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The Regulation of Kit Ligand Expression in Rat Granulosa Cells By Ovarian Factors

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THE REGULATION OF KIT LIGAND EXPRESSION IN RAT GRANULOSA CELLS BY OVARIAN FACTORS

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Submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

The aim of this research was to clarify the actions and interactions between factors implicated in the regulation of kit ligand (KITL) expression in rat granulosa cells by measuring *Kitl1* and *Kitl2* mRNA levels through quantitative PCR. Our findings included the observation that induction of the transcripts in primary granulosa cells by follicle-stimulating hormone (FSH), was not mimicked by dibutyryl cAMP and forskolin in spontaneously immortalized granulosa cells (SIGC). Transforming growth factor-β1 (TGF-β1) suppressed *Kitl1* and *Kitl2* mRNA levels in primary granulosa cells and SIGC - an effect mediated via Smad2 and Smad3. Exposure of primary granulosa cells to estrogen *in vivo*, via diethylstilbestrol (DES)-priming of immature rats and *in vitro*, by culturing in the presence of 17β-estradiol, inhibited *Kitl1* and *Kitl2* mRNA expression, an effect mediated via TGF-β1. Therefore, three levels of control are involved in regulating *Kitl* mRNA expression in the ovary: gonadotropins, local ovarian factors and steroid hormones.

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LIST OF ABBREVIATIONS

ACK-2	KIT neutralizing antibody
Akt	signalling molecule downstream phosphatidylinositol 3'-kinase
ALK	activin-like receptor kinase
ATP	adenosine triphosphate
3β-HSD	3β-hydroxysteroid
17β-HSD	17β-hydroxysteroid dehydrogenase
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CRE	cyclic adenosine monophosphate-responsive element
CREB	cyclic adenosine monophosphate-responsive element binding protein
COC	cumulus-oocyte cell complexes
dbcAMP	dibutyryl cyclic adenosine monophosphate
DES	diethylstilbestrol
DMEM/F12	Dulbecco's modified Eagle's medium/F12
DMSO	dimethylsulfoxide
ER	estrogen receptor
ERK1/2	extracellular signal-regulated kinase1/2
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gdf9	growth/differentiation factor-9 gene or mRNA
GDF-9	growth/differentiation factor-9 protein
GnRH	gonadotropin-releasing hormone
GSK-3	glycogen synthase kinase-3
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H-89	inhibitor of cAMP-dependent protein kinase A
HCl	hydrochloric acid
hCG	human chorionic gonadotropin

IgY	immunoglobulin Y
kb	kilobase
kDa	kiladaltons
KGF	keratinocyte growth factor
Kit	kit ligand receptor gene or mRNA
KIT	kit ligand receptor protein
Kitl1	soluble kit ligand gene or mRNA
Kitl2	membrane-bound kit ligand gene or mRNA
KITL-1	soluble kit ligand protein
KITL-2	membrane-bound kit ligand protein
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LIF	leukemia inhibitory factor
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
MAPK	mitogen-activated protein kinase
MGF	mast cell growth factor
mRNA	messenger RNA
MSK	mitogen and stress activated kinases
ng	nanogram
nm	nanometer
nM	nanomolar
OGC	oocyte-granulosa cell complexes
P	probability
PAGE	polyacrylamide gel electrophoresis
p450arom	cytochrome p450 aromatase enzyme
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGC	primordial germ cells
PI3K	phosphatidylinositol 3'-kinase
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PKI	PKA-selective inhibitory peptide
PMSG	pregnant mares' serum gonadotropin

p450scc	cytochrome p450 cholesterol side-chain cleavage enzyme
p70S6K	p70 ribosomal S6 protein kinase
QPCR	quantitative polymerase chain reaction
RPM	revolutions per minute
R-Smads	receptor-regulated Smads
RT	reverse transcription
SC1494	KIT neutralizing antibody
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SIGC	spontaneously immortalized granulosa cells
Sl	Steel loci; encodes *Kitl* gene
18S rRNA	18S ribosomal RNA
StAR	steroid acute regulatory protein
TβRI	TGF-β receptor type I
TβRII	TGF-β receptor type II
TBST	tris-buffered saline tween
TGF-β	transforming growth factor-β
U	units
UV	ultraviolet
V	volts
W	White spotting loci; encodes *Kit* gene

CHAPTER 1 - INTRODUCTION

1.1 Ovarian Follicle Development Overview

The assembly of the primordial follicle early in mammalian ovarian development and the subsequent stages through which it transitions to eventually become an ovulatory follicle or to undergo degeneration are critical processes for determining the reproductive capacity of a female. This process begins with the formation of primordial germ cells (PGC) during the early stages of embryonic development. PGC migrate from the extra-embryonic mesoderm and through the hindgut to eventually colonize the gonadal ridge, undergoing mitotic divisions throughout their path (Ginsburg et al., 1990). Once situated in the developing ovary, proliferation of the PGC ceases and the entire population enters prophase of the first meiotic division (meiosis I) - a unique cell division that occurs exclusively in gametes (Ginsburg et al., 1990; McLaren and Southee, 1997). This change from mitotic proliferation into the first meiotic division defines the transition from oogonia to oocytes (McLaren and Southee, 1997).

During meiosis I, homologous chromosomes segregate and fetal oocytes arrest at the diplotene stage of meiotic development, the final stage of prophase I of meiosis (Hartshorne et al., 1994). Oocytes arrested at prophase I are characterized by an intact nuclear membrane and the nucleus is called a germinal vesicle (GV) (Hartshorne et al., 1994). Completion of the first meiotic division will occur only after the oocyte and the surrounding ovarian follicle have undergone extensive growth (McGee and Hsueh, 2000).

In rodents, follicle formation and development occur fairly synchronously during the first few days after birth (McGee and Hsueh, 2000). During the first phase of

development, individual, meiotically-arrested oocytes become surrounded by an incomplete layer of flattened precursors of the granulosa cells (pre-granulosa cells), generating a large pool of dormant primordial follicles (McGee and Hsueh, 2000). The oocytes that do not become surrounded by follicular somatic cells undergo atretic degeneration (Hsueh et al., 1994). Intraovarian and/or other unknown factors stimulate only some primordial follicles to initiate growth. These initially quiescent primordial follicles are recruited in a continuous manner into the growing follicle pool (McGee and Hsueh, 2000). Their granulosa cells develop a cuboidal appearance and form a complete layer around the oocyte, which in turn begins an extensive growth phase (McGee and Hsueh, 2000). A glycoprotein layer, called the zona pellucida, is laid down around the oocyte shortly after initiation of follicular growth (Wassarman et al., 1996). An extensive network of adherent junctions and gap junctions metabolically couples the granulosa cells with each other and with the oocyte, providing a mechanism for coordinating their function (Grazul-Bilska et al., 1997).

As follicles progress to the secondary stage, multiple layers of granulosa cells form, generating a stratified epithelium around the oocyte now at mid-growth, and beginning the second developmental phase to produce the actively growing secondary follicle (McGee and Hsueh, 2000). At this stage of development, follicles also gain an independent blood supply and consequently become directly exposed to factors circulating in the blood (Gougeon, 1996). Some stromal cells near the basal lamina, a basement membrane that envelopes the granulosa cells separating them from the surrounding stromal elements, become aligned parallel to each other giving rise to a layer of theca cells (Gougeon, 1996). As the follicle enlarges, this theca stratifies and

differentiates in two parts: the theca externa, the outer part that consists of cells forming the external cellular envelope of the follicle; the theca interna, in the inner part, which consists of some fibroblast-like precursor cells that assume the appearance of typical steroid-secreting cells (Gougeon, 1996). From the time of the appearance of these cells, the secondary follicle is defined as a preantral follicle. At the end of the preantral stage, the oocyte has almost completed its growth, but it is unable to resume meiosis I to progress beyond the diplotene stage and is therefore referred to as meiotically incompetent (McGee and Hsueh, 2000).

As this proliferative phase ends, unknown signals initiate the formation of a fluid filled cavity, termed the antrum, within the granulosa cell layers and the follicle becomes known as early antral (Salustri et al., 2006). The formation of this structure divides the population of granulosa cells into two main groups: cumulus cells, closely associated with the oocyte forming the cumulus-oocyte cell complex (COC), and mural granulosa cells, in the periphery lining the follicular wall (Salustri et al., 2006). Furthermore, the mural granulosa cells acquire differing morphological and functional properties depending on their location in the follicle. Those located most peripherally and adjacent to the basement membrane elongate, stop proliferating, synthesize enzymes involved in estrogen production, and acquire luteinizing hormone (LH) receptors (LHR; Channing et al., 1981). In contrast, the innermost (periantral) layers lining the antrum and the cumulus cells remain polyhedral, continue to proliferate, show limited steroidogenic activity, and acquire fewer LHR (Channing et al., 1981). The transition from the preantral to the antral stage is a significant point in time for the oocyte during which it acquires the capacity to resume meiosis (Hartshorne et al., 1994).

Despite the significant developmental achievements, most of the follicles produced up to this antral stage undergo atretic degeneration (Hsueh et al., 1994). Of the population that remains, a cohort may be recruited in response to a critical threshold of circulating follicle-stimulating hormone (FSH) (Gougeon, 1996; McGee and Hsueh, 2000). This third developmental phase of FSH-dependent progression can only be initiated after pubertal onset (Gougeon, 1996; McGee and Hsueh, 2000). Among this group of preovulatory follicles, additional proliferation of the granulosa cell layers and fluid accumulation in the antrum results in increased follicular diameter. Within this cohort, certain preovulatory follicles are apt to grow faster than the rest to become the dominant Graafian follicles (singular in human) that respond to a systemic surge of LH and undergo LH-responsive maturation (McGee and Hsueh, 2000).

Following the preovulatory (mid-cycle) LH surge, granulosa cell proliferation terminates and the fully grown oocyte of the Graafian follicle completes meiosis I. Resumption of meiosis is characterized by GV breakdown (GVBD), chromosome condensation, the assembly of a bipolar metaphase I spindle, and extrusion of the first polar body (Hartshorne et al., 1994). As oocytes proceed through to metaphase of meiosis II, a second meiotic spindle is assembled (Hartshorne et al., 1994); oocytes then remain arrested in meiosis II until fertilization. The cumulus cells start to secrete hyaluronic acid, a non-sulphated glycosaminoglycan (Salustri et al., 1989). When the hyaluronic acid becomes hydrated, spaces between cumulus cells become enlarged and the process of cumulus expansion or mucification takes place as the cells become embedded in a sticky, mucified matrix (Salustri et al., 1989). About the time the oocyte reaches metaphase of the second meiotic division and the cumulus cells are uniformly

dispersed in the matrix, the follicle wall breaks and the expanded COC is extruded from the ovary. The mural granulosa cells together with the theca cells undergo luteinization and form the corpus luteum, which produces the high levels of progesterone required for successful implantation of the embryo and maintenance of pregnancy (Gougeon, 1996). In *figure 1* the transition of the ovarian follicle throughout the major developmental stages is demonstrated.

1.2 Follicle-Stimulating Hormone

FSH is the primary pituitary glycoprotein hormone essential for the regulation and mature function of the ovary. As shown in *figure 2*, its production is stimulated by the action of gonadotropin-releasing hormone (GnRH) from the hypothalamus on the pituitary gland. Within the ovary, FSH interacts with its target tissue, the granulosa cells, to which specific receptors for this gonadotropin have been localized (Gougeon, 1996). The primary mechanism for FSH mode of action is by stimulating its membrane-associated receptor (FSHR), part of a large family of transmembrane receptors that regulate the heterotrimetric G proteins (Richards, 1994). The post-receptor signalling system that relays FSH action into the cell nucleus rests mainly on adenylate cyclase stimulating an increase in cyclic adenosine monophosphate (cAMP) production and activation of cAMP-dependent protein kinase A (PKA).

FSH binds to its receptor with high affinity initiating a conformational change that activates the G-proteins and results in the activation of the adenylate cyclase enzyme to generate cAMP from adenosine triphosphate (ATP; Richards, 1994). Elevation of intracellular cAMP levels causes binding of cAMP to the regulatory subunits of PKA, a

Figure 1. **Ovarian Follicle Development**

This figure illustrates the developmental process that a primordial follicle undergoes once it is recruited into the pool of growing follicles. As the granulosa cells proliferate and gradually differentiate, attaining the capacity for steroid hormone production, supporting structures, such as blood vessels and theca cells are also formed, while the meiotically-arrested oocyte grows in preparation for attaining meiotic competence.

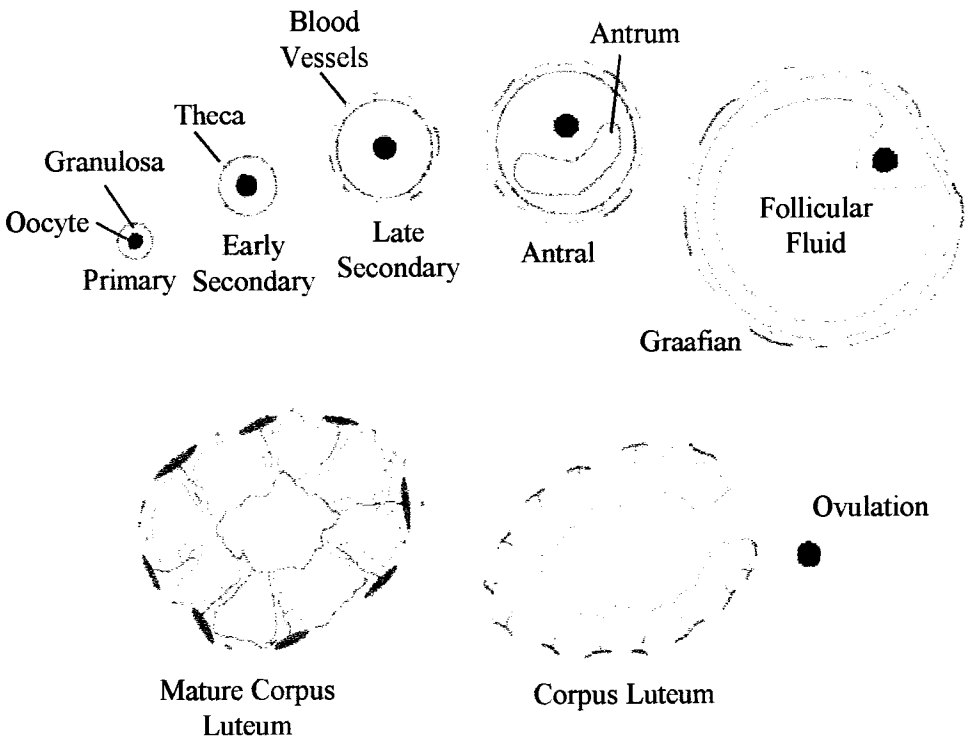
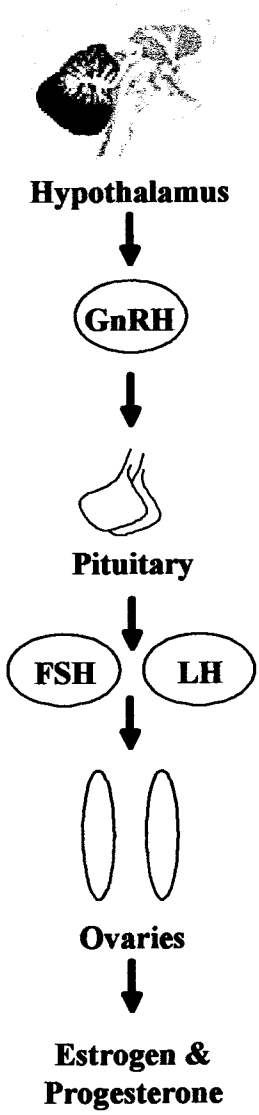


Figure 2. **The Hypothalamic-Pituitary-Gonadotropin Axis**

This figure is an illustration of the hypothalamic-pituitary-gonadotropin axis. The hypothalamus in the brain produces GnRH which targets the pituitary gland and stimulates the production and secretion of FSH. In turn, FSH acts on the granulosa cells in the ovary to bring about the many changes its actions are associated with.



cytosolic, tetrameric holoenzyme that is composed of two regulatory subunits associated with two catalytic subunits. This leads to a dissociation of the tetrameric complex, thus allowing the free catalytic subunit to be active as a serine/threonine kinase in the cytoplasm and nucleus. The catalytic subunits can then affect cell physiology via phosphorylation of a wide variety of substrates, such as the transcription factor cAMP-responsive element (CRE) binding (CREB) protein (Richards, 1994), which can in turn bind to upstream DNA regulatory sequences to regulate gene activity. Therefore, FSH action within the ovary is mediated by stimulation of granulosa cells via the FSHR and by the actions of the intracellular cAMP that is generated (*figure 3*).

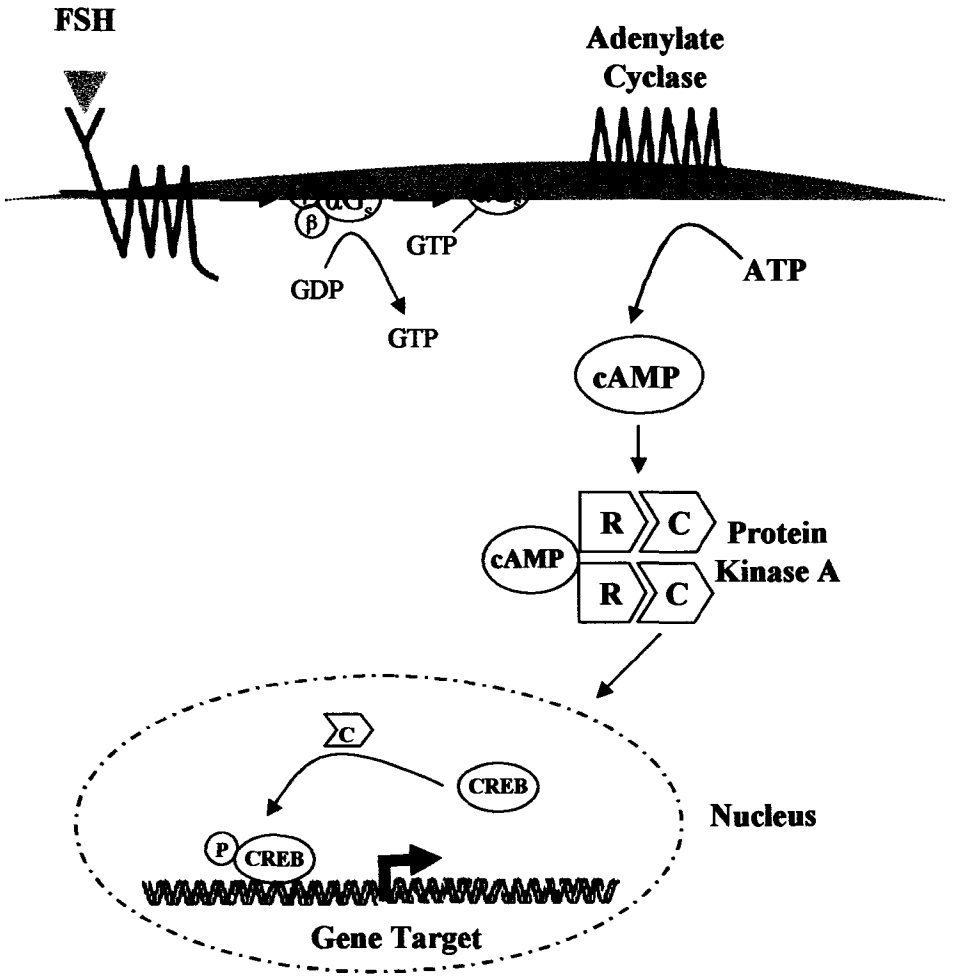
A secondary rise in plasma FSH is the underlying mechanism for the process of dominant follicle growth and development to the preovulatory stage. Progressively, FSH accumulates in the follicular fluid in the microenvironment of the dominant follicle (while its levels remain low or undetectable in the non-dominant cohort of follicles), providing an obligatory stimulus for selection. In response to FSH stimulation, a sustained period of high proliferation of the granulosa cells is observed, a characteristic of the developing dominant follicle. In addition to proliferating, granulosa cells in antral and preovulatory follicles undergo extensive cytodifferentiation through activation of genes required for these processes, such as attaining the ability for LH receptor expression. The potential to aromatize androgens to estrogens through FSH induction of cytochrome p450 aromatase (p450arom) expression, seen only in the granulosa cells of the dominant follicle, is also acquired (Richards, 1994). The physiological mechanism by which the dominant follicle produces estradiol is called "the two-cell two-gonadotropin" concept. The action of LH on theca cells leads to the synthesis and

Figure 3. **The Traditional FSH-Regulated Signalling Pathway**

This figure is a schematic diagram of the cAMP-dependent PKA pathway that has been shown to mediate FSH signalling within granulosa cells. Stimulation of granulosa cells via the FSHR leads to the generation of intracellular cAMP that binds to and activates PKA. This in turn leads to the phosphorylation of molecules, most notably CREB, that act as transcription factors within the nucleus.

R = Regulatory subunit, C = Catalytic subunit

P indicates phosphorylated status



secretion of androstenedione. Some androstenedione is transported into granulosa cells, where in response to p450arom, is aromatized to estrone, which is then converted to estradiol by the type I 17β-hydroxysteroid dehydrogenase (17β-HSD), constitutively expressed in granulosa cells from the primary to the preovulatory stage (Sawetawan et al., 1994). Therefore, by virtue of expression of the two enzymes, the granulosa cells become highly active in converting theca-derived androstenedione to estradiol.

Continuous stimulation of granulosa cells by FSH appears to be involved in their acquisition of the potential to produce increasing amounts of progesterone and luteinize by stimulating their expression of steroid acute regulatory (StAR) protein, the cytochrome p450 cholesterol side-chain cleavage (p450scc) enzyme and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Richards, 1994); however, this potential remains suppressed until just prior to ovulation. Therefore, increased and sustained levels of circulating FSH play an obligatory role in the mechanisms of selection and development of the dominant follicle.

In addition to its importance for somatic cell proliferation and the cyclic recruitment of antral follicles, FSH is an important trophic factor for the survival of follicular granulosa cells. Unless rescued by FSH, most multilayer preantral and early antral follicles undergo atresia and ovulation does not occur (Richards, 1994). Suppression of serum gonadotropins after hypophysectomy leads to atresia and apoptosis of developing rat follicles, whereas FSH treatment of early antral follicles prevents the spontaneous onset of follicular apoptosis (Chun et al., 1996). Additionally, estrogens, whose production is dependent upon both, FSH stimulation of aromatase in granulosa cells and LH stimulation of androstenedione production by the theca (Gougeon, 1996),

are potent antiapoptotic hormones in early antral follicles (Billig et al., 1993). The importance of FSH in preventing apoptosis has lead to the concept that FSH is a follicle survival factor essential for preventing the programmed demise of early follicles in rodents (Hsueh et al., 1994).

It is well established that the continued development of antral and larger follicles is dependent on the presence of FSH. In contrast, the growth of preantral follicles has been considered to be gonadotropin-independent because follicles can develop to the antral stage with minimal circulating FSH or defective FSHR (Aittomaki et al., 1995; Kumar et al., 1997). However, since granulosa cells express FSHR during preantral follicle development (as early as the primary follicle stage) (Dunkel et al., 1994; Rannikki et al., 1995), it has been suggested that the necessity for this gonadotropin may occur earlier than is widely supposed. This is supported by several reports. For example, serum FSH levels are elevated in rodents during a time of rapid preantral follicle growth (Dahl et al., 1988). Additionally, in hypophysectomized rats, larger pools of resting follicles indicate decreased initial recruitment as compared to non-operated controls, suggesting a requirement for FSH (Wang and Greenwald, 1993). Furthermore, in hypophysectomized rats (Hirshfield, 1985) and hypogonadol mice (Halpin et al., 1986), although ovarian follicles can develop to the secondary and early antral stages, this happens more slowly and in fewer numbers. Treatment of infantile rats with pregnant mares' serum gonadotropin (PMSG), which exhibits FSH-like activity, increases ovarian weight (Goldenberg et al., 1973), whereas treatment of neonatal rats with a GnRH antagonist reduces the number of growing ovarian follicles found at puberty (Meijs-Roelofs et al., 1990). Finally, treatment of intact, hypophysectomized, or GnRH

antagonist-treated juvenile rats with FSH increases ovarian weight and preantral follicle development (McGee et al., 1997).

Although the data is conflicting, *in vitro* evidence also indicates that preantral follicles may be gonadotropin-dependent since FSH is observed to facilitate their development. Primordial follicles that initiate growth in whole newborn mouse ovaries develop to the secondary stage without the requirement for FSH in the medium (Eppig and O'Brien, 1996). FSH also has no effect on growth or estrogen secretion by cultured medium-sized preantral follicles isolated from immature mouse ovaries, although it synergizes positively with several intra-ovarian regulators (Liu et al., 1998). However, when preantral follicles are isolated from adult mouse ovaries, FSH alone is effective in promoting growth to the preovulatory stages (Liu et al., 1999), although other studies indicate this only to be the case when the follicles are >200μm (Hartshorne et al., 1994). In cultures of primordial follicles from immature rats, FSH treatment promotes follicle progression to the antral stage (Cain et al., 1995); yet, in a similar study, FSH treatment alone does not enhance granulosa cell division or steroidogenesis (Li et al., 1995). Therefore, the question of whether or not FSH plays a role in the development of early follicles requires further investigation; however, that it is pertinent for controlling the reproductive cycle and therefore female fertility, is indubitable.

1.3 Paracrine Factors in Ovarian Follicle Development

Folliculogenesis and oocyte maturation involve complex networks of cell-cell interactions. It is thus to be expected that apart from extraovarian hormonal regulation these ovarian cells will function in response to factors produced by the cells within their

local environment. There is a vast amount of evidence indicating the role of several locally produced factors in the regulation of ovarian follicle development and function. These are factors that commonly act as messengers to regulate proliferation and differentiation of ovarian cells. In this section, the roles of kit ligand and transforming growth factor-β as factors that exert local effects with implications on overall follicle development will be discussed.

1.3.1 Kit Ligand

It has been suggested that a putative follicular organizer produced by granulosa cells, is important for the granulosa cell-oocyte interactions involved in the initiation and progression of primordial follicle development, as well as in the differentiation of theca cells from stromal cells (Packer et al., 1994). Kit ligand (KITL), also known as stem cell factor (SCF) and mast cell growth factor (MGF), which is part of a ligand-receptor interaction, is a postulated candidate based on its pattern of expression and numerous actions within ovarian follicles.

Kitl mRNA expression is initially detected in the mouse embryonic ovary along the migratory path of PGC and in the gonadal ridge (Matsui et al., 1990; Keshet et al., 1991). Expression of the mRNA for its receptor, the type III tyrosine kinase KIT, is detected at high levels in migrating PGC (Manova et al., 1990; Manova and Bachvarova, 1991), making it likely that the KITL/KIT interaction facilitates their directed migration. Once colonization of the genital ridge is complete, *Kitl* mRNA expression becomes confined to the fetal gonads (Matsui et al., 1990; Keshet et al., 1991; Motro et al., 1991),

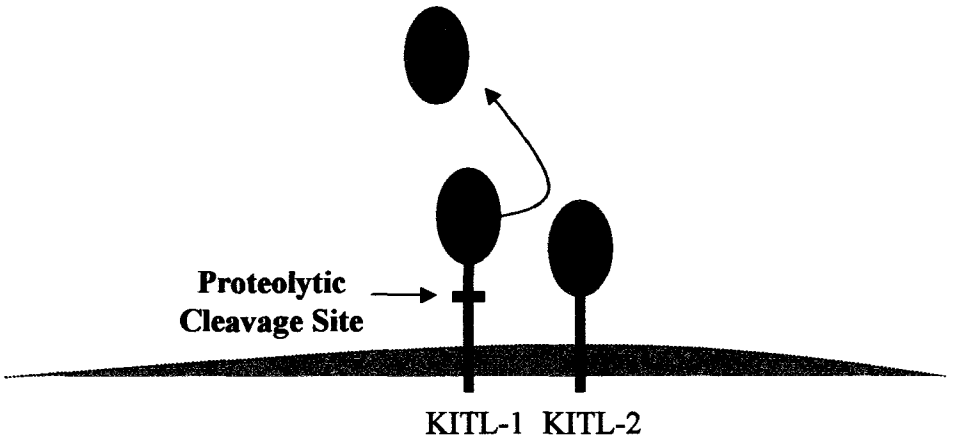
while the now mitotic PGC continue to express *Kit* mRNA transcripts (Manova et al., 1990; Manova and Bachvarova, 1991).

In the post-natal mouse ovary, *Kitl* mRNA expression is concentrated in the central cords. Thereafter, expression of *Kitl* mRNA and its protein become specifically associated with granulosa cells, where levels remain low in primordial follicles, but progressively increase during the primary and secondary stages of development (Keshet et al., 1991; Motro et al., 1991; Manova et al., 1993; Motro and Bernstein, 1993). By the late antral stage, when growth of the oocyte has ceased, expression of *Kitl* mRNA and its protein becomes confined to the mural granulosa cell layer (Manova et al., 1993; Motro and Bernstein, 1993). Expression of KIT receptor mRNA is detected in the oocytes of the post-natal mouse ovary at all stages of follicle development, as well as on undifferentiated stromal cells and the theca interna cell layer of antral follicles (Horie et al., 1991; Yoshinaga et al., 1991; Motro and Bernstein, 1993).

KITL proteins are derived from alternatively spliced mRNA yielding the soluble (KITL-1) and membrane-bound (KITL-2) isoforms, both of which are expressed in the ovary (*figure 4*; Huang et al., 1992; Manova et al., 1993). The full-length *Kitl* mRNA encodes a 248-amino acid soluble protein that is initially membrane-bound but immediately undergoes proteolysis at a cleavage site encoded by the 6^{th} exon, releasing a 165-amino acid protein into the extracellular domain (Huang et al., 1992). The alternatively spliced mRNA lacks the 6^{th} exon containing the peptidase cleavage site, and is therefore translated into a 220-amino acid membrane-bound protein (Huang et al., 1992).

Figure 4. **Alternative Splicing of *Kitl* mRNA**

This figure demonstrates the production of the two isoforms of KITL in the ovary. The full-length *Kitl* mRNA encodes a protein that is initially membrane-bound but immediately undergoes proteolysis at a cleavage site, releasing the soluble protein (KITL-1) into the extracellular domain. The alternatively spliced mRNA lacks this cleavage site, and is therefore translated into a membrane-bound protein (KITL-2).



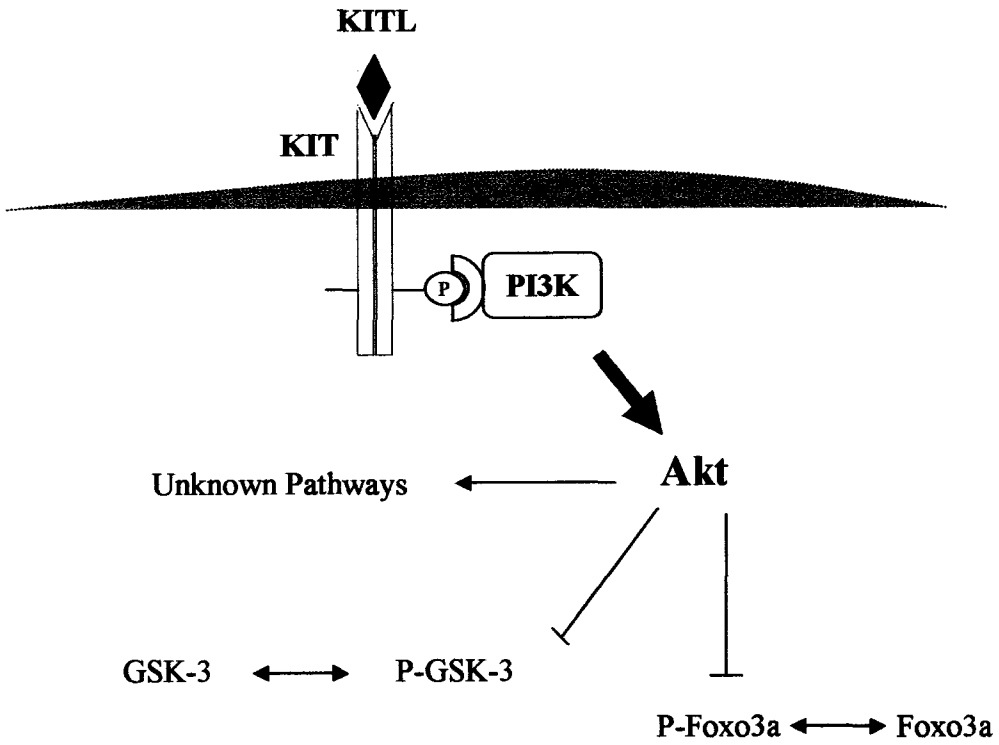
The signalling process involves dimers of KITL binding to adjacent KIT receptors resulting in dimerization and autophosphorylation on tyrosine residues, and hence activation of the KIT molecule (Lemmon et al., 1997). Although the pathway mediated by KITL/KIT within its target cell types in the ovary remains largely unknown, in the oocyte the phosphorylated tyrosine on the KIT molecule provides a docking site for phosphatidylinositol 3'-kinase (PI3K), leading to its recruitment to the oocyte cell membrane. This lipid kinase then phosphorylates a key molecule, Akt, which in turn targets various signalling molecules such as glycogen synthase kinase-3 (GSK-3) and forkhead transcription factors of the FOXO family, as illustrated in *figure 5* (Liu et al., 2006).

The study of mice carrying mutations within the Steel (*Sl*) and White spotting (*W*) loci, which encode the *Kitl* (Chabot et al., 1988; Huang et al., 1990) and *Kit* gene (Geissler et al., 1988), respectively, has provided the greatest insight into the function of the system in the ovary. There is considerable evidence from these mice mutants to suggest a role for the ligand-receptor pair in PGC survival, migration and proliferation, as well as in general follicle development. For example, mice homozygous for the viable Steel dickie (Sl^{d}) allele, which involves an intragenic deletion that includes the transmembrane domain and C-terminus of KITL, are sterile, a consequence of the failure of PGC to successfully migrate to the fetal genital ridge (Flanagan et al., 1991; Brannan et al., 1991). In the case of mice carrying the Steel panda (Sl^{pan}) or Steel contrasted (Sl^{con}) mutations, although follicles are present in the ovary, they do not progress beyond the primary stage of development (Huang et al., 1993; Bedell et al., 1995). Less severe mutations that result in reduced expression of KITL, such as Steel transfer (Sl^{t}), permit

Figure 5. **The KITL Signalling Pathway in the Oocyte**

This figure is a general illustration of KITL signalling in the oocyte. When dimers of KITL bind to adjacent KIT receptors, this causes dimerization and autophosphorylation on KIT receptors, and hence activation of the KIT molecule. The phosphorylated tyrosine on the KIT molecule provides a docking site for PI3K - a lipid kinase that phosphorylates Akt, which in turn targets various signalling molecules such as GSK-3 and members of the FOXO family.

P indicates phosphorylated status



follicle growth to the antral stage, although these animals ovulate sporadically and have limited fertility (Kuroda et al., 1988). Therefore, the phenotypes of the Sl^{t}/Sl^{t} and Sl^{pan}/Sl^{pan} ovaries, which exhibit early developmental arrest of the follicle and oocyte, provide the strongest evidence for a role of KITL/KIT in the ovary, indicating that a specific form, amount or site of expression of the ligand is required at the early stages. It is worthy to note that mutations in either *Kitl* or *Kit* genes have not been detected in cases of human female infertility (Hutt et al., 2006).

1.3.1.1 KITL in the Initiation of Follicle Growth

Although the levels of *Kitl* mRNA and its protein appear to be low in primordial to primary follicles, there is evidence to indicate detrimental effects on growth initiation upon blockade of the KITL/KIT interaction. Studies of the Sl^{pan}/Sl^{pan} mutant mice provided the first evidence. Although the ovaries of these mice contain primordial follicles and follicular growth is initiated, development past the primary stage is rare (Huang et al., 1993), indicating a requirement for KITL. The importance of the KITL/KIT interaction for growth initiation is further demonstrated based on injections of mice with the KIT neutralizing antibody, ACK-2. A daily injection of ACK-2 from birth to day 12 completely blocks the onset of primordial follicle development, although only the first injection (at birth) appears significant since, when it is omitted, growth initiation proceeds normally (Yoshida et al., 1997). *In vitro* studies produce supporting data. In a rat ovarian organ culture system, it is reported that the ability of fresh ovaries from neonatal rats to undergo spontaneous primordial follicle development is blocked in the presence of ACK-2 (Parrott and Skinner, 1999). Furthermore, the authors directly

verified the role of KITL in promoting the activation of primordial follicles through their finding that treatment of ovaries with recombinant KITL increases the percentage of follicles in the growing pool relative to ovaries in control medium (Parrot and Skinner, 1999).

1.3.1.2 KITL in the Growth of Early Follicles

Several types of evidence support a role for KITL/KIT interactions in preantral follicular development. General descriptive evidence supports a growth factor-like action of KITL on the oocyte. This includes (1) the observation that oocyte growth begins in follicles formed in the medulla in the late fetus at a time when *Kitl* mRNA expression is restricted to this area; (2) *Kitl* mRNA expression in primary and secondary follicles, and KITL uptake into oocytes increases as the rate of growth of the oocyte increases; and (3) production of *Kitl* mRNA in adjacent follicular cells and uptake of KITL into oocytes is greatly reduced in follicles containing fully grown oocytes (Manova et al., 1993). *In vitro* evidence also supports a role for KITL in promoting oocyte growth. The addition of 10-50ng/ml recombinant KITL to growing mouse follicles cultured in collagen gels causes a dose-dependent increase in the rate of oocyte growth (Packer et al., 1994). This effect is blocked by the addition of ACK-2, before or early in the growth phase of the oocyte, and genistein, an inhibitor of tyrosine kinases including KIT, later in the growth phase (Packer et al., 1994). KITL has also been shown to be effective in promoting the growth of denuded oocytes obtained from mouse embryos (Klinger and De Felici, 2002). Furthermore, recent results have shown that FSH promotion of oocyte growth in cultured

mouse preantral follicles is KIT-dependent since it is blocked in the presence of Gleevec, a drug that blocks the tyrosine kinase active site of KIT (Thomas et al., 2005).

Besides promoting oocyte growth, KITL appears to be involved in promoting the survival of both primordial and preantral follicles. It is reported to inhibit apoptosis in oocytes maintained in primordial follicles in whole mouse ovaries cultured *in vitro* (Jin et al., 2005). This pro-survival effect is correlated with the up-regulation of antiapoptotic proteins Bcl-2 and Bcl-cL and down-regulation of proapoptotic factor Bax (Jin et al., 2005). In cultures of preantral follicles, others have demonstrated that inhibition of the KITL/KIT interaction using the KIT neutralizing antibody, SC1494, promotes the death of oocytes (Reynaud et al., 2005). The *in vivo* findings of Yoshida et al. (1997) lend further credence to this notion of an important effect of the ligand-receptor interaction in the inhibition of preantral follicle atresia. Their results indicate that two days of injecting mice with ACK-2 results in a dramatic (10-fold) reduction in the population of large preantral follicles.

1.3.1.3 KITL in the Growth and Maturation of Antral Follicles

The KITL/KIT interaction may be important for antrum formation as indicated by the work of Yoshida et al. (1997), who demonstrated that *in vivo* administration of ACK-2 to mice suppresses antrum formation. Additionally, they have shown that when preovulatory follicles from mice are injected with ACK-2, 12-24 hours before inducing ovulation using LH, the preovulatory follicle becomes atretic and there is subsequent ovulation failure. Driancourt et al. (2000) state that this observation denotes that LH receptor differentiation occurs during that 12-24 hour period; blocking KIT during that

time period prevents granulosa cell differentiation and consequently, their ability to respond appropriately to LH.

Previous studies have also suggested a role for KITL in maintenance of meiotic arrest. When oocytes from either unprimed or PMSG-primed rats are cultured in the presence of recombinant KITL, meiosis is transiently blocked; pretreatment of oocytes with ACK-2 prior to culturing in KITL-supplemented media results in the rate of spontaneous meiotic maturation becoming similar to controls cultured without KITL (Ismail et al., 1996). In later experiments, it was demonstrated that when *Kit* antisense oligonucleotides are injected into meiotically arrested rat oocytes, their ability to resume meiosis is significantly increased (Ismail et al., 1997), supporting the possibility that KITL may be relevant in controlling nuclear maturation of the oocyte. This possible effect of KITL on oocyte maturation is further supported by the observation of a positive correlation between *Kitl* mRNA concentrations in follicular fluid and pregnancy rate after *in vitro* fertilization (Smikle et al., 1998). Additionally, human chorionic gonadotropin (hCG)-induced meiosis not only results in an increase in the quantity of *Kitl* mRNA (Ismail et al., 1996) but also precipitates a dramatic redistribution in its expression with levels becoming undetectable in cumulus granulosa cells and increasing further in mural granulosa cells (Ismail et al., 1996). Therefore, KITL appears to be important in maintenance of oocyte meiotic arrest prior to the LH surge - an event that triggers a decrease or redistribution in *Kitl* mRNA by those granulosa cells adjacent to the oocyte, thereby allowing meiosis to resume. Hence, the significance of the KITL/KIT system to ovarian follicle maturation is well established.

1.3.1.4 Differential Roles for KITL-1 and KITL-2

The levels of soluble *Kitl1* mRNA and membrane-bound *Kitl2* mRNA are differentially regulated between tissues (Huang et al., 1992), between the ovaries of mice of differing ages (Manova et al., 1993) and between granulosa cells of preovulatory and ovulatory rat follicles (Ismail et al., 1997). Differential roles for KITL-1 and KITL-2 have been demonstrated in other cellular systems, with KITL-1 mediating cellular migration and KITL-2 contributing to cell survival and proliferation (Wehrle-Haller and Weston, 1995; Tajima et al., 1998; Kunisada et al., 1998). There is now also mounting evidence that within the ovary the two isoforms have functions that vary in accordance with the phase of germ cell or follicular development.

Initial support for this notion of differential roles comes from the study of mice with a natural mutation in the *Sl* locus that affects the relative expression of KITL-1 versus KITL-2. Sl/Sl^{d} mice produce only the soluble isoform (KITL-1) as a result of an intragenic deletion that includes the transmembrane domain and the C-terminus (Brannan et al., 1991; Flanagan et al., 1991). In these mice, PGC fail to successfully migrate to the fetal genital ridge resulting in infertility (Brannan et al., 1991; Flanagan et al., 1991), an effect that is almost as severe as in mice carrying deletions including the *Kitl* gene (Huang et al., 1993). Based on the phenotype of these mice, it appears that KITL-2 is preferentially required for the early function of the ligand in germ cell development during the time when PGC migrate from the yolk sac to the genital ridge in the embryo. In contrast, mice carrying the Sl^{Kitl2} allele (generated through gene targeting), which produce KITL-2 exclusively, are fertile (Tajima et al., 1998).

Recent evidence indicates that the role of KITL in primordial germ cell development may be dependent on a complex balance between its soluble and membrane-bound forms. In the absence of KITL-2, KITL-1 interacts with fibroblast growth factor (FGF) to promote primordial germ cell proliferation *in vitro* (Kawase et al., 2004). By contrast, in the presence of KITL-2, KITL-1 inhibits FGF-induced proliferation (Kawase et al., 2004). These findings indicate that the role of KITL-1 in (either promoting or inhibiting primordial) germ cell proliferation depends on the presence or absence of KITL-2, which may depend on the location of the primordial germ cells on their migratory path towards the gonad.

Interestingly, the ability of KIT to promote adhesion of primordial germ cells to somatic cells *in vitro*, is dependent on KITL-2 (Pesce et al., 1997). A specific functional role for KITL-2 in supporting cellular survival is suggested by *in vitro* studies showing that the survival of PGC is supported by KITL-2, whereas KITL-1 has limited impact (Dolci et al., 1991). Although both isoforms have similar binding affinities for KIT (Flanagan and Leder, 1990), because KITL-2 is membrane-anchored, it may prevent subsequent down-regulation and internalization of activated receptor, as has been demonstrated in mast cells (Yee et al., 1994) and a myeloid cell line (Miyazawa et al., 1995).

Evidence from the *in vitro* culture of mouse preantral follicles suggests that FSH-stimulated-oocyte growth is also dependent on the relative expression of KITL-1 and KITL-2. A low concentration of FSH decreases the *Kitl1*/*Kitl2* mRNA ratio by increasing *Kitl2* mRNA levels, and this is associated with increased oocyte growth (Thomas et al., 2005). Additionally, when the KITL-1/KITL-2 ratio is increased by the

addition of soluble KITL-1 to cultures in the presence of low FSH, FSH-induced oocyte growth is suppressed (Thomas et al., 2005), stressing that the ratio itself is important rather than the total amount of KITL present. In fact, it has been observed that fully grown oocytes increase the *Kitl1/Kitl2* mRNA ratio in preantral granulosa cells (Joyce et al., 1999). In light of findings that a low *Kitl1/Kitl2* mRNA ratio is associated with increased follicle growth, it follows that a fully grown follicle would not require this.

Finally, it has been found that hCG-induced meiosis not only results in an increase in the quantity of *Kitl* mRNA (Ismail et al., 1996) and a dramatic redistribution in its expression within rat granulosa cell populations (Ismail et al., 1997), but moreover, these changes are accompanied by a shift from *Kitl2* to *Kitl1* predominating (Ismail et al., 1997); the result is an increase in the ratio of *Kitl1/Kitl2* mRNA. Considering the proposal that KITL may be important in maintaining meiotic arrest, this suggests that it is specifically KITL-2 that is the functional inhibitor; a shift to the less effective KITL-1 allows meiosis to proceed.

1.3.1.5 A Role for FSH in the Regulation of KITL Expression

There is convincing evidence in support of a role for FSH in regulating KITL expression, as well as an indication that KITL is responsible for mediating some of the actions of FSH. It has previously been demonstrated that *Kitl* mRNA levels are increased in cultured mouse Sertoli cells in the presence of FSH (Rossi et al., 1991; 1993). Additionally, treatment of Sertoli cells with the cell permeable cAMP analogue dibutyryl cAMP (dbcAMP) produces a dramatic increase in *Kitl* mRNA expression, reminiscent of FSH action (Rossi et al., 1991; 1993). Similarly, dbcAMP treatment of mouse granulosa

cells in culture stimulates accumulation of *Kitl* mRNA (Packer et al., 1994). A significant increase in the levels of *Kitl* mRNA is also observed in granulosa cells from PMSG-primed rats as compared to age-matched, unprimed controls (Ismail et al., 1996).

Indirect evidence further suggests that FSH may function through KITL in order to bring about some of its effects. Gonadotropin treatment of rat whole ovarian cultures increases the percentage of preantral follicles per section, an effect reminiscent of KITL actions and that is, in fact, blocked using ACK-2 (Parrott and Skinner, 1999). A more recent study reports that a low concentration of FSH (0.05ng/ml) is effective in increasing *Kitl2* mRNA expression, although a similar increase is not observed in the presence of a higher concentration of FSH (0.5ng/ml), nor does either concentration have any effect on *Kitl1* mRNA expression (Thomas et al., 2005). Additionally, the lower concentration of FSH, associated with an increase in *Kitl2* mRNA expression, induces oocyte growth, an effect that is blocked upon use of the KIT inhibitor Gleevec (Thomas et al., 2005).

The proximal promoter region of the *Kitl* genes in human, rat and mouse has been studied and found to contain elements that allow induction by cAMP in Sertoli cells (Taylor et al., 1996; Bedell et al., 1995; Jiang et al., 1997; Grimaldi et al., 2003). This indicates that the hormonal effects on the increase of endogenous *Kitl* mRNA levels are likely exerted at the transcriptional level. However, there is a lack of CRE sequences in the proximal 5'-flanking regions of all these *Kitl* genes, suggesting that cAMP-induction is mediated by transcription factors different from CREB. Studies of the 5'-flanking region of the mouse *Kitl* gene show that mutations in the canonical TATA box sequence and immediately downstream GC-rich element within the minimal promoter region (-55/+8) result in abrogation of cAMP responsiveness (Grimaldi et al., 2003). Further

analysis demonstrated that for cAMP-induced transcription from the *Kitl* gene promoter, an interaction among an Sp1-binding region, factors recognizing the TATA box sequence and an unidentified cAMP-induced factor binding the GC-rich element were necessary. Therefore, although the PKA pathway is involved in stimulating KITL expression, it does so with the involvement of other transcription factors and possibly signalling pathways.

That KITL serves a pertinent role throughout follicle development is well established. Considering that FSH may be the primary signal fueling the production of this vital factor, we were interested in initially demonstrating FSH regulation of KITL expression within our rat granulosa cell system. Furthermore, although it is well documented that FSH stimulates adenylate cyclase with a resultant increase in cAMP synthesis, it is unclear whether activation of the PKA signalling pathway alone is sufficient to account for the expression pattern of the many genes that are activated during FSH-dependent follicular maturation, such as *Kitl*. Since the intracellular signalling system used by the FSHR to regulate KITL expression remains uncertain, it was particularly our interest to investigate the involvement of PKA signalling.

1.3.2 Transforming Growth Factor-β1

Transforming growth factor-β1 (TGF-β1) is a member of the TGF-β superfamily that is comprised of a number of alternative isoforms encoded by individual genes. It shares a high level of sequence conservation and almost identical tertiary structure with its two isoforms, TGF-β2 and TGF-β3 (Ingman and Robertson, 2002). Despite employing the same receptors for signal transduction and sharing many biological functions, the isoforms are not functionally redundant as the promoter sequence within

each gene shows distinguishing features and differential expression is evident *in vivo* (Ingman and Robertson, 2002). The synthesis of TGF-β ligand and receptors appears to be almost ubiquitous in reproductive tissues and evidence for its local production in the follicle has been found in a number of species. The following review will focus mainly on rodents since both its expression and function appear to vary between species, in addition to stage of follicular development and cycle day.

As early as 1987, studies on the expression of TGF-βs in the ovary suggested that the theca cells are the major source of TGF-β1 in the rat and mouse ovary (Skinner et al., 1987). General studies looking at the expression of TGF-β1 immunohistochemically localized it in the ovarian theca cells of immature rats and adult mice (Thompson et al., 1989; Hernandez et al., 1990). However, more recent studies have shown expression of TGF-β1 in granulosa cells. During development of the ovarian follicle, immunostaining for TGF-β1 is first evident in the neonatal rat ovary, in the pre-granulosa cells that surround the oocyte (Levacher et al., 1996) and in the granulosa cells of preantral follicles from gonadotropin-primed mice (Ghiglieri et al., 1995; Juneja et al., 1996). Its levels decrease in the granulosa cells of primordial and primary follicles and it is completely absent in the granulosa cells from subsequent stages of follicle development (Levacher et al., 1996); however, in gonadotropin-induced follicular maturation, TGF-β1 immunoreactivity is reported to be evident in granulosa cells into the antral stage. The theca cells, formed during the secondary follicle stage, immediately begin to express TGF-β1 (Levacher et al., 1996); however, in the large preantral follicles from gonadotropin-primed mice, it is reported that immunoreactivity for TGF-β1 drops in the thecal layers until after the LH surge when some follicles evolve into preovulatory

follicles (Ghiglieri et al., 1995). Nonetheless, the thecal cells appear to be the major cell type containing immunoreactive TGF-β1 protein with intensity increasing relative to the size of the follicle (Juneja et al., 1996). Over the course of gonadotropin-induced maturation, the oocytes of preantral follicles of mice are also observed to have TGF-β1 immunostaining that remains present in all follicles throughout the various stages of development (Ghiglieri et al., 1995; Juneja et al., 1996).

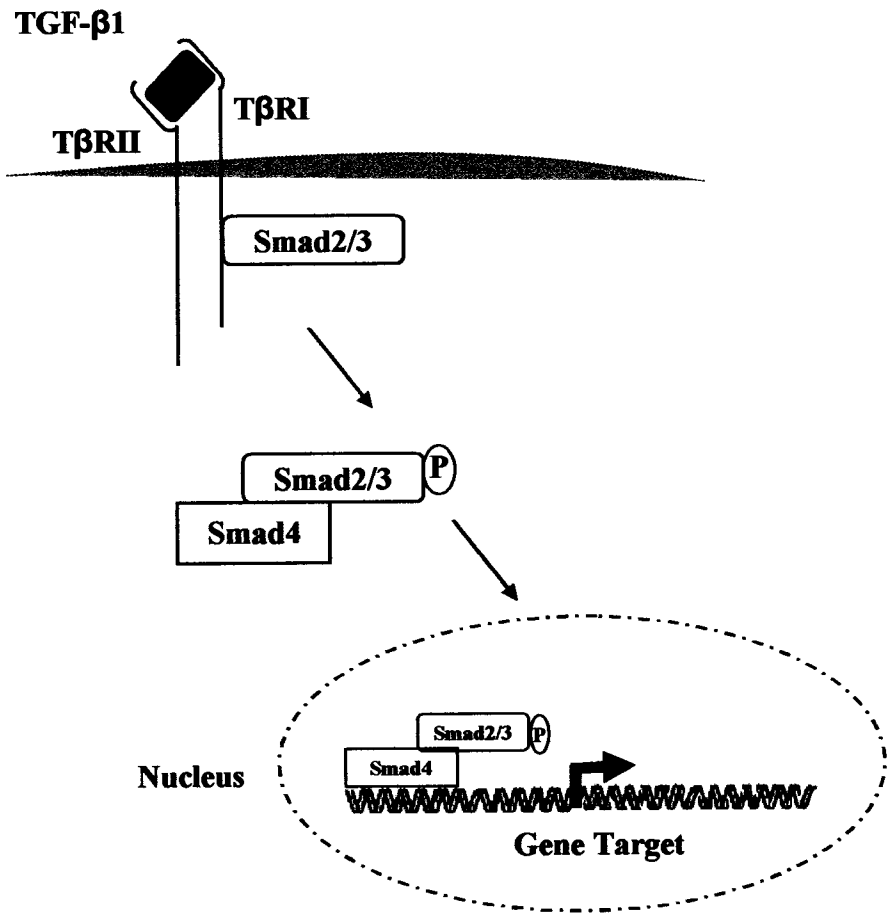
Receptors for the majority of TGF-β superfamily members consist of two closely related subsets of transmembrane serine/threonine kinase receptors, the type I (TβRI; activin receptor-like kinases – ALK), and type II (TβRII) receptors (Massague, 1998). The TβRI has been detected in granulosa cells of the mouse ovary in all follicles at various stages of development, although its main sites of immunoreactivity are theca cells and oocytes (Juneja et al., 1996). TβRII is equally present in all ovarian cell types (Juneja et al., 1996). The ligand must bind both receptor types, although TβRI are the primary transducers of the signal and specify the signalling pathway (Macias-Silva et al., 1998).

As depicted in *figure 6*, the TGF-β signalling pathway is initiated upon binding of the ligand with high affinity to TβRII receptor kinases, which then phosphorylate TβRI receptors at specific serine/threonine residues, thereby activating these (Massague, 1998). The resultant receptor complex then phosphorylates intracellular signalling molecules called Smads, a family of related intracellular proteins critical for transmitting to the nucleus signals from the receptor after binding of the ligand (Miyazono et al., 2001). These receptor-regulated Smads (R-Smads) can be grouped into two subsets based on the subfamily of ligands that can activate specific Smad molecules. The TGF-β/activin

Figure 6. **The TGF-β1-Induced Smad Signalling Pathway**

This figure is a schematic diagram of the Smad signalling pathway that mediates TGF-β/activin function. TGF-β1 binding to and activation of the TβRI/TβRII receptor complex leads to the phosphorylation of intracellular Smad2 and Smad3 that interact with Smad4 and act as transcription factors within the nucleus.

P indicates phosphorylated status



responsive ALK4 and ALK5 receptors phosphorylate R-Smads Smad2 and Smad3, whereas the bone morphogenetic protein (BMP)-responsive ALK2, ALK3 and ALK6 receptors phosphorylate R-Smads Smad1, Smad5 and Smad8 (Massague, 1998). Therefore, two distinct downstream Smad-signalling pathways can be initiated. Once phosphorylated, the R-Smads form heteromeric complexes with the common mediator Smad4; this complex then translocates into the nucleus where it activates gene transcription by binding directly to DNA and/or by interacting with other DNA-binding proteins that target genes for transcriptional regulation (Massague, 1998).

1.3.2.1 Role and Functions of TGF-β1 in Follicle Development

TGF-β plays important autocrine and paracrine roles in modulating ovarian cell functions. However, investigations of its function in isolation are limited with the majority of studies reviewed pertaining to its actions in ovarian follicle development suggesting a role that is closely tied to that of FSH. As the following review will discuss, TGF-β facilitates gonadotropin-induced proliferation and differentiation, especially of granulosa cells, and aides in the preovulatory process to produce mature follicles.

TGF-β appears to have a role in the acquisition of gonadotropin-sensitivity in the ovary. In rat granulosa cells cultured in the presence of TGF-β1 there is an increase in FSHR mRNA and protein expression (Dunkel et al., 1994). TGF-β1 alone marginally elevates basal levels of FSH binding to the receptor and, in the presence of FSH, considerably enhances binding by increasing FSHR number (Gitay-Goren et al., 1993).

A role for TGF-β in modulating LH receptor expression has also been suggested. In cultures of rat granulosa cells, TGF-β augments the stimulatory effect of a low dose

(5ng/ml) of FSH on LH receptor expression while attenuating the stimulatory effect of a high dose (50ng/ml) of FSH (Knecht et al., 1986; 1987). Other reports indicate that TGF-β dose-dependently augments the stimulatory effect of FSH on LH receptor expression (Inoue et al., 2002). This role of TGF-β is further supported by studies indicating that TGF-β1 augments FSH stimulated LH/hCG receptor binding (Dodson and Schomberg, 1987; Gitay-Goren et al., 1993) an effect that is due to an increase in binding capacity rather than binding affinity (Gitay-Goren et al., 1993).

TGF-β1 can act alone or synergistically with FSH to stimulate DNA synthesis, which translates into increased cell number in cultured rat granulosa cells (Skinner et al., 1987; Dorrington et al., 1988; Saragueta et al., 2002). It is also found that a TGF-β neutralizing antibody blocks the ability of conditioned medium, generated by primary cultures of theca cells, to stimulate DNA synthesis in rat granulosa cells and augment the effect of FSH on DNA synthesis (Bendell and Dorrington, 1988).

TGF-β is functional in supporting steroidogenic activities of granulosa cells. It augments FSH-induction of aromatase activity in rat primary granulosa cells (Adashi and Resnick, 1986; Hutchinson et al., 1987; Adashi et al., 1989). Similar results are noted in granulosa cells from hypophysectomized DES-treated rats in culture where TGF-β augments the effect of FSH on estrogen accumulation (Ying et al., 1986). Studies have also demonstrated that TGF-β1 exerts an additive modulatory effect on FSH-stimulated progesterone production. In cultures of rat granulosa cells, FSH-stimulated progesterone production is increased by TGF-β1 in a dose-dependent manner (Knecht et al., 1987; Dodson and Schomberg, 1987; Ke et al., 2004; 2005). Others have reported this effect of TGF-β, although only over an extended culture period during which time it also causes

maximum FSH-induced progesterone synthesis to peak earlier than with FSH alone (Hutchinson et al., 1987). Moreover, in cultured rat theca cells, TGF-β also enhances progesterone production in the presence of LH (Magoffin et al., 1989; Hernandez et al., 1990) while inhibiting LH-induced androgen production (Magoffin et al., 1989). This ability of TGF-β to enhance FSH-stimulated progesterone production is likely mediated through modulation of the levels of StAR protein and/or the p450scc enzyme, both involved in progesterone production. TGF-β1 appears to play a permissive role by enhancing FSH-stimulated StAR protein levels and inducing FSH-stimulation of p450scc enzyme in cultured granulosa cells from PMSG-primed rats (Ke et al., 2004; 2005).

TGF-β is also involved in regulating the production of other modulatory factors in the ovary. Zhang et al. (1988) observed that in cultured rat granulosa cells TGF-β causes a dose-dependent increase in both basal and FSH-stimulated inhibin production. TGF-β is also reported to stimulate EGF receptor expression (Feng et al., 1986). Additionally, in granulosa cells from antral follicles of PMSG-primed rats, TGF-β1 augments the FSH-induced increase in the expression levels of phosphorylated forms of connexin 43 protein and therefore gap junctional communication (Ke et al., 2005).

Finally, TGF-β1 may serve a role in preventing the resumption of meiosis to allow completion of oocyte cytoplasmic maturation prior to ovulation. Individual preovulatory follicles from PMSG-primed rats incubated with TGF-β in the absence or presence of LH display partial inhibition of LH-induced meiosis resumption (Tsafriri et al., 1989). Additionally, fewer number of ova are recovered from these TGF-β-treated ovaries in the presence of LH and those recovered are of poorer quality as determined by *in vitro* fertilization studies (Tsafriri et al., 1989). A second finding in support of this

concept is that intrabursal administration of TGF-β1 into the ovaries of superovulated mice one hour prior to hCG administration inhibits follicular rupture (Juneja et al., 1996).

1.3.2.2 A Role for TGF-β1 in the Regulation of KITL Expression

It is evident that locally produced factors serve a pertinent role in coordinating the proliferation and differentiation of follicle cells and oocyte maturation. It is therefore to be expected that within this environment of tight cell-cell interactions, a locally produced factor may also have a role in modulating KITL expression and function in the way that FSH is known to do. Some early evidence for the role of paracrine factors in regulating the expression of KITL comes from studies of mouse granulosa cell/oocyte cocultures where it was demonstrated that oocytes regulate the steady-state levels of *Kitl1* and *Kitl2* mRNA in a developmental stage-dependent manner (Joyce et al., 1999). Several other reports also highlight the pertinent role of specific local factors, such as growth/differentiation factor-9 (GDF-9), BMP-15 as well as keratinocyte growth factor (KGF) and leukemia inhibitory factor (LIF), in influencing the expression and function of the ligand.

The influence of GDF-9 on KITL first became evident when ovaries from *Gdf9*-deficient mice were found to have elevated levels of *Kitl* mRNA, suggesting its role as a negative regulator (Elvin et al., 1999). This hypothesis was later corroborated by Joyce et al. (2000) when they demonstrated that GDF-9 inhibits overall *Kitl* mRNA expression in both preantral and mural granulosa cells from mice. A second, exclusively oocyte-derived factor, BMP-15 (Dube et al., 1998; Otsuka et al., 2000), also appears to regulate KITL; incubation of rat granulosa cell/oocyte cocultures with BMP-15 stimulates an

increase in both *Kitl1* and *Kitl2* mRNA levels in a dose-dependent manner (Otsuka and Shimasaki, 2002). More recently, Thomas et al. (2005) found that addition of recombinant BMP-15 to cultures of mouse preantral oocyte-granulosa cell complexes (OGC) results in elevated levels of *Kitl1* and *Kitl2* mRNA expression. Finally, KGF and LIF have been shown to stimulate *Kitl* mRNA expression in a rat ovarian organ culture system and in cultured granulosa cells, respectively (Nilsson et al., 2002; Kezele et al., 2005). Therefore, local factors not only influence the development of the follicle, but may do so, at least in part, by regulating the expression of KITL.

A role for TGF-β as a regulator of KITL expression has been shown in previous studies. In bone marrow stromal cells, TGF-β1 down-regulates *Kitl* mRNA expression by repressing gene transcription (Heinrich et al., 1995). More recently, work from our lab demonstrated a suppressive action of TGF-β on overall *Kitl* mRNA expression in rat ovarian surface epithelial cells (Ismail et al., 1999). However, whether TGF-β1 serves as a regulator of KITL expression in granulosa cells remains to be investigated. Therefore, we were interested in determining whether TGF-β1 could act as a regulator of KITL expression in cultured rat granulosa cells. Additionally, we aimed to explore the signalling pathway that is activated in rat granulosa cells in response to TGF-β1 stimulation.

1.3.2.3 TGF-β1 in the Modulation of FSH Action

A complex interaction between extraovarian and intraovarian factors that act as stimulatory and inhibitory signals is involved in mediating follicle development. Since these factors are all found in the milieu of the follicle, modulatory interactions between

them are to be expected, thus allowing fine balance of the developmental process. That local factors, such as those produced by the oocyte, modulate the function of the extraovarian hormone FSH in the ovary is well established (Eppig et al., 1997; Erickson and Shimasaki, 2000). For example, BMP-15 suppresses the expression of FSHR mRNA in granulosa cells (Otsuka et al., 2001). By doing so, BMP-15 may thwart the stimulatory action of FSH on KITL expression *in vivo*. Similarly, in rat ovarian surface epithelial cells, TGF-β has been shown to attenuate cAMP-induced *Kitl* mRNA expressionresulting in its suppression below the levels seen in untreated controls (Ismail et al., 1999). Therefore, we were interested in determining whether a similar modulatory role for locally produced TGF-β1 on FSH-induced KITL expression exists within rat granulosa cells. It was our intention to determine how an interaction between extraovarian FSH and locally produced TGF-β1 would modulate KITL expression levels.

1.4 Spontaneously Immortalized Granulosa Cells

Because whole follicle cultures and even primary cultures of granulosa cells are not always convenient and practical systems in which to investigate gene regulation and molecular signalling involved in the regulatory processes of follicular development, it was advantageous to identify a cell line that could serve as a more efficient model to manipulate. The Spontaneously Immortalized Granulosa Cells (SIGC) are an immortalized clonal granulosa cell line derived from primary rat ovarian granulosa cell cultures (Stein et al., 1991), and are thus comparable to primary cultures of rat granulosa cells. The cells grow in a homogenous epithelial monolayer with a cobblestone appearance and have the ability to proliferate rapidly and divide indefinitely in culture, as

a result of constitutive expression of p53, which in normal cells, is present in low levels and only during cell division (Stein et al., 1991). Despite their indefinite growth in culture, SIGC do not form clones in soft agar nor tumors in nude mice (Stein et al., 1991) indicating that they are reasonably "normal." In contrast to primary cultures of granulosa cells, which terminally differentiate (luteinize) after a few days in culture, SIGC exhibit an undifferentiated morphology and grow indefinitely without luteinization (Stein et al., 1991), which suggests that they are ideal for studying the early (preantral) stages of follicle development. Ultrastructural studies conducted to examine the morphology of SIGC reveal the presence of a rough endoplasmic reticulum as well as gap junctions, abundant desmosomes and intermediate filaments, indicating that the cells have a relatively normal epithelial phenotype. Finally, although they may not necessarily function syncytially as in the case of primary granulosa cells, the immortalized population remains coupled by gap junctions, allowing intercellular communication, although at a slightly reduced rate (Stein et al., 1991). We were therefore interested in evaluating SIGC as a model system in which to investigate the mechanisms controlling KITL expression. The SIGC cell line has been used extensively for the study of normal reproductive processes involving granulosa cells (Peluso et al., 2001a; Peluso et al., 2001b; Peluso et al., 2002; Peluso, 2003; Peluso et al., 2003; Peluso and Pappalardo, 2004; Peluso et al., 2005); however, it has not previously been exploited for KITL studies. Therefore, certain characterizations of the cell line were necessary to further understand the feasibility of their use for our interest in studying the regulation of KITL expression.

1.5 Rationale

Initially detected in the mouse embryonic ovary and continuously present throughout post-natal follicle development, KITL serves a vital role during folliculogenesis. Although the data regarding the functions of KITL are vast, investigations into the processes that regulate its expression, which ultimately control its functions at specific stages, are limited. The mechanisms that have been shown to regulate its expression include hormonal, paracrine and autocrine, but the consequences on its expression from possible interactions between these mechanisms and particularly whether differential effects on the two KITL isoforms are resultant, remain to be elucidated. Therefore, it was of particular interest to clarify the role of certain factors active in follicle development (FSH and TGF-β1), in regulation of KITL expression in granulosa cells. This project further aimed to determine the consequence of the interaction between these factors on the expression of KITL. The goal of this research was achieved by addressing the specific objectives that follow:

1. Develop SIGC as a model system in which to investigate the mechanisms controlling KITL-1 and KITL-2 expression.

Our approach involved initially screening the cell line for the expression of *Kitl1* and *Kitl2* mRNA transcripts and the protein isoforms. Proliferation assays were conducted in order to examine the growth characteristics of the cell line and more importantly, to determine whether *Kitl* mRNA expression varied with changes in cell density thus characterizating the cell line's expression of the isoforms. Finally, the expression of *Kitl1* and *Kitl2* mRNA in SIGC was compared to the expression in primary cultures of rat granulosa cells. The purpose of this was to determine whether down-

regulation of expression occurred in the immortalized cell line, thus making it less likely that we would be able to observe quantifiable responses to particular treatments where a down-regulatory response would be expected. In this way, we determined their usability as a substitute for primary cultures of rat granulosa cells for the study of the regulation of KITL expression.

2. Demonstrate the regulatory effect of FSH on KITL-1 and KITL-2 expression and investigate the involvement of the PKA pathway.

Our approach to this problem was to initially test whether FSH induces *Kitl1* and *Kitl2* mRNA expression in primary cultures of rat granulosa cells. We then determined whether blocking the PKA pathway using a specific kinase inhibitor would prevent FSH regulation of *Kitl1* and *Kitl2* mRNA expression. In addition to using an inhibitor of PKA to investigate the involvement of the pathway, supposed effectors of the pathway that have been previously shown to mimic the effects of FSH in granulosa cells were utilized in SIGC to determine whether they could mimic FSH regulation of *Kitl* mRNA expression. In this way, we intended to demonstrate for the first time FSH-induced KITL expression in cultured rat granulosa cells. Additionally, we aimed to provide insight into the FSH-mediated cellular signalling pathway with resultant regulation of KITL expression, thus clarifying the role of PKA signalling.

3. Determine whether a regulatory effect of TGF-β1 on KITL-1 and KITL-2 expression exists and identify the signalling components involved.

Our approach to this problem involved initially determining how TGF-β1 treatment of SIGC would affect *Kitl1* and *Kitl2* mRNA expression. We then tested the effect of TGF-β1 on *Kitl1* and *Kitl2* mRNA expression in primary cultures of rat

granulosa cells. After screening the immortalized granulosa cell line for the expression of the proteins traditionally implicated in the TGF-β/activin signalling pathway, we determined whether TGF-β1 treatment of the cells would result in the activation by phosphorylation of these proteins. Additionally, by experimentation, we excluded the involvement of proteins not traditionally associated with TGF-β/activin signalling in TGF-β1 signalling in rat granulosa cells. Through these studies, our aim was to determine whether TGF-β1, produced by all the major cell types of the ovary at varying points throughout follicle development, is, like GDF-9, BMP-15, KGF and LIF, involved in regulating KITL expression. Additionally, by investigating the downstream signalling pathway, we expected to shed light on the proteins required to mediate TGF-β1 regulation of KITL expression in rat granulosa cells.

4. Determine the consequence of simultaneous treatment with FSH and TGF-β1 on KITL-1 and KITL-2 expression.

Our approach to this problem involved treatment of rat primary granulosa cells with both FSH and TGF-β1, followed by assessment of the effects on *Kitl1* and *Kitl2* mRNA expression. Through this study, we anticipated shedding light on the complexities of the regulation of the expression of KITL that may exist *in vivo*.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Culture of Spontaneously Immortalized Granulosa Cells (SIGC)

SIGC were kindly provided by Dr. Robert Burghardt of Texas A&M University (College Station, TX) and routinely cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12; Invitrogen Canada Inc., Burlington, ON) containing 5% serum [3:1 fetal bovine serum: fetal calf serum (FBS:FCS)] and supplemented with 75μg/ml penicillin, 10μg/ml streptomycin, 25μg/ml gentamycin (Sigma-Aldrich Canada Ltd., Oakville, ON) and maintained in a humidified incubator at 37°C with 5% CO_2.

2.2 Detecting *Kitl1* and *Kitl2* mRNA Transcripts and the Protein Isoforms in SIGC

For experiments to assess the expression of *Kitl1* and *Kitl2* mRNA transcripts and protein isoforms in SIGC, cells were cultured in 10mm cell culture plates and grown to confluence. At the end of the culture period, one of two protocols was performed: (1) medium was aspirated and cells were rinsed with 1× phosphate-buffered saline (PBS), followed by trypsinization and collection; cells were then flash-frozen in a dry ice-ethanol bath and stored at -80°C for later isolation of total cellular RNA; or (2) protein extraction from cultured cells was performed immediately according to the methods outlined (*see* **SDS PAGE and Western Blot Analysis**).

2.3 SIGC Proliferation Assays

SIGC were plated at a density of 7.5×10^4 cells/well in 6-well cell culture plates. After 24, 48 and 72 hours of culture, medium was aspirated and cells were rinsed with 1×PBS, followed by trypsinization, collection and counting using a Coulter Counter®

(Beckman Coulter Canada Inc., Mississauga, ON). Cells were then flash-frozen and stored at -80°C for later isolation of total cellular RNA and analysis (*see* **RT-PCR and QPCR**) to assess differences in *Kitl1* and *Kitl2* mRNA expression between the different time points.

2.4 Treatment of SIGC

2.4.1 To Assess Changes in *Kitl1* and *Kitl2* mRNA Expression

For assessing the individual effects of dbcAMP (Roche Diagnostics Canada, Laval, Quebec), forskolin (Sigma-Aldrich Canada Ltd., Oakville, ON), an activator of adenylate cyclase (Seamon et al., 1981) and recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) on *Kitl1* and *Kitl2* mRNA expression, SIGC were plated at a density of 3×10^5 cells/well in 12-well cell culture plates in 5% serum-containing DMEM/F12 and allowed to adhere for approximately 8 hours. Medium was then replaced with serum-free DMEM/F12 and serum-starvation was carried out overnight. The following day, medium was changed to fresh serum-free DMEM/F12 and cells were cultured with vehicle or in the presence of the following reagents at the concentration(s) specified: dbcAMP (75, 150 or 300μM) for which the maximum concentration tested has been shown to induce an increase in overall *Kitl* mRNA expression in mouse primary granulosa cells (Packer et al., 1994); forskolin (2.5, 5.0 or 10μM; diluted from a 25mM stock dissolved in DMSO), for which the maximum concentration tested has been shown to be effective at mimicking the effects of FSH in rat primary granulosa cells (Hsueh et al., 1984; Das et al., 1996); or TGF-β1 (1.0, 5.0 or 10ng/ml; diluted from a 20μg/ml stock dissolved in 4mM HCl/0.1% BSA), for which the maximum concentration tested is most commonly

used to treat rat primary granulosa cells (Hernandez et al., 1990; Ke et al., 2004; 2005). Treatments were carried out for 24 hours and terminated by aspirating medium and rinsing cells with 1×PBS, followed by trypsinization and collection. Cells were flash-frozen and stored at -80°C for later isolation of total cellular RNA and analysis (*see* **RT-PCR and QPCR**).

2.4.2 To Assess Changes in Smad Activation

For experiments to detect changes in the expression of Smad or phosphorylated (phospho)-Smad proteins, cells were plated at a density of 1.8×10^6 cells/well in 6-well cell culture plates in 5% serum-containing DMEM/F-12 for approximately 8 hours followed by serum starvation overnight. The following day, cells were treated with 10ng/ml TGF-β1 for 0, 15 or 30 minutes, or 100ng/ml BMP-2 (gift of Dr. I. Lorimer) for 0 or 30 minutes, a time course that has previously been used for the detection of phospho-Smad proteins in primary granulosa cells post-treatment with TGF-β family members (Kaivo-Oja et al., 2003; Mazerbourg et al., 2004). Extraction of total cellular protein was performed immediately following the treatment period according to the methods outlined (*see* **SDS PAGE and Western Blot Analysis**).

2.5 Animals

Female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and housed at the University of Ottawa (Ottawa, ON) animal care facilities under controlled conditions and provided food and water *ad libitum*. Animals were handled in accordance with Guidelines for the Care and Use of Laboratory Animals

of the Canadian Council on Animal Care using protocols approved by the University of Ottawa Animal Care Committee.

2.6 Isolation and Preparation of Primary Granulosa Cells

Immature female rats (17- or 19-days-of-age) were subcutaneously injected for 3 consecutive days with 1mg diethylstilbestrol (DES), an estrogen analogue that stimulates granulosa cell proliferation (Goldenberg et al., 1972). Granulosa cells of large preantral follicles were collected and prepared according to previously described methods (Das et al., 1996) with some modifications. Briefly, ovaries were trimmed to remove the bursa, fat and oviducts and incubated for approximately 10-15 minutes at 37°C, 5% CO_2 in 6mM EGTA in DMEM/F-12 followed by a 10-15 minute incubation in 0.5M sucrose in DMEM/F-12. A 30-gauge needle was used to puncture each ovary repeatedly; granulosa cells were then forced out using a heat-polished and sealed Pasteur pipette. Cells were collected into a 50ml Falcon tube, the volume of media doubled with 5% serum-containing DMEM/F-12 and supplemented with 75μg/ml penicillin, 10μg/ml streptomycin, 50μg/ml gentamycin. This was followed by a brief vortex, then centrifugation at 1,000 RPM for 5 minutes. Media was aspirated and granulosa cells were resuspended in 5ml of 5% serum-containing DMEM/F-12 for cell number determination.

2.7 To Assess Differences in *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells

For experiments to assess the expression of *Kitl1* and *Kitl2* mRNA expression in primary granulosa cells, cells were either flash-frozen immediately post-ovarian

collection or plated at a density of 2×10^6 cells/well in 6-well cell culture plates for 24 and 48 hours. At the end of the culture period, cells were collected, flash-frozen and stored at -80°C for later isolation of total cellular RNA and analysis (*see* **RT-PCR and QPCR**).

2.8 Treatment of Primary Granulosa Cells

For assessing the individual effects of FSH (National Hormone and Pituitary Program), TGF-β1 and 17β-estradiol (Sigma-Aldrich Canada Ltd., Oakville, ON) on *Kitl1* and *Kitl2* mRNA expression, cells were plated at a density of 1×10^6 cells/well in 12-well cell culture plates and maintained in a humidified incubator at 37°C and 5% CO_2 for overnight. The following day, medium was changed to fresh 5% serum-containing DMEM/F12 to remove any non-attached cells and cells were cultured with vehicle or in the presence of the following reagents at the concentration(s) specified: FSH (0.5, 5.0 or 50ng/ml) a range of concentrations that is commonly used to treat rat primary granulosa cells (Haynes-Johnson et al., 1999; Gonzalez-Robayna et al., 2000); TGF-β1 (10ng/ml); or 17β-estradiol (500nM), a concentration previously shown to stimulate proliferation of rat primary granulosa cells (Bendell and Dorrington, 1988).

To determine the combined effects of FSH and H-89 (Sigma-Aldrich Canada Ltd., Oakville, ON) on *Kitl1* and *Kitl2* mRNA expression, cells were cultured with vehicle or in the presence of FSH (50ng/ml) and/or H-89 (10μM; diluted from a 5mM stock dissolved in DMSO) with which cells were pretreated for 1 hour prior to the addition of FSH. The concentration of H-89 chosen to block PKA activity was based on the recommendation of the manufacturer; additionally, this is the concentration most

commonly used to block the action of FSH and forskolin in rat primary granulosa cells (Haynes-Johnson et al., 1999; Gonzalez-Robayna et al., 2000).

To test the efficacy of the anti-TGF-β1 antibody (R&D Systems, Minneapolis, MN) in preventing TGF-β1 action on *Kitl1* and *Kitl2* mRNA expression, cells were cultured with vehicle or in the presence of TGF-β1 (10ng/ml) and/or the anti-TGF-β1 antibody (5μg), a concentration previously shown to neutralize the actions of TGF-β on granulosa cell growth (Bendell and Dorrington, 1988). For evaluating the effect of the anti-TGF-β1 antibody on 17β-estradiol action, cells were cultured with vehicle or in the presence of 17β-estradiol (500nM) and/or anti-TGF-β1 antibody (5μg) or immunoglobulin Y (IgY; 5μg; R&D Systems, Minneapolis, MN), the backbone of the anti-TGF-β1 antibody. Finally, to determine the combined effects of FSH and TGF-β1, cells were cultured with vehicle or in the presence of FSH (50ng/ml) and/or TGF-β1 (10ng/ml). All treatments were carried out for 24 hours and terminated by aspirating medium and rinsing cells with 1×PBS, followed by trypsinization and collection. Cells were then flash-frozen in a dry ice-ethanol bath and stored at -80°C for later isolation of total cellular RNA and analysis (*see* **RT-PCR and QPCR**).

2.9 RNA Analysis

2.9.1 Reverse Transcription (RT)

For the generation of complementary DNA (cDNA), total cellular RNA was isolated from flash-frozen cells using the RNeasy® Mini Kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol and eluted with 30μl of RNase-free water. To minimize genomic DNA contamination, deoxyribonuclease treatment of each RNA

sample was performed using the DNase-free® Kit (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. RNA levels were then quantified through spectrophotometry using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). cDNA was generated from purified RNA by RT using the Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany). Initially, a 7µl reaction consisting of 1µg RNA, 1µl deoxynucleotide triphosphate mix (dNTPs; 10mM stock) and 1µl random primers (0.3µg/µl stock) was incubated at 65°C for 5 minutes, followed by an incubation at 4°C for 1 minute. This reaction was then brought to a total volume of 20µl by supplementing with 4µl First Strand Buffer (5× stock), 1µl dithiothreitol (0.1M stock), 1µl RT Superscript™ III (200U/µl stock) (Invitrogen Canada Inc., Burlington, ON) and 1µl RNase Out (10U/µl stock). Reactions were then incubated at 25°C for 5 minutes, followed by 50°C for 50 minutes and deactivated at 70°C for 15 minutes. To further exclude the possibility of genomic DNA contamination in RT reactions, controls were generated in the absence of reverse transcriptase. All reagents were purchased from Invitrogen Canada Inc. (Burlington, ON) unless stated otherwise.

2.9.2 Primer Design

For the detection of the *Kitl1* and *Kitl2* mRNA transcripts in SIGC or rat primary granulosa cells, primers were designed and ordered from Sigma Genosys (Oakville, ON) based on the nucleotide sequence published for rat *Kitl1* (GenBank accession #: NM_021843) and *Kitl2* (GenBank accession #: AF071205) mRNA transcripts. In order to differentiate between the expression of the two isoforms, each primer set amplified either the region of cDNA that encompasses exon 6 (for detection of *Kitl1*) or flanks the region encompassing the deleted exon 6 (for detection of *Kitl2*). The primer sequences

were as follows: *Kitl1* (5' -TGC AGC CAG TTC CCT TAG GA- 3' and 3' -CCA GTA TAA GGC TCC AAA AGC AA- 5'); *Kitl2* (5' -GAG AAA GAA AGC CGC AAA G- 3' and 3' -CCA GTA TAA GGC TCC AAA AGC AA- 5'), and were expected to yield products of 148bp and 114bp corresponding to rat *Kitl1* and *Kitl2*, respectively. For the detection of the *Kitl1* and *Kitl2* mRNA transcripts in mouse cell lines, primers previously designed based on the mouse *Kitl* mRNA sequence were utilized and were expected to yield products of 191bp and 400bp corresponding to mouse *Kitl1* and *Kitl2*, respectively (Thomas et al., 2005). For the detection of the FSHR mRNA in SIGC or rat primary granulosa cells, primers previously designed (Rubina Ismail, unpublished) based on the rat FSHR mRNA sequence were utilized. The primer sequences were as follows: 5' -CAC TGG CTG TGT CAT TGC TCT- 3' and 3' -CCC CAT CTT GTC TTG ATC CTC TTA- 5' and were expected to yield a product of 1812bp.

2.9.3 Polymerase Chain Reaction (PCR)

Each 50µl PCR reaction consisted of 50ng cDNA, 1× PCR reaction buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.05U Platinum® Taq and 0.5µM each of the forward and reverse primers. In some reactions, sterile double-distilled water was used in place of the cDNA template to serve as a negative control. PCR was carried out using the Eppendorf Mastercycler® Gradient Thermal Cycler. For the detection of rat and mouse *Kitl1* and *Kitl2* mRNA transcripts, the following cycling parameters were utilized: 1 cycle of predenaturation (94°C for 1 minute), 35 cycles of amplification alternating between denaturation (94°C for 10 seconds), annealing (58°C for 15 seconds) and extension (72°C for 1 minute), followed by a final extension step (72°C for 5 minutes). For the detection of the FSHR mRNA, the cycling parameters utilized were as follows: 1

cycle of predenaturation (94°C for 1 minute), 30 cycles of amplification alternating between denaturation (94°C for 45 seconds), annealing (55°C for 45 seconds) and extension (72°C for 1 minute), followed by a final extension step (72°C for 10 minutes).

2.9.4 Gel Electrophoresis

To detect *Kitl1* and *Kitl2* mRNA transcripts in SIGC or rat primary granulosa cells, PCR products and 100bp DNA ladder (2.5μg) were then loaded onto 1.2% agarose gel containing ethidium bromide (0.1μg/ul) and separated at 80V for approximately 2 hours, followed by visualization using a UV transilluminator. Bands were subsequently excised for extraction of the DNA using the QIAquick® Gel Extraction Kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol. Templates were sent for sequencing to the Ottawa Health Research Institute StemCore Laboratories DNA Sequencing Facility (Ottawa, ON) to ensure consistency with sequences previously reported for rat *Kitl1* and *Kitl2* mRNA transcripts. All reagents were purchased from Invitrogen Canada Inc. (Burlington, ON) unless stated otherwise. For experiments to detect *Kitl1* and *Kitl2* mRNA transcripts in mouse cell lines, PCR products and 100bp DNA ladder (2.5μg) were loaded onto 1.0% agarose gel containing ethidium bromide (0.1μg/ul) and separated at 80V for approximately 1 hour, followed by visualization using a UV transilluminator. Finally, for experiments to detect the FSHR mRNA in SIGC or rat primary granulosa cells, PCR products and 100bp DNA ladder (2.5μg) were loaded onto 0.6% agarose gel containing ethidium bromide (0.1μg/ul) and separated at 60V for approximately 1 hour, followed by visualization using a UV transilluminator.

2.9.5 Quantitative PCR (QPCR)

The oligonucleotide primers described above for the detection of rat *Kitl1* and *Kitl2* mRNA transcripts were designed to span only expressed regions of the coding sequence (exons), which prevents the detection of genomic *Kitl* sequence, making them ideal for quantifying *Kitl1* and *Kitl2* mRNA expression levels. For relative quantification, real-time QPCR with melting curve analysis was performed in an Applied Biosystems 7500 PCR system (Applied Biosystems, Foster City, CA). Each 25µl QPCR reaction consisted of 200ng cDNA, 12.5µl 2× SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 0.5µM each of the forward and reverse primer. The endogenous control gene 18S ribosomal RNA (18S rRNA) was amplified as a reference for mRNA quantification; for its detection, the reaction consisted of 0.01ng cDNA, 12.5µl 2× SYBR® Green PCR Master Mix and 2µl of the 18S Universal Primer (Cedarlane Laboratories Ltd., Burlington, ON). Negative control samples generated in the absence of reverse transcriptase were also included in the QPCR runs. The cycling parameters were as follows: 1 cycle of incubation (50°C for 2 minutes), 1 cycle of AmpliTaq Gold® DNA polymerase activation (95°C for 10 minutes), followed by 40 cycles of amplification alternating between denaturation (95°C for 15 seconds) and annealing/extension (60°C for 1 minute). The SYBR Green I fluorescent signal was obtained once per cycle at the end of the extension step. After amplification, melting curve analysis was performed for distinguishing non-specific products and primer dimers from the target genes. Samples were heated to 95°C for 15 seconds, then cooled to 70°C for 1 minute, followed by a linear temperature increase to 95°C at a rate of 0.5°C/second, with continuous monitoring of the fluorescent signal. Data was analyzed using the

Applied Biosystems 7500 System SDS Software version 1.2 (Applied Biosystems, Foster City, CA). Additionally, periodic analysis of the QPCR products was performed through DNA gel electrophoresis. This was to confirm product sizes as well as to ensure the absence of genomic DNA contamination as determined by lack of product in the negative control samples. QPCR products were loaded onto 1.2% agarose gel containing 0.1μg/ul ethidium bromide and separated at 80V for approximately 1 hour followed by visualization using a UV transilluminator.

2.10 Protein Analysis

2.10.1 Preparation of Samples

Monolayers of SIGC were rinsed two times with ice cold 1×PBS. Approximately 1ml of 1×PBS was then used to lift the cells by scraping using a "rubber policeman". The cell suspensions were then placed into separate Eppendorf tubes and centrifuged at 4,000 RPM for 3 minutes at 4°C. The fluid in the tubes was then aspirated and the pellet resuspended using up to 500μl of either Whole Cell buffer (50mM Tris-Cl @ pH 7.6, 150mM NaCl, 1mM EDTA, 0.5% NP40, 0.1% Triton X, 1× Protease Inhibitor Cocktail) for non-phosphorylated proteins or RIPA buffer (50mM HEPES @ pH 7.4, 150mM NaCl, 10% Glycerol, 1.5mM $MgCl_2$, 1mM EGTA, 1% Triton X, 0.1% SDS and, prior to use: 1mM sodium vanadate, 10mM sodium pyrophosphate, 10mM sodium fluoride, 1% sodium deoxycholate, 1mM PMSF, 1× Protease Inhibitor Cocktail) for phosphorylated proteins. The samples were sonicated for 15-30 seconds and centrifuged at 14,000 RPM for 10 minutes at 4°C. The supernatants from each sample were transferred into fresh Eppendorf tubes; protein levels were then quantified using the Bio-Rad Protein Assay

Dye Reagent system (BioRad, Mississauga, ON) and determination of the absorbance at a wavelength of 595nm through the Beckman Coulter DU 640 Spectrophotometer (Beckman Coulter Canada Inc., Mississauga, ON).

2.10.2 SDS-PAGE

Equal amounts of proteins (25μg) containing 1× SDS-PAGE load buffer (62.5mM Tris-Cl @ pH 6.8, 2% SDS, 11% Glycerol, 0.0008% Bromophenol Blue, 5% β-mercaptoethanol) and sterile double-distilled water to a volume of 40μl were loaded onto a pre-cast one-dimensional NuPage Novex Bis-Tris acrylamide gel with a 4-12% gradient (Xcell SureLock Mini-Cell; Invitrogen Canada Inc., Burlington, ON) in a vertical electrophoresis system containing 1× NuPage MOPS running buffer (50mM MOPS, 50mM Tris, 3.5mM SDS, 1.0mM NaEDTA). 5μl of PageRuler™ Prestained Protein Ladder (Fermentas Canada Inc., Burlington, ON) was also included to determine the molecular mass in kDa of protein bands. Separation was carried out at 120V for approximately 2 hours at room temperature.

2.10.3 Western Blot

Proteins were then transferred at 30V for 3 hours at room temperature onto Hybond™-C Extra nitrocellulose membranes (Amersham Biosciences, Arlington Heights, IL) using the Xcell II Blot Module (Invitrogen Canada Inc., Burlington, ON) according to the manufacturer's protocol. Membranes were blocked in 5% milk TBST (20mM Tris, 137mM NaCl, 0.05% Tween-20, @ pH 7.6) for 1 hour at room temperature followed by overnight incubations at 4°C with the appropriate primary antibodies diluted in 5% milk TBST to the concentrations specified: goat polyclonal anti-SCF (1:250; R&D Systems Inc., Minneapolis, MN) and (1:50; Santa Cruz Biotechnology Inc., Santa Cruz,

CA), both for the assessment of KITL-1 and KITL-2 protein expression in SIGC; rabbit polyclonal anti-human Smad2 (1:200; Zymed Laboratories, South San Francisco, CA); rabbit polyclonal anti-human Smad3 (Zymed Laboratories, South San Francisco, CA); goat polyclonal anti-human Smad4 (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA); rabbit polyclonal anti-human phospho-Smad2 (1:500; Cell Signaling Technology Inc., Boston, MA); rabbit polyclonal anti-human phospho-Smad3 (1:250; Cell Signaling Technology Inc., Boston, MA); rabbit monoclonal anti-human phospho-Smad1/5 (1:250; Cell Signaling Technology Inc., Boston, MA). The antibodies for phosphorylated Smads detect Smads that are C-terminally phosphorylated by distinct ligand-activated type I serine/threonine receptors. These antibodies are specific to the phosphorylated forms of the Smad proteins and do not recognize the unphosphorylated proteins. Blots were washed in TBST three times for 10 minutes each time both prior and post incubation with the appropriate secondary antibody for 1 hour at room temperature. Protein bands were then visualized using the Amersham Enhanced Chemiluminescence® kit (Amersham Life Sciences, Oakville, ON) and the Kodak™ X-Omat™ Blue XB-1 film (PerkinElmer Life and Analytical Sciences, Woodbridge, ON). To confirm equivalent loading of protein samples, all membranes were reprobed using a monoclonal mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:2000; Abcam Inc., Cambridge, MA).

2.11 Luciferase Assays

SIGC were plated at a density of 7.5×10^4 cells/well in 6-well cell culture plates in 5% serum-containing DMEM/F12 supplemented with 75μg/ml penicillin, 10μg/ml

streptomycin, 25μg/ml gentamycin and maintained in a humidified incubator at 37°C with 5% CO_2, for overnight. The following day, medium was replaced with fresh 5% serum-containing DMEM/F12. Cells were then transiently transfected using FuGENE® 6 (Roche Diagnostics Canada, Laval, Quebec) according to the manufacturer's protocol. Each well was transfected with a constant amount of DNA containing reporter plasmid 4×CRE-Luciferase (3μg) (gift of Dr. D. Gray; Stratagene, La Jolla, CA) and *Renilla* expression vector (3ng) (gift of Dr. D. Gray; Promega, Madison, WI). At 24 hours post-transfection, medium was replaced with fresh 5% serum-containing DMEM/F12 and cells were cultured in the presence of H-89 (0, 2.5, 5.0 or 7.5μM) or DMSO vehicle (0.05, 0.10 or 0.15%) corresponding to the amount of DMSO found in the H-89 treated cells. Treatments were carried out for 24 hours and terminated by aspirating medium and rinsing cells with 1×PBS. Cells were lysed and analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol, and the Lumat LB 9507 luminometer (EG&G Berthold, Bundoora, VIC, Australia).

2.12 Statistical Analysis

Each experiment performed was repeated at least three times using different, independent cell preparations. All experiments were conducted in triplicate. Levels of *Kitl1* and *Kitl2* mRNA (target genes) were standardized to 18S rRNA (reference gene), which corrected for differences in both RNA quality and quantity between samples, and were then expressed as fold-differences compared with the vehicle-treated control sample in the experiment. The levels of expression of both transcripts in all treatment groups

from one experiment were quantified simultaneously in a single real-time QPCR run. All values are represented as the fold change ± standard error of the mean (SEM).

For statistical evaluation of the effect of multiple treatments on either *Kitl1* and *Kitl2* mRNA expression or CRE-luciferase activity, one-way ANOVA (parametric) was performed. When whole group differences were noted, a Bonferroni Multiple Comparison post-test was used to determine significance with P values < 0.05 accepted as statistically significant. For experiments consisting of only two treatment groups (ie. control and treated sample), a two-tailed paired t-test with a 95% confidence interval was utilized to compare the treated group with its respective control. Data for the two transcripts was analyzed separately. All statistical analyses were performed using GraphPad Prism Software (Version 3.2m GraphPad Software, San Diego, CA).

3.1 Characterization of the Expression of *Kitl1* and *Kitl2* mRNA Transcripts and the Protein Isoforms in SIGC

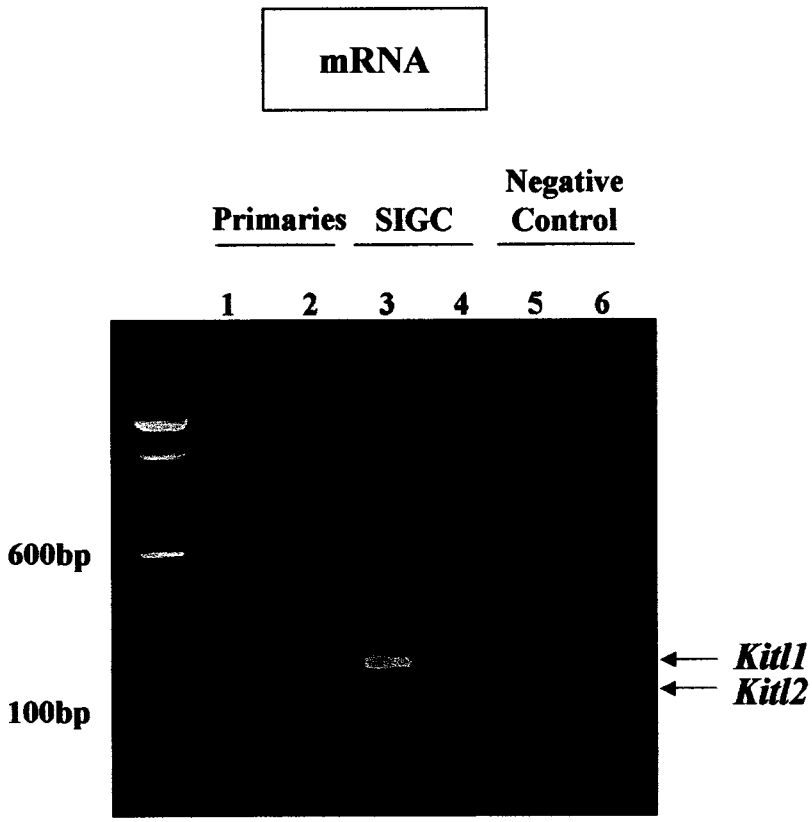
3.1.1 SIGC Express the *Kitl1* and *Kitl2* mRNA Transcripts

In order to determine whether SIGC express detectable levels of the *Kitl1* and *Kitl2* mRNA transcripts, RNA was isolated from cultured SIGC and monolayers of primary granulosa cells, which served as a positive control. A product 148bp in length, corresponding to the *Kitl1* mRNA transcript, was amplified in the 3rd lane (*figure 7*), consisting of cDNA from SIGC; this matched the size of the product detected in the 1st lane (positive control) consisting of cDNA from primary granulosa cells. A product 114bp in length, corresponding to the *Kitl2* mRNA transcript, was amplified in the 4th lane, also consisting of cDNA from SIGC; this matched the size of the product detected in the 2nd lane (positive control) consisting of cDNA from primary granulosa cells. Sequencing of these bands confirmed their homology to the reported sequences for the rat *Kitl1* (GenBank accession #: NM_021843) and *Kitl2* (GenBank accession #: AF071205) mRNA transcripts. These findings made it possible to proceed with using SIGC as an alternative to primary granulosa cells in some experiments.

To demonstrate the ability of *Kitl* transcripts to be translated into protein, the expression of KITL-1 and KITL-2 protein isoforms in SIGC was examined by Western blot analysis. To date, there has been only one study that utilized this method for demonstrating KITL protein expression and this was in a malignant mesothelioma cell line (Catalano et al., 2004). We used an anti-mouse SCF antibody (R&D Systems Inc) to assess the expression of KITL-1 and KITL-2 protein isoforms in untreated SIGC and

Figure 7. **Expression of *Kitl1* and *Kitl2* mRNA Transcripts in SIGC**

RT-PCR was performed using 1μg of RNA extracted from SIGC cultured for 24 hours. RNA from primary granulosa cells (Primaries) of DES-primed rats was included as a positive control. The two sets of rat *Kitl* PCR primers, designed to amplify either the region of cDNA that encompasses exon 6 (for detection of *Kitl1*) or flanks the region encompassing the deleted exon 6 (for detection of *Kitl2*), were utilized. PCR products and 100bp DNA ladder (2.5μg) were loaded onto a 1.2% agarose gel containing ethidium bromide (0.1μg/ul), and separated at 80V for approximately 2 hours. A product 148bp in length, corresponding to the *Kitl1* mRNA transcript, was amplified in the 1st (positive control) and 3rd lanes, consisting of cDNA from primary granulosa cells and SIGC, respectively. A product 114bp in length, corresponding to the *Kitl2* mRNA transcript, was amplified in the 2nd (positive control) and 4th lanes, consisting of cDNA from primary granulosa cells and SIGC, respectively.

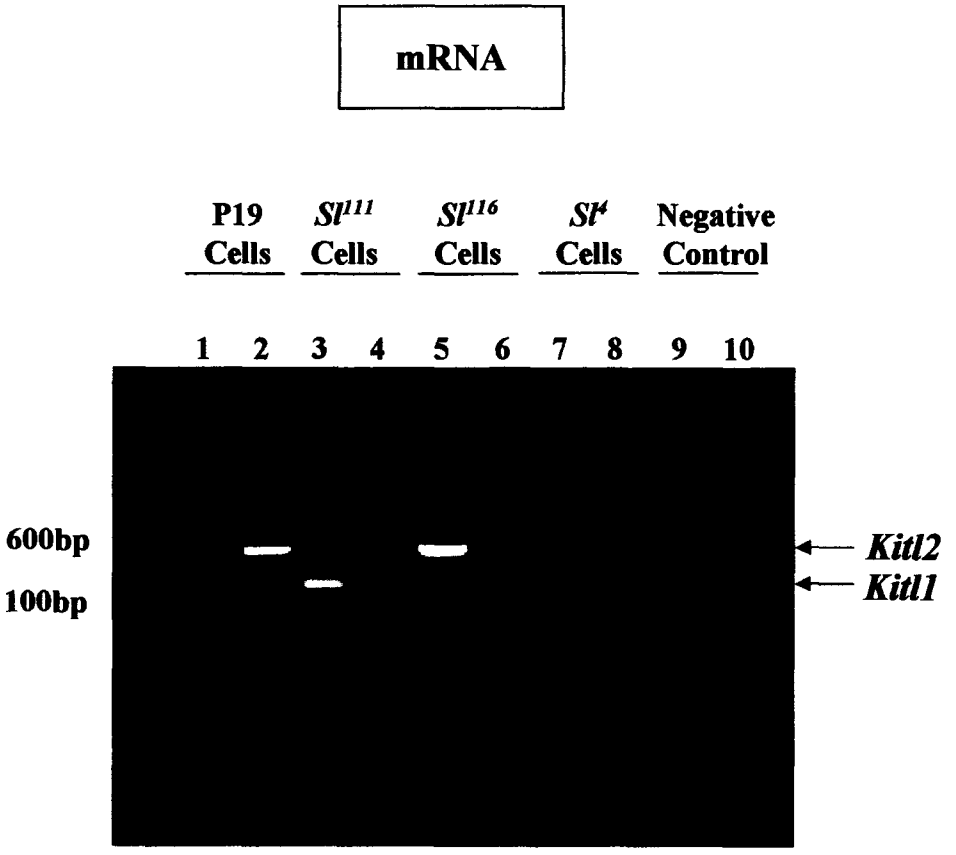


three genetically modified 3T3 fibroblast cell lines: Sl^{111} (KITL-1 positive control), Sl^{116} (KITL-2 positive control) and Sl^{4} (KITL-1 and KITL-2 negative control) cells (Thomas et al., 2007). In total protein extract from SIGC, two bands at 35kDa and 25kDA were detected, corresponding approximately to the expected sizes of the KITL-1 (31kDa) and KITL-2 (23kDa) protein isoforms (results not shown). However, these bands were also present in the Sl^{4} cells carrying the Steel (*Sl*) mutation, which renders the cell incapable of expressing KITL-1 and KITL-2 (Huang et al., 1990), as verified through RT-PCR (*figure 8*) thus leading us to conclude that the bands were non-specific. In *figure 8*, we have shown that in the 1st and 2nd lanes, containing cDNA from P19 cells (positive control), the primer set for the detection of the *Kitl1* mRNA transcript amplified a product 191bp in length (1st lane), while that for the detection of the *Kitl2* mRNA transcript amplified a product 400bp in length (2nd lane). In the 3rd lane, which contained cDNA from Sl^{111} cells, a product 191bp in length was amplified, while no product was amplified in the 4th lane, confirming the lack of *Kitl2* mRNA expression by Sl^{111} cells. In the 5th lane, which contained cDNA from Sl^{116} cells, a product 400bp in length was amplified confirming the presence of *Kitl2* mRNA expression by Sl^{116} while no product was amplified in the 6th lane, confirming the lack of *Kitl1* mRNA expression by Sl^{116} cells. Finally, in the 7th and 8th lanes, which both contain cDNA from Sl^{4} cells, products of either size were not amplified, confirming the lack of *Kitl1* and *Kitl2* mRNA expression in this cell line.

Attempts were also made to detect KITL protein expression by Western blot analysis using a second anti-mouse SCF antibody (Santa Cruz); however, this antibody completely failed to detect protein bands of the expected sizes in protein from all three *Sl*

Figure 8. **Expression of *Kitl1* and *Kitl2* mRNA Transcripts in *Sl* Cells**

RT-PCR was performed using 1μg of RNA extracted from either Sl^{111} (*Kitl1* positive control), Sl^{116} (*Kitl2* positive control) or Sl^{4} (*Kitl1* and *Kitl2* negative control) cells. RNA from P19 embryonic carcinoma cells was included as a positive control for both the *Kitl1* and *Kitl2* mRNA transcripts. The two sets of mouse *Kitl* PCR primers, designed to amplify either the region of cDNA that encompasses exon 6 (for detection of *Kitl1* mRNA transcript) or flanks the region encompassing the deleted exon 6 (for detection of *Kitl2* mRNA transcript), were used. PCR products and 100bp DNA ladder (2.5μg) were loaded onto 1.0% agarose gel containing ethidium bromide (0.1μg/ul), and separated at 80V for approximately 1 hour. A product 191bp in length, corresponding to the *Kitl1* mRNA transcript, was amplified in the 1st (positive control) and 3rd lanes, consisting of cDNA from P19 and Sl^{111} cells, respectively. A product 400bp in length, corresponding to the *Kitl2* mRNA transcript, was amplified in the 2nd (positive control) and 5th lanes, consisting of cDNA from P19 and Sl^{116} cells, respectively. No products were amplified in the lanes consisting of cDNA from Sl^{4} cells.



cell lines tested as well as mouse ovaries, rat ovaries, primary granulosa cells, SIGC and P19 cells (data not shown). This was likely a result of the antibody being of poor quality.

3.1.2 *Kitl1* and *Kitl2* mRNA Expression in SIGC is Dramatically Elevated After 24 Hours of Culture as Compared to 48 and 72 Hours

In order to determine whether the levels of *Kitl1* and *Kitl2* mRNA expression vary with increasing cell density, SIGC proliferation assays were conducted. At 24, 48 and 72 hours after re-plating, SIGC were counted and levels of *Kitl1* and *Kitl2* mRNA expression at each time point were assessed via real-time QPCR. It was noted that the most rapid rate of increase in the population size of SIGC occurred in the initial 24 hours after re-plating, during which time the cell population size increased 86% above the density of cells used to seed the cell culture plates. In the following 24 hour period (48 hours after re-plating), cell numbers increased 46% above the density of cells present at 24 hours after re-plating, and in the subsequent 24 hours (72 hours after re-plating), cell numbers increased 67% above the density of cells present 48 hours after re-plating, during which time confluence was also attained. Therefore, in the initial 24 and 48 hours after re-plating, the cell population size reached 18% and 33% respectively, of the total size attained after 72 hours (confluence). *Figure 9A* is a growth curve demonstrating the growth behaviour of SIGC as described.

With regards to the expression of *Kitl* mRNA during these time points, it was observed that after 24 hours in culture, expression of *Kitl1* mRNA was 86% greater than at the later time points of 48 and 72 hours, when cells were nearing and had reached confluence, respectively (*figure 9B*); expression of *Kitl2* mRNA was 88% and 90%

greater in the initial 24 hours as compared to 48 and 72 hours, respectively (*figure 9C*). No statistical significance was noted in the expression levels of *Kitl1* and *Kitl2* mRNA between the 48 and 72 hour time points ($P > 0.05$). The dramatically elevated levels of *Kitl1* ($n = 3$ experiments, $P < 0.001$ by ANOVA) and *Kitl2* ($n = 3$ experiments, $P < 0.001$) mRNA expression during the initial 24 hour culture period (as compared to the later time points) corresponded with the time period during which the most rapid increase in cell number was noted. At the later time points of 48 and 72 hours, despite their being a greater number of SIGC present, the expression levels of *Kitl1* and *Kitl2* mRNA were significantly lower. Therefore, it is possible that the effect of cell density on *Kitl* mRNA expression is such that when the granulosa cell population is low, as sensed by few cell-cell contacts for example, *Kitl* expression is up-regulated.

3.1.3 Expression Levels of *Kitl1* and *Kitl2* mRNA in SIGC is Comparable to the Levels in Primary Granulosa Cells

Since SIGC would be serving as a model of primary granulosa cells, it was also important to compare their capacity for expression of the *Kitl1* and *Kitl2* mRNA transcripts at different time points in their growth with that of primary cultures of granulosa cells. *Figure 10* demonstrates that levels of *Kitl1* (*A*) and *Kitl2* (*B*) mRNA expression in SIGC cultured for 24 hours (non-confluent) were 98% and 97%, respectively, above those seen in primary granulosa cells cultured for 24 hours (confluent; $n = 4$ experiments, $P < 0.05$ by ANOVA). However, after 48 and 72 hours in culture, there was no longer a statistical difference detected ($P > 0.05$ by ANOVA) in the levels of *Kitl1* and *Kitl2* mRNA expression between SIGC and primary cultures of granulosa cells; the expression of *Kitl1* and *Kitl2* mRNA decreased in the former as previously discussed.

***Figure 9. Kitl1* and *Kitl2* mRNA Expression During SIGC Proliferation**

SIGC were plated at a density of 7.5×10^4 cells/well in 6-well cell culture plates. After 24, 48 and 72 hours of culture, cells were collected, counted and flash frozen. The SEM of data obtained from three independent counts at each time point was plotted **(A)**. RT-PCR was performed using 1μg of RNA extracted from SIGC plated at a density of 7.5×10^4 cells/well in 6-well cell culture plates and collected at 24, 48 and 72 hours post-culture. Relative quantification of the **(B)** *Kitl1* and **(C)** *Kitl2* mRNA expression levels at each time point was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from three independent counts. Bars indicated with different letters from the control are significantly different (ANOVA).

Kitl1: b - $P < 0.001$

Kitl2: b - $P < 0.001$

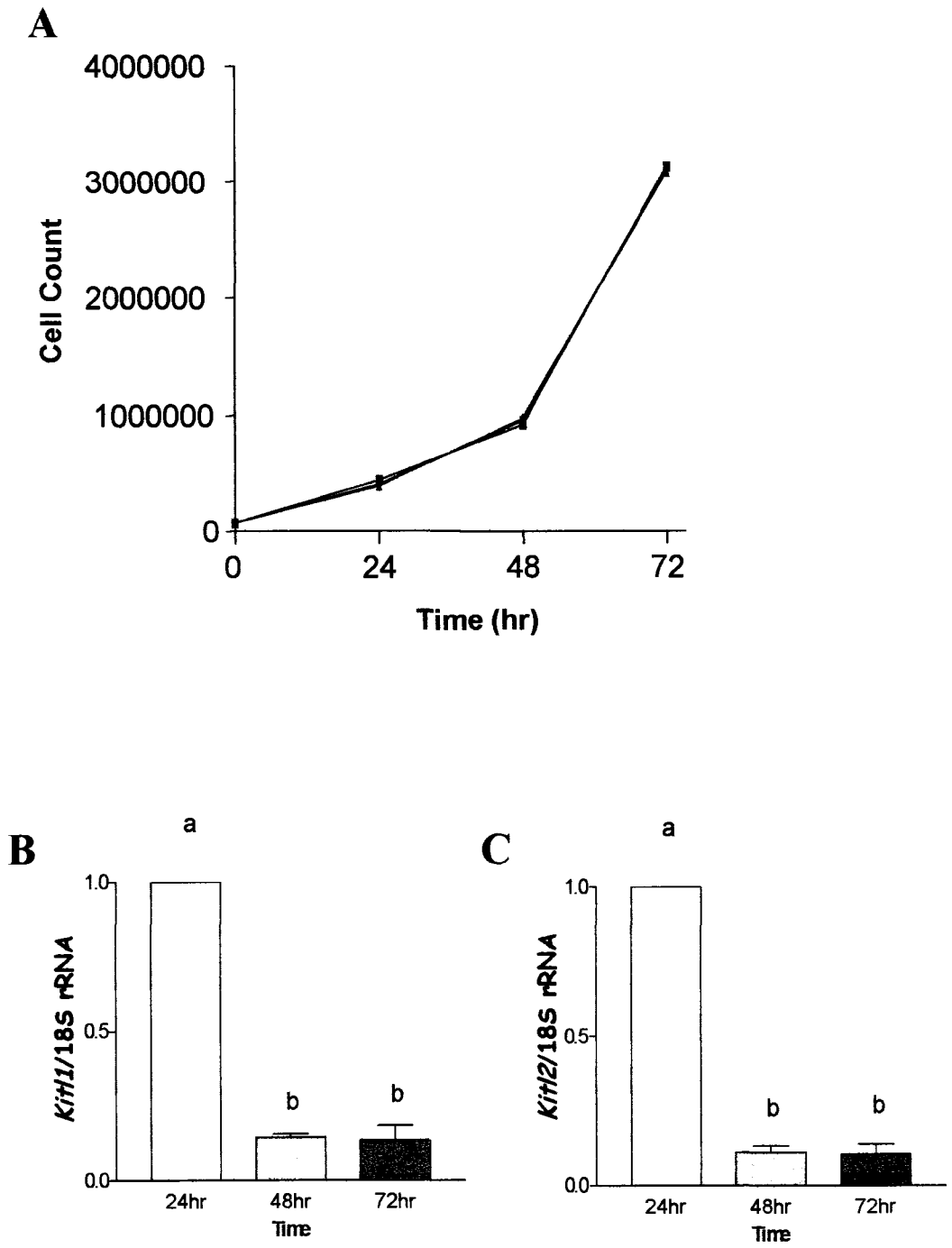


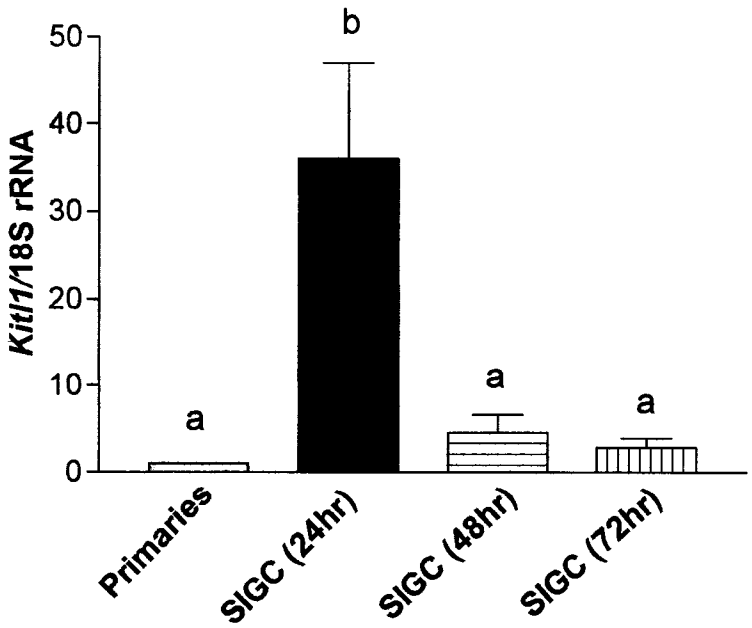
Figure 10. **Comparison of *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells and SIGC**

SIGC were plated at a density of 7.5×10^4 cells/well in 6-well cell culture plates. Total cellular RNA was extracted from 24, 48 and 72 hour cultured SIGC as well as from a confluent plate of primary granulosa cells (Primaries) cultured for 24 hours. Relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

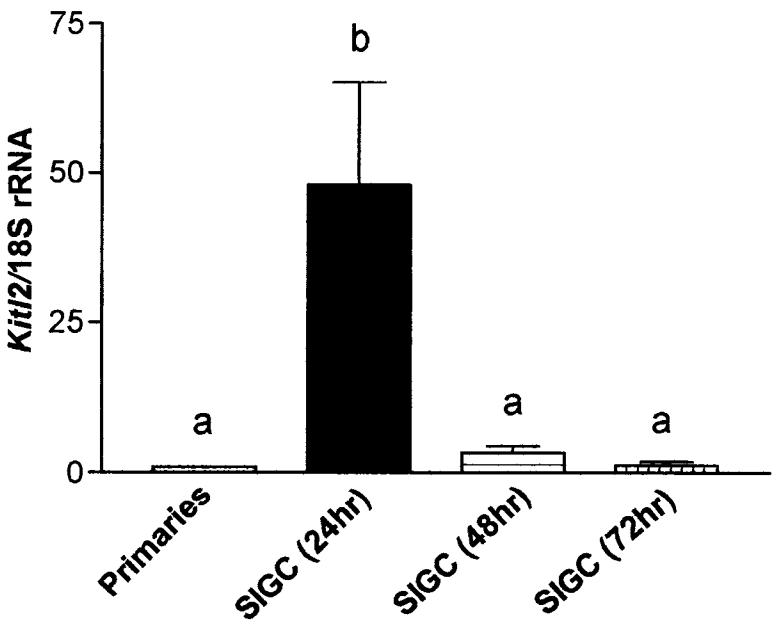
Kitl1: b - $P < 0.05$

Kitl2: b - $P < 0.05$

A



B



Finally, it is worthy to mention that granulosa cells in culture often lose the expression of FSHR over time (Keren-Tal et al., 1993). In order to determine whether this too was the case with SIGC, RT-PCR was performed using RNA from SIGC and primary granulosa cells, which served as a positive control. FSHR mRNA expression was not detected in the 2nd lane containing cDNA from SIGC (*figure 11*), necessitating other means of stimulating the pathway downstream of FSH in this immortalized granulosa cell line.

3.2 FSH and/or Downstream Effectors: Effect and Pathway of Action

3.2.1 FSH Stimulates *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells

In order to gain some understanding of the FSH-stimulated signalling pathway that regulates KITL expression, it was initially necessary to demonstrate that FSH could directly stimulate *Kitl1* and *Kitl2* mRNA expression in primary cultures of rat granulosa cells, as has been demonstrated in other cell culture systems (Rossi et al., 1993; Thomas et al., 2005). Treatment of granulosa cells with FSH (50ng/ml) for 24 hours increased the levels of both *Kitl1* ($n = 4$ experiments, $P < 0.01$ by ANOVA) and *Kitl2* ($n = 4$ experiments, $P < 0.05$) mRNA expression 75% and 62%, respectively, above vehicle-treated controls (*figures 12A* and *12B*).

3.2.2 H-89 Is Not Effective in Preventing FSH-Stimulated *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells

FSH action within granulosa cells is believed to be mediated primarily through the activation of PKA (Richards, 1994). Therefore, we were interested in determining whether inhibition of PKA would hinder FSH-stimulated *Kitl1* and *Kitl2* mRNA

Figure 11. **Expression of FSHR mRNA in SIGC**

RT-PCR was performed using 1μg of RNA extracted from SIGC cultured for 24 hours. RNA from primary granulosa cells (Primaries) of DES-primed rats was included as a positive control. PCR products and 100bp DNA ladder (2.5μg) were loaded onto a 0.6% agarose gel containing ethidium bromide (0.1μg/ul), and separated at 60V for approximately 1 hour. A band 1812bp in length was amplified in the positive control lane although not in the lane containing cDNA from SIGC.

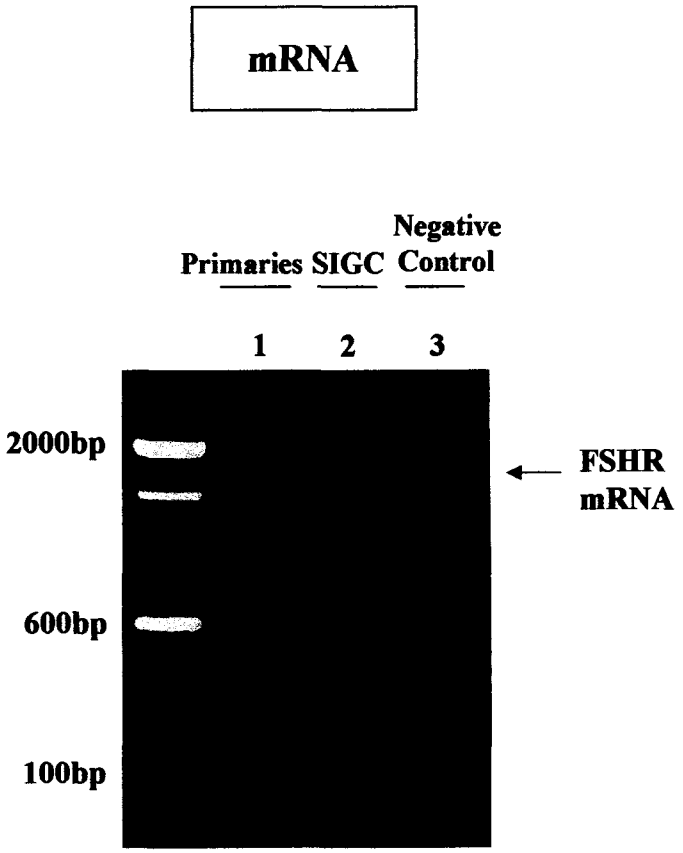


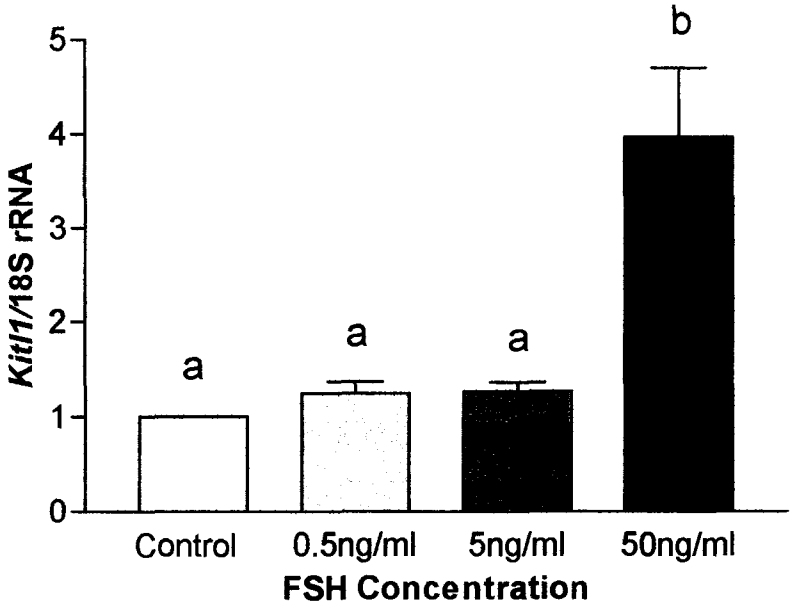
Figure 12. **Effect of FSH on *Kitl1* and *Kitl2* mRNA Expression**

Rat primary granulosa cells, plated at a density of 1×10^6 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of FSH (0.5, 5.0 and 50ng/ml) for 24 hours. Total cellular RNA was extracted from flash-frozen cells collected at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

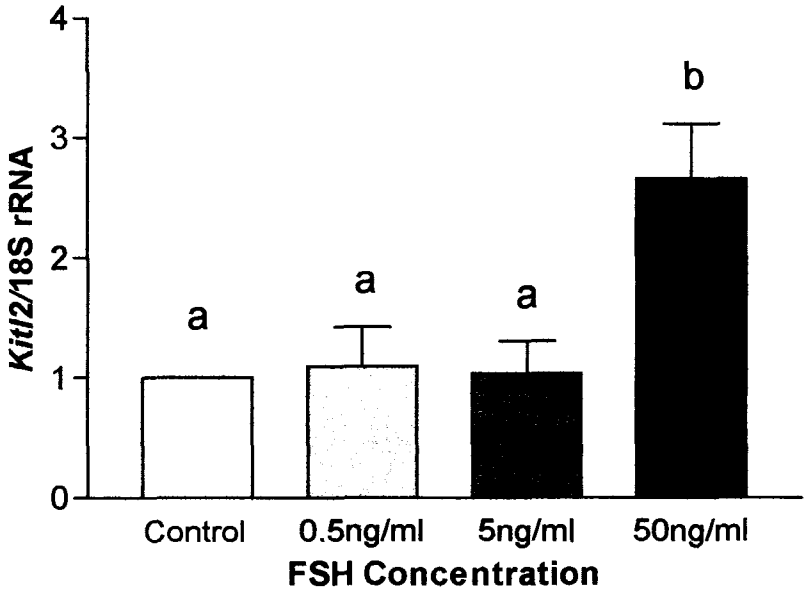
Kitl1: b - $P < 0.01$

Kitl2: b - $P < 0.05$

A



B



expression. In addition to providing insight into the signalling pathway, this would allow the studies to be furthered in SIGC which lack FSHR, thus necessitating other means of PKA pathway stimulation. Pretreatment of cultured primary granulosa cells with the PKA pathway inhibitor H-89 (10μM) for 1 hour prior to treatment with FSH (50ng/ml) for 24 hours, did not prevent the FSH-induced increase in *Kitl1*(46%; n = 4 experiments, $P < 0.05$ by ANOVA) and *Kitl2* (62%; n = 4 experiments, $P < 0.05$) mRNA expression above vehicle-treated controls (*figures 13A* and *13B*).

To determine whether this inability of H-89 to inhibit FSH-stimulated *Kitl1* and *Kitl2* mRNA expression was a result of an inactive reagent, SIGC transfected with the CRE-luciferase construct were treated for 24 hours with H-89 (2.5, 5 or 7.5μM) or DMSO vehicle control (0.05, 0.1 or 0.15%) corresponding to the amount of DMSO in the H-89 treated samples. With transfected SIGC, we were not able to test the concentration of H-89 used in primary granulosa cells (10μM) as the combination proved toxic to the cells. In *figure 14*, we show that in fact H-89 may not be effectively suppressing PKA activity in SIGC as it did not reduce base levels of CRE-luciferase below those seen in vehicle-treated controls. Although the results were not significant (n = 4 experiments, $P > 0.05$ by ANOVA), there does appear to be a trend towards increased CRE-luciferase activity in the presence of 0.15% DMSO, which is found in the highest concentration of H-89 (7.5μM) used to treat transfected SIGC. This suggests that DMSO may be a hindrance to the inhibitory effect of H-89 if it is present at a substantial quantity and may explain why 10μM H-89 was not effective in the primary cultures of granulosa cells.

Figure 13. **Effect of H-89 on FSH-Stimulated *Kitl1* and *Kitl2* mRNA Expression**

Rat primary granulosa cells, plated at a density of 1×10^6 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of FSH (50ng/ml) and/or H-89 (10μM) with which cells were pretreated for 1 hour prior to the addition of FSH. Total cellular RNA was extracted from flash-frozen cells collected at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

Kitl1: b - $P < 0.05$

Kitl2: b - $P < 0.05$

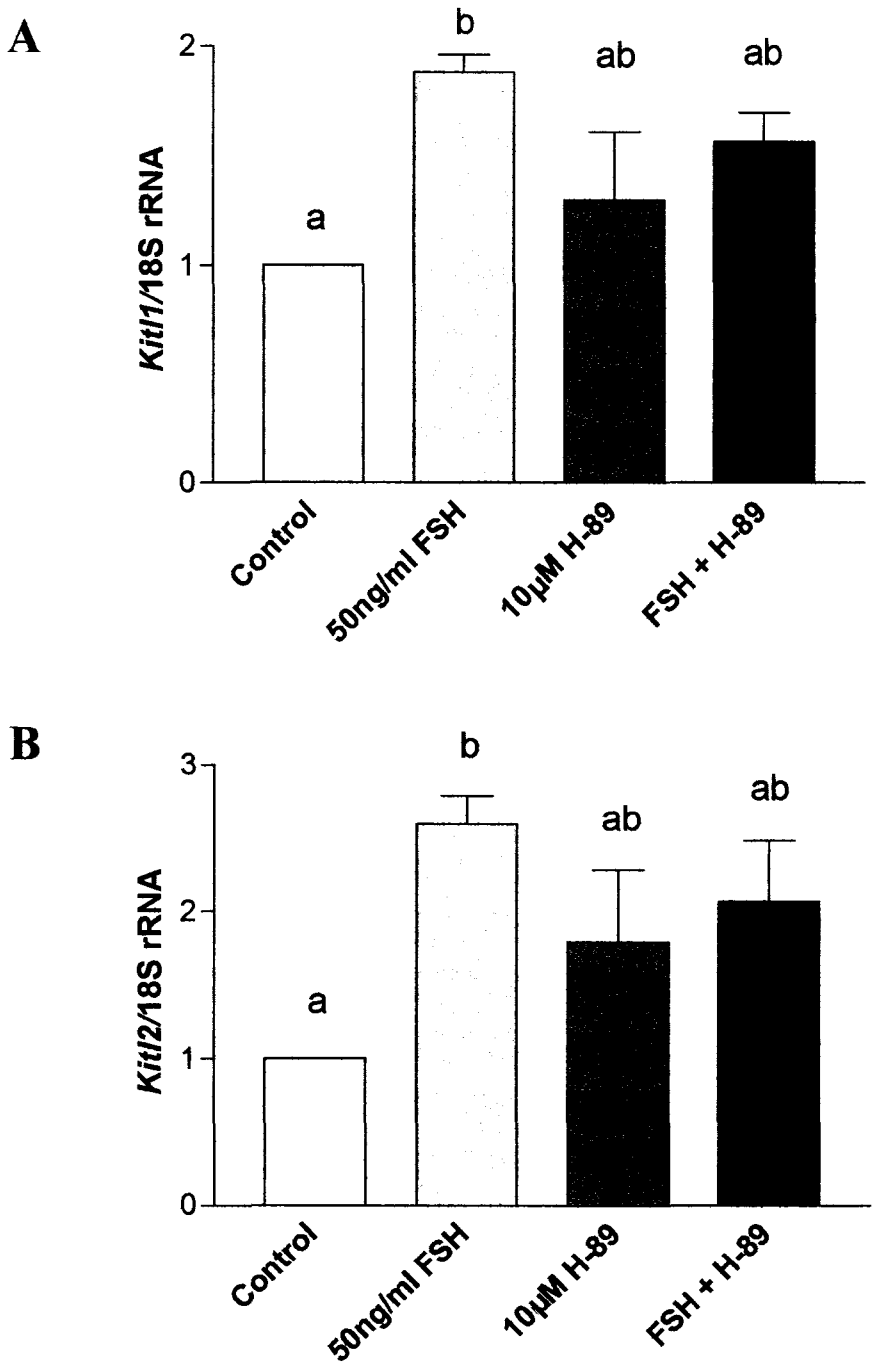
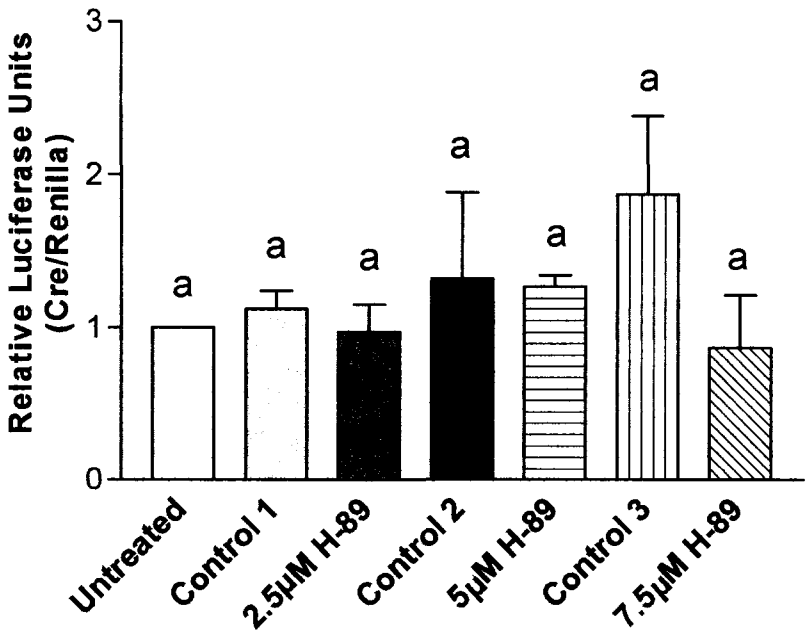


Figure 14. **Effect of H-89 on CRE-Luciferase Activity in SIGC**

SIGC were plated at a density of 7.5×10^4 cells/well in 6-well cell culture plates and the following day, transiently transfected with reporter plasmid 4×CRE-Luciferase (3μg) and *Renilla* expression vector (3ng) using FuGENE® 6. At 24 hours post-transfection, cells were treated with H-89 (0, 2.5, 5.0 or 7.5μM) or DMSO vehicle control (0.05, 0.10 or 0.15%) corresponding to the amount of DMSO in the H-89 treated samples. Treatments were carried out for 24 hours, cells were lysed and analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay System and the Lumat LB 9507 luminometer. Results are shown as the fold change ± (SEM) from untreated cells with data obtained from four independent experiments. No significant differences were detected (ANOVA).



3.2.3 DbcAMP Suppresses *Kitl1* and *Kitl2* mRNA Expression in SIGC

Despite not being able to demonstrate conclusively PKA pathway involvement in FSH-induced *Kitl1* and *Kitl2* mRNA expression, cAMP accumulation in response to FSH-treatment of granulosa cells is well documented (Richards, 1994) and treatment of granulosa cells with cell-permeable cAMP analogues mimics FSH-induced effects (Gonzalez-Robayna et al., 2000; Alam et al., 2004). Furthermore, it has previously been shown that dbcAMP treatment specifically increases overall *Kitl* mRNA expression in cultures of mouse Sertoli cells and granulosa cells (Rossi et al., 1991; 1993; Packer et al., 1994). Therefore, we deemed it appropriate to attempt to stimulate *Kitl1* and *Kitl2* mRNA expression in SIGC, which do not express the FSHR (*figure 11*), using dbcAMP. Interestingly, rather than mimicking the effect seen with FSH in primary cultures of granulosa cells (*figures 12A and 12B*), as would be expected if (1) dbcAMP simply acts downstream of FSH and (2) SIGC are similar to primary granulosa cells, dbcAMP treatment of SIGC for 24 hours resulted in the suppression of *Kitl1* and *Kitl2* mRNA expression (*figures 15A* and *15B*). Specifically, the *Kitl1* mRNA expression level decreased below vehicle-treated controls in a dose-dependent manner at concentrations of 75μM (21%; n = 4 experiments, $P < 0.01$ by ANOVA), 150μM (34%; n = 4 experiments, $P < 0.001$) and 300μM (42%; n = 4 experiments, $P < 0.001$) dbcAMP (*figure 15A*); the *Kitl2* mRNA expression level decreased significantly below vehicle-treated controls only at a concentration of 300μM dbcAMP (53%; n = 4 experiments, $P < 0.05$ by ANOVA; *figure 15B*). The idea to use dbcAMP as a means to stimulate *Kitl1* and *Kitl2* mRNA expression in SIGC was therefore abandoned.

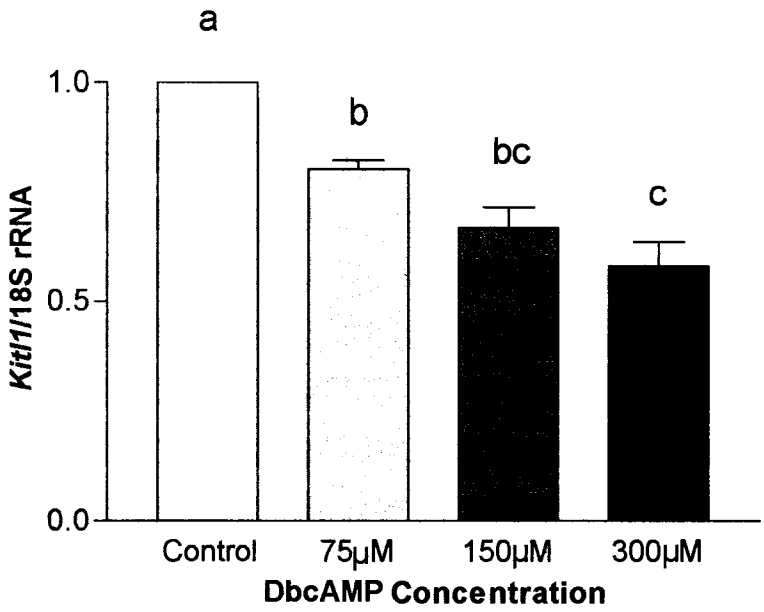
Figure 15. **Effect of DbcAMP on *Kitl1* and *Kitl2* mRNA Expression in SIGC**

SIGC, plated at a density of 3×10^5 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of dbcAMP (75, 150 and 300μM) for 24 hours. Total cellular RNA was extracted from flash-frozen cells collected at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

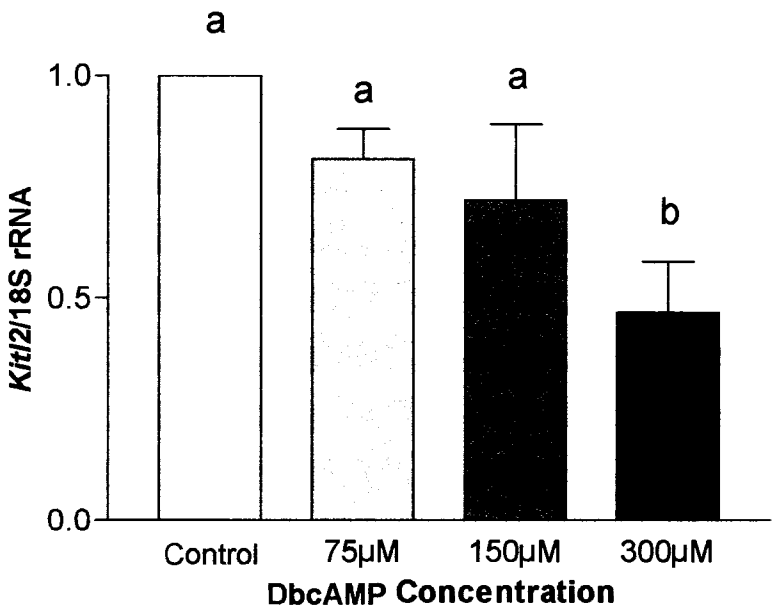
Kitl1: b - $P < 0.01$; c - $P < 0.001$

Kitl2: b - $P < 0.05$

A



B



3.2.4 Forskolin Stimulates *Kitl2* But Not *Kitl1* mRNA Expression in a Biphasic Manner in SIGC

Since we were not able to induce *Kitl1* and *Kitl2* mRNA expression in SIGC using dbcAMP, an alternative method of stimulating in SIGC the FSH-induced effect seen in primary granulosa cells was required. Forskolin is a direct activator of adenylate cyclase (Seamon et al., 1981) and thus promotes the accumulation of cAMP (and PKA activation) independent of FSHR involvement. A multitude of studies have demonstrated the ability of forskolin to mimic the effects of FSH in cultured granulosa cells (Hsueh et al., 1984; Das et al., 1996). It was therefore useful for our objective to stimulate *Kitl1* and *Kitl2* mRNA expression in SIGC, in the way we observed FSH to stimulate the expression of the mRNA transcripts in primary cultures of granulosa cells. At a concentration range of 0-10μM for 24 hours, a forskolin-induced increase in the *Kitl1* mRNA expression level above vehicle-treated controls was not observed ($n = 4$ experiments, $P > 0.05$ by ANOVA; *figure 16A*). However, 5μM forskolin increased the *Kitl2* mRNA expression level 68% above vehicle-treated controls ($n = 4$ experiments, $P < 0.05$; *figure 16B*). Interestingly, a concentration of 10μM forskolin did not further increase the *Kitl2* mRNA level as would be expected if its effect was dose-dependent. This indicates that 5μM forskolin induced the accumulation of the specific amount of cAMP necessary to bring about the maximum stimulation of *Kitl* mRNA possible whereas 10μM, like lower concentrations of forskolin, did not.

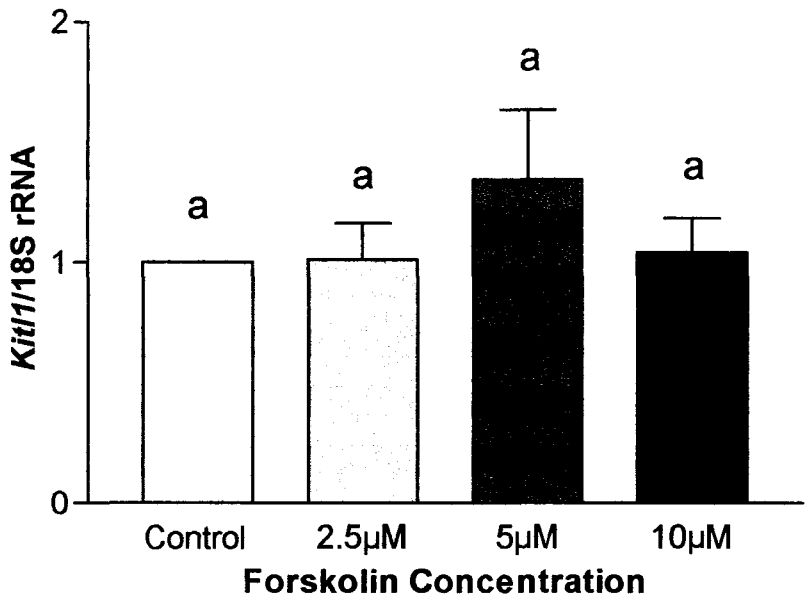
Figure 16. **Effect of Forskolin on *Kitl1* and *Kitl2* mRNA Expression in SIGC**

SIGC, plated at a density of 3×10^5 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of forskolin (0, 2.5, 5.0 and 10μM) for 24 hours. Total cellular RNA was extracted from flash-frozen cells collected at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

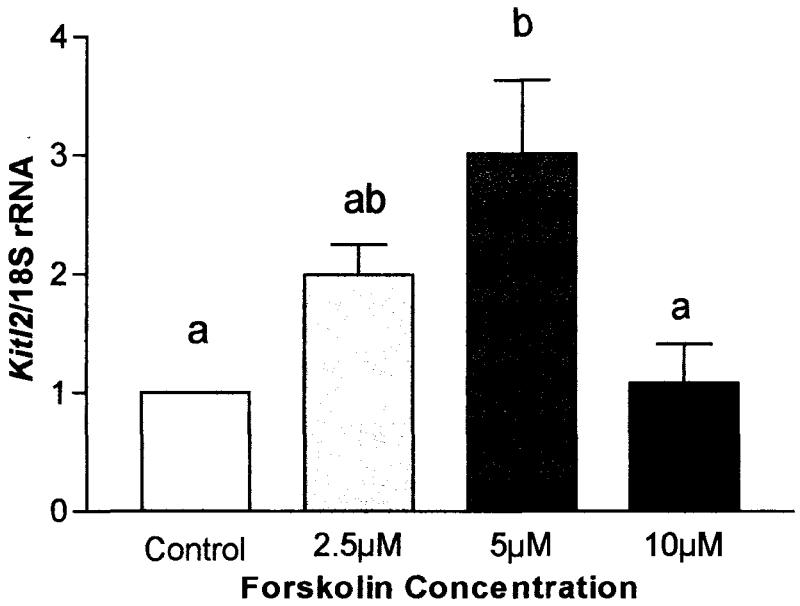
Kitl1: $P > 0.05$

Kitl2: b - $P < 0.05$

A



B



3.3 TGF-β1: Effect and Pathway of Action

3.3.1 TGF-β1 Suppresses *Kitl1* and *Kitl2* mRNA Expression in SIGC and Primary Granulosa Cells

In order to determine whether TGF-β1 could suppress *Kitl1* and *Kitl2* mRNA expression levels in SIGC, a dose-response experiment was performed using TGF-β1 at a concentration range of 0-10ng/ml. We found that 24 hours of treatment with the maximum dose of TGF-β1 used (10ng/ml) was effective in suppressing the expression levels of both *Kitl1* and *Kitl2* mRNA (*figures 17A and 17B*). Specifically, *Kitl1* mRNA expression was suppressed 38% below vehicle-treated controls ($n = 4$ experiments, $P < 0.05$ by ANOVA) while that of *Kitl2* was suppressed 58% below vehicle-treated controls ($n = 4$ experiments, $P < 0.01$). Having found the concentration effective in suppressing *Kitl* mRNA expression in SIGC, we tested whether this concentration would also inhibit the expression of the transcripts in primary cultures of granulosa cells. The expression level of *Kitl1* mRNA was suppressed 66% below vehicle-treated controls ($n = 4$ experiments, $P < 0.001$ by paired t-test; *figure 18A*), while that of *Kitl2* was suppressed 52% below vehicle-treated controls ($n = 4$ experiments, $P < 0.01$; *figure 18B*).

3.3.2 TGF-β1 Treatment Stimulates the Phosphorylation of Smad2 and Smad3, But Not Smad1/5 in SIGC

Having established that TGF-β1 has this inhibitory effect on *Kitl1* and *Kitl2* mRNA expression levels, it was next of interest to determine whether this regulation was mediated via Smad2 and Smad3, traditionally utilized by the activin/TGF-β-mediated pathway. Since SIGC have not been previously used for studying TGF-β signalling and due to some of the unexpected behaviour that they exhibited in response to dbcAMP and forskolin, we initially thought it necessary to ensure the expression of Smads, prior to any

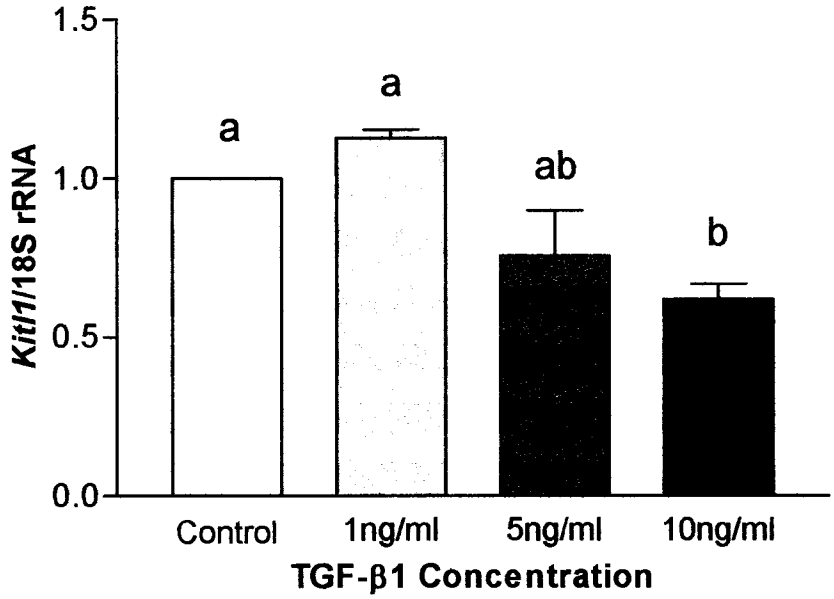
Figure 17. **Effect of TGF-β1 on *Kitl1* and *Kitl2* mRNA Expression in SIGC**

SIGC, plated at a density of 3×10^5 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of TGF-β1 (1.0, 5.0 or 10ng/ml) for 24 hours. Total cellular RNA was extracted from flash-frozen cells collected at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

Kitl1: b - $P < 0.05$

Kitl2: c - $P < 0.01$

A



B

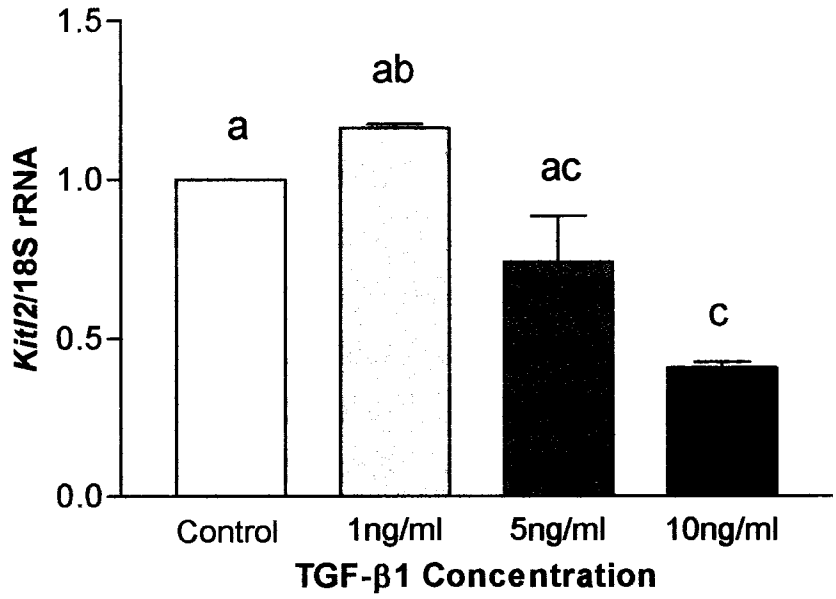


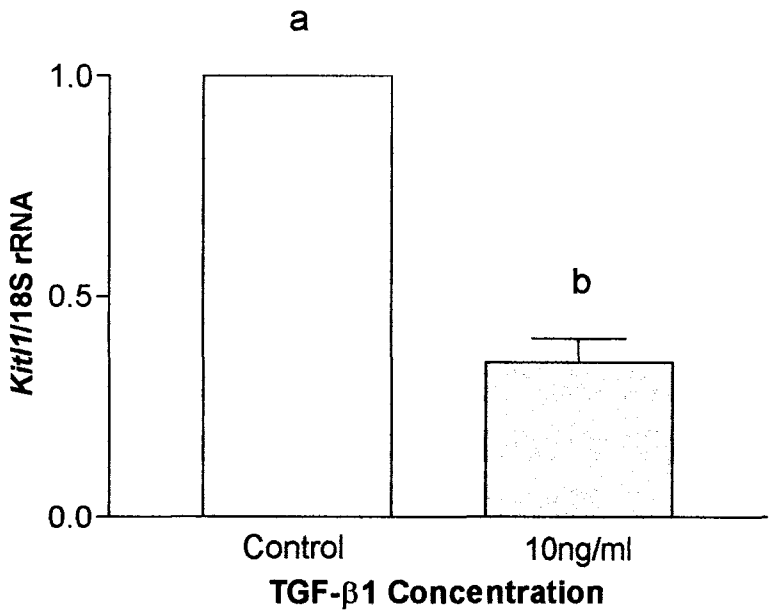
Figure 18. **Effect of TGF-β1 on *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells**

Rat primary granulosa cells, plated at a density of 1×10^6 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of TGF-β1 (10ng/ml) for 24 hours. Total cellular RNA was extracted from flash-frozen cells at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (paired t-test).

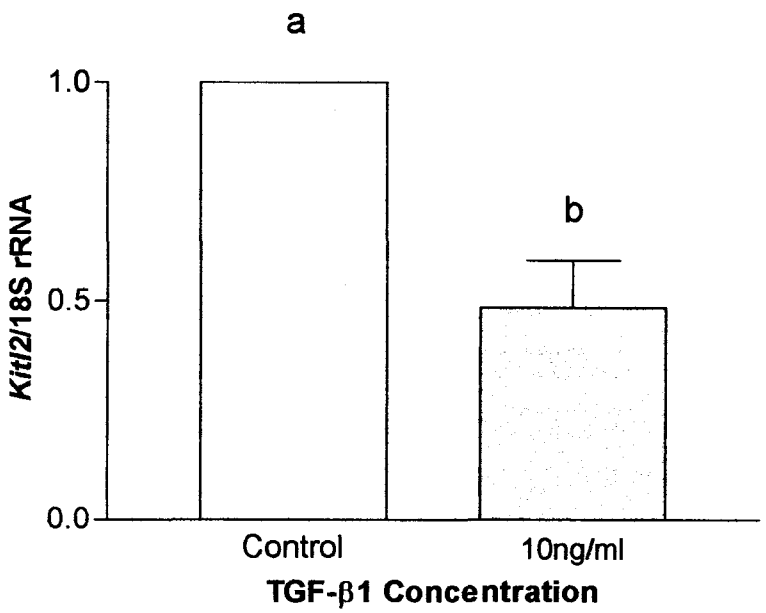
Kitl1: b - $P < 0.001$

Kitl2: b - $P < 0.01$

A



B



attempts at induction and analysis for the activated Smads. As is shown in *figure 19*, SIGC expressed A) Smad2 B) Smad3 and C) Smad4, indicating the retention of the traditional proteins required for the functioning of TGF-β signalling.

We were next interested in determining whether TGF-β1 treatment would result in the activation/phosphorylation of Smad2 and Smad3, but not Smad1/5, which is traditionally associated with the BMP-mediated signalling pathway. SIGC were treated with TGF-β1 (10ng/ml) or BMP-2 (100ng/ml) and total cellular protein was collected at 0, 15 and 30 minutes or at 0 and 30 minutes post-treatment. SDS PAGE and Western blot analysis were performed for the detection of Smad2 and Smad3, and for phospho-Smads 2, 3 and 1/5. TGF-β1 induced up-regulation of the phosphorylated forms of both Smads after 15 minutes of treatment, and the activity was still noticeable after 30 minutes of treatment; *figures 20A* and *20C*). The phosphorylated bands corresponded to the expected molecular masses (58kDa) of the phosphorylated forms of Smad2 and Smad3, respectively. In control samples harvested at the same time points, no phospho-Smads reactivity was detected. Importantly, TGF-B1 treatment did not induce the appearance of phospho-Smad1/5 in the way that BMP-2 (100ng/ml) did, as previously described (Kaivo-Oja et al., 2003; *figure 21)*. This indicates the specificity of the cellular response to TGF-β1. We also observed that TGF-β1 treatment resulted in increased levels of total Smad2 and Smad3 (*figures 20B* and *20D*); however, whether this is to be expected is unknown since two previous studies on Smad activation (Kaivo-Oja et al., 2003; Mazerbourg et al., 2004) only mentioned the effect of the particular treatment on the phosphorylated forms of the Smads. We concluded that as the granulosa cell recruits Smads for phosphorylation, it also up-regulates the expression of the unphosphorylated

Figure 19. **Expression of Smad2, Smad3 and Smad4 in SIGC**

SIGC cultured in 10mm cell culture plates and grown to confluence were collected and protein extraction was performed immediately according to the procedure outlined in the **MATERIALS AND METHODS** section. Equal amounts of proteins extracted from SIGC as well as from SKOV3 cells, which served as a positive control, were separated by 4-12% gradient SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were probed with **(A)** anti-Smad2 antibody **(B)** anti-Smad3 antibody or **(C)** anti-Smad4 antibody. To confirm equivalent loading of protein samples, all membranes were reprobed using a GAPDH antibody.

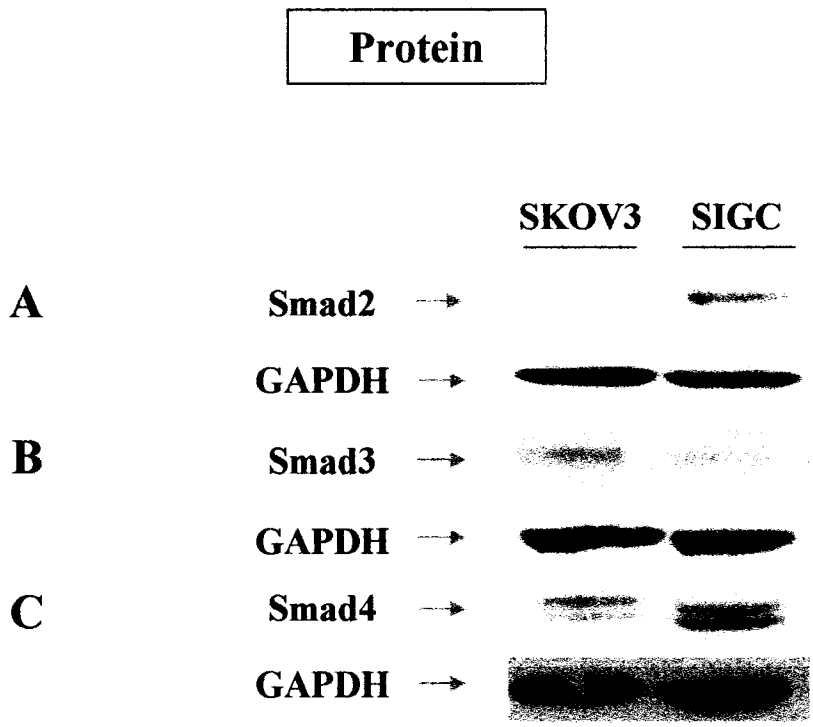


Figure 20. **Effect of TGF-β1 on Smad2 and Smad3 Activation in SIGC**

SIGC, plated at a density of 1.8×10^6 cells/well in 6-well cell culture plates, were cultured with vehicle or in the presence of TGF-β1 (10ng/ml) for 0, 15 or 30 minutes. SKOV3 cells cultured with vehicle or in the presence of TGF-β1 (10ng/ml) for 0 or 30 minutes were included as a positive control. Extraction of total cellular protein was performed immediately following the treatment period according to the procedure outlined in the **MATERIALS AND METHODS** section. Equal amounts of proteins were separated by 4-12% gradient SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were probed with **(A)** anti-Smad2 and **(B)** anti-phospho-Smad2 antibodies or **(C)** anti-Smad3 and **(D)** anti-phospho-Smad3 antibodies. Both phospho-Smad2 and phospho-Smad3 were 58kDa. To confirm equivalent loading of protein samples, all membranes were reprobed using a GAPDH antibody. The Western blot shown is representative of four independent experiments.

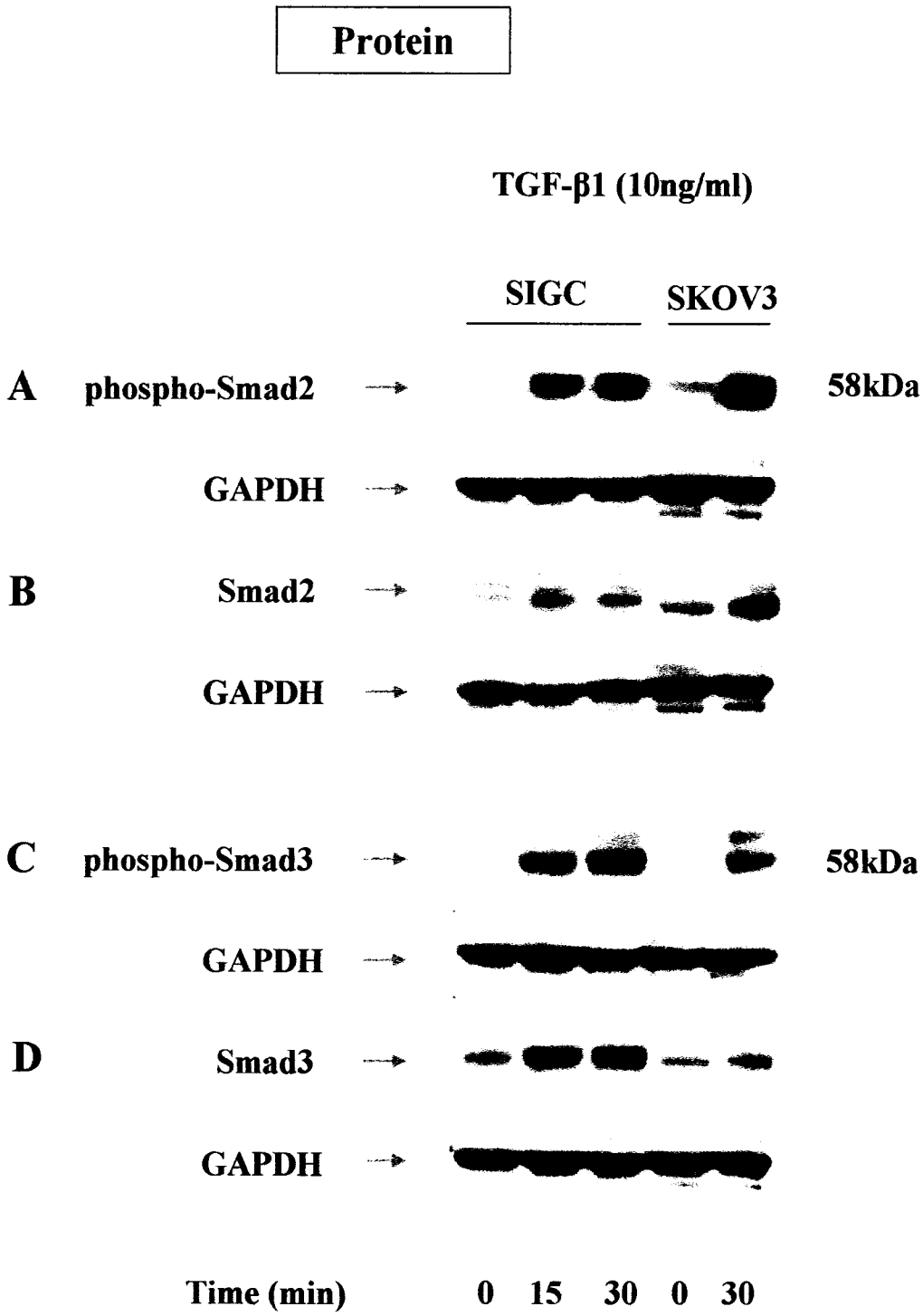
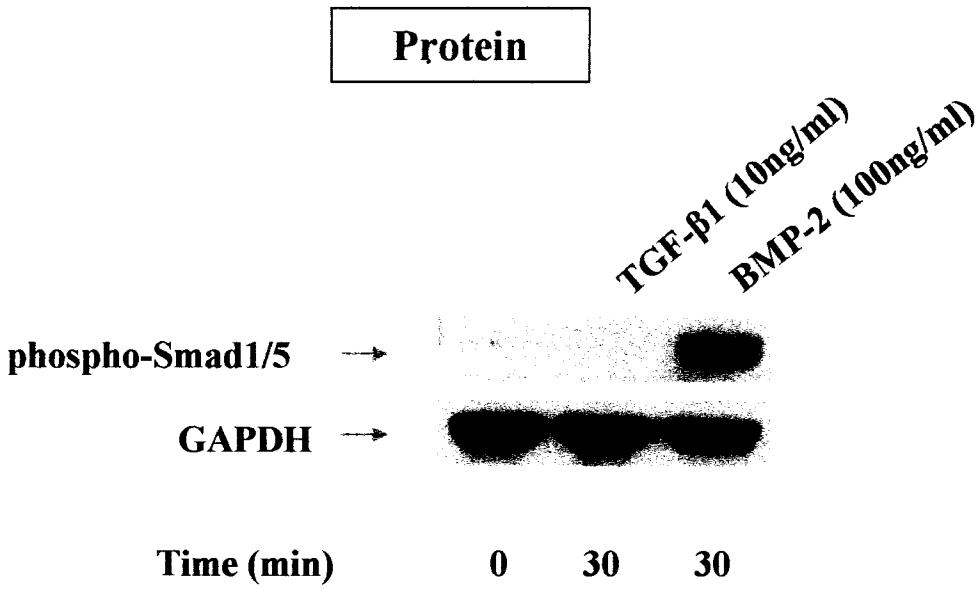


Figure 21. **Effect of TGF-β1 on Smad1/5 Activation in SIGC**

SIGC, plated at a density of 1.8×10^6 cells/well in 6-well cell culture plates, were cultured with vehicle or in the presence of TGF-β1 (10ng/ml) for 0 or 30 minutes or BMP-2 (100ng/ml) for 30 minutes. Extraction of total cellular protein was performed immediately following the treatment period according to the procedure outlined in the **MATERIALS AND METHODS** section. Equal amounts of proteins were separated by 4-12% gradient SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was probed with anti-phospho-Smad1/5 antibody. To confirm equivalent loading of protein samples, all membranes were reprobed using a GAPDH antibody. The Western blot shown is representative of four independent experiments.



forms in order to replenish the supply. Therefore, the treatment of SIGC with TGF-β1 and the resultant effect of *Kitl1* and *Kitl2* mRNA transcript inhibition is correlated with the activation of both Smad2 and Smad3 and not Smad1/5.

3.3.3 17β-Estradiol Suppresses *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells

Previous work in our lab had demonstrated that granulosa cells obtained from untreated, immature rats exhibit elevated levels of overall *Kitl* mRNA expression compared to granulosa cells obtained from DES-primed rats (and flash-frozen immediately post-collection) (Rubina Ismail, unpublished observations). We found that granulosa cells obtained from DES-primed rats and flash-frozen immediately post-collection also display lower levels of *Kitl1* and *Kitl2* mRNA expression as compared to those cultured for 24 or 48 hours post-collection (*figures 22A* and *22B*). Specifically, the expression levels of *Kitl1* and *Kitl2* mRNA in cells cultured for 24 hours post-collection were 96% and 93%, respectively, greater than those observed in cells flash-frozen post-collection (*Kitl1*: $n = 3$ experiments, $P < 0.01$ by ANOVA; *Kitl2*: $n = 3$ experiments, $P < 0.001$).

The observation that *Kitl1* and *Kitl2* mRNA expression levels were significantly elevated in cultured cells, in conjunction with the finding that granulosa cells from untreated rats exhibit significantly higher levels of overall *Kitl* mRNA expression than from DES-primed rats, suggested that perhaps DES, an estrogenic compound, was inhibiting *Kitl1* and *Kitl2* mRNA expression. To test this hypothesis, primary granulosa cells were cultured for 24 hours in the presence of 17β-estradiol (500nM). In *figures 23A* and *23B*, we have shown that 17β-estradiol suppressed the levels of *Kitl1* and *Kitl2* mRNA expression 36% ($n = 3$ experiments, $P < 0.05$ by ANOVA) and 59% ($n = 3$

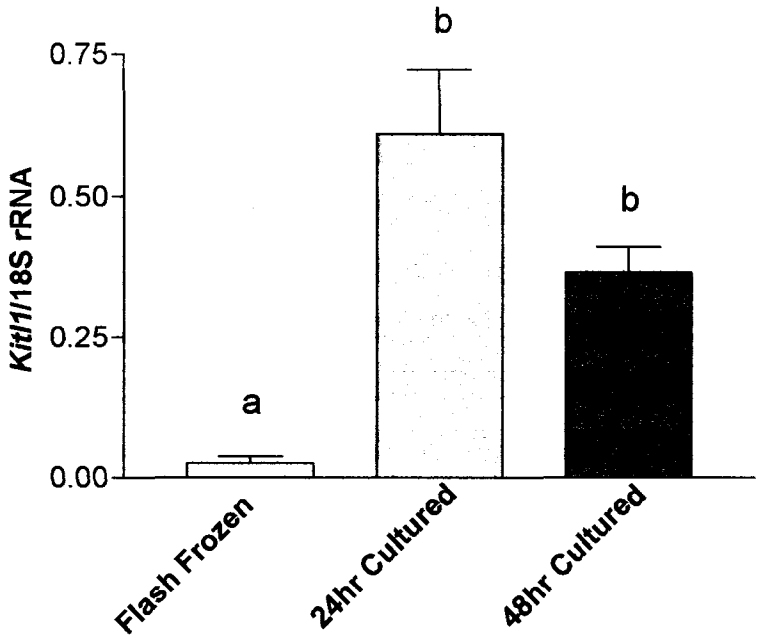
***Figure 22.* *Kitl1* and *Kitl2* mRNA Expression in Granulosa Cells Flash-Frozen or Cultured Post-Collection from DES-Primed Rats**

Total cellular RNA was extracted from primary granulosa cells isolated from DES-primed rats and either flash frozen immediately post-collection or cultured for 24 and 48 hours. Relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from three independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

Kitl1: b (24 hours) - $P < 0.01$; b (48 hours) - $P < 0.05$

Kitl2: b - $P < 0.001$; c - $P < 0.05$

A



B

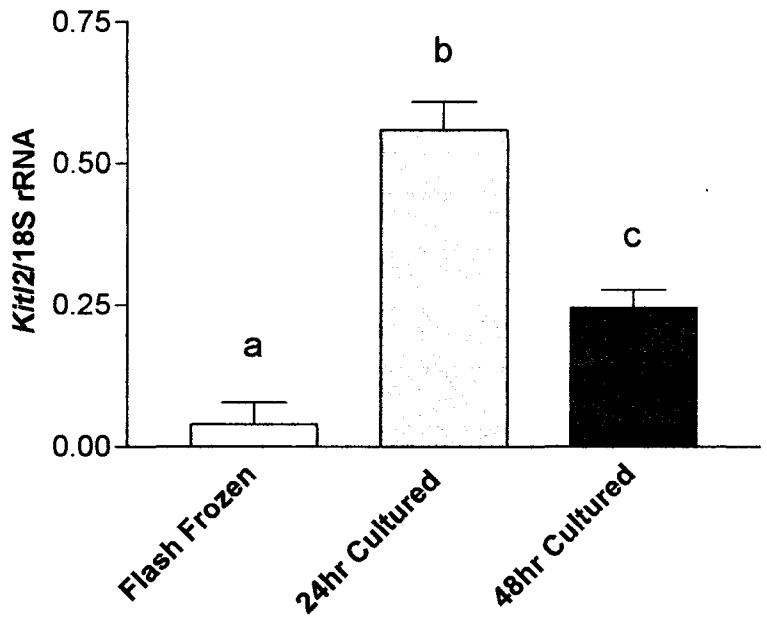


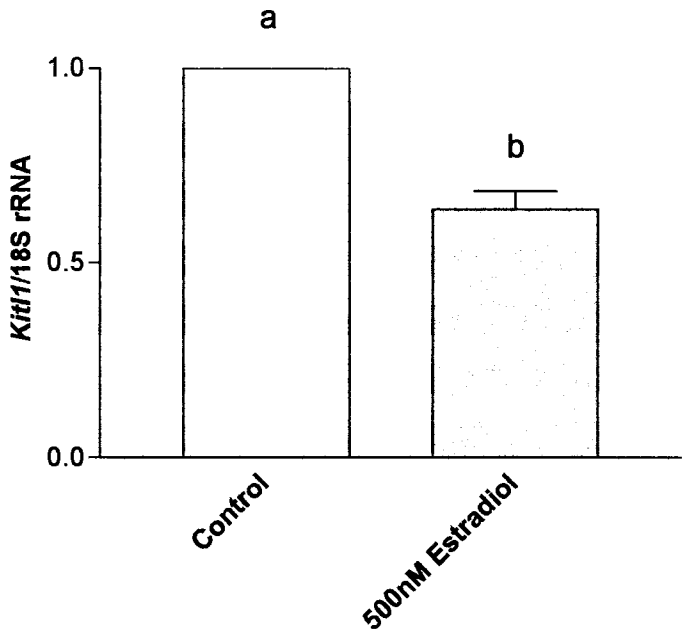
Figure 23. **Effect of 17β-Estradiol on *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells**

Rat primary granulosa cells, plated at a density of 1×10^6 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of 17β-estradiol (500nM) for 24 hours. Total cellular RNA was extracted from flash-frozen cells collected at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from three independent experiments. Bars indicated with different letters from the control are significantly different (paired t-test).

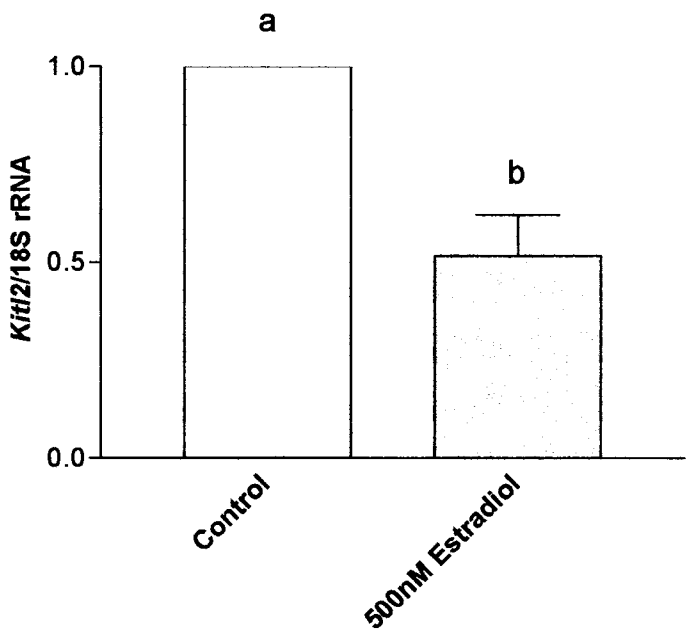
Kitl1: b - $P < 0.05$

Kitl2: b - $P < 0.01$

A



B



experiments, $P < 0.01$), respectively, below vehicle-treated controls.

3.3.4 The Effect of 17β-Estradiol on *Kitl1* and *Kitl2* mRNA Expression is Mediated Via TGF-β1

We found that TGF-β1 can inhibit *Kitl1* and *Kitl2* mRNA expression levels in both SIGC and primary cultures of granulosa cells, and that estrogen had a similar effect in primary granulosa cells. Previous studies have demonstrated that 17β-estradiol regulates the secretion of TGF-β by rat granulosa cells (Dorrington et al., 1988). Additionally, the use of a neutralizing anti-TGF-β antibody prevented 17β-estradiol-stimulated rat granulosa cell proliferation in culture (Bendell and Dorrington, 1991). Considering these findings, we were interested in determining whether using a neutralizing antibody against TGF-β1 would prevent 17β-estradiol suppression of *Kitl1* and *Kitl2* mRNA expression levels. Initially, we tested the efficacy of the anti-TGF-β1 antibody in preventing TGF-β1 mediated suppression of *Kitl1* and *Kitl2* mRNA expression levels by treating primary granulosa cells with TGF-β1 and/or TGF-β1 antibody for 24 hours. Our results indicate that the antibody was effective in preventing TGF-β1 suppression of *Kitl1* (63% below vehicle-treated controls; n = 3 experiments, $P < 0.05$ by ANOVA) and *Kitl2* (60% below vehicle-treated controls; n = 3 experiments, $P < 0.05$) mRNA expression, such that the expression levels of the two transcripts returned to those of vehicle-treated controlscultured for the same time period (*figures 24A* and *24B*).

Next, we found that treatment of primary granulosa cells with the anti-TGF-β1 antibody in combination with 17β-estradiol for 24 hours prevented the suppressive effect of 17β-estradiol on *Kitl1* (31% below vehicle-treated controls; n = 4 experiments, $P < 0.05$ by ANOVA) and *Kitl2* (43% below vehicle-treated controls; n = 4 experiments, $P < 0.05$) mRNA expression, such that levels of the transcripts returned to those of vehicle-

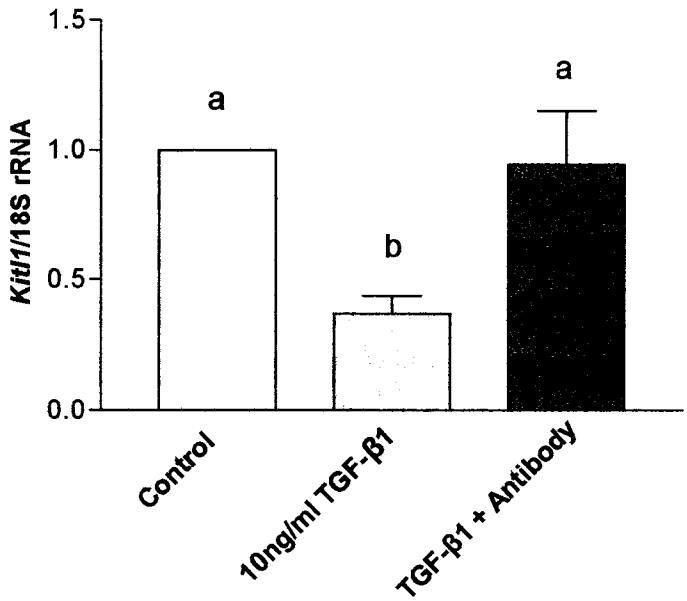
Figure 24. **Effect of the TGF-β1 Antibody on TGF-β1 Suppression of *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells**

Rat primary granulosa cells, plated at a density of 1×10^6 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of TGF-β1 (10ng/ml) and/or the anti-TGF-β1 antibody (5μg) for 24 hours. Total cellular RNA was extracted from flash-frozen cells collected at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from three independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

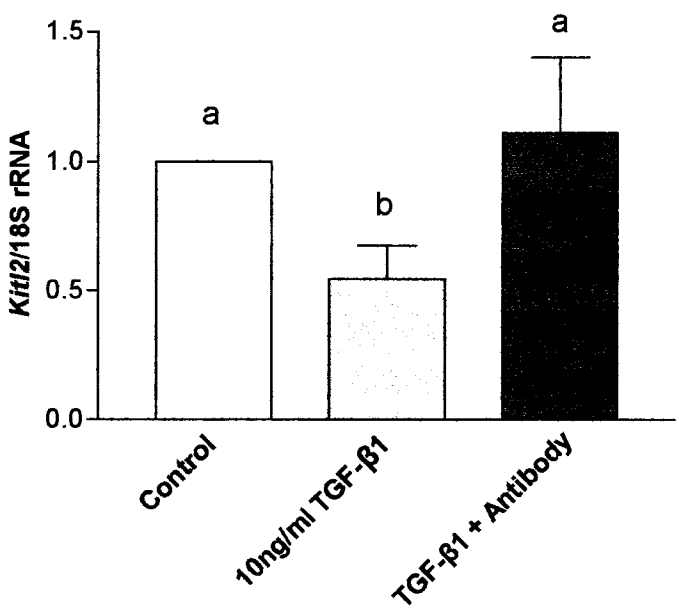
Kitl1: b - $P < 0.05$

Kitl2: b - $P < 0.05$

A



B



treated controls (*figures 25A* and *25B*). We have also shown that this preventive effect of anti-TGF-β1 antibody on the suppressive action of 17β-estradiol was not a result of the IgY backbone of the antibody and therefore, an effect of the antibody itself. Therefore, it appears that the effect of 17β-estradiol on *Kitl1* and *Kitl2* mRNA expression levels is mediated via TGF-β1.

3.3.5 TGF-β1 Inhibits FSH-Stimulated *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells

Having established that FSH acts as an inducer and that TGF-β1 acts as an inhibitor of *Kitl* mRNA expression in primary cultures of granulosa cells, it was further of interest to determine what their effect on expression would be in the event of cotreatment. To determine what the combined effect would be, we used primary granulosa cells, thus allowing the use of FSH which stimulated the expression of both *Kitl1* and *Kitl2* mRNA, unlike forskolin in SIGC, which only stimulated *Kitl2* mRNA expression. Primary cultures of granulosa cells were treated with a combination of FSH (50ng/ml) and TGF-β1 (10ng/ml) for 24 hours. In *figures 26A* and *26B*, we have shown that TGF-β1 prevented the 53% ($n = 4$ experiments, $P < 0.01$ by ANOVA) and 57% ($n = 4$ experiments, $P < 0.001$) FSH-induced increase in *Kitl1* and *Kitl2* mRNA expression level, respectively, returning the levels of *Kitl* mRNA to those of vehicle-treated controls. Therefore, it appears that TGF-β1 antagonizes the FSH-induced effect on *Kitl1* and *Kitl2* mRNA expression.

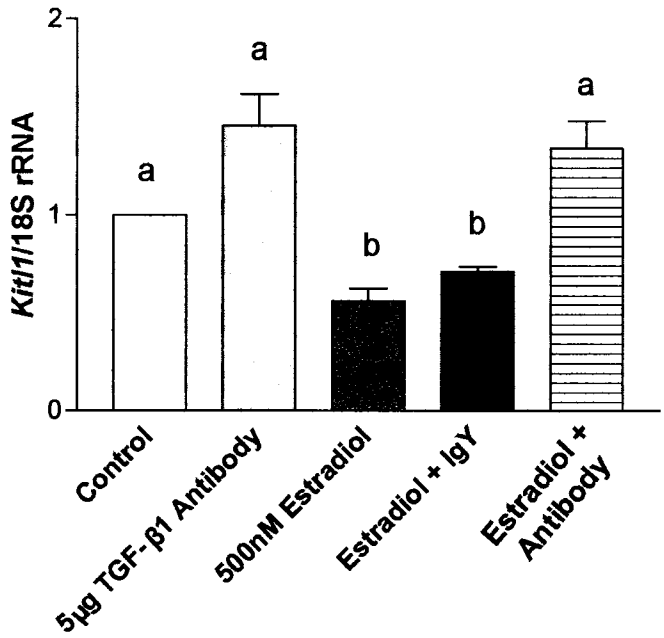
Figure 25. **Effect of the TGF-β1 Antibody on 17β-Estradiol Suppression of *Kitl1* and *Kitl2* mRNA Expression in Primary Granulosa Cells**

Rat primary granulosa cells, plated at a density of 1×10^6 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of 17β-estradiol (500nM) and/or anti-TGF-β1 antibody (5μg), or IgY, the backbone of the anti-TGF-β1 antibody for 24 hours. Total cellular RNA was extracted from flash-frozen cells at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

Kitl1: $P < 0.05$

Kitl2: $P < 0.05$

A



B

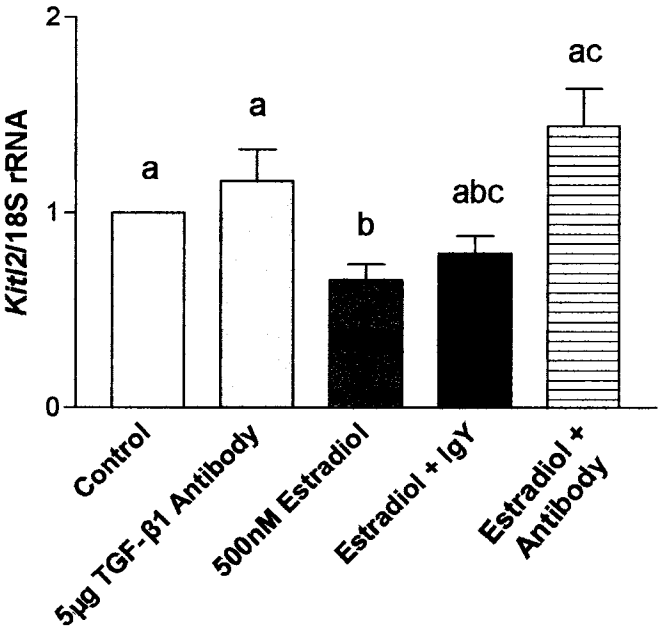


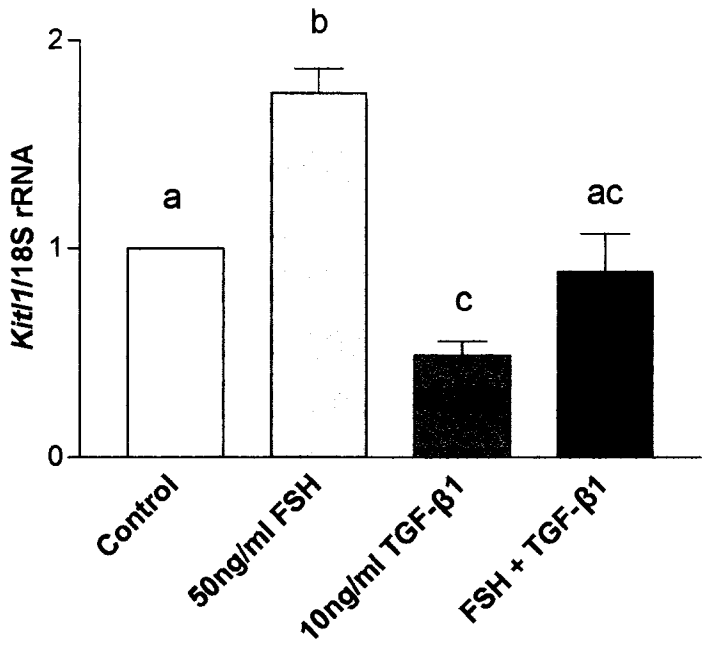
Figure 26. **Effect of TGF-β1 on FSH-Stimulated *Kitl1* and *Kitl2* mRNA Expression**

Rat primary granulosa cells, plated at a density of 1×10^6 cells/well in 12-well cell culture plates, were cultured with vehicle or in the presence of FSH (50ng/ml) and/or TGF-β1 (10ng/ml). Total cellular RNA was extracted from flash-frozen cells at the end of the 24 hour culture period and relative quantification of the **(A)** *Kitl1* and **(B)** *Kitl2* mRNA expression levels was performed through real-time QPCR using the Applied Biosystems 7500 PCR system and SYBR® Green PCR Master Mix. The expression levels of *Kitl1* and *Kitl2* mRNA were standardized to 18S rRNA in each sample. Results are shown as the fold change ± (SEM) of data obtained from four independent experiments. Bars indicated with different letters from the control are significantly different (ANOVA).

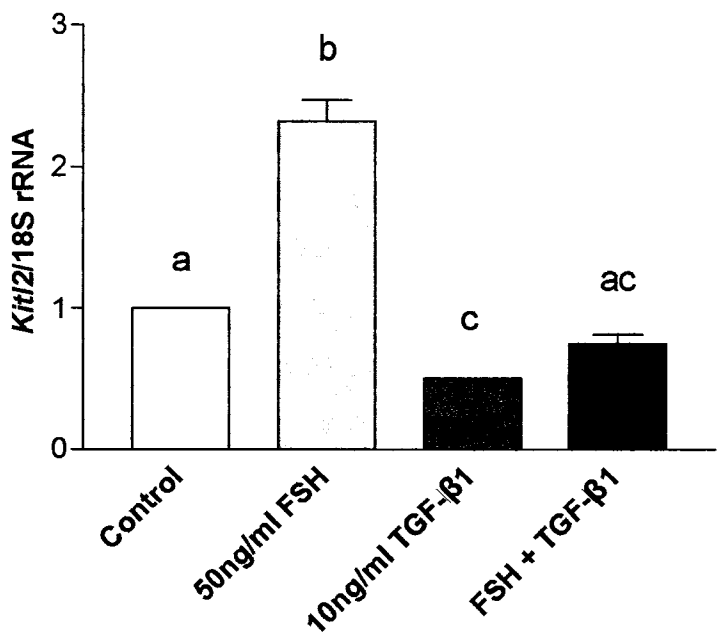
Kitl1: b - $P < 0.01$; c - $P < 0.05$

Kitl2: b - $P < 0.001$; c - $P < 0.01$

A



B



CHAPTER 4 - DISCUSSION

4.1 Characterizing a Granulosa Cell Line to be Used in Studies of KITL

The results of this research have demonstrated the expression of *Kitl1* and *Kitl2* mRNA transcripts in SIGC, like in primary rat granulosa cells, although we were not able to find an antibody functional in detecting the specific protein band of either of the isoforms in various cell lines and tissues tested. This has been a persistent problem in this field and, as a consequence, the vast majority of studies reported to date have relied upon RT-PCR and QPCR methods for both the detection and quantification of *Kitl* expression. Visualization of KITL protein has been performed by a few groups only utilizing the immunohistochemistry technique. A polyclonal rabbit serum against recombinant mouse KITL protein was produced (Huang et al., 1992) and used for Western blot detection of KITL-1 and KITL-2 protein immunoprecipitated from COS-1 cells transfected with *Kitl1* and *Kitl2* expression plasmids (Huang et al., 1992). This anti-KITL serum was also used successfully in a more recent study for Western blot detection of KITL in protein from 15P-1 mouse Sertoli cell culture system (Vincent et al., 1998). Since these two studies remain the only ones that have used Western blotting for the detection of KITL protein in rodent cell culture systems, better anti-KITL antibodies remain to be developed.

Through SIGC proliferation assays, we noticed an interesting pattern of *Kitl1* and *Kitl2* mRNA expression, whereby levels were dramatically elevated (86% and 88%, respectively) in the first 24 hours after re-plating as compared to 48 hours after re-plating. This period coincided with the time during which SIGC proliferation was more rapid than

at the later time points, with cell numbers increasing by 86% from the time of initial re-plating. Our observation of elevated *Kitl1* and *Kitl2* mRNA levels during the first 24 hours after re-plating, a period when (1) the SIGC proliferation rate was at its greatest and (2) when the cells may be considered in a state of disorganization, brings to mind the concept that KITL possibly serves as the putative follicular organizer. *In vivo*, the levels of *Kitl* mRNA and its protein progressively increase as the follicle attempts to achieve greater complexity and organization, serving a role in promoting the development of theca cells from stromal cells, proliferation of granulosa cells and growth of the oocyte (the latter two being mediated via KIT receptors on the oocyte). Despite the lack of KITL target cells in our *in vitro* culture system, it is possible that even within immortalized granulosa cells (SIGC), a period of disorganization when for example, granulosa cells have few cell-cell contacts, serves as a signal to up-regulate the expression of KITL such that it may function to bring about the necessary organization. Therefore, it is possible that KITL is up-regulated in SIGC post-culturing to promote increased cellular organization, thus reflecting its function to aid in follicle growth and development *in vivo*.

By comparing the expression levels of *Kitl1* and *Kitl2* mRNA in SIGC with that of primary granulosa cells, we determined that when SIGC were cultured for 48 and 72 hours, expression levels of the two isoforms were not significantly different from 24 hour cultured primary granulosa cells. Therefore, we would not need to be concerned with levels in the immortalized cells being incomparable to normal granulosa cells. However, unlike primary granulosa cells, we found that SIGC have not retained expression of the FSHR. This finding is consistent with (1) their unresponsiveness to FSH treatment (Stein

et al., 1991) and (2) with the noted loss of FSH receptor expression when granulosa cells are cultured for an extended period (Keren-Tal et al., 1993). This finding was also an indication that we would not be able to use FSH directly in SIGC to study its downstream signalling in the regulation of *Kitl1* and *Kitl2* mRNA expression.

4.2 Understanding the Regulation of KITL Expression by FSH

With respect to our aim to further the understanding of FSH regulation of KITL expression in rat granulosa cells, our findings have demonstrated that FSH stimulates an increase in *Kitl1* and *Kitl2* mRNA expression in rat primary granulosa cells. This is the first time that direct stimulation with FSH has been shown to result in the up-regulation of both *Kitl* mRNA transcripts in cultured granulosa cells, and is consistent with previous reports using Sertoli cell-enriched cultures, whereby treatment with FSH was observed to stimulate an increase in the overall *Kitl* mRNA expression level (Rossi et al., 1993). However, our current findings differ from what has been previously reported regarding the regulation by FSH of *Kitl1* and *Kitl2* mRNA expression in mouse preantral OGC. In that system, it was found that while FSH had no effect on *Kitl1* mRNA expression, a biphasic effect on *Kitl2* mRNA expression was induced. Specifically, FSH at a low dose (0.05ng/ml) produced an increase in *Kitl2* mRNA expression whereas a higher dose (0.5ng/ml) had no effect (Thomas et al., 2005). In our current study, we found that only 50ng/ml of FSH produced an effect and moreover, an increase in both *Kitl* mRNA transcripts was induced. The explanation for this may lie in the different culture systems used since the Thomas et al. (2005) study utilized cultures of mouse preantral OGC whereas our system consisted of monolayers of rat granulosa cells. Therefore, it is

possible that the oocytes present in the former study modified the effects of FSH on *Kitl1* and *Kitl2* mRNA expression in addition to possibly exerting a direct influence. In fact, that the oocyte regulates *Kitl* mRNA expression through secretion of specific factors is a well documented observation (Elvin et al., 1999; Joyce et al., 1999; 2000; Otsuka and Shimasaki, 2002). Additionally, these factors produced exclusively by the oocyte throughout folliculogenesis are also found to modulate FSH action (Otsuka et al., 2000; 2001) and thus may have modulated the effect of FSH in the mouse preantral OGC system.

The ability of FSH to induce *Kitl1* and *Kitl2* mRNA expression in primary granulosa cells raises interesting possibilities as to the role of FSH in regulating the *in vivo* expression of KITL and the implications of this induction. As previously mentioned, a role is indicated for KITL in the initiation of follicular growth by Sl^{pan}/Sl^{pan} mutant mice which have follicles arrested at the primary stage of development (Huang et al., 1993). Moreover, an antibody blocking KIT prevents primordial follicle development when either injected into newborn mice or added to the culture of early post-natal ovarian fragments (Yoshida et al., 1997; Parrot and Skinner, 1999). Considering our findings of the ability of FSH to induce *Kitl1* and *Kitl2* mRNA expression, it is plausible that FSH is the signal that up-regulates this vital factor in granulosa cells of early follicles. Furthermore, our findings in conjunction with reports that ACK-2 blocks the FSH-induced increase in the percentage of preantral follicles (Parrot and Skinner, 1999), indicate that the actions of FSH are mediated at least in part via KITL.

4.3 Investigating the Pathway Through Which FSH Regulates KITL Expression

Despite demonstrating an inductive action of FSH on *Kitl1* and *Kitl2* mRNA expression, we were not successful in determining whether this effect was mediated at least in part via PKA since the inhibitor H-89 did not attenuate FSH-induced *Kitl* mRNA expression. Therefore, whether FSH functions via this pathway to stimulate *Kitl1* and *Kitl2* mRNA expression in rat granulosa cells remains unclear. FSH has been shown to promote the rapid activation of PKA (DeManno et al., 1999) and PKA selective inhibitors abrogate the effects of FSH to activate target genes and signalling pathways that lead to granulosa cell differentiation (DeManno et al., 1999; Haynes-Johnson et al., 1999; Gonzalez-Robayna et al., 2000; Salvador et al., 2001; Cottom et al., 2003; Silva et al., 2006). For example, FSH-stimulated Cyp19 mRNA accumulation and estradiol secretion in granulosa cells is inhibited by H-89 (Haynes-Johnson et al., 1999; Silva et al., 2006). Additionally, in granulosa cells, FSH stimulates histone H3 phosphorylation and the rapid but transient phosphorylation of p38 MAPK (Salvador et al., 2001) and ERK1/2 (Cottom et al., 2003), effects which are all abrogated by the PKA-selective inhibitory peptide (PKI) or H-89. Taken together, these results point to PKA as an initial protein kinase activated in response to FSH and suggest that cAMP signals are mediated largely via PKA leading to the activation of a wide array of pathways that become involved in FSH-mediated granulosa cell growth and differentiation.

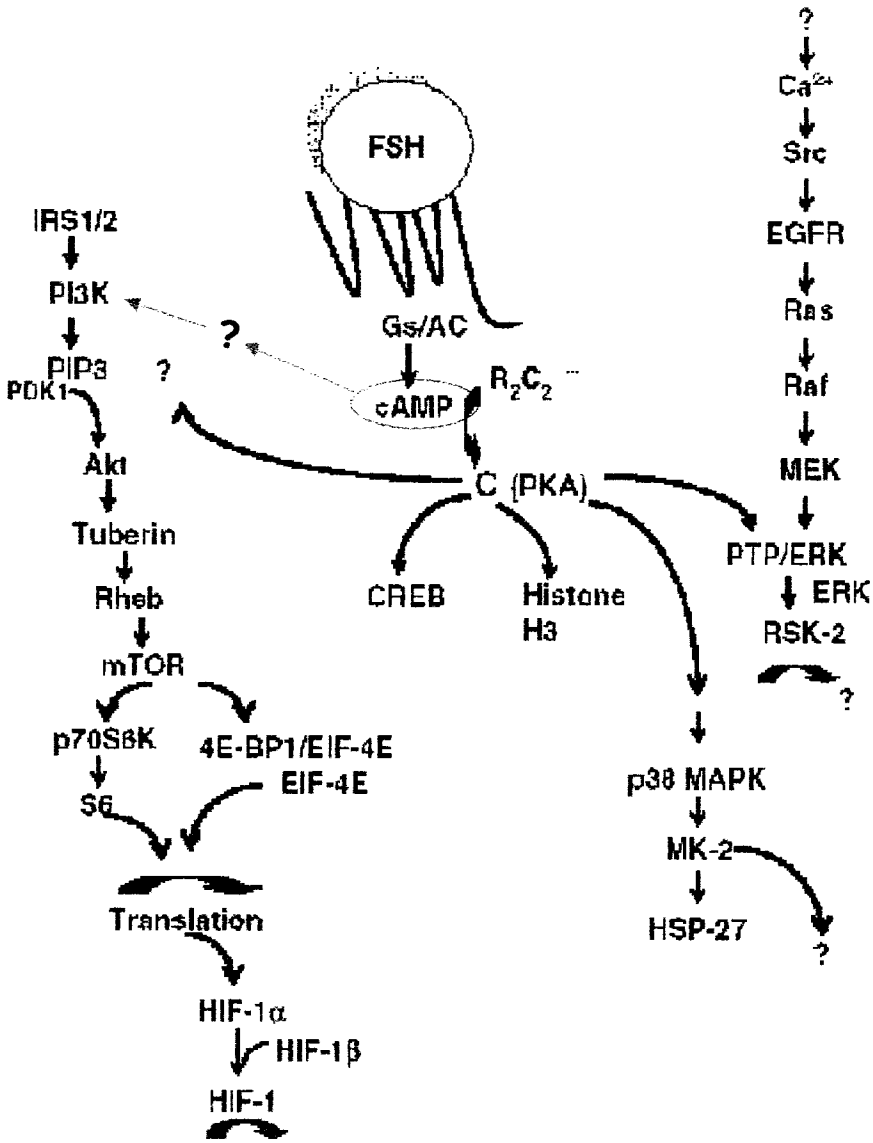
However, there are other reports that indicate the possibility that FSH induction of cAMP production by adenylate cyclase may not result in PKA activation. For example, although FSH promotes rapid activation of the phosphatidylinositol 3'-kinase (PI3K)

pathway in rat granulosa cells, resulting in phosphorylation of downstream Akt (Gonzalez-Robayna et al., 2000; Cottom et al., 2003), the effect is not inhibited by H-89 (Gonzalez-Robayna et al., 2000). According to Hunzicker-Dunn and Maizels (2006), this inability of H-89 could reflect its propensity to inhibit p70 ribosomal S6 protein kinase (p70S6K) preferentially over other kinases including PKA (Davies et al., 2000; Alam et al., 2004) and thus to inhibit an unidentified negative feedback pathway from p70S6K. In contrast, the authors of the study (Gonzalez-Robayna et al., 2000) interpreted the observation of the failure of H-89 to inhibit FSH-induced PI3K pathway activation as suggesting that FSH may signal to PI3K via a PKA-dependent pathway (Gonzalez-Robayna et al., 2000; Ongeri et al., 2005). Since FSH-induced PI3K pathway activation was mimicked by forskolin and cell-permeable cAMP analogues (Gonzalez-Robayna et al., 2000), this would suggest that a branch point in the FSH induced pathway exists, not only downstream of PKA, but also (upstream to that) immediately downstream of cAMP (*figure 27*). Therefore, it is possible that in our study of FSH regulation of *Kitl1* and *Kitl2* mRNA expression, there is no PKA pathway involvement.

Nonetheless, we did not simply assume that the failure of H-89 indicated PKA-independent stimulation of *Kitl1* and *Kitl2* mRNA expression by FSH and sought to verify the H-89 reagent's ability to inhibit PKA activity by testing its capacity to inhibit a CRE-luciferase construct stimulated by endogenous PKA activity in SIGC. The main reason for doing so had to do with a previous study that reported the stimulatory effect of dbcAMP on overall *Kitl* mRNA expression in cultures of mouse granulosa cells could be inhibited using 25μM H-89 (Packer et al., 1994), thus suggesting that the cAMP effect (and therefore the effect of FSH) is mediated via this kinase. It is to be noted however,

Figure 27. **The FSH-Regulated Signalling Pathway Expanded**

This figure is a schematic diagram that represents a current understanding of the complexity associated with the FSH-regulated signalling pathway responsible for inducing the program of growth and differentiation within granulosa cells. This figure was obtained from Hunzicker-Dunn and Maizels, 2006 and modified with permission from the publisher, Elsevier.



that such concentrations of H-89 (>10μM) result in the inhibition of not only PKA, but also isoforms of mitogen- and stress-activated kinase (MSK) and the p70S6K (Alam et al., 2004) based on studies of protein kinase inhibitors by Davies et al. (2000). Therefore, such a high concentration would not be specific to PKA.

In our primary granulosa cell studies, 10μM of H-89 was used to inhibit FSH-stimulated *Kitl1* and *Kitl2* mRNA expression. However, it was not possible to treat transfected SIGC with this dose, as it proved toxic to the cells; therefore a maximum dose of 7.5μM H-89 was used. Although our luciferase data indicated no significant effects, there appeared to be a trend toward increased luciferase activity with increased presence of DMSO in a sample. Since the construct consisted only of the CRE promoter upstream the luciferase reporter gene, it can be assumed that DMSO stimulated the luciferase activity through an influence on cellular activity that in turn stimulated the CRE promoter, thus possibly explaining the inability of H-89 to act as a proper inhibitor of PKA activity in SIGC. Considering that DMSO as a vehicle may be problematic, future studies that aim to characterize this pathway in greater detail could involve the use of other inhibitors of PKA such as PKI, which does not require DMSO as a solvent.

Since SIGC do not express FSHR (*figure 11*), dbcAMP was used to induce *Kitl1* and *Kitl2* mRNA expression within this cell line, in the way that FSH was used in primary granulosa cells. If dbcAMP would induce the same effect in SIGC as that seen using FSH in primary granulosa cells, this would establish SIGC as an alternative to the use of primary granulosa cells for studying FSH signalling downstream cAMP and allow further investigation of the pathway using cells that are more readily available, and in greater quantities. However, we found that rather than increasing the expression of *Kitl1*

and *Kitl2* mRNA, dbcAMP was actually suppressive. Inhibition of KITL expression in granulosa cells by cell-permeable cAMP analogues has not previously been reported. The accumulation of cAMP in response to FSH-treatment of granulosa cells is well documented (Richards, 1994) and treatment of granulosa cells with cell-permeable cAMP analogues mimics FSH-induced effects (Gonzalez-Robayna et al., 2000; Alam et al., 2004). Additionally, the ability of dbcAMP to increase overall *Kitl* mRNA expression by 1.56-fold, at a concentration as low as 250μM, has previously been reported in cultured granulosa cells from mouse preantral follicles (Packer et al., 1994). We therefore expected similar results in SIGC, especially considering the report that they maintain an undifferentiated phenotype (Stein et al., 1991), an important observation since once granulosa cells have terminally differentiated to luteal cells, they no longer respond to changes in cAMP (Gonzalez-Robayna et al., 2000). There is the possibility that the down-regulatory effect induced by dbcAMP resulted from the dibutyryl component of the reagent since, despite the common use of this tool for proving the responsibility of cAMP for certain biological effects, there appear to be serious drawbacks associated with its use. In order for the reagent to stimulate the pathway, it needs to split off one of the butyryl groups to release the kinase active monobutyryl cAMP. However, the released butyrate induces effects which often interfere with second messenger pathways. Butyrate is reported to act as a stimulator of gene expression (Graham and Buick, 1988), differentiating agent (Kawamoto et al., 1998), inducer of apoptosis (Soldatenkov et al., 1998) and disturber of the focus on the PKA pathway by activating protein kinase C (PKC) (Rivero and Adunyah, 1998).

We therefore attempted to bypass the need for a cell-permeable cAMP analogue by using forskolin, which acts directly on the adenylate cyclase enzyme to stimulate endogenous production of cAMP (Seamon et al., 1981). We would assume that if PKA is solely responsible for mediating the FSH-induced program of growth and differentiation within granulosa cells, than stimulating PKA upstream with forskolin rather than FSH would simply produce the same effect on *Kitl* mRNA expression that FSH has. While forskolin had no effect on the expression of *Kitl1* mRNA, it produced a biphasic effect on the expression of *Kitl2* mRNA with a lower dose (5μM) increasing its expression, while a higher dose (10μM) produced no effect. These findings paralleled the previously reported effects seen by Thomas et al. (2005) in mouse preantral OGC using FSH. However, the findings did not reflect what we observed when using FSH to directly stimulate *Kitl1* and *Kitl2* mRNA expression in primary granulosa cells. We attributed the difference between the results we saw using FSH in our system of cultured granulosa cells and those of Thomas et al. (2005) to the moderating effects of oocytes present in the culture system of the latter study. However, it is baffling how a similar modification could occur in SIGC, which consist only of granulosa cells.

Evidence from the literature indicates that forskolin in the concentration range we tested mimics the effects of FSH in granulosa cells (Hsueh et al., 1984; Das et al., 1996). However, it is possible that in each different culture system, highly specific concentrations of the stimulant are necessary to induce expression of a specific amount of cAMP, which in turn brings about the biphasic effect observed in SIGC (using forskolin) and in mouse preantral OGC (using FSH; Thomas et al., 2005). Therefore it is possible that we did not select the appropriate concentrations of FSH capable of inducing a

biphasic effect in primary granulosa cells while the forskolin concentrations we selected were precise for bringing about the biphasic effect seen in SIGC. In fact, it is hypothesized that the different patterns of gene expression induced by FSH are the result of a combination of subtle differences in the intensity, duration and subcellular compartmentalization of the cAMP signal (Schwartz, 2001). This indicates that what may be an identical initial cAMP signal can then lead to stimulation of different pathways. Therefore, it is possible that cAMP concentration specificity, that is, accumulation of a precise amount of cAMP within the cell, is also responsible for the differences we see when using FSH in primary granulosa cells and when using dbcAMP or forskolin in SIGC.

4.4 The Implications of the Divergent Effects of FSH, DbcAMP and Forskolin on the Use of SIGC as a Granulosa Cell Model System

Our observations of the disparity in the induced expression of *Kitl1* and *Kitl2* mRNA between primary granulosa cells and SIGC also led us to suspect that perhaps SIGC do not function as normal granulosa cells. Although they are reported to act and appear as non-differentiated granulosa cells, the cell line was attained by continuous passaging in culture, during which time they were observed to undergo luteinization, a characteristic that nonetheless disappeared over a period of several months (Stein et al., 1991). However, analysis of the potential for steroidogenesis in the SIGC has indicated the presence of the p450scc enzyme, which converts cholesterol to pregnenolone. All SIGC stain positively and uniformly for this enzyme, in contrast to the mitochondrial staining pattern in primary granulosa cells that exhibit variable stages of differentiation

(Stein et al., 1991). This would indicate retention of a differentiated granulosa cell feature, despite the cells appearing fairly undifferentiated.

cAMP signalling is involved in most aspects of differentiation and maturation of granulosa cells in the ovarian follicle. However, differences exist in the signalling pathway stimulated by cAMP in, for example, preantral and preovulatory follicles. In granulosa cells from preantral follicles, FSH promotes growth of granulosa cells and stimulates androgen aromatization via activation of cAMP signalling (Richards, 1994); conversely, in granulosa cells from preovulatory follicles, the cAMP signalling pathway (stimulated by LH) promotes the exit from the cell cycle and suppression of aromatase activity (Richards, 1994). This is believed to be possible due to the dependency of cAMP signalling on the cell context, such as the state of granulosa cell differentiation (Conti, 2002), which is the feature that allows FSH and LH to use the same pathway for bringing about apparently contrasting effects. Therefore, this may explain our observation of differences between the two groups of granulosa cells in response to reagents that should be stimulating the accumulation of cAMP. These divergent outcomes of cAMP activation would not be possible if the signalling pathway was simply linear (Conti, 2002). Therefore, if cAMP accumulation is being stimulated in granulosa cells at a later stage of differentiation, when they usually are not responsive to FSH, then they will not respond to cAMP stimulation as they do at stages when they are FSH-responsive.

4.5 TGF-β1 Regulation of KITL Expression

We have also demonstrated that TGF-β1 acts as a negative regulator of *Kitl1* and *Kitl2* mRNA expression in both SIGC and primary granulosa cells. Previous work from

our lab has demonstrated a suppressive action of TGF-β on overall *Kitl* mRNA expression in rat ovarian surface epithelial cells (Ismail et al., 1999). Additionally, TGF-β1 has been shown to down-regulate overall *Kitl* mRNA expression in marrow stromal cells by repressing gene transcription (Heinrich et al., 1995). However, the current finding that TGF-β1 acts as a regulator of *Kitl1* and *Kitl2* mRNA expression in granulosa cells is novel. Our finding is especially significant considering that TGF-β is implicated in almost every aspect of reproductive function, with a vital role that is supported by the positive correlation between the TGF-β1 content of follicular fluid and pregnancy success in human *in vitro* fertilization (Fried and Wramsby, 1998).

This ability of TGF-β1 to inhibit *Kitl* mRNA expression in granulosa cells coincides with the known function for GDF-9. This is an oocyte-specific factor that suppresses KITL expression according to both *in vivo* studies, that have shown dramatically elevated levels of *Kitl* mRNA expression in ovaries from *Gdf9*-deficient mice (Elvin et al., 1999), and *in vitro* studies demonstrating GDF-9 inhibition of *Kitl* mRNA expression in preantral and mural granulosa cells from mice (Joyce et al., 2000). Therefore, a major role of GDF-9 can be interpreted as allowing the oocyte, its site of production, to regulate the expression of KITL either through direct action on the factor, or indirectly by influencing granulosa cell activity. TGF-β1 protein has been detected in the granulosa cells, theca cells and oocytes, and its receptors are also found on all three major cell types of the ovary throughout various stages of follicle development (Ghiglieri et al., 1995; Levacher et al., 1996; Juneja et al., 1996). Furthermore, all three cell types are influenced by KITL either directly, such as in the case of theca cells (Parrott and Skinner, 1997) and oocytes (Packer et al., 1994) or indirectly, such as in the case of

granulosa cells (Otsuka et al., 2002). Therefore, ovarian follicular cells that produce TGF-β1 and whose function is regulated by KITL have the capacity to control their own fate. Employed as a powerful negative regulator that may act in both an autocrine and paracrine manner, TGF-β1 may keep KITL production under control until other, either local regulators independently, or perhaps under the influence of FSH, overcome its effect. Given the vital role of KITL in regulating follicle development, the ability of a cell to regulate its expression would serve as a tool for that cell. Although a suppressive action of the oocyte on granulosa cell *Kitl* mRNA production has been demonstrated (Joyce et al., 1999), this has been attributed mainly to GDF-9 (Joyce et al., 2000); whether a similar suppressive action of theca cells and even granulosa cells on granulosa cell production of *Kitl* mRNA expression exists, has not been reported.

During the primordial to primary stages of follicle development, *Kitl* mRNA and levels of its protein in granulosa cells are reported to be low in primordial follicles, but progressively increase (Keshet et al., 1991; Motro et al., 1991; Manova et al., 1993; Motro and Bernstein, 1993); it is possible that TGF-β1 is one of the factors responsible for maintaining the low expression levels during these initial stages. However, TGF-β1 expression appears to be ubiquitous and continuous; thus, if it was the only element involved, then KITL levels would remain eternally suppressed. Therefore, several factors that neutralize or antagonize the effects of TGF-β1 are likely present; based on the evidence, there is a degree of complexity in the signalling factors produced locally with oocytes secreting a range of different proteins (Eppig et al., 1997; Erickson and Shimasaki, 2000) that are likely involved in influencing follicle development. For example, in addition to GDF-9, the oocyte is the exclusive producer of BMP-15, which

has been shown to stimulate *Kitl1* and *Kitl2* mRNA expression in rat granulosa cell/oocyte cocultures and in mouse preantral OGC (Otsuka and Shimasaki, 2002; Thomas et al., 2005). Nonetheless, our finding suggests an interesting role for TGF-β1 in regulating *Kitl* mRNA expression in granulosa cells. Combined with the evidence that KITL and TGF-β1 are both important local factors in regulating follicle growth and development, it would be interesting to determine whether KITL is in turn able to regulate the expression of TGF-β1 thus revealing the presence of a regulatory feedback loop involving TGF-β1 and KITL/KIT signalling. Finally, given the evidence presented here that TGF-β1 down-regulates *Kitl1* and *Kitl2* mRNA expression in granulosa cells, it would be interesting to measure the ovarian *Kitl* mRNA levels in the TGF-β1 null mouse (Shull et al., 1992) to determine whether like in the *Gdf9*-null mutant (Elvin et al., 1999), its levels are elevated.

4.6 Investigating the Pathway Through Which TGF-β1 Regulates KITL Expression

In order to determine how this suppressive effect of TGF-β1 may be mediated, our present study also focused on the post-receptor effects of TGF-β1 by determining whether the Smad signalling system used by many members of the TGF-β superfamily would be activated. Our findings clearly indicate that TGF-β1 stimulates Smad2 and Smad3 phosphorylation in SIGC. Nuclear localization of Smad2 and Smad3 following TGF-β treatment of cultured granulosa cells has been previously reported (Xu et al., 2002), although phosphorylation of Smads induced by TGF-β1 has not been. Furthermore, whereas BMP-2 activated endogenous Smad1/5 phosphorylation, TGF-β1 did not induce a similar effect. The correlation between suppression of *Kitl1* and *Kitl2*

mRNA expression and the phosphorylation of Smad2 and Smad3 (*figure 20*), but not Smad1/5 by TGF-β1 (*figure 21*) implicates the traditional TGF-β/activin pathway in mediating TGF-β1 modulation of *Kitl* mRNA expression. It therefore seems clear that at least part of the effects of TGF-β1 on *Kitl1* and *Kitl2* mRNA expression are mediated through specific Smads. Additionally, this suggests that the mechanism through which TGF-β1 is down-regulating *Kitl1* and *Kitl2* mRNA expression in granulosa cells may be transcriptional, controlled via the Smad signalling pathway. Smad3 and Smad4 are found to preferentially recognize the 5'-GTCT-3' sequence, also termed the Smad binding element (SBE) (Dennler et al., 1998). SBE-like sequences are critically important for TGF-β induced activation of multiple TGF-β-responsive genes (Derynck, 1998; Chen et al., 2002). Smad3 and Smad4 also interact with several AP-1 family members and regulate transcription via AP-1 transcription factor binding sites (Liberati et al., 1999). Additionally, in certain genes, the transcription factor binding site Sp1 appears to be required for regulation by Smads, suggesting a further possibly critical response element for Smad action (Moustakas and Kardassis, 1998). A brief examination of the 5'-flanking region of the *Kitl* gene reveals four possible SBE binding regions, five AP-1 binding regions, three AP-2 binding regions, and four Sp1 binding regions, suggesting that the gene has the required elements for regulation by Smads.

Smad2 and Smad3 are expressed in the ovary at follicular stages during which TGF-β1 may serve a regulatory role (Xu et al., 2002; Drummond et al., 2003). However, despite having an understanding of the expression pattern of Smads, it is difficult to say what the implication of TGF-β1 action would be since it is not the only factor in the ovary with influence on KITL expression that utilizes Smad signalling. Furthermore, our

findings that Smads are activated in response to TGF-β1 does not rule out the possibility that Smad-independent pathways could also be involved in the cellular effects of TGF-β1. In fact, evidence over the past few years suggests that TGF-β may signal through various other pathways. For example, TGF-β can activate several mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinases (Erks), c-Jun N-terminal kinases (JNKs) and p38 kinases (Hartsough and Mulder, 1995; Hanafusa et al., 1999; Engel et al., 1999). In the developing follicle, various extraovarian and intraovarian factors are involved in regulation, therefore, extensive cross-talk between the pathways is likely; this possibility complicates predicting the response to a signal. Nonetheless, what is clear is that TGF-β1 inhibits *Kitl1* and *Kitl2* mRNA expression and that this action is likely mediated via Smad2 and Smad3.

4.7 A Role for Estrogen in Regulating KITL Expression

The data presented here also demonstrates that the expression of *Kitl1* and *Kitl2* mRNA in granulosa cells obtained from DES-primed rats and flash-frozen immediately post-collection is low as compared to granulosa cells that are cultured for 24 or 48 hours. Previous work in our lab had demonstrated that overall levels of *Kitl* mRNA in granulosa cells obtained from DES-primed rats are actually lower than in granulosa cells obtained from untreated rats (Rubina Ismail, unpublished observations). This finding in conjunction with our own suggested that DES may be inhibiting *Kitl* mRNA expression. DES is an estrogenic compound and therefore a mitogen, that stimulates the follicles to increase in size while the granulosa cells remain largely undifferentiated, resembling those of large preantral follicles (Goldenberg et al., 1972). It induces its action via

binding to one of two estrogen receptor subtypes – estrogen receptor α (ERα; Jensen and DeSombre, 1973) and estrogen receptor β (ERβ; Kuiper et al., 1996) which act as nuclear transcription factors responsible for transmitting the genomic actions of estrogenic compounds. The ovary contains both ER subtypes, with a predominance of ERβ over ERα in granulosa cells (Sar and Welsch, 1999).

In attempting to determine whether the observation of low *Kitl* mRNA expression in flash frozen granulosa cells from DES-primed rats was due to estrogen, we treated primary granulosa cells with 17β-estradiol and found that the treatment did in fact result in the suppression of *Kitl1* and *Kitl2* mRNA expression. This verified that estrogen was at least partially responsible for the lower levels in the granulosa cells from DES-primed rats. This role for estrogen in the regulation of *Kitl* mRNA has previously been suggested by preliminary experiments in cultures of rat granulosa cells, where 17β-estradiol induced a 2.6-fold decrease in overall *Kitl* mRNA expression as compared to untreated controls (Rubina Ismail, unpublished observations).

Whether this effect of estrogen is direct, through action on *Kitl* gene transcription for example or indirect, through changes that are induced in the granulosa cells by estrogen treatment, is unknown. Nonetheless, it appears that elevated levels of estrogen, which is normally produced at the antral follicle stage once granulosa cells have differentiated, may be acting as a signal for down-regulating *Kitl* mRNA expression. Estrogen is a well established potent mitogen for rat granulosa cells *in vivo*. Therefore, if this effect of estrogen on *Kitl* mRNA expression was mediated by its known action on granulosa cells (stimulation of proliferation) than we would expect that there would be an increase in *Kitl* mRNA expression with DES-priming. A possible explanation for the

observed suppressive action may be that, since estrogen is normally produced only by differentiated granulosa cells in the antral stage of follicle development, exposure of undifferentiated granulosa cells to high levels of estrogen may indicate some sort of imbalance in cell function. Given that KITL plays an important role in follicle growth and development, down-regulating it may prevent the further development of that possibly unhealthy follicle.

An alternative explanation is as follows: it is noted that in the late antral stage of follicle growth, when estrogen levels are increasing, there is a decline in KITL expression. Therefore, an increase in estrogen in the granulosa cell environment at any stage may be a sign of differentiation and thus a signal for down-regulation of KITL expression. This would indicate a negative regulatory role for estrogen on KITL expression, one that is possibly present and functional in late antral follicles, and may be partly responsible for the down-regulation of its expression that is noted at that stage (Manova et al., 1993; Motro and Bernstein, 1993). It is also possible that when granulosa cells proliferate too rapidly without a change in differentiated status, KITL is down-regulated to suppress the indirect proliferative effect on granulosa cells that it is known to have (Otsuka et al., 2002). Those latter two ideas are illustrated as a proposed model in *figure 28.*

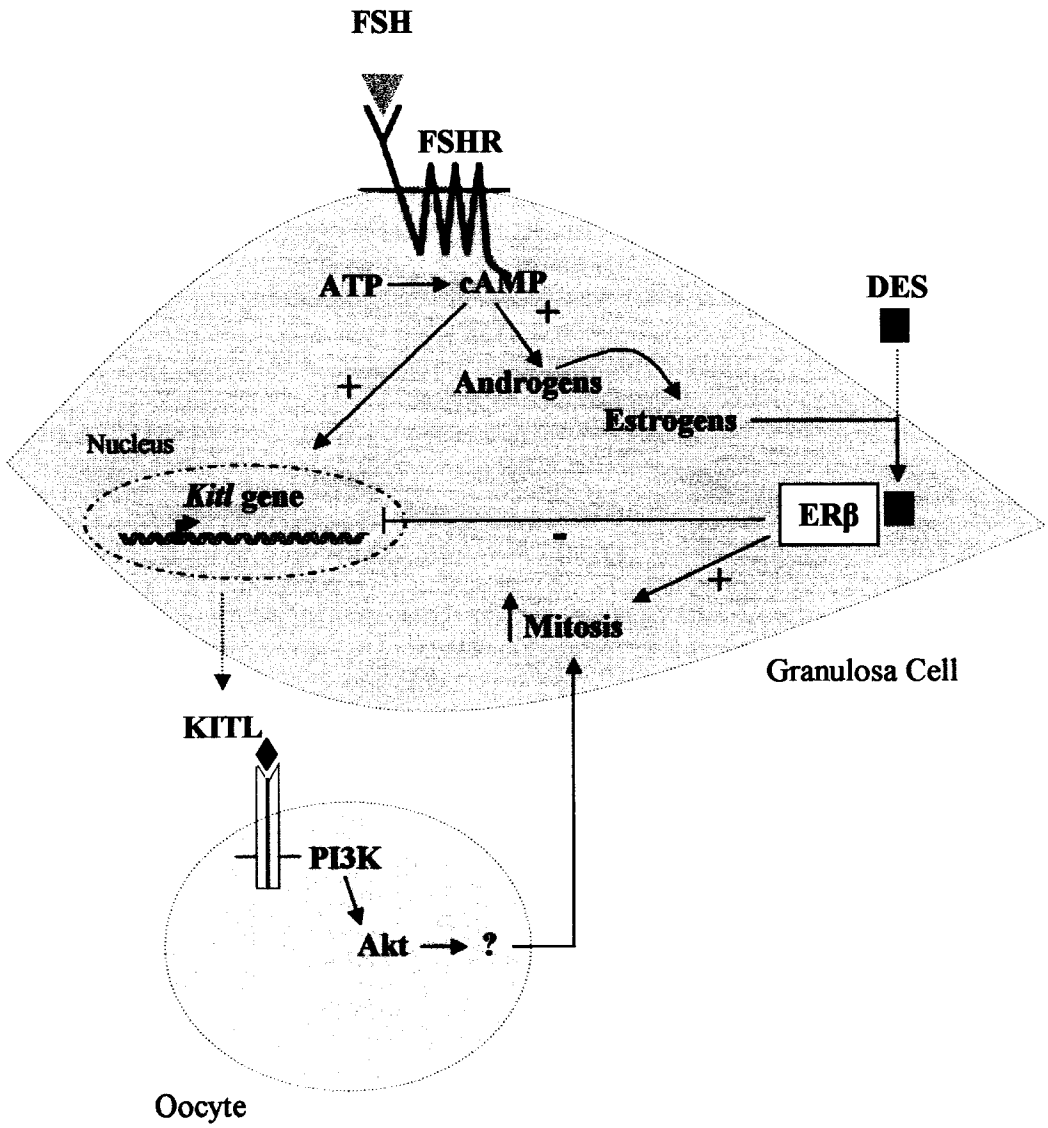
4.8 TGF-β1 as a Mediator of Estrogen Action

Results from our current study show that TGF-β1 suppresses *Kitl1* and *Kitl2* mRNA expression levels in both SIGC and primary granulosa cell cultures, and that estrogen suppresses *Kitl1* and *Kitl2* mRNA expression both *in vivo* and *in vitro*. Previous

Figure 28. **Proposed Model for Suppression of KITL Expression by Estrogen**

This is a model that illustrates a possible explanation for the observation that estrogen suppresses *Kitl1* and *Kitl2* mRNA expression. In the late antral stage of follicle growth, when estrogen levels are increasing, a decline in KITL expression is noted. Therefore, an increase in estrogen in the granulosa cell environment at any stage may be a sign of differentiation and thus a signal for down-regulating KITL expression resulting in a negative regulatory role for estrogen on KITL expression, one that is possibly present and functional in late antral follicles, and is partly responsible for the down-regulation of its expression that is noted at that stage. Additionally, it is possible that when granulosa cell numbers increase without a change in differentiated status, KITL expression is down-regulated to prevent the indirect proliferative effect on granulosa cells KITL is known to have, as a method of preventing continued proliferation.

ERβ - estrogen receptor β

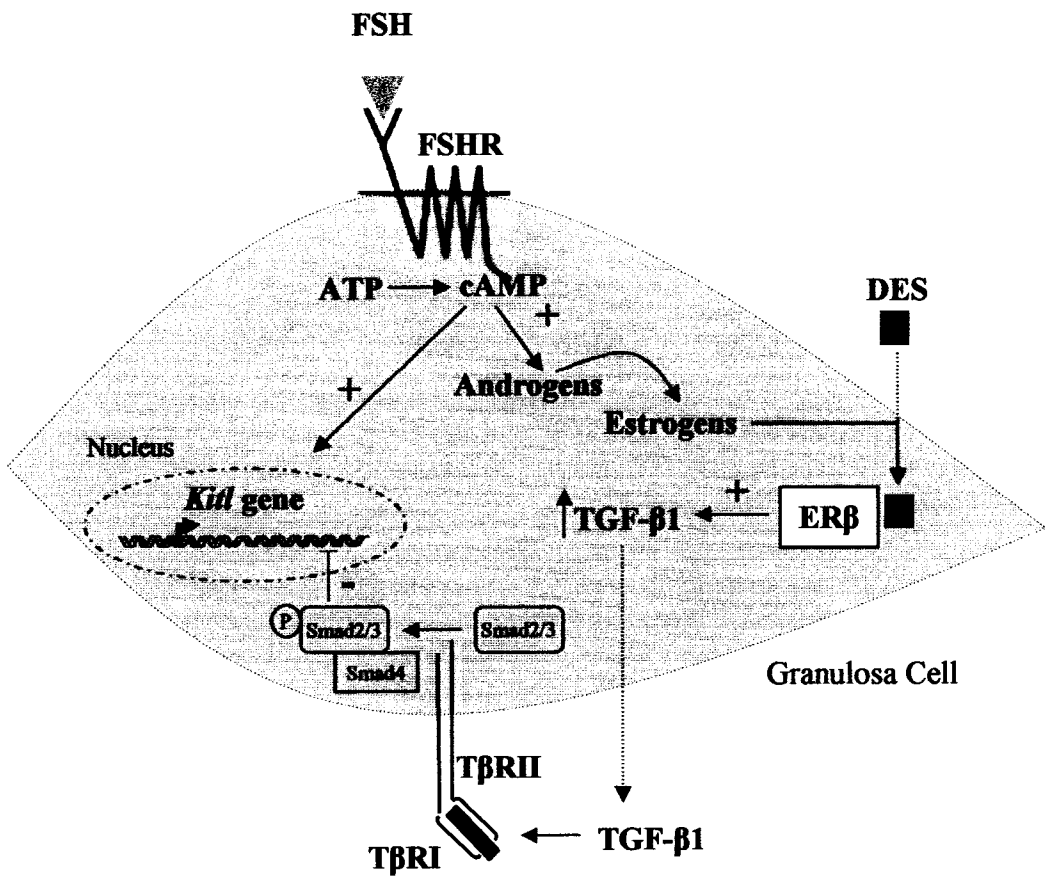


reports indicate that not only does TGF-β stimulate rat granulosa cell proliferation in culture like estrogen, but neutralization of TGF-β using an antibody, significantly reduces cell proliferation in 17β-estradiol treated granulosa cells (Bendell and Dorrington, 1991). Considering the findings of these previous studies and given the similarity we noted between the action of TGF-β1 and estrogen, we considered the possibility that the estrogen effects on *Kitl1* and *Kitl2* mRNA expression were also mediated at least in part by TGF-β1. A neutralizing antibody against TGF-β1 prevented the suppressive effect of 17β-estradiol on *Kitl1* and *Kitl2* mRNA expression, suggesting that the action of 17β-estradiol is actually mediated via TGF-β1. Therefore, if our idea for the role of estrogen is as we illustrated in *figure 28*, and if the effect of estrogen on *Kitl1* and *Kitl2* mRNA expression is exerted at the level of transcription, then TGF-β1 is the effector of the estrogen action and this is likely mediated via Smad2 and Smad3. 17β-estradiol has been shown to stimulate the secretion of TGF-β by rat granulosa cells (Dorrington et al., 1988). Therefore, estrogen stimulates the secretion of TGF-β that in turn acts to inhibit FSH-stimulated KITL expression. The increased levels of TGF-β generated by the rat granulosa cells exposed to estrogen will augment the actions of FSH on aromatase activity, since this is known to be one of the roles of TGF-β (Adashi and Resnick, 1986; Ying et al., 1986; Hutchinson et al., 1987; Adashi et al., 1989), thereby establishing an auto-amplifying mechanism to up-regulate estrogen synthesis in late antral follicles. By this mechanism, KITL expression would decrease. A model that illustrates this idea is shown in *figure 29*. In the future, it would be interesting to measure TGF-β1 levels in our 17β-estradiol treated primary granulosa cells and vehicle-treated controls obtained from

Figure 29. **Proposed Model for Estradiol Stimulation of TGF-β1 Secretion Leading to Suppression of KITL Expression**

This is a model that illustrates the concept that, if estrogen is directly responsible for suppressing *Kitl1* and *Kitl2* mRNA expression, then it is doing so by stimulating the production of TGF-β1. In turn, TGF-β1 may be acting directly to suppress *Kitl* gene transcription. This mechanism may be through a paracrine manner, with TGF-β1 secreted by a particular granulosa cell acting on adjacent granulosa cells. Alternatively, it may occur in an autocrine manner, with TGF-β1 secreted by a particular granulosa cell acting back on the same cell.

ERβ - estrogen receptor β; P indicates phosphorylated status



DES-primed rats to determine whether TGF-β1 levels are elevated as compared to primary granulosa cells obtained from untreated rats.

4.9 The Relative Strength of FSH and TGF-β1 in Regulating KITL Expression

Nonetheless, in order for estrogen to be effective in suppressing *Kitl* mRNA expression *in vivo*, important to both of our proposed models (*figures 28* and *29*), with levels of circulating FSH still high in the antral follicle, it would have to overcome the stimulatory effect that FSH has on KITL expression throughout follicle development. Only in this way could follicular KITL expression levels be limited despite continued stimulation by FSH. Interestingly, we found that when primary granulosa cells were treated with a combination of FSH and TGF-β1, the stimulatory effect FSH has on *Kitl1* and *Kitl2* mRNA expression was in fact prevented. Therefore, it would appear that estrogen via TGF-β1 may be powerful enough to overcome the stimulatory effect of circulating FSH on *Kitl1* and *Kitl2* mRNA expression. Thus, in the late antral stage of follicle development, it is possible that the levels of KITL decrease as a result of the action of estrogen, as mediated via TGF-β1 making the proposed models more likely to be reflective of the situation *in vivo*. Overall, the implications of this down-regulatory effect of TGF-β1 on *Kitl1* and *Kitl2* mRNA expression despite the presence of FSH, is dependent on its local production and the timing of this production. For example, a dramatic reduction of TGF-β secretion in granulosa cells would allow the gonadotropin to amplify its stimulatory action on KITL expression.

Determining the combinatorial effect of TGF-β1 and FSH was additionally of special interest because of the vast amount of data indicating a main role for TGF-β1 in augmenting FSH action in rat granulosa cells. This is in contrast to previous studies from our lab indicating an ability of TGF-β to abrogate the dbcAMP-induced increase in overall *Kitl* mRNA expression in rat ovarian surface epithelial cells (Ismail et al., 1999). Our current finding indicating an antagonistic role for TGF-β1 on FSH action are also in contrast with what has generally been reported regarding the relationship of TGF-β and FSH. Nonetheless, a similar antagonistic action of TGF-β1 on FSH-induced aromatase activity and lactate production has been shown in porcine Sertoli cells (Morera et al., 1992).

We did not attempt to determine the cellular mechanisms through which TGF-β1 attenuation of FSH-stimulated *Kitl1* and *Kitl2* mRNA expression were mediated. However, in the porcine Sertoli cell model mentioned (Morera et al., 1992) the effects of TGF-β1 were unrelated to a change in Sertoli cell number, nor did they involve a change in specific FSH binding to Sertoli cells; rather, the effect appeared to be due to a decrease in the accumulation of cAMP in the presence of FSH. This effect was mediated, not by direct action of TGF-β1 on cAMP, but instead, by its stimulation of cAMP-phosphodiesterase activity in the cell (Morera et al., 1992). Whether TGF-β1 modulation of the activity of this enzyme may play a physiologically relevant role in attenuating the action of FSH in our primary granulosa cell system, remains to be studied. Since it is likely that the effects of TGF-β1 in target cells are mediated via several parallel pathways, direct cross-talk with the gonadotropin-activated pathways at various stages of folliculogenesis is anticipated. TGF-β1 modification of FSH action may therefore be tied

closely to its action on the downstream pathway of FSH. In fact, although TGF-β1 alone is often without effect on the basal accumulation of extracellular cAMP by cultured rat granulosa cells, in studies where it is shown to augment FSH action, it enhances cAMP activity (Adashi and Resnick, 1986; Knecht et al., 1987; Adashi et al., 1989). Further evidence in support of this idea comes from a mesangial cell model (Wang et al., 1998), whereby TGF-β1 was shown to significantly increase PKA activity and to mediate phosphorylation of CREB, effects that were completely reversed by PKI and H-89, respectively. Interestingly, PKA activation induced by TGF-β occurred in the absence of a detectable rise in cAMP levels (Wang et al., 1998). Furthermore, it has been shown that a TGF-β-induced Smad3/Smad4 complex is capable of directly activating PKA, a process that is cAMP-independent (Zhang et al., 2004) in accordance with what has been reported in the Sertoli cell model (Morera et al., 1992) and the mesangial cell model (Wang et al., 1998). Therefore, in future attempts to determine the cellular mechanisms whereby TGF-β1 attenuates FSH hormonal action, consideration must be given to sites of action concerned with cAMP generation, breakdown or action.

4.10 Effect of Treatments on Alternative Splicing of *Kitl* mRNA

Finally, considering the importance that has been associated with the relative expression of KITL-1 and KITL-2, it is worthy to note that almost all reagents utilized in our experiments induced an effect in a similar direction on the expression of *Kitl1* and *Kitl2* mRNA. That is, a treatment that increased *Kitl1* mRNA expression also did likewise for *Kitl2*, whereas a treatment that decreased *Kitl1* mRNA expression also did likewise for *Kitl2*, although the degree of increase or decrease generally differed slightly.

However, as mentioned, we did observe a differential effect of forskolin on *Kitl1* and *Kitl2* mRNA expression in SIGC. While a lower dose of forskolin increased the expression of *Kitl2* mRNA, no effect was observed on *Kitl1* mRNA. To notice that a particular treatment results in a differential effect on the expression of two isoforms is an indication of its influence on alternative splicing of the pre-mRNA. The literature reports only two previous observations of regulation of alternative splicing of *Kitl* mRNA. An acidic environment in cultured mouse Sertoli cells results in a switch in *Kitl* mRNA splicing that favours the expression of *Kitl2* over *Kitl1* (Mauduit et al., 1999). Additionally, as previously discussed, it has also been noted that a low concentration of FSH stimulates an increase in the expression of *Kitl2* in mouse preantral OGC while having no effect on *Kitl1* mRNA expression (Thomas et al., 2005). Therefore, similar to these two cases, a specific concentration of forskolin induced exon 6 skipping to increase the expression of *Kitl2* and produce a lower *Kitl1*/*Kitl2* ratio in granulosa cells.

CHAPTER 5 - CONCLUDING REMARKS

Because of the pertinent role that KITL serves in the milieu of the ovarian follicular environment, an understanding of its regulation is critical for understanding its function. To briefly summarize the main points of this research, the expression of *Kitl1* and *Kitl2* mRNA transcripts was detected in SIGC, like in primary granulosa cells although the expression of the two isoforms varied depending on the time after re-plating of cells, with initial levels being dramatically elevated. Once expression levels stabilized, 24 and 48 hours after re-plating, we observed that the expression of the two isoforms was not significantly different from that detected in primary granulosa cells. For the reasons outlined, this immortalized granulosa cell line can therefore serve as a practical, alternative model for the study of *Kitl* mRNA expression allowing for the possibility of less reliance on primary cultures.

We were able to demonstrate that FSH, the key hormone involved in regulation of ovarian maturation, does induce the expression of *Kitl1* and *Kitl2* mRNA expression in primary granulosa cells. Our findings are the first to demonstrate that direct FSH stimulation increases *Kitl1* and *Kitl2* mRNA expression in cultured granulosa cells. However, this effect of FSH was not mimicked by dbcAMP and forskolin (traditional activators of the pathway downstream FSH) in SIGC. This may indicate that the condition of the immortalized granulosa cell line does not fully reflect that of primary cultures of granulosa cells.

TGF-β1 generally plays an important local role in modulating ovarian cell functions. Perhaps some of its functions are implemented through its action on KITL

since we found it to function as a negative regulator of *Kitl1* and *Kitl2* mRNA expression in both SIGC and primary granulosa cells; this elucidates a role for the local factor that has not previously been demonstrated in granulosa cells. Our findings also offered some insight into the signalling molecules required for mediation of this effect suggesting the involvement of the traditional TGF-β/activin signalling pathway since TGF-β1 treatment of SIGC resulted in the phosphorylation of Smad2 and Smad3 but not Smad1/5. Additionally, we have introduced the possibility of using an immortalized granulosa cell line for the study of *Kitl* mRNA regulation by TGF-β1, since the cells were responsive and contained the appropriate signalling molecules.

Exposure of primary granulosa cells to estrogen both *in vivo*, via DES-priming of immature rats and *in vitro*, by culturing in the presence of 17β-estradiol, inhibited *Kitl1* and *Kitl2* mRNA expression, an effect that we found to be mediated via TGF-β1. Since estrogen is normally produced by the antral follicle, at a point in folliculogenesis when KITL expression almost completely ceases, this lead us to propose that estrogen via TGF-β1 may be in part responsible for this decrease *in vivo*.

Finally, when primary granulosa cells were treated with a combination of FSH and TGF-β1, TGF-β1 prevented the stimulatory effect FSH has on *Kitl1* and *Kitl2* mRNA expression. This suggests that *in vivo*, TGF-β1 may act as a powerful modulator of FSH action and may explain how in the late antral stage, there is a decline in KITL expression despite the presence of circulating FSH.

Through this research, we have demonstrated a complex interaction between gonadotropins, local factors and steroid hormones, three levels of control, that are involved in the regulation of *Kitl* mRNA expression in the ovary. Insight has therefore

been provided into the factors that may be serving as regulators of KITL expression *in vivo*. Considering the impact that these ovarian factors have on the expression of *Kitl* mRNA, further intricacy is introduced into the process of female fertility. To search for mutations in *Kitl* genes as an explanation for a possible cause of infertility is clearly the simple approach. In light of our findings and in the complex milieu of the ovarian environment, the problem is likely to be more complicated.

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