NOTE TO USERS

DISSERTATION

EXPRESSION OF FOXL2 IN THE MURINE OVARY; A FORKHEAD PROTEIN IMPLICATED IN PREMATURE OVARIAN FAILURE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JODI L. HALLER ENTITLED EXPRESSION OF FOXL2 IN THE MURINE OVARY; A FORKHEAD PROTEIN IMPLICATED IN PREMATURE OVARIAN FAILURE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Committee on Graduate Work

Advisor

Department Head

ABSTRACT OF DISSERTATION

EXPRESSION OF FOXL2 IN THE MURINE OVARY; A FORKHEAD PROTEIN IMPLICATED IN PREMATURE OVARIAN FAILURE

The exact mechanism propelling primordial ovarian follicles to emerge from the resting pool and resume development remains elusive. Furthermore, a vague understanding of the molecular interaction between germ and somatic cells during primordial follicle formation currently exists. As such, some novel insights into ovarian follicular development have been realized through the study of relevant knockout mouse models. More precisely, targeted disruption of the winged-helix/ forkhead domain transcription factor, FoxL2, has indicated it to be essential in differentiation and development of granulosa cells and all major somatic cell lineages surrounding growing oocytes from the onset of primordial follicle formation. In this regard, I have identified both murine FoxL2 mRNA and protein to be highly granulosa cell specific in the adult animal and that the onset of this expression occurs at the critical transition from the primordial into the primary stage of follicular development. Next, the exact of mechanism of FoxL2 regulation is yet poorly defined. However, FoxL2 has been shown to directly interact with Smad3, signaling mediator of the TGF-β family of growth and differentiation factors. With this in mind, I confirmed the presence of BMPRII in our FoxL2 expressing granulosa cell model, identified as a functional receptor for

TGF-β signaling specifically mediated by Smads 2 and 3. At issue, then, was the identity of a suitable ligand capable of regulating FoxL2 expression. Toward this end, my studies have characterized FoxL2 as perhaps exhibiting an internuclear rather than intercellular redistribution event in contrast to the nucleocytoplasmic shuttling typifying related forkhead family members. I specifically noted the presumed internuclear reorganization of Foxl2 in response to forskolin (PKA mediated) induced stimulation. However, the functional implication of the resultant reorganization of FoxL2 in the nucleus remains to be seen. Upon doing so, I might better be able to forge a connection between the observed internuclear activity of FoxL2 and the differential expression of specific genetic markers that I have conveyed as the outcome of FoxL2 cDNA microarray analysis.

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I also wish to take this opportunity to briefly reminisce:

The 17th day of September 1995 marked another monumental occasion in my life, as I became the first female baseball player to ever play men's baseball in Japan. I consider myself very fortunate to have been invited to play for Meiji University's Men's baseball team that year and consider it to have been a challenge of epic proportion, not unlike that of pursuing my doctorate degree. Furthermore, I can recall being truly shaken that day, as I had never played before a crowd so large. It was an event receiving both national and international coverage. As my catcher approached me on the mound to calm my nerves, I couldn't imagine what he would convey. May I emphasize that he, like his teammates, could not speak English. Unknown to me, my pitching coach had prepared him with a simple phrase. It was an acronym of sorts. The product of a lengthy attempt at conversation that had transpired between the coach and I, in what I like to refer to as "Japanglish." Simply told, it was the secret of life as we saw it. Thus, despite the roar of the crowd and intense pounding of my heart, muffled by a catcher's mask came the words: "Hara-san, (that's me) three C's. My three C's were and will always remain CHRIST, COMMITMENT AND CONFIDENCE. They claim my every victory.

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CHAPTER ONE

INTRODUCTION

FoxL2 is a member of the winged helix / forkhead family of transcription factors. Characterized by a unique highly conserved 100 amino acid DNA binding domain (figure 1), these proteins are capable of great versatility in DNA recognition and diverse biological function. Important roles for Forkhead proteins have been defined in embryogenesis, development and aging within all eukaryotes. Among more than 20 human forkhead genes now characterized, mutant forms of several have already been identified as responsible for tumorigenesis, and / or hereditary disorders (1). In regard to FoxL2, of particular interest is the role of this protein in folliculogenesis. In humans, the initial stage of folliculogenesis is coincident with prophase of the oocyte and at which point the follicle remains in a state of arrest for an average of 13 to 50 years. Those follicles which are unable to leave the resting pool and resume development have no potential for ovulation and will ultimately undergo atresia. The exact mechanism propelling primordial follicles to leave the resting pool remains elusive. However data indicates that onset of FoxL2 expression to be at the critical transition from primordial into primary stage follicles.

Initial interest in FoxL2 was prompted by the discovery of specific mutations in FoxL2 that result in two phenotypes in women. First a characteristic set of cranio-facial abnormalities termed blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) and, second, premature ovarian failure (POF). This

FOXL2 Amino Acid Sequence Comparison

```
human    1 MMASYPEPED AAGALLAPET GRTVKEPEGP PPSPGKGGGG GGGTAPEKPD  50
mouse    1 MMASYPEPED TAGTLLAPES GRAVKEAEAS PPSPGKGGG    TTPEKPD  46
                     *  *     *   **  * **          * *** *

human   51 PAQKPPYSYV ALIAMAIRES AEKRLTLSGI YQYIIAKFPF YEKNKKGWQN 100
mouse   47 PAQKPPYSYV ALIAMAIRES AEKRLTLSGI YQYIIAKFPF YEKNKKGWQN  96

human  101 SIRHNLSLNE CFIKVPREGG GERKGNYWTL DPACEDMFEK GNYRRRRRMK 150
mouse   97 SIRHNLSLNE CFIKVPREGG GERKGNYWTL DPACEDMFEK GNYRRRRRMK 146

human  151 RPFRPPPAHF QPGKGLFGAG GAAGGCGVAG AGADGYGYLA PPKYLQSGFL 200
mouse  147 RPFRPPPAHF QPGKGLFGSG GAAGGCGVPG AGADGYGYLA PPKYLQSGFL 196
                               *          *

human  201 NNSWPLPQPP SPMPYASCQM AAAAAAAAAA AAAAGPGSPG AAAVVKGLAG 250
mouse  197 NNSWPLPQPP SPMPYASCQM AAAAAAAAAA AAAAGPGSPG AAAVVKGLAG 246

human  251 PAASYGPYTR VQSMALPPGV VNSYNGLGGP PAAPPPPP    HPHPHPHAH 297
mouse  247 PAASYGPYSR VQSMALPPGV VNSYNGLGGP PAAPPPPPPP PHPHPHPHAH 296
                   *                                ** *

human  298 HLHAAAAPPP APPHHGAAAP PPGQLSPASP ATAAPPAPAP TSAPGLQFAC 347
mouse  297 HLHAAAAPPP APPHHGAAAP PPGQLSPASP ATVAPPAPAP TSAPGLQFAC 346
                                           *

human  348 ARQPELAMMH CSYWDHDSKT GALHSRLDL                      376
mouse  347 ARQPELAMMH CSYWDHDSKT GALHSRLDL                      375
```

Figure 1. **Alignment of the amino acid sequences of human and mouse FoxL2.** The shaded region represents the winged-helix DNA binding domain. Asterices indicate sequence divergence. Amino acids in red depict predicted phosphorylation sites. Blue box indicates putative NLS.

POF is revealed as either primary (never ovulates) or secondary (premature menopause) amenorrhea, despite existing elevated levels of gonadotropins. Thus, FoxL2 is the first gene that has been identified in mediating POF. Haploinsufficiency and loss-of-function mutation in one FoxL2 allele may be associated with BPES type I POF. More recent data suggest that FoxL2 is required for forming a full complement of eggs in the ovary before birth and so in the event of FoxL2 mutation, there exist fewer eggs than necessary to sustain a normal reproductive life span (1-3).

It is also interesting to note that forkhead transcription factors are recognized as important mediators of cellular differentiation and often are involved in mediating nuclear responses to cellular activation by Transforming Growth Factor Beta (TGF-β) family members (4). This would necessitate interaction with Smad proteins that function as signal transducers of the TGF-β family members ranging from worms to humans (5-8).

Over the course of the research that follows, I have investigated the expression of FoxL2 within the murine ovary. I have identified both FoxL2 mRNA and protein to be highly granulosa cell specific and that the onset of this expression occurs at the critical transition from primordial into primary stage follicles. I have also begun to evaluate potential ligand-induced regulation of FoxL2 cellular localization within murine granulosa cells (KK1 cell line). Specifically, I have confirmed the presence of Bone Morphogenetic Protein receptor type II, (BMPRII), capable of mediating TGF-β family type signaling

within KK1 cells and have investigated the potential impact of any such signaling events upon *in vitro* expression and/ or translocation of FoxL2.

CHAPTER TWO

REVIEW OF LITERATURE

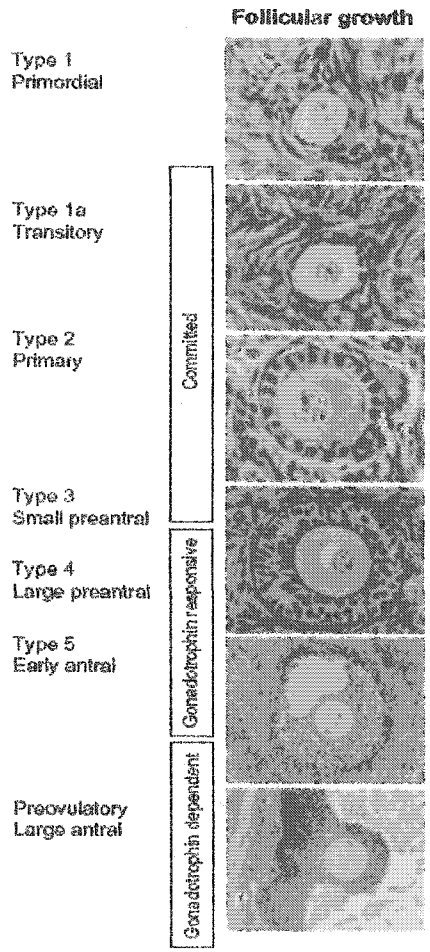
I. Ovarian Folliculogenesis

A. Overview

Follicles are the basic functional units of mammalian ovaries. Individual mammalian follicles are composed of a central oocyte bordered by granulosa cells and an outermost layer of thecal cells. By way of the process termed "folliculogenesis" each follicle proceeds through the developmental stages of primordial, primary, and secondary (preantral) before acquiring an antral cavity (9).

Although, follicular classification schemes vary slightly across species, generally a first category consists of small, non-growing follicles (primordial and primary). Next, medium-sized, non-growing follicles can be appropriately termed either small or large pre-antral or secondary and to consist of up to 6 layers of granulosa cells. It is at this time that the presence of a growing oocyte as well as distinguishable thecal layers and a visible zona pellucida (protective muccopolysaccharide halo secreted by granulosa cells) serve as criteria for follicle classification. Lastly, antrum formation is used to designate follicular type 6-8 (mice), that are generally perceived as large follicles (of maximum diameter) bearing a fully grown oocyte. The term "Graafian" is often applied as a descriptor for the most fully developed pre-ovulatory stage in mice (10) (figure 2b). Regardless of species, a critical initial step in folliculogenesis is when a type I follicle (an oocyte surrounded by a single layer of flattened granulosa cells (pre-

A



B

Classification Scheme of Murine Follicular Development

Classification	Layers Granulosa Cells	Number Granulosa Cells	Oocyte Diameter
Type 1 (primordial)	1	<10 (flattened)	<20 mcirons
Type 2	1-<2	<10	<20 microns
Type 3a	1-2	<20 (cuboidal)	<20 microns
Type 3b	1-2	21-60 (cuboidal/ zona pellucida present)	<20 microns
Type 4	2-3	60-100	>20 microns
Type 5a	4-6	101-200	>20 microns
Type 5b	4-6	201-400	>20 microns
Type 6	4-6	401-600 (antrum developing)	70 microns
Type 7	>6	>600 (antrum present)	70 microns
Type 8	>6	>600 (antrum present)	70 microns

Figure 2. Stages of Follicular Development A). A critical first step involves the transition of the type I follicle (oocyte surrounded by a single layer of flattened gransulosa cells) into the type II "committed" follicle (single layer of cuboidal granulosa cells), all further development occurs under exogenous gonadotropin dependence. **Classification Scheme of Murine Follicular Development B)** Follicles in mouse ovary are classified according to the number of granulosa cells in the largest cross section (Pedersen and Peters 1968). A growing oocyte and visible zona pellucida are also criteria for classification. Antrum formation designates type 6-8 or large pre-ovulatory follicle,of maximum diameter with a fully- grown oocyte.

granulosa cells) begins to grow into a type II follicle (an oocyte surrounded by a single layer of cuboidal granulosa cells). This transition is one that delineates a "committed" follicle that will continue to grow until either it is ovulated or undergoes atresia (figure 2a). Granulosa cell proliferation, zona pellucida development, and progressive increase in oocyte diameter are landmarks of the committed growth phase, which proceeds independent of the influence of gonadotropins. Only upon achieving the small antral stage with more than 5 layers of granulosa cells present and an antral cavity does a follicle become acutely dependent upon gonadotropins and remain so throughout final maturation potentially culminating with ovulation (11-13). At the antral stage, most follicles undergo atretic degeneration, however, under cyclic gonadotropin stimulation "selected" follicles are enabled to proceed through final maturation into Graafian follicles that are ovulated to release a mature oocyte. The remaining granulosa and thecal cells develop into the progesterone secreting corpus luteum (14).

B. Hormonal Regulation of Folliculogenesis

Although follicle stimulating hormone (FSH) and LH do not act directly upon primordial follicles because functional gonadotropin receptors are not yet present, preantral follicles do respond to gonadotropins with cell division and differentiation even though otherwise capable of advancing to the antral stage in absence of gonadotropins. For instance, pre-granulosa cells and primordial follicles do respond to activators of cyclic AMP pathways (e.g., forskolin and

cAMP analogs) with increased expression of aromatase and FSH receptors (15-20). However, regarding any such growth response that occurs in follicles prior to the age of puberty, ovulation will not occur and the fate of the developing follicles will be atresia. Following the onset of puberty, cyclic activity of gonadotropins (FSH, LH) allows a limited number of antral follicles to continue to grow to a preovulatory stage and so escape apoptosis. FSH specifically directs this process perhaps owing to increased sensitivity of a given follicle or its elevated FSH receptor expression. Regardless, the dominant follicle itself releases estrogen and inhibin into the hormonal milieu, serving as negative feedback upon any further production of FSH from the anterior pituitary. In this way, other antral follicles among the growing cohort will no longer survive (21;22).

C. Growth Factors

In the absence of gonadotropin influence, the onset of folliculogenesis and subsequent development of preantral follicles is mediated by other intragonadal signals and growth factors (12;23;24). *In vitro* studies have identified granulosa-oocyte communication as essential for normal oocyte growth and development in early follicles. The integrity of properly maintained gap junctions between the two entities appears necessary for attainment of full size competent oocytes. Oocyte secreted factors regulate granulosa cell division, luteinizing hormone (LH) receptor formation, and steroidogenesis of granulosa cells (25-30). First, the only growth factors currently known to actively participate in folliculogenesis

during the critical transition from primordial into type 2 follicles is the tyrosine kinase receptor, c-kit, and its ligand, stem cell factor. Additionally, stem cell factor appears necessary for primordial germ cell culture survival in mice and is expressed in granulosa cells at all stages of follicular development from primordial through antral stages in sheep (31-34). Administration of an anti-c-kit antibody disrupts onset of follicular growth in a stage specific manner, similar to that observed in mutation of the transforming growth factor beta family member, growth and differentiation factor 9 (GDF-9) in mice. Moreover, GDF-9 may be an oocyte-derived signal for granulosa cell proliferation, which is activated via stem cell factor and c-kit interaction to ultimately promote follicular growth and survival (35). Furthermore, as just indicated, members of the transforming growth factor beta (TGFβ) superfamily of growth and differentiation have been implicated as serving critical roles in the initiation and organization of folliculogenesis and are considered more thoroughly below.

D. The Transforming Growth Factor Beta Superfamily

1.Overview

This family consists of more than 35 members including: TGF-β's, growth differentiation factors, bone morphogenetic proteins, activins, inhibins, and glial cell line-derived neurotrophic factor (36). This family exhibits diverse functions during both embryonic development and adult tissue homeostasis (5). As such, TGF-β ligands, their regulatory proteins and downstream signaling elements have been identified in association with embryogenesis, reproduction, growth,

and maintenance of appropriate body composition and tumor suppression. Of particular relevance, is the role of TGF-β family members in embryonic regression of the Mullerian duct, proliferation of primordial germ cells, as well as gonadal growth and development postnatally (5).

Posttranslational processing is critical in acquisition of biological activity by TGF-β family members. That is, all members of this family are translated as large preproproteins composed of a signal peptide, prodomain, and the mature domain. Upon cleavage of the signal peptide, dimerization of the proprotein occurs. Lastly, proteolytic enzymes target the RXXR site of the dimerized protein generating the biologically active dimeric mature protein (5).

2. Intracellular Mediation of TGF-β signaling

Upon activation, the TGF-β intracellular signaling cascade commences with the binding of ligand to transmembrane serine/threonine kinase type 1 or type 2 receptors. The type-1 receptor transphosphorylates the type-2 receptor (or vice versa) at an intracellular juxtamembrane GS domain rich in glycine and serine residues. The phosphorylated receptor next transphosphorylates a set of intracellular substrate signaling proteins called Smads that are capable of binding DNA and recruiting transcriptional co-activators and co-repressors (37-40). These receptor-regulated Smads (R-Smads) include Smad 1,2, 3, 5, and 8 all of which interact with a common Smad (Co-Smad), Smad4, such that the resultant R-Smad/Co-Smad complex is capable of nuclear translocation and (39;40) interaction with specific transcription factors regulating downstream target gene

expression. When inactivated, the R-Smads are predominantly cytoplasmic and Smad 4 is distributed throughout the cytoplasm and nucleus. Following activation (TGF-β), all Smads are predominantly nuclear (41). Most recently, Nicolas et al provided strong evidence for the continuous shuttling of Smad 2 and Smad 4 between cytoplasm and nucleus in both uninduced and TGF-β induced cells. Their model suggests that the unphosphylated R-Smad (Smad 2) may be retained in the cytoplasm via retention factors, whereas Smad 4 remains there because of its export (facilitated via the nuclear exporter CRM 1) from the nucleus is dominant over its import. Under exporter CRM 1 activation R-Smads are released from cytoplasmic tethering, form complexes with Smad 4 and accumulate in the nucleus. The Smads are exported from the nucleus following dephosphorylation and release from Smad complexes (41).

For the duration of signaling, this cycle of phosphorylation and activation in the cytoplasm and dephosphorylation and deactivation in the nucleus continues. In the nucleus, Smads participate in gene transcription by recruiting co-activators such as p300 and CBP (histone acetyltransferases HATs) (5;42) as well as co-repressors Sloan-Kettering Institute proto-oncogne (SKI) and Ski-related novel gene N (SnoN) (the histone deacetylases HDACs) in order to either activate or repress gene expression, respectively (5;43;44).

Also noteworthy is the divergence that exists in the Smad signaling pathway between the BMP's and activins/TGF-β's (37;45;46) (figure 3). Intracellular signaling for activin's and TGF-β's is mediated by Smad2 and 3, (47-52). Recent investigation of Smad activation by GDF-9 in granulosa cell culture

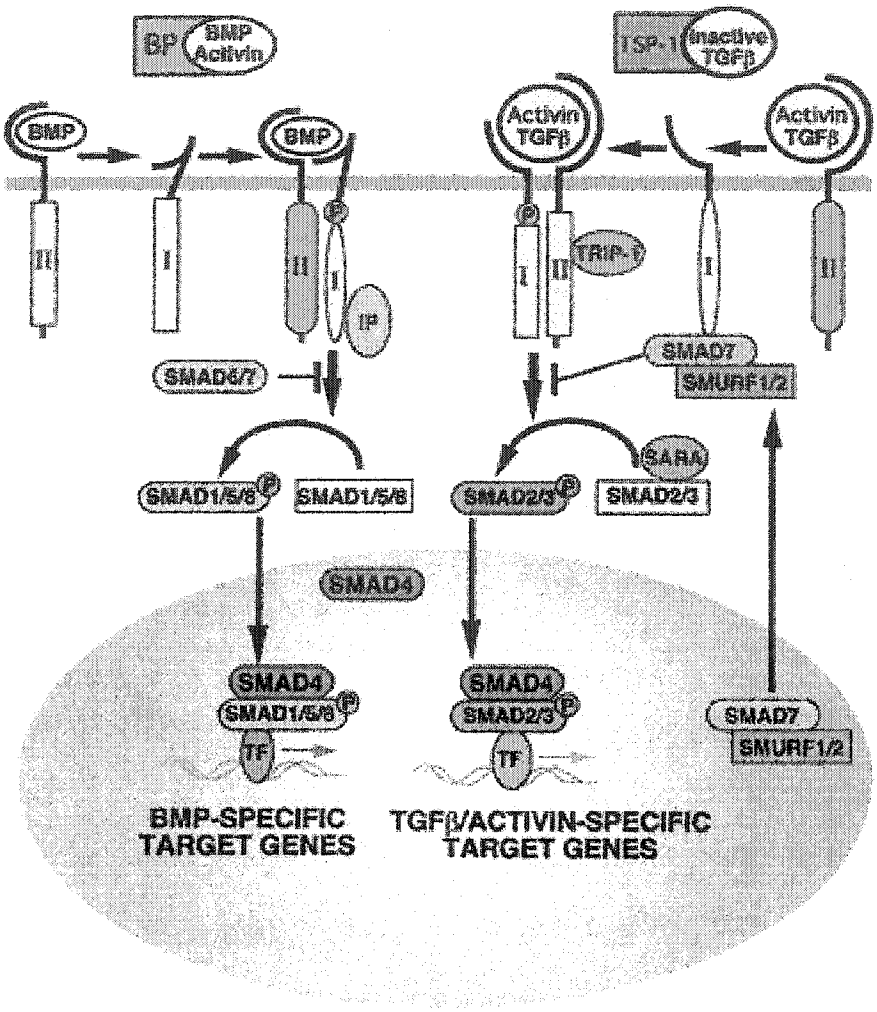


Figure 3. Regulation of TGF-β Superfamily Signal Transduction: BMP's bind either type I or type II serine/threonine kinase receptors. Type II receptor phosphorylates and activates the type I receptor that phosphorylates SMAD proteins 1,5, or 8. The phosphorylated Smad, moves into the nucleus, interacts with"the common Smad," Smad4, and regulates BMP-specific genes. Activins and TGF-β's must first the bind type II serine/threonine kinase receptor that phosphorylates the Type I receptor. This enables phosphorylation of Smad 2 or 3 and movement into nucleus to permit interaction with Smad 4 and activation of activin/TGF- β type target genes. Smad ubiquination regulatory factor-1 (**SMURF**) is an intracellular signaling inhibitor responsible for ubiquitination and degradation of smads 1 and 5 {Zhu, Kavsak, et al. 1999 5583 /id} Smad anchor for receptor activation (**SARA**) functions in the the retention of Smads in the cytoplasm when not under TGF-β stimulation. Smads 6 and 7 are inhibitors, competing with R-smads for binding to activated type I receptors {Hata, Lagna, et al. 1998 5582 /id}.

has demonstrated that GDF-9 treatment increases Smad 2 phosphorylation, leading to dose dependent increase in phospho-Smad 2 levels (53). Likewise failure of GDF-9 to activate the phosphorylation of Smad1 in human granulosa-luteal cells also suggests that GDF-9 mediates its effect through the pathway commonly activated by TGF-β and activin, but not that activated by many BMP's (53). Conversely, that of most BMP ligands as well as growth and differentiation factor 5 (GDF5) and mullerian inhibitory substance (MIS) is enabled by Smads 1,5, and/ or 8 (37;54-59).

3. Inhibitory Regulation of TGF-β Signaling

In contrast to Smads 1,2,3,5 and 8 that activate gene expression, Smads 6 and 7 are inhibitors (figure 3). That is, Smads 6 and 7 compete with R-Smads for binding to activated type I receptors (60). This selective inhibition of TGF-β and BMP signaling has been indicated as an important source of negative feedback in morphogenesis and tissue homeostasis particularly in cardiac and respiratory development (5). Next, TGF-β signaling may also be prevented via Smad ubiquitination in the nucleus and subsequent proteasome-mediated degradation. Specifically, Smad ubiquitination regulatory factor-1 (SMURF) is an intracellular signaling inhibitor responsible for ubiquitination and degradation of Smads 1 and 5 (61) (figure 3). Additionally, in the region joining the Smad MH1 (amino-terminal) and MH2 (carboxy-terminal) domains SMURF-1 binding sites have been identified (62). SMURF has been further characterized as

demonstrating specificity for BMP type signaling because it has not been proven capable of mediating degradation of either Smad 2 or 4 (61).

Another opportunity for restraint from Smad signaling is evident in a non-TGF-β stimulated state as Smads remain anchored to the protein SARA (Smad anchor for receptor activation) in the cytoplasm via the Smad MH2 domain (figure 3). It is the receptor-mediated phosphorylation event that then releases the Smad from SARA, readily exposing the Smad nuclear import signal to Smad 4 in preparation for nuclear translocation (54;63).

Lastly, nuclear translocation of activated Smads may be prevented by the negative-feedback regulator BAMBI (BMP and Activin membrane-bound inhibitor) a protein that may actually be functioning as a dominant negative receptor (5;64;65). The BAMBI protein is a truncated kinase-deficient type I receptor that competes with type I receptors for ligand binding and inhibits the signaling of TGF-βs, BMPs and activn (5;64;65) . From the perspective of extracellular regulation of TGF-β pathways, it is at the level of production and secretion of the involved cytokine that the major opportunity for control exists. For instance, many different proteins have been shown to bind to TGF-β family factors preventing their interaction with signaling receptors: LAP (latency associated protein), Follistatin, Noggin, and Chordin (5). However, the opposite is also true of several membrane –anchored factors, including Betaglycan, Endoglin, and Crypto that have been shown to facilitate ligand binding to TGF-β family receptors (5).

4. Cross-talk With Alternative Signaling Cascades

Although Smads are the only TGF-β receptor substrates and signaling transducers currently known to exist, increasing evidence suggests that other signaling pathways may participate in mediating TGF-β and BMP action (66-70). For instance, a particularly relevant link between the antagonistic TGF-β/Smad and PI3K/Akt pathways has recently been suggested (71). FoxO proteins have been identified as key partners of Smad3 and Smad4 in the TGF-β dependent generation of a p21Cip1 (growth inhibitory gene) activation complex. Conversely, a hyperactive PI3K/Akt pathway and high levels of FoxG1 (telencephalic development factor) in glioblastoma cells cooperate to prevent p21Cip1 induction and cytostasis by the TGF-β/Smad-FoxO pathway (71).

Also noteworthy, is interaction with mitogen-activated protein kinase (MAPK) signaling molecules, extra-cellular related kinase (ERK) 1/2, p38, and stress-activated protein kinase / Jun N-terminal kinase have been implicated in both positively transducing BMP signals (37) or inhibiting Smad signaling (37). Furthermore, the region that links the MH1 and MH2 domains of the Smad proteins possesses mitogen-activated protein kinase phosphorylation sites (5;72;73). TGF-β has also been reported to activate JNK through MKK4 (MAP kinase kinase 4) or p38 through MKK3 dependent upon the specific cell line surveyed (74-76). Additionally, JNK has been identified as capable of Smad phosphorylation, although the phosphorylation sites remain to be identified (5). Thus, numerous accounts (5;75-78), have indicated that TGF-β-activated MAPKs may converge on Smads along with the direct effect of TGF-β receptors on these

proteins. This suggests the potential for classical activators of MAPK pathways such as cytokines (tumor necrosis factor, TNF-α), cellular stress (ionizing radiation) and hormones to be capable of modification of Smad transcriptional complexes. Conversely, TGF-β induced Smad interactions might modify the cellular responses to other cytokines and stress signals (5).

Also noteworthy is the ability of the ERK activated Ras pathway to modify TGF-β signaling at different levels. For instance, a hyperactive Ras pathway, (in cells expressing Ras oncogene) is able to counteract the normal anti-mitogenic activity of TGF-β by down regulating its receptors and preventing Smad accumulation in the nucleus(37).

Most recently, Conery et al. demonstrated that cross talk between Akt/PKB and Smad 3 may regulate sensitivity to TGF-β induced apoptosis (79). That is, Akt directly interacts with unphosphorylated Smad 3 in the cytoplasm, preventing its phosphorylation and nuclear translocation, inhibiting Smad 3 mediated transcription and apoptosis (79). Additionally, a correlation between the intercellular ratio of Smad 3 to Akt and the corresponding sensitivity of cells to TGF-β has been predicted (79). Specifically, the scenario may be that, in cells with a high Smad 3:Akt ratio a threshold level of Smad 3 enters the nucleus to initiate apoptosis, however when the ratio favors Akt instead, a sufficient level of Smad 3 is sequestered from the nucleus and apoptosis as well as other Smad 3 mediated responses may be prevented (79).

5. TGF-β Involvement in Folliculogenesis

Members of the TGF-β superfamily of growth and differentiation factors, namely bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9) have been implicated in initiating and organizing folliculogenesis. Expression of both of these factors is restricted to the oocytes of developing follicles (in mice, from primary to ovulatory follicles but not primordial) and based on genetic "knock out" studies, appear to act synergistically to direct normal follicular growth and development and, in the absence of GDF9 and BMP15, folliculogenesis aborts at the primary follicle stage. Similar to mice, expression of these factors is also exclusive to the ovine oocyte and mutations of the BMP15 gene have been noted to produce the Inverdale fecundity phenotype in sheep (80). Although intra-ovarian mechanisms of action have not been determined for GDF9, granulosa cells do bear receptors for this growth factor (81). The presence of a potential signal sequence for secretion suggests that GDF9 is secreted and has paracrine and/or autocrine actions (81).

Yet another report of a functional BMP system operating in the mammalian ovary was made by Shimasaki et al (82). Theca cell expression of BMP-4 and BMP-7 mRNAs as well as granulosa cell specific localization of BMPR-II, ALK-3 and ALK-6 (their corresponding receptors) was identified (82). Regarding the functionality of BMP-4 and BMP-7 in the ovary, studies performed in primary cultures of rat granulosa cells using recombinant forms of these growth factors demonstrated modulation of FSH signaling to increase estradiol production and inhibit of progesterone biosynthesis (82). This suggests that

through inhibition of progesterone synthesis, essential for ovulation, BMP-7 may be causally connected to the mechanisms normally inhibiting ovulation (83). Also noteworthy is the potential for involvement of BMP-7 in the poorly understood period of recruitment of primordial follicles into the growing follicle pool (83). That is, injection of BMP-7 into the rat ovary has been shown to decrease the number of primordial follicles but increase the number of primary, secondary, and antral stage follicles (83). Thus, this data may indicate that BMP-7 is also necessary for normal folliculogenesis (83).

II. Forkhead Proteins and Folliculogenesis

A. FoxL2

1. Overview

The winged helix / forkhead family of transcription factors is characterized by a unique, highly conserved 100 amino acid DNA binding domain, these proteins are capable of great versatility in DNA recognition and diverse biological function. Forkhead proteins have been noted as serving important roles in embryogenesis, development, and aging within all eukaryotes. For instance, establishment of the body axis and development of tissues from all three germ layers in animals reflects regulation by this family (1). FoxL2 is a member of the winged helix / forkhead family of transcription factors. FoxL2 expression has been delineated as nearly exclusive to the developing eyelid and ovarian follicle. However, recently several different reports have noted FoxL2 expression very early in development, nearly coinciding with the onset of sex

determination (84-86). Specifically, Loffler et al. provided evidence of FoxL2 expression in the developing ovary at 12.5 days post coitum (dpc) (85). Furthermore, FoxL2 has been characterized as a highly conserved and earliest known marker of ovarian differentiation in mammals (84). Lastly, immunohistochemical evidence currently suggests that FoxL2 is specifically associated with both somatic and germ cell populations (85), where it may participate in somatic cell differentiation and further follicle development and/ or maintenance (84).

2. Related Pathogenesis

Of particular relevance, specific mutations in FoxL2 have been reported as resulting in 2 phenotypes in women, first, a characteristic set of cranio-facial abnormalities termed blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) and, second, premature ovarian failure (POF). This POF is revealed as either primary (never ovulates) or secondary (premature menopause) amenorrhea, despite existing elevated levels of gonadotropins. Although the underlying mechanism(s) of POF are not known, it appears that FoxL2 represents a new member of the expanding class of genes implicated in the regulation of follicular growth and development (1-3). Furthermore, Schmidt et al have both indicated the murine FoxL2 gene as critical for granulosa cell differentiation and maintenance as well as proposed a potential mechanism for premature ovarian failure in BPES. That is, FoxL2 lacZ homozygous mutant ovaries do not manifest squamous to cuboidal transition of granulosa cells

resulting in subsequent absence of secondary follicles and oocyte atresia (87). Most recently however, FoxL2 null mice may indicate a similar method of POF (88). That is, FoxL2 appears to be a selective determinant of perinatal ovarian histogenesis as all major somatic cell lineages fail to develop around growing oocytes from the onset of primordial follicle development (88).

C. Related Forkhead Proteins

1. Overview

In consideration of other closely related forkhead proteins it has been documented that the FoxO family: FKHR (FoxO1), FKHRL1 (FoxO3), and AFX (FoxO4) are expressed in the rodent ovary at specific stages of follicular development and luteinization (89). FoxO1 is specifically localized to granulosa cells of growing follicles, while FoxO3 and FoxO4 show higher expression in theca cells and corpora lutea (90). Furthermore, direct evidence links the FoxO family of proteins to the normal process of ovarian folliculogenesis. That is, suppression of ovarian follicle activation in mice has been attributed to the transcription factor FoxO3 (91). A FoxO3 knock-out mouse model has been shown to exhibit marked elevation in numbers of early- growing follicles by post-natal day 14. However, subsequent oocyte death, depletion of functional follicles and secondary infertility was observed at 8.5 weeks of age. This evidence then strengthens a case for the possible role of accelerated follicular initiation in the onset of premature ovarian failure (POF) (91).

Involvement of the FoxO family of forkhead transcription factors has also been implicated in the activation of target genes that serve roles in cell survival, cell cycle progression, DNA repair and insulin sensitivity (92). For expample, Fas ligand (FasL), an inducer of apoptosis, p27 Kip, an inhibitor of cell cycle progression, and IGF BP-1, a presumed inhibitor of IGF-1 are examples of genes believed to be regulated by FoxO1, FoxO3, and FoxO4 (89). Specifically, in mammalian cells, activation of FoxO transcription is capable of inducing either cell cycle arrest or apoptosis most likely depending upon the specific physiological conditions and cell type involved (93). Recent studies also demonstrate that FoxO proteins regulate the gene expression of catalase and manganese superoxide dismutase, serving as cellular protection from oxidative stress (94-96). FoxO proteins have also been indicated as capable of mediating transcriptional activity of nuclear hormone receptors as either coactivators or corepressors (97-99).

2. Ligand Induced Intracellular Trafficking of Forkhead Proteins

It has been well established that intraovarian growth factors, such as IGF-1 and estrogen, as well as the pituitary gonadotropins, FSH and LH are all critical regulators of follicular development (89). Specifically, FSH is known to be active in the IGF-1 pathway through stimulation of the phosphatidyl inositol 3 (PI3) kinase leading to phosphorylation of protein kinase B (PKB)/ Akt and the PKB related kinase, serum- and glucocorticoid- induced protein kinase (Sgk) (89).

Subsequently, a number of substrates that are phosphorylated by PKB are inactivated and/or degraded such as GSK-3β, BAD, and caspase 9 (89).

FoxO proteins are regulated by insulin and other cytokines via activation of PKB/ Akt. That is, in a current model, insulin or the closely related growth factor, IGF-1, initiates the phosphorylation of forkhead proteins via PKB/Akt at 3 different Ser/Thr residues in specific PKB phosphorylation motifs (100). As a result, the phosphorylated forkheads interact with 14-3-3 proteins and are rendered inactive presumably by masking their nuclear localization signal and sequestering them in the cytoplasm (101). Thus, negative regulation of FoxO mediated transcription is achieved, attenuating expression of such gene products as those mediating gluconeogenesis or apoptosis. (102). Furthermore, regarding the nature of this nuclear exclusion, it has been reported that the transcription factor FoxO4 is phosphorylated by protein kinase B at Thr-28 and Ser-193 to create the necessary dual 14-3-3 binding motifs (103). Thus in a phosphorylation dependent manner, both the stable association of 14-3-3 proteins with FoxO4 as well as complete inhibition of FoxO4 binding its target DNA (the insulin response element) are achieved (103).

Lastly, although this concept of nuclear exclusion as the principal mechanism of FoxO regulation has been widely accepted (92), it may also prove noteworthy that FoxA1 and FoxA3 of the FoxA subfamily are not regulated by insulin. Unlike FoxA2, they lack the single threonine site that is phosphorylated by Akt upon insulin activation causing cytoplasmic localization of FoxA2. Additionally, FoxO6 compared with its group members lacks a highly conserved

region containing multiple phosphorylation sites. Although the nuclear export of FoxO6 is similarly mediated through the PI3 kinase/ PKB pathway, FoxO6 generally demonstrates an unexpectedly high nuclear localization upon stimulation with growth factors, in contrast to the primarily cytosolic localization of other FoxO family members (100). Additionally it has been noted that under serum-free conditions the localization of FoxO6, FoxO1 and FoxO3 alike is predominantly nuclear (100). This condition has been used to simulate growth factor deprivation during which the PI3 kinase pathway is inactivated and forkhead factors remain unphosphorylated. Thus, activation of genes associated with programmed cell death, such as insulin-like growth factor-binding protein 1 (IGFBP-1) (104;105), Fas ligand (FasL) (106) Bim (107) and p27 (108) are enabled (104).

Also relevant to the discussion of localization of forkhead proteins, is new evidence that suggests an Akt/PKB – independent mechanism of regulating FoxO proteins. More specifically, a mechanism has been proposed whereby, IκB kinase (IKK) physically interacts with, phosphorylates, and inhibits FoxO3a independent of Akt and causes proteolysis of FoxO3a via the Ub-dependent proteosome pathway (93). Furthermore, the data suggests that negative regulation of FoxO factors by IKK is a key mechanism promoting tumor cell growth in the pathogenesis and development of cancer (93).

Lastly, the ability of insulin to regulate forkhead –stimulated transcription may not entirely depend upon altering the subcellular distribution of the transcription factor (92). For example, FoxO1 bearing a mutated nuclear export

signal remains in the nucleus upon insulin stimulation however, insulin remains capable of inhibiting mutant FoxO1-stimulated transcription to the same extent as transcription stimulated by wild-type FoxO1 (92).

Current literature also suggests that more elusive modes of transcriptional regulation may be in operation among the forkhead proteins. First, recent reports of regulation of FoxO transcription factors by silencing information regulator genes or SIRTs of the sirtuin family of nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases (109-111) is perhaps one potential venue of future investigation. Reports already exist of nuclear sirtuins deacetylating transcription factors such as p53, significantly inhibiting transcription and allowing cells to escape p53-dependent death (112-114). Furthermore, sirtuin gene targets have now been proposed to include FoxO3 as well (109). More specifically, FoxO3 is normally acetylated in response to cellular stress by acetyl-transferase activity of nuclear hormone receptor coactivators CBP/ p300 and expression of Sirt1 results in FoxO3 deacetylation (109;112;115). Additionally, Motta et al has reported that under conditions of FoxO3 deacetylation, expression of p27kip, a FoxO target regulating cell cycle progression, decreases (115).

In conclusion, it is the natural scheme of ovarian physiology that less than 1% of oocytes present in the mammalian ovaries at birth will ever ovulate. Alternatively, remaining oocytes and follicles become atretic and exhibit programmed cell death (89). The cyclic activity of gonadotropins (FSH/LH) allows only a limited number of antral follicles to assume a preovulatory stage and

escape apoptosis (21;22). Furthermore, the FoxO genes, expressed in a cell specific manner at defined stages of follicular growth, are likely candidates for mediation of such apoptotic signals. Thus, precise control over the spatio-temporal expression of these forkhead proteins is necessary and at least partially achieved by transcriptional regulation as well as phosphorylation (89). The forkhead transcription factors must remain sensitive to their own set of extra-cellular signals (FSH, insulin, pharmacological activators PKA, estrogen, etc.) in order to swiftly orchestrate a response to growth factor combinations and enable dynamic processes such as embryogenesis and folliculogenesis. Furthermore, the interaction of forkhead proteins with members of the Smad family has been well documented. Likewise it has been reported that many Smad gene responses depend upon the specific cell type and existing conditions (116). As such, cell specific cofactors, like forkhead family members, often dictate the choice of Smad target genes. Ultimately, it is through protein-protein interaction and regulation of nuclear transcription factors that the repertoire of genes differentially regulated by a given ligand, such as a steroid hormone is expanded and additional impact upon processes like the cell cycle and apoptosis is realized (98).

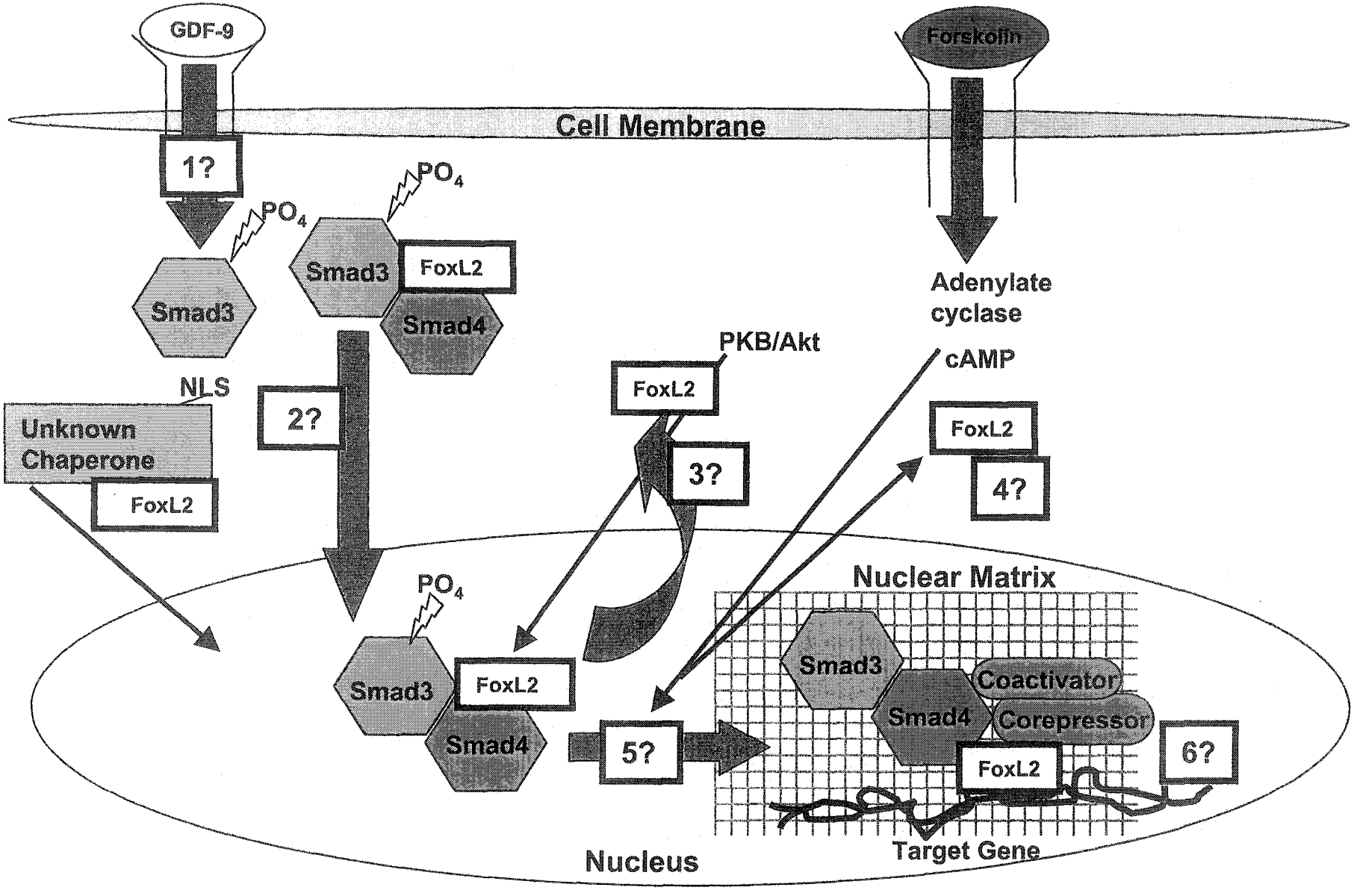


Figure 4. At the time of puberty, a first cohort of primordial follicles rises from the resting pool to resume development. The exact mechanism propelling primordial follicles to leave the resting pool remains elusive (117). Likewise, the precise role of the novel gene, FoxL2, in folliculogenesis in the adult ovary is not yet understood. Toward this end, I have outlined a set of experiments intended to address the mechanism(s) underlying the regulation of FoxL2 (figure 4). First, my data has indicated the onset of FoxL2 expression to be at the critical transition from primordial into primary stage follicles. I have also noted that the onset of temporal expression of TGF-β family member, GDF-9, critical in the normal course of folliculogenesis (23), is coincident with FoxL2. Thus, I was motivated to further investigate the possibility that FoxL2 may participate in mediating TGF-β family signaling in the ovary (1?, 2?). Additionally, nucleocytoplasmic shuttling is important for regulation of the forkhead proteins (100). Thus, I also sought to determine if activation of established signaling cascades, (PKB, cAMP) lead to cellular redistribution of FoxL2 (3?-5?). Finally, the biological role of FoxL2 must be to regulate the expression of specific target genes. Towards this end, I initiated a preliminary study to begin to identify the potential FoxL2 regulated transcriptome (6?).

CHAPTER THREE

EXPRESSION OF THE INTRA-OVARIAN FORKHEAD PROTEIN FOXL2 IS DETECTABLE IN GRANULOSA CELLS OF ALL POST-PRIMORDIAL FOLLICLES

ABSTRACT

A newly identified intra-ovarian transcription factor, FoxL2, is a member of a family of transcription factors named for a conserved DNA binding domain termed a winged-helix or forkhead domain. Although first identified within the embryonic pituitary gland, FoxL2 has, more recently, been implicated in regulation of ovarian function. In particular, specific mutations in FoxL2 results in a characteristic set of cranio-facial abnormalities termed either type I or type II Blepharophimosis-ptosis epicanthus inversus syndrome, (BPES). Type I but not type II BPES is also associated with infertility and premature ovarian failure. Thus, FoxL2 may contribute to normal reproductive function at both the pituitary and ovarian level. To further investigate the latter, we cloned the gene encoding mouse FoxL2 and utilized the sequence encoding the DNA binding domain as a radioactive probe for *in situ* analysis of FoxL2 gene expression in mouse ovarian sections. Consistent with a potential role in folliculogenesis, we find FoxL2 mRNA expression predominantly localized to granulosa cells of all post-primordial stages of follicular development in mice. Thus, FoxL2 may contribute to granulosa cell growth and differentiation in developing follicles.

INTRODUCTION

Ovarian folliculogenesis is fundamental to the production of fertilizable female gametes. This process is dependent on a complex interaction of hormones and intraovarian factors that cooperate to control the growth and differentiation of the 3 basic follicular components – granulosa cells, theca cells and the oocyte. Although a number of classification schemes have been developed to characterize follicular growth and development across multiple mammalian species, all of these generally represent 4 primary stages that include primordial, primary, secondary (preantral) and antral (9;10). The critical first step in initiation of folliculogenesis begins when a primordial or Type 1 follicle, an oocyte surrounded by a single layer of flattened pre-granulosa cells, enters the growth phase to become a primary or Type 2 follicle typically characterized as an oocyte surrounded by a single layer of cuboidal granulosa cells. Once this transition into a Type 2 follicle is initiated the follicle is referred to as "committed". As such, follicular growth proceeds continuously culminating either in ovulation or atresia (10). The initial growth phase of follicles is characterized by proliferation of the granulosa cells that surround the oocyte and an increase in oocyte diameter (12;13;118). Thus, the number and appearance of granulosa cells, the number of granulosa cell layers surrounding the oocyte and follicle and oocyte diameter all serve as objective criteria for defining the specific stages of follicle development. Growth of preantral follicles and the earliest stages of antrum formation occur independent of gonadotropin input from the pituitary gland (12;13;118). Gonadotropin input is, however, necessary for

the final maturation events and ovulation. At issue then is the identity of the intra-follicular signals that mediate both gonadotropin-independent and gonadotropin-dependent follicular growth and development. In this regard, it is clear that bi-directional communication between granulosa cells and the oocyte is critical (119;120).

The role of gonadotropins in the final stages of follicular development has been well documented (11;80;121). Specifically, upon achieving the small antral follicle stage, defined by the presence of more than five layers of granulosa cells surrounding an antral cavity, a follicle becomes acutely dependent upon gonadotropins and remains so for the subsequent duration of its development (11;13). Although the intraovarian signals and mechanisms by which primordial follicles are prompted from a state of growth arrest to growth to growth commitment are less clear (122), the past decade has witnessed much progress in the identification of ovarian factors that play a crucial role in initiating and organizing follicular growth and development. For example, the tyrosine kinase receptor c-kit and its ligand, stem cell factor, have been recognized as key players in follicular growth and development and appear to participate in folliculogenesis during the critical transition from the primordial stage of development (31-34). Additionally, stem cell factor appeared to be necessary for survival of mouse primordial germ cells in culture and is expressed in granulosa cells at all stages of follicular development in sheep(31-34). More recently, particular interest has been focused on GDF9 and BMP15 - two members of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation

factors. Expression of both of these proteins is restricted to oocytes of developing follicles in multiple species and, based on genetic "knock-out" studies, appear to act synergistically to direct normal follicular growth and development (80;123-126). Specifically, mice deficient in GDF9 are infertile and ovarian follicular growth is arrested at the primary or Type 2 stage of development(125;127). In contrast, mice deficient in BMP15 are fertile but display reduced fecundity (126). Similar to mice, expression of these factors is also exclusive to oocytes in the sheep ovary (80;123;128) and, based on immunoneutralization, both proteins appear to be critical for follicular development past the primary stage (129). Interestingly, however, the higher ovulation rates associated with the *Inverdale* fecundity phenotype in sheep results from an inactivating mutation in one of the BMP15 alleles (130).

Although the intraovarian mechanisms of action have not been determined for GDF9 and BMP15, the presence of amino-terminal signal sequences suggests that both proteins are secreted by oocytes and likely regulate follicular growth via paracrine mechanisms. In support of this notion, granulosa cells appear to harbor receptors for both GDF9 and BMP15 (81;131). Furthermore, treatment of granulosa cells with either GDF9 or BMP15 leads to a rapid and transient phosphorylation of Smad proteins – the intracellular mediators of TGF-β family signaling (6-8;132). Thus, members of the Smad family of signaling proteins are likely key components of GDF9 and BMP15 signaling in granulosa cells. While Smads possess both DNA binding and transcriptional activation domains (5;36) it is clear that the functional activity of

these proteins often require interaction with non-Smad protein partners. In this regard, it is interesting to note that functional interactions have been reported between Smad proteins and FoxL2 (1-3) – a gene recently implicated in premature ovarian failure in women(133).

Originally termed pituitary forkhead factor or pFrk, FoxL2, a member of the winged-helix or forkhead family of DNA-binding proteins, was first identified in the embryonic pituitary gland (134) and appears to be one of the earliest markers of ovarian differentiation in mammals (84). Of particular interest, however, has been the identification of specific mutations in FoxL2 that are associated with two phenotypes in women. First, a characteristic set of cranio-facial abnormalities termed blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) and, second, in a subset of women, premature ovarian failure (POF) (1-3). This POF is revealed as either primary or secondary amenorrhea (1-3). Although the precise mechanisms underlying the POF are unknown, in extreme cases the POF may result from excessive attrition of ovarian follicles in fetal life such that follicles remaining at birth are insufficient to sustain a full reproductive lifespan (1-3). Thus, FoxL2 appears to represent a new member of the expanding class of genes and forkhead DNA-binding proteins implicated in the regulation of follicular growth and development (89). Accordingly, the goal of the present study was to characterize the spatial and temporal patterns of expression of FoxL2 in the adult mouse ovary.

MATERIALS AND METHODS

Materials

All experiments were performed using adult female nonpregnant mice of the *FVB* inbred strain and carried out under approved protocols from the Colorado State University Animal Care and Use Committee. ^{35}S nuclide was purchased from Amersham Biosciences Corp. (Piscataway, NJ). Sac II and Sal I restriction endonucleases were obtained from New England BioLabs (Beverly, MA). Ribonuclease inhibitor was purchased from Fisher Scientific (Houston TX). *Maxiscript SP6/T7 Invitro transcription kit* Ambion Inc., (Austin TX) was used for production of sense and anti-sense FoxL2 riboprobes. All polymerase chain reactions (PCR) were performed using the *Robo Cycler* instrumentation from Stratagene (La Jolla, Ca).

Antibodies

A rabbit anti-peptide FoxL2 antibody specific for the mouse was obtained through Affinity Bio-Reagents (ABR) (Golden, CO). Specific nucleotide sequences of murine FoxL2 were used to generate a synthetic peptide representing the 14 N-terminal amino acids for immunization. An antiserum was collected, tested for titer and used in western analysis and immunocytochemistry. An HRP conjugated rabbit anti-goat secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Ca).

Cloning and Analysis of FoxL2

Messenger RNA isolated from the gonadotrope derived αT3-1 cell line (135) was used as the template in a reverse transcription (RT)-polymerase chain reaction (PCR) utilizing primers directed against the DNA binding domain of FoxL2 (forward primer: 5' CCGTACTCGTACGTGGCGCTCATCGCC 3'; reverse primer: 5' TTCTCGAACATGTCCTCGCAGGC 3'). This reaction yielded a 263 bp cDNA fragment of murine FoxL2 that was confirmed by sequence analysis. The RT-PCR product representing the FoxL2 DNA binding domain was subsequently used to screen a mouse genomic library (136) by phage-plaque hybridization (136). A single genomic clone was identified that contained an approximately 2.2 kbp XhoI fragment containing the entire, uninterrupted coding sequence of FoxL2. The same primer pairs were used to amplify the DNA binding domain of ovine FoxL2 from 200 ng of ovine genomic DNA.

In situ Hybridization

The 263 bp fragment representing the DNA binding domain of murine FoxL2 was sub-cloned into the plasmid vector pGEM –Teasy (Promega Corporation, Madison, WI). Plasmid DNA was linearized with either Sal I (T7; antisense) or Sac II (SP6; sense) restriction endonucleases for the preparation of riboprobes. The ^{35}S labeled riboprobes were produced by in vitro transcription reactions using the MAXIscript Invitro transcription kit (Ambion Inc., Austin, TX) using either the T7 (antisense) or SP6 (sense) promoters. The distribution of FoxL2 mRNA was analyzed by *in situ* hybridization (128;137). Paraffin

embedded tissue sections (5-7 μm thick) were deparaffinized by immersion in Americlear xylene substitute (twice for ten minutes each) and dehydrated by passage through graded ethanol washes (100 –70%) each twice for 5 minutes. The ethanol washes were followed by a final 5 minute wash in diethyl pyrocarbonate (DEPC) treated water. Prior to hybridization, slides were subjected to a 0.2 M HCl wash for 20 minutes at room temperature followed by a 30 minute wash in 2X SSC (1X SSC = 0.15M sodium chloride and 0.15M sodium citrate) at room temperature. Proteinase K (10 mg/ml proteinase K in 0.2 M Tris-HCL pH 7.2, 50 mM EDTA prepared in DEPC treated water) digestion was then conducted for 5 minute at 37°C. Slides were then dipped in triethanolamine (0.1M pH 8.0) and incubated in 0.25% acetic anhydride in 0.1M triethanolamine for 10 minutes at room temperature, dipped in double strength SSC, dehydrated through graded ethanols (50 –100%) for 3 minutes each and air dried. Sixty μl (2 x 10^7cpm/ml) of RNA probe in hybridization buffer (for 40 ml, 25 ml deionized formamide, 3.75 ml 4M NaCl, 0.5 ml 1 M Tris pH 8.0, 1ml 50 strength Denhardt's solution, 10ml 50% dextran sulfate, 0.75 ml water and 200mM DTT) was applied to each section and covered with a glass coverslip. Slides were hybridized with riboprobe overnight at 55°C in a humidified chamber. Double strength SSC was used to rinse off coverslips. Slides were then washed with 2X SSC for 10 minute wash and then incubated in a RNase-A solution (Sigma Chemical, St. Louis, MO) containing 600 μl of 10 mg/ml RNase-A in 300 ml 2X SSC at 37°C for 30 minutes. The incubation in RNase-A was followed by washing in 2X SSC for 10 minutes at room temperature and a 2 hour incubaton at 55°C in a stringent wash

solution (0.1X SSC, 0.09% β-mercaptoethanol, 1mM EDTA). Slides were rinsed two final times in 0.5X SSC for 10 minutes each and dehydrated through graded ethanol-7.5M ammonium acetate solutions, (50,70, 95, and 100% ethanols at 3 minutes each) and air-dried. Slides were then coated with type NTB2 emulsion (Eastman Kodak, Rochester, NY) and stored for 14 days at 4°C. The emulsion was developed and tissue sections were stained with hematoxylin and eosin and examined using both brightfield and darkfield optics. Three repetitions of this *in situ* hybridization experiment were performed each using ovarian sections from 3 adult, non-pregnant mice.

Classification of Follicles

Criteria used to classify murine follicles into specific developmental stages included the number and appearance of granulosa cells, layers of granulosa cells, and follilce/oocyte diameters. In mice, eight well-defined stages from primordial to large antral stages have been established (10). Primordial follicles are classified as type 1 folicIles and are characterized by a singe layer of squamous granulosa cells. Additionally, preantral follicles (types 1-5b) all contain an oocyte of approximately 20 microns in diameter. Type 2 follicles feature one to two layers of flattened granulosa cells. The transitional type 3a stage that follows marks the first appearance of cuboidal granulosa cells. Next, in the type 3b follicle, the presence of the zona pellucida about the oocyte is first evident. A third layer of granulosa cells is noted in type 4 follicles, while 4-6 layers may be present in types 5a, 5b, and type 6. The latter also demonstrates early antrum

formation. Significant enlargement of the oocyte to a maximum diameter of approximately 70 microns is evident in the type 6 follicle. Follicular types 7 and 8 represent full size pre-ovulatory follicles classified by a fully developed antral cavity and more than 6 layers of granulosa cells.

Western Analysis

Cell lysates were collected from αT3-1 pituitary gonadotrope derived cell line and the CHO and KK1 ovarian cell lines. Lysates were homogenized in RIPA buffer and assayed for total protein (Pierce BSA). Five μg of total protein /cell line was combined with 2x SDS, denatured and subjected to SDS polyacrylamide gel electrophoresis (acrylamide: bis-acrylamide ratio of 29:1). This was followed by electro-blotting to nitrocellulose (Bio-Rad Hercules, Ca), membrane. Membranes were then blocked in 5% non-fat dried milk in Tris buffered saline (TBS). Anti-FoxL2 antibody (1:5000 dilution in 1% milk) was applied for an 8 hour incubation at 4° C on an orbital shaker. The blot was washed for 30 minutes (3 washes X 10 minutes) with TBS and then incubated with a goat anti-rabbit HRP conjugated secondary (1:5000) for 2 hours. The blot was washed for 60 minutes (6 x 10 minutes) with TBS following the secondary antibody incubation and then visualized by chemiluminescence using Pierce Super Signal reagents.

Immunocytochemistry

Murine ovarian tissue was immersion fixed in 4.0% paraformaldehyde. The fixed tissue was washed dehydrated and embedded in paraffin. Slides were prepared of 5-micron thick tissue sections to be processed for ICC. First, paraffin was melted in a 55° C oven and then rehydrated through a series of decreasing strength xylene and ethanol washes, ending with tap water. Next, tissue was incubated in a sodium citrate buffer in a 90° C water bath for 40 minutes for the purpose of target retrieval. An additional room temperature incubation in the same buffer continued for 20 minutes. Following three consecutive phosphorous- buffered saline (PBS) washes, a five-minute .03% peroxidase solution was applied for blocking of endogenous peroxidases and rinsed with nanopure water. Next, a 10-minute block in goat serum (1:1000 dilution) was followed by application of primary anti-amino terminal peptide FoxL2 antibody (mouse, 1:5000, sheep 1:100) for 1 hour at 37° C. Following a PBS wash, tissue was incubated in a horseradish peroxidase (HRP) conjugated rabbit anti-goat secondary antibody at a 1:200 dilution. The tissue was washed in PBS and exposed to an avidin-biotinylated HRP reagent (Vectastain Elite ABC kit) for 30 minutes for immunostaining enhancement. A final PBS wash preceded chromagen, (diaminobenzidine tetrahydrochloride,DAB), application until desired stain intensity developed. Lastly, we performed a hematoxylin and eosin counterstaining, followed by clearing (dehydration through increasing strength ethanol washes), and mounting with coverslips for viewing.

RESULTS

Molecular Cloning of the Murine FoxL2 Gene

A 263 base pair (bp) fragment encoding the murine FoxL2 forkhead DNA binding domain was cloned by reverse transcription (RT)-polymerase chain reaction (PCR) methodology using mRNA isolated from the gonadotrope derived αT3-1 cell line (135). Subsequent screening of a mouse genomic library yielded a single clone containing a 2.2. kbp XhoI fragment that contained an uninterrupted 1100 bp open reading frame that represented the entire coding sequence of FoxL2. Thus, like the human gene, murine FoxL2 appears to be intronless. The predicted amino acid sequence of murine FoxL2 displays greater than 90% homology with the human sequence (Fig 1). Almost perfect conservation of sequence within the forkhead DNA binding domains of murine and human FoxL2 suggests a strongly conserved role for this transcription factor across multiple species.

Expression of FoxL2 mRNA in the mouse ovary

In situ hybridization was used to determine the distribution and relative abundance of mRNA for murine FoxL2. The distribution of silver grains across sections of murine ovaries incubated with ^{35}S radiolabeled antisense probe was granulosa cell specific (figure 5). Hybridization to granulosa cells was observed beginning at the primary follicle stage and was evident at all subsequent stages of follicle development. Specific hybridization of FoxL2 probe to oocytes or

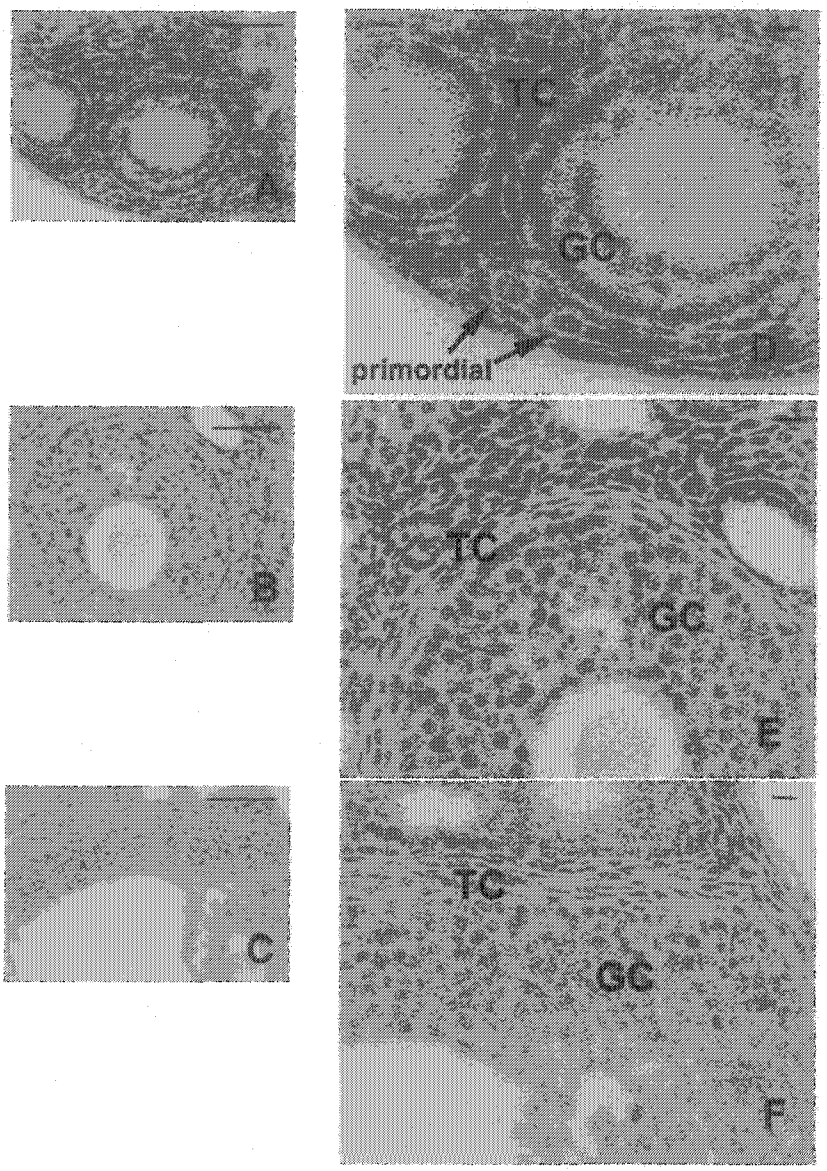


Figure 5. FoxL2 localizes to granulosa cells of all post primordial stages of follicular development. In situ localization of mRNA for FoxL2 in granulosa cells of murine follicles. A-C) 40x magnification of murine ovarian sections hybridized to murine FoxL2 ^{35}S-labeled antisense probe (scale bars = 50 µm). D-F) corresponding 60x magnifications of the same ovarian sections demarcating thecal cell (TC) and granulosa cell (GC) regions (scale bars = 30 µm). D) murine preantral type 3a (lefthand) and 3b (righthand) follicles, and primordial follicles. E) murine preantral (type 5) follicle. F) murine large antral type 8 follicle.

thecal cell components was not observed. Specific labeling was not observed in sections incubated with ^{35}S-radiolabeled sense probe for FoxL2 (figure 6).

Western analysis

Consistent with the role of FoxL2 in ovarian and pituitary development, protein was extracted from cells of ovarian (CHO and KK1) and pituitary (αT3-1) origins. Following western blot analysis using the anti-FoxL2 antibody directed against the N-terminus of FoxL2, specific bands were detected at 45 kD and 90 kD but not in choriocarcinoma cells (BeWo) (figure 7).

Expression of FoxL2 protein in the mouse ovary

Anti-FoxL2 immunocytochemical analyis revealed a pattern of staining in the murine ovary similar to FoxL2 mRNA expression (figure 8). FoxL2 was highly expressed in the granulosa cell layer and also weakly detected in the oocyte of all post primordial stages of development. Immunolabeling was not observed when antisera was preabsorbed with an excess of FoxL2 terminal blocking peptide.

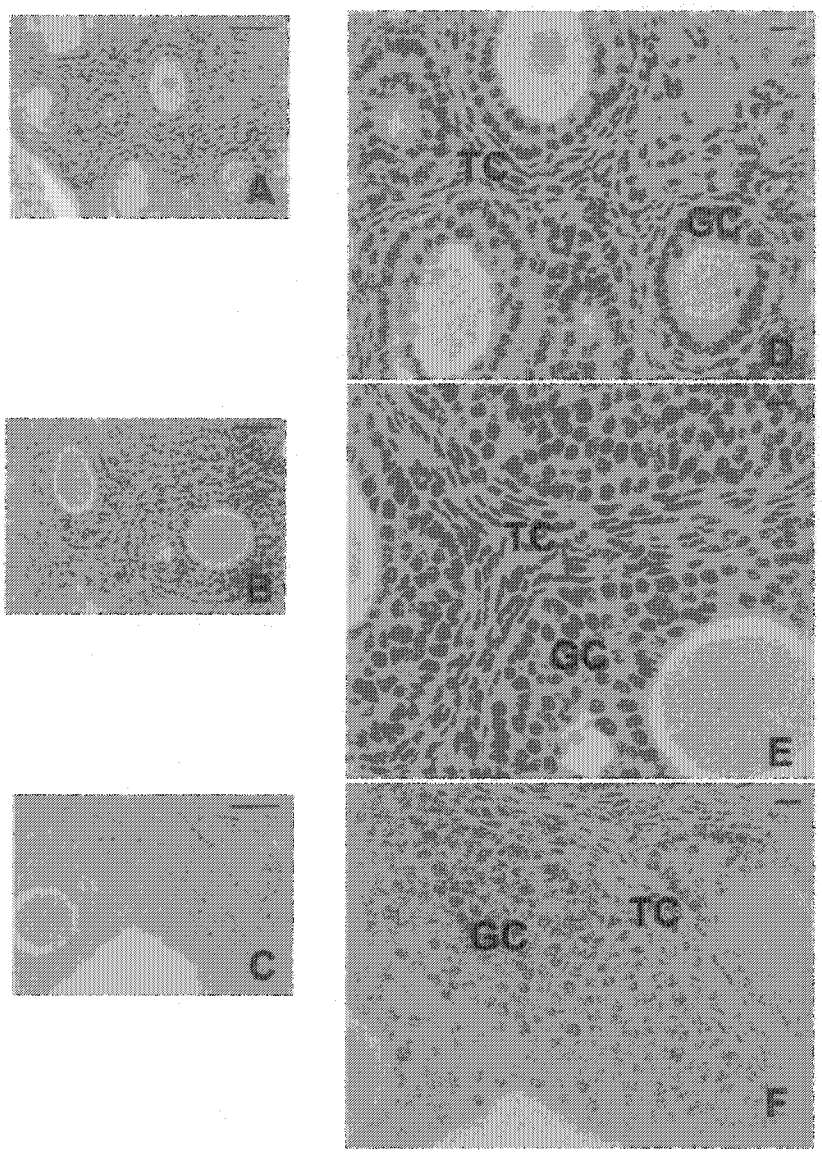


Figure 6. Controls for FoxL2 hybridization. **A-C)** Murine ovarian sections incubated in presence of murine FoxL2 ^{35}S-labeled sense probe (scale bars = 50µm). **D-F)** 60x magnifications of the same ovarian section demarcating thecal cell (**TC**) and granulosa (**GC**) regions (scale bars = 30µm). **D)** murine preantral (type 3b) follicles. **E)** murine preantral (type 5) follicle. **F)** murine large antral (type 8) follicle.

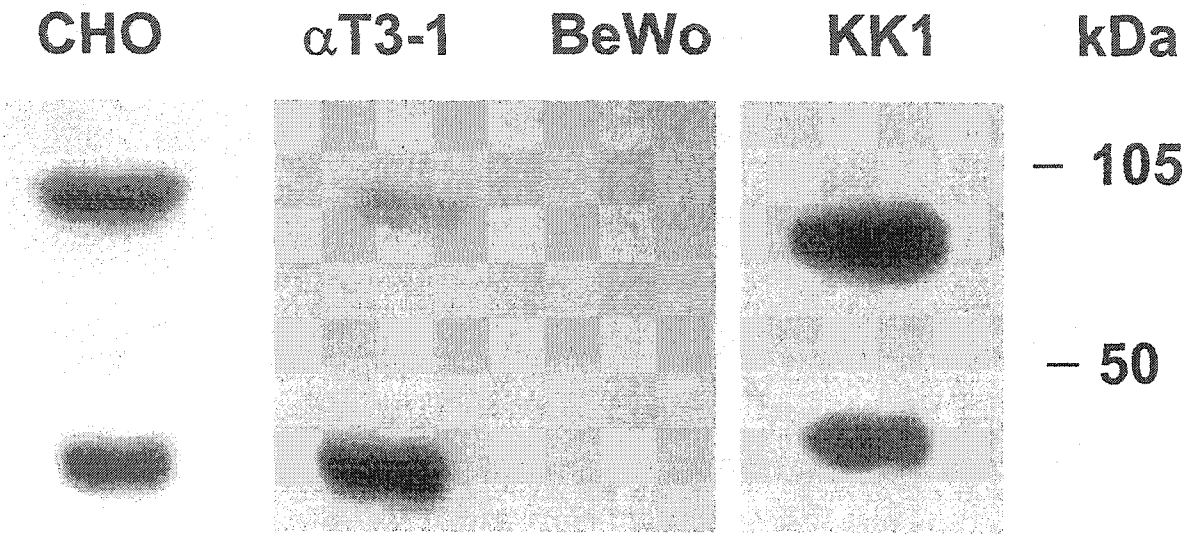


Figure 7. Generation of an anti-FoxL2 antibody. Western blot analysis of cell lysates from each of the respective cell lines were homogenized in RIPA buffer and assayed for total protein. Five micrograms of total protein /cell lysate was combined with 2x SDS, denatured and loaded onto a 10% SDS page gel demonstrating resultant 45 and 90 kilodalton bands. Serving as a negative control, antisera was preabsorbed with an excess of FoxL2 N terminal peptide that blocked all bands from detection (data not shown).

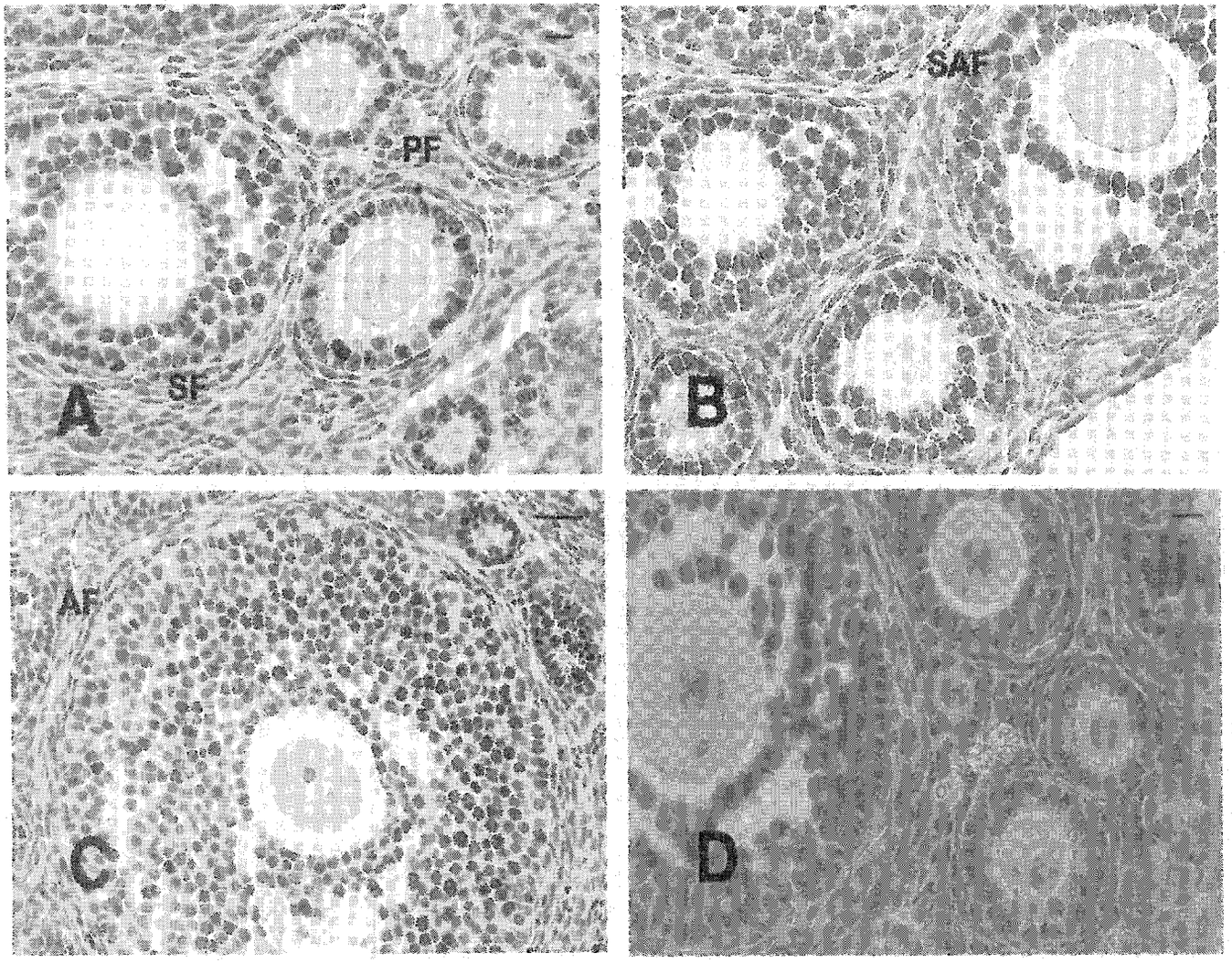


Figure 8. Immunohistochemical localization of FoxL2. Anti-FoxL2 (N terminal antibody) was incubated with murine ovarian sections representing different stages of follicular development. FoxL2 is highly expressed in the granulosa cell layer and also weakly in the oocyte. A-C) Anti-FoxL2 x60-fold magnification, D) Antisera preabsorbed with an excess of FoxL2 N terminal blocking peptide x60-fold magnification

DISCUSSION

Ovarian folliculogenesis requires a complex interaction of hormones and intraovarian growth and differentiation factors. In regard to endocrine regulation, considerable information is available as to the role of gonadotropins in later stages of follicle development and ovulation (11;80;121). In contrast, much less is known regarding the factors that mediate the earliest, gonadotropin-independent stages of folliculogenesis including the transition of primordial or Type I follicles from a growth arrested state to a growth committed state. Thus, elucidation of the signals and mechanism(s) involved in the initiation and maintenance of gonadotropin independent folliculogenesis remains an important issue in our understanding of the growth and development of ovarian follicles. The past 5 years have however, witnessed several promising developments including the identification of GDF-9 and BMP-15, two members of the TGFβ family of growth and differentiation factors, as key intraovarian signaling proteins (80;126). Expression of both proteins appears to be restricted to oocytes of developing follicles in both mice and sheep (80;123;124). Genetic knockout studies in mice and immunoneutralization studies in sheep have revealed a critical role for these proteins in early follicular growth (125;129). Similarly, progress in the characterization of the intraovarian expression patterns of intracellular signaling proteins and transcription factors, including members of the forkhead family of DNA binding proteins, represents another important development in our understanding of the regulatory factors that may mediate

ovarian folliculogenesis (89). In this light, the isolation of FoxL2 was an intriguing development.

A member of the winged-helix or forkhead family of transcription factors FoxL2 was first implicated in the emergence of ventral cell types in the embryonic pituitary gland (134). Consistent with this potential role in development, FoxL2 is expressed in a pituitary derived cell line and interacts with a binding site located in the promoter of the gonadotropin releasing hormone receptor gene. Of particular interest, however, was the identification of naturally occurring mutations in human FoxL2 that result in premature ovarian failure (POF). Now recognized as perhaps the earliest known marker of ovarian differentiation in mammals, FoxL2 has been implicated in ovarian somatic cell differentiation and follicle development and maintenance in the adult ovary (84).

Similar to the sequence reported for human FoxL2, murine FoxL2 appears to be intronless with the entire coding region contained within a single, uninterrupted genomic fragment. While human and murine FoxL2 display greater than 90% conservation at the amino acid level, even greater conservation is evident in the amino acid sequence of the DNA binding domain that is almost perfectly conserved across mice, humans and sheep. Also of interest is the conservation of a polyalanine tract that resides approximately 80 amino acids downstream of the forkhead DNA binding domain. Mutations involving expansion of the polyalanine tract are implicated as a causative factor in cases of BPES type II (1;3;138). In contrast, mutations that lead to a truncation of the polyalanine tract are more typical of type I BPES – the form of BPES associated

with POF. Finally, the risk of developing POF appears particularly high with mutations that truncate FoxL2 before the polyalanine tract (139). While the precise mechanisms underlying BPES and POF, it is likely that the pleiotropic effects associated with mutations in FoxL2 reflect haploinsufficiency resulting from either a null or hypomorphic allele (1;3;138). However, whether some of the truncated proteins may also act in a dominant-negative fashion is an issue that has not been resolved (1).

A critical first step in elucidating the potential biological role(s) of a protein is characterization of its expression patterns. In this regard, we have implemented immunocytochemistry to localize FoxL2 protein expression primarily to granulosa cells of murine follicles representing all stages of follicular development beyond primordial (Type I) to large antral (Type VIII) follicles. This ICC data is in agreement with our *in situ* results and would suggest that expression of FoxL2 occurs upon transition of a primordial follicle to the growth committed stages of follicular development. Expression appears to persist at all subsequent stages of follicular development. Thus, in addition to its putative role in fetal ovarian development (84-86), FoxL2 may also contribute to granulosa cell proliferation and differentiation in the adult ovary. Finally, given the temporal pattern of FoxL2 expression in the adult ovary, it is intriguing to speculate that FoxL2 may be a factor that contributes to the critical transition of a growth arrested to growth-committed follicle. In this regard, it is particularly interesting to note that the temporal expression of FoxL2 is identical to that reported for GDF9 in mouse oocytes (123;140). As a number of forkhead DNA binding proteins

contribute to intracellular signaling by TGFβ family members it is plausible that FoxL2 may be one of several factors that mediate GDF9 signaling to the level of gene expression. If correct, then one would predict that FoxL2 would interact with members of the Smad family of transcription factors. Consistent with this possibility, FoxL2 has been shown to interact with both Smad4 and Smad3 in yeast and mammalian 1 and 2-hybrid studies (141).

Forkhead DNA binding proteins have long been recognized as mediators of cellular growth and differentiation (1). As such, it is not surprising that expression of multiple members of this family of transcription factors has been characterized in different cell types of the mammalian ovary (89). For example, FoxO1 (FKHR) expression is detectable in granulosa cells of growing follicles and immature oocytes, whereas FoxO3 (FKHRL1) and FoxO4 (AFX) mRNA was detected in granulosa cells of selected follicles, throughout the thecal cell layer and in luteal cells(89). Although partially overlapping expression patterns of these forkhead proteins raises some concern as to the specificity of hybridization, it is important to note that the riboprobe generated for use in FoxL2 *in situ* analyses performed in this study displays only 68% sequence identity with that of FoxO1 (accession no. NM 019739) in the most highly conserved 70 bp region of the forkhead protein DNA binding domain. Only 43% conservation is evident over the sequence comprising the entire DNA binding domain (approximately 263 base pairs) of FoxL2 and FoxO1. As such, it seems unlikely that the expression patterns we have observed for FoxL2 represent cross-hybridization to mRNA for other forkhead DNA binding proteins.

In summary, members of the winged-helix or forkhead family of DNA-binding proteins are critical mediators of embryogenesis and development in all eukaryotes (1;4). A novel member of this family, FoxL2, has been implicated in POF and appears to participate in modeling of both the developing eyelid and ovary. Our studies indicate that expression of FoxL2 is granulosa cell specific in murine follicles, from primary through pre-ovulatory stages of development. The presence of FoxL2 exclusively within the granulosa cell layer is consistent with a potential role for FoxL2 in granulosa cell maintenance and proliferation and may represent a factor that contributes to transition of primordial follicles to growth committed stages of follicular development.

CHAPTER FOUR

EVIDENCE OF INTRANUCLEAR SHUTTLING IN REGULATION OF FORKHEAD PROTEIN FOXL2 IN GRANULOSA CELLS

INTRODUCTION

Members of the forkhead/winged-helix family of transcription factors share a characteristic 100 amino acid forkhead DNA binding domain and are implicated as important mediators of folliculogenesis, and multiple roles in the processes of embryogenesis, development and aging. In the past several years, considerable attention has been directed toward forkhead family member, FoxL2. Initially identified as a transcript expressed in the embryonic pituitary and implicated in mediating the final differentiation of gonadotropes (134), FoxL2 expression is now established in the developing eyelid and ovarian follicular cells. In regard to the latter, multiple FoxL2 mutations have been shown to underlie the human condition termed blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) (1-3). This autosomal dominant disorder in its type II form manifests as eyelid abnormalities. BPES type I is associated with these same eyelid deformities but also with premature ovarian failure (POF). Recent "knock-out" studies have indicated that the murine FoxL2 gene is essential for granulosa cell differentiation and ovarian maintenance (87;88). The phenotypic features of FoxL2 null animals suggest a new mechanism of POF. Namely, mice lacking FoxL2 ($FoxL2^{-/-}$) show lack of development of all major somatic cell lineages around growing oocytes from the onset of primordial follicle formation, suggenting that FoxL2 disruption serves as a potential model for histogenesis and reproductive competence of the

ovary (88). Furthermore, the severity of the abnormalities associated with FoxL2 mutation suggest that this protein contributes to key mechanistic events in ovary formation, maturation and follicular development (88).

Our understanding of the intracellular regulation of FoxL2 is hampered by the lack of mechanistic details regarding biochemical and cell biological properties of FoxL2 in regulating gene expression. Many have speculated that FoxL2 may participate in transforming growth factor beta (TGF-β) related signaling pathways involved in both eyelid development and gonadal function (142). In support of this notion, FoxL2 has been shown to interact with a member of the Smad family of intracellular TGFβ signaling molecules, Smad 3 (133;143-145). However, our current understanding of the regulatory mechanisms underlying FoxL2 as well as that of its gene targets pales in comparison to that which is known of the related FoxO family members. These later proteins are insulin sensitive transcription factors regulated by several signal transduction cascades including phosphoinositide 3-kinase (PI3K), casein kinase 1 (CK1) and dual-specificity regulated kinase 1A (DYRK1A) (94;146-148). In particular, phosphatidylinositol 3 (PI3)-kinase appears to underlie protein kinase B (PKB) mediated phosphorylation of FoxO proteins. This event results in nuclear exclusion as the phosphorylated targets remain bound to 14-3-3 proteins in the cytoplasm where they are effectively sequestered from their target genes (94;149-153).

The goal of these studies was to determine if a similar regulatory paradigm might be evident in FoxL2 and to identify structural features needed for appropriate intracellular trafficking.

MATERIALS AND METHODS

Construction of Plasmids

A FoxL2 cDNA was excised from a FoxL2 VP-16 construct (141) with EcoRI and BamHI and cloned into EcoRI and BamHI sites of pGFP^2-C1 (Biosignal Packard, Montreal, Canada), to express a FoxL2 protein with "humanized" GFP on its amino terminus (GFP-FoxL2).

The NLS (-) mutant FoxL2 was generated by a PCR-based strategy (*Robo Cycler* instrumentation from Stratagene La Jolla, Ca). First, the putative NLS localizes to amino acids (aa) 140-144, five consecutive arginine residues of which the first 4 of 5 or last 4 of 5 may represent a canonical NLS. We began by designing two overlapping primers to mutate arginine residues141-143, replacing them with alanine and incorporating a *Pvu*II site (indicated in bold lettering within primer sequences 1 and 2 below) for subsequent diagnostic purposes). Alternative codons were also introduced to incorporate a *Stu*I site corresponding to amino acids 147-148, to later assist in screening. Two internal primers were designed: primer 1: 5' GCAGCTGCACGCATGAAGAGGCCTTTCCGGCCGC 3' and primer 2: 5' CATGCGTGCAGCTGCCCGGTAGTTGCCCTTC 3'. Two separate PCR reactions were implemented using wild type GFP-FoxL2 (described above) as starting material. That is, primer 1 was specifically used with upstream primer 3 (5' AGCTTATGATGGCCAGCTACCC 3') that conserved the start codon and initiated priming downstream of the 5' untranslated region. Next, primer 2 was used with downstream primer 4 (5' GACCACCGCGGCTGCACCG 3'), corresponding with amino acid codons 235-

241, also including the last nucleotide base of codon 234. The PCR reactions generated 450bp and 700bp products, respectively that were gel isolated and entered into a third PCR reaction using primers 3 and 4. Sequence analysis of this PCR product confirmed that the intended mutation was present (Davis Sequencing, Davis Ca). GFP-FoxL2 (Packard Biosigna, Montreal, Canada) was digested with *Ppum*I and *BamH*I (vector shell and N-terminus of FoxL2) and again with *Nar*I and *BamH*I (C-terminus of FoxL2). The PCR product was cut with *PpuM*I and *Nar*I (generating 520 bp containing the mutation). (A second NarI site in wild type FoxL2 was removed by the *Stu*I mutation addressed above). The appropriate fragments were then gel isolated, ligated and the resulting clone was screened by sequence analysis to confirm the mutation (Davis Sequencing).

Cell Culture / Transient Transfection / Treatments

KK1 cells were maintained with DMEM (cellgro, Mediatech, Herndon, VA) with 10% fetal bovine serum (Gemini Bio-Products, Woodland, Ca), plus antibiotics/antimycotics 1% penicillin/streptomycin and antimycotic, 1% glutamine (cellgro, Mediatech, Herndon, VA) in a 5% CO_2 incubator at 37˚C .

KK1 cells were transfected with pGFP^2-C1-FoxL2 (wild type) or pGFP^2-C1-FoxL2 (mutant) at 50% confluency on 35mm glass bottom culture dishes (Mat Tek Ashland, MA) by Superfect (Qiagen Inc. Valencia, CA) liposome-mediated transient transfection. After 2 hours, cells were washed with PBS and provided fresh media. Twenty-four to Forty-eight hours later, an 8-10 hour serum starvation was performed before growth factor or hormone treatments: 100nm

insulin (Sigma-Aldrich, St. Louis, MO), 100ng/ml recombinant human follicle stimulating hormone (Dr. A.F. Parlow, Harbor-UCLA Med Ctr, Torrance, CA), or 10μm forskolin/DMSO (Calbiochem, La Jolla, CA). The intracellular localization either GFP-FoxL2 or endogenous FoxL2 was noted using confocal imaging of each respective treatment group.

Immunocytochemistry

KK1 cells were plated in 6 well plates containing glass coverslips (Mat Tek Ashland, MA) at 2 x 10^5 cells/well overnight. The second day three 15 minute washes of the cells in phosphate buffered saline (PBS) pH 7.4 were performed. Next, cells were fixed in fresh frozen 4% paraformaldehyde solution/PBS for 15 minutes at room temperature (rt). At this time fix was removed and excess aldehyde was quenched by 15 minute incubation at rt in 1mg/ml sodium borohydride prepared fresh in PBS pH 8. Cells were permeabilized at rt by 10 minute incubation in 0.25% tritonx-100 in PBS (pH 8) and blocked for 30 min at rt in 3% bovine serum albumin and 10% goat serum in tris-buffered salt solution with tween (TTBS) (0.3 M NaCl, 20 mM TrisCl pH 8.0, 0.1% v/v tween-20). Rabbit polyclonal anti-FoxL2 (Affinity Bioreagents, Golden, Colorado) diluted 1:500 in blocking solution was applied to coverslips for overnight incubation at 4° C in a moist chamber. On day three, three 15 minute TTBS were performed and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon), was applied at 7ug/ml for 2 hr at rt in a dark cabinet. Coverslips were washed again 3

times at 15min/ wash and mounted on microscope slides (VWR Scientific, West Chester, PA) using Gel Mount mounting media (biomedia corp, Foster City, CA).

Confocal Laser Scanning Microscopy

Both live and fixed cell micrscopy was performed on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc. Thornwood, NY). GFP and Alexa Fluor 488 were excited with the 488-nm laser line of an air-cooled Ar laser. Images were captured using the 63x oil immersion objective and processed using LSM 510 Meta software.

Image Analysis

Quantitative analysis of the effect of ligand treatment upon GFP-FoxL2 nuclear distribution was performed as follows. Statistical evaluation of the observed fluctuation in fluorescence intensity was performed by determining the coefficient of variation. Toward this end, the average mean and average standard deviation (stdev) values of the fluorescence intensity of three given lines intersecting the nucleus, two perpendicular to each other and a third crossing at a 45° angle, was determined for a large population of nuclei treated in a similar manner. In the case of the serum control sample the mean fluorescence intensity was 135 with a stdev of 47 and the corresponding values for the serum starved condition reflected a mean of 127 with a stdev of 39. Meanwhile, the corresponding mean and stdev of the forskolin treated group was 92 and 40, respectively. Next, calculation of the coefficient of variation allowed for

normalization of the stdev by the mean and direct comparison of the magnitude of change in fluorescence intensity for samples with different mean values. Employing the coefficients of variation, a mean coefficient of variation as well as its stdev was derived corresponding to the populations of cells representing each treatment group. More specifically, the mean coefficient of variation/ stdev for serum (control) cells was 0.35 +/- 2.4, that of the serum starved cells 0.31+/- 3.5 and the same values reported for the serum starved/ forskolin treated cells was 0.43 +/- 4.2. Statistical T test (SAS Institute Inc., 1989) provided a value of 0.325 ($p > 0.05$) indicating that the means of the two groups, serum and serum starved, do not reflect a statistically significant redistribution of GFP-FoxL2. However, the value 0.044 ($p < 0.05$) indicates that a high degree of statistical significance exists between those cells serum starved only and those serum starved prior to forskolin treatment. Thus, the forskolin treatment has produced an increase in the mean coefficient of variation reflecting a significant redistribution of GFP-FoxL2.

RESULTS

Expression of the GFP-FoxL2 Fusion Protein

To follow the subcellular localization and trafficking of FoxL2-GFP in living cells, we tagged the amino terminus of the 375 amino acid murine FoxL2 with the humanized pGFP^2-C1 under control of the cytomegalovirus (CMV) promoter. (BioSignal Packard, Montreal, Canada).

Previous investigations of the FoxO family of proteins has shown that upon ligand-induced phosphorylation these proteins are restricted in the cytoplasm. When the pathway(s) responsible for these phosphorylation events are not engaged, for example, serum-free conditions, the FoxO proteins accumulate in the nucleus. In contrast to this mechanism, the localization of GFP-tagged FoxL2 appeared to be constitutively nuclear. Figure 9 depicts an optical section of GFP-FoxL2 expressing KK1 cells grown in absence of any specific treatment obtained through confocal microscope. Despite differences in overall level of brightness due to cell specific variation in level of GFP-FoxL2 expression, the tagged transcription factor is expressed in a diffuse pattern evenly distributed throughout the nucleus with the exception of the nucleoli.

Neither serum starvation nor inclusion of ligand or growth factor (100nm insulin, 100ng/ml rhuman FSH, 10μm forskolin) had any apparent effect on the nucleocytoplasmic compartmentalization of GFP-FoxL2 (figure 10). Similarly, immunocytochemistry using a rabbit polyclonal anti-FoxL2 antibody and Alexa Fluor 488 goat anti-rabbit secondary for detection revealed the same predominantly nuclear distribution of FoxL2 (figure 9). Thus the presence of the

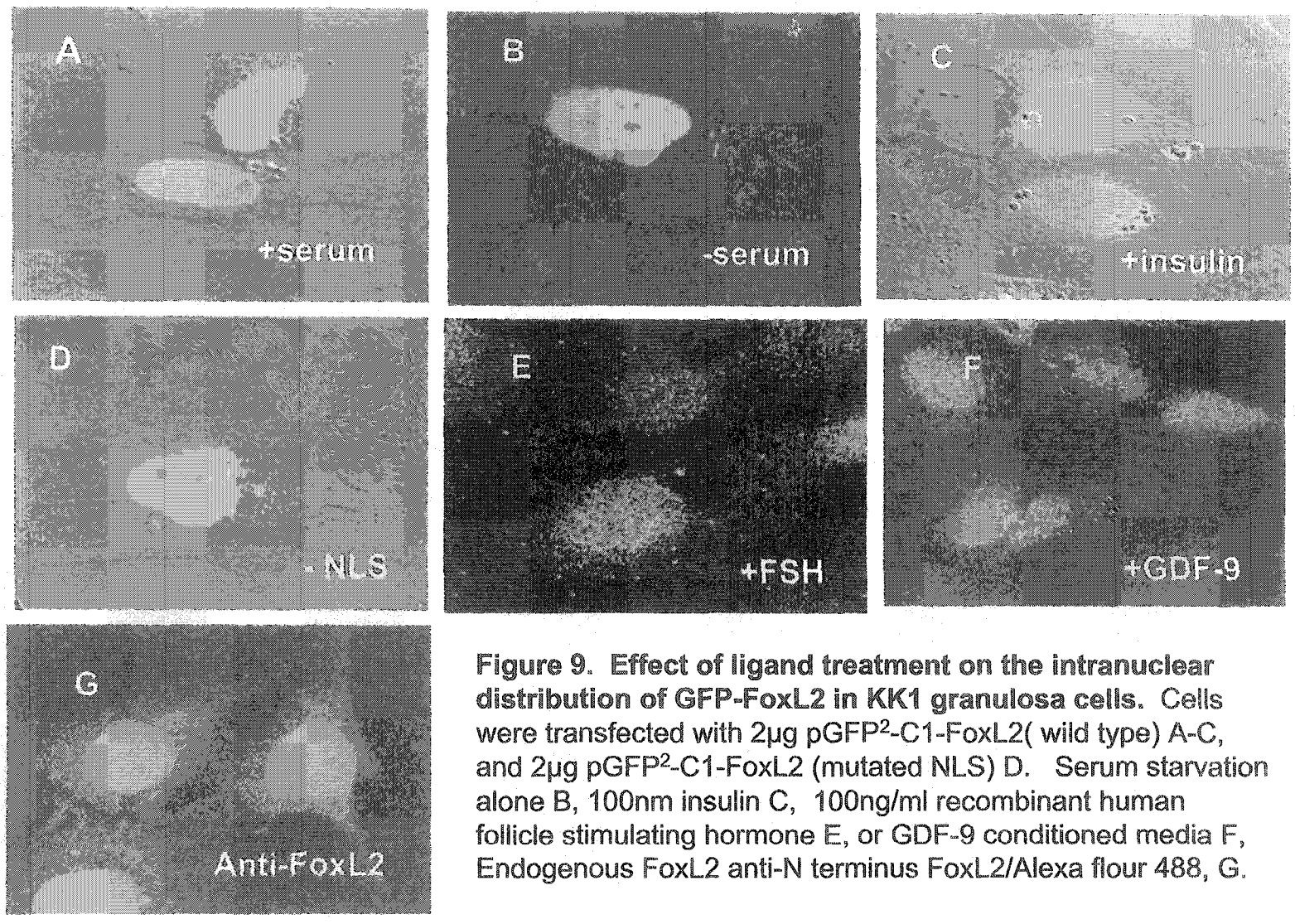


Figure 9. Effect of ligand treatment on the intranuclear distribution of GFP-FoxL2 in KK1 granulosa cells. Cells were transfected with 2µg pGFP^2-C1-FoxL2(wild type) A-C, and 2µg pGFP^2-C1-FoxL2 (mutated NLS) D. Serum starvation alone B, 100nm insulin C, 100ng/ml recombinant human follicle stimulating hormone E, or GDF-9 conditioned media F, Endogenous FoxL2 anti-N terminus FoxL2/Alexa flour 488, G.

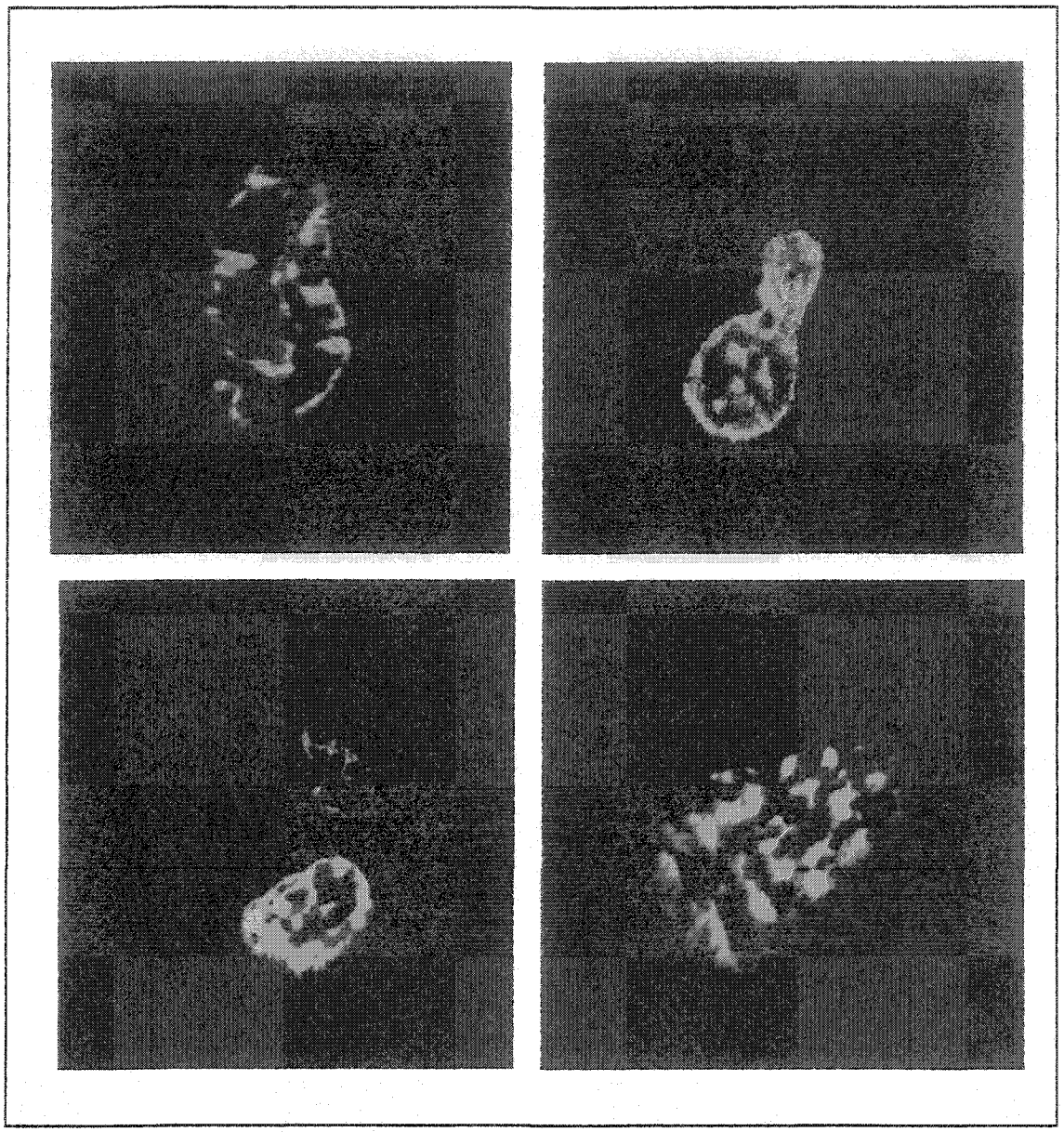


Figure 10. GFP-FoxL2 undergoes forskolin-induced intranuclear redistribution. Confocal image analysis of KK1 granulosa cells following 3h incubation in10μm forskolin/DMSO under serum free conditons, reflects more punctate, perinuclear distribution of GFP-FoxL2. (DMSO vehicle only treatment displayed the same exclusively nuclear distribution (data not shown)

approximate 30kDa GFP tag of the GFP-FoxL2 fusion protein does not appear to influence the trafficking pattern of the endogenous protein.

Although no change in nuclear versus cytoplasmic compartmentalization of GFP-FoxL2 was observed in response to 10μm forskolin, the intranuclear localization of GFP-FoxL2 may have been affected. Treatment with 10μm forskolin for 3-5 hours leads to redistribution of FoxL2 from a diffuse uniform distribution to a punctate and highly structured pattern (figures 10, 11, 12).

Mutation of the Canonical Nuclear Localization (NLS) in FoxL2 Does Not Prevent Nuclear Trafficking

We have analyzed the sequence of murine FoxL2 (PSORT II server http://psort.ims.u-tokyo.ac.jp) and identified a conserved nuclear localization signal consisting of five adjacent arginine residues approximately 270 base pairs upstream of the polyalanine tract. To determine whether this is the signal responsible for FoxL2 localization to the nucleus, we used site-directed mutagenesis to replace three of the five arginine residues with alanine, generating RAAAR as the sequence present at this location in our FoxL2 (NLS-). In order to monitor its localization, we fused the mutated FoxL2 with GFP at its N terminus. Introduction of the intended mutations was validated through sequencing of our final product. Confocal analysis of its subcellular localization demonstrated that GFP-FoxL2 in absence of its putative nuclear localization signal (NLS-) remained entirely nuclear and did not redistribute to the cytosolic compartment (figure 9).

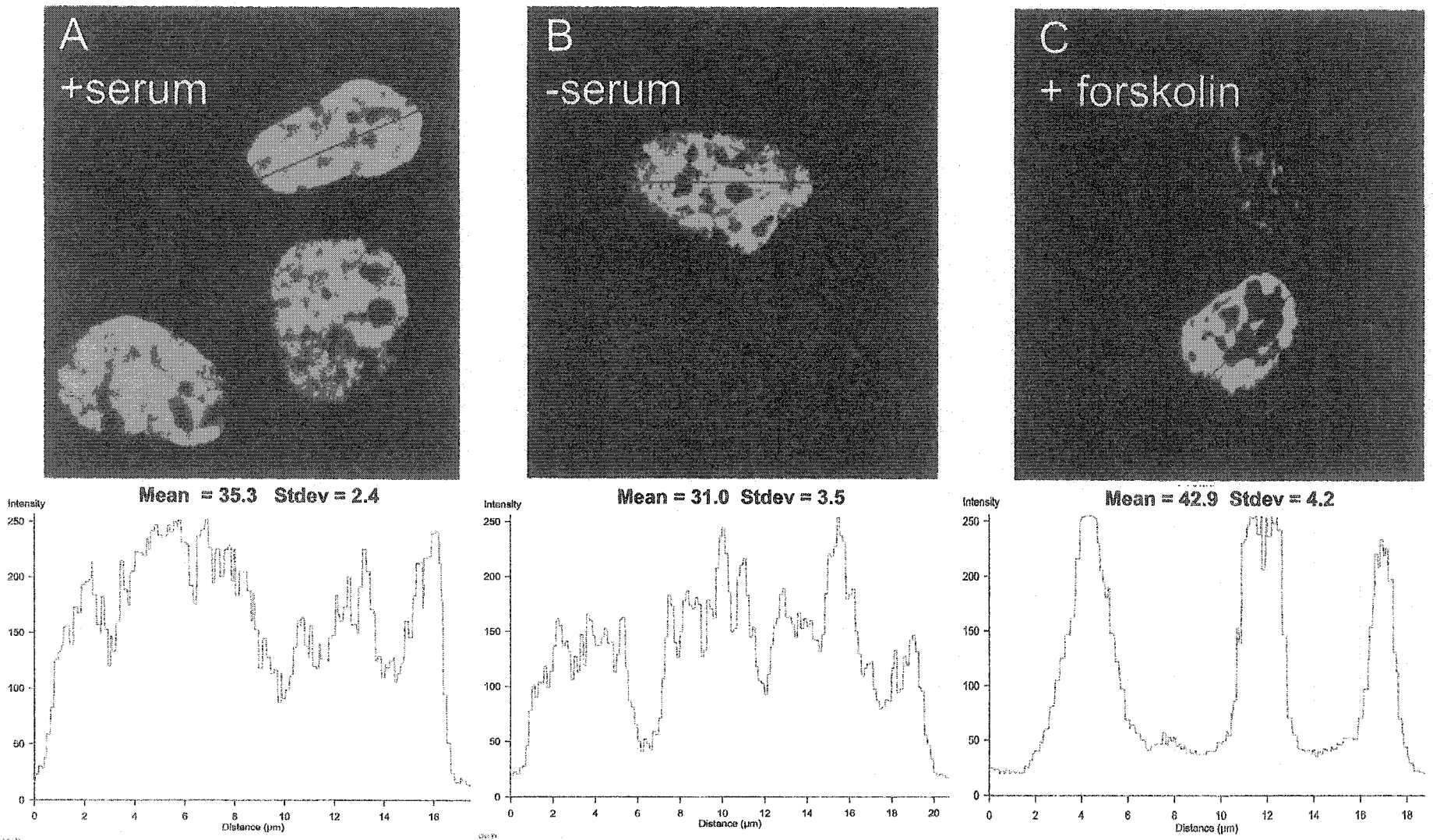


Figure 11. Quantitative analysis of the effect of ligand treatment upon GFP-FoxL2 nuclear distribution. Visualization of GFP-FoxL2 expressing KK1 cells was performed by confocal laser scanning microscopy and images were processed using LSM 510 Meta software. Graphs of fluorescence intensity along the red line drawn through each respective nucleus (A-C) are represented, arrowheads point in the direction of the plot from left to right. A mean coefficient of variation as well as its stdev was derived corresponding to the populations of cells representing each treatment group (A-C).

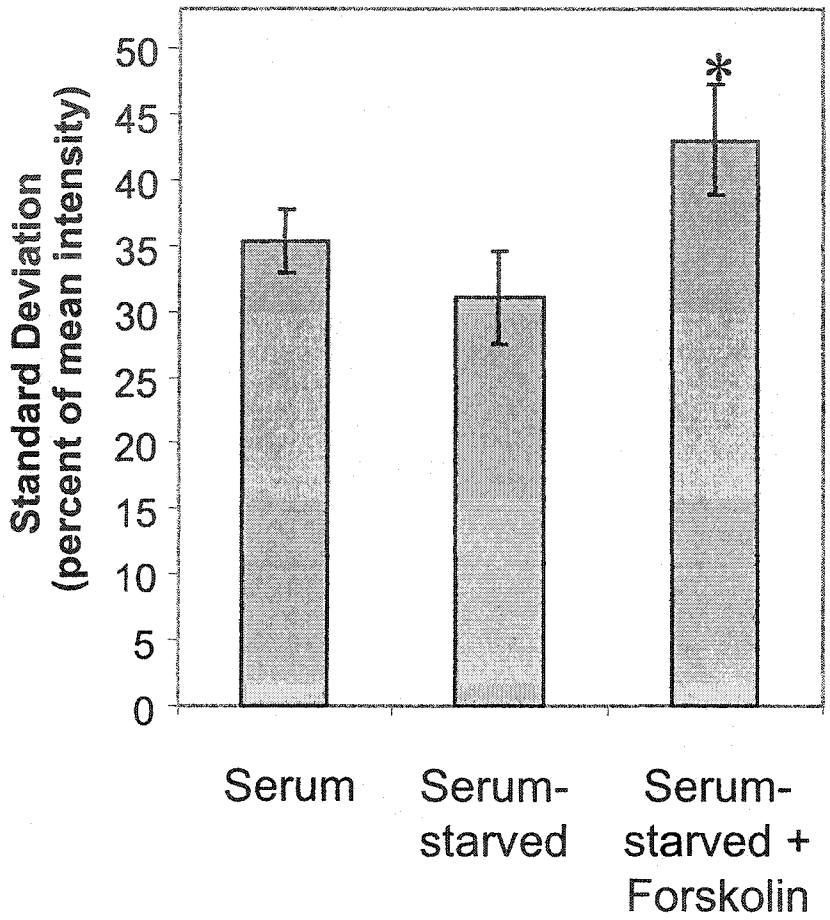


Figure 12. Statistical Evaluation of GFP-FoxL2 Under Conditions of Forskolin Treatment. T test provided a value of 0.325 ($p > 0.05$) indicating that the means of the two groups, serum and serum starved, do not reflect a statistically significant redistribution of GFP-FoxL2. However, the value 0.044 (*, $p < 0.05$) indicates that statistical significance exists between those cells serum starved only and those serum starved prior to forskolin treatment.

DISCUSSION

Experimental evidence now suggests that FoxL2, expressed in granulosa cell precursors, regulates the fate of all major ovarian cell lineages, including oocytes and stroma (88). Furthermore, the defects observed in FoxL2 $^{-/-}$ mice occur earlier in development than those observed in null mutants for other previously identified regulatory genes (88;91;125;154;155). In the absence of FoxL2, defects are first realized at the time of primordial follicle formation when oocyte nests are intercalated by somatic cells and fragment into single follicles (156-158). This is in contrast to null mutants for GDF-9 and FSH receptor that later impact the growth of competent primordial follicles or FoxO3a believed to be associated with recruitment of primordial follicles (91;125;154;155). Thus, much is known regarding the key role of FoxL2 in initiation of ovarian function. However, virtually nothing has been reported of the intracellular regulation of this protein. In these studies we sought to determine if FoxL2 undergoes ligand-induced nucleocytoplasmic shuttling similar to FoxO forkhead family members. Our data suggest that this is not the case and find endogenous FoxL2 protein and FoxL2 expressed as a fusion protein with GFP remain exclusively nuclear in KK1 murine granulose cell line, regardless of treatments known to induce the nucleocytoplasmic shuttling of FoxO proteins (100). However, FoxL2 may exhibit some degree of rearrangement within the confines of the nucleus itself, particularly upon administration of forskolin (10μm). More specifically, the pattern of green fluorescence, although remaining entirely confined to the nucleus, appears to be organized into distinct clusters within the nuclear compartment.

At issue is whether the nuclear redistribution of FoxL2 is biologically relevant. This may be the case as multiple proteins including, estrogen receptor α (ER α) and PPARS, peroxisome proliferator (PP)-activated receptors (PPARs), both ligand-activated transcription factors of the nuclear hormone receptor superfamily (159) have demonstrated intranuclear redistribution in response to ligand stimulation. The estrogen receptor α GFP fusion (ER α-GFP) exhibits a diffuse nuclear pattern in the absence of ligand, yet becomes concentrated at specific locations within the nucleus when in the presence of 17-β estradiol (160;161). Under these circumstances, additional studies have verified that GFP-ER α and steroid receptor coactivator (SRC-1) colocalize to nuclear matrix bound foci (160). Similarly, PPAR-GFP fusion proteins display an intranuclear redistribution characterized by a transition from a diffuse to reticulated pattern of GFP-labeled PPAR within the nucleus under conditions of coexpression of the heterodimeric partner of PPARs, 9-cis retinoic acid receptor (RXR). (159;162). Furthermore, since PPARγ/RXRα coexpression correlated with an increase in transcriptional activity, the regions of strong fluorescence likely represent PPARγ/RXRα heterodimers in association with DNA or proteins composing a transcriptional complex (159).

The functional significance of the nuclear matrix-bound foci within the nuclear compartment has been more thoroughly addressed. For instance, steroid receptors, RNA polymerase II, and even actively transcribed genes have previously been recognized as associated with the nuclear matrix (160;163). However, recent findings of Stenoien et al reveal that most ER-α foci do not

overlap with transcription sites (163). In fact, regarding the transcription factors currently investigated, the observed minimal overlap of expression with that of RNA synthesis foci has been speculated to be an indication of the dynamic nature of the assembly/disassembly of the transcription factor containing complexes in which very little time may be directed toward active transcription(160;163).

Finally, the role of dimerization as just noted in the case of PPARγ/RXRα as well as that also observed with thyroid receptor GFP fusions TR/RXR (164) appears to be necessary in order for these nuclear transcription factors to redistribute themselves within the nuclear compartment and remain in the nucleus in an unliganded state (164). The potential for FoxL2 homodimerizaton has also been suggested by two-hybrid analyses conducted in our laboratory (Escudero,unpublished). This may indicate a similar mechanism by which FoxL2 remains and redistributes within the nucleus while associated with perspective DNA target sites. Dimerization would significantly enhance the affinity of FoxL2 for its DNA binding sites that may prove particularly relevant given its suspected weak transcriptional activation capacity (Escudero, unpublished). A thorough investigation of this scenario, perhaps first employing a FRET based approach may be particularly enlightening.

If PKA elicits a functionally relevant nuclear redistribution of FoxL2 then a key issue is the identity of an extracellular ligand that could be linked to cAMP production. A logical candidate is perhaps, activation of PKA, follicle- stimulating hormone (FSH) is a logical candidate, FSH. However, following treatment of

KK1 cells with recombinant human FSH, no redistribution of FoxL2 protein within the nuclear compartment was observed. Importantly, however, while FSH receptor expression was detectable by Kananen et al, no specific binding of FSH could be detected in KK1 cell membranes (165). As such, FSH only produces a modest increase in [cAMP] in KK1 cells while forskolin elevated cAMP production nearly 40-fold (165). Thus, it may be necessary to repeat this study using primary cultures of granulosa cells.

Perhaps another ligand capable of initiating PKA mediated nuclear redistribution of FoxL2 is growth and differentiation factor (GDF)-9. The induction of cumulus cell expansion in murine follicles has been reported as a mitogen-activated protein kinase (MAPK) dependent event occurring downstream of both cAMP elevation as well as GDF-9 (oocyte-secreted, cumulus expansion enabling factor) (166). FoxL2 might also be mediated by protein kinase A (PKA) and/or mitogen-activated protein kinase (MAPK). If correct then GDF-9 should, like forskolin, induce FoxL2 nuclear redistribution. This, however, did not appear to be the case (figure 9). The phosphorylation of Smad 2 observed in western analyses of conditioned media (figure 13) confirmed the biological activity of our ligand, however, it would be important to repeat this analysis using purified GDF-9 so that a pre-determined quantity might be administered in our treatment. Also, FoxL2 regulation as proposed here would necessitate involvement of Smads 2 or 3 specifically serving in activin/TGF-β type signaling events (5) and FoxL2 interaction with Smad3 has already been reported (141).

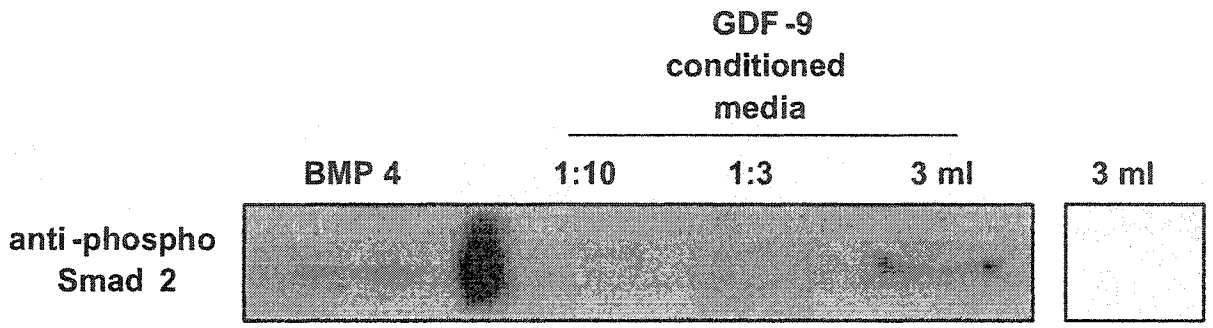


Figure 13. GDF9 conditioned media leads to Smad2 phosphorylation in KK1 cells. Lanes: BMP4=treatment with purifed BMP4 (positive control); 1:10, 1:3 = dilutions of conditioned media; 3 ml = 3 ml full strength conditioned media; last lane = 3 ml of media from non-transfected HEK293 cells.

Our interest in the putative NLS for FoxL2 was based on recent reports that this approach may represent an avenue for producing a dominant negative variant of FoxL2 (167). Surprisingly, however, mutation of the candidate NLS has no impact on FoxL2 nuclear localization. Whether this fusion protein retained biological activity remains to be determined. If, as the data suggest, the NLS is not functionally relevant then FoxL2 must possess alternative nuclear trafficking information or associate with a chaperone protein that indicates nuclear localization. In regard to the latter, a functional NLS exists in the MH1 domain of Smad3 (41;168) as well as a constitutively active NLS in the Smad4 MH1 domain (169). Additionally, although Smads 2,3 and 4 generally exhibit continuous nucleocytoplasmic shuttling, upon stimulation Smad mobility in the nucleus is significantly decreased. This may suggest that Smad complexes are actively retained in the nucleus with associated transcriptional co-activators and/or co-repressors acting as retention factors (169;170). Similarly, ovarian sulfated glycoprotein (SGP)-2, is a candidate chaperone protein known to be regulated by FSH (171). Specifically, a shortened isoform of SGP-2 is retained intracellularly and targeted to the nucleus via a NLS (172). Thus, FoxL2 nuclear localization might initially occur through interaction with another protein (Smad3, SGP-2, etc.) that possesses a functional NLS. Subsequently, FoxL2 may remain tethered to the nuclear matrix as part of a large immobilized complex of signal transduction molecules and additional unidentified co-activators or co-repressors.

CHAPTER FIVE

CONCLUSIONS

In the spring of 2001 FoxL2 was identified as the first human gene required for the maintenance of ovarian follicles and implicated in idiopathic premature ovarian failure (1;2;138). Mutations in FoxL2 were shown to be responsible for the rare genetic disorder, BPES, types I and II (1;2;138). In addition to the characteristic eyelid malformations of the syndrome, premature ovarian failure was exclusively linked to the type I form.

Originally termed pituitary forkhead factor or pFrk, FoxL2, was first identified in the embryonic pituitary gland (134). At this time mouse FoxL2 expression was identified in the ovary follicle cells and developing eyelid (1;2). Thus, my initial goal was to characterize the spatial and temporal expression of FoxL2 in the adult mouse ovary. For this purpose, I used the 263 base pair FoxL2 DNA binding domain to generate a ^{35}S labeled riboprobe for *in situ* hybidization reactions in parrafin sections of mouse ovary. I found the transcript to be clearly localized to granulosa cells of all post primordial stages of development. A low level of transcript may also be present in the oocyte and terminally differentiated luteinzed granulosa cells of the corpora lutea. Lastly, owing to the highly conserved nature of the forkhead DNA binding domain I was also able to confirm similar localization of FoxL2 to the granulosa cell region of the sheep ovary using the same murine FoxL2 probe. Thus, FoxL2 is not only one of the earliest markers of ovarian differentiation but is clearly expressed in both somatic and germ cells of the adult ovary in multiple species.

I next wished to confirm whether the expression of FoxL2 protein paralleled that of its mRNA. For this purpose, an N-terminus peptide antibody was generated and implemented in immunocytochemistry (ICC). Consistent with the *in situ* analysis, these data demonstrated that FoxL2 protein is present in granulosa cells in all post primordial follicles. As with the mRNA, low levels of FoxL2 staining were evident in oocytes. I should emphasize that my work focused predominantly upon FoxL2 expression in the adult mouse ovary which, in contrast to embryonic specimens (84;85), contain relatively few primordial follicles.

Forkhead transcription factors are widely recognized as key mediators of growth and differentiation (4) and are often involved in mediating nuclear responses to cellular activation by transforming growth factor beta (TGFβ) family members (173;174). Thus, it was encouraging to discover that a functional interaction between FoxL2 and Smad3 (mediator of TGFβ type signaling) is detectable in both yeast and mammalian two-hybrid protein interaction screens (133). Also, my ICC results confirmed that FoxL2 expression in mouse granulosa cells is identical to the temporal pattern of expression of the TGFβ family member growth and differentiation factor nine (GDF-9) in mouse oocytes.

Thus, my next goal was to further explore the possibility that FoxL2 may participate in GDF9 signaling. In the preliminary stages, I was able to identify the presence of bone morphogenetic protein receptor type II (BMPRII) (175)in our murine KK1 granulosa cell line (176) and also to construct a human embryonic kidney (HEK) 293 cell line that stably expresses GDF9. At this point, I began

Western blot analyses (using an antibody specific for phosphorylated Smad2 (53) and found that conditioned media from the GDF9 expressing cell line led to Smad2 phosphorylation in KK1 cells. Media from non-transfected HEK293 cells had no effect (5;36). Thus, biologically active GDF9 could be produced in HEK293 cells and a receptor for GDF-9 signaling is present in the KK1 model system.

One of the fundamental mechanisms for regulating functional activity of the forkhead proteins is regulation of nuclear-cytoplasmic shuttling (92;146;177). As such, I sought to determine the potential for intracellular trafficking of FoxL2 upon ligand treatment. I initiated these studies by performing ICC on KK1 cells. I determined that the FoxL2 protein is exclusively nuclear in either serum containing or serum free conditions. Moreover, FoxL2 distribution remained entirely nuclear under treatment conditions ranging from GDF9 conditioned medium to ligands known to induce nucleocytoplasmic transitioning of the FoxO proteins (serum starvation, insulin, follicle stimulating hormone) (92;146;177). I also investigated the intracellular trafficking of FoxL2 using a GFP tagged construct. These data supported the notion that FoxL2 appears to be constitutively localized to the nucleus.

As nuclear-cytoplasmic shuttling of FoxL2 did not appear to be a regulated step, I next focused on the possibility of nuclear redistribution. In this regard, the application of forskolin appears to lead to intranuclear reorganization. Specifically, FoxL2 distribution appeared more punctate and concentrated at perinuclear foci. Similar behavior has been reported for GFP tagged estrogen

receptor alpha (ER α)(161) and the proxisome proliferator activated receptors (PPARs) (159). From my perspective, additional investigation of the potential implications of the intranuclear reorganization of FoxL2 is warranted. For example, others have speculated that the sites of GFP- ER concentration represent transient loci of ER dependent transcription "factories" or perhaps ER processing sites, storage sites, or sites of interaction with nuclear matrix (160).

Given constitutive localization of FoxL2 in the nucleus, I next sought to determine the structural attributes of this protein that direct nuclear trafficking. Towards this end, I constructed a GFP-FoxL2 fusion protein in which the putative nuclear localization signal (PSORT II server http://psort.ims.u-tokyo.ac.jp) is mutated (GFP-FoxL2 NLS-). Following evaluation of this construct in KK1 granulosa cells using confocal microscopy, the FoxL2 protein remained exclusively nuclear similar to the wild-type FoxL2.

In summation, I have identified predominantly granulosa cell expression of murine FoxL2 mRNA and protein in the adult animal. Unlike the behavior noted for other forkhead transcription factors, I found FoxL2 protein expression to be constitutively nuclear and unregulated at the level of nucleo-cytoplasmic shuttling. However, I propose that intranuclear redistribution is likely and indicate such as a potential mechanism regulating the expression of FoxL2. Following further investigation of the nature of the nuclear localization of FoxL2, I was unable to identify the presence of a functional NLS. As such, I suggest that nuclear entry is likely to be determined by other structural information inherent to the FoxL2 protein. At this time, I believe that additional investigation of the

potential implications of the intranuclear reorganization of FoxL2 is necessary. Moreover, residing in the nuclear compartment, the biological role of the transcription factor, FoxL2, must, in a global sense, be to regulate the expression of specific gene programs in the fetal and adult ovary. Thus, I initiated the first attempt to identify FoxL2 target genes using microarray technologies (appendix). Potentially promising are a number of genetic markers that have been indicated as FoxL2 responsive and currently warrant further investigation.

CHAPTER SIX

APPENDIX: IDENTIFICATION OF THE FOXL2 MEDIATED "TRANSCRIPTOME" USING MICROARRAY TECHNOLOGIES

INTRODUCTION

Fertility in women depends on complex molecular events that are initiated in utero with ovary organogenesis and primordial follicle formation and, postnatally, require the highly coordinated actions of the hypothalamic-pituitary axis (178). Unfortunately, we currently have a very vague understanding of the molecular interaction between germ and somatic cells during primordial follicle formation or the molecular mechanisms that trigger the selective growth of particular follicles in vivo. Novel insights into ovarian follicular development have however, been gained through the study of relevant knockout mouse models. For example, targeted disruption of the winged-helix/forkhead domain transcription factor, FoxL2, has provided evidence for a key role for this protein in differentiation and development of granulosa cells and all major somatic cell lineages surrounding growing oocytes from the onset of primordial follicle formation (87;88). Interestingly, mice lacking FoxL2 have also been shown to recapitulate features of the human condition blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), in that both males and females show distinctive craniofacial abnormalities with absence of upper eyelids. Sterility was evident only in the females (88).

As a transcription factor, the biological role of FoxL2 must, in a global sense, be to regulate transcription of a family of genes. The key issue of interest is the identity of this FoxL2 mediated "transcriptome." To begin to address this issue we have used gene profiling technology in an attempt to identify and characterize FoxL2-dependent genes that are differentially expressed as a result of activation or repression of FoxL2 in KK1 murine granulosa cells(176).

MATERIALS AND METHODS

Three groups of three 150mm tissue culture plates of KK1 cells seeded at approximately 50% confluency received the following treatments: 1-3 mock transfected (implying 30µl Fugene transfection reagent (Roche Molecular Biochemical, Switzerland), 4-6 FoxL2 -VP16 10µg in 30µl Fugene and 7-9 FoxL2-MAD 10µg in 30µl Fugene. Thus, FoxL2 was analyzed under activated conditions using full-length FoxL2 fused to the strong transcriptional activation domain of VP16 (133). Similarly, FoxL2-MAD SID fusion was included in order to simulate strong transcriptional repression of FoxL2. This features the 40 amino acid N-terminus of the MAD mSin3 interaction domain or SID (putative mammalian corepressor interacting with Mad family proteins) (179-182), fused to the entire mouse FoxL2 open reading frame. These constructs were validated in transient transfections of KK1 cells with a FoxL2 responsive luciferase reporter construct (3X-GRAS-LUC) (gonadotropin releasing hormone receptor activation sequence fused to luciferase) in the presence or absence of either FoxL2 VP16 or the FoxL2-SID fusion protein. Consistent with our previous results, overexpression of FoxL2-VP16 produced greater than 50-fold increase in luciferase expression. In contrast, coexpression of FoxL2-SID dramatically attenuated the ability of FoxL2-VP16 to activate the luciferase reporter. Following 24h incubation at 37°C the transfected cultures were washed in phosphate buffered saline (pH 7.4) (PBS) and trypsinized. A total of 10µg of PolyA+ RNA (1 µg/µl) was isolated using the Qiagen Rneasy RNA isolation kit per manufacturers instructions and submitted to the Colorado State Affymetrix

core facility for application to GeneChip expression array analysis. Specifically, nine Affymetrix mouse genome 430 2.0 gene chips arrays were used in these experiments. The chips used contain probe sequences for over 34,000 well-substantiated mouse genes. Data analysis was performed using GeneSifter.Net 2003-2004 VizX Labs, LLC, Seattle, WA. *Pairwise analysis* function was implemented, applying mean normalization and t-test statistical analysis.

RESULTS

To characterize changes in gene expression that result from specific transcriptional activation and/or repression of FoxL2, we used cDNA microarray technology to identify mRNA species that are over- or underexpressed in FoxL2 VP-16 and/or FoxL2-mad transfected cells relative to untransfected controls. The data identified approximately 600 genes/ESTs under each of three transfection conditions (tables 1,2,3,4) as having at least two-fold change in expression. Corresponding to FoxL2-VP16 transfection, 190 genes were activated and 408 repressed relative to control cells. Similarly, FoxL2-mad transfection resulted in upregulation of 210 genes and downregulation of 388 genes as compared to control cells. A third comparison of FoxL2-VP16 transfection versus that of FoxL2-mad featured 314 activated genes and 286 repressed. These genes represent three major ontologies including genes implicated in physiological processes, cellular processes and development. Herein, we have focused upon specific genes based upon differential regulation under conditions of both FoxL2-VP16 and FoxL2-mad overexpression and/or genes associated with patterns of gene activity regarded as particularly relevant to folliculogenesis. We have directed our evaluation of specific candidate genes in the following manner: 1) those involved with developmental processes (embryogenesis and organogenesis), 2) cytoskeletal elements and markers of cell adhesion and, 3) those implicated in ovulation and luteinization.

Perhaps most striking among genetic markers of embryogenesis organogenesis are changes in expression of members of the Wnt family

Table 1. Changes in gene expression detected by Microarray analysis of KK1 granulosa cells.

GenBank Accession no.	Gene ID	Function	Activated- 1 Basal	Repressed- 2 Basal	Activated- 3 Repressed	Repressed- 4 Activated
U20658	Wnt10b	Organogenesis Frizzled-2 signaling			3.1 fold induction	
NM_009290	Wnt8a	Frizzled-2 signaling	2.8 fold induction			
BB129109	Wnt7a	Organogenesis Frizzled-2 signaling	2.1 fold suppression			
NM_015814	Dkk3	Negative regulation Wnt receptor signaling		8.0 fold suppression	4.37 fold induction	
NM_010051	Dkk1	Negative regulation Wnt receptor signaling				8.1 fold induction
NM_013834	Sfrp1	Transmembrane receptor activity	4.5 fold induction	5.7 fold induction		
NM_007556	Bmp6	BMP signaling pathway, cell growth/maintenance			3.5 fold suppression	
BB698679	Bmpr1b	Transmembrane receptor protein serine/threonine kinase activity	7.52 fold induction			
JO3770	Hoxd3	Organogenesis , transcription factor		9.9 fold suppression		
NM_008711	Nog	Pattern specification skeletal, CNS		9.54 fold suppression		
NM_057173	Lmo1	Development, transcription factor		13.6 fold supression		
BB665367	Ntng1	Neurogenesis, axonogenesis				3.9 fold induction
NM_019740	Foxo3	Initiation primordial ovarian follicle growth, apoptotsis				4.8 fold induction
BB555419	Sox5	SRY-box containing gene 5, transcription factor activity		3.9 fold suppression		
U18673	Fgf8	Regulation cell cycle, fibroblast growth factor receptor binding		4.7 fold suppression		
NM_010514	Ifg2	Regulation of cell cycle, growth factor activity				2.4 fold suppression
NM_008341	Igfbp1	Regulation of cell cycle, growth factor binding		4.6 fold suppression		
NM_021543	Pcdh8	Morphogenesis of embryonic epithelium	9.5 fold induction	7.0 fold suppression		
NM_053127	Pcdhb2	Ceil adhesion				9.5 fold suppression
BB077413	Pcdh10	Cell adhesion	7.4 fold induction			
NM_053138	Pcdhb13	Cell adhesion				7.4 fold induction
X53177	Itga4	Cell-matrix adhesion		7.9 fold suppression		
BB280360	Mtap4	Cytoskeletal regulatory protein binding		22.9 fold suppression		
BB166188	Ube3a	Ubiqutin dependent protein catabolism	4.6 fold induction			
NM_008769	Otc	Amino acid metabolism ornithine carbamooyltransferase activity		7.8 fold suppression		
BB047737	Ube2b	Ubiqutin dependent protein catabolism	5.6 fold induction			

GenBank Accession no.	Gene ID	Function	Activated- 1 Basal	Repressed- 2 Basal	Activated - 3 Repressed	Repressed- 4 Activated
BE373450	Ak1	ATP metabolism, cell cycle arrest, adenylate kinase activity	4.7 fold induction			
BC024810	Guca1b	Receptor guanylyl cyclase signaling pathway	6.4 fold induction			
AV100992	Pparbp				4.6 fold induction	
NM_010323	Gnrhr	G-protein coupled receptor signaling pathway, rhodopsin-like receptor activity		3.2 fold suppression		
M22958	Prlr	(PRLR1) Lactation,epithelial cell differentiation, prolactin receptor activity	7.3 fold suppression			1.9 fold suppression
M22959	Prlr	(PRLR2) Lactation,epithelial cell differentiation, prolactin receptor activity		7.2 fold suppression		
BG067261	Star	C21-steroid hormone biosynthesis, cholesterol transporter activity	2.73 fold induction			
NM_007817	Cyp2f2	Cytochrome P450 family 2 subfamily 2 polypeptide 2 Electron transport, oxidoreductase activity				2.9 fold induction
NM_008294	Hsd3b4	Hydroxysteroid dehydrogenase-4, C21 steroid hormone biosynthesis, oxidoreductase activity		7.1 fold suppression		
BC005440	Ptger2	Prostaglandin E receptor 2 (subtype EP2)			16.6 fold induction	

Table 1. Changes in gene expression detected by Microarray analysis of KK1 granulosa cells. Gene accession numbers are from the GenBank database (http://www.ncbinlm.nih.gov/genbank/genbankoverview.html) Fold values are represented as mean from 3 Mouse Genome 430 2.0 genechip arrays per treatment condition with pairwise comparisons drawn between 1 activated (FoxL2-VP16 transfected)/ basal (mock transfected), 2 repressed (FoxL2-MAD transfected)/ basal, 3 FoxL2-VP16/ FoxL2-MAD, 4 FoxL2-MAD/ FoxL2-VP16

Table 2. FoxL2-VP16 Versus Control

Ontology	Total Number Genes/ ESTs	Activated	Repressed
Physiological Process	295	88	207
Cellular Process	221	74	147
Development	82	28	54

Table 3. FoxL2-Mad Versus Control

Ontology	Total Number Genes/ ESTs	Activated	Repressed
Physiological Process	311	111	200
Cellular Process	213	79	134
Development	74	20	54

Table 4. FoxL2-VP-16 Versus FoxL2-Mad

Ontology	Total Number Genes/ ESTs	Activated	Repressed
Physiological Process	304	161	143
Cellular Process	220	115	105
Development	76	38	38

signaling cascade. Fold changes in expression of wingless related MMTV integration sites or Wnts -7a, -8a and -10b, as well as secreted frizzled-related sequence protein 1 or sFRP-1 and dickkopf homologs of Xenopus laevis or Dkks -1, -3 are represented (table 1). Also noteworthy is suppression of homeobox D3 (9.9 fold), bone morphogenetic protein (BMP) specific binding protein Noggin (9.5 fold) and Lim domain only 1 (13.6 fold) under conditions of FoxL2-mad expression. BMP-6 also exhibited 3.5 fold suppression under conditions of FoxL2 VP16 expression relative to that of FoxL2 (FoxL2-mad).

Induction and/or suppression of protocadherins suggests potential involvement of FoxL2 in regulating expression of cytoskeletal elements and markers of cellular adhesion, ranging from 7-9 fold changes over basal levels. Protocadherins (Pcdh) 8 and 10 were markedly inducted under conditions of FoxL2 activation. In contrast, expression of FoxL2-mad led to repressed expression of protocadherins 8, beta 2 and integrin α-4 (8, 9.5 and 7.9 fold, respectively, while protocadherin β-13 exhibits 7.4 fold induction). Nearly 23 fold suppression of microtubule-associated protein 4 was also evident in RNA isolated from KK1 cells overexpressing FoxL2-mad.

Finally, change in expression of several genetic markers of terminal differentiation/luteinization of granulosa cells was evident, indicating nearly identical 7 fold suppressions of prolactin receptor, PRLR1 and PRLR2 under FoxL2 activation and repression, respectively (table 1). Through differential regulation of these two genes (PRLR1 and PRLR2), 2 of the 3 short isoforms currently identified for the mouse prolactin receptor are represented. Factors

involved in the acquisition of steroidgenic capacity of the postovulatory follicle also show fold changes. Specifically, the steroidgenic acute regulatory gene (StAR) was induced 2.7 fold under Foxl2 activation and 3β-hydroxysteroid dehydrogenase-4 (3β-HSD), presented 7.1 fold suppression under repression of FoxL2 (table 1). Lastly, prostaglandin E2 (PGE2), displays approximately 17 fold induction under FoxL2 activation.

DISCUSSION

Development

A number of genes known to serve as intercellular signaling molecules associated with developmental events, in particular members of the Wnt family, (specifying cell fates and tissue types, etc.) appear as candidate genes for FoxL2 regulation. In particular, members of the wnt family of signaling molecules have previously been implicated in follicular development and ovarian function (183). As FoxL2 is currently the earliest known marker of ovarian differentiation in mammals, (184), it is intriguing that Wnt 7a (table 1) has been implicated in regulating sex specific development of the Mullerian duct and its derivatives (185). Similarly, other components of the wnt signaling cascade (wnt-2, wnt-2b and wnt receptor frizzled-4) have been localized to specific compartments of the immature rat ovary (183). Thus, this signaling cascade may contribute to remodeling and patterning processes in the ovary throughout reproductive life (183). Additionally, there may be a functional connection between FoxL2 and the Wnt proteins mediated by the transforming growth factor beta superfamily (TGF-β). For example, the possibility of FoxL2 participating in regulation of TGF-β responsive genes has already been proposed (1). Consistent with this, FoxL2 been shown to interact with Smad 3 in both mammalian and bacterial two-hybrid assays (133). Thus, TGF-β and Wnt do represent two distinctly different groups of secreted proteins that signal through unique downstream effectors. However, cooperative interaction between these two pathways has been reported. For instance, both TGF-β and Wnt pathways have been shown to be capable of

regulating lymphoid enhancer binding factor (LEF1) target genes (186-188). Moreover, this may suggest that involvement of FoxL2 in transcriptional regulation by TGF-β could conceivably impact wnt target genes. Conversely, the influence of FoxL2 upon the expression of Wnt family members may impact TGF-β mediated events. In support of the latter, BAMBI (BMP and activin membrane bound inhibitor) overexpression has been shown to inhibit the normal growth suppressing role of TGF-β signaling in tumor cells, specifically via wnt signaling mediator, β-catenin, induced transactivation of BAMBI. (189) Thus, in light of such precedents we remain encouraged in our pursuit of functional involvement of FoxL2 in either or both TGFβ or Wnt/ Beta catenin signaling cascades.

Also potentially relevant for discussion at this time is TGF-β family ligand BMP-6 as a FoxL2-responsive gene (3.5 fold suppression, under conditions of FoxL2 VP16 expression, table 1). BMP-6 activity in the ovary includes reduction of FSH-induced progesterone synthesis and down regulation of FSH responsive genes up until the dramatic decline in BMP-6 expression noted to overlap with dominant follicle selection (190). BMP-6 inhibits FSH- and forskolin- induced cAMP production by suppressing adenylate cyclase activity (190) Thus, as FoxL2 may be responsive to forskolin-induced adenylate cylclase activity, indicated by its resultant intranuclear redistribution (chapter 3). BMP-6 regulation may normally oppose the FoxL2 responsive gene program, independently involved in the timely onset of follicular development and rise of a dominant follicle.

It is also interesting to note that another forkhead family member, FoxO3, is induced in cells overexpressing FoxL2-mad. FoxO3 has been implicated in

suppression of follicular activation and as such counteracts a potential route of premature ovarian failure, specifically through accelerated follicular initiation (91). Similarly, Schmidt et al has shown that nearly all primordial follicles are activated in FoxL2^{lacz} homozygous mutant ovaries (87). Thus, the observed phenotype of the null model may reflect the cumulative loss of both FoxL2 and FoxO3 proteins. As, FoxL2 may participate in regulation of the expression of FoxO3 in the common interest of maintaining an adequate number of primordial follicles throughout the reproductive life span.

Cytoskeleton/Cellular Adhesion

The protocadherins and related cadherin family members also emerge as potential candidates for FoxL2 regulation. These proteins are known to be active in early develoment, specifically in differentiation of the embryonic ectoderm as shown in Xenopus (191), however, the ovarian cadherins are also involved in the modulation of gonadotropin-stimulated signal transduction and granulosa cell differentiation (183;192). This has attracted our attention in light of the FoxL2 null phenotype that suggests an essential role for FoxL2 in differentiation and development of all major somatic cell lineages surrounding growing oocytes ((87;88). In further support of the involvement of FoxL2 in ovarian protocadherin expression is the potential participation of FoxL2 in gonadotropin (FSH)-induced transcriptional events as indicated by the observed intranuclear redistribution of FoxL2 in granulosa cells following forskolin treatment (chapter 3). Lastly, the abnormal histogenesis specifcally noted in murine FoxL2$^{-/-}$ ovaries may suggest

the involvement of two separate gene families, the Wnt signaling molecules and cadherins, both potentially influenced by FoxL2. As the Wnt proteins and cadherins normally compete for a mutual signaling mediator, β-catenin, (193), the observed FoxL2$^{-/-}$ phenotype may reflect a scenario in which either insufficient levels of protocadherin expression or a hyperactive wnt signaling pathway results in failure to sequester β-catenin at the plasma membrane in the cadherin adhesion complex (183), essential for adhesion and maintenance of the ovarian epithelium (193).

Ovulation/Luteinization

Several markers of terminal differentiation and luteinization of granulosa cells appeared to be differentially regulated by the FoxL2 fusion proteins. First, suppression of prolactin receptor expression has been indicated. In this regard, it is interesting to note that similar phenotypic characteristics are evident in the ovaries of mice deficient in either PRLR $^{-/-}$ or FoxL2 $^{-/-}$ (194) (88). It may be that under the influence of FoxL2 the respective programs for the differentiation of granulosa cells are impacted at both the onset of follicular development (FoxL2 null ovary) and in the course of terminal differentiation (PRLR null ovary).

The acquisition of steroidgenic morphology of luteinizing follicles is dependent upon the steroidgenic acute regulatory gene (StAR) that is necessary for the conversion of cholesterol into pregnenolone (195). The induction of StAR under conditions of FoxL2-VP-16 overexpression (table 1) has been further validated by transfection studies in which wild type murine FoxL2 results in

activation of the StAR promoter (Escudero unpublished). We should note that this finding is in contrast to results of Hsueh et al. in which FoxL2 mediated repression of the StAR promoter (196). The disparity that exists between our results and those of Hsueh et al may partially reflect the cell model. That is, the normal expression level of the transcription factor FoxL2 would be highest in granulosa cells of small/medium growing follicles and much diminished in luteinizing granulosa cells. Our data used KK1 cells that are a more luteinized line than the Chinese hamster ovary line (CHO) that Hsueh et al. studied. Thus, regulation may vary at different stages of follicular development.

In conclusion, we recognize that overexpression of fusion proteins is imperfect and might reflect "potential," rather than "natural" gene targets (197). As such, future studies should be directed at developing "knock-down" strategies and over expression of wild-type protein. We should point out, however, that wild type FoxL2 alone is a very weak transcriptional activator. Thus, wild type FoxL2 overexpression may not provide a sufficiently robust model of FoxL2 activation. At this time, the need for validation of the altered expression levels of these genes by Northern blot and/or quantitative RT-PCR is recognized. Nonetheless, as the data stands, some related clusters of genetic markers have been indicated that may at least suggest a potential entry point and provide license for future endeavors in the quest for ovarian targets of FoxL2.

In short, we acknowledge the preliminary nature of these data. However, at present the gene "programs" regulated by FoxL2 are essentially undefined. As such, our hope is that these data provide potential targets for future analogies

and, perhaps, the first glimpse of the FoxL2 responsive transcriptome in granulosa cells.

CHAPTER SEVEN

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