDa, however, it is usually seen as 120 to 160 kDa isoforms due to varying degrees of receptor glycosylation on asparagine residues within the carboxy terminal half of the extracellular domain (Yarden et al., 1987; Qiu et al., 1988; Majumder et al., 1988). Due to the Ig-like motifs in the extracellular domain, Kit is recognized as a member of the Ig gene superfamily. The cytoplasmic tail of Kit composed mainly of tyrosine kinase sequences is the site of interaction with downstream effector molecules. Upon ligand binding, the receptor dimerizes and autophosphorylates at specific tyrosine residues which in turn provide docking sequences for Src homology 2 (SH2)-containing proteins. Numerous substrates have been identified for Kit including phospholipase C γ -subunit (PLCγ), PI-3K, GTPase activating protein, guanine dissociation stimulator, the guanine nucleotide exchange factor Vav, and SHP-1 (discussed below) (Qui et al., 1988; reviewed in Lev et al., 1994).

3.3 Expression of the Kit tyrosine kinase

The Kit receptor is thought to be important in the migration and proliferation of undifferentiated cells (Russell, 1979). With respect to ovarian germ cell and follicle development, Kit mRNA is expressed in PGCs from embryonic day 7½ to 13½ and decreases to undetectable levels as the germ cells enter prophase of meiosis (Manova and Bachvarova, 1991). Kit expression reappears in oocytes at the diplotene stage and remains present throughout all stages of oocyte development and into early embryogenesis (Manova et al., 1990; Horie et al., 1991). Kit is also expressed in the theca cells and interstitial/stromal cells throughout follicle development although its function in these cells is not understood.

FIGURE 4. The Kit Tyrosine Kinase.

Kit is a receptor tyrosine kinase that primarily functions as a growth factor receptor and is composed of an extracellular, a transmembrane and a cytoplasmic domain. The extracellular domain contains five immunoglobulin (Ig)-like repeats thus Kit is recognized as a member of the Ig gene superfamily. The molecular weight of this protein varies according to the degree of glycosylation on asparagine residues in this extracellular domain. Upon receptor activation, tyrosine residues become phosphorylated in the cytoplasmic domain and provide binding sites for SH2-containing substrates like PI-3K, and SHP-1. Figure modified from Lev et al. (1994).

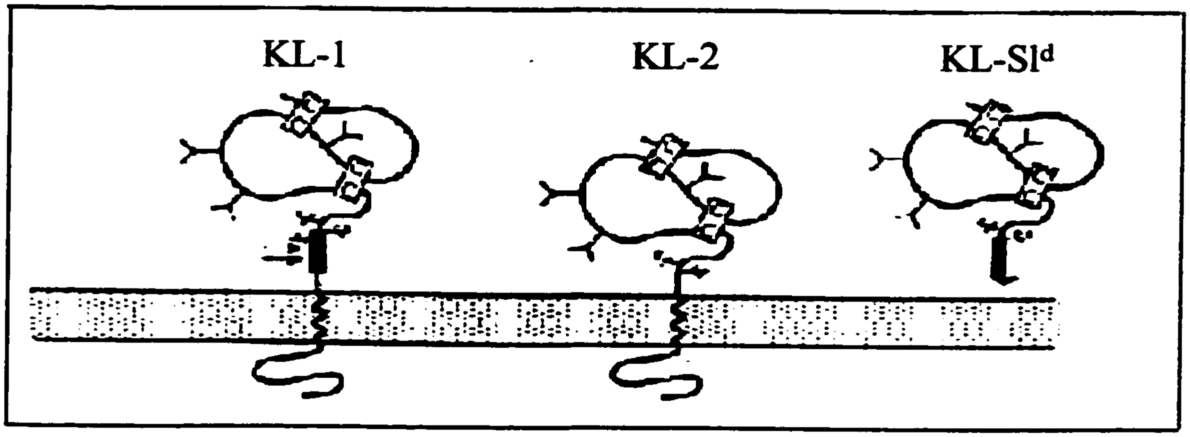

Extracellular domain
569 SHP-1 binding site
Juxtamembrane domain
Bissected Tyrosine Kinase Domain
C Terminus

3.4 Structure of Kit Ligand

The *Kitl* gene located in the *Sl* locus on chromosome 10 in mice encodes the ligand for the Kit receptor, KL (Copeland et al., 1990; Huang et al., 1990). KL is a protein that is present in either soluble (KL-1) or membrane-associated (KL-2) forms due to the alternative splicing of mRNA (Huang et al., 1992). This splicing is dependent upon the presence (KL-1) or absence (KL-2) of a major proteolytic cleavage site that includes a segment of 84 nucleotides of the coding region in exon 6 of the *Kitl* gene (Martin et al., 1990; Huang et al., 1992). KL-1, which is initially generated in a membrane-associated form, is efficiently cleaved from the membrane, thus yielding a soluble product, due to the presence of a proteolytic cleavage site spanning the transmembrane region. KL-2, which is membrane-associated, lacks the proteolytic cleavage site and remains attached to the membrane (Huang et al., 1992). KL has a molecular weight of 28 to 35 kDa that varies according to heterogeneous glycosylation, however these sugars are not required for receptor binding (Zsebo et al., 1990). The soluble form of KL contains 4 potential *N*-linked glycosylation sites, is generally 164 – 165 amino acids in length, and normally exists in non-covalently held dimers. In contrast, the membrane-associated form has an internal deletion in the region that encodes for the carboxy terminus of KL-1 and only one of the four *N*-linked glycosylation sites (Fig. 5).

FIGURE 5. Structure of Kit Ligand.

Diagram of membrane-bound and soluble Kit ligand (KL). The presence of a proteolytic cleavage site in exon 6 of the *Kitl* gene results in the release of the extracellular portion of KL to generate the soluble isoform KL-1. KL-2 is generated in the absence of this cleavage site and is believed to the the most potent form of KL. Several mutations are known to arise in the *Kitl* gene and can exert deleterious effects to varying degrees on melanogenesis, hematopoiesis and gametogenesis. Steel dickie mutants (KL-Sl^{d}) have an internal deletion for the entire coding region of the cytoplasmic and transmembrane domains. Thus, only the soluble form of KL is produced in the animals. Subsequently, these mice suffer from, among other problems, severely reduced PGC populations rendering them infertile. This figure was modified from Lev et al. (1994).


KL-1
KL-2
KL-Sl^d

3.5 Expression of Kit ligand

The expression of the KL isoforms is controlled by alternative splicing which is regulated in a tissue-specific manner. KL-2 is predominantly expressed in all tissues while the abundance of KL-1 varies more according to the site of expression. With respect to germ cell and follicle development, KL mRNA is expressed along the path of migration of PGCs, in the gonadal ridge and fetal gonads (Matsui et al., 1990; Keshet et al., 1991; Motro et al., 1991). Post-natally, KL is expressed in the cells adjacent to Kit expressing cells, ie., in the granulosa cells and in the surface epithelium (Keshet et al., 1991; Motro et al., 1991; Ismail et al. 1996). In rat ovaries, KL has been demonstrated to be most abundantly expressed in the cumulus granulosa cells surrounding the oocyte and its mRNA expression is hormonally regulated as evidence by its increase with hCG stimulation in both rats and mice (Motro and Bernstein, 1993; Ismail et al., 1996).

In normal mice, both KL-1 and KL-2 are expressed in the ovaries (Manova et al., 1993), however, these two proteins differ greatly in their tissue distribution, regulation and function. In rat ovaries, KL-1 and KL-2 have been shown to differ in their distribution in granulosa cells of preovulatory and ovulatory follicles (Ismail et al., 1997) suggesting that the transcripts can be differentially regulated. This study was able to demonstrate that hCG stimulation resulted in a shift from KL-2 to KL-1 in the mural granulosa cells and a complete loss of both isoforms in the cumulus granulosa cells surrounding the oocytes. It is generally believed that KL-2 is capable of eliciting a more potent effect than KL-1, although both forms are biologically active (Huang et al., 1992). Thus, the shift in isoforms from a highly potent KL to a less potent KL in the mural granulosa cells and the complete loss of KL in the cumulus granulosa cells, as mentioned above, coincide with the role for Kit-KL signaling in the regulation of oocyte growth, since growth stimulatory signals are no longer required at this stage in follicular development.

3.6 Regulation of Kit signaling

Kit signaling may be regulated by: 1) the expression and isoforms of KL, 2) the expression of Kit, 3) downstream effector molecules involved in the dephosphorylation of the active receptor. *In vivo*, Kit signaling has been well studied in the context of *Kit* (formely *W*) and *Kitl* (formely *Sl*) mutants. These animals provide excellent models to better understand the importance of Kit activity in follicle development. Mutant mice that lack Kit or KL display similar phenotypes when in the homozygous state. These animals are black-eyed white, have varying degrees of macrocytic anemia and mast cell deficiency, and both males and females are infertile (Russel, 1979; Silvers, 1979). Although the phenotypes are similar, it is thought that *Kitl* mutations cause defects in the microenvironment in which melanogenesis, hematopoiesis and gametogenesis occur while *Kit* mutations affect intrinsic functions to these three developmental lineages (Mayer, 1970). Mice that are affected by weak mutations of *Kit* and *Kitl* have germ cells post-natally but do not have the required environment for later stages of germ cell development suggesting an important role for Kit and KL in the initiation of follicle development.

3.6.1. Phenotypes of *Kit* mutants

Mutations in the *Kit* gene have provided us with important information regarding the role of Kit activity in germ cell and follicle development. For instance, W^v/W^v mice are sterile due to a missense mutation that causes a reduction in the tyrosine kinase activity of the Kit protein (Nocka et al., 1990). Consequently, these animals are infertile due to decreased numbers of PGCs and impaired oocyte growth. As well, other mutants like the W^e/W^e animals are sterile due to reduced survival rates of PGCs during early oogenesis (Buehr et al., 1993). Recently, Reynaud et al. (2001) generated a *Kit* mutation by inserting the LacZ gene in the first exon of *Kit*. This mutation resulted in a 15-50 % decrease in Kit protein in the ovaries of mice heterozygous for the mutation ($Kit^{W\text{-}lacZ/+}$) in comparison to wild-type mice. Interestingly, the amount of KL was decreased by 15 % in these same animals suggesting that Kit expression may regulate KL expression in ovaries. Oocyte growth, granulosa cell proliferation and antrum formation were impaired by the reduced Kit and KL proteins supporting previous findings of a requirement for Kit activity in oocyte growth and follicle development (Packer et al., 1994; Yoshida et al., 1997; Kissel et al., 2000). Thus the regulation of Kit activity through changes in its expression and intrinsic kinase activity can have serious consequences on germ cell and follicle development to the point of infertility.

3.6.2. Phenotypes of *Kitl* mutants

In the same manner that *Kit* mutants can improve our understanding of the functions of the Kit receptor in the ovary, *Kitl* mutants are invaluable to determining the role of KL in the regulation of Kit activity. There are many different types of KL mutants whose phenotypes vary greatly in their severity. The most commonly discussed mutants are the Sl^{pan}/Sl^{pan} and the Sl/Sl^{t} mice. In the homozygous state, the females animals are sterile due to reduced germ cell numbers and impaired oocyte and follicular development (Kuroda et al., 1988; Huang et al., 1993). Another important *Kitl* mutant is the Sl^{d} mouse. This mutation produces shorter transcripts due to a 4.0 kilobase intragenic deletion of the coding region for the entire cytoplasmic and transmembrane domains of KL. Thus, these animals can produce the soluble form of KL but are completely devoid of membrane-associated KL. Consequently, Sl/Sl^{d} mice, similar to W/W^{v} mutants, are sterile due to severely reduced PGC populations suggesting that KL-2 is required for PGC proliferation (Flanagan et al., 1991). Thus, KL expression, in terms of both abundance and isoforms, is critical to the germ cell proliferation and follicle development, and thus is essential for fertility.

4 The SHP-1 Tyrosine Phosphatase

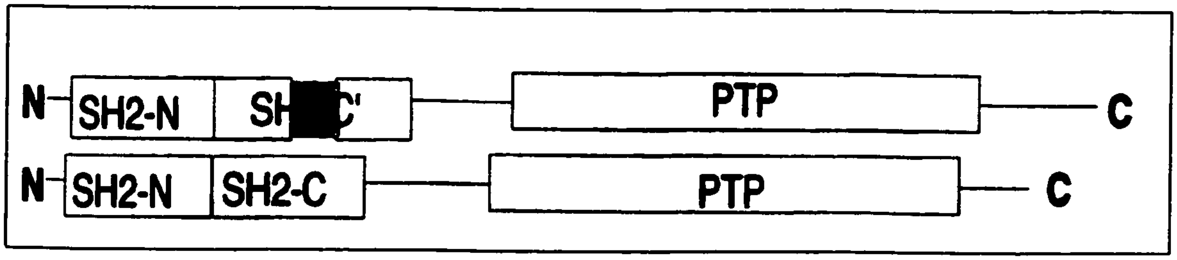
4.1 Structure of SHP-1

Protein tyrosine phosphatases (PTPs) are known to catalyze the dephosphorylation of phosphotyrosine peptides and are essential in regulating the intracellular level of phosphotyrosines. PTPs can be either receptor-like or cytosolic. The receptor-like PTPs have intracellular catalytic domains that are arranged in a conserved tandem sequence as well as extracellular domains that are thought to play a role in cell signaling (Denu et al., 1996). Cytosolic PTPs are composed of only one catalytic domain in conjunction with non-catalytic segments like the SH2 domains that are only found in the SHPs. These phosphatases are a subfamily of the cytosolic PTPs that consist of SHP-1, SHP-2 and Csw. Although all three enzymes contain two tandem SH2 domains, a catalytic domain and a conserved C terminus, they differ considerably in their biological activity (Yang et al., 1998).

SHP-1, also known as PTP1C, SH-PTP1, HCP, SHP, and PTPN6, is a cytosolic protein tyrosine phosphatase that is a known regulator of many signal transduction pathways (discussed below) (Fig. 6). In most cases, SHP-1 provides the negative signals required to maintain a balance of stimulation and inhibition for cellular homeostasis (Cambier et al., 1997). It has a conserved catalytic domain that is linked to two non-catalytic N terminal SH2 domains, or Src homology 2 domains, and a carboxy terminal regulatory domain. SHP-1 is primarily inactive within resting cells due to the presence of a D'-E loop of the amino terminal SH2 domain within the substrate binding pocket (Yang et al., 1998). Cell stimulation allows for the translocation of SHP-1 from the cytosol to

FIGURE 6. The SHP-1 protein tyrosine phosphatase.

The SHP-1 phosphatase contains 2 non-catalytic N-terminal SH2 domains in tandem sequence, a catalytic domain and a regulatory C-terminal domain. SHP-1 is thought to have 2 isoforms that were generated by alternative splicing within the C-terminal SH2 domain, thus generating proteins in the range of 67 and 71 kDa. SHP-1 has been shown to bind and dephosphorylate Kit at tyrosine 569 on the receptor, an interaction that occurs via the SH2-N and SH2-C′ domains. In hematopoietic cells, this relationship is inhibitory to Kit activity. Our hypothesis is that Kit activity is regulated by SHP-1 in mouse oocytes. This figure was modified from Kozlowski et al. (1998).



N
SH2-N
SH
C'
PTP
C
N
SH2-N
SH2-C
PTP
C

the plasma membrane which is followed by the direct binding of the phosphatase to receptor and non-receptor tyrosine kinases. This interaction occurs via the SH2 domains which form a pocket that binds phosphotyrosine moieties (Burshtyn et al., 1997; Yang et al., 1998).

4.2 Expression and function of SHP-1

SHP-1 is mainly expressed in hematopoietic cells where it functions as an inhibitor of several signal transduction pathways. Loss of SHP-1 has been characterized by a marked over-expansion of the CD5+ subpopulation of peripheral B cells, reduced T-cell and NK cell function, a decrease in the number of B cell progenitor cells and an increase in granulocytes, monocytes and macrophages (Kozlowski et al., 1993). SHP-1 is known to bind non-receptor tyrosine kinases such as the p85 subunit of PI-3K (Cuevas et al., 1999), as well as a variety of receptors such as those for erythropoietin (Sharlow et al., 1997), interleukin-3 (IL-3) (Haque et al., 1998), B lymphocyte (Cyster and Goodnow, 1995), PDGF (Yu et al., 1998), EGF (Tenev et al. 1997), and KL (Yi and Ihle, 1993). A recent study by Russell and Richards (1999) showed the expression of SHP-1 in granulosa cells of rat ovaries. They found that SHP-1 levels were hormonally regulated, decreased by estrogen and FSH and increased with luteinization. Interestingly, SHP-1 localized to the cytoplasm of atretic granulosa cells and to the nuclei of luteal cells. The hormonal regulation and differential sub-cellular localization patterns suggest the possibility of multiple roles for the phosphatase in follicle development.

4.3 *Motheaten* mice

The loss of SHP-1 is exemplified in the *motheaten* mice where a single mutation results in an early onset of a lethal autoimmune and immunodeficiency disease. A 101 base pair frame-shift deletion of the SHP-1 gene (*me*) on chromosome 6 underlies the expression of the *motheaten* phenotype which leads to death by the age of 3 weeks (Kozlowski et al., 1993). This mutation, located within the coding region of the amino-terminal SH2 domain, originated by altered mRNA splicing due to a point mutation that consequently altered the splice sequence signal (Tsui et al., 1993). Kozlowski et al. (1993) showed that *motheaten* mice do not express SHP-1 RNA or protein in various hematopoietic tissues in contrast to wild-type mice that have two distinct bands reported to be approximately 67 and 70 kDa and that are believed to have arisen from alternative splicing events. A variety of sizes of SHP-1 isoforms have been reported and it is thought that they may be differentially expressed in various cell types (Kozlowski et al., 1993; Tsui et al., 1993; Uchida et al., 1993; Yeung et al., 1992).

4.4 Interactions between Kit and SHP-1

In hematopoietic cells, SHP-1 dephosphorylates specific tyrosine residues of the Kit receptor and inhibits Kit signaling. Paulson et al. (1996) demonstrated that mice homozygous for both *motheaten* and W^v mutations had a lessened mast cell deficiency compared to what was normally associated with W^v mutations confirming a functional interaction between Kit and SHP-1. Their study extensively examined cells from the hematopoietic system but did not address the possibility of compensatory mechanisms from the *motheaten* phenotype that could alter the consequences of W^v mutations in the gonads, notably infertility due to decreased numbers of PGCs and impaired oocyte growth. Around the same time, Lorenz et al. (1996) performed a similar experiment in which they cross-bred W^v/+ and *me*/+ mice and examined the F2 generation for intergenic complementation. Their study revealed similar results to those published by Paulson et al. (1996) in that the *motheaten* phenotype was able to rescue the animals from the W^v phenotype. However, they also showed that mast cells from W^v/+:*me/me* were more responsive to KL stimulation than mast cells derived from W^v/+:+/+ mice suggesting that SHP-1 may have a direct interaction with Kit. In 1998, Kozlowski et al. demonstrated that SHP-1 can directly bind Kit and dephosphorylate the receptor in hematopoietic cells. Specifically, the SH2 domain of SHP-1 attaches to the phosphorylated tyrosine 569 that is located in the juxtamembrane domain of Kit and inhibits Kit signaling. This project is the first to address the possibility that SHP-1 may also inhibit Kit activity in the ovary and provide negative signals in the regulation of oocyte growth.

5 Hypothesis and Objectives

Knowing and understanding the factors involved in the normal balance of stimulation and inhibition of various kinases is important for proper recognition of abnormal conditions that may lead to infertility. The development of healthy mature oocytes and large preovulatory follicles is crucial to the successful fertilization of ovulated eggs. Although numerous studies have proposed an important role for Kit signaling in oocyte growth and follicle development, the regulation of this signaling pathway is not understood.

The hypothesis for this research project was that SHP-1 can inhibit Kit activity in mouse oocytes and that its inhibition of other kinases may impact granulosa cell functions (Fig. 7). Therefore, the objectives were as follows:

(1) To determine the expression of SHP-1 in reproductive tissues, specifically in the different cell types of the ovary.

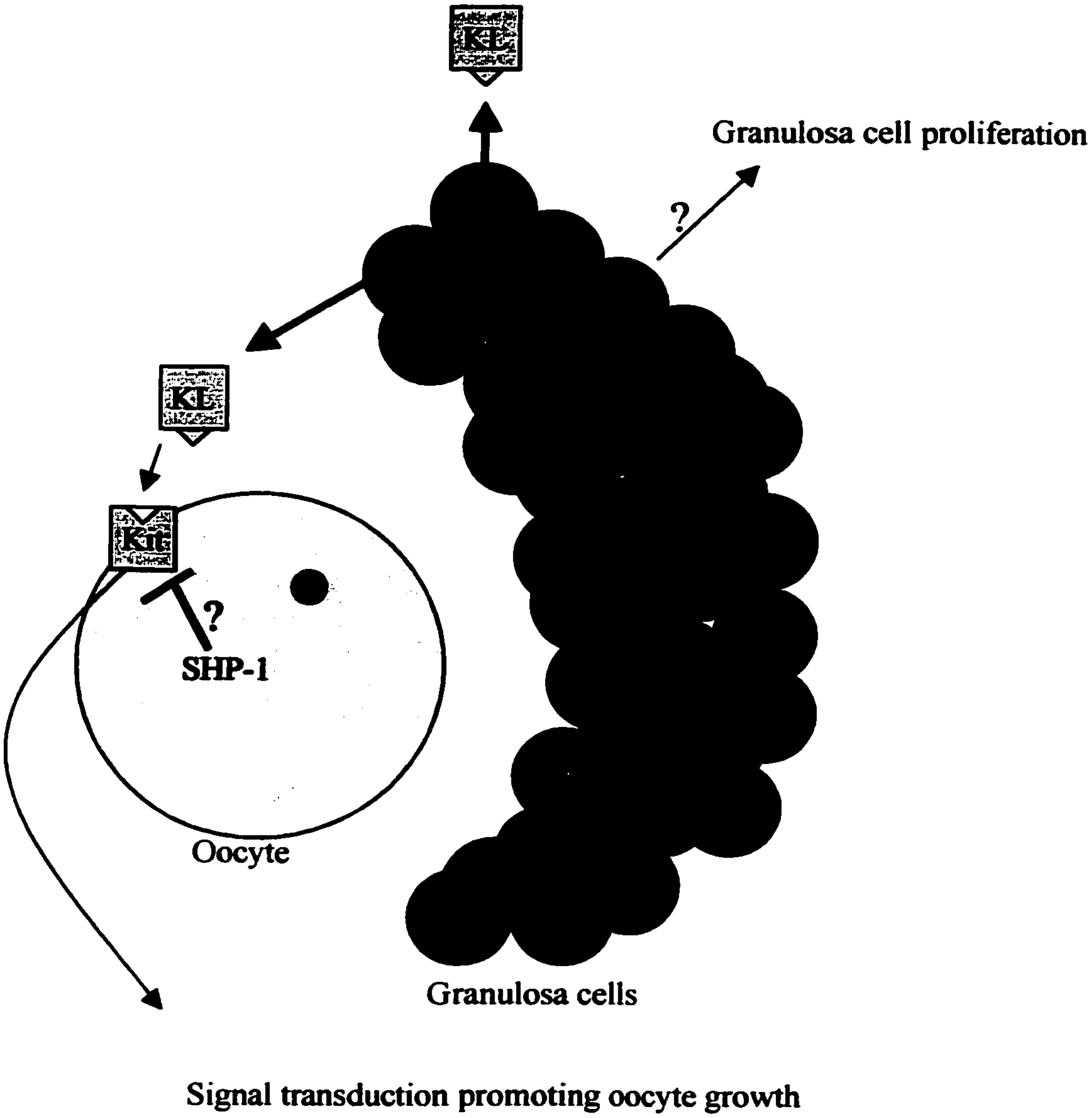
(2) To determine an association between SHP-1 and Kit in the ovary.

(3) To examine the consequences of SHP-1 deficiency on Kit activity in oocytes through its effects on oocyte growth at all stages of development.

(4) To examine the effects of SHP-1 deficiency on granulosa cell proliferation and differentiation at all stages of follicle development.

FIGURE 7. Model of hypothesis.

The present hypothesis is that SHP-1 inhibits Kit signaling in mouse oocytes and that this interaction is required to provide inhibitory signals in the regulation of oocyte growth. SHP-1 may also interact with other kinases in the oocytes or elsewhere in the follicle and contribute to the regulation of follicle development.


KL
Granulosa cell proliferation
?
KL
Kit
?
SHP-1
Oocyte
Granulosa cells
Signal transduction promoting oocyte growth

6. Animals

Prepubertal female CD1 and SCID mice were obtained from Charles River Canada (St. Constant, Quebec). Animals were allowed free access to food and water, and lighting was provided for 14 hours (h) daily. CD1 mice were injected intraperitoneally at 24-25 days of age with 5 I.U. PMSG (Folligon, Intervet) and 40 h later injected with 5 I.U. of hCG (Sigma Chemical Co., St. Louis, MO). Animals were sacrificed at 40-48 h post PMSG injection and 5-9 h post-hCG treatment. Tissues from *motheaten (me/me)* animals (C3HeBFeJ) and their age-matched controls (wild-type, +/+; or heterozygous, +/-) were obtained from the animal colony at Health Canada (Ottawa, Canada). All animals were treated in accordance with the policies and regulations of the Animal Care and Veterinary Service of the University of Ottawa.

7. Antibodies

The SHP-1 antibody was raised in rabbits against the wild-type GST-SHP-1 SH2 domain fusion protein or with a GST-fusion protein containing the two SH2 domains (Kozlowski et al., 1993). Kit antibodies, purchased from Oncogene Research (Cambridge, MA) and Santa Cruz Biotechnologies (C-19; SC; Santa Cruz, CA), are rabbit polyclonal antibodies raised against the C-terminal end of Kit to recognize human and mouse epitopes respectively.

8. Immunohistochemistry

8.1 Tissue collection and preservation of samples

Oocyte size, granulosa cell proliferation, and the number of primordial and growing follicles were assessed in hematoxylin and eosin (H&E) stained sections of 10-13 day old (d.o.) wild-type (C3HeBFeJ) and *motheaten* ovaries. SHP-1 and Kit proteins were immunolocalized in sections of wild-type ovaries (CD1) at different stages of follicle development and in sections of 10-12 d.o. *motheaten* ovaries. Ovaries from 12 and 27 d.o. wild-type mice, as well as wild-type mice primed with PMSG alone and PMSG followed by hCG, were fixed in 160 mM phosphate buffered saline (PBS) (pH 6.9) with 4% paraformaldehyde and 0.2% saturated picric acid. The ovaries were subsequently embedded in paraffin blocks for sectioning.

8.2 Tissue processing

Sections (4 μm in thickness) were deparaffinized in 4 changes of Xylene (5 min each) and partially rehydrated in 2 changes of 100 % ethanol (5 min each). Sections were either stained with H&E or processed for immunohistochemistry as follows. Endogenous peroxidase activity was blocked by treating the sections with 3 % H_2O_2 in 100 % ethanol for 15 min in the dark. The tissue sections were then rehydrated in 95 % ethanol for 5 min followed by 80 % ethanol for 5 min. The sections were washed under tap H_2O and briefly rinsed in distilled H_2O. The sections were permeabilized in a 0.01 M sodium citrate buffer for 5 min in a microwave at 50 % maximum intensity and allowed to cool to room temperature. Subsequently, half the buffer was discarded and replaced with fresh citrate buffer and heated again for 5 min. Sections were cooled under tap water to room temperature, washed in PBS for 5 min, and subjected to immunodetection (Histostain-SP Kit; Zymed Laboratories Inc., San Francisco, CA).

8.3 Immunodetection

Sections were blocked with 10 % non-immune goat serum for up to 1 h in a covered humidity chamber and incubated with either 1:300 dilution of the SHP-1 antibody or 1:15 dilution (6μg/ml) of the Kit antibody (Oncogene) in PBS. Sections on which the primary antibody was omitted were included as controls for the specificity of any observed staining. After washing for 3 x 10 min in PBS, sections were incubated with a biotinylated secondary antibody for 30 min. Following 3 x 10 min washes in PBS, sections were incubated with a streptavidin-peroxidase conjugate for 25 min. Following 3 x 10 min washes in PBS, the sections were subjected to a substrate-chromogen mixture (substrate buffer, AEC chromogen solution and H_2O_2) in the dark for 4 min. The sections were then rinsed in distilled H_2O (dH_2O), counterstained with hematoxylin for 2 minutes, rinsed under tap water for 10 sec, washed in PBS for 30 sec, and rinsed in dH_2O before being coverslipped.

9. Western Analysis

9.1 Tissue collection and isolation of proteins.

SHP-1 protein expression was assessed by western analysis in various reproductive tissues. All tissues were isolated from the animals immediately after their death and flash frozen in liquid nitrogen. The samples were either used immediately or stored at -80°C for future use. Tissues were mechanically sheared in Frack's buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA (pH 8.0), 1% Triton X-100, 20% glycerol) with freshly added protease and phophatase inhibitors (2 mM NaF, 2 mM NaPP, 500 μM Na_3Vo_4, 200 μg ml^{-1} PMSF, 2 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin). Lysates were incubated on ice for 20-30 min with occasional vortexing, centrifuged for 10 min at 4°C and 14,000xg. Supernatants were collected and stored at -80°C. The concentration of protein in the lysates was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

9.2 Collection of growing and fully grown oocytes

Ovaries were removed from 24 d.o. female mice (CD1) that were injected 40-44 h previously with 5 I.U. PMSG. Oocyte-cumulus complexes were immediately collected from these ovaries in Waymouth MB 752/1 (WAY; Sigma) with 5 % fetal bovine serum (FBS; Hyclone, Logan, UT) by puncturing the large antral follicles with 25-gauge needles. Cumulus granulosa cells were detached from the fully grown oocytes by gently pipetting and 500 denuded oocytes were collected to determine the level of SHP-1 expression by western blot.

Ovaries were removed from 12 d.o. female mice (CD1) and growing denuded oocytes were collected. In Ca^{2+}-free PBS supplemented with 0.5 % collagenase (Worthington Biochemical Corp.) and 0.01% bovine pancreas DNase I (Sigma), dissociated follicles from the ovaries were pipetted at 4-5 minute intervals, and 500 growing denuded oocytes were collected to determine the level of SHP-1 expression by western blot.

9.3 Electrophoresis and electroblotting

Protein lysates were mixed with equal volumes of 2X SDS gel loading buffer with 1/10th volume 1 M DTT. Prior to electrophoresis, the samples were heated at 95°C for 5 min and subsequently loaded into gels. The sample proteins and protein markers (Bio-Rad) were separated on 8% polyacrylamide gels and electroblotted to Hybond C extra nitrocellulose membranes (Amersham) in 0.2 M glycine, 0.02 M Tris-HCl, 20% (v/v) methanol.

9.4 Immunodetection

The blots were blocked in TBS-T (10 mM Tris-HCl, 180 mM NaCl, 0.05% (v/v) Tween-20) containing 5% skim milk powder for 1 h with shaking at room temperature or overnight at 4°C with shaking. Blots were incubated with a primary antibody in TBS-T 1:2000 SHP-1 for 1 h at room temperature with shaking or overnight at 4°C with shaking. After washing for 3 x 10 min in TBS-T, blots were incubated with a goat anti-rabbit horseradish-peroxidase conjugated secondary antibody (Kirkegaard and Perry, Gaithersburg, MD; diluted 1:5000 in TBS-T) for 1 h at room temperature with shaking. After washing 3 x 10 min in TBS-T, immunoreactive bands were visualized using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard and Perry) and X-Omat XB-1 film (Eastman Kodac Company, Rochester, NY).

10. Immunoprecipitation

10.1 Tissue collection and protein isolation

SHP-1 and Kit were co-immunoprecipitated from mouse ovaries. Tissues were collected and sheared, as described above, in Frack's buffer with added protease and phosphatase inhibitors. Lysates were incubated on ice for 60 min, centrifuged for 10 min at 4°C and 14,000 rpm. Supernatants were either used immediately or stored at -80°C for the immunoprecipitation.

10.2 Immunoprecipitation of Kit

Supernatants were collected and pre-cleared with 30 µL of a 50% slurry of protein A sepharose beads (Amersham) overnight at 4°C on a shaker. The beads were then pelleted by 30 sec centrifugation at 10, 000 rpm and the lysates were incubated overnight at 4°C with Kit antibody (SC) and with constant shaking. Thirty µL of 50% protein A slurry was then added to each lysate for another overnight incubation at 4°C. Subsequently, the beads were pelleted, resuspended in 2X SDS gel loading buffer with 1/10th volume 1 M DTT.

10.3 Electrophoresis and electroblotting

Prior to electrophoresis, the samples were heated for 5 minutes at 95°C and loaded onto 8% polyacrylamyde gels. Proteins were then electroblotted onto Hybond C nitrocellulose membranes (Amersham) in 0.2 M glycine, 0.02 M Tris-HCl, 20% (v/v) methanol.

10.4 Immunodetection

The blots were blocked in TBS-T containing 5% skim milk powder for 1 h with shaking at room temperature or overnight at 4°C with shaking. Blots were incubated with a primary antibody in TBS-T (1:2000 SHP-1) for 1 h at room temperature with shaking or overnight at 4°C with shaking. After washing for 3 x 10 min in TBS-T, blots were incubated with a goat anti-rabbit SHP-1 horseradish-peroxidase conjugated secondary antibody (Kirkegaard and Perry; diluted 1:5000 in TBS-T) for 1 h at room temperature with shaking. After washing 3 x 10 min in TBS-T, immunoreactive bands were visualized using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard and Perry) and X-Omat XB-1 film (Rochester, NY).

11. Ovary Transplantation

11.1 Ovary collection

Ovaries from 10-12 d.o. *motheaten* mice and their littermate controls (+/- or +/+) were collected in WAY with 10% FBS and washed several times in this media. Subsequently, the ovaries were transferred onto Millicell-PC inserts (3 μm) (Millipore Corp., Bedford, MA) in 6 well tissue culture plates (Falcon) containing 1.5 ml of media under the inserts. The ovaries were then covered by a drop of media to maintain them in culture for up to 3 h until transplantation.

11.2 Transplantation

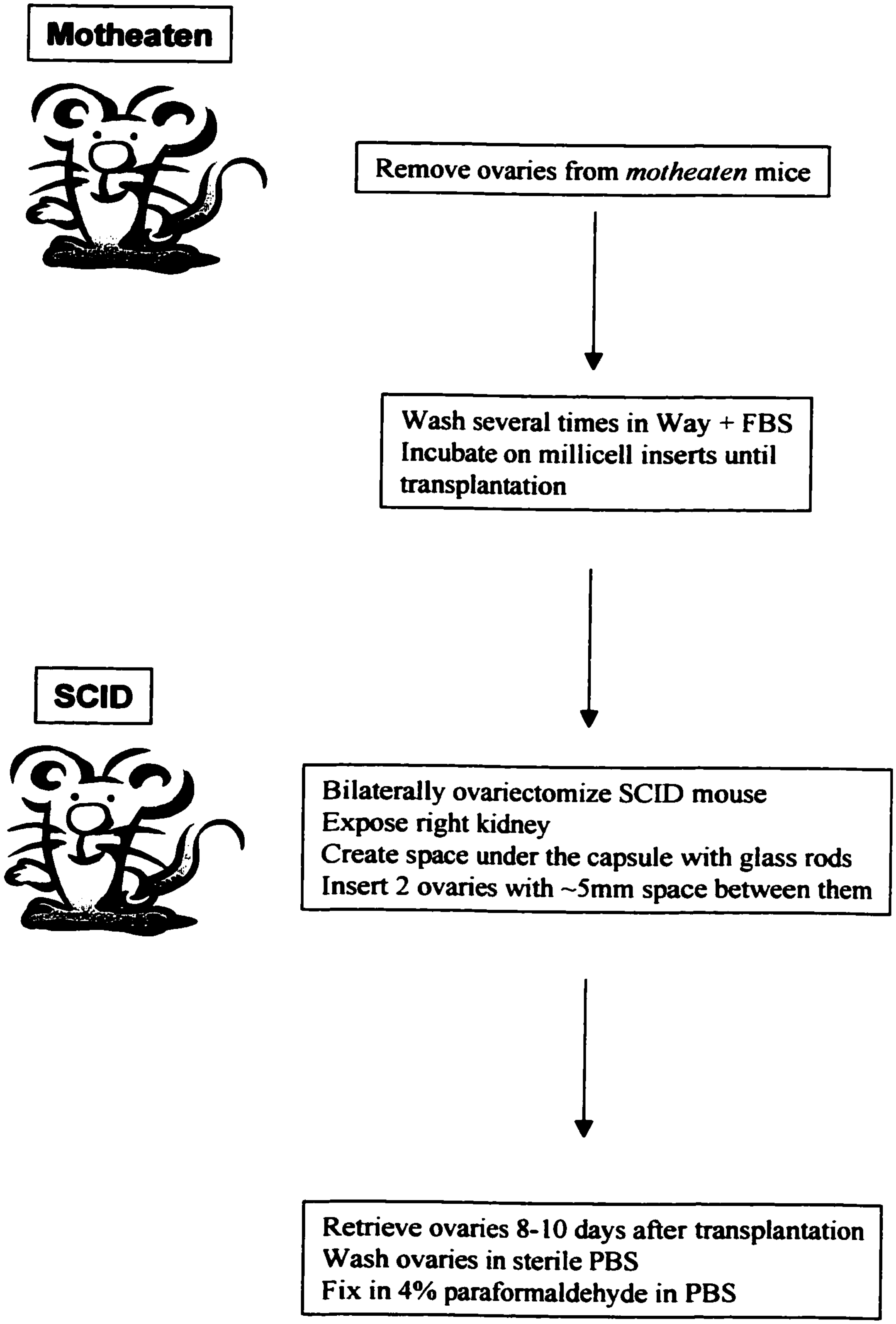
Under sterile conditions in a barrier facility, ovaries were transplanted under the kidney capsule of bilaterally ovariectomized SCID mice (at least 8 weeks of age). SCID mice were anaesthetized by either an intraperitoneal injection of Avertin or by continual administration of halothane. These animals were also given 0.3 mg kg^{-1} buprenorphine subcutaneously 30 min prior to surgery and 6 h post surgery, as well as 1 ml of saline subcutaneously pre- and post surgery (Fig. 8).

11.3 Recovery

Ovaries were recovered 8-10 days post surgery to ensure that all the ovaries were 20 d.o. at the time of collection. Upon removal, the ovaries were rinsed in room temperature sterile PBS and immediately fixed in cold 4% paraformaldehyde in PBS overnight at 4°C. Ovaries were then rehydrated in an ethanol gradient, embedded in paraffin, and cut into serial sections for H&E staining. The oocytes and follicles were measured throughout the ovaries in sections in which the germinal vesicle was visible in the oocyte. Oocyte and follicle size were assessed in large antral follicles ≥ 150 μm in order to examine changes pertaining to the termination of growth. Also, the ability to form antra, and the distribution of follicle size were assessed in all antral follicles from wild-type and *motheaten* animals.

FIGURE 8. Ovary transplantation procedure.

Ovaries were collected from *motheaten* animals between the age of 10 and 12 days. Once collected, they were washed several times and incubated on a membrane covered by a very thin layer of media until the transplant procedure. SCID mice were used as recipients so as to not reject the graft. These animals were anaesthetized, bilaterally ovariectomized and their right kidney was exposed. Glass tubes served as tools to insert the *motheaten* ovaries under the kidney capsule. The transplanted ovaries were left in the SCID mice for 8 to 10 days so that the final day of recovery would be equivalent to day 20 of age, a time point that would normally allow for antrum formation and the presence of preovulatory follicles. Ovaries were then removed and processed histologically.



Remove ovaries from *motheaten* mice

Wash several times in Way + FBS
Incubate on millicell inserts until transplantation

Bilaterally ovariectomize SCID mouse
Expose right kidney
Create space under the capsule with glass rods
Insert 2 ovaries with ~5mm space between them

Retrieve ovaries 8-10 days after transplantation
Wash ovaries in sterile PBS
Fix in 4% paraformaldehyde in PBS

12. In Vitro Maturation of Oocytes

Transplanted ovaries were collected in WAY with 5% FBS. The large antral follicles were punctured using 27 gauge needles to release the oocyte-cumulus cell complexes. In both control and *motheaten* groups, several denuded oocytes were also released from the ovaries in higher numbers than the complexes that were collected. Thus, the denuded oocytes were tested for their ability to undergo GVBD while the complexes were examined on the basis of their ability to undergo cumulus expansion.

Both denuded oocytes and complexes were washed 2-3 times and pooled in groups of 10-20 in 50 μl of WAY with 5% FBS and 240 ng ml^{-1} of ovine FSH (oFSH-19; NIH, Bethesda, MA) under equilibrated oil in a 35 mm petri dish (Falcon). Subsequently, they were incubated at 37°C in 5% CO_2 for 16 h and oocyte maturation was evaluated based on the ability of the cumulus granulosa cells from the complexes to undergo expansion and on the ability of denuded oocytes to undergo GVBD.

13. Statistical Analysis

All data are expressed as mean ± standard error of the mean and analyzed by an unpaired two-tailed t-test (2 groups of data) or analysis of variance (one-way ANOVA) with Bonferroni's multiple comparison test (more than 2 groups of data). When specified, Chi square test with Yates comparison was used to compare categorical variables. A value of $p < 0.05$ was considered to be statistically significant.

14. Phenotype of 10-13 day old *motheaten* mouse ovaries

Ovaries from 10-13 day old *motheaten* mice and age-matched controls were microscopically examined after H&E staining. *Motheaten* ovaries displayed obvious morphological differences with wild-type ovaries including reduced size and an apparently large number of primordial and primary follicles (Fig. 9) and no differences were observed in the phenotypes of wild-type and heterozygous controls. The apparent difference in size was confirmed by weighing the ovaries. *Motheaten* ovaries (134.5±8.9 µg; $n=4$) had on average 48% less mass than those of control animals (281.3±32.6 µg; $n=3$) ($p<0.005$) (Fig. 10). Also, *motheaten* mice (6.0 ± 0.13 g; $n=6$) had on average 23.6% less total body mass in comparison to controls (8.0 ± 0.27 g; $n=6$) $p<0.0001$. Thus, on average, the mass of one wild-type ovary represented 3.5 % of the total body mass while the mass of one *motheaten* ovary was 2.25 % of the total body mass, suggesting that although *motheaten* animals are smaller than controls, their ovaries are further disproportionately reduced in mass.

FIGURE 9. Phenotype of 10-13 d.o. wild-type and *motheaten* ovaries.

Ovaries were obtained from 12 day old mice and were embedded in paraffin, sectioned and stained with H&E. *Motheaten* ovaries (B) were morphologically smaller than controls (A) and contained numerous primordial follicles in addition to many growing follicles with few layers of granulosa cells.

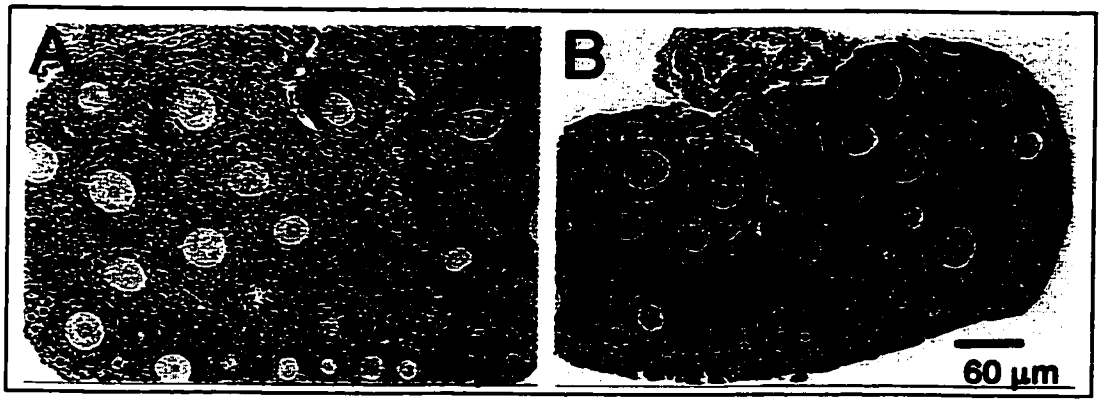
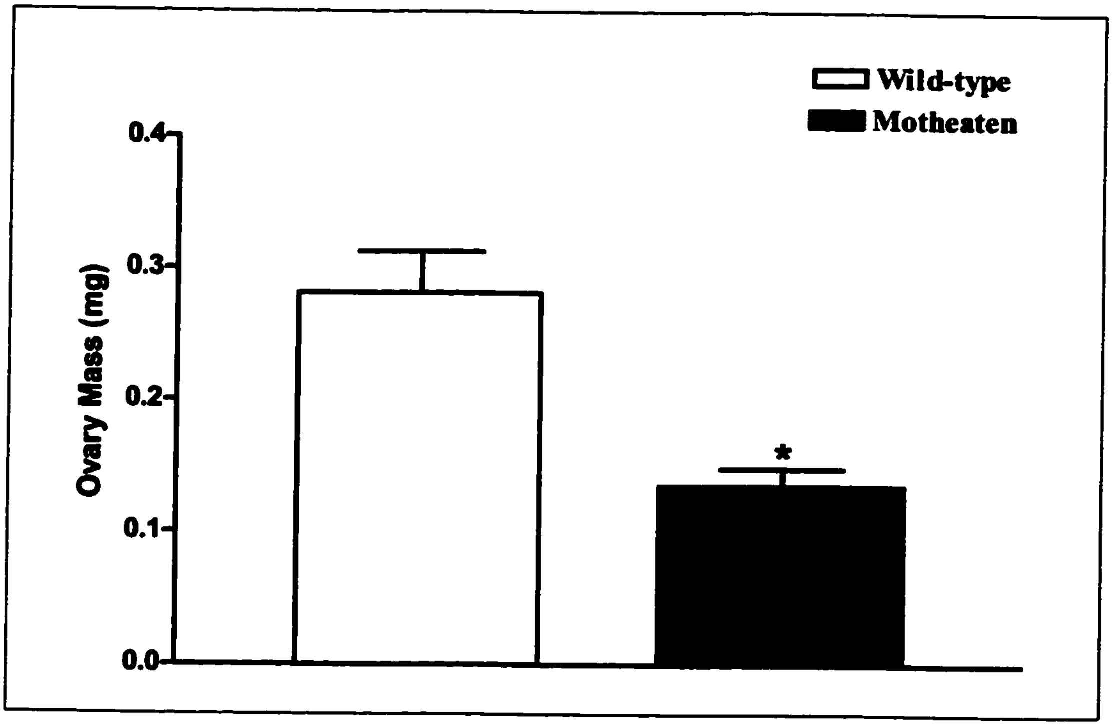

A
B
60 µm

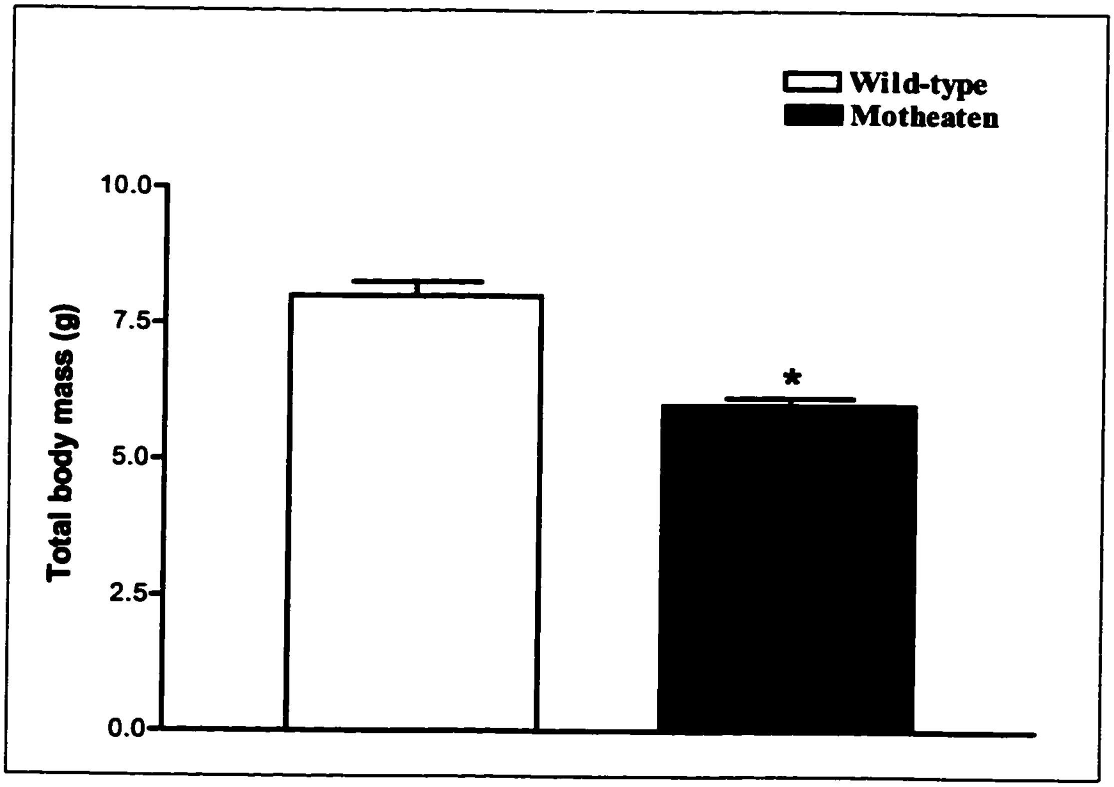
FIGURE 10. Mass of 10-12 day old wild-type and *motheaten* ovaries.

Freshly isolated ovaries from 10-12 d.o. mice were weighed on a micro-balance in order to compare masses. *Motheaten* ovaries had 48% less mass than that of sibling control animals (A) ($p<0.005$). Similarly, *motheaten* and controls were weighed in order to compared ovary mass to body mass. Consequently, *motheaten* mice had on average 23.6 % less mass than controls (B) ($p<0.0001$). Thus, regardless of the difference in total body weight, *motheaten* ovaries were further disproportionately reduced in mass compared to controls.

A


Wild-type
Motheaten
Ovary Mass (mg)
0.4
0.3
0.2
0.1
0.0
*

B


Wild-type
Motheaten
Total body mass (g)
10.0
7.5
5.0
2.5
0.0
*

15. SHP-1 expression in mouse reproductive tissues

Ovaries, uteri, granulosa cells, and spleen (used as a positive control) were collected from mature animals and protein was isolated from the tissues in order to determine SHP-1 expression in reproductive tissues. SHP-1 proteins were detected by western blot analysis in wild-type ovaries (CD1), granulosa cells, uteri, and spleen (Fig. 11). This experiment was performed in triplicate and generated similar results with each trial.

Cellular localization of expression was then determined in ovaries obtained from 27 day old mice, as well as PMSG and PMSG- and hCG-primed animals (CD1). 12 day old *motheaten* ovaries were used as negative controls. Animals were primed with PMSG in order to stimulate follicle development to the late preantral and early antral stages. Others were primed with both PMSG and hCG in order to stimulate follicle development to the preovulatory stage. SHP-1 proteins were expressed in all cell types of the ovary throughout all stages of follicle development and was most clearly visible in the oocytes (Fig. 12). Kit expression was also evaluated by immunohistochemistry and the proteins were visible in oocytes from both groups as well as faintly visible in the stromal tissue (Fig. 13). These experiments were performed in triplicate and generated similar results with each trial.

Five hundred fully grown and 500 growing oocytes were collected to compare the levels of SHP-1 expression. Western blot analysis showed that fully grown oocytes had more SHP-1 proteins than growing oocytes. Although the immunodetection of β-actin on the same blot showed that higher amounts of protein were present in the lane corresponding to the fully grown oocytes, densitometric analysis revealed that SHP-1 expression was increased 1.43-fold in fully grown oocytes compared to controls (Fig. 14). These data were generated by Claude Barchéchat, a former student in our lab. This experiment was only performed once and thus statistical significance of the data was not assessed.

FIGURE 11. Western blot analysis of SHP-1 expression in mouse reproductive tissues.

Protein lysates were obtained from various tissues of 4-6 week old mice and proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose paper. SHP-1 protein was detected by Western blot analysis as 68 and 70 kDa bands in spleen (positive control), ovaries, granulosa cells and uteri.

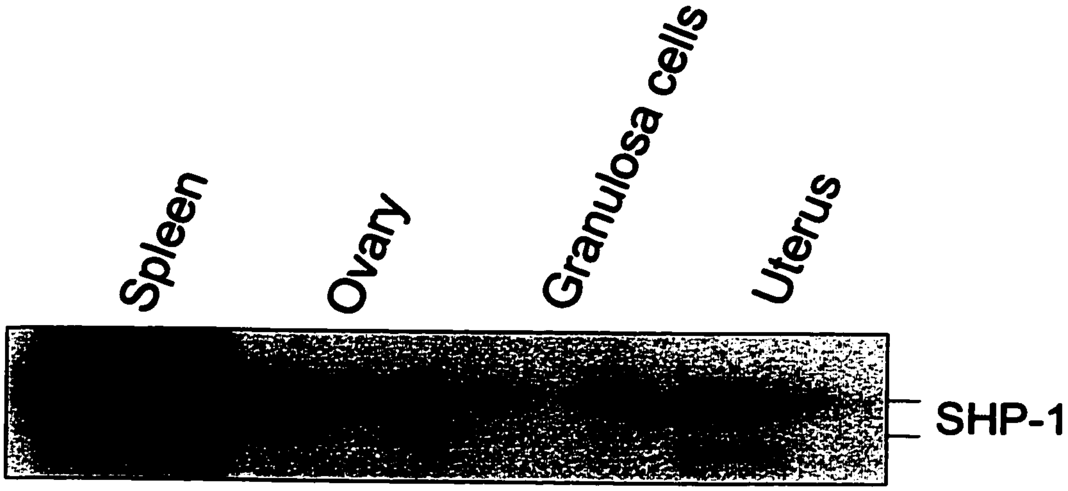

Spleen
Ovary
Granulosa cells
Uterus
SHP-1

FIGURE 12. Immunohistochemistry detecting SHP-1 protein in mouse ovaries. Ovaries were fixed, embedded in paraffin and cut into serial sections. Immunohistochemistry was performed with or without anti-SHP-1 antibodies. A) 27 day old wild-type, B) 12 day old *motheaten*, C) 27 day old wild-type, D & E) PMSG-primed wild-type, F) PMSG- and hCG-primed wild-type. Sections B-F were incubated with anti-SHP-1 antibodies. SHP-1 was found in all cell types throughout the stages of follicle development and was most clearly visible in the oocytes.

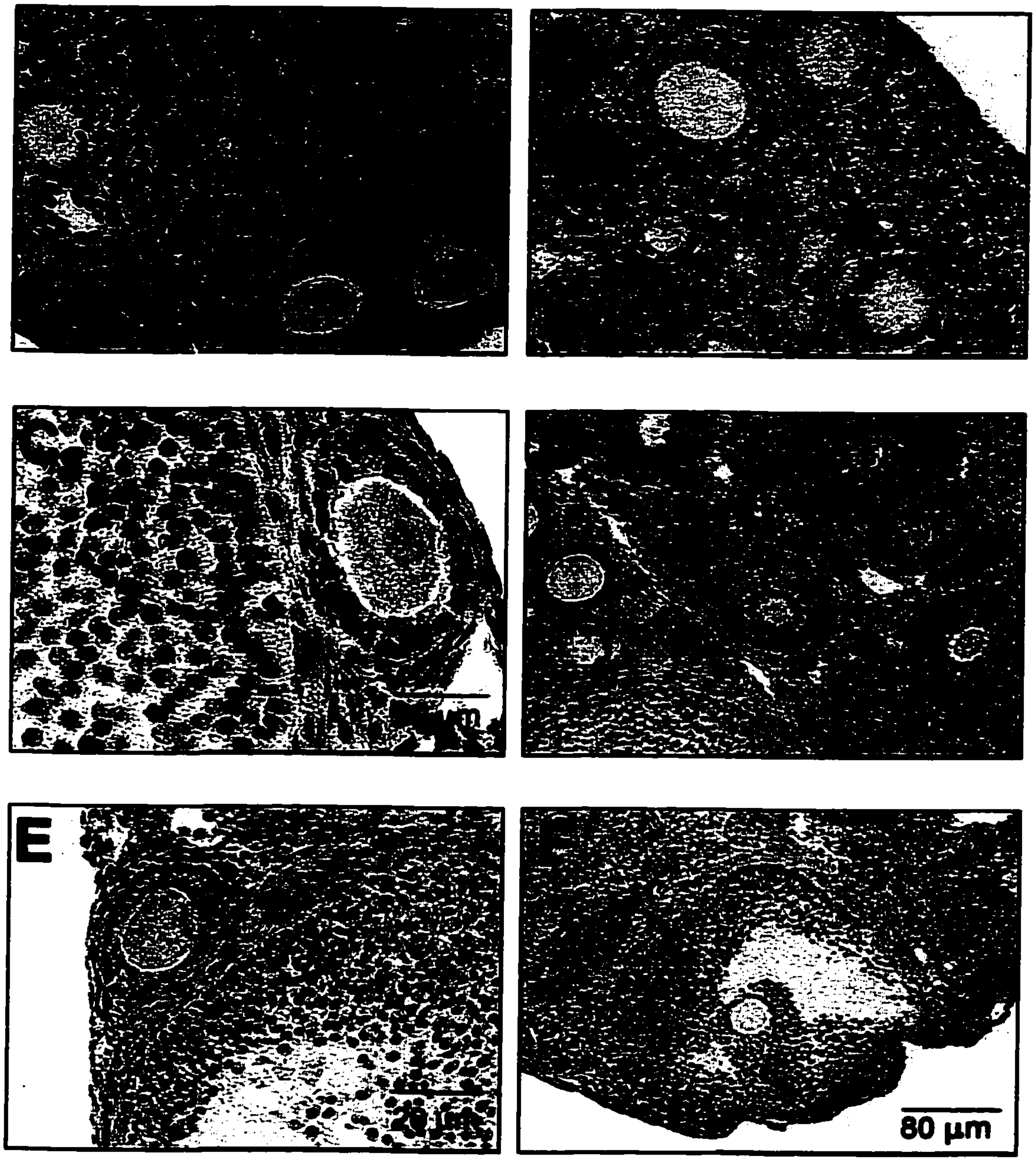

E
20 µm
80 µm

FIGURE 13. Immunohistochemistry detecting Kit protein in mouse ovaries. Ovaries were fixed, embedded in paraffin and cut into serial sections. Immunohistochemistry was performed with or without anti-Kit antibodies. A) 12 day old wild-type, B) 12 day old wild-type, C & D) 12 day old *motheaten*, E) PMSG-primed wild-type, F) PMSG- and hCG-primed wild-type. Sections B-F were incubated with anti-Kit antibodies (Oncogene). Kit proteins were clearly visible in the oocytes of both wild-type and *motheaten* ovaries and faintly detected in the stromal tissue.

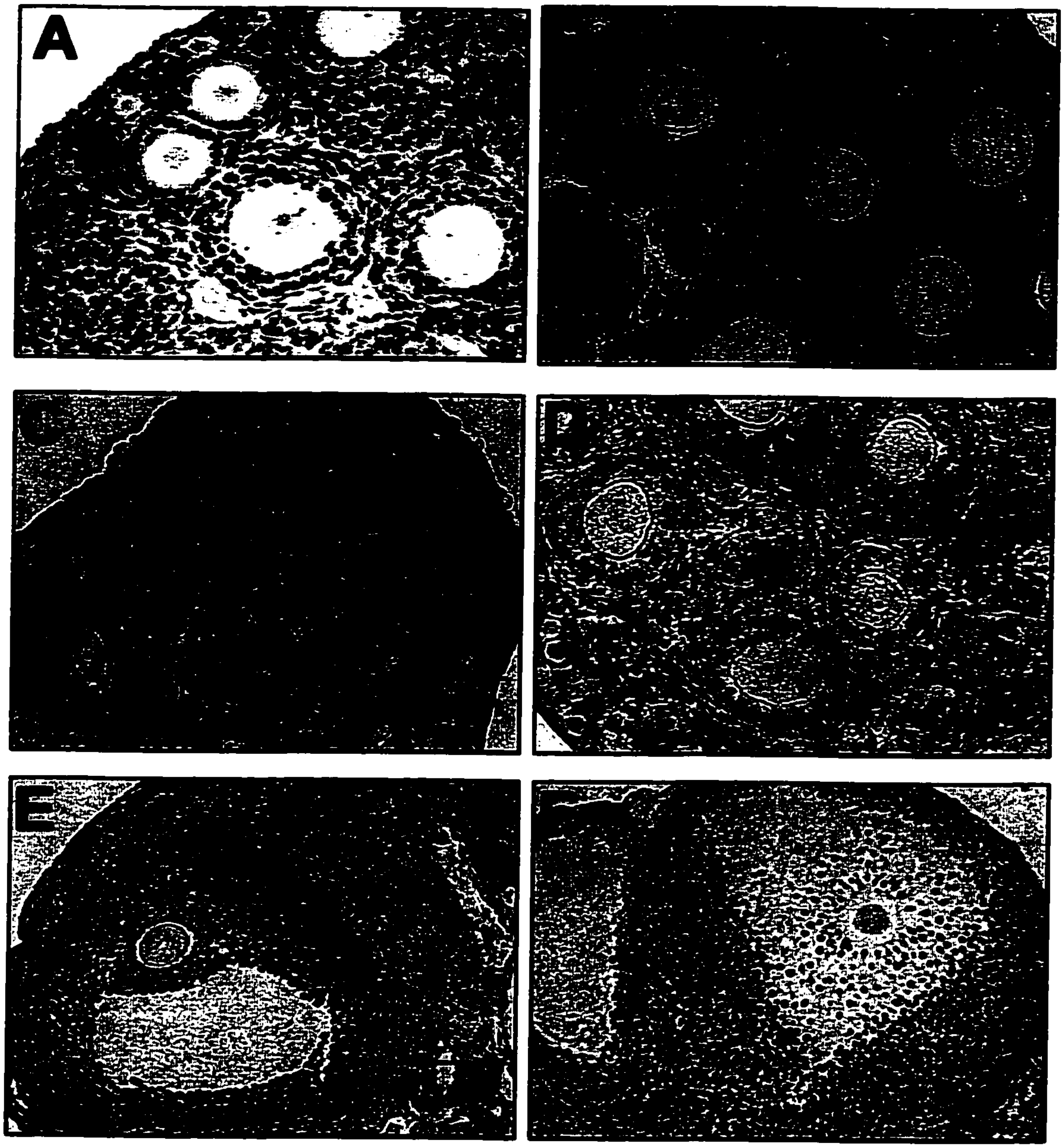
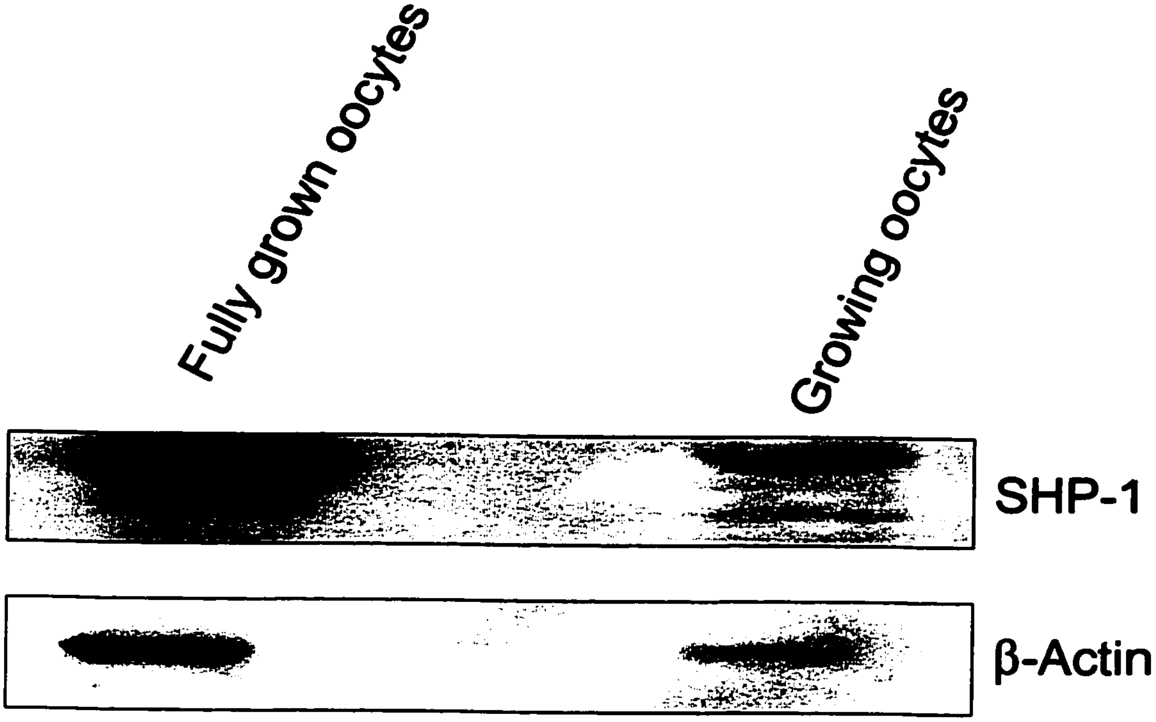

A
E

Figure 14. SHP-1 expression in growing vs. fully grown oocytes.

Five hundred growing and 500 fully grown oocytes, all of which were denuded, were analysed by western blot to compare differences in the levels of SHP-1 expression. Immunodetection of β-actin was also performed on the same blot and those protein levels were compared to SHP-1 expression in order to normalize the data based on the total amount of proteins in each lane. Densitometric analysis revealed that regardless of increased β-actin expression in fully grown oocytes, these germ cells had 1.43-fold more SHP-1 expression than growing oocytes. This data was generated by Claude Barchéchat, a former student in our lab.

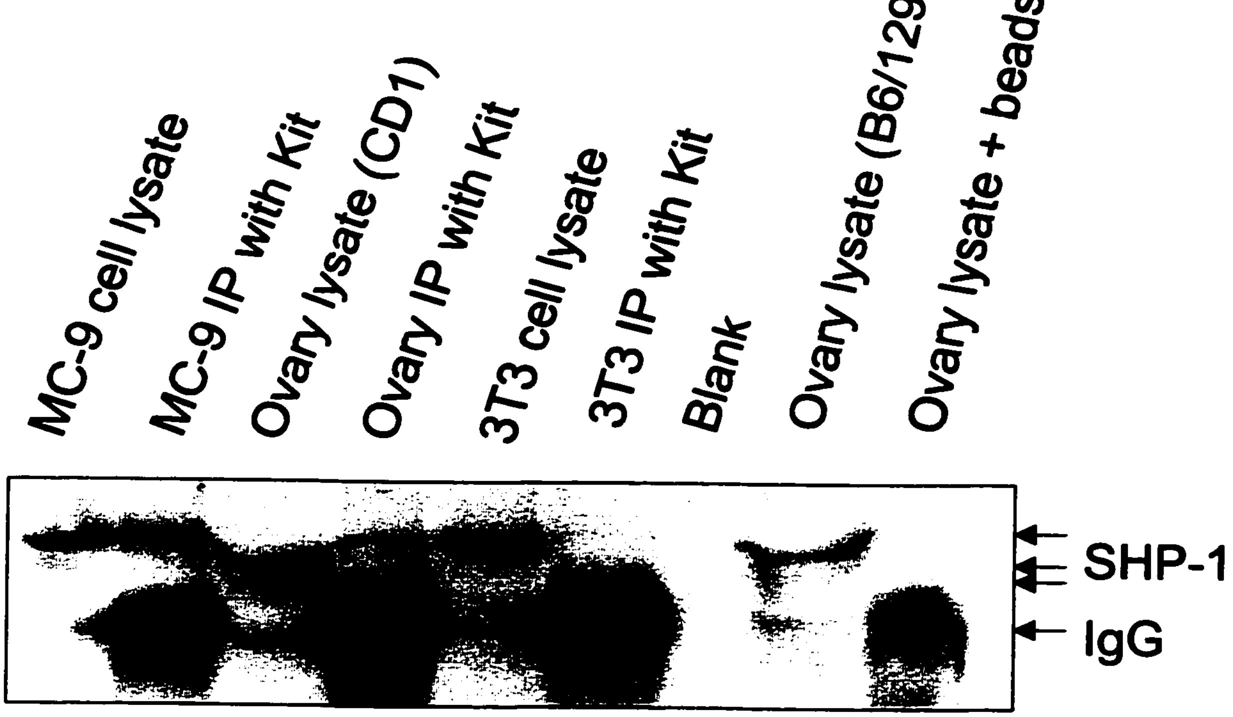

Fully grown oocytes
Growing oocytes
SHP-1
β-Actin

16. SHP-1 and Kit co-immunoprecipitate in the mouse ovary

In hematopoietic cells, Kit and SHP-1 have a known association at tyrosine 569 in the juxtamembrane domain of the Kit receptor (Kozlowski et al., 1998). Here, we have established a direct association between the two proteins in the ovary by immunoprecipitation. MC-9 mast cells which express both Kit and SHP-1 were used as a positive control for the co-immunoprecipitation of these proteins. 3T3 cells served as a negative control since they express SHP-1 but not Kit. Ovary lysate was incubated with beads and with the omission of the Kit antibodies in order to serve as a control for the specificity of the binding. As well, an ovary lysate from another mouse strain was included in the blot to control for the migration of the proteins from that tissue. The ovary lysates showed two isoforms of SHP-1 that migrated at approximately 65 and 68 KDa. The presence of these isoforms is consistent with previously reported findings of differentially expressed splice variants of SHP-1 (Kozlowski et al., 1993; Tsui et al., 1993; Uchida et al., 1993; Yeung et al., 1992). The cell lines showed SHP-1 proteins migrating at approximately 70 kDa. The difference in the rate of migration of SHP-1 is likely due to different salt concentrations in tissue samples versus cell line samples. After immunoprecipitation with Kit antibodies, a SHP-1 band was visible in the MC-9 cells and ovary samples but not in the 3T3 cells indicating that SHP-1 and Kit have a direct binding interaction in the mouse ovary (Fig. 15). These experiments were performed in triplicate and generated similar results with each trial. The reciprocal experiment involving the immunoprecipitation of SHP-1 and western blotting of Kit was not performed due to the lack of good anti-Kit antibodies for western blot analysis.

FIGURE 15. Co-immunoprecipitation of Kit and SHP-1 in mouse ovaries.

Protein lysates were obtained from adult wild-type mice (ovary), 3T3 cells (negative control), and MC-9 mast cells (positive control). Lysates were incubated with Protein A sepharose beads and immunoprecipitated (IP) with anti-Kit antibodies. Subsequently, proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose paper and SHP-1 protein was detected by western blot analysis. SHP-1 and Kit co-immunoprecipitated in ovary and MC-9 lysates, but not in 3T3 lysates. Ovary lysates were incubated with beads and the omission of anti-Kit antibodies to serve as a control for the specificity of the interaction. An ovary lysate from a different strain of mice was used to demonstrate the increased rate of migration in all ovary lysates, compared with lysates derived from cell lines.


MC-9 cell lysate
MC-9 IP with Kit
Ovary lysate (CD1)
Ovary IP with Kit
3T3 cell lysate
3T3 IP with Kit
Blank
Ovary lysate (B6/129)
Ovary lysate + beads
SHP-1
IgG

17. Effect of SHP-1 deficiency on oocyte and follicle development

17.1 Total number of follicles vs. number of growing follicles

Serial sections were obtained from 10-13 d.o. wild-type (n=11) and *motheaten* ovaries (n=11) and stained with H&E. Every third section was stained and the four largest stained sections were used to evaluate the total number of follicles and the number of growing follicles in each section. Follicles were considered growing upon the transition of their squamous pre-granulosa cells to cuboidal granulosa cells and total follicle counts included both growing and primordial follicles. The average number of growing follicles per section was not different between *motheaten* and wild-type ovaries, however the total number of follicles per section was significantly greater in the *motheaten* group (92.1 ± 10.0) compared to wild-type (69.8 ± 11.1) ($p<0.05$) indicating that increased numbers of primordial follicles were present in *motheaten* ovaries (Fig. 16).

17.2 Oocyte Size

Serial sections were obtained from 10-13 d.o. wild-type (n=11) and *motheaten* ovaries (n=11) and all sections were stained with H&E. All growing follicles in the ovaries were included in the evaluation. Each oocyte was measured in the histological section in which its germinal vesicle was visible. *Motheaten* and wild-type oocyte sizes were then compared based on follicle size. Growing oocytes in preantral follicles (no antral follicles were present in these ovaries) had the same distribution of size in wild-type and *motheaten* ovaries (Fig. 17).

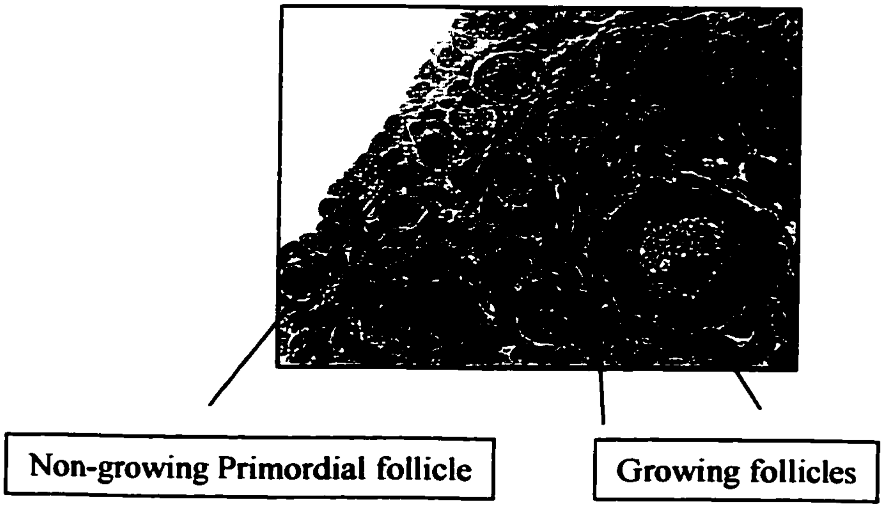
17.3 Granulosa cell proliferation

Serial sections were obtained from 10-13 d.o. wild-type (n-11) and *motheaten* ovaries (n=11) and all sections were stained with H&E. Granulosa cell proliferation was assessed throughout the mouse ovaries. All growing follicles present in the ovaries were included in the evaluation. Each follicle was measured in the histological section in which the germinal vesicle of the enclosed oocyte was visible. *Motheaten* ovaries had a greater number of follicles with three layers of granulosa cells or less ($p<0.05$) and fewer follicles with four layers of granulosa cells or more ($p<0.0001$) (Fig. 18).

FIGURE 16. Quantitative assessments of follicle growth in 10-13 d.o. ovaries.

Wild-type (n=11) and *motheaten* ovaries (n=11) were fixed, cut into serial sections and stained with H&E. Every third section was stained with H&E and the four largest stained sections were used to evaluate the number of growing follicles as well as the total number of follicles (growing and primordial) per section (A). Although the number of growing follicles was the same in both groups, the total number of follicles was greater in the *motheaten* sections than in controls ($p<0.05$) (B). Statistically significant results are denoted by an asterisk.

A


Non-growing Primordial follicle
Growing follicles

B

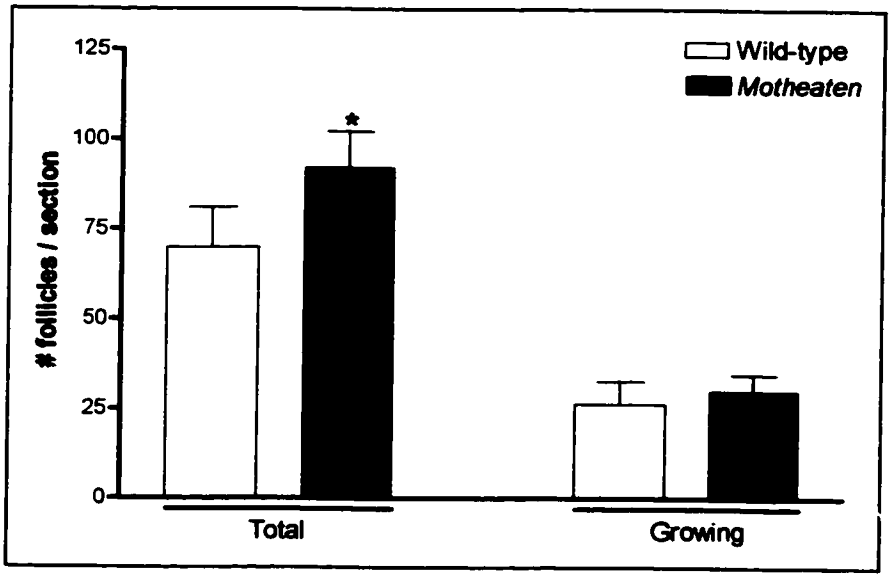
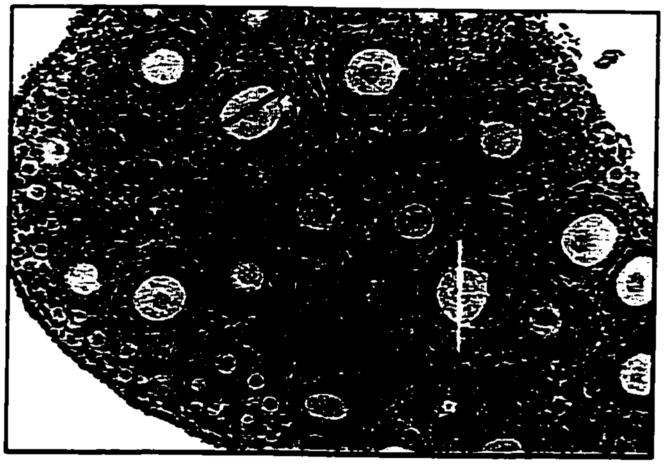

□ Wild-type
■ *Motheaten*
follicles / section
125
100
75
50
25
0
*
Total
Growing

FIGURE 17. Quantitative assessments of oocyte growth in 10-13 d.o. ovaries. Wild-type (n=11) and *motheaten* ovaries (n=11) were fixed, cut into serial sections and stained with H&E. Growing oocytes and their follicles were counted throughout the ovary in serial sections in which the germinal vesicles of the enclosed oocytes were visible (A; oocytes measured in black; follicles measured in white). Follicles were divided according to size and oocyte measurements showed no difference between *motheaten* and wild-type ovaries regardless of follicle size (B).

A



B

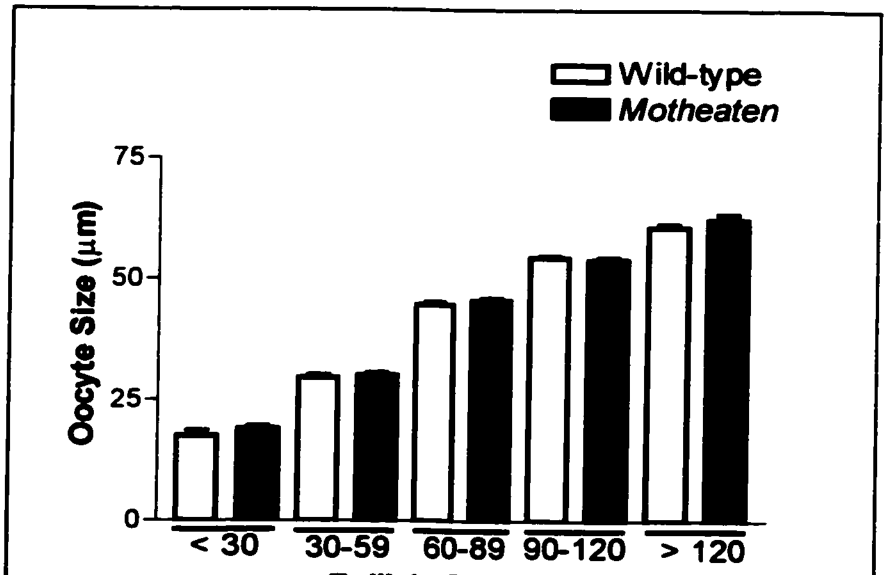
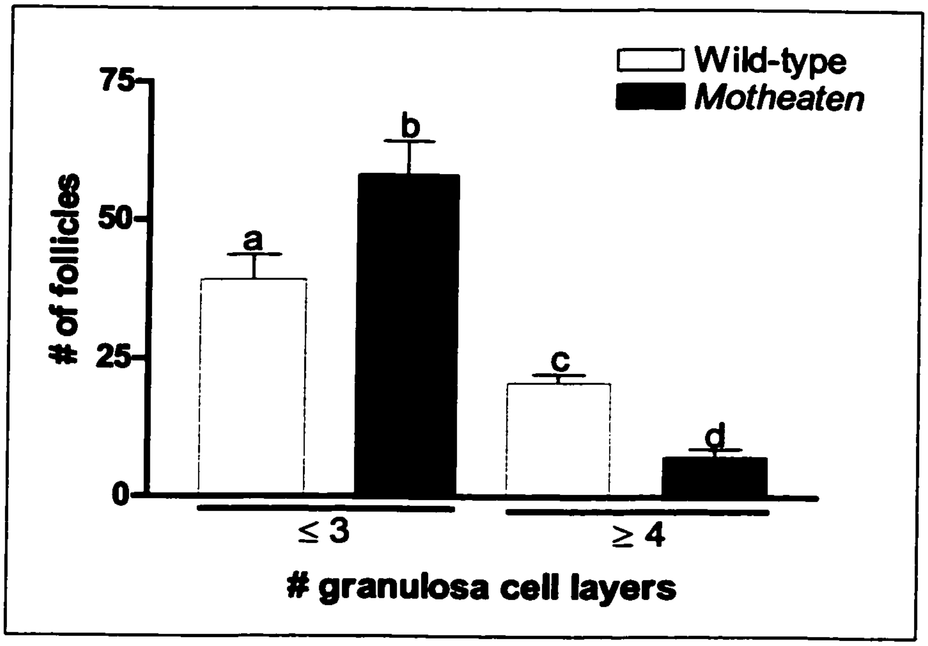

▭ Wild-type
■ *Motheaten*
Oocyte Size (µm)
75
50
25
0
< 30
30-59
60-89
90-120
> 120
Follicle Size (µm)

FIGURE 18. Quantitative assessments of granulosa cell proliferation in 10-13 d.o. ovaries.

Wild-type (n=11) and *motheaten* ovaries (n=11) were fixed, cut into serial sections and stained with H&E. Granulosa cell proliferation was assessed by counting the number of growing follicles containing 3 layers of granulosa cells or less and the number of follicles with four layers or more. In *motheaten* ovaries, there were significantly more follicles with three layers of granulosa cells or less in comparison with wild-type ovaries ($p<0.05$). Similarly, there were fewer *motheaten* ovaries containing follicles with four layers of granulosa cells or more compared to controls ($p<0.0001$). Statistically significant results are denoted different letters.

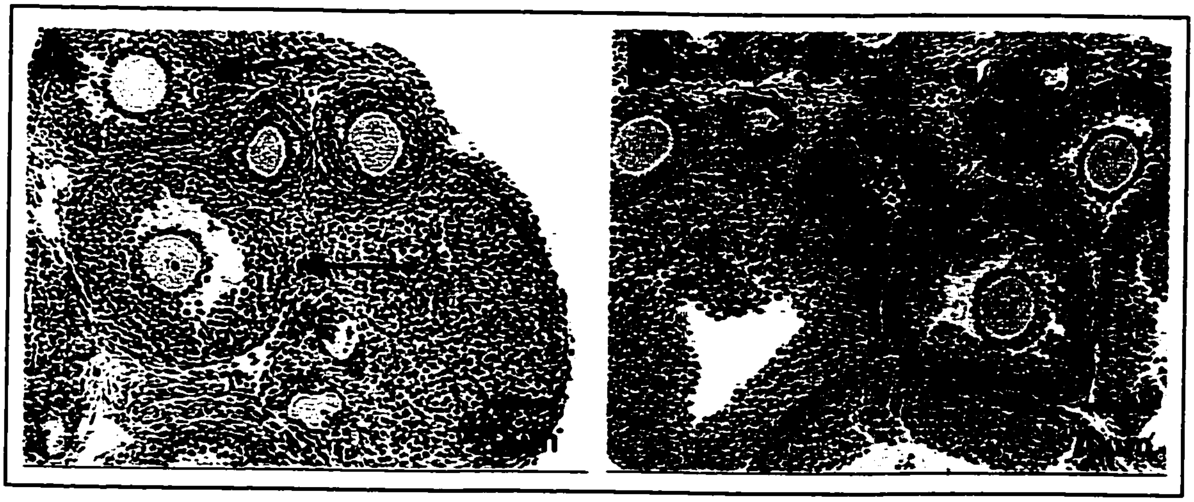

Wild-type
Motheaten
75
50
25
0
# of follicles
a
b
c
d
≤ 3
≥ 4
granulosa cell layers

18. Motheaten ovaries after transplantation

18.1 Phenotype

Unfortunately, complete oocyte and follicle development cannot be studied using the *motheaten* animals because these mice die before puberty. Thus, we transplanted the ovaries of 10-12 d.o. *motheaten* mice and littermate controls under the kidney capsule of ovariectomized SCID mice. Under blinded conditions, oocyte size, follicle size, and the ability to form antra were examined in histological sections of post-transplantation ovaries. *Motheaten* ovaries were fully capable of forming antral follicles (Fig. 19).

FIGURE 19. Phenotype of wild-type and *motheaten* ovaries after transplantation. 10-12 day old wild-type (A) and *motheaten* (B) ovaries were transplanted under the kidney capsule of SCID mice for up to 10 days. The ovaries were then fixed, embedded in paraffin, cut into serial sections and stained with H&E. The arrows indicate the presence of antral follicles. Under blinded conditions, we were unable to distinguish the difference between control and *motheaten* ovaries. Both were able to produce large antral follicles with the appearance of healthy oocytes.



18.2 Follicle Size and distribution

Serial sections were obtained from the transplanted ovaries and were stained with H&E. All antral follicles present in the ovary were included in the evaluation. Each follicle was measured in the histological section in which the germinal vesicle of the enclosed oocyte was visible. There was no difference in the size of antral follicles (> 150 μm) between control (236.9 ± 8.7 μm) (n=7) and *motheaten* ovaries (252.1 ± 10.2 μm) (n=7) (Fig. 20). As well, the distribution of antral follicles in each ovary was the same for control (n=7) and *motheaten* (n=7) ovaries (Fig. 21).

18.3 Oocyte growth

Serial sections were obtained from the transplanted ovaries and were stained with H&E. All antral follicles ≥ 150 μm present in the ovaries were included in the evaluation. Each oocyte was measured in the histological section in which its germinal vesicle was visible. Measurement of oocytes in large antral follicles showed that *motheaten* oocytes (63.0 ± 1.2 μm) (n=7) were significantly larger than their controls (56.1 ± 0.8 μm) (n=7) ($p < 0.0005$) (Fig. 22).

FIGURE 20. Quantitative assessments of follicle growth in *motheaten* ovaries and controls following transplantation.

Eight to ten days following transplantation under the kidney capsule of SCID mice, *motheaten* (n=7) and control (n=7) ovaries were fixed, embedded in paraffin, cut into serial sections and stained with H&E. Each follicle was measured in the histological section in which the germinal vesicle of the enclosed oocyte was visible (A). There was no difference in the size of large antral follicles (≥ 150 μm) between *motheaten* and control ovaries (B).

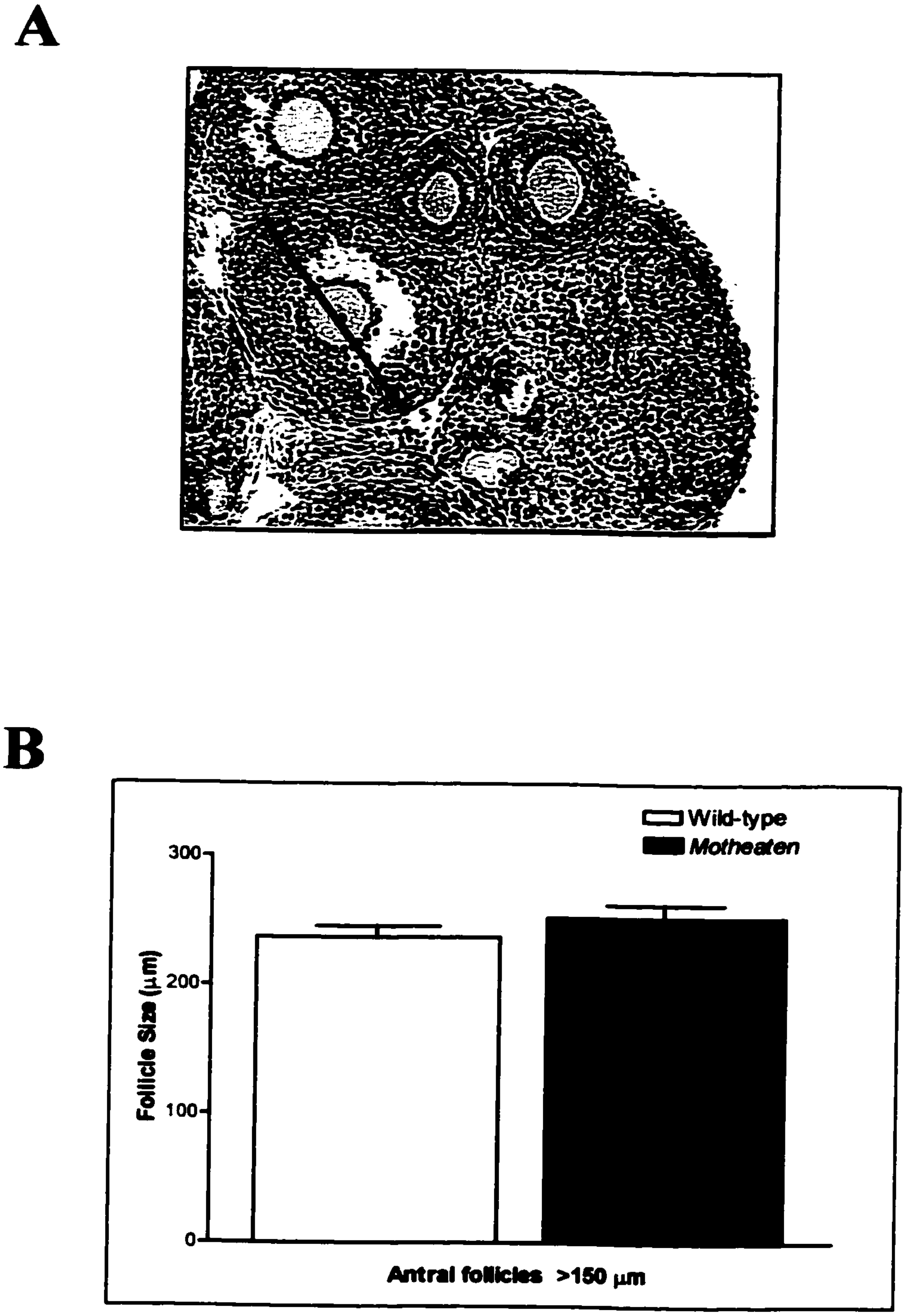
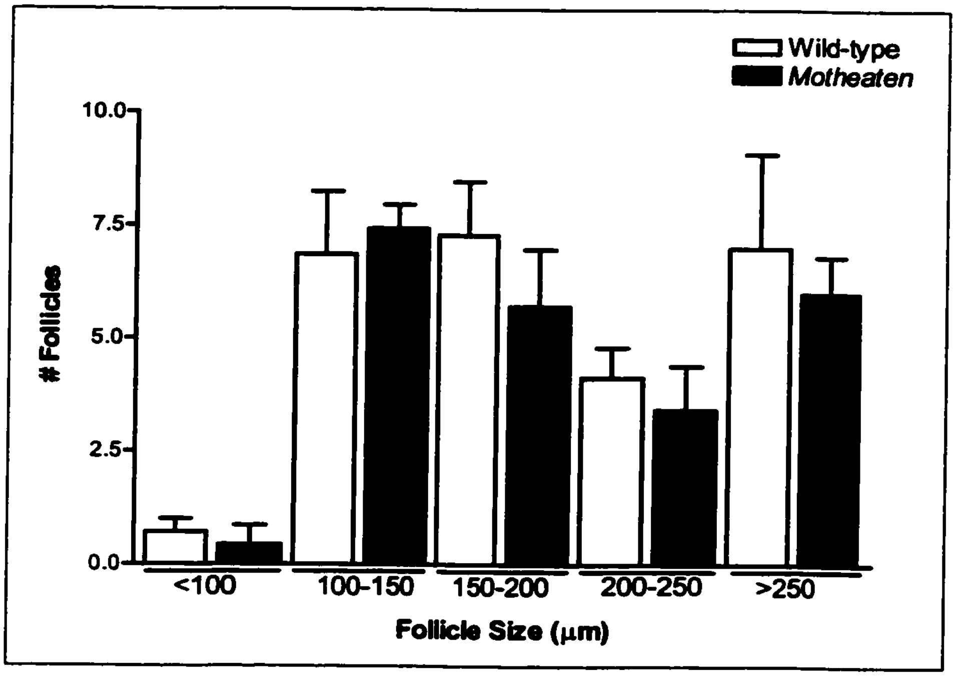

A
B
Wild-type
Motheaten
300
200
100
0
Follicle Size (µm)
Antral follicles >150 µm

FIGURE 21. Quantitative assessments of the distribution of follicle size in *motheaten* ovaries and controls following transplantation.

Eight to ten days following transplantation under the kidney capsule of SCID mice, *motheaten* (n=7) and control (n=7) ovaries were fixed, embedded in paraffin, cut into serial sections and stained with H&E. Each follicle was counted in the histological section in which the germinal vesicle of the enclosed oocyte was visible. There was no difference in the size distribution of antral follicles in *motheaten* and control ovaries.

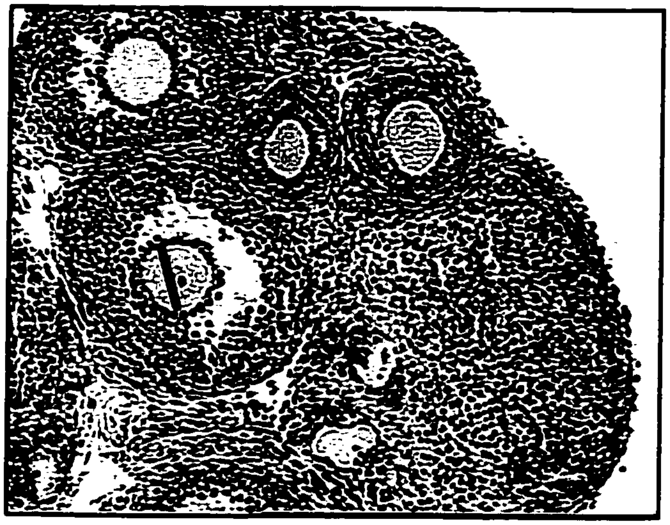


Wild-type
Motheaten
10.0
7.5
5.0
2.5
0.0
Follicles
<100
100-150
150-200
200-250
>250
Follicle Size (μm)

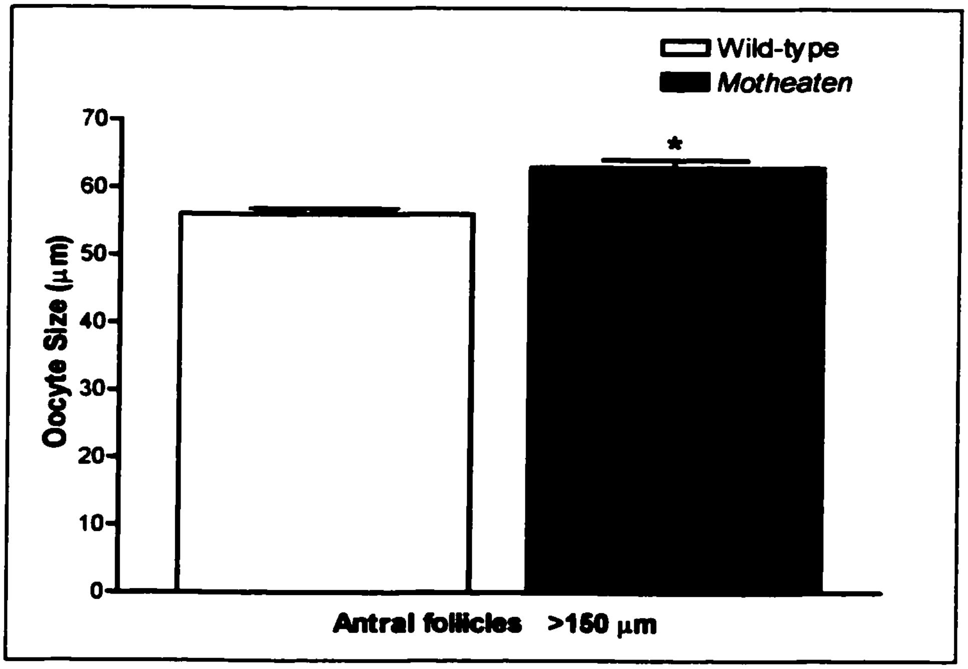
FIGURE 22. Quantitative assessments of oocyte growth in *motheaten* ovaries and controls following transplantation.

Eight to ten days following transplantation under the kidney capsule of SCID mice, *motheaten* (n=7) and control (n=7) ovaries were fixed, embedded in paraffin, cut into serial sections and stained with H&E. Each oocyte was measured in the histological section in which its germinal vesicle was visible (A). *Motheaten* oocytes in large antral follicles ($\geq$ 150 µm) were larger than their controls ($p<0.005$) (B).

A



B


Wild-type
Motheaten
70
60
50
40
30
20
10
0
Oocyte Size (μm)
*
Antral follicles >150 μm

19. In vitro maturation of oocytes

Oocyte-cumulus cell complexes and denuded oocytes from both control (n=7) and *motheaten* (n=7) ovaries were collected after transplantation and their ability to undergo cumulus cell expansion and GVBD was assessed. In the presence of FSH, complexes from both controls and *motheaten* ovaries were able to undergo cumulus expansion. No differences were noticed in the proportion of complexes that were able to undergo expansion. Also, the degree to which the complexes expanded was independent of whether they came from control or *motheaten* ovaries. However, the ability of the complexes to expand was somewhat variable within each group. Similarly, denuded oocytes from both control and *motheaten* ovaries were capable of GVBD. No differences were noticed in the proportion of control or *motheaten* oocytes that were able to undergo GVBD (Table 1). These sets of data were analyzed by Chi square analysis with Yates correction.

TABLE 1. **Cumulus expansion and germinal vesicle breakdown in oocytes grown in transplanted ovaries.**

The relative ability of *motheaten* vs. age-matched control oocyte-cumulus complexes (OCC) and denuded oocytes (DO) from transplanted ovaries to undergo cumulus expansion (CE) and germinal vesicle breakdown (GVBD) following *in vitro* maturation was assessed. The results showed no differences in meiotic maturation between the groups or in the ability of their cumulus cells to undergo expansion. Interestingly, the majority of oocytes that we were able to obtain from the ovaries were denuded rather than surrounded by cumulus cells.

Cumulus expansion and germinal vesicle breakdown in oocytes grown in transplanted ovaries.

	# OCC	# CE	%	# DO	# GVBD	%
Control	39	32	82.1	78	53	67.9
Motheaten	35	32	91.4	102	73	71.6

DISCUSSION

Communication between oocytes and granulosa cells is absolutely essential for the production of a healthy preovulatory follicle with a mature oocyte. The goal of this project was to examine the role of the SHP-1 tyrosine phosphatase in the regulation of Kit activity which is thought to be important for oocyte growth. It is believed that KL, located in the granulosa cells, interacts with the Kit receptors on oocytes to transmit intercellular signals that contribute to oocyte growth (Manova et al., 1990; Horie et al., 1991; Packer et al., 1994). Although these studies examined the relationship between Kit and KL, they did not examine the possible regulation of Kit activity by means of receptor dephosphorylation. In the hematopoietic system, Kit activity is known to be regulated by SHP-1 (Paulson et al., 1996; Kozlowski et al., 1998). The loss of SHP-1 can compensate for mast cell deficiencies observed in *W* mutants (Paulson et al., 1996), therefore we postulated that SHP-1 may play a role in the mouse ovary as a regulator of Kit function in oocytes and that it may contribute to granulosa cell function through its interactions with other tyrosine kinases. Thus, the relationships between SHP-1, Kit and oocyte growth were examined as were the consequences of the absence of SHP-1 on follicle development in *motheaten* mouse ovaries.

The experiments reported here demonstrated that the SHP-1 tyrosine phosphatase was present in all ovarian cell types throughout all stages of follicle development and that SHP-1 co-immunoprecipitated with the Kit receptor in mouse ovaries. This evidence suggested a role for SHP-1 in regulating Kit activity in oocytes as well as other potential functions like meiotic maturation and follicle development. Ovaries from 10-13 d.o.

motheaten animals, which do not express SHP-1, were compared to age-matched wild-type ovaries and revealed differences in mass, granulosa cell proliferation and the number of primordial follicles. Specifically, *motheaten* ovaries were smaller, had fewer layers of granulosa cells in their growing follicles and more primordial follicles than controls.

Although *motheaten* animals displayed a smaller total body mass than controls, their ovaries were disproportionately reduced in size, suggesting a possible role for SHP-1 in the regulation of ovarian functions. However, it is possible that this phenotype is due to the lack of SHP-1 in *motheaten* animals leading to the deterioration in the health of these mice. Interestingly, despite the fact that the *motheaten* ovaries were smaller than their controls, we noted an increase in the number of primordial follicles per section. During embryonic development, Kit signaling is required for PGC proliferation and survival (Mintz and Russell, 1957; Orr-Urtreger et al., 1990; Dolci et al., 1991). During their migration from the genital ridge, PGCs proliferate rapidly to produce a pool of follicles that serve as the only source of adult germ cells. Mice that have mutations at either the *W* or *Sl* loci, thus lacking Kit and KL respectively, show the presence of PGCs during embryonic development, however these germ cells fail to proliferate and subsequently ovaries from these animals have decreased numbers of primordial follicles, if any, at the time of birth. Assuming that the direct association between SHP-1 and Kit may inhibit Kit activity in the ovaries as it does in other systems (Yi and Ihle, 1993; Paulson et al., 1996; Kozlowski et al., 1998), it is not surprising that *motheaten* ovaries contained more primordial follicles since the lack of inhibition of the Kit signaling pathway could allow for increased PGC proliferation and/or survival.

Similarly, enhanced Kit signaling post-natally could confer increased oocyte sizes in growing follicles. Based on the hypothesis that SHP-1 inhibits Kit signaling in mouse oocytes, it was expected that *motheaten* ovaries would contain oocytes of larger sizes than those in wild-type animals. However, 10-13 d.o. *motheaten* animals did not have increased oocyte sizes or increased numbers of growing follicles suggesting that at the preantral stage of development the absence of SHP-1 does not affect the rate or initiation of oocyte growth.

In 10-13 d.o. animals, *motheaten* ovaries showed decreased granulosa cell proliferation as indicated by an increase in small preantral follicles and a decreased number of large preantral follicles. This difference in the number of somatic cells was likely, in part, accountable for the reduction in ovary mass. Although it is possible that the unhealthy physiology of *motheaten* animals contributed to the decrease in granulosa cell proliferation, it is also likely that SHP-1 plays an important role in granulosa cell signaling. In 1999, Russell and Richards demonstrated in rat granulosa cells that SHP-1 was hormonally regulated by estrogen and FSH and was differentially expressed in both cytoplasm and nuclei. A nuclear localization sequence has been identified in SHP-1 and supports the hypothesis of roles for SHP-1 in granulosa cell signaling (Craggs and Kellie, 2001).

As well, SHP-1 is known to interact with a number of kinases such as PI-3K, Janus kinases (JAKs), EGF, as well as with signal transduction activators of transcription

(STATs). These proteins have important functions in the oocyte and granulosa cells and the possibility of associations between SHP-1 and these proteins in the ovary is likely.

PI-3K, present in oocytes and granulosa cells, is known to be an important factor in many cellular functions including anti-apoptosis and is likely part of the downstream pathway from Kit leading to oocyte growth. Mice that have PI-3K mutations show impaired follicle development at the cuboidal stages (Kissel et al., 2000). Interestingly, in T lymphocytes, SHP-1 has been shown to dephosphorylate PI-3K, a process which results in decreased PI-3K enzyme activity (Cuevas et al., 1999). It is possible that this association also exists in the ovary. Such a relationship has not yet been investigated and could further contribute to our understanding of the regulation of cell survival and/or other ovarian cell functions. However, it is difficult to study Kit signaling in oocytes due to the limited number of cells and the fact that they are not mitogenic.

Similarly, EGF receptor signaling is known to be important for granulosa cell proliferation (Liang et al., 1994) and meiotic maturation (Lonergan et al., 1996). In 3T3 and COS cells, SHP-1 has the ability to dephosphorylate the EGF receptor and inhibit its signaling (Tenev et al., 1997). Another study has shown that transfection of A431 cells with SHP-1 results in EGF receptor dephosphorylation and subsequently causes decreased DNA-binding by STATs (Tenev et al., 2000), proteins that have been shown by others to play a role in the transcriptional regulation of steroid receptors (Stoecklin et al., 1999). However, there has also been evidence of a stimulatory role for SHP-1. In 1996, Su et al. demonstrated that SHP-1 could exert a positive effect on EGF-stimulated mitogenic signaling in 293 cells. Thus, it would be very interesting to examine the relationship between SHP-1 and EGF receptors and/or the JAK-STAT pathway in the mammalian ovary to determine whether or not SHP-1 may directly regulate granulosa cell proliferation and perhaps even the transcription of steroid receptors.

In the present study, although the *motheaten* animals were very useful tools in observing *in vivo* consequences of the lack of SHP-1, they also presented several obstacles to our understanding of Kit regulation in ovaries: 1) the mice are unhealthy, 2) they do not survive beyond 3 weeks of age, 3) oocyte and follicle development beyond the preantral stage cannot be examined. Therefore ovary transplantation was used to examine the consequences of the absence of SHP-1 on later stages of oocyte and follicle growth. Ovaries of 10-12 d.o. *motheaten* and age-matched control mice were transplanted under the kidney capsule of SCID mice for up to 10 days and the phenotypic changes were examined histologically. The transplanted ovaries were presumed to be exposed to high gonadotropin conditions because the SCID recipients were ovariectomized. This environment was selected to provide ample stimulation for follicle development, as has been reported previously (Eppig and Wigglesworth, 2000; Liu et al., 2000).

The results showed that ovaries from *motheaten* animals were capable of achieving antrum formation and of producing large mature follicles comparable to controls. Thus, in comparison with 10-13 d.o. ovaries, those that were transplanted to a healthy recipient SCID mouse showed normal granulosa cell proliferation regardless of the absence of SHP-1 suggesting that either SHP-1 may not be required for granulosa cell proliferation and antrum formation or that the downregulation of SHP-1 may be required for these functions to progress normally.

SHP-1 may be involved in the signaling pathways contributing to or inhibiting granulosa cell proliferation but *in vivo* effects may not be evident due to compensatory pathways within the ovary itself. Assuming that SHP-1 is required for granulosa cell proliferation, it is possible that the transplanted ovaries received sufficient growth factors from the kidney to override the SHP-1 deficiency and promote further follicle development. The mammalian kidney is known to secrete numerous growth factors, including EGF and hepatocyte growth factor (HGF) (Ruff et al., 1997) and it is possible that the transplanted ovaries received abundant quantities of these proteins via the extensive vascularization from the renal cortex. However, the fact that antrum formation and granulosa cell proliferation were not impaired after transplantation does suggest that either SHP-1 was not required for these functions or that endocrine signals from the kidney or the elevated levels of gonadotropins were able to rescue the ovaries and stimulate further development.

However, the more likely scenario is that follicle development may normally require suppression of SHP-1 in which case it is not surprising that follicle development progressed normally in the absence of the phosphatase. Evidence in rats has shown that FSH and estrogen stimulation decrease SHP-1 mRNA in granulosa cells (Russell and Richards, 1999). Thus, assuming no species variability, late in follicle development, when gonadotropin stimulation is high, SHP-1 is likely suppressed. Therefore, in the transplanted ovaries, which are subjected to high gonadotropin stimulation, it is also likely that SHP-1 levels in the control ovaries were suppressed and it is not surprising that no differences were observed in the follicle development of control or *motheaten* ovaries.

Although no differences were noted in the number of antral follicles and the distribution of follicle size, ovaries from the *motheaten* animals had larger oocytes in their antral follicles than controls. This information suggests that SHP-1 may be involved in regulating the termination of oocyte growth. Futhermore, preliminary evidence from our lab (generated by Claude Barchéchat) showed that fully grown oocytes expressed SHP-1 in higher levels than growing oocytes suggesting that SHP-1 may play a more important role in fully grown oocytes. This evidence also reinforces a role for SHP-1 in the termination of oocyte growth. One possible explanation to account for the larger oocytes from transplanted *motheaten* ovaries involves the hypothesis that inhibition of Kit signaling is normally required for the termination of oocyte growth and that it is not inhibited within the oocytes of *motheaten* animals which in turn may result in increased oocyte size in large antral follicles of *motheaten* ovaries. The fact that, in the absence of SHP-1, the initiation of oocyte growth, as well as the rate of growth itself were unaffected before transplantation whereas oocyte size was increased in large antral follicles after transplantation suggests that SHP-1 may be most active towards the end of oocyte growth and/or that Kit signaling requires negative regulation by SHP-1 for the termination of oocyte growth.

Another explanation for enhanced Kit activity could be an increase in KL expression in *motheaten* granulosa cells from large antral follicles. Fully grown oocytes have recently been shown to secrete a factor that suppresses KL expression in granulosa cells isolated from both preantral and antral follicles (Joyce et al., 2000). In addition, recombinant GDF-9 has been shown to decrease KL expression in granulosa cells (Joyce et al., 2000). Therefore, GDF-9 secreted from fully grown oocytes may suppress KL expression and contribute to decreased oocyte growth. We suspect that the increased oocyte size in the *motheaten* ovaries is due to enhanced Kit signaling. If this model is correct, we can speculate that ovaries from the *motheaten* animals may have increased or maintained KL expression due to undefined signaling cascades in the granulosa cells or possibly as a result of diminished GDF-9 secretion from oocytes. Another possibility is that the theca cells may be involved in the regulation of KL. Theca cells, on their own and in response to hCG, produce HGF which has been shown to stimulate KL expression in bovine granulosa cells (Parrott and Skinner, 1998). As mentioned above, the transplanted ovaries were well vascularized under the kidney capsule and it is likely that they received stimulation from the kidney in the form of growth factors like HGF. It is possible that SHP-1, present in theca cells, may normally inhibit HGF receptors or interfere with their downstream signals thus preventing or decreasing KL production. Therefore, consistent with the notion that SHP-1 may be required for the termination of oocyte growth, the absence of this phosphatase in *motheaten* animals could result in increased oocyte growth due to impaired regulation of granulosa cell KL production by the theca cells.

Finally, in order to assess the quality of the oocytes grown in the absence of SHP-1, we obtained oocytes from the transplanted *motheaten* and control ovaries and subjected them to *in vitro* maturation. No differences were observed in their ability to undergo GVBD or cumulus expansion in response to FSH suggesting that under favorable conditions SHP-1 is not required for these functions. However, it is possible that meiotic maturation and cumulus expansion normally require suppression of SHP-1. As mentioned above, FSH suppresses SHP-1 in rat granulosa cells (Russell and Richards, 1999) and since the *in vitro* maturation requires FSH, it is not surprising that no differences were observed in the ability of *motheaten* and control complexes and oocytes to undergo expansion and GVBD respectively. Preliminary attempts to fertilize the matured oocytes were complicated by high rates of parthenogenic cleavage and fragmentation in both control and *motheaten* groups, thus indicating that the conditions require further optimization. It would be very interesting, once the conditions are optimized, to determine if these oocytes are fertilizable and whether or not they can progress to the blastocyst stage, both of which are good indicators of the developmental capacity of the oocytes.

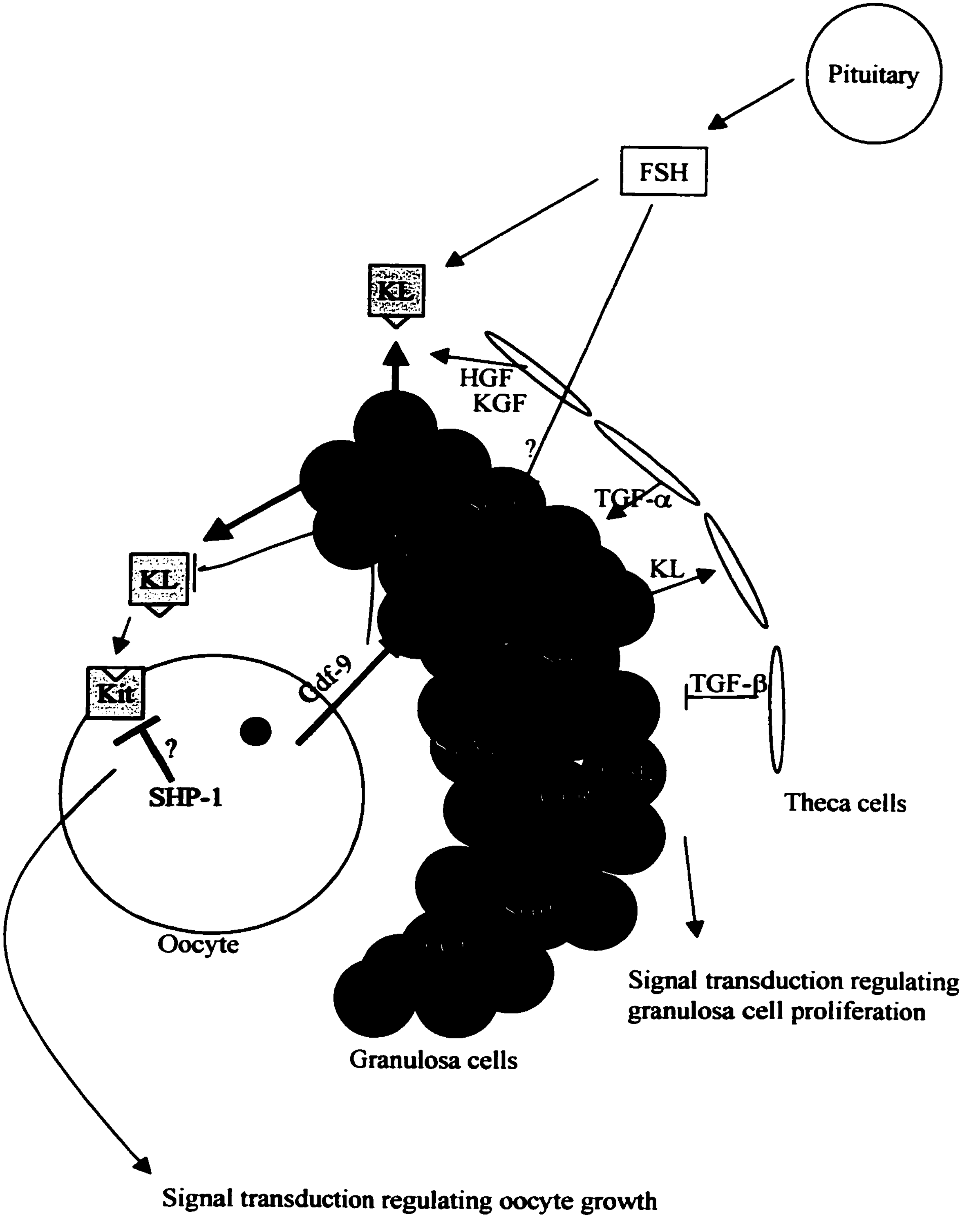
Oocyte growth and follicle development involve the complex interplay of numerous stimulatory and inhibitory factors. This balance is exquisitely fine-tuned *in vivo* in order for the ovaries to achieve mature and healthy preovulatory follicles. Although a role for SHP-1 in mouse granulosa cells has not yet been defined, it is likely that SHP-1 has other functions in the ovary since it is known to interact with ovarian factors like the EGF receptor (Tenev et al., 2000), STATs (Ram and Waxman, 1997), and

PI-3K (Yu et al., 1998; Cuevas et al., 1999) (Fig. 23). The fact that SHP-1 is differentially expressed and hormonally controlled in rat granulosa cells (Russell and Richards, 1999) as well as the presence of SHP-1 in all cell types of the ovary also support the hypothesis that SHP-1 likely has multiple roles in the ovary that remain to be elucidated.

In this study, we demonstrated the expression of SHP-1 in mouse ovaries and proposed that SHP-1 negatively regulates Kit activity, thus oocyte growth, and that this phosphatase may play other important roles in the regulation of follicle development. This hypothesis was supported by the increased number of primordial germ cells and increased oocyte size in animals lacking SHP-1. It was further supported by the co-immunoprecipitation of Kit and SHP-1 in wild-type ovaries. As well, the fact that the oocytes in large antral follicles were larger in *motheaten* ovaries in comparison to controls as well as the fact that increased SHP-1 expression was detected in fully grown oocytes compared to growing oocytes supports the hypothesis that SHP-1 may be required for the termination of oocyte growth.

FIGURE 23. Possible interactions between SHP-1 and tyrosine kinases in the follicle.

Although this research project was mainly focuses on the regulation of Kit signaling by the SHP-1 phosphatase, the are many possible interactions between SHP-1 and other tyrosine kinases, such as PI-3K and the EGF receptor, in the ovary. This model postulates potential interactions between SHP-1 and estrogen (E_2), STATs and growth factors that have not yet been addressed in the ovary whose study could reveal interesting regulatory functions for the phosphatase.


Pituitary
FSH
KL
HGF
KGF
?
TGF-α
KL
KL
Kit
Gdf-9
?
SHP-1
TGF-β
Theca cells
Oocyte
Granulosa cells
Signal transduction regulating granulosa cell proliferation
Signal transduction regulating oocyte growth

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