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Investigating the role of the extracellular matrix protein, Spondin 1, in ovarian folliculogenesis

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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INVESTIGATING THE ROLE OF THE EXTRACELLULAR MATRIX PROTEIN, SPONDIN 1, IN OVARIAN FOLLICULOGENESIS

(Thesis format: Integrated Article)

by

Caitlin Sarah Elizabeth O’Flynn

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Folliculogenesis is the ovarian process in which a follicle, consisting of an oocyte surrounded by granulosa cells (GCs), theca cells, a fluid-filled antrum, and a basal lamina separating GCs from TCs, grows and differentiates, culminating in development of a fertilizable oocyte. TCs under the influence of luteinizing hormone (LH) produce androgens. GCs under the influence of follicle stimulating (FSH) hormone proliferate, produce steroid hormones, and differentiate to become responsive to the surge of LH that initiates ovulation. The cellular processes of folliculogenesis require cell-extracellular matrix (ECM) interactions. Spondin 1 (SPON1) is an ECM protein primarily studied for its role in nervous system development as a nerve outgrowth signalling molecule, but is also known to affect cell viability, differentiation, and migration in non-neural tissues and cells. Evidence that Spondin 1 is functional within the ovary includes its discovery in bovine follicular fluid, its increased mRNA expression in response to estrogen in uterus and mammary gland, its decreased mRNA expression in GCs of mice null for estrogen receptor β, and its overexpression in ovarian cancer. Despite the possible importance of Spondin 1 in the ovary it has never been characterized in this tissue. This study was undertaken with the goal of elucidating roles of Spondin 1 in the ovary. Experiments with the human GC tumour cell line, KGN, found that Spondin 1 increases cell viability and proliferation possibly by activating the mTORC1 complex, and decreases cAMP-induced progesterone production by inhibiting cAMP-induced STAR transcription. Experiments with mouse primary GCs corroborated the effects of Spondin 1 on granulosa cell viability and again found a role for Spondin 1 in steroidogenesis, however, progesterone production was increased in these cells. Interestingly, Spondin 1 co-localized with vasculature markers in the mouse ovary and uterus, two of only a few tissues where the vascular network is dynamic, suggesting a role in angiogenesis. Finally, characterization of the reproductive phenotype of Spon1−/− females revealed that loss of Spondin 1 results in subfertility marked by smaller litter sizes, decreased ovulation capacity, and smaller ovarian weight. These findings support an important role for Spondin 1 in ovarian folliculogenesis and maintenance of optimal fertility.
Keywords

Spondin 1, F-spondin, ovary, granulosa cell, folliculogenesis, ovulation, fertility, ovarian angiogenesis, corpus luteum, steroidogenesis
Co-Authorship Statement

Chapters 1, 4 and 5 were written by Caitlin O’Flynn and edited by Dr. Bonnie Deroo.

Chapters 2 and 3 was co-written and edited by Caitlin O’Flynn and Dr. Bonnie Deroo.

All experiments in Chapters 2, 3, 4, and 5 were performed by Caitlin O’Flynn with the exception of confocal microscopy in Figure 3-3 and Appendix F which was performed by Dr. Macarena Pampillo. Dr. Pampillo also helped to maintain the mouse colony and monitor the breeding study in Chapter 4.

The red-green intensity plots in Appendix C were created by Dr. Andrew Fernandes who also consulted on statistical analyses throughout the thesis.
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Thank you to my committee members, Dave Edgell and Joseph Torchia, for your guidance, criticism (constructive!), and engaging discussions throughout my degree. Thank you, also, to my examiners, James Squires, Harvey Goldberg, Dan Hardy, and Eva Turley for your thoughtful questions and critiques in helping me prepare my final thesis.

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I would like to thank my family, my Mum, my Dad, and my sister. You have loved and supported me my entire life and always taught me that I could do and be whatever I wanted. I would unequivocally not have accomplished this feat without you. Thank you.

And finally, I would like to dedicate my thesis to my husband, Eamon, my best friend. I could not have made it through without you and your support, encouragement, understanding, and love. Words cannot describe my gratitude.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>8-CPT</td>
<td>8-(4-chlorophenylthio)-adenosine-3’,5’-cyclic monophosphate</td>
</tr>
<tr>
<td>ACTA2</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>a disintegrin and metalloproteinase with thrombospondin motifs</td>
</tr>
<tr>
<td>Akr1c18</td>
<td>alpha-keto reductase family 1, member C18/20α-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>AKT(1)</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMH</td>
<td>anti-mullerian hormone</td>
</tr>
<tr>
<td>ANG</td>
<td>angiopoietin</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ApoER2</td>
<td>apolipoprotein E receptor 2/LRP8</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<td>AREG</td>
<td>amphiregulin</td>
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<tr>
<td>BCT</td>
<td>betacellulin</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA125</td>
<td>cancer antigen 125</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>CG</td>
<td>ciliary ganglion</td>
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<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<td>-----------</td>
</tr>
<tr>
<td>CL</td>
<td>corpus luteum</td>
</tr>
<tr>
<td>COC</td>
<td>cumulus oocyte complex</td>
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<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
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<td>CREB</td>
<td>cAMP response-element binding protein</td>
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<tr>
<td>CS</td>
<td>cholesterol sulfate</td>
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<tr>
<td>Cyp11a1</td>
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<td>Dazl</td>
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<tr>
<td>dbcAMP</td>
<td>dibutyryl cAMP</td>
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<tr>
<td>DEPTOR</td>
<td>DEP domain-containing mTOR-interacting protein</td>
</tr>
<tr>
<td>DES</td>
<td>desmin</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dpc</td>
<td>days post coitus</td>
</tr>
<tr>
<td>eCG</td>
<td>equine chorionic gonadotropin, an FSH analog</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ERE</td>
<td>estrogen responsive element</td>
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<td>EREG</td>
<td>epiregulin</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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</table>
ERβ  estrogen receptor β
Esr2  estrogen receptor β (gene)
FBS   fetal bovine serum
Foxl2 forkhead box L2
Foxo3 forkhead box O3
FSH   follicle stimulating hormone
FSHR  FSH receptor
GAG   glycosaminoglycans
GC    granulosa cell
Gdf9  growth and differentiation factor 9
GL    granulosa lutein cells
GnRH  gonadotropin releasing hormone
HA    hyaluronan
hCG   human chorionic gonadotropin, an LH analog
Hsd17b1 17β- hydroxysteroid dehydrogenase 1
Hsd17b3 17β- hydroxysteroid dehydrogenase 3
Hsd3b1 3β- hydroxysteroid dehydrogenase 1
HUVEC human umbilical vein endothelial cell
IGF1  insulin-like growth factor 1
IL-6  interleukin 6
IU    international unit
kb    kilo-base pairs
kDa   kilodaltons
<table>
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<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>Lhcg</td>
<td>luteinizing hormone receptor (gene)</td>
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<td>LHR</td>
<td>luteinizing hormone receptor</td>
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<td>steroid acute regulatory protein</td>
</tr>
<tr>
<td>TC</td>
<td>theca cell</td>
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<td>transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloproteinases</td>
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<tr>
<td>TL</td>
<td>theca lutein cells</td>
</tr>
<tr>
<td>Tnfaip6</td>
<td>tumour necrosis factor, alpha induced protein 6</td>
</tr>
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<tr>
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<td>very low-density lipoprotein receptor</td>
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<td>vascular smooth muscle cell growth promoting factor or Spondin 1</td>
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<tr>
<td>WNT</td>
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Chapter 1: Introduction

1.1 Folliculogenesis Overview

Ovarian folliculogenesis is the coordinated growth and development of somatic and germ cells from primordial germ cells surrounded by a single layer of squamous pre-granulosa cells to a preovulatory follicle comprised of theca cells (TCs), cuboidal differentiated granulosa cells (GCs) and an oocyte capable of fertilization. An oocyte proceeds through several follicular stages before ovulation occurs: primordial germ cell, primordial follicle, primary follicle, secondary follicle, and antral/preovulatory follicle (Figures 1-1 and 1-2). After ovulation follicular remnants that were not expelled become the corpus luteum which produces progesterone to support pregnancy. A primordial follicle consists of an oocyte surrounded by a single layer of squamous pre-granulosa cells. In a primary follicle there is still only a single layer of cells surrounding the oocyte but they have differentiated to become cuboidal GCs. Once these GCs multiply to form a few layers and are surrounded by TCs (separated from GCs by a basal lamina) the follicle is known as a secondary follicle. An antral or preovulatory follicle consists of several layers of GCs and a fluid filled antrum with cumulus GCs surrounding the oocyte. The corpus luteum is highly vascularized and consists of luteinized granulosa and theca cells. Specific details of each stage are discussed in the following paragraphs.

The process of folliculogenesis is dependent on the cooperative hypothalamic-pituitary-gonadal axis in which the hypothalamus, pituitary and ovaries act as endocrine glands. At puberty, the hypothalamus begins releasing gonadotropin releasing hormone (GnRH) in pulses, signalling the pituitary to release the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Exposure of the ovary to FSH and LH results in the growth of follicles and follicular production of estrogen (primarily 17β-estradiol) which feeds back to the pituitary affecting release of gonadotropins. Before ovulation occurs a pulse of LH is released by the pituitary and pre-ovulatory follicles expressing adequate levels of the LH receptor (LHR) undergo ovulation.
Figure 1-1. The Follicle and Follicular Stages

A) A representative antral follicle demonstrating parts of the follicle including: the oocyte, granulosa cells, theca cells, and the antrum. B) Stages of folliculogenesis.
Figure 1-2. Overview of Folliculogenesis

An overview of folliculogenesis from primordial follicle to corpus albicans. The oval represents the ovary and the tube-like structure above and to the right represent the uterus and a fallopian tube. 1) primordial follicles 2) primary and secondary follicles 3) antral or preovulatory follicle 4) ovulation 5) corpus luteum 6) corpus albicans.

GCs and TCs that are not lost when ovulation occurs differentiate under the influence of LH to granulosa lutein and theca lutein cells. Lutein cells produce high levels of progesterone required by the uterus during early pregnancy. Only a small number of oocytes complete folliculogenesis and are ovulated. The majority are lost to atresia at various stages throughout the process.

1.1.1 Epiblast to Primordial Follicle

During early mammalian embryonic development primordial germ cells (PGCs) arise from the epiblast. Differentiation of epiblasts into PGCs in mice is determined by signalling of bone morphogenetic proteins (BMP), BMP2, BMP4, and BMP8B [1]. These PGCs migrate toward the undifferentiated gonad from the yolk sac. Once at the gonad they proliferate from only a few dozen cells to several thousand in mice [2] and several million in humans [3]. If their fate is to become oogonia they undergo several rounds of mitosis and then differentiate into oocytes and enter and arrest in prophase I of meiosis [4, 5], while spermatogonia proliferate and then become quiescent. PGC proliferation is regulated by several different signalling proteins including activin, inhibin, BMPs, and transforming growth factor β, (TGFβ) [6-9]. However, there is evidence that the specific pathways that affect proliferation are not conserved between different animals, as activin increases proliferation in humans [8] but decreases it in mice [6].

Differentiation of PGCs to either oogonia or spermatogonia is determined by several sex determining factors. The process of sex determination has recently been revealed to be much more complex than the previously accepted notion of a default female pathway. In the previous model of sex determination presence of the sex determining region Y (SRY) gene in the genome resulted in suppression of development of female structures by anti-mullerian hormone (AMH) [10, 11] and initiation of testis development, while absence of SRY resulted in the default development of ovaries and other female anatomy. SRY is indeed required to activate the signalling cascade that begins testis development but now it is known that the developing and developed ovary expresses male-suppressing genes. Notably, in the absence of either wingless type MMV integration site family, member 4 (WNT4) or R-spondin (RSPO1) in mice the
ovary undergoes sex-reversal to a more testicular phenotype [12-15]. While loss of forkhead box L2 (FOXL2) in adulthood results in partial sex reversal with ovarian cells differentiating to testis cell types [16]. Interestingly, male-to-female sex reversal in seen in genotypically XY mice stably overexpressing β-catenin (a downstream target of WNT) [17]. It is likely these above mentioned proteins signal to the PGCs to differentiate to oogonia or spermatogonia.

Proliferation of PGCs in the mammalian primordial ovarian gonad results in the formation of syncytia or “nests of cells” that arise due to incomplete cytokinesis producing intercellular bridges ([18], reviewed in [19]). After nests are formed cells enter meiosis. In mice this occurs around 13.5 days post coitus (dpc) [20] and in humans it is around 13 weeks of gestation [21]. These nests break down to generate primordial follicles perinatally in mice [22] and in the second trimester in humans [23]. At this stage the majority of PGCs are lost to atresia with only 33% of mouse cells [22] and only two million cells in humans surviving to become primordial follicles [3]. This loss of cells is not random and mechanisms are in place to ensure that the “healthiest” cells go on to become primordial follicles. In Drosophila nests, all cells save one become nurse cells and provide cellular components such as nutrients and mitochondria to the future oocyte before undergoing apoptosis [24]. A similar mechanism has been seen in mice where programmed synchronous nest breakdown is accompanied by movement of mitochondria through intercellular bridges [22, 25]. While the nests are breaking down, surrounding somatic cells invade the nests and eventually surround the oocyte. This forms the primordial follicle consisting of a single oocyte with a single layer of epithelial pre-granulosa cells [4, 22].

1.1.2 Primordial Follicle to Secondary Follicle

To maintain fertility primordial follicles remain quiescent for months or even decades, depending on the species, before being activated to become primary follicles. The exact mechanisms that allow for activation of one primordial follicle but not its neighbour are still being discovered. Several proteins and pathways are known to be involved in suppressing activation of primordial follicles. AMH, while not expressed in
the ovary during development, is expressed postnatally [26] and suppresses formation of primary follicles [27]. Loss of AMH results in over-activation of primordial follicles [28] while overexpression of AMH inhibits activation and decreases FSH responsiveness of early follicles [29]. Over-activation of the primordial follicle pool is also seen in mice lacking forhead box O3, Foxo3a [30] and mice lacking phosphatase and tensin homolog, Pten in oocytes [31]. The mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway is involved in maintaining dormancy as loss of tuberous sclerosis 1, Tsc1 [32] or -2, Tsc2 [33], repressors of mTOR, results in activation of the entire pool. Further studies into these pathways should yield insight into the dynamics of primordial follicle activation.

When a primordial follicle is activated the somatic, squamous pre-granulosa cells differentiate into a single layer of cuboidal GCs forming a primary follicle. In mice with inactivation of FOXL2 oocytes prematurely grow while pre-granulosa cells do not differentiate to GCs [34], suggesting that FOXL2 is one of the factors responsible for initiating granulosa cell differentiation. Secondary follicles are formed when the GCs proliferate to more than one layer of cells and when TCs surround GCs. Granulosa cell proliferation requires oocyte-produced growth and differentiation factor 9, (GDF9) as follicles in mice null for Gdf9 never grow to the secondary stage [35]. The process of theca cell recruitment is not very well understood but signals from the GCs are believed to recruit stromal cells to differentiate into TCs [36]. Endothelial cells are also recruited to form a vascular network inside the TCs and outside of the basal lamina of the follicle. This vasculature delivers hormones and nutrients and is essential for the survival and further growth of follicles [37, 38].

1.1.3 Secondary Follicle to Pre-ovulatory Follicle

Growth of a follicle to the pre-ovulatory stage is dependent on the ability of GCs to respond to FSH and TCs to respond to LH, and therefore depends on the expression of follicle stimulating hormone receptor (FSHR) and LHR. Mice lacking either the ability to produce FSH (Fshb−/−) or the ability to respond to FSH (Fshr−/−) fail to produce follicles past the secondary stage [39, 40]. Follicles in mice unable to respond to LH only progress
to the early antral stage [41]. Through the combined actions of FSH and LH, follicles grow, produce steroids, and form a fluid-filled antrum.

The primary function of TCs after the secondary follicle stage is to collect cholesterol from the blood supply and produce androgens, specifically androstenedione and testosterone as precursors for 17β-estradiol production in GCs. GCs also produce progesterone, which may be used by TCs as a substrate for androgen production. The expression of steroidogenesis enzymes in TCs is reliant on the response of TCs to LH. Several factors are known to increase LHR levels in TCs, including insulin-like growth factor 1 (IGF1), stem cell factor (SCF), and inhibin. These proteins are produced by GCs suggesting that GCs continue to regulate TC differentiation after recruitment [42]. Granulosa cells respond to the increase in androgens by expressing FSHR [43]. Then in response to FSH, GCs proliferate and express steroidogenesis genes to produce 17β-estradiol. Cooperative steroid hormone production by TCs and GCs in response to LH and FSH, respectively, is known as the two-cell, two-gonadotropin theory (Figure 1-3).

The formation of the antrum is not well understood but both GCs and TCs are thought to be involved. One theory is that GCs create an osmotic gradient by producing molecules such as hyaluronan and versican which drives movement of fluid from the vascular network within the TCs into the follicle [44]. Follicular fluid is similar to serum in its composition although plasma proteins larger than 100 kDa are found at lower concentrations suggesting a barrier to movement of larger molecules [45]. The GCs that surround the oocyte after formation of the antrum are known as cumulus cells or the cumulus oophorus while the cells closer to the basal lamina are termed mural cells. In response to FSH, mural GCs produce high levels of 17β-estradiol and express LHR. Cumulus GCs do not produce as many steroids as mural GCs but provide nutrients and protection to the oocyte [46].

Feedback from the follicle to the pituitary is required for the follicle to reach the pre-ovulatory stage. As granulosa cells proliferate, 17β-estradiol levels increase and affect production of GnRH at the hypothalamus. High levels of 17β-estradiol result in lower production of FSH and higher production of LH. Granulosa cells also affect gonadotropin production through signalling of TGFβ family members, activin and inhibin, so named for their effects on FSH. Small follicles and early antral follicles
Figure 1-3. The two-cell, two-gonadotropin model.

Schematic diagram showing a theca cell (TC) and a granulosa cell (GC) responding to luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to make steroid hormones. TCs respond to LH by increasing cAMP signalling to make testosterone from cholesterol. Testosterone travels through the basal lamina to GCs where it is made into 17β-estradiol (estradiol) under the influence of FSH. Estradiol is then released into circulation. GCs also make progesterone from cholesterol to be released into circulation and as a substrate for TC androgen production.
express more activin while late-antral/pre-ovulatory follicles express more inhibin and therefore suppress production of FSH. The end result of gonadotropin-dependent follicular growth is a pre-ovulatory follicle that produces high levels of 17β-estradiol and inhibin to stimulate a large surge of LH from the pituitary and expresses enough LHR to respond to that surge.

1.1.4 Ovulation – Corpus Luteum – Corpus Albicans

In follicles capable of response, the LH surges results in oocyte maturation, expansion of the cumulus GCs, ovulation, and luteinization of GCs and TCs. LH rapidly changes expression of a myriad of genes [47] and activates several pathways that work both cooperatively and parallel to each other to begin these processes. The initial signal that activates these pathways is signalling of the LHR in mural granulosa cells [48] to guanine nucleotide-binding proteins (G proteins) which increase the levels of cAMP [49]. The pathways activated by this signalling include the protein kinase A (PKA), phosphoinositide-3 kinase/protein kinase B (PI3K/AKT), and RAS pathways [4]. Several molecules within these pathways are known to be required for a full response to LH. For example, secretion, in mural GCs, of the epidermal growth factor (EGF)-like growth factors, epiregulin (EREG), amphiregulin (AREG) and betacellulin (BCT) is induced by LH signalling [50]. These factors activate EGF receptors in cumulus cells [51], and disruption of the EGF-pathway blocks ovulation [52]. LH-dependent activation of extracellular signal-regulated kinase (ERK)1/2 via multiple signalling pathways is essential for oocyte maturation, cumulus expansion, and ovulation [53].

**Oocyte Maturation.** During primordial germ cell formation, the oocytes arrest at Prophase I of meiosis. Following the LH surge oocytes resume meiosis and then arrest again at Metaphase II until fertilization or death. Maintenance of arrest and resumption of meiosis following the LH surge is not fully understood but factors derived from the follicle have been discovered. The first evidence that follicle-derived factors were involved in maintenance of arrest was that oocytes removed from large follicles of rabbits and cultured in nutrients spontaneously resumed meiosis [54], suggesting that loss of
some follicle factor(s) results in oocyte maturation. This finding has since been confirmed in numerous species. The cyclic nucleotides, cAMP and cGMP, regulate oocyte maturation. High levels of cAMP are required to maintain arrest and must be produced by the oocyte itself [55] as cumulus GC cAMP production alone is not sufficient to maintain arrest [56]. Resumption of meiosis is coincident with reduction in cAMP levels and maintaining high levels of cAMP stops spontaneous meiosis in cultured oocytes [57, 58]. Meiotic resumption is also accompanied by a decrease in cGMP, and injection with cGMP maintains arrest in rat oocytes [59]. cGMP agonists have no effect in oocyte culture but impair arrest in cumulus oocyte complex (COC) culture suggesting that cGMP is derived from somatic cells [60]. cGMP inhibits breakdown of cAMP in oocytes [61]. High levels of cGMP in cumulus GCs are produced through the action of natriuretic peptide receptor B/guanylate cyclase B, NPR2 which is stimulated by natriuretic peptide precursor C, NPPC, produced in mural GCs [62]. The LH surge decreases activity of NPPC and NPR2, decreasing cGMP levels, which in turn decreases cAMP levels and re-activates meiosis [63, 64].

**Cumulus Expansion and Dissociation.** Following the LH surge, cumulus GCs produce proteins to enrich their extracellular matrix (ECM) in preparation for ovulation. The process is termed cumulus expansion due to the increased volume of the COC. The enriched ECM contains high amounts of hyaluronan (HA) as well as ECM proteins such as laminin, collagen IV, fibronectin, and proteoglycans. The production of these proteins is dependent on altered signalling in both cumulus GCs and the oocyte [65, 66] and several knockout mouse models have elucidated genes essential to this process. Prostaglandin signalling, activated by ERK1/2 [67], induces cumulus GCs to produce ECM proteins [68]. Prostaglandin endoperoxide synthase 2, Ptgs2, null mice are infertile due to failure of cumulus expansion and ovulation [69] and prostaglandin E2 receptor, Ptger2, null mice are subfertile due to incomplete cumulus expansion [70]. Loss of the tumour necrosis factor, alpha induced protein 6, Tnfaip6, which interacts with hyaluronan, in mice results in infertility as well [71]. Isolated COCs with poor cumulus expansion or oocytes with loss of cumulus GCs are unfertilizable [72]; the former due to an inability of sperm to degrade the hyaluronan-rich ECM while the latter suggests that
cumulus GCs maintain viability of the oocyte. Concurrent with expansion, the COC dissociates from the surrounding follicle resulting in an oocyte surrounded by a single layer of expanded cumulus GCs [73].

**Ovulation.** Ovulation involves expulsion of the COC from the follicle and from the ovary itself. Several changes within the follicle must occur before ovulation including cumulus expansion and dissociation, breakdown of the ECM surrounding mural GCs and TCs, and possibly, increases in intrafollicular pressure. Following the LH surge the ECM is degraded by proteinase activity [74-76], primarily matrix metalloproteinases (MMPs) [77] and tissue inhibitors of metalloproteinases (TIMPs) [77, 78]. The ECM surrounding apical (closer to the ovarian epithelium) GCs, TCs, and cells of the surface epithelium of the ovary is degraded to allow for rupture and expulsion of the COC [77, 79]. The specific mechanism for localized degradation is still unclear but may result from localized expression of TIMPs [78]. The a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family has recently begun to be studied for its role in ECM degradation during ovulation. Currently only ADAMTS-1 has been found to be essential for ovulation [80-82] but other family members are hypothesized to be involved. A much-debated hypothesis is that follicular fluid increases following LH surge and increases intrafollicular pressure providing the force for ovulation to occur [83, 84].

**Luteinization and Angiogenesis.** After ovulation, GCs and TCs undergo differentiation to granulosa lutein (GL) and theca lutein (TL) cells in a process called luteinization. Endothelial cells enter the follicle and form capillaries by angiogenesis, the growth of new blood vessels from existing ones. The new structure created by luteinization and angiogenesis is called the corpus luteum (CL). Corpus luteum, meaning yellow body, is named due to the high amount of cholesterol required to produce high levels of progesterone to prepare the endometrium for implantation and pregnancy. If fertilization does not occur the CL regresses and becomes a corpus albicans (white body), and if fertilization occurs the CL continues to make progesterone until the placenta can produce its own. Following successful fertilization and implantation, the length of time
that the CL remains functional is dependent on the species but in all cases loss of the CL in early pregnancy results in loss of pregnancy.

The process of luteinization varies between species but the overall mechanisms are thought to be similar [85]. Again in luteinization, the LH surge signal is thought to be propagated by an increase in cAMP. The first marker of differentiated GLs and TLs is exit from the cell cycle as the main purpose of luteal cells is to produce progesterone. In most species, a marker of luteinization is loss of steroidogenesis enzymes responsible for steroid production downstream of progesterone: androgens in TLs and estrogens in GLs. However, in many primates production of androgens and estrogens is maintained following luteinization [86-89]. Genes involved in uptake and metabolism of cholesterol are upregulated in both TLs and GLs. For example, in GLs, production of steroid acute regulatory protein, StAR, not highly expressed in non-luteinized GCs, is essential for luteinization as GLs acquire the capability to produce large amounts of progesterone.

Within species there is variation in the extent to which GLs and TLs can be distinguished. In several species, the GLs are larger hypertrophied cells that produce more progesterone and the TLs remain smaller, while in rodents the large and small luteal cell populations differ in progesterone output but cannot be traced to initial GC and TC populations [86, 90-93].

To acquire large amounts of cholesterol as a substrate for progesterone and to better act as a paracrine gland the corpus luteum must become vascularized. Prior to ovulation, the vasculature of the follicle exists only in the TCs and does not penetrate the basal lamina. After ovulation, endothelial cells create new capillaries that invade the follicle along with the TCs. The primary factor that initiates angiogenesis is vascular endothelial growth factor, VEGF. VEGF expression is turned on during differentiation of GCs to GLs, while endothelial cells express the VEGF receptors, VEGFR1 and VEGFR2 suggesting that these cells signal to the endothelial cells to invade the CL [94-97]. While VEGF initiates angiogenesis, several other factors are important in regulating capillary growth including angiopoietins (ANGPT), vascular endothelial-cadherin (VE-cadherin) and thrombospondins. Angiopoietins, ANGPT-1 and ANGPT-2 stabilise new vessels and destabilise existing vessels, respectively [98, 99]. VE-cadherin is a cell adhesion molecule that is required for proper vessel development and whose inhibition by antibody
in mice results in loss of follicular vascularization [100]. Thrombospondins, TSP-1 and TSP-2, are anti-angiogenic factors within the ovary and expressed in GCs of small follicles. Expression patterns of TSP-1 and -2 differ by species, with bovine CLs having no expression [101] while rats express it in early but not late CLs [102]. Although currently unknown, it is likely that downregulation of TSP-1 and -2 is required for normal CL vascularization.

1.1.5 ECM Proteins and Folliculogenesis

The complex process of folliculogenesis involves proper functioning of the extracellular matrix (ECM) both between cells and at the basal lamina. As mentioned in 1.1.5, changes to the ECM are essential for cumulus expansion and ovulation. But the frequent tissue remodelling, cell growth, and differentiation required for preparing an oocyte for fertilization necessitates compositional changes and altered signalling of the ECM throughout folliculogenesis. ECM proteins affect cellular proliferation, survival, morphology, and steroidogenesis. ECM proteins are highly variable, with some proteins serving as structural components and others primarily as signalling molecules.

The ECM of each tissue is unique but within the follicle there is a highly specialized type of matrix called the focimatrix [103]. The ECM usually completely surrounds cells or cellular structures in most tissues but the focimatrix exists as small deposits of unique matrix between GCs. These deposits increase in size and number as the follicle grows [103] and are found in different amounts depending on cellular location [104]. As well, expression of focimatrix genes has been correlated with expression of steroidogenesis genes [104, 105]. These data suggest that the focimatrix plays a significant role in granulosa cell function although that role is not yet clear.

Proper functioning of the basal lamina is essential for maintaining the blood-follicle barrier until ovulation occurs. Although the blood-follicle barrier was first noted almost 50 years ago, its exact mechanisms remain largely unknown [106]. It is generally understood that size is a determinant of permeability; however different molecular weights from 100-500 kDa have been reported as the minimum size. The charge of a
molecule is also important with negatively charged proteins being excluded from transiting the barrier.

Structural proteins within the ovarian ECM include, but are not limited to, collagens, fibronectin, laminins, nidogens, and perlecan. Along with proteoglycans (which are mostly heparin sulfate proteoglycans in the follicle), these proteins form the scaffolding of the matrix. A second role of these proteins is cellular signal transduction achieved through direct or indirect interaction with cell surface proteins, primarily integrins. Several studies of artificial matrices containing various ECM proteins have shown the effects of these proteins on proliferation, adhesion, and morphology of GCs or ovarian tissue as well as steroid production of GCs [107-112]. As previously mentioned, matrix metalloproteinases (MMPs), and their inhibitors, TIMPs are required for ECM breakdown and therefore turnover and remodelling of ECM components [113].

Over the past two decades, the ECM has been recognized for its importance not only in structural integrity but in signal transduction as well. A second group of proteins not involved in structure within the ECM has begun to be extensively studied. Examples of important ECM signalling molecules within the ovary include thrombospondins, TSP-1 and TSP-2, R-spondin, secreted protein acidic and rich in cysteine (SPARC), and ADAMTS proteins.

Several null mouse models of ECM proteins show that specific proteins are essential for normal folliculogenesis. However, even more numerous are examples of ECM proteins whose functions are so important that loss is lethal or results in mild or no phenotypic changes to fertility due to redundancy.

1.2 Spondin 1

1.2.1 The Protein

1.2.1.1 Identification

Spondin 1 (protein symbol SPON1), encoded by Spon1, was first identified in 1992 as a secreted ECM protein highly expressed in the rat floorplate where several aspects of the developing nervous system are controlled [114]. Therefore it is
unsurprising that currently the majority of published research about Spondin 1 concerns its role in the early nervous system. Spondin 1 was initially named F-spondin [114] after its location in the floorplate of the neural tube (F) and its thrombospondin repeats (spondin) (see Structure below) and is also known as vascular smooth muscle cell growth promoting factor or VSGP [115]. Spondin 1 is a thrombospondin type 1 repeat superfamily member and is considered part of the neuronal subclass of that family. Spondin 1 is highly conserved, with a homologous protein found in over 140 species of mammals, birds, fish, amphibians and insects.

1.2.1.2 Structure

Spondin 1 is comprised of 807 amino acids, with a hydrophobic N-terminal leader sequence, a consensus signal peptide cleavage site and no other hydrophobic regions [114]. For these reasons it was initially determined and then proven to be a secreted protein. The 807 amino acids can be divided into three regions. The first 1-200 amino acids are similar to a region in Reelin which has been referred to in Spondin 1 as the “reelin domain” and in Reelin as the “F-spondin domain” and will be referred to here as the “reeler domain” [116]. Amino acids 201-400 share similarity with a domain in Spondin 2, also known as Mindin. This domain is sometimes divided into two smaller domains and referred to as the FS1 and FS2 domains or is treated as a single domain called the FS or spondin domain [117-119]. Amino acids 441-807 comprise six thrombospondin type 1 repeat (TSR) domains. Studies of the crystal structure of the reeler domain found several arginine and lysine residues that provide surface positive charges for heparin binding and potentially other glycosaminoglycan (GAG) binding [120]. Evidence of weak dimerization of two reeler domains was also observed and it has been hypothesized that through binding of GAGs, Spondin 1 interaction with other GAG-binding proteins could be facilitated [120]. The spondin domain, FS, is not well-studied but may play a role in nerve outgrowth signalling [121]. TSR domains are found in several proteins, including thrombospindins 1 and 2 (TSP-1, encoded by Thbs1; TSP-2, encoded by Thbs2), R-spondins, and ADAMTSs and are responsible for cellular adhesion, anti-angiogenic activity, tumour progression, and activation of TGFβ [122]. There are several regions within these TSRs that provide insight into its possible
functions. A CSVCTG motif is present in the fourth TSR while CSVSCG and CSACTG are found in the second and third. This motif inhibits angiogenesis and binds CD36 in TSP-1 [123, 124]. TSRs one, three, five, and six all contain basic residues and the crystal structures of five and six show that these residues all are located on one face of the domain. Therefore, these TSRs are predicted to bind heparin and other GAGs like the reeler domain [114, 122]. Each TSR contains a WxxW sequence, known to bind TGFβ. These tryptophan residues have also been indicated as potential sites for C-mannosylation, while several serine and threonine residues are potentially O-fucosylated [125]. In TSRs one, four, and five these sequences are WSxWS which is also found in CNTF, LIF, and ILs, and mutation in IL2 results in loss of transmembrane signalling [114]. WSxW and a KRKF motif are found in the sixth TSR [122], and are required for latent TGFβ activation by Tsp-1 [126].

1.2.1.3 Proteins with Similar Domains

Spondin 1 shares domains with several proteins, most of which are members of the thrombospondin type 1 repeat domain (TSR) superfamily (Fig. 1-4). These proteins are ECM or transmembrane proteins and contain between one to twenty-five TSR domains [127]. A major role for many TSR proteins is in the nervous system [127]. A few TSR family members are discussed below, as well as the non-thrombospondin protein, Reelin.

**Thrombospondin 1 and 2** – TSP-1 and TSP-2 are thrombospondin family members defined as matricellular proteins because they exist within the ECM but do not contribute to its structural integrity. Instead, they are involved in cell-matrix signalling. TSP-1 and -2 are primarily recognized for their role as anti-angiogenic molecules but are also involved in cell adhesion and wound healing [128]. Tsp-1 also contains the WxxW and KRKF motifs responsible for binding and activating TGFβ [126]. As mentioned in 1.1.4, both TSP-1 and TSP-2 are expressed in the ovary in a species-dependent manner. In rat, TSP-1 is expressed in GCs of early and late antral follicles and early but not late corpora lutea, while TSP-2 is seen in early corpora lutea [102]. Mice null for Thbs1 produce fewer and smaller litters; however, these matings were homozygous and therefore it cannot be concluded if one or both sexes are subfertile [129].
Figure 1-4. Spondin 1 and similar proteins; domain diagrams.

Representative model of Spondin 1, showing its reeler, spondin, and TSR domains. Representative models of thrombospondin 1 (Tsp-1), R-spondin, Spondin 2, and Reelin, showing their reeler, spondin, or TSR domains, with other domains shown in grey.
**R-spondin** – The four R-spondins, R-spondin 1-4, are also neuronal TSR family members. Their name originates from the location of R-spondin 1 in the roofplate of the developing neural tube. The R-spondins have been primarily studied for their roles in Wnt signalling during embryogenesis [130]. Of the four R-spondins, R-spondin 1 is the most extensively studied for its role in gonadal development, and its loss can result in partial female-to-male sex reversal [12, 13]. R-spondin 3 and 4 have no known function in the ovary, but R-spondin 2 is expressed in the oocytes of primary to antral follicles and promotes follicle growth [131].

**Spondin 2** - The spondin domain is only found in Spondin 1 and Spondin 2. Spondin 2 is the only protein with two domains of homology with Spondin 1 as it contains one TSR domain. Spondin 2 has not been extensively studied in any species but is known to bind integrins and block AKT signalling. It is also involved in disease processes in the immune system, heart, and kidney [132].

**Reelin** - Only Reelin, Spondin 1, and the minimally studied ferric-chelate reductase 1, (FRRS1) are known to share the reeler domain. The term reeler comes from a mutant mouse that had a “reeling” gait that was due to lack of functional Reelin [116]. Like Spondin 1, Reelin is involved in neural development and disorders. Recently, Reelin was found to be expressed in the bovine and chicken ovaries, primarily in the theca cells of larger follicles, and to promote chicken granulosa cell proliferation in culture [133, 134].

1.2.2 Neural Development and Growth

As mentioned above, the majority of research published for Spondin 1 concerns its role in the nervous system. In 1992, the first paper to identify Spondin 1 did so in the floorplate of the embryonic day 13 (E13) rat embryo [114]. Along with mRNA expression in the floorplate, Spondin 1 was also found to be expressed in the spinal cord and peripheral motor and sensory nerves during their development [114]. Cells were transfected with myc-tagged Spondin 1 and this fusion protein was localized to the cell surface and in the medium suggesting that Spondin 1 is a membrane or ECM-associated secreted protein [114]. In one of the first demonstrations of its function, dorsal root
ganglia cells plated on purified Spondin 1 grew to a greater length than those plated on control proteins. Dorsal spinal cord cells adhered to Spondin 1 more than control bovine serum albumin (BSA) and this adhesion was diminished in the presence of heparin suggesting that Spondin 1 binds heparin [114]. These results were the first evidence that Spondin 1 regulates neural cell adhesion and nerve outgrowth. Subsequent work corroborated these findings in several different nerve types. Adhesion and outgrowth of neurons in the embryonic hippocampus is promoted by Spondin 1 [117]. Although expression diminishes after embryonic development, upregulation of Spondin 1 occurs during axon outgrowth of peripheral nerves following injury [121]. Spondin 1 is also a chemoattractant that promotes correct “pathfinding” of commissural axons which cross the floorplate [135].

Cleavage of Spondin 1 into smaller polypeptides also plays a role in signalling. In HEK293 (human embryonic kidney) but not COS (green monkey kidney) cells (suggesting a cell type-dependent process), Spondin 1 is cleaved into two half proteins, with the reeler and spondin domains in one half and the TSRs in the other [121]. The TSRs can also be cleaved between the fourth and fifth repeats [121]. This cleavage is achieved by plasmin and releases the more N-terminal part of the protein from the ECM as the fifth and sixth TSRs bind the ECM but TSRs 1-4 do not [136]. The TSR half of the protein is responsible for commissural axon guidance [137], while the reeler/spondin half is responsible for outgrowth [121]. Further study of the two TSR fragments shows that the 5-6 fragment remains at the basement membrane and the 1-4 fragment localizes to the cell surface of the floorplate where it is tethered by its interaction with apolipoprotein E receptor 2, ApoER2, also known as low-density lipoprotein receptor 8, LRP8, and other low-density lipoprotein receptor proteins, LDLRs [137]. Surprisingly, the Spondin 1-like protein Reelin also associates with LRP8 and other lipoprotein receptors, although it does not contain TSRs, suggesting that the reeler domain may still play a functional role in this interaction. The TSR 1-4 fragment inhibits growth of commissural axons into the floorplate while the 5-6 fragment promotes outgrowth below the floorplate [137]. Motor neuron outgrowth is also affected by Spondin 1 in a contact-repellent manner in vitro and again the TSR region was found to be more important than the N-terminal half of the
protein for this effect [138]. Spondin 1 also acts as a repellent molecule in the caudal half of somites in chicken embryos where it inhibits the migration of neural crest cells [139].

Spondin 1 not only affects nerve growth and adhesion but promotes nerve cell differentiation and viability as well. In a rat adult hippocampal precursor cell line, AHP, treatment with Spondin 1 promoted differentiation from neural precursor to nerve-like cells and this function was attributed to the reeler/spondin domains rather than the TSRs [140]. Spondin 1 increases the viability of chicken ciliary ganglion (CG) cell in culture via the activation of TGF-β signalling by the sixth TSR via its KRFK motif [141]. However, loss of the reeler and spondin domains resulted in decreased CG survival while reeler, spondin, and the sixth TSR without TSRs 1-5 was sufficient to maintain survival, suggesting that although the KRFK motif activates TGFβ, the reeler and spondin domains are required for complete signalling [141]. This increased survival was also a result of increased disabled-1 (DAB-1) signalling achieved through interaction with the amyloid precursor protein (APP) receptor [141].

In summary, within the nervous system, Spondin 1 regulates cell adhesion, nerve outgrowth, axon guidance, cell viability and differentiation and as a whole protein and as cleavage products is an attractant and repellent molecule as well as being involved in cell signalling.

1.2.3 Other Functions and Mechanisms of Action

The known functions of Spondin 1 outside of the nervous system are similar to those within it. Spondin 1 that was isolated and purified from bovine follicular fluid promoted growth of rat aortic vascular smooth muscle cells in vitro [115]. However, Spondin 1 also inhibits vascular endothelial growth factor (VEGF)-stimulated migration and tube formation of human umbilical vein endothelial cells (HUVECs) as well as neovascularization within the rat cornea [142]. Inhibition of HUVEC migration may result from blocked signalling of integrin αvβ3 and decreased activation of AKT [142]. Interestingly, Spondin 1 has been implicated in both promoting and inhibiting angiogenesis and therefore it is possible that different domains and locations of the protein may play a role as previously identified in nerve outgrowth. These seemingly
opposed actions may indicate that Spondin 1 is involved in guiding or limiting growth of new blood vessels.

Cell adhesion in neural and non-neural tissues in *C. elegans* is affected by Spondin 1 [143]. Spondin 1 localizes to integrin expressing regions and basement membranes in the *C. elegans* embryo and mutants with a defective Spondin 1 lose attachment of muscle to the epidermis along with loss of cell adhesion in axons [143].

As mentioned in 1.2.2, Spondin 1 interacts with LDLRs, specifically LRP8 and VLDLR. It also interacts with amyloid precursor protein, APP. β-secretase cleavage of APP results in Aβ products whose accumulation is associated with Alzheimer’s disease. However, APP interaction with Spondin 1 creates a complex of APP, Spondin 1, and LRP8 that results in decreased production of Aβ cleavage products [144, 145], making Spondin 1 an intriguing target for Alzheimer’s therapeutics.

Studies with chicken ciliary ganglion suggest a role for Spondin 1 in cell survival (see 1.2.2). Further evidence of this function is seen in murine neuroblastoma cells, Neuro-2a, when exposed to adverse conditions [146]. Knockdown of Spondin 1 in these cells results in decreased levels of interleukin-6 (IL-6), and decreased survival after serum starvation or Aβ-peptide treatment. This decrease in IL-6 was correlated with decreases in NF-κB and p38 mitogen-activated protein kinase (MAPK) [146]. Treatment with Spondin 1 or IL-6 rescues cell survival. This suggests that not only does Spondin 1 inhibit cleavage of APP but also reduces the effects of Aβ peptides.

Alterations in TGFβ signalling due to Spondin 1, also seen in chicken ciliary ganglion cells, was first observed in chondrocytes in osteoarthritic cartilage *in vitro* [147]. Increased activation of latent TGFβ results in increased expression of matrix metalloproteinase 13 (MMP-13), and prostaglandin E2 (PGE2) leading to increased collagen degradation and decreased proteoglycan synthesis [147]. In embryonic cartilage Spondin 1 is highly expressed and via TGFβ induces chondrocyte maturation and mineralization by increasing MMP-13 and alkaline phosphatase (AP) expression [148]. These two proteins are also upregulated by Spondin 1 during cementoblast differentiation [149]. The only paper to date that studies the Spondin 1 knockout mouse addresses the
effect of Spondin 1 loss in bone and cartilage. At six months of age, these mice have increased bone mass most likely due to elevated bone synthesis. Levels of TGFβ1 are decreased in serum while the levels of phosphorylated SMAD1/5, bone morphogenetic protein (BMP) regulatory SMADs, is increased. The authors hypothesize that decreased TGFβ signalling leads to increased BMP signalling and bone deposition in adult mice [150].

Outside of the nervous system, Spondin 1’s functions and mechanisms are analogous to those within. The effects on angiogenesis in other tissues are similar to those seen in outgrowth of nerves. As in the nervous system, cell adhesion is lost with loss of Spondin 1 in muscle. Cell survival and differentiation are affected in neuroblastoma and cartilage, respectively, and both Spondin 1-regulated TGFβ and LRP8 signalling are also observed outside of the CNS.

1.2.4 Spondin 1 and the Ovary

Evidence for a role of Spondin 1 in the ovary is sparse but compelling. First, Spon1 gene expression increases in the uterus and mammary gland of ovariectomized mice treated with 17β-estradiol [151, 152]. Spon1 expression is decreased 2.5-fold in granulosa cells of eCG-treated estrogen receptor β (ERβ) knockout mice compared to eCG-treated mice heterozygous for ERβ [153]. Second, as mentioned in 1.2.3, Spondin 1 was isolated and purified from bovine follicular fluid and robustly promoted the growth of vascular smooth muscle cells, suggesting a role in the ovary in angiogenesis or vascularization [115]. A role for Spondin 1 in angiogenesis in the ovary is further indicated by the fact that Spondin 1 is expressed in the boar testis during development when vascularization and angiogenesis are occurring but is not expressed post-puberty [154].

Spondin 1 is highly expressed in ovarian cancer and is considered to be a biomarker for the disease [155]. Positive expression of Spondin 1 may be a negative prognostic indicator and its expression is higher in late stage ovarian cancer versus early stage [156]. No work has been done to determine what role Spondin 1 may play in oncogenesis or progression of ovarian cancer; however, the increased survival of
neuroblastoma cells in the presence of Spondin 1 may suggest that it plays a similar role in ovarian cancer cells [146].

1.3 Rationale

Since Spondin 1 was first identified in 1992, its functional and mechanistic roles in nerve outgrowth, cell adhesion, cell survival, cell differentiation, and angiogenesis have been studied in various tissues, both within the nervous system and several tissues without. However, its role in the ovary has never been characterized, despite evidence that i) it is present in the ovary, ii) it may be an ovarian factor for angiogenesis, iii) its mRNA expression is regulated by 17β-estradiol, and iv) it is highly overexpressed in ovarian cancer. If Spondin 1 is functional within the ovary it is likely to act in the same manner as in other tissues. Therefore, it could function in cell survival or proliferation, cell differentiation, angiogenesis, or cell adhesion/migration during the process of folliculogenesis. The discovery of a role for Spondin 1 in the ovary could have implications in the fields of infertility and ovarian cancer.

1.4 Hypothesis and Objectives

The work presented in this thesis was executed to test the hypothesis that Spondin 1 plays a functional role in ovarian folliculogenesis.

To test this hypothesis I pursued the following objectives:

1) To characterize functions of Spondin 1 in granulosa cell tumour cell lines to discover roles for Spondin 1 in granulosa cells and possibly ovarian cancer.

2) To characterize expression and functions of Spondin 1 in mouse primary granulosa cells and the mouse ovary to discover the possible role of Spondin 1 in ovarian folliculogenesis.

3) To characterize the reproductive phenotype of the Spon1−/− female mouse to determine if loss of Spondin 1 affects fertility.
1.5 Bibliography


49. Rajagopalan-Gupta RM, Lamm MLG, Mukherjee S, Rasenick MM and Hunzicker-Dunn M. Luteinizing hormone/choriogonadotropin receptor-mediated
activation of heterotrimeric guanine nucleotide binding proteins in ovarian follicular membranes. Endocrinology 1998; 139:4547-4555.


59. Tornell J, Billig H and Hillensjo T. Resumption of rat oocyte meiosis is paralleled by a decrease in guanosine 3', 5'-cyclin monophosphate (cGMP) and is inhibited by microinjection of cGMP. Acta Physiol Scand 1990; 139:511-517.


63. Robinson JW, Zhang M, Shuhaibar LC, Norris RP, Geerts A, Wunder F, Eppig JJ, Potter LR and Jaffe LA. Luteinizing hormone reduces the activity of the Npr2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. Developmental Biology 2012; 366:308-316.


65. Tirone E, D'Alessandris C, Hascall VS, Siracusa G and Salustri A. Hyaluron synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor b1). The journal of Biological Chemistry 1997; 272:4787-4794.


67. Shimada M, Hernadez-Gonzalez I, Gonzalez-Robayna I and Richards JS. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: Key roles for prostaglandin
synthase 2 and progesterone receptor. Molecular Endocrinology 2006; 20:1352-1365.


73. Leibfried ML and First NI. Preovulatory changes in cumulus-oocyte complex of the hamster. The Anatomical Record 1982; 202:339-348.


86. Ohara A, Mori T, Taii S, Ban C and Narimoto K. Functional differentiation in steroidogenesis of two types of luteal cells isolated from mature human corpora


95. Shweiki D, Itin A, Neufeld G, Gitay-Goren H and Keshet E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in
mice suggest a role in hormonally regulated angiogenesis. Journal of Clinical Investigation 1993; 91:2235-2243.


130. Lau WBMd, Snel B and Clevers HC. The R-spondin protein family. Genome Biology 2012; 13:


150. Palmer GD, Attur MG, Yang Q, Liu J, Moon P, Beier F and Abramson SB. F-spondin deficient mice have a high bone mass phenotype. PLOS One 2014; 9:


Chapter 2 – Spondin 1 regulates steroidogenesis and viability in granulosa cell lines

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2.1 Introduction

Folliculogenesis is the process whereby immature primordial follicles develop into mature preovulatory follicles capable of releasing a mature oocyte. This process is complex, and tightly-regulated by a variety of signals, both extra- and intra-ovarian, as well as extracellular and intracellular. These signals include, but are not limited to gonadotropins, steroid hormones, growth factors, peptides, miRNAs, and extracellular matrix (ECM) proteins. While these and other factors regulating folliculogenesis have been identified, due to its complexity, the factors responsible for successful folliculogenesis are not yet fully understood. However, identifying these factors and their functions is critical for understanding and treating infertility resulting from defects in folliculogenesis and ovulation.

The majority of folliculogenesis intra-ovarian factors identified to date are oocyte-derived factors that regulate granulosa cell or cumulus cell function. Less work has been undertaken regarding *somatic*-cell derived factors [1]. Known examples of major granulosa cell-derived secreted factors essential for follicle development include Anti-Müllerian hormone (AMH), inhibins and activins, activin-binding-protein (follistatin), prostaglandins, and the wingless-type MMTV integration site family (WNT), bone morphogenetic protein (BMP), and epidermal growth factor (EGF) families of proteins. However, despite the growing body of knowledge indicating that in many tissues, the ECM provides not only structural support to the cells it surrounds, but initiates signal transduction pathways in the same cells [2, 3], little is known about how signalling by
ECM proteins to ovarian cells contributes to folliculogenesis. In this study, we examine F-Spondin/Spondin 1, hereafter referred to as SPON1, an ECM protein that is present within granulosa cells of the human and mouse ovary, but whose function within the ovary remains unknown.

SPON1, encoded by SPON1, is a secreted glycoprotein that was originally identified in, and studied for its role in, the central nervous system (CNS). SPON1 is a member of the thrombospondin family, and contains six C-terminal thrombospondin repeats (TSRs), an N-terminal reeler domain, and a spondin domain [4, 5]. The reeler domain (aa 1-200) is homologous with a domain in the protein, Reelin (RELN), which, like SPON1, regulates neuronal migration [4, 6]. The spondin domain (aa 201-440) is homologous with regions in Spondin 2, proteins known to bind the ECM [4, 6]. In the CNS, SPON1 promotes the outgrowth of embryonic hippocampal neurons [6] and the outgrowth of sensory neurons in peripheral nerves after injury, but inhibits outgrowth of embryonic motor neurons by contact repulsion [7]. SPON1 is required for pathfinding of commissural axons [6] and is a signalling molecule for segmentation of neural crest cells in somites [8]. SPON1 induces both morphological and biochemical differentiation of CNS precursor cells into nerve cells [9].

SPON1 also carries out similar functions in other tissues. In C. elegans, SPON1 localizes to integrin-containing tissues, body muscle, and basement membranes, and functions via integrins to maintain ECM adhesion [10]. SPON1 promotes growth of vascular smooth muscle cells [11] and increases cell survival of murine neuroblastoma [12] and chicken ganglion cells [13]. SPON1 also promotes chondrocyte terminal differentiation [14], cementoblast (HCEM, human cementoblast-like cell line) differentiation [15], and inhibits osteoclast differentiation [16]. Finally SPON1 inhibits migration of HUVECs [17] and human cementoblast-like (HCEM) cells [15]. Thus, SPON1 regulates adhesion, outgrowth, differentiation, proliferation, migration and viability in many different tissues and species.

SPON1 is highly expressed in the human ovary relative to other human tissues [11], is overexpressed in ovarian cancer [18], and is currently under investigation as a
new marker for detection of ovarian cancer [18-20]. However, the biological function of SPON1 in the ovary is currently unknown. A potential functional role for SPON1 in granulosa cells of the ovary is supported by several intriguing lines of evidence. First, SPON1 isolated from bovine follicular fluid increases proliferation of endothelial cells [17]. Secondly, in primary rat granulosa cells, SPON1 is regulated by follicle-stimulating hormone (FSH) [21]. FSH and a constitutively-active protein kinase A (PKA) mutant increase Spon1 mRNA levels 3-fold and 4-fold, respectively in this model [21]. Interestingly, the secreted ECM glycoprotein, thrombospondin-1 (TSP-1/THBS1), is produced by granulosa cells and regulates ovarian angiogenesis in various species [22]. Thus, although compelling evidence suggests that SPON1 may play a role during folliculogenesis by regulating granulosa cell function and may be involved in ovarian cancer progression, little is known about SPON1’s functional role within granulosa cells of the ovary.

Therefore, to test the hypothesis that SPON1 regulates granulosa cell function, we examined the effect of exogeneous recombinant human SPON1 on the steroidogenesis, viability, and proliferation of the metastatic human granulosa cell tumor-derived cell line, KGN. We also carried out a screen to identify potential signalling pathways involved in SPON1’s effect on human granulosa cell function.

2.2 Materials and Methods

2.2.1 Cell Culture

The metastatic human granulosa cell tumor-derived cell line, KGN, was kindly provided by Drs. T. Yanase and Y. Nishi [23] via the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. KK-1 granulosa cells were derived from a tumor in a transgenic mouse expressing the Simian Virus 40 (SV40) T antigen under the control of the mouse inhibin α-subunit promoter [24], and kindly provided by Dr. Douglas Stocco (Texas Tech University Health Sciences Center). Both KGN and KK-1 cell lines were maintained in Dulbecco Modified Eagle medium: Nutrient Mixture F-12 (DMEM/F12) (Wisent Inc.) supplemented with 10% fetal bovine serum (FBS) (Wisent) and 1% Penicillin/Streptomycin (Pen/Strep) with 1 mM glutamine (Wisent) and
maintained in 5% CO\textsubscript{2} at 37°C. Huh-7 cells, a human hepatoma line, were kindly provided by Jason Matthews (The University of Toronto, Toronto, ON) and cultured in Dulbecco Modified Eagle medium (DMEM) supplemented with 10% FBS, 1% Pen/Strep, and 1 mM glutamine. Huh-7 cells were maintained in 5% CO\textsubscript{2} at 37°C. Both granulosa cell lines were initially treated with both 1 μg/ml and 5 μg/ml recombinant human SPON1 (SPON1) (R&D Systems, made is a mouse cell line and contains a His-tag); however only 5 μg/ml treatment resulted in a significant increase in viability compared to vehicle-treated cells, and thus 5 μg/ml was used for all subsequent experiments. These doses are comparable to those in other studies in which recombinant SPON1 is used to treat cell lines. SPON1 doses range from 0.5 μg/ml [14, 16] to 10 μg/ml [12]. Ovarian antral and/or circulating concentrations of SPON1 in humans or mice are currently unknown.

2.2.2 Western blots: Human and mouse tissues and cell lines

To determine SPON1 protein levels in human tissues, Human INSTA-Blot membranes were purchased from EMD Millipore (product no. 407211-1BLOT), and incubated with a rabbit polyclonal anti-SPON1 antibody (2 μg/ml) (product no. ab40797; Abcam) for one hour. After incubation for one hour with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) (product no. 934; GE Healthcare, NA), SPON1 levels were visualized with Pierce ECL Plus Western Blotting Substrate (Fisher). To determine SPON1 protein levels in mouse tissues, Mouse INSTA-Blot membranes were purchased from Novus Biologicals Canada (product no. NBP2-30111) and incubated under the same conditions used for the Human INSTA-Blot. Both INSTA-Blots are received pre-stained with amido black, which served as a loading control.

To determine SPON1 levels in the following cell lines: GFSHR-17 (rat granulosa), KGN (human granulosa cell tumour), KK-1 (mouse granulosa), SKOV3 (human ovarian adenocarcinoma), HEC-1 (human endometrial adenocarcinoma), MCF7 (human mammary adenocarcinoma) and U2OS (human osteosarcoma), cells were cultured in DMEM/F12 supplemented with 5% FBS, 1% Pen/Strep, and 1 mM glutamine.
GFHSR-17), DMEM/F12 supplemented with 10% FBS and 1% Pen/Strep with 1 mM glutamine (KGN, KK-1), DMEM (Wisent) supplemented with 5% FBS and 1% Pen/Strep with glutamine (SKOV3), DMEM supplemented with 10% FBS and 1% Pen/Strep with 1 mM glutamine (HEC1, MCF7, U2OS). The GFHSR-17 cell line [25] is an immortalized gonadotropin-responsive rat granulosa cell line that we obtained from Dr. Abraham Amsterdam (Weizmann Institute of Science). The SKOV3 cell line was graciously provided by Dr. Gabriel DiMattia (Lawson Health Research Institute, London, ON). The HEC-1 cell line was purchased from the ATCC (American Type Culture Collection). The MCF7 cell line was kindly provided by Dr. Joseph Torchia (Lawson Health Research Institute, London, ON). The U2OS cell line was obtained from Dr. Frederick Dick (University of Western Ontario).

Protein lysates were prepared using Radioimmunoprecipitation assay buffer (RIPA, 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0) and lysate concentrations were measured using the DC protein assay (Bio-Rad Laboratories (Canada) Ltd). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with SPON1 antibody under the same conditions used for the INSTA-Blot membranes. Immunizing peptide blocking experiments were carried out using the blocking peptide provided by the manufacturer (product no. ab41539, Abcam) for the SPON1 primary antibody we used. α-tubulin was used as a loading control.

2.2.3 Progesterone Assay

KGN cells were plated at 80 000 cells per well into a 24-well plate in DMEM/F12 supplemented with charcoal stripped FBS (Wisent). After 24 hours, cells were treated with bovine serum albumin (BSA) control (250 μg/mL; BioShop Canada Inc.), recombinant human SPON1 (SPON1) (5 μg/mL; R&D Systems, Minneapolis, MN), dibutyryl-cAMP (dbcAMP) (1 mM) (Enzo Life Sciences), or both SPON1 (5 μg/mL) and dbcAMP (1 mM). Media was removed after 48 hours and frozen at -80°C until further analysis. Protein lysates were prepared using RIPA buffer supplemented with a protease inhibitor cocktail (1:1000 dilution, product no. P8340-5ml; Sigma). Protein lysate
concentrations were determined using the DC protein assay (Bio-Rad). Cell culture media was sent to the University of Virginia Centre for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA) and the progesterone concentration determined by progesterone radioimmunoassay (RIA). Progesterone concentrations were calculated by normalizing ng/μl progesterone/well to the total protein/well. Differences in progesterone levels between the four treatments, ie. vehicle, dbcAMP, SPON1, or dbcAMP + SPON1 were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey Multiple Comparison post-hoc test (Tukey test) (PRISM 6.0e for Mac OSX; GraphPad).

2.2.4 Quantitative RT-PCR

KGN cells were seeded into six-well plates and treated as for progesterone assays. RNA was extracted using the Qiagen RNeasy mini kit, the second step of which requires the Qiashredder kit (product nos. 74104 and 79654, respectively; Qiagen). RNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies). Relative gene expression levels were determined using TaqMan Fast Advanced Master Mix (product no. 4444963; Life Technologies) and TaqMan probe sets in 384-well plates with a final reaction volume of 10 μl/well. cDNA levels were detected by quantitative PCR using the Applied Biosystems 7900HT Fast Real Time PCR System. TaqMan Gene Expression Assays (product no. 4331182; Life Technologies) probe/primer sets were used to analyze CYP11A1, STAR, CYP17A1, CYP19A1, HSD3B1, HSD17B1, HSD17B3, and RPL7 (endogenous control). Fold changes in gene expression were determined by quantitation of cDNA from target (treated) samples relative to a calibrator sample (vehicle). The gene for ribosomal protein L7 (RPL7) was used as the endogenous control for normalization of RNA levels. Expression ratios were calculated using the ΔΔCT model. Data averaged from three independent experiments were analyzed by one-way ANOVA followed by a Tukey multiple comparison post-hoc test, with the exception of STAR, for which a single representative experiment is shown. The reason a single representative experiment is shown for STAR is that although SPON1 significantly reduced dbcAMP-induced STAR mRNA levels in each of three independent experiments (data not shown), when the data from all three experiments were combined, this effect of
SPON1 was no longer statistically significant. We believe this variability between experiments is largely due to the variable response of the KGN cell line to dbcAMP. All data was analyzed using PRISM 6.0e for Mac OSX; GraphPad.

2.2.5 Western blot: STAR expression in SPON1-treated KGN cells

To determine SPON1’s effect on steroidogenic acute regulatory protein (STAR) expression in KGN cells, cells were plated into six-well dishes in DMEM/F12 supplemented with 10% charcoal stripped FBS, incubated for 24 hours, then treated with dibutyryl-cAMP (dbcAMP) (1mM) (Enzo Life Sciences, Inc.) or both SPON1 and dbcAMP for 8 or 24 hours. Protein lysates were prepared using RIPA buffer and protein concentrations were measured using a DC Protein assay (Bio-Rad). Proteins were separated using SDS-PAGE and transferred to PVDF membrane for 2 hours at 100V at 4°C. The membrane was blocked in 5% milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) and incubated in STAR primary antibody (1:500 dilution; product no. sc-25806; Santa Cruz) overnight at 4°C, then incubated with anti-rabbit HRP-conjugated secondary (1:10 000 dilution, product no. NA 934; GE Healthcare) for 1 hour at room temperature (rt). α-tubulin was used as a loading control; α-tubulin antibody (1:8000 dilution, product no. T5168; Sigma) and anti-mouse HRP conjugated (1:10 000 dilution, product no. NA 931; GE Healthcare) were applied to the membrane under the same conditions used for the STAR Western blot. STAR: α-tubulin ratios were calculated using ImageJ software [26].

2.2.6 STAR Promoter Luciferase Assays

A STAR promoter reporter plasmid (STAR-luc) containing 966 bp of the STAR proximal promoter driving firefly luciferase expression on a pGL3 backbone was kindly provided by Dr. Douglas Stocco (Texas Tech University Health Sciences Center). Luciferase assays were carried out using Huh-7 cells (a human liver hepatoma cell line). Huh-7 cells were seeded into 24-well plates at 60 000 cells/well in DMEM supplemented with 10% charcoal stripped FBS, 1% Pen/Strep and 1 mM glutamine. After twenty-four hours, the cells were transfected with STAR-luc (250 ng/well), a Renilla luciferase control plasmid pRL-TK (20 ng/well), and pcDNA3 (230 ng) at a 1:3 ratio with FuGENE
HD (product no. PRE 2311; Fisher) (500 ng DNA: 1.5 μl FuGENE HD). DNA and FuGENE HD were suspended in antibiotic-free DMEM supplemented with 10% charcoal stripped FBS. Twenty-four hours after transfection, the cells were treated with SPON1 (5 μg/ml), 8-CPT-cAMP (2.5 mM, product no. BML-CN130; Enzo Life Sciences), or both. Forty-eight hours after treatment, STAR promoter activity was assessed using the Promega Dual Luciferase Reporter Assay System (product no. E1960; Fisher). Differences in firefly/Renilla luciferase ratios between vehicle treatment alone or treatment with SPON1 were analyzed by a two-tailed, unpaired, Student’s t-test (PRISM 6.0e for Mac OSX; GraphPad).

2.2.7 Cell Viability Assays and Cell Counting

KGN and KK-1 cells were seeded at 5000 cells per well into 96-well plates, incubated for one hour, then treated with recombinant human SPON1. Cell viability was measured after 24, 48, and 72 hours using the Cell Titer AQueous Non-Radioactive Cell Proliferation Assay (MTS) (product no. PRG3582; Promega). Cell counts were measured at 24, 48, and 72 hours by trypsinizing the cells, then counting an aliquot of the trypsinized cells using a Coulter counter. Differences in viability and cell number between vehicle treatment alone or treatment with SPON1 at each time-point were analyzed by a two-tailed, unpaired, Student’s t-test (PRISM 6.0e for Mac OSX; GraphPad).

2.2.8 Phospho-kinase Arrays and PRAS40 phosphorylation Western blot

To screen for potential downstream targets of SPON1 that may act in the signal transduction pathways regulating granulosa cell function in response to SPON1, a Proteome Profiler Human Phospho-kinase array was purchased from R&D Systems (product no. ARY003B). This array allows for the simultaneous analysis of the relative site-specific phosphorylation levels of 43 different kinase proteins or their targets. KGN cells were grown as described (see section 2.2.1) in a 10 cm dish and the media replaced three hours prior to treatment with serum-free medium. Cells were then treated with BSA (250 μg/ml; Bioshop) or SPON1 (5 μg/ml) for 15 minutes. This time-point was chosen
based on published studies [12, 13] in which recombinant SPON1 is applied to cultured cells and SPON1-induced kinase activation is detected (10 to 30 minutes). For the phospho-kinase array, 400 μg of protein was applied to the blots.

To confirm the SPON1-induced increase in proline-rich AKT1 substrate 1 (PRAS40/AKT1S1) levels that we observed in the phospho-kinase array (see Phospho-kinase Methods section), KGN cells were plated into six-well plates, incubated for 24 hours, then serum-starved for three hours. The cells were then treated with SPON1 (5 μg/ml) or BSA (vehicle control) (250 μg/ml; Bioshop) for 0, 15, or 60 minutes. Protein lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors (1:10 000 dilution, product no. P2850-1 mL; Sigma) and protein concentrations were measured using the DC protein assay (Bio-Rad). Proteins were separated using SDS-PAGE and transferred to a PVDF membrane for 2 hours at 100V at 4°C. The membrane was blocked in 5% BSA in TBST and incubated in pPRAS40 antibody (1 μg/ml, product no. 2640S; Cell Signalling technology, Inc.) overnight at 4°C, followed by incubation with anti-mouse HRP conjugated (1:10 000 dilution, product no. NA 931; GE Healthcare) for 1 hour at rt. Total PRAS40 levels were determined using a second Western blot onto which the same amount of lysate was loaded as for the pPRAS40 blot. The resulting membranes were incubated with PRAS40 antibody (0.5 μg/ml, product no. 2610; Cell Signalling Technology, Inc.) overnight at 4°C. Histone deacetylase 4 (HDAC4) was used as a loading control; HDAC4 antibody (1:1000 dilution, product no. sc-11418; Santa Cruz), followed by incubation with anti-rabbit HRP conjugated (1:10 000 dilution, product no. NA 934; GE Healthcare) incubated under the same conditions used for the PRAS40 Western blots.

2.3 Results

2.3.1 SPON1 expression in human and mouse tissues and cell lines

Because little is known about SPON1 protein levels in human and mouse tissues and granulosa cells, we first investigated SPON1 levels in these models. We found that
Figure 2-1. SPON1 expression in human and mouse tissues and immortalized cell lines.

A. A human tissue INSTA-Blot™ was incubated with an anti-Spondin 1 antibody to detect SPON1 (arrow, upper). Lanes: 1) Brain; 2) Heart; 3) Small Intestine; 4) Kidney; 5) Liver; 6) Lung; 7) Muscle; 8) Stomach; 9) Spleen; 10) Ovary; 11) Testis. Amido black staining as a control (bottom).

B. A mouse tissue INSTA-Blot™ was incubated with an anti-SPON1 antibody to detect SPON1 (arrow, upper). Lanes: 1) Brain; 2) Heart; 3) Small Intestine; 4) Kidney; 5) Liver; 6) Lung; 7) Muscle; 8) Stomach; 9) Spleen; 10) Ovary; 11) Testis. Amido black staining as a control (bottom).

C. SPON1 expression in a panel of reproductive or estrogen-responsive mouse and human immortalized cell lines. Lanes: 1) Rat GFSHR-17 granulosa cell line; 2) Human KGN human granulosa cell tumor cell line; 3) Mouse KK-1 granulosa cell line; 4) Human SKOV3 ovarian cancer cell line; 5) Human HEC1 endometrial carcinoma cell line; 6) Human MCF7 breast cancer cell line; 7) Human U2OS osteosarcoma cell line. α-tubulin was used as a loading control (bottom).
SPON1 expression was similar in most human and mouse tissues, with a few notable differences (Fig. 2-1). In human tissues (Fig. 2-1 A), SPON1 (arrow) was most highly expressed in the ovary and testis, with robust expression also in brain, heart, small intestine, and liver. Weaker expression was observed in kidney, lung, skeletal muscle, and stomach. In mouse tissues (Fig. 2-1 B), SPON1 was most highly expressed in the brain, heart, kidney, muscle, spleen, ovary, and testis. Comparing human and mouse SPON1 tissue expression, the main difference was that SPON1 was easily detectable in human small intestine, liver, and lung, but only weakly expressed in these tissues in the mouse (Fig. 2-1 A and B). Conversely, in mouse tissues, SPON1 was easily detectable in kidney and spleen, but barely detectable in these human tissues. We also detected SPON1 in a variety of different granulosa cell lines and cell lines of other reproductive tissue origin (Fig. 2-1 C). These included the GFSHR-17 cell line (an immortalized gonadotropin-responsive rat granulosa cell line) [16, 25], the human granulosa cell-like cell line, KGN [17, 23], the KK-1 cell line, an immortalized mouse ovarian granulosa tumor cell line [24], HEC-1 endometrial adenocarcinoma cells, MCF7 breast cancer cells, SKOV3 ovarian cancer cells, and U2OS osteosarcoma cells (a non-reproductive cell type). SPON1 was robustly expressed in all cell lines (Fig. 2-1 C), with the greatest expression in the granulosa and ovarian cancer cell lines (Lanes 1-4). Immunizing peptide blocking experiments confirmed that this binding was specific (data not shown).

2.3.2 SPON1 reduces progesterone levels in response to cAMP stimulation of KGN cells

We next investigated whether SPON1 would affect one of the primary functions of granulosa cells: steroidogenesis. KGN cells produce progesterone in response to stimulation by cAMP and therefore are a good model to study progesterone production; however, they do not produce significant levels of 17β-estradiol. Therefore, we did not measure 17β-estradiol levels. We treated KGN cells with the cAMP analog, dbcAMP, in the presence or absence of SPON1 and measured progesterone levels in the media after 48 hours. cAMP treatment increased progesterone levels 5.3-fold over vehicle-treated KGN cells (Fig. 2-2), while SPON1 treatment alone had no significant effect. However,
Figure 2-2. SPON1 inhibits the cAMP-mediated increase in progesterone levels in KGN cells.

Cells were treated for 48 hours with vehicle, dbcAMP (1 mM), SPON1 (5 μg/mL), or dbcAMP (1 mM) + SPON1 (5 μg/mL). Progesterone levels in the culture media were determined by radioimmunoassay and normalized to total protein levels. Data are expressed as average progesterone levels in KGN medium (± SEM of three independent experiments). Differences in progesterone levels between the four treatments, ie. vehicle, dbcAMP, SPON1, or dbcAMP + SPON1were analyzed by a one-way ANOVA followed by a Tukey Multiple Comparison post-hoc test (Tukey test). There was a statistically significant difference between the four treatments as determined by one-way ANOVA, F (3, 8) = 120, P < 0.0001. A Tukey test revealed that there were statistically significant differences (P < 0.05) between: vehicle and dbcAMP; vehicle and dbcAMP + SPON1; dbcAMP and SPON1; dbcAMP and dbcAMP + SPON1; SPON1 and dbcAMP + SPON1. a = P < 0.05. n=3.
SPON1 reduced the cAMP-mediated increase in progesterone from 5.3-fold to 3.3 fold; a reduction of 38%.

2.3.3 SPON1 inhibits the cAMP-induced expression of CYP11A1, CYP17A1 and STAR in KGN cells

To determine if SPON1 might reduce cAMP-mediated progesterone levels by inhibiting the expression of steroidogenic enzymes key to the production of progesterone or its conversion to other steroids (see Appendix A – Steroidogenesis Pathway), we measured the mRNA levels of CYP11A1, CYP17A1, CYP19A1, HSD3B1, HSD17B1, HSD17B3, and STAR (which regulates the rate-limiting step of steroidogenesis by regulating cholesterol movement within the mitochondria) in KGN cells. Cells were treated with dbcAMP in the presence or absence of SPON1 for 24 hours (Fig. 2-3). Interestingly, SPON1 treatment reduced the mRNA levels of both CYP11A1 and CYP17A1 by 45% and 40%, respectively (Fig. 2-3 A and C). STAR mRNA levels were also significantly reduced by SPON1 (Fig. 2-3 B). However, mRNA levels of CYP19A1 (Fig. 2-3 D), HSD3B1, HSD17B1, and HSD17B3 (data not shown) were not significantly altered following SPON1 treatment.

Because STAR is upstream of CYP11A1 and CYP17A1, we chose to pursue its regulation by SPON1 further. Protein levels of cAMP-induced STAR were significantly reduced by SPON1 treatment (Fig. 2-4). After 8 hours of dbcAMP treatment, STAR protein levels increased 2.6-fold relative to vehicle, and co-treatment with SPON1 reduced this by 50% (Fig. 2-4 A and B). After 24 hours of dbcAMP treatment, STAR protein levels increased 7.1-fold, and co-treatment with SPON1 reduced this by 89%, similar to the vehicle-treated levels of STAR (Fig. 2-4 A and B).

2.3.4 SPON1 inhibits the cAMP-induced activation of the human STAR promoter

To determine if SPON1 inhibits cAMP-induced STAR promoter activity, we carried out a luciferase assay in the Huh-7 human liver hepatoma cell line. Huh-7 cells were transfected with a construct containing the firefly luciferase gene driven by 966 bp of the human STAR proximal promoter, and a Renilla luciferase control plasmid. The
Figure 2-3. SPON1 antagonizes the cAMP-mediated increase in CYP11A1, CYP17A1 and STAR mRNA levels, but not CYP19A1 mRNA levels in KGN cells.

Cells were treated for 24 hours with vehicle, dbcAMP (1 mM), SPON1 (5 μg/mL), or dbcAMP (1 mM) + SPON1 (5 μg/mL), and mRNA levels of (A) CYP11A1, (B) STAR, (C) CYP17A1 and (D) CYP19A1 determined by quantitative RT-PCR. mRNA data are expressed as average gene expression relative to the RPL7 control (± SEM of three independent experiments, except for STAR). A. There was a statistically significant difference in CYP11A1 mRNA levels between the four treatments as determined by one-way ANOVA, F (3, 8) = 26, P < 0.001. A Tukey test revealed that there were statistically significant differences (P < 0.05) between: vehicle and dbcAMP; vehicle and dbcAMP + SPON1; dbcAMP and SPON1; dbcAMP and dbcAMP + SPON1; SPON1 and dbcAMP + SPON1. B. There was a statistically significant difference in STAR mRNA levels between
the four treatments as determined by one-way ANOVA, \( F (3, 4) = 63.7, P < 0.0001 \). A
Tukey test revealed that there were statistically significant differences \( (P < 0.01) \)
between: vehicle and dbcAMP; vehicle and dbcAMP + SPON1; dbcAMP and SPON1;
dbcAMP and dbcAMP + SPON1. One representative experiment is shown for \textit{STAR}. C. 
There was a statistically significant difference in \textit{CYP17A1} mRNA levels between the
four treatments as determined by one-way ANOVA, \( F (3, 8) = 1.2, P < 0.0001 \). A Tukey
test revealed that there were statistically significant differences \( (P < 0.05) \) between:
vehicle and dbcAMP; vehicle and dbcAMP + SPON1; dbcAMP and SPON1; dbcAMP
and dbcAMP + SPON1; SPON1 and dbcAMP + SPON1. D. There was no statistically
significant difference in \textit{CYP19A1} mRNA levels between the four treatments as
determined by one-way ANOVA.
Figure 2-4. SPON1 antagonizes the cAMP-induced increase in steroidogenic acute regulatory (STAR) protein levels.

A. KGN cells were plated in DMEM/F12 supplemented with 10% charcoal stripped FBS, incubated for 24 hours, then treated with dibutyryl-cAMP (dbcAMP) (1mM) or both recombinant human SPON1 (SPON1) and dbcAMP for 8 or 24 hours. Protein lysates were separated by SDS-PAGE and STAR protein levels assessed by Western blot analysis. α-tubulin was used as a loading control. n=2 B. Densitometric analysis of Western blot data (ImageJ) showing ratio of STAR: α-tubulin.
cells were then treated with 8-CPT-cAMP with vehicle or SPON1 for 48 hours, and the firefly luciferase activity measured and normalized to Renilla luciferase activity (Fig. 2-5). Compared to treatment with 8-CPT-cAMP alone, SPON1 modestly but significantly reduced this increase in STAR promoter activity by 17% (P < 0.0001).

2.3.5 SPON1 increases granulosa cell viability and proliferation in KGN and KK-1 cells

To determine whether SPON1 might affect GC viability, we treated both KGN and KK-1 cells with human recombinant SPON1 or bovine serum albumin (BSA) as vehicle for 24, 48, or 72 hours. We measured viability using an MTS assay, which detects the activity of NAD(P)H-dependent cellular oxidoreductase enzymes, and serves as a quantitative measure of the number of viable cells, not of the proliferation of cells. We found that SPON1 modestly, but significantly, increased cell viability in both KGN and KK-1 cells (Fig. 2-6 A and B). SPON1 increased KGN cell viability after 24, 48 and 72 hours by 33%, 20% and 75%, respectively. KK-1 viability increased significantly only after 72 hours (32%). To determine if the change in viability in both cell lines was a result of increased cellular proliferation, we assessed cell proliferation by counting cells under conditions corresponding to the viability assay treatments and time-points (Fig. 2-6 C and D). We found that the number of KGN cells increased significantly compared to controls following SPON1 treatment at 48 and 72 hours by 27% and 32%, respectively.

The increased viability and proliferation of KGN cells following SPON1 treatment could also result from decreased apoptosis. In order to determine if apoptosis was affected by SPON1 treatment we used a Proteome Profiler Human Apoptosis Array Kit to look for changes in apoptosis markers in KGN cells following SPON1 treatment under the same conditions used for the viability assays. No differences were observed in any apoptosis marker proteins (Appendix B), suggesting SPON1 is unlikely to be affecting apoptosis in these cells.
Figure 2-5. SPON1 inhibits the cAMP-induced activation of the proximal STAR promoter.

Huh-7 hepatoma cells were transfected with a STAR promoter luciferase construct (STAR-luc) containing 966 bp of the STAR proximal promoter. After 24 hours, the cells were treated with SPON1 (5 μg/ml), 8-CPT-cAMP (2.5 mM) or both. Forty-eight hours after treatment, STAR promoter activity was assessed using the Promega Dual Luciferase Reporter Assay System. Differences in firefly/Renilla luciferase values between vehicle treatment alone or treatment with SPON1 were analyzed by a two-tailed, unpaired, Student’s t-test. a: P < 0.0001. n=3.
Figure 2-6. SPON1 increases granulosa cell proliferation and cell viability.

Assessment of cell viability by MTS assay in KGN (A) and KK-1 (B) granulosa cells in the presence or absence of SPON1. Cells were cultured in BSA vehicle or human recombinant SPON1 (5 μg/mL) as indicated. Each data point represents the mean ± SD of triplicate measurements within one representative experiment. Differences in viability between vehicle treatment alone or treatment with SPON1 at each time-point were analyzed by a two-tailed, unpaired, Student’s t-test. a: P < 0.01. Assessment of cell number in KGN (A) and KK-1 (B) granulosa cells in the presence or absence of SPON1. Cells were cultured in BSA vehicle or human recombinant SPON1 (5 μg/mL) for 24 to 72 hours as indicated. Each data point represents the mean ± SD of triplicate measurements within one representative experiment. Differences in cell number between
vehicle treatment alone or treatment with SPON1 at each time-point were analyzed by a two-tailed, unpaired, Student’s t-test. b: $P < 0.01$ n=3.
2.3.6 SPON1 increases pPRAS40 levels in KGN cells

To screen for potential intracellular targets of SPON1 in GCs that could be responsible for increased viability and/or proliferation, we used the Proteome Profiler Human Phospho-kinase Array Kit, which simultaneously detects the relative levels of phosphorylation of 43 phosphorylation sites on 43 proteins and 2 kinase-related (non-phosphorylated) total proteins, and/or their targets. KGN cells were serum-starved for three hours to reduce background kinase activity, then treated for 15 minutes with vehicle or SPON1, after which cell lysates were prepared. These lysates were then applied to the Human Phospho-Kinase array, and the blots processed and spot intensities quantified according to the manufacturer’s directions. Phosphorylation of two proteins, PRAS40/AKT1S1 (T246) and WNK-1 (T60), increased 4-fold and 2-fold, respectively, after SPON1 treatment compared to control (Fig. 2-7). Of these two proteins identified by this analysis, we chose PRAS40 for further investigation. Proline Rich AKT1 Substrate of 40 kDa (PRAS40) (Official name: AKT1 substrate 1 (proline-rich); Official symbol: AKT1S1) [27] is a member of the mTORC1 complex, which is associated with increased cell growth, proliferation and protein production [27, 28].

To validate the SPON1-mediated increase in pPRAS40 that we observed in the array, we conducted Western blot analysis with lysates from KGN cells serum starved for three hours and treated with vehicle or SPON1 for 15 or 60 minutes. After fifteen minutes, the ratio of pPRAS40 to total PRAS40 levels increased 2-fold following SPON1 treatment compared to 15 minutes of control (Fig. 2-8 A and B). Similarly, after 60 minutes, the ratio of pPRAS40 to total PRAS40 levels increased approximately 3-fold following SPON1 treatment compared to 60 minutes of vehicle treatment alone (Fig. 2-8 A and B).
Figure 2-7. SPON1 phospho-kinase targets

KGN cells were serum starved for three hours and then treated with BSA (as control) or Spondin 1. KGN cell lysates were incubated with the phospho-kinase array and the array protocol followed. The left two blots were BSA-treated and the right two were Spondin 1-treated. The top two white boxes represent pPRAS40 and the bottom two represent pWNK1. The assay was performed twice with identical results, n=2.
Figure 2-8. SPON1 increases protein levels of phosphorylated PRAS40.

A. KGN cells were serum-starved for three hours, then treated with SPON1 (5μg/ml) or BSA (vehicle control) (250 μg/ml) for 0, 15, or 60 minutes. Protein lysates were separated using SDS-PAGE and protein levels assessed by Western blot analysis. Histone deacetylase 4 (HDAC4) was used as a loading control. B. Densitometric analysis of Western blot data showing ratio of pRAS40: total PRAS40 (ImageJ).
2.4 Discussion

2.4.1 SPON1: an ECM protein that regulates human granulosa cell steroidogenesis

The ECM is composed of a complex array of glycoproteins, collagens, glycosaminoglycans, and proteoglycans. While ECM proteins are well-known for their role in providing mechanical and structural support to cells, giving tissues shape and strength, a growing body of evidence indicates that a major role for ECM proteins includes triggering signalling pathways in the cells they surround [2]. Evidence indicates that ECM protein-induced signalling can alter cell function, behaviour, migration, proliferation, differentiation, and viability [2]. SPON1 is an example of such a signalling ECM protein, and we show that altering steroidogenesis in granulosa cells may be one of its functions within the ovary.

In our study, SPON1 reduced cAMP-induced progesterone levels in KGN cells by 38% - (Fig. 2-2). The extent of this reduction correlated with the SPON1-mediated reduction of cAMP-induced CYP11A1 mRNA levels by 45%, and the reduction of steroidogenic acute regulatory protein (STAR) protein expression by 40-89% (Fig. 2-3 and 2-4). STAR transfers cholesterol from the outer mitochondrial membrane to the inner, while CYP11A1 cleaves the cholesterol side chain to form pregnenolone—the first enzymatic reaction in steroidogenesis (Appendix A). The SPON1-mediated reduction of cAMP-induced CYP11A1 and STAR expression is consistent with the reduction in progesterone levels that we observed after SPON1 treatment, given that both CYP11A1 and STAR participate in converting cholesterol to pregnenolone (the substrate for progesterone). We also observed that SPON1 reduced the cAMP-mediated increase in CYP17A1 mRNA levels by 40% (Fig. 2-3). Because CYP17A1 converts pregnenolone and progesterone to 17α-hydroxyprogrenolone and 17α-hydroxyprogesterone, respectively, a reduction in CYP17A1 mRNA levels could be predicted to increase progesterone levels. However, there are potential explanations for this not occurring, the simplest being that expression of CYP17A1 following dbcAMP + SPON1 treatment is decreased due to a lack of progesterone resulting from decreased STAR and CYP11A1 activity. The effect of loss of
CYP17A1 on progesterone levels may also be negligible compared to loss of STAR and CYP11A1.

While much remains to be understood about ECM proteins in the ovary, there is evidence of their involvement in steroidogenesis. The focimatrix is a specialized ECM that is located between granulosa cells and is composed of basal lamina ECM proteins [29]. The focimatrix is observed in the follicles of multiple species, including humans [29-35]. Expression of focimatrix proteins has been linked with steroidogenesis in the bovine ovary [29, 30, 36]. mRNA levels of focimatrix proteins have been highly correlated with CYP11A1 mRNA levels, suggesting that focimatrix proteins may regulating CYP11A1 expression. [30]. These and other studies [37] have pioneered our understanding of how ECM proteins may regulate folliculogenesis by showing that the gene expression levels of focimatrix proteins correlate with the expression of steroidogenic genes in specific model systems. In our study, we extend and expand these previous studies by showing that SPON1 alters the expression of CYP11A1 mRNA levels and STAR mRNA protein expression in granulosa cells when SPON1 is added to the culture medium of granulosa cell lines.

2.4.2 Inhibition of cAMP-induced STAR and CYP11A1 expression in granulosa cells by other biological molecules

In addition to SPON1, several other proteins and biological molecules inhibit the cAMP-induced increase in STAR mRNA or protein levels. Exogenous bone morphogenetic protein 15 (BMP15) inhibits progesterone production in SVOG cells (immortalized human granulosa cells) by reducing STAR mRNA and protein levels, and this is consistent with the levels of progesterone in the culture medium [38]. A second example of a biological molecule that inhibits STAR and CYP11A1 expression is cholesterol sulfate (CS) [39], a component of the cell membrane, that inhibits the cAMP-induced increase in progesterone production and STAR and CYP11A1 mRNA levels in KGN cells—the same cells used in our study. STAR protein levels are also reduced by CS treatment [39]. However, the mechanism for this reduction was not investigated. Finally, in human primary granulosa cells [40], Yamamoto et al. find that oocyte-derived growth differentiation factor 9 (GDF-9) inhibits the cAMP-mediated increase in the
mRNA levels of STAR, CYP11A1, and CYP19A1. Because all three genes are affected, the authors propose that GDF-9 must block a key step in the cAMP-dependent/PKA pathway [40]. Thus, SPON1 can now be added to the short list of factors that inhibit the increase of STAR and CYP11A1 by cAMP; however, SPON1 is unique among these examples because it is an ECM protein, while the others are not. It should be noted that at least one study has demonstrated that ECM proteins can regulate STAR expression in granulosa cells, although the inhibition of cAMP was not shown [37].

2.4.3 SPON1 regulation of the human STAR promoter

To understand how SPON1 might regulate STAR mRNA levels, we postulated that SPON1 might, through an unknown mechanism, regulate the STAR promoter. We chose to use Huh-7 cells for these experiments after many unsuccessful attempts to transfect the KGN and KK-1 cell lines, which are generally considered very difficult to transfect. In addition, Huh-7 cells are cAMP-responsive [41-45], and thus we considered them a good model to study STAR promoter activity. We observed a modest (17%), but significant, reduction in cAMP-induced STAR promoter activity by SPON1. cAMP is the predominant regulator of the STAR gene in the adrenals and gonads [46]. While multiple transcription factors and signalling pathways have been identified that regulate STAR transcription (see Manna et al. [46] and Stocco et al. [47] for excellent reviews), the exact mechanisms by which cAMP increases STAR transcription remain uncertain [46, 47].

Two of the best-characterized factors regulating STAR transcription include Steroidogenic factor 1 (SF-1) [48, 49], and CCAAT/enhancer-binding protein β (C/EBPβ) [47, 50], which can cooperate with SF-1 to regulate transcription of the STAR promoter [51]. CREB/CREM/ATF, AP-1, and more recently, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [52] also regulate the STAR promoter. How SPON1 might interact with these factors that regulate the STAR promoter remains to be determined. One possible mechanism is that SPON1 interaction with receptors on the granulosa cell triggers a signal transduction pathway in which one or several kinases is activated or de-activated. Because SF-1 and C/EBPβ are active only when phosphorylated, it is possible that SPON1 reduces activity that phosphorylates these transcription factors, resulting in reduced SF-1 and C/EBPβ activity, and thus reduced
transcription of STAR. Many possible mechanisms can be postulated, and further experiments are necessary to dissect the mechanism linking SPON1 to reduced STAR promoter activity.

2.4.4 Other mechanisms by which SPON1 may reduce STAR protein levels in KGN cells

Because SPON1 had only a modest effect on reducing STAR promoter activity (17%), SPON1 may have mechanisms to regulate progesterone levels other than regulating STAR promoter activity. Here we suggest two such possibilities: a) SPON1 may stabilize STAR protein levels, and b) SPON1 may regulate cAMP levels or activity. There is evidence that STAR protein levels are regulated by post-translational events [46]. STAR undergoes proteolytic processing in the mitochondria, although what effect this has on steroidogenesis remains to be understood [46]. STAR is also post-translationally modified by several kinases, and phosphorylation of STAR on Ser194 is essential for maximal activity [53-55]. Secondly, because in our experiments cAMP is added directly to the medium, and SPON1 inhibits the effect of this cAMP, it is likely that SPON1 does not inhibit cAMP production, but rather a step downstream of cAMP production. For example, SPON1 may increase the expression of phosphodiesterases (PDEs), which degrade cAMP, resulting in reduced levels of cAMP, and a reduction in STAR and progesterone levels.

2.4.5 Mechanisms by which SPON1 regulates cellular function in other model systems

A limited number of studies in a wide variety of model systems have explored the mechanisms and signal transduction pathways by which SPON1 regulates cellular function, but no common mechanism of SPON1 action has been identified. Some studies suggest that SPON1 begins the signal transduction process by interacting with integrins. In C. elegans, SPON1 binds to muscle α-integrin and is localized to integrin-containing sites [10]. In a human umbilical vein endothelial cell (HUVEC) model, SPON1 inhibits migration by blocking integrin αvβ3 [2, 17]. Interestingly, kinases are well-known to associate with integrins to propagate intracellular signals [2]. Results suggesting SPON1 may regulate kinase activity include: inhibiting phosphorylation of focal adhesion kinase
and VEGF-stimulated protein kinase B (AKT) [17], and increasing phosphorylation of p38 MAPK in murine neuroblastoma cells [12]. In chick ciliary ganglion, SPON1 increases the phosphorylation of the intracellular adaptor protein, disabled-1 (Dab1) and phosphorylation of AKT [13]. SPON1 also interacts with LRP8 (low-density lipoprotein receptor-related protein 8) and APP (amyloid precursor protein) [56-58] but the functional significance of this is not yet known [16]. Thus, a variety of biological factors, signalling molecules, kinases, and proteins have been implicated in the mechanism of action of SPON1 in very diverse model systems. Understanding SPON1’s mechanism of action remains a field rich for discovery in many tissues, including the ovary.

2.4.6 SPON1, PRAS40 and regulation of cell viability and/or proliferation

In our study, we observed that SPON1 modestly but significantly increased both KGN (human) and KK-1 (mouse) cell line viability and significantly increased proliferation in KGN cells. Our findings suggest a role for PRAS40 in granulosa cell viability and proliferation. PRAS40 is a member of mTORC1 (mammalian target of rapamycin complex 1), which regulates cell growth, proliferation, viability, protein synthesis and motility [59, 60]. Significantly, mTORC1 controls ovarian follicle growth by regulating proliferation of granulosa cells [28] and mTORC1 has been implicated in ovarian cancer cell proliferation [34, 61]. mTORC1 is composed of the kinase and catalytic subunit, mTOR, regulatory-associated protein of mTOR (Raptor/RPTOR), mammalian lethal with SEC13 protein 8 (MLST8), PRAS40 (which inhibits mTORC1 activity), and DEP domain containing MTOR-interacting protein (DEPTOR).

mTORC1 promotes cell survival and tumourigenesis and is positively correlated with proliferative disease progression [61]. Within the mTORC1 complex, unphosphorylated PRAS40 binds Raptor and mTOR under adverse conditions, such as low nutrients, and inhibits activity of mTORC1 by blocking AKT1 signalling to mTOR and directly inhibiting binding of substrates such as the translational repressor, eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) [62, 63]. Phosphorylation of
PRAS40 by AKT1 and mTOR physically removes PRAS40 from mTOR and activates the mTORC1 complex [63].

A linear signalling pathway linking cell surface to function has not yet been established for SPON1. However, a signal transduction pathway has been established for the SPON1-like protein RELN. [64-67] RELN is expressed in the adult mouse ovary [65] and in the chicken follicle where it promotes granulosa cell proliferation [67]. The RELN pathway provides us with a potential mechanism of action for SPON1 because SPON1 and RELN bind the same cell surface receptors, VLDLR and LRP8 [64, 68, 69]. RELN binding to VLDLR and LRP8 leads to DAB1 phosphorylation [70] which induces activation of AKT1, resulting in activation of mTORC1 [71]. Interestingly, SPON1 phosphorylates both DAB1 and AKT1 in chicken ciliary ganglion [13]. Thus, there are many parallels between the established RELN pathway, and the emerging details of SPON1 signalling. Further experiments are required to investigate this intriguing potential pathway of SPON1 signalling.

2.4.7 Limitation of Methods

One of the limitations of this study is the lack of a loss-of-function approach to establish a requirement of SPON1 for the functions we have demonstrated. This was not due to an oversight or lack of effort. For over two years, we attempted to reduce SPON1 levels using various siRNA approaches. We tried a number of different protocols, including multiple primer sets from different suppliers, a number of different cell lines, culturing conditions, and transfection reagents. The most success we had with this approach was a 50% reduction of SPON1 protein that lasted only 24 hours before SPON1 levels were restored to pre-knockdown levels. Thus, we were unable to use this loss-of-function approach to further support the results and conclusions of this study. Another limitation worth noting is that although granulosa cell lines are very useful for initial studies of function, they are not as physiologically relevant as rodent models, for example, and in vivo studies are required to complement our current data.
2.4.8 Conclusion

In summary, we have made several novel discoveries in the present study. We identify potential functions for SPON1 in the ovary, namely, regulation of: a) granulosa cell steroidogenesis, b) STAR and CYP11A1 expression, c) STAR promoter activity, and d) granulosa cell viability and proliferation. We also identify PRAS40 as a kinase target that is phosphorylated in the presence of SPON1, and is potentially involved in the signalling pathways regulating these functions. In addition, we demonstrate that cAMP-induced STAR protein levels are reduced by an ECM protein, and very few ECM proteins with this ability have been identified. These results strongly suggest that in addition to its role in other tissues, SPON1 plays a role in ovarian folliculogenesis by regulating granulosa cell function. We believe that these findings may help explain why SPON1 is highly overexpressed in ovarian cancer, and provide further support for its utility as an ovarian cancer marker.
2.5 Bibliography


44. Park SW, Schonhoff CM, Webster CRL and Anwer MS. Protein kinase c differentially regulates cAMP-dependent translocation of ntcp and mrp2 to the plasma membrane. AJP: Gastrointestinal and Liver Physiology 2012; 303:G657-G665.

45. Schonhoff CM, Webster CRL and Anwer MS. Cyclic amp stimulates mrp2 translocation by activating p38 mapk in hepatic cells. AJP: Gastrointestinal and Liver Physiology 2010; 298:G667-G674.


Chapter 3: Characterization of Spondin 1 function in mouse ovarian folliculogenesis

3.1 Introduction

Folliculogenesis, followed by successful ovulation, is a complex process that transforms a primordial follicle, an oocyte surrounded by a single layer of pre-granulosa cells, into a fully-mature pre-ovulatory follicle, consisting of an oocyte surrounded by several layers of cumulus granulosa cells, multiple layers of mural granulosa cells, a fluid-filled antrum, and a layer of vascularized theca cells separated from the rest of the follicle by a basal lamina. In response to luteinizing hormone, ovulation occurs and the oocyte is released, resulting in formation of a highly-vascularized corpus luteum (CL). The CL produces progesterone to prepare the uterus for implantation and maintain pregnancy, should it occur. This process requires the action of a large number of factors, including pituitary hormones, steroid hormones produced by the ovary itself, the extracellular matrix (ECM), glycoproteins, and growth factors. Despite extensive characterization of many of these factors, many more remain to be discovered, as indicated by the numerous genome-wide expression analyses that have been carried out using follicles at various stages of folliculogenesis. Identification of new regulatory factors may point to new directions of therapeutic intervention for women since unexplained sub-infertility or infertility contributes to a large proportion of female infertility, and defects in ovulation account for the greatest proportion of causes of infertility.

Spondin 1 (SPON1) was first characterized as a secreted (ECM) protein highly expressed in the mouse embryonic floorplate [1]. SPON1 has been primarily studied for its role in the central nervous system, where it regulates migration of neurons during early development and cell adhesion in many cell types of the central nervous system [2-6]. In C. elegans SPON1 maintains cell-ECM adhesion in several tissues, both neural and non-neural [7]. SPON1 also increases cell viability in neural cells. Chicken ciliary ganglion cells treated with recombinant SPON1 in culture have increased viability [8]. In a mouse neuroblastoma cell line knockdown of SPON1 resulted in decreased survival under adverse conditions that was rescued by SPON1 treatment [9].
Several lines of evidence suggest that SPON1 regulates angiogenesis, and in fact, may inhibit it. Angiogenesis is the growth of new blood vessels from pre-existing vessels and consists of several steps. Existing blood vessels are destabilized by separation of endothelial cells from mural cells, including pericytes and vascular smooth muscle cells (vSMC). The ECM must be degraded by proteases to allow migration of endothelial and mural cells. These cells then migrate towards the angiogenic stimuli. And finally, depending on the size of the vessel, mural pericytes (small and large vessels) and vSMC (large vessels) reattach to promote structural integrity. With the exception of tissue injury or tumour progression, angiogenesis only occurs in a few adult tissues, including the CL, the endometrium of the uterus, and the placenta. In relation to angiogenesis, SPON1 inhibits migration and tube formation by human umbilical vein endothelial (HUVEC) cells, and neovascularization in rat cornea [10]. SPON1 also promotes vSMC proliferation [11], and was isolated from bovine ovarian follicular fluid in a search for a factor that promoted vSMC proliferation in the ovary [11].

Despite the fact that SPON1 mRNA is highly expressed in the ovary in humans, as shown by Northern blot [11] there have been few reports describing SPON1 gene expression in the mammalian ovary or uterus [12]. As mentioned, SPON1 was identified in bovine follicular fluid [11]. In addition, when ovariectomized mice are treated with 17β-estradiol, Spon1 mRNA expression increases in both the uterus [13] and mammary gland [14], suggesting SPON1 may play a role in these reproductive tissues. Interestingly, SPON1 is also highly overexpressed in ovarian cancer [15, 16]. Our previous microarray studies [17] comparing the gene expression profiles of granulosa cells isolated from gonadotropin-treated immature (PND 23-29) C57Bl/6 wildtype (WT) and estrogen receptor β (ERβ)-null mice identified SPON1 (Spon1) expression to be lower in ERβ-null granulosa cells than in WT cells (Gene Expression Omnibus accession number GSE11585). Together this information points to a potentially significant role for SPON1 in the ovary. Given that there is evidence for a role for SPON1 in cell signalling in survival, differentiation and angiogenesis in other tissues, we also hypothesized that SPON1 may play a role in these functions in the uterus and ovary. Currently, it is not known in what cells of the ovary or uterus SPON1 is expressed. It is also not known
whether SPON1 expression is regulated by gonadotropins, or what functional roles it plays in these tissues.

Therefore, to further characterize the localization, potential function, and regulation of SPON1 by gonadotropins in the mouse, we examined SPON1 expression, localization, and regulation by follicle stimulating hormone and luteinizing hormone in the mouse ovary and uterus and SPON1 function in survival and steroidogenesis in mouse primary granulosa cells. We also examined the potential co-localization of SPON1 with markers of endothelial cells, pericytes, and vascular smooth muscle cells. Interestingly, our data point to a novel role for SPON1 in regulating cell survival and steroidogenesis in the ovary and angiogenesis in both the ovary and uterus.

3.2 Materials and Methods

3.2.1 Mice and treatments

Experiments were performed in compliance with the guidelines set by the Canadian Council for Animal Care, and the policies and procedures approved by the University of Western Ontario Council on Animal Care (Protocol Number: 2007-042). All mice used were of the strain C57Bl/6. All females were weaned at PND 21, and for mRNA analysis or immunofluorescence were treated at PND 22-23 with saline vehicle, 5.0 IU eCG (Sigma Chemical Co.) or 5.0 IU eCG followed 48 h later with 5.0 IU hCG (Sigma) unless stated otherwise. Both gonadotropins were dissolved in phosphate-buffered saline solution and injected subcutaneously in a total volume of 0.1 ml. At necropsy, animals were killed by CO₂-asphyxiation; and the ovaries and uterus were dissected, trimmed of surrounding tissue, and frozen and stored as described below.

3.2.2 Granulosa cell isolation and culture

To isolate granulosa cells ovaries were removed from females and immediately placed in a 60-mm cell culture dish on ice containing M199 medium (316-010; Wisent) supplemented with 1mg/ml of bovine serum albumin (BSA) (ALB003; Bioshop) 2.5 μg/ml of amphotericin B (15240-062; Life Technologies), and 50 μg/ml of gentamicin (450-135-XL; Wisent). Ovaries were pooled by genotype and the GCs were then isolated.
by manual puncture with 25-gauge needles followed by manual squeezing with a sterile spatula. Follicular debris was removed by filtration through a 150-nm Nitex nylon membrane (Sefar America, Inc.) mounted in a 25-ml syringe. The GCs were pelleted by centrifugation at 250 x g for 5 min at 4°C, washed in DMEM/F12 medium containing 1% penicillin/streptomycin solution (15070-063; Invitrogen) and re-centrifuged. The final cell pellet was re-suspended in DMEM/F12 with 1% pen/strep and counted using Trypan Blue and a haemocytometer. Cells were cultured in DMEM/F12 with 1% pen/strep for viability assays, progesterone assays, and qPCR of treated cells.

### 3.2.3 RNA isolation and quantitative RT-PCR

Ovaries and uteri were removed from virgin adult or treated prepubertal mice (see 3.2.1) and frozen. Uteri were pulverized using a mortar and pestle on dry ice with liquid nitrogen prior to RNA isolation. Whole ovaries and pulverized uteri were homogenized in Trizol (Invitrogen) and RNA was isolated according to the manufacturer’s protocol. RNA was further treated with DNaseI, then reverse-transcribed using Superscript II (Invitrogen). Complementary DNA (cDNA) levels were detected using quantitative RT-PCR with the Viia7 Sequence Detection System (Applied Biosystems) and SYBR Green I dye. Primers were generated using Applied Biosystems Primer Express Software version 2.0. The Rpl7 primers were: forward 5’-AGCTGGCCTTTGTCATCAGAA-3’, reverse 5’-GACGAAGGAGCTGCAGAACCT-3’. The Spon1 primers were: forward primer - 5’-TTGACTGTGAACTCAGCGAGTGGT-3’, reverse primer - 5’TCCGGGTTCGAATCATGTGACCTT-3’. Fold changes in gene expression were determined by quantitation of cDNA from treated samples relative to saline samples. Experiments were done in triplicate. Relative gene expression was analyzed with Rpl7 as the control gene using the ΔΔ-CT method. Differences in gene expression between treatments were measured using a two-tailed, unpaired Student’s t-test.

### 3.2.4 Western blot analysis

To determine SPON1 protein levels in mouse ovaries, and granulosa cells, tissues were homogenized in ice-cold radioimmunoprecipitation (RIPA) buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate,
50 mM Tris, pH 8.0) and lysate concentrations measured using the DC protein assay (product nos. 500-0113, 500-0114, 500-0115; Bio-Rad Laboratories (Canada) Ltd.). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with a rabbit polyclonal anti-SPON1 antibody (2 µg/ml) (product no. ab40797; Abcam) for one hour at room temperature (rt). After incubation with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) (product no. 934; GE Healthcare) for one hour at rt, SPON1 levels were visualized with Pierce ECL Plus Western Blotting Substrate (product no. 32132; Fisher Scientific) on Hyperfilm (product no. CA95017-653L, VWR).

3.2.5 Immunofluorescence

Primary antibodies used were: Anti-SPON1 (Santa Cruz Cat. No. sc-49004), Anti-PECAM1/CD31 (Abcam Cat. No. ab28364), Anti-Desmin (Abcam Cat. No. ab15200), anti-α Smooth muscle actin (Abcam Cat. No. ab5694). Secondary antibodies used were: FITC-conjugated rabbit anti-goat (Sigma Cat. No. F7367), and TRITC-conjugated donkey anti-rabbit (Abcam Cat. No. ab6799); and for confocal microscopy: Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 568 donkey anti-goat (Invitrogen, Cat. Nos. A21206, A11057). The Anti-SPON1 antibody detects a region in the C-terminus of the protein. Dissected ovaries were embedded in Cryomatrix (Fisher Scientific Inc.) and frozen using liquid nitrogen. Uteri horns and whole ovaries were embedded in Cryomatrix and frozen using liquid nitrogen. Tissues were cut into 6 µm sections (in the case of the uterus, cross-sections), mounted onto slides (Fisher) and stored at -80°C until use. Sections were fixed with 4% formaldehyde for 10 minutes, rinsed three times with phosphate-buffered saline (PBS), then permeabilized with 0.1% Triton X-100 for 15 minutes. Sections were again rinsed three times with PBS and then blocked for 30 minutes with blocking solution (5% BSA in 0.1% Triton X-100). The tissue was then incubated overnight with primary antibody diluted in blocking solution. For co-localized sections slides were incubated in primary antibodies simultaneously. Sections were then rinsed three times in blocking solution and incubated in secondary antibody. For co-localized sections slides were first incubated in the TRITC-conjugated donkey anti-rabbit secondary then rinsed three times with blocking solution and
incubated in FITC-conjugated rabbit anti-goat secondary. The slides were then washed twice in PBS and mounted with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) diluted 1:1 with Vectashield (Vector Laboratories). Slides were visualized within 24 hours with an Olympus Provis AX70 upright microscope. Images were captured using Image-Pro 6.2 Software. Confocal analysis was performed using an Olympus Fluoview 1000 laser-scanning confocal microscope (Olympus).

3.2.6 Viability Assay

Primary granulosa cells were seeded at 40,000 cells per well in 96-well plates, incubated for one hour, then treated with recombinant human SPON1 (5 μg/ml; R&D Systems). Cell viability was measured after 24 and 48 hours using the Cell Titer AQeuous Non-Radioactive Cell Proliferation Assay (MTS) (product no. PRG3582; Promega). Differences in viability between vehicle treatment alone or treatment with SPON1 at each time-point were analyzed by a two-tailed, unpaired, Student’s t-test. (PRISM 6; GraphPad).

3.2.7 Progesterone Assay

Primary granulosa cells were plated at 500,000 cells per well in a 24-well plate in DMEM/F12 supplemented with 1% Penicillin/Streptomycin (Wisent). After 24 hours, cells were treated with bovine serum albumin (BSA) control (250 μg/mL; BioShop Canada Inc.), recombinant human SPON1 (SPON1) (5 μg/mL; R&D Systems, Minneapolis, MN), dibutyryl-cAMP (dbcAMP) (1 mM) (Enzo Life Sciences), both SPON1 and dbcAMP, FSH (recombinant human FSH from National Hormone and Peptide Program) and testosterone (National Hormone and Peptide Program), or FSH, testosterone, and SPON1. Media was removed after 48 hours and frozen at -80°C until further analysis. Protein lysates were prepared using RIPA buffer supplemented with a protease inhibitor cocktail (1:1,000 dilution, product no. P8340-5ml; Sigma). Protein lysate concentrations were determined using the DC protein assay (Bio-Rad). Cell culture media was sent to the University of Virginia Centre for Research in Reproduction Ligand Assay and Analysis Core and the progesterone concentration determined by Progesterone radioimmunoassay (RIA). Progesterone concentrations were calculated by normalizing
ng/μl progesterone/well to the total protein/well. Differences in progesterone levels between all treatments were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey Multiple Comparison post-hoc test (Tukey test) (PRISM6.0; GraphPad).

3.3 Results

3.3.1 Expression of Spon1 mRNA and protein in the mouse female reproductive organs

Our first goal was to determine if SPON1 was expressed in the mouse ovary and uterus and if it was regulated by gonadotropins there. To determine if Spon1 mRNA levels in the ovary and uterus were regulated by the gonadotropins, mice were treated with equine chorionic gonadotropin (eCG), which activates the follicle stimulating hormone (FSH) receptor, and human chorionic gonadotropin (hCG), which stimulates the luteinizing hormone (LH) receptor, and induces ovulation. Prepubertal mice (PND 21-23) were treated with saline vehicle (48h), eCG (5 IU, 48h), or eCG followed by hCG (5 IU, 24h). These treatments mimic the FSH-stimulated stage of folliculogenesis and the LH-stimulated ovulation stage of folliculogenesis. Ovaries and uteri were isolated, and analyzed for Spon1 mRNA levels by quantitative RT-PCR analysis. In ovaries, eCG treatment stimulated a six-fold increase in Spon1 mRNA levels over saline-treated ovaries, and was reduced to 2-fold after hCG treatment (Fig. 3-1 A). In the uterus, eCG treatment stimulated a five-fold increase in Spon1 mRNA levels over saline-treated uteri, and was also reduced to 2-fold after hCG treatment (Fig. 3-1 B). Western blots performed with extracts of both the ovary and granulosa cells showed high levels of SPON1 (Fig. 3-1 C, D).

3.3.2 SPON1 localization in the adult mouse ovary and uterus

The location of SPON1 within the ovary and uterus, to our knowledge, has never been identified (other than in bovine follicular fluid [11]). Therefore, we determined SPON1 localization using immunofluorescence in the ovaries and uterus of adult female mice (Fig. 3-2 A, B). In the adult mouse ovary, SPON1 localized to the theca cells (Fig. 3-2 A, open arrow) and the interstitium between small to large follicles (Fig. 3-2 A) with
Figure 3-1. Expression of SPON1 mRNA in mouse ovary and uterus and protein in ovary and granulosa cells.

A and B. Post-natal day (PND) 21-23 C57Bl/6 mice were treated with saline, equine chorionic gonadotropin (eCG) (5.0 IU per mouse) for 48 hours, or eCG (5.0 IU) for 48 hours followed by human chorionic gonadotropin (hCG; 5.0 IU per mouse) for 24 hours. Ovaries and uteri from three mice were used to isolate mRNA and prepare cDNA for analysis by quantitative RT-PCR compared to an Rpl7 control. eCG treatment increases Spon1 mRNA levels in the ovary and uterus and further treatment with hCG reduces this expression. Data are expressed as average gene expression compared with the Rpl7 control (± SEM of three mice). Differences in average mRNA levels between treatments were analyzed by two-tailed, unpaired, Student’s t-test. A. In the ovary there were
significant differences between saline and eCG, and eCG and eCG + hCG, P < 0.001, and P < 0.01, respectively. B. In the uterus there was a significant difference between saline and eCG, P < 0.001. ** = P < 0.01. *** = P < 0.001. C. Western blot of whole ovary and granulosa cells with anti-SPON1 antibody showing high expression of SPON1. For both ovary and granulosa cell, lanes are duplicates of the same lysate.
Figure 3-2. SPON1 localization in the mouse ovary and uterus.

Immunofluorescence with an anti-SPON1 antibody was used to detect SPON1 localization and expression in the mouse ovary (A) and uterus (B) of mice treated with eCG followed by hCG. A. In the ovary SPON1 was localized to the theca cells (A c, open triangle), corpus luteum (A a-d, higher magnification in b) and weakly in granulosa cells (square). SPON1 is also present in the ovarian epithelium (A c, white arrow) B. In the uterus SPON1 was detected in the stroma of the endometrium, and weakly in the epithelial cells lining the lumen or endometrial glands (B a, b, white triangle). SPON1 was weakly detectable in the inner or outer layers of the myometrium (B a, b, open triangle).
triangle). Interestingly, SPON1 was also found in the stratum vasculare, the layer of large blood vessels located between the inner and outer layers of the myometrium (B, b, open square). All images are 100x magnification except A, b which is 200x.
weak staining in the outer granulosa layer of various follicular sizes (Fig. 3-2 A, square in panel a). SPON1 staining was also strikingly high within corpora lutea (Fig. 3-2 A panel b), and also in the ovarian epithelium (Fig. 3-2A panel a, white arrow).

In the uterus, SPON1 was localized to the stroma of the endometrium, and weakly in the epithelial cells lining the lumen or endometrial glands (Fig. 3-2 B, panels a and b, white arrow). It was also weakly detectable in the inner and outer layers of the myometrium (Fig. 3-2 B, panels a and b, open arrow). Interestingly, SPON1 was expressed in the stratum vasculare, the layer of blood vessels located between the inner and outer layers of the smooth muscle of the myometrium (Fig. 3-2 B, open square).

3.3.3 SPON1 localizes with endothelial cell markers in the mouse ovary

Due to the localization of SPON1 to what appeared to be the vasculature of the follicle and corpus luteum of the ovary, and its expression in the uterine stratum vasculare and the highly-vascularized stroma, we hypothesized that SPON1 may localize with the vasculature in both tissues, and perhaps play a role in regulating angiogenesis in these tissues. To test this hypothesis, we investigated the potential co-localization of SPON1 with three traditional markers of vasculature, namely: a) platelet endothelial cell adhesion molecule 1 (PECAM1, also called CD31), a glycoprotein used as a general endothelial cell surface marker including pericytes b) desmin (DES), an intermediate filament protein that is a marker of pericytes, and c) alpha smooth muscle actin (αSMA), officially known as “actin, alpha 2, smooth muscle” (ACTA2), a microfilament present in high amounts in vascular SMC. ACTA2 is found in larger blood vessels walls, but not in smaller capillaries which do not contain smooth muscle. Co-localization of SPON1 with all markers was also examined by plotting the intensity of green (SPON1) vs. red (marker) pixels on an XY graph for the merged images (Appendix C).

In the mouse ovary, PECAM1 has been shown to localize to the blood vessels in the theca cell compartment that surrounds the basal lamina and in the vasculature of the CLs [18]. As shown in Figure 3-3, we observed similar localization of PECAM1.
Figure 3-3. Co-localization of SPON1 and PECAM1 in the mouse ovary.
Immunofluorescence with an anti-SPON1 and anti-PECAM1 antibody was used to detect SPON1 and PECAM1 localization and expression in the mouse ovary. A. Both SPON1 (green) and PECAM1 (red) localized to the theca cell layer (white arrow) in two large pre-antral/preovulatory follicles. A is 100x magnification. B. A mature corpus luteum (CL) beside a secondary follicle. Both SPON1 (green) and PECAM1 (red) localized to the theca cell layer of the secondary follicle, and throughout the CL. Overlap between SPON1 and PECAM1 at these sites was observed (an open arrow indicates one example of overlap). C. A developing CL. PECAM1 (red) shows overlap with SPON1 (green) (white arrows indicate examples of overlap). B and C are 200x magnification. D. Confocal microscopy of the thecal cell layer and a developing CL (bottom) show overlap of SPON1 (red) and PECAM1 (green) in these areas. Mouse treatments were as follows: (A) eCG, 48h; (B, C, and D) eCG, 48h, followed by hCG for 24 hours.
Co-localization experiments using both antibodies showed a significant overlap of PECAM1 and SPON1 in the theca cell layer (see Fig. 3-3 A, white arrows), and in mature (Fig. 3-3 B, open arrow) and developing corpora lutea (Fig. 3-3 C, open arrow). This overlap is even more striking under the high magnification of a confocal microscope (Fig. 3-3 D).

Similarly, DES localized to the theca cell layer of developing follicles (Fig. 3-4 A, open arrows) and the corpora lutea, but not granulosa cells. DES, like PECAM1 showed overlap with the SPON1 signal in the theca cells (Fig. 3-4 A, open arrows) and in the corpus luteum (Fig. 3-4 A, white arrows). However, co-localization of DES with SPON1 was less dramatic than with PECAM1 (Appendix C). Also, while SPON1 showed some expression surrounding granulosa cells, DES did not (Fig. 3-4 A, open square).

Finally, ACTA2 was also expressed in the theca cell layer surrounding follicles (Fig. 3-4 B), but not in the corpus luteum where large vessels are rarely present. Again, we observed co-localization between the SPON1 and ACTA2 signal in the theca cell layer, although, like DES, it was less dramatic than with PECAM1 (Appendix C).

### 3.3.4 SPON1 localizes with endothelial cell markers in the mouse uterus

In the uterus, we observed a high degree of co-localization between PECAM1 and SPON1 (Fig. 3-5, white arrows, Appendix C). An exception was that faint SPON1 expression was observed in the luminal and glandular epithelial cells while PECAM1 was not (Fig. 3-5).

Between DES and SPON1 there was only co-localization in the stroma (Fig. 3-6 A, Appendix C). Interestingly, DES was highly expressed in inner and outer layers of the myometrium and not the stratum vasculare (Fig. 3-6 A, white arrow), while SPON1 is expressed in the only in the stratum vasculare of the myometrium.
Figure 3-4. Co-localization of SPON1 with DES and ACTA2 in the mouse ovary.

Immunofluorescence with an anti-SPON1 antibody and antibodies against vascular/pericyte markers, DES and ACTA2, were used to characterize the overlap between SPON1 and the ovarian vasculature. Panels A, a-c and B, a-c are 100x
magnification, A, d-f, and B, d-f are 200x. A. DES (red) localized to the blood vessels in the theca cell layer around a large antral follicle, and in the vasculature of the corpora lutea (CL), and showed a significant overlap (A, c and f) with SPON1 (green) in the theca cell layer (open arrow) and CL (white arrows), but not in granulosa cells, where only SPON1 was expressed (open square). B. ACTA2. ACTA2 localized solely to the theca cell layer surrounding follicles, but unlike DES, was not detected in CLs. Mouse treatments for all images were eCG, 48h, followed by hCG for 24 hours.
Figure 3-5. Co-localization of SPON1 and PECAM1 in the mouse uterus.

Immunofluorescence with an anti-SPON1 antibody and anti-PECAM antibody was used to characterize the potential for overlap between SPON1 and the uterine vasculature. Panels a-c are 100x magnification and d-f are 200x. PECAM1 was robustly detected throughout the uterus, but not in the luminal and ductal epithelium. As shown in Figure 3-2 B, SPON1 was detected in the stroma of the endometrium, and weakly in the epithelial cells lining the lumen or endometrial glands. Co-localization of PECAM1 with SPON1 appeared almost total, except for the weak luminal and ductal epithelial staining by SPON1 where PECAM1 was undetectable. An example of overlap in the stroma is shown with white arrows. Mouse treatment for all images was eCG, 48h, followed by hCG for 24 hours.
Figure 3-6. Co-localization of SPON1 with DES and ACTA2 in the mouse uterus.

Immunofluorescence with an anti-SPON1 antibody and antibodies against vascular/pericyte markers, DES and ACTA2, were used to characterize the overlap between SPON1 and the uterine vasculature. A. Desmin (DES) staining was found throughout the stroma but not in the luminal and ductal epithelium. Co-localization with
SPON1 (panel c) occurred in the stroma. B. ACTA2, as expected, was expressed primarily in the myometrium. Little overlap was observed between SPON1 and ACTA2. Mouse treatment for all images was eCG for 48h, followed by hCG for 24 hours. For both A and B magnification for all panels a-c was 100x and for d-f 200x.
Finally, ACTA2 was, unsurprisingly, expressed in both the inner and outer layers of the myometrium, and there was almost no co-localization between ACTA2 and SPON1 (Fig. 3-6 B, Appendix C).

### 3.3.5 SPON1 treatment increases viability of mouse granulosa cells

A function of ECM proteins in the ovary is to regulate cell survival and SPON1 has been shown to affect survival of neural cell types. Therefore we measured the viability of mouse primary granulosa cells in the absence or presence of recombinant SPON1. To measure the effect of SPON1 on cell survival, isolated wildtype granulosa cells isolated from prepubertal mice were treated with recombinant SPON1 and viability was measured using an MTS assay. SPON1-treated cells were significantly more viable than control-treated cells after both 24h and 48h (Fig.3-7).

### 3.3.6 SPON1 treatment increases dbcAMP- and FSH-induced progesterone production

SPON1 affects differentiation of nerve cells, a function known to be carried out by ECM proteins in ovarian cell types during folliculogenesis. An indicator of differentiation in granulosa cells is the ability to produce 17β-estradiol and progesterone but the effects of SPON1 on steroid production have never been measured in any cell type. Therefore we quantified 17β-estradiol and progesterone production of mouse primary granulosa cells by measuring both steroids in culture medium following various treatments. SPON1 treatment increased progesterone production in cells co-treated with either dibutyryl-cAMP (dbcAMP) or FSH and testosterone but had no effect on progesterone production by itself (Fig. 3-8). However, SPON1 treatment had no effect on 17β-estradiol production (data not shown).
Figure 3-7. SPON1 treatment increases viability of mouse primary granulosa cells.

Measurement of cell viability by an MTS assay at 24 and 48 hours following treatment with control BSA (bovine serum albumin) or recombinant SPON1. Each bar represents the mean ± SD of triplicate measurements within one representative experiment. Three experiments were performed and all were significant. Differences in viability between vehicle treatment alone or treatment with SPON1 at each time-point were analyzed by a two-tailed, unpaired Student’s t-test, \( P < 0.05 \). There were significant differences between treatment at both 24h and 48h, \( P < 0.05 \). * = \( P < 0.05 \).
Figure 3-8. SPON1 treatment increases progesterone output of mouse primary granulosa cells.

Measurement of progesterone levels by radioimmunoassay normalized to protein levels. Each bar represents mean ± SEM of triplicate experiments. Differences in progesterone level were measured by one-way ANOVA followed by a Tukey Multiple Comparison post-hoc test (Tukey test). There was a statistical significance between treatments as determined by one-way ANOVA, $F(6, 14) = 39.01$, $P > 0.0001$. There were no statistical differences between BSA (SPON1 control), water (dbcAMP and FSH + T control), and SPON1 treatment. A significant difference was found between water and dbcAMP, $P < 0.05$, between dbcAMP and dbcAMP + SPON1, $P < 0.05$, between water and FSH + T, $P < 0.01$, and between FSH + T and FSH + T + SPON1, $P < 0.001$. * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$
3.4 Discussion

3.4.1 Summary of findings

In this study, we have shown that SPON1 protein is highly expressed in the ovary and uterus. We show that eCG increases the mRNA levels of Spon1 in both mouse ovary and uterus, and that hCG following this treatment reduces Spon1 levels almost to pre-treatment levels. In the ovary, we show that SPON1 strongly localizes to the theca cell layer and the corpus luteum, as well as weakly in the granulosa cell layer. In the uterus, SPON1 is strongly present in the stroma of the endometrium, but only weakly in the epithelial cells of the lumen or glands. SPON1 is very minimally expressed in the inner and outer layers of the myometrium, but is expressed in the stratum vasculare between these layers. Interestingly, in both ovary and uterus, SPON1 co-localizes with the vascular markers, PECAM1 and DES, but not a marker of larger vessels containing smooth muscle actin (ACTA2). These results suggest that SPON1 may play a role in regulating angiogenesis in the ovary and uterus, particularly in developing capillaries. We show that SPON1-treated granulosa cells have increased viability and that both dbcAMP- and FSH with testosterone-induced production of progesterone is elevated with SPON1 co-treatment. Taken together these results strongly suggest an important role for SPON1 in folliculogenesis and pregnancy.

3.4.2 Regulation of SPON1 expression by gonadotropins

Given our discovery of a potential role for SPON1 in female reproductive angiogenesis and granulosa cell function, it is not surprising that Spon1 mRNA levels are regulated by gonadotropins. Dynamic angiogenesis occurs throughout the menstrual or estrus cycle in both ovary and uterus, while FSH induced cell growth and differentiation of granulosa cells is required for folliculogenesis. In the ovary, cAMP signalling may increase Spon1 transcription, as is common for many eCG-regulated genes. It is particularly interesting that after hCG treatment (mimicking the LH surge), Spon1 levels are reduced, suggesting that Spon1, like many other genes, is downregulated following the LH surge that transitions the follicle from growth to ovulation to luteinization. In the uterus, it is also possible that circulating estrogens (resulting from eCG treatment)
increase Spon1 levels, rather than cAMP-mediated events, given that Spon1 mRNA levels are increased by estradiol in the mouse uterus [13].

Many questions remain to be answered to understand the exact role of SPON1 in the ovary and uterus. First, further studies using in situ hybridization are needed to determine which cells within the ovary produce SPON1. Our data (unpublished) indicate that isolated granulosa cells produce both Spon1 mRNA and protein. The presence of SPON1 in the theca cell layer suggests either that theca cells express SPON1 or that granulosa cells secrete SPON1 to theca cells across the basal lamina.

3.4.3 SPON1 localizes with PECAM1 and DES, but not ACTA2 in the mouse ovary and uterus

In the mouse ovary, PECAM1, a marker of both endothelial cells and pericytes, localizes to the blood vessels in the theca cell layer and in the vasculature of the corpora lutea [18]. We observed similar localization of SPON1 in the ovary by co-localization experiments. In the uterus, PECAM1 has been shown to localize to the highly-vascularized stromal endometrium [19], and we observe almost identical colocalization of SPON1. Co-localization with PECAM1 suggests that SPON1 plays a role in both corpus luteum angiogenesis and in the vascularization of the theca cell layer as follicles grow and progress through folliculogenesis.

DES is a marker of pericytes or smooth muscle cells [20] in a tissue-dependent manner. DES localization has previously been characterized in the ovaries of various avian and mammalian species, including the emu, ostrich, rat, and African Giant Rat [21-25]. The localization of DES we observed in the mouse ovary is consistent with that published for the rat [25]. In the rat, DES was “dispersed in the CL”, and in the “arteries around the CL, as well as elsewhere in the ovary” [25], including the theca cell layer. Thus, the partial co-localization we observed between DES and SPON1 in the ovary would be consistent with its previously published location within the ovary. In the adult mouse, “the developing endometrial stroma … strongly expressed DES”, as did the myometrial layers [26]. As we also observed, there was no DES staining in the endometrial epithelium, where we observed only weak SPON1 staining. Thus, the DES
localization we observe in the uterus is consistent with what has previously been published, and suggests SPON1 may play a role in uterine angiogenesis due to its proximity to this marker.

The localization of ACTA2, which is a marker of the internal structure of smooth muscle cells and pericytes in blood vessel walls, has also been studied in various species. In the mouse ACTA2 has previously been shown to be present in the theca cell layer surrounding follicles but not expressed in the CL [27]. In contrast, in the African Giant Rat ACTA2 is still strongly present in the theca cell layer, but is also seen within limited pockets within the CLs [24]. Consistent with the previous work, in our study, we found ACTA2 in the theca and interstitium but not the CLs. The lack of presence of ACTA2 suggests that only capillaries, which do not contain smooth muscle, are present in the mouse CL. In the uterus of mouse and rat, DES and ACTA2 localization has also been characterized. In the uterus, by far the predominant location of ACTA2 is reported to be in the myometrial layers [26, 28, 29], and this is consistent with our studies, as we saw expression of ACTA2 solely in the myometrium.

3.4.4 A role for SPON1 in ovarian and uterine angiogenesis?

These localization studies suggest that SPON1 associates most closely with capillaries and smaller vessels where we observed high co-localization with PECAM1. The lesser co-localization of SPON1 with ACTA2 in the theca cell matrix suggests SPON1 may also associate with larger blood vessels although not as closely. Ovulation results in angiogenesis from pre-existing vessels of the theca cell layer into the newly formed corpora lutea, producing an abundant vasculature that touches almost every cell in the corpus luteum. We have observed the presence of SPON1 early in this process and once the vasculature has formed suggesting that SPON1 is involved in the entire process. Angiogenesis also takes place in the developing follicle before ovulation, in the interstitial and theca cell layers. We have observed SPON1 in both ovarian sites of angiogenesis.

The lack of ACTA2 in the mouse CL suggests that there are no smooth muscle cells and only capillaries are present. Therefore, although DES is a marker of smooth
muscle cells and pericytes, in the CL in our study, it is most likely only showing the presence of pericytes. Thus, when SPON1 and DES are co-localized in the CL, we feel this is supportive of SPON1’s role in angiogenesis, as pericytes are critical for the process of angiogenesis.

We therefore postulate that SPON1 regulates angiogenesis in the ovary and uterus. While SPON1 may either promote or inhibit angiogenesis, there is more evidence in the literature to support an inhibitory role for angiogenesis in the ovary, especially since SPON1 mediated vSMC growth could be interpreted as pro- or anti-angiogenic, as vSMCs surrounding mature vessels is a last step in angiogenesis. Treatment of HUVECS with recombinant human SPON1 inhibits the spreading of HUVECs plated onto anti-α,β3 integrin antibodies, and also inhibits VEGF-mediated HUVEC tube formation [10]. Intriguingly, endostatin, a naturally-occurring 20 kDa angiogenesis inhibitor found in humans, also inhibits angiogenesis by binding to integrin α,β3 [30]. Therefore, we speculate that in both ovary and uterus, SPON1 may participate in regulating the size or length of blood vessels, possibly limiting the size of vessels in the corpus luteum to only capillaries. This provides a possible explanation of SPON1 overexpression in ovarian cancer, ie. SPON1 may be attempting to inhibit angiogenesis, but failing.

3.4.5 SPON1 affects cell survival and differentiation of primary granulosa cells

SPON1 has been shown to affect cell survival and cell differentiation in neural and non-neural cells. We show that treatment of wildtype mouse granulosa cells with recombinant SPON1 led to increased viability. We could not determine if there was increased proliferation as primary granulosa cells that are not treated with hormones or cultured with fetal bovine serum do not proliferate. As mentioned above, SPON1 promotes cell viability of chicken ciliary ganglion[8] and mouse neuroblastoma cells [9]. In chicken ciliary ganglion this was achieved through increased active TGFβ levels [8] while in neuroblastoma cells loss of viability following SPON1 knockdown was attributed to lower IL-6 levels [9]. With current knowledge a likely candidate for the effect of SPON1 on cell viability in granulosa cells is alteration of TGFβ signalling, as TGFβ is an important protein in folliculogenesis and also regulates IL-6.
Differentiation of early granulosa and theca cells to steroid producing cells is an essential step in folliculogenesis. Differentiation of cells due to SPON1 has been observed in nerve cells [5], cementoblasts [31], and chondrocytes [32]. The ability of SPON1 to alter gene expression has been seen in chondrocytes where expression of proteins involved in ECM remodelling is increased by SPON1 [32, 33]. Again, these changes in gene expression were brought about by increased levels of TGFβ [32, 33]. Granulosa cells treated with SPON1 and either dbcAMP or FSH and testosterone resulted in increased production of progesterone compared to cells that were not treated with SPON1. Because SPON1 affects both viability and steroid production of wildtype granulosa cells we hypothesize that it may play an important role in vivo in these functions.

3.4.6 Conclusion

In summary, we have shown for the first time that SPON1 is expressed in both the ovary and uterus and that expression changes in response to gonadotropins. We have also shown that SPON1 localizes to the theca cell layer and corpus luteum in the mouse ovary, and to the endometrial stroma and the myometrial stratum vasculare of the mouse uterus. Through co-localization studies with vascular cell markers we have shown that SPON1 is present at small and, to a lesser extent, large blood vessels. This is the first in vivo evidence showing that SPON1 is associated with blood vessels and therefore may have a role in angiogenesis. We have also found possible functional roles for SPON1 within ovarian folliculogenesis as a mediator of FSH-induced granulosa cell proliferation and differentiation. These studies suggest the enticing possibility that SPON1 may be a new regulator of angiogenesis and folliculogenesis with potential therapeutic implications.
3.5 Bibliography


4 Chapter 4 – Spondin 1 is required for normal ovarian function in \textit{Mus musculus}

This chapter is based on a manuscript that has been prepared but not yet submitted.

4.1 Introduction

The ovary functions to prepare oocytes for ovulation and fertilization via folliculogenesis and as an endocrine gland producing steroid hormones to act on itself and other organs. These processes require involvement of the extracellular matrix (ECM). The ovary is constantly undergoing tissue remodelling as follicles grow and follicular cells differentiate, and the ECM modulates these changes [1]. ECM proteins affect follicle growth and development [2] and are involved in granulosa cell proliferation, survival, morphology and steroidogenesis [3-6]. Numerous studies have found that granulosa cells or ovarian tissue cultured with ECM proteins, such as laminin, fibronectin, and collagen IV, or Matrigel (a gelatinous protein mixture rich in ECM proteins) are more viable than those grown with other growth media [2, 3, 7].

As folliculogenesis progresses, the composition of the ECM changes and several different types of ECM proteins are required for normal ovarian cell differentiation and proliferation. These include the above mentioned laminins, fibronectins, and collagens, as well as proteoglycans with heparan sulfate or chondroitin sulfate side chains. Proteoglycans are involved in several cellular processes including cell adhesion, ligand-receptor signalling, and basement membrane organization [8]. Matrix metalloproteinases (MMPs) degrade ECM proteins to remodel the ECM [1, 5]. In addition to supporting cells physically, ECM proteins are also important for signal transduction [9]. Integrins are often responsible for signal transduction in cell-ECM interactions. There are several examples of impaired fertility in knockout mouse models where ECM genes are disrupted, including MMP9 [10], integrin-β1 [11], Tnfaip6 [12] and ADAMTS-1[13-15]. However, because the ECM is important for normal function in all tissues, mutations or deletions of specific ECM proteins are often lethal and effects on fertility cannot be evaluated, or the deletion results in no phenotypic changes due to functional redundancy.
Spondin 1 (SPON1) is a secreted ECM glycoprotein encoded by the gene Spon1 [16]. It was initially studied for its role in neuron outgrowth during embryonic development [17-22]; however, Spon1 is also expressed in other tissues such as ovary, kidney, bone, and small intestine, where several other functions for SPON1 have been uncovered. In C. elegans Spondin 1 interacts with integrins to maintain cell adhesion [23]. In bone, chondrocyte differentiation and endochondral bone formation are regulated by SPON1 in mice [24] and expression of SPON1 is high in human osteoarthritic cartilage [25]. In chondrocytes and cartilage, treatment with SPON1 increased levels of MMP-13 [24, 25] and in cartilage, SPON1 increased the active levels of TGFβ [25].

There is compelling evidence that SPON1 plays a role in the ovary. Spon1 expression increases in response to 17β-estradiol in the mouse mammary gland [26] and the uterus [27]. SPON1 isolated from bovine ovarian follicular fluid increases proliferation of rat vascular smooth muscle cells activity, and inhibits tube formation of human umbilical vein endothelial cells, suggesting a role in angiogenesis [28, 29]. SPON1 has also been identified as an ovarian cancer marker [30-32].

The SPON1 protein is comprised of: a) an N-terminal reeler domain, homologous to a domain in the protein, Reelin, b) a spondin domain homologous to domains in Spondin 2 (Mindin/M-spondin), and c) six thrombospondin type 1 repeats (TSRs). Several members of the thrombospondin superfamily are known to be involved in normal ovarian function. Thrombospondin -1 and -2 are both expressed in the ovary and regulate angiogenesis [33, 34]. R-spondin is expressed in the developing ovary and female knockout mice have masculinized gonads and impaired ovulation [35-37]. A disintegrin and metalloproteinase with thrombospondin repeats 1 (ADAMTS-1) knockout mice have defective follicular growth, abnormal steroid gene mRNA expression, and are subfertile [13, 15].

The SPON1 knockout mouse (Spon1−/−) is viable and so far has been studied for its potential role in osteoarthritis [38] but its fertility has not been rigorously examined. Spon1−/− mice have increased bone mass compared to controls, possibly brought about due to reduced levels of TGFβ-1 resulting in increased Smad1/5 phosphorylation. In the
present study we sought to elucidate the role of SPON1 in female fertility and the ovary using Spon1-null (Spon1\(^{-/-}\)) mouse as a model.

4.2 Materials and Methods

4.2.1 Mice and Treatments

Experiments were performed in compliance with the guidelines set by the Canadian Council for Animal Care and the policies and procedures approved by The University of Western Ontario Council on Animal Care. Investigations were conducted in accordance with the National Research Council’s Guide for Care and Use of Laboratory Animals. Wildtype C57Bl6 (Spon1\(^{+/-}\)) and Spon1\(^{-/-}\) mice were kindly provided by Dr. Steven Abramson (New York University). Genotyping was performed as in Palmer et al. 2014 [38]. Mice were bred heterozygous x heterozygous and litter mates were used for all experiments, with the exception of counting COCs. For superovulation experiments involving gonadotropin treatment, postnatal day 23-28 Spon1\(^{+/-}\) and Spon1\(^{-/-}\) female mice were treated with saline or 5.0 IU equine chorionic gonadotropin (eCG) (Sigma) for 48 hours followed by 5.0 IU human chorionic gonadotropin (hCG) (Sigma) for the indicated times.

4.2.2 Granulosa Cell Isolation and Cell Culture

To isolate granulosa cells (GCs), ovaries were removed from Spon1\(^{+/-}\) or Spon1\(^{-/-}\) females and immediately placed in a 60-mm cell culture dish on ice containing M199 medium (product no. 316-010; Wisent) supplemented with 1 mg/ml of bovine serum albumin (BSA) (product no. ALB003; BioShop), 2.5 μg/ml of amphotericin B (product no. 15240-062; Life Technologies), and 50 μg/ml of gentamicin (product no. 450-135-XL; Wisent). Ovaries were pooled by genotype and manually punctured with 25-gauge needles followed by pressure applied with a sterile spatula. Follicular debris was removed by filtration through a150-nm Nitex nylon membrane (Sefar America, Inc.) mounted in a 25-ml syringe. GCs were pelleted by centrifugation at 250 x g for 5 min at 4°C, washed in DMEM/F12 medium containing 1% penicillin/streptomycin solution (pen/strep) (product no. 15070-063; Invitrogen) and centrifuged as previously described. The final cell pellet was re-suspended in DMEM/F12 containing 1% pen/strep and the cells
counted using Trypan Blue and a haemocytometer. Cells were cultured in DMEM/F12 with 1% penicillin/streptomycin for viability assays, progesterone assays, and quantitative RT-PCR (qPCR).

4.2.3 Quantitative RT-PCR

To measure mRNA levels of Spon1 in Spon1<sup>+/+</sup> mice ovaries, uterus, and isolated granulosa cells, RNA was isolated using Trizol (product no. 15596; Life Technologies). RNA was reverse transcribed using Superscript II reverse transcriptase (product no. 18064-014; Life Technologies). Rpl7, a ribosomal protein, was used as a control. Relative gene expression levels were measured using SYBR Green Master Mix and Rpl7 (forward primer – 5’-AGCTGGCCTTTGTCATCAGAA-3’, reverse primer –5’-GACGAAGGAGCTGCAGAACCT-3’) and Spon1 (forward primer - 5’-TTGACTGTGAACTCAGCGAGTTGGT-3’, reverse primer - 5’TCCGGGTTCGAATCATGTGACCTT-3’) cDNA levels were determined in 384-well plates with 10 μl reactions using the Applied Biosystems 7900HT Fast Real Time PCR System. Relative gene expression was analyzed with Rpl7 as the control gene using the ΔΔ-CT method. To measure mRNA levels of other genes in ovaries of Spon1<sup>+/+</sup> and Spon1<sup>−/−</sup> mice, RNA was isolated using the Qiagen RNeasy mini kit, the second step of which requires the Qiashredder kit (product nos. 74104 and 79654, respectively; Qiagen). Relative mRNA levels were determined using TaqMan Fast Advanced Master Mix (product no. 4444963; Life Technologies) and TaqMan Gene Expression Assays (product no. 4331182; Life Technologies) probe/primer sets. cDNA levels were determined as described for Spon1. For all qPCR experiments, differences in expression between genotypes or treatments were measured using a two-tailed, unpaired Student’s t-test with Welch’s correction or a two-way ANOVA with Fisher’s LSD post-hoc test (PRISM6; GraphPad).

4.2.4 Western Blot

To determine SPON1 protein levels in mouse ovaries, and granulosa cells, tissues were homogenized in ice-cold Radioimmunoprecipitation (RIPA) buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0) and lysate concentrations measured using the DC protein
assay (product nos. 500-0113, 500-0114, 500-0115; Bio-Rad Laboratories (Canada) Ltd.). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with a rabbit polyclonal anti-SPON1 antibody (2 μg/ml) (product no. ab40797; Abcam) for one hour at room temperature (rt). After incubation with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) (product no. 934; GE Healthcare) for one hour at rt, SPON1 levels were visualized with Pierce ECL Plus Western Blotting Substrate (product no. 32132; Fisher Scientific) on Hyperfilm (product no. CA95017-653L, VWR).

4.2.5 Immunofluorescence

*Spon1*+/+ and *Spon1*−/− mice were treated with saline or 5.0 IU equine chorionic gonadotropin (eCG) (Sigma) for 48 hours followed by 5.0 IU human chorionic gonadotropin (hCG) (Sigma) for 24 hours. Ovaries were removed and immediately frozen in Cryomatrix (product no. 67-690-06, Fisher Scientific). Ovaries were sectioned at 5 μm and placed on glass slides (product no. 12-550, Fisher Scientific), then stored at -80°C until further use. Prior to exposure to antibody, slides were allowed to adjust to rt. Ovaries were fixed in 4% formaldehyde (product no. FOR201, BioShop) and rinsed three times for 5 minutes with PBS. Sections were permeabilized with 0.1% Triton X-100 (product no. X100, Sigma) in PBS and rinsed three times with PBS. Sections were blocked with 5% BSA and 0.1% Triton X-100 in PBS. Slides were incubated in primary antibody (goat anti-SPON1, product no. sc-49004, Santa Cruz Biotechnology, detects a region in the C-terminus of Spondin 1) in blocking solution for one hour at rt, rinsed with blocking solution, and then incubated in secondary antibody (FITC-conjugated rabbit anti-goat, product no. F7357, Sigma) in blocking solution for one hour at rt. Slides were rinsed twice for five minutes in PBS and then dried and mounted with Vectashield Mounting Medium with DAPI (product no. H-1200, Vector Laboratories) 1:1 with Vectashield (product no. H-1000, Vector Laboratories). Slides were stored at 4°C and visualized with an Olympus Provis AX70 upright microscope. Images were captured using Image-Pro 6.2 Software.
4.2.6 Progesterone Assay

Serum isolated from treated mice was sent to the University of Virginia Centre for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA) and the progesterone concentration determined by Progesterone radioimmunoassay (RIA). Differences in progesterone levels between all treatments were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey Multiple Comparison post-hoc test (Tukey test) (PRISM6.0; GraphPad).

4.2.7 Histology

Ovaries were removed from Spn1+/+ and Spn1−/− mice and fixed in 4% formaldehyde (BioShop) for 48 hours and transferred to 70% ethanol. Ovaries were then processed (through ethanol rinses), embedded, sectioned at 5 μm, and stained with hematoxylin and eosin (Molecular Pathology Core Facility, Robarts Research Institute, London, ON, Canada). Slides were scanned using the Aperio CS2 and visualised using Aperio Imagescope software (Leica Biosystems).

4.2.8 Continuous Breeding Study

To determine if Spn1−/− female mice were as fertile as Spn1+/+ females, 8-week old female Spn1+/+ and Spn1−/− mice were bred for six months with Spn1+/+ male mice who were proven breeders. Nine pairs for each genotype were individually housed, and cages were checked for pups every morning. Differences between genotypes in litter size and days between litters were analyzed using a two-tailed, unpaired Student’s t-test (PRISM6; GraphPad).

4.2.9 Assessment of ovulation capacity

To determine if Spn1−/− mice have a diminished ovulation capacity, six-week old Spn1+/+ and Spn1−/− mice were treated with eCG (5 IU) for 48 hours followed by hCG (5 IU) for 14 hours. Oviducts were removed and placed in M2 medium with hyaluronidase warmed to 37°C. Oviducts were punctured to release cumulus-oocyte complexes (COCs), and the complexes allowed to dissociate to facilitate counting of oocytes. Differences
between genotypes in number of oocytes were analyzed using a two-tailed, unpaired Student’s t-test (PRISM6; GraphPad).

4.2.10 Ovarian Weight Analysis

To determine if Spon1<sup>−/−</sup> ovaries differed in weight from Spon1<sup>+/+</sup> ovaries, 4-week, 8-week, and 12-week old mice were euthanized and then weighed to obtain body weight. Ovaries were then removed and weighed. Ovarian weight was normalized to body weight, and the differences between genotypes analyzed using a two-tailed, unpaired Student’s t-test (PRISM6; GraphPad).

4.3 Results

4.3.1 SPON1 expression and localization in the Spon1<sup>+/+</sup> mouse ovary

To determine if SPON1 is expressed in the whole ovary and in granulosa cells, Western blots and qPCR were performed. SPON1 was expressed at both the gene and protein levels in granulosa cells and whole ovary (Fig. 4-1 A). Spon1 mRNA levels were also altered following treatment with eCG or eCG + hCG (Fig. 4-1 B). eCG increased Spon1 mRNA levels, while hCG reduced this increase when administered after eCG. Immunofluorescence showed that in the ovaries of pre-pubertal, untreated Spon1<sup>+/+</sup> mice, SPON1 was localized in mural granulosa cells and more strongly in thecal cells (Fig. 4-1 C). In ovaries treated with eCG for 48 hours followed by hCG for 24 hours (to induce ovulation) SPON1 was additionally localized within corpora lutea (Fig. 4-1 D).

4.3.2 Spon1<sup>−/−</sup> female mice are subfertile

Spon1<sup>−/−</sup> mice are viable but no long-term study has determined if they are as fertile as Spon1<sup>+/+</sup> mice. To determine breeding capacity, a long-term breeding study comparing Spon1<sup>−/−</sup> and Spon1<sup>+/+</sup> mice was conducted. Eight-week old females were housed with proven Spon1<sup>+/+</sup> males for six months. Spon1<sup>−/−</sup> females produce significantly less pups per litter (7.9 ± 0.32, n=9) than their Spon1<sup>+/+</sup> counterparts (10.1 ± 0.46, n=8) (Fig. 4-2 A). However there was no significant difference in the number of
Figure 4-1. SPON1 is expressed in mouse granulosa cells, ovary, and uterus.

A. mRNA expression of Spon1 in ovary isolated from immature mice that were untreated, or treated with 48h eCG, or 48h eCG followed by 24h hCG (also in Fig. 3-1). Differences in gene expression between treatments were measured using a two-tailed, unpaired Student’s t-test, P < 0.05. There was a significant difference between control and eCG treatment, P < 0.001, and a significant difference between eCG and eCG + hCG treatment P < 0.01, but no difference between saline and eCG + hCG. B. Western blots of SPON1 in granulosa cells and ovary (also in Fig. 3-1). C. Immunofluorescence of SPON1 in the ovary isolated from immature mice treated for 48h with eCG. D. Immunofluorescence of SPON1 in the ovary isolated from immature mice treated following 48h eCG and 24h hCG treatment. ** = P < 0.01, *** = P < 0.001.
Differences between genotype for A, B and C were measured using a two-tailed, unpaired Student’s t-test, \( P < 0.05 \). A. Mean litter size per female in a six-month breeding study. Each data point represents one female. \( Spon1^-/^- \) females (7.8 ± 0.32) has significantly less pups per litter than \( Spon1^+/+ \) females (10.1 ± 0.46), \( P < 0.01 \). B. Mean days between litters per female. Each data point represents one female. There was no significant difference between genotypes (\( Spon1^+/+ \) 28.5 ± 2.3, \( Spon1^-/^- \) 27.3 ± 2.1). C. Ovulatory capacity was measured by counting COCs (cumulus oophorus complexes) following 48h eCG and 14h hCG treatment. Each data point represents one female (two ovaries). \( Spon1^-/^- \) females ovulated significantly less oocytes (45.6 ± 4.6) than \( Spon1^+/+ \) females (61.8 ± 0.48), \( P < 0.01 \). D. Ovarian weight per body weight at 4, 8, and 12 weeks. Ovarian weight per body weight was analyzed using a two-way ANOVA followed by a Tukey test, \( P < 0.05 \). There was no effect of age but the effect of genotype was significant, \( F (2, 24) = 21.4 \), \( P = 0.0001 \). There were no significant differences between

**Figure 4-2. \( Spon1^-/^- \) females are subfertile and have smaller ovaries.**
genotype at 4 weeks, but at 8 and 12 weeks $Spon1^{-/-}$ ovaries were significantly smaller than $Spon1^{+/+}$, $P < 0.05$ and $P < 0.01$ respectively. * = $P < 0.05$; ** = $P < 0.01$
days between litters (29.0 for $Spon1^{+/+}$, 27.3 for $Spon1^{-/-}$) (Fig. 4-2 B) or between litters per breeding female (4.8 for $Spon1^{+/+}$, 5.4 for $Spon1^{-/-}$).

4.3.3 $Spon1^{-/-}$ female mice have diminished ovulatory capacity and smaller ovaries

To determine if a lack of ovulated oocytes contributed to female $Spon1^{-/-}$ subfertility, superovulation studies were performed. $Spon1^{+/+}$ mice ovulated an average of 61.8 oocytes/mouse while $Spon1^{-/-}$ ovulated 45.6 oocytes/mouse (Fig. 4-2 C). This significant decrease in ovulation strongly mirrors the decrease in number of pups observed in the breeding study, as $Spon1^{-/-}$ females ovulated 73% of oocytes compared to $Spon1^{+/+}$ females and have litters 77% that of $Spon1^{+/+}$ females. Because $Spon1^{-/-}$ mice have a diminished ovulatory capacity, ovarian size was measured and corrected for body weight (body weight was not significantly different by genotype).

No difference in the ovary/body weight ratio was observed at four weeks; however at eight and twelve weeks, $Spon1^{-/-}$ ovaries were significantly smaller than $Spon1^{+/+}$ ovaries, relative to body weight, ($Spon1^{-/-}$ ovary/body weight is 65% that of $Spon1^{+/+}$ at both eight and twelve weeks) (Fig. 4-2 D).

4.3.4 Ovaries from cycling $Spon1^{-/-}$ mice contain abnormal corpora lutea

To determine the cause of the subfertility and diminished ovulatory capacity of $Spon1^{-/-}$ mice, we examined the histology of ovaries from normally cycling $Spon1^{+/+}$ and $Spon1^{-/-}$ mice at various ages. In $Spon1^{-/-}$ ovaries, follicles at all stages of growth were observed. At three weeks of age there were no differences seen between genotypes (Fig. 4-3 A, B). However, at four months, $Spon1^{-/-}$ ovaries, although largely normal otherwise, (Fig. 4-3 C, D), contained abnormal CLs with granulosa cells that failed to luteinize (Fig. 4-3 E, F), though the thecal cells appeared to have luteinized normally. At eight and twelve months, no differences in ovarian histology between genotypes were observed (not shown). When mice were treated between 23-28 days of age with eCG for 48 hours, or eCG for 48 hours followed by hCG for 4, 16, 24, 72 or 120 hours, no differences in histology were observed between genotypes.
Figure 4-3. *Spon1*<sup>−/−</sup> female mice are grossly normal with some abnormal corpora lutea.

*Spon1*<sup>+/−</sup> and *Spon1*<sup>−/−</sup> ovaries at three weeks and four months of age. A. Three-week old *Spon1*<sup>+/−</sup> ovary. B. Three-week old *Spon1*<sup>−/−</sup> ovary. C. Four-month old *Spon1*<sup>+/−</sup> ovary. D. Four-month old *Spon1*<sup>−/−</sup> ovary. E. Four-month old *Spon1*<sup>−/−</sup> ovary with un-luteinized CL. F. Close-up of un-luteinized CL in four-month old *Spon1*<sup>−/−</sup> ovary.
4.3.5 Sex determination genes are dysregulated in Spon1<sup>−/−</sup> mice

Because female Spon1<sup>−/−</sup> mice are subfertile, have a diminished ovulatory capacity, and in a subgroup of CLs, have a granulosa cell-to-luteal cell transition defect, we investigated the ovarian mRNA levels of several genes important to the process of folliculogenesis in untreated mice (Appendix D – List of Genes). Despite subtle histological abnormalities in corpora lutea of cycling mice, we did not see any differences in gene expression between genotypes for the luteinizing hormone/choriogonadotropin receptor (Lhcgr), or LH-responsive genes: progesterone receptor (Pgr), prostanlin F receptor (Ptgfr), prostaglandin-endoperoxide synthase 2 (Ptgs2) and 20α-hydroxysteroid dehydrogenase (Akr1c18) (Fig. 4-4 A, B, data not shown). No differences between genotypes were observed in the mRNA levels of the steroidogenesis genes, scavenger receptor B1 (Scarb1), steroid acute regulatory protein (StAR), cholesterol side chain cleavage enzyme (Cyp11a1), and aromatase (Cyp19a1) (Fig. 4-4 C, D, E, F) following eCG +/- hCG treatment, which is consistent with the similar serum progesterone levels observed under the same treatment conditions in Spon1<sup>+/+</sup> and Spon1<sup>−/−</sup> females (Fig. 4-5).

Surprisingly, expression of Scarb1 and Star was higher in Spon1<sup>−/−</sup> pre-pubertal untreated ovaries than Spon1<sup>+/+</sup> (Fig. 4-6 A, B). Scarb1 and Star are involved in uptake of cholesterol and localization. At this stage of ovarian development, steroidogenesis is minimal and the lack of difference in Cyp11 expression at the same time suggests steroidogenesis has not been prematurely activated in Spon1<sup>−/−</sup> ovaries. However, because we see this dysregulation before puberty, we decided to measure expression of genes involved in earlier ovarian processes. Interestingly, mRNA levels of the sex determination and primordial follicle activation inhibitor, Amh, were higher in immature Spon1<sup>−/−</sup> ovaries than in Spon1<sup>+/+</sup> ovaries. mRNA levels of Sox9, another sex determination gene, were also significantly higher in immature Spon1<sup>−/−</sup> ovaries than in Spon1<sup>+/+</sup> ovaries (Fig. 4-6 C, D).
Figure 4-4. Expression of steroidogenesis genes following gonadotropin treatment is unchanged in Spon1−/− ovaries.

Mice were treated with eCG for 48h or eCG for 48h followed by hCG for 4h then ovaries were removed and mRNA levels were determined by reverse transcription and qPCR. Gene expression for all genes was normalized to Rpl7. Differences of genotype in genes A–F were analyzed by a two-tailed, unpaired Student’s t-test with Welch’s correction, P < 0.05. There was no difference between genotypes for genes A–F. The specific gonadotropin treatment was chosen for genes based on when they would initially increase in vivo. A and C–F were measured following 48h eCG treatment. B was measured after 48h eCG followed by 4h hCG treatment. A. Lhcgr, luteinizing hormone receptor. B. Pgr, progesterone receptor. C. Cyp11a1, cholesterol side-chain cleavage enzyme. D. Cyp19a1, aromatase. E. Scarb1, scavenger receptor class B, member 1. F. Star, steroid acute regulatory protein.
Figure 4-5. Serum progesterone levels are unchanged following gonadotropin treatment of *Spon*1/+ females.

Mice were treated with eCG for 48h or eCG for 48h followed by hCG for 4h or 24h. Serum was removed and progesterone was measured by radioimmunoassay. Differences in progesterone levels between treatments were measured by a two-way ANOVA which found a significant effect of treatment but not genotype, F (2, 16) =13.92, P=0.0003. There were no significant differences between genotype at any treatment as measured by Tukey multiple comparison post-hoc test.
Figure 4-6. Expression of steroidogenesis and sex determination genes is altered in early folliculogenesis in Spon1+/− ovaries.

Ovaries were removed from prepubertal mice (23-27 days old) that were untreated or treated for 48h with eCG. mRNA levels were determined by reverse transcription and qPCR. For genes A and B differences in gene expression were analyzed by a two-tailed, unpaired Student’s t-test with Welch’s correction, P < 0.05. C and D were analyzed using a two-way ANOVA with a Fisher’s LSD post-hoc test comparing genotype means of each treatment. A. Gene expression of Scarb1 in prepubertal mice was significantly different between Spon1+/+ and Spon1−/−, P < 0.01. B. Gene expression of Star in prepubertal mice was significantly different between Spon1+/+ and Spon1−/−, P < 0.05. C. Gene expression of Amh was not affected by treatment but was significantly affected by genotype F (1, 19) = 13.42, P = 0.0017. Between Spon1+/+ and Spon1−/− prepubertal mouse expression was significantly different, P < 0.05, and between Spon1+/+ and Spon1−/− mice treated for 48h with eCG expression was significantly different, P < 0.01. D. Gene expression of Sox9 was not affected by treatment but was significantly affected by genotype F (1, 19) = 12.13, P = 0.0025. Between Spon1+/+ and Spon1−/− prepubertal mouse expression was significantly different, P < 0.05 , and between Spon1+/+ and Spon1−/− mice
treated for 48h with eCG expression was significantly different, P < 0.05. * = P < 0.05; ** = P < 0.01
4.4 Discussion

There has been previous work reporting that SPON1 is found within the ovary and that it may play a role in ovarian cancer but no work has examined the role of SPON1 in fertility. We report for the first time that SPON1 plays a role in normal functioning of the ovary \textit{in vivo}.

4.4.1 SPON1 expression is regulated by gonadotropins

We have shown that \textit{Spon1} expression in the ovary and uterus responds to follicle stimulating hormone (FSH) and luteinizing hormone (LH), which strongly suggests a role for SPON1 in normal ovarian function. The pattern we see of LH downregulating the FSH-induced expression of \textit{Spon1} is a well-established observation in ovarian biology. When the LH surge occurs and begins the process of ovulation, FSH-dependent expression of many steroidogenesis genes and granulosa cell growth-promoting factors is terminated. This suggests that SPON1 is involved in one or more FSH-dependent processes such as steroid production or cell growth.

4.4.2 SPON1 localization suggests a role in angiogenesis

The location of SPON1 within the follicle changes as the follicle grows and differentiates. This is seen following treatment of the mouse with eCG and hCG. When a prepubertal mouse is treated with eCG to initiate growth of follicles SPON1 is seen in the theca cell compartment and in the mural granulosa cells. We have shown that SPON1 is expressed by granulosa cells and by the whole ovary but can only speculate on its function in theca cells. Theca cells may produce their own SPON1 or granulosa cells may secrete SPON1 that could passively diffuse through the basal lamina to theca cells as a signalling molecule. In its function as a signalling molecule in nerve outgrowth, SPON1 is known to be cleaved, and the resulting two polypeptides localize to different areas [17, 18, 39]. Therefore, we may be detecting the localization of only one cleavage product of SPON1 (the antibody used recognizes a C-terminal region), although we did not see any cleavage products on Western blots in the ovary or granulosa cells.
Following ovulation, angiogenesis occurs rapidly within the corpus luteum to provide cholesterol substrate for progesterone production. After treatment with hCG to induce ovulation, SPON1 localizes to string or tube-like structures within the corpus luteum. The location of SPON1 within these structures suggests a potential role in angiogenesis. This is supported by the fact that other thrombospondin family members are known to have a role in fertility and ovarian angiogenesis [40, 41] and by its previous discovery as a molecule within bovine follicular fluid that affects growth of rat aortic vascular smooth muscle cells [28]. The dual localization of SPON1 to theca cells where vascularization occurs and the corpus luteum where angiogenesis occurs further strengthens this hypothesis.

4.4.3 Subfertility of Spon1−/− female mice

We found that Spon1−/− female mice are subfertile as they produce litters 23% smaller than Spon1+/+ females. However, there was no difference in the average number of days between birth of litters. This suggests that the subfertility arises from a lack of fertilizable oocytes or an inability to maintain embryos/fetuses and not from an inability to become pregnant or an altered estrous cycle. Indeed, when we measured ovulatory capacity following superovulation, Spon1−/− females ovulated 27% less oocytes than Spon1+/+ females. Interestingly, although there are only minor and incompletely penetrant histological differences between Spon1+/+ and Spon1−/− ovaries, Spon1−/− ovaries are smaller post-puberty while the mice themselves are the same size regardless of genotype.

A decrease in ovulatory capacity could arise from defects at any stage of folliculogenesis; loss of primordial germ cells or improper nest breakdown, improper primordial follicle formation, inhibition of primordial follicle activation, inability of secondary follicles to respond to FSH or LH, and inability of pre-ovulatory follicles to respond to LH could all result in less or no oocytes ovulated. Since Spon1−/− ovaries are smaller but contain all classes of follicle it is plausible that less primordial follicles are recruited and the overall pool of follicles is smaller. Examples of knockout mice with smaller ovaries compared to body weight are few and, most often, are the result of arrested folliculogenesis at some stage prior to ovulation. Fshb+/+ and Fshr−/− ovaries do not contain any follicles beyond the secondary stage and Lhcgr−/− ovaries contain no
follicles that mature beyond the early antral stage. Ovaries treated with a bone morphogenetic protein-4 (BMP-4) antibody are smaller than untreated ovaries due to loss of oocytes and follicles and increased apoptosis of all cell types [42]. Examples of mouse models with smaller ovaries that contain all classes of follicles are not common, but a subfertile mouse null for E6-associated protein (E6-AP), an estrogen receptor co-activator, has decreased ovarian size compared to wildtype until 10 weeks of age [43].

However, there are examples of mice with histologically normal ovaries with impaired ovulatory capacity. Bmp15−/− knockout mice are subfertile due to decreased ovulation but are otherwise histologically normal compared to wildtype [44].

### 4.4.4 Corpus luteum abnormalities

The histological differences in CLs consisted of granulosa cells that failed to luteinize although theca cells did. This feature suggests a Spon1-dependent difference between theca cells and granulosa cells within the same follicle and possible altered gene expression of steroidogenesis genes. However, subsequent mRNA expression analysis of genes involved in the process of luteinization, Lhcgr, Pgr, Ptgrfr, Ptgs2, and Akr1c18, revealed no differences between Spon1+/+ and Spon1−/− ovaries. This lack of differences could be explained by variation between CLs or incomplete penetrance of the failure to luteinize phenotype, as normal CLs that are present could mask any gene expression differences within the abnormal structures. It is also unlikely that these abnormal CLs are affecting fertility as normal CLs could compensate for lack of progesterone and therefore minor malfunctions in luteinization would not affect fertilization or pregnancy.

### 4.4.5 Increased expression of Amh and Sox9 in the immature mouse ovary

AMH, anti-mullerian hormone, and SOX9, SRY-box 9, are both sex determining proteins involved in the development of the testes. SOX9 regulates the transcription of AMH, while AMH suppresses the formation of the mullerian duct and therefore the development of the female reproductive system. Therefore the elevated mRNA levels of Amh that we see in Spon1−/− mice prior to and following FSH treatment may result from the overexpression of Sox9 following the same treatment. Genotypically XY mice null
for Sox9 undergo male to female sex reversal [45] and XX mice over-expressing Sox9 undergo female to male sex reversal [46]. Genotypically XX transgenic mice overexpressing Amh develop no uterus, have a blind vagina, and although ovaries are present at birth, they lose germ cells and develop seminiferous tubule-like structures after two weeks and by adulthood are lost completely [47]. Female mice lacking Amh were initially thought to be phenotypically normal [48], however, it was found that these mice experience over-activation of primordial follicles [49]. In contrast, primordial follicle activation is inhibited in wildtype ovaries treated with AMH [50]. This suggests a model where the increased expression of Amh we see in Spon1−/− ovaries could be responsible for the decrease in ovulated oocytes and the smaller number of CLs at eight weeks in untreated females either by lack of activation of primordial follicles or decreased sensitivity to FSH of pre-antral follicles. These changes could also explain the decrease in ovarian size seen in Spon1−/− females and of note is the fact that Amh−/− ovaries weigh 1.8x their wildtype counterparts at four months [49].

Of note, in previous work characterizing the Spon1−/− mouse, TGFβ1 serum levels were decreased and this was proposed as the reason for higher bone mass [38]. AMH is a TGFβ family member and therefore, the increased expression of AMH we observe is the second example of dysregulation of a TGFβ family member in the Spon1−/− mouse. SOX9 is directly regulated by TGFβ and, although most often upregulated [51, 52], has been shown to be downregulated by TGFβ as well [53]. Therefore, a model where lower TGFβ1 levels increase Sox9 expression and subsequently Amh expression is possible.

4.4.6 Conclusion

We have reported for the first time a role for SPON1 in mammalian fertility. The Spon1−/− female mouse is subfertile, and has less ovulatory capacity and smaller ovaries post-puberty than Spon1+/+ females. Because the phenotype is subtle, future experiments measuring the expression of proteins homologous to SPON1 could be performed to discover compensatory mechanisms. We found that loss of Spon1 results in early dysregulation of Amh and Sox9 expression which may be causing decreased ovulatory capacity. This is the second instance of Spon1−/− mice demonstrating dysregulation of a TGFβ family member protein, and therefore future work should be undertaken to
determine how SPON1 might be involved in TGFβ superfamily signalling in this model. This work is a first step towards defining the role of SPON1 within the healthy ovary.
4.5 Bibliography


29. Terai Y, Abe M, Miyamoto K, Koike M, Yamasaki M, Ueda M, Ueki M and Sato Y. Vascular smooth muscle cell growth-promoting factor/F-spondin inhibits angiogenesis via the blockade of integrin avb3 on vascular endothelial cells. Journal of Cellular Physiology 2001; 188:


33. Fraser HM. Regulation of the ovarian follicular vasculature. Reproductive Biology and Endocrinology 2006; 4:


plays an essential role in ovarian development through positively regulating Wnt-4 signaling. Human Molecular Genetics 2008; 17:1278-1291.

38. Palmer GD, Attur MG, Yang Q, Liu J, Moon P, Beier F and Abramson SB. F-spondin deficient mice have a high bone mass phenotype. PLOS One 2014; 9:


53. Handorf AM and Li W-J. Fibroblast growth factor-2 primes human mesenchymal stem cells for enhanced chondrogenesis. PLOS One 2011; 6:
5 Chapter 5 - Discussion

5.1 Summary: A Role for Spondin 1 in the Ovary

Prior to this investigation, Spondin 1 had not been extensively studied in the ovary. The first indication that Spondin 1 is functional within the ovary was its discovery in bovine follicular fluid and classification as a major vSMC growth promoting factor [1]. The first evidence that Spondin 1 may play a role in fertility in mice was its downregulation by 2.5-fold in granulosa cells (GCs) of infertile estrogen receptor β Esr2⁻/⁻ females versus fertile Esr2⁺/⁻ females [2]. This Esr2⁻/⁻ data along with data indicating that 17β-estradiol increases Spon1 mRNA levels in the mammary gland [3] and uterus [4] imply that Spon1 is, at least indirectly, regulated by estrogens. Spondin 1 is also a biomarker for ovarian cancer suggesting that it may be important in progression of the disease [5, 6]. Founded on this evidence, we hypothesized that Spondin 1 is important in ovarian folliculogenesis.

In this work I establish that Spondin 1 does indeed play a role within the ovary (Fig. 5-1). This was accomplished by characterizing its expression and function in granulosa cell lines, wildtype mouse primary GCs and whole ovary, and Spon1⁻/⁻ whole ovaries. We were unable to prove direct regulation of Spon1⁺/⁻ transcription by 17β-estradiol (Section 5.2), but uncovered the first evidence that Spon1 transcription is regulated by gonadotropins in the ovary and uterus, with expression increasing after eCG treatment (which mimics FSH) and decreasing following hCG treatment (which mimics LH). In Chapters 2 and 3, I show that Spondin 1 promotes viability of human (KGN) and mouse (KK-1) granulosa tumour cell lines, as well as mouse primary GCs. This increased viability may result from increased signalling of the mTORC1 complex following phosphorylation of PRAS40. Spondin 1 affects steroidogenesis by altering the mRNA levels of steroidogenic genes (Chapters 2, 3, and 4), protein levels (Chapter 3), and progesterone levels (Chapter 2 and 3). In Chapter 3, we provide the first in vivo evidence that Spondin 1 may play a role in angiogenesis by demonstrating its co-localization with protein markers of vascularization in the ovary and uterus. Finally, in Chapter 4, we show that the Spon1⁻/⁻ female mouse is subfertile, producing litters that are smaller than Spon1⁺/⁺, possibly due to decreases in ovulated oocytes and ovarian size.
<table>
<thead>
<tr>
<th>Effects of Spondin 1</th>
<th>Granulosa Cell Tumour Cell Line (KGN)</th>
<th>Mouse Primary Granulosa cells and Whole Ovary</th>
<th>Knockout of Spondin 1 in mouse (ovarian effects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroidogenesis</td>
<td>↓ Progesterone secretion; Spondin 1 decreased mRNA and protein expression of STAR</td>
<td>↑ Progesterone secretion</td>
<td>No effect on serum levels of progesterone; Spon1&lt;sup&gt;−/−&lt;/sup&gt; prepubertal ovaries had increased expression of Star, Scarb1</td>
</tr>
<tr>
<td>Viability</td>
<td>↑ cell viability and proliferation; Spondin 1 increased phosphorylation of PRAS40, an inhibitor of mTORC1</td>
<td>↑ cell viability</td>
<td>Not measured; no histological indication of decreased growth</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>N/A</td>
<td>Spondin 1 co-localized with PECAM1/CD31 and DESMIN (endothelial and vascular mural cell markers) in the ovary</td>
<td>No histological indication of abnormal angiogenesis; no differences in mRNA expression of VEGF or PECAM</td>
</tr>
<tr>
<td>Fertility</td>
<td>N/A</td>
<td>N/A</td>
<td>Subfertile as measured by litter size; decreased ovulation; decreased ovarian weight; luteinization defects; Spon1&lt;sup&gt;−/−&lt;/sup&gt; prepubertal ovaries had increased expression of Sox9, and Amh</td>
</tr>
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**Figure 5-1. Summary of Results.**
5.2 Gonadotropin Responsiveness of Spon1 in the Female Reproductive System

In this investigation we present evidence that Spon1 mRNA expression is upregulated by FSH and downregulated by LH in both the mouse ovary and uterus. This expression pattern mimics that of many other genes in the ovary that are involved in FSH-induced follicle growth and steroidogenesis. These genes are either not essential or function in a different role when FSH-regulated processes are halted following the LH surge. A classic example of this is Cyp19a1, which encodes aromatase, the enzyme responsible for 17β-estradiol production, whose expression decreases dramatically after the LH surge. Prior to this investigation, examples of Spon1 gene regulation within the mouse female reproductive system included possible estrogen regulation in the uterus, mammary gland, and ovary. In both the uterus and mammary gland, expression of Spon1 following 17β-estradiol treatment was increased roughly ten-fold [3, 4]. In the ovaries of Esr2<sup>−/−</sup> mice expression of Spon1 was 2.5-fold lower than those of heterozygous mice [7]. This led to the hypothesis that Spon1 may be directly regulated by estrogens and subsequent experiments to identify an estrogen-responsive region in the Spon1 promoter were performed.

Within the mouse promoter we identified four identical non-conserved, non-canonical estrogen response elements (EREs) upstream of the transcriptional start site (TSS). The canonical ERE sequence is 5’-GGTCAnnTGACC-3’ and the four sequences within the Spon1 mouse promoter, found approximately 2 kb from the TSS, are 5’-GGTTAGATGATG-3’. Within the same region two half-EREs 5’-GGTCA-3’ are conserved in Gorilla gorilla, Homo sapiens, and Pan troglodytes. We cloned this region into a luciferase construct to conduct promoter-reporter assays, but found no evidence of estrogen responsiveness. As this experiment was performed in vitro and with a partial promoter it cannot conclusively be stated that there is no direct effect of estrogen on Spon1. This result is not unexpected given that often EREs are not found within the proximal promoter of genes, but are well-known to be located great distances from the genes they regulate [8]. However, because transcription of a gene by FSH is most often propagated by signalling through cAMP, and estrogen is also known to increase levels of
cAMP [9], it is probable that \textit{Spon1} is a cAMP responsive gene. This theory is strengthened by the presence in the \textit{Spon1} promoter of two half-cAMP response elements (CREs), 5’-CGTCA-3’, one 2kb upstream of the TSS and one in the 5’ untranslated region. These sequences are conserved in \textit{Gorilla gorilla}, \textit{Homo sapiens}, and \textit{Pan troglodytes}. Of note is the fact that LH also accomplishes signalling through cAMP [10], so although \textit{Spon1} is likely cAMP-responsive, additional factors must control its differential regulation by FSH and LH.

## 5.3 Functional Roles of Spondin 1 in the Ovary

### 5.3.1 Viability

Prior to this investigation, a role for Spondin 1 in increasing cellular viability was seen in chicken ciliary ganglion [11] and a neuroblastoma cell line [12]. We have now shown that Spondin 1 increases viability of primary mouse GCs (Chapter 3), as well as human and mouse granulosa tumour cell lines (Chapter 2). The increase in viability of Spondin 1-treated GCs coincides with increased \textit{Spon1} mRNA expression following eCG/FSH treatment. Chicken ciliary ganglion studies implicated both TGFβ activity and APP signalling through DAB-1 in increasing viability, while blocking both the reeler/spondin domains and the TSR domains inhibited Spondin 1’s effects [11]. In neuroblastoma cells, viability was attributed to changes in levels of IL-6 [12], which, as discussed in Chapter 3, can be regulated by TGFβ. TGFβ is a promoter of granulosa cell proliferation [13, 14] and is likely a factor in Spondin 1-induced viability.

Here we provide the first evidence that the mTORC1 complex, via its activation following PRAS40 phosphorylation, may be involved in Spondin 1-induced increases in viability of GCs. As discussed in Chapter 2, Reelin promotes granulosa cell viability in chicken ovaries by binding LRP8 or VLDLR, which phosphorylate DAB-1 and activate the AKT pathway [15]. Reelin has also been shown to activate mTOR via increased AKT phosphorylation [16]. Our new evidence that Spondin 1 increases phosphorylation of PRAS40, coupled with past studies showing parallels between Spondin 1 and Reelin receptor binding, and DAB-1 and AKT phosphorylation, strongly suggests a similar mechanism of action for Spondin 1.
To date, identified functional Spondin 1 receptors include LRP8, VLDLR, and APP. Spondin 1 can induce clustering of these receptors with other proteins, including LRP8 with APP, but not LRP8 with VLDLR [17]. In the chicken ciliary ganglion APP but not LRP8 or VLDLR was expressed and therefore APP was identified as the Spondin 1 receptor responsible for increased viability. Expression of Spondin 1’s receptors within the ovary has not been extensively studied, but during chicken folliculogenesis VLDLR and LRP8 are present in GCs at all stages [18] and in the cow LRP8 is present in GCs of dominant/preovulatory follicles [15]. Expression of these receptors in rodent models has been shown but their locations are unknown [19, 20].

I propose that Spondin 1, like Reelin, activates either LRP8 or VLDLR, possibly both, in our granulosa cell models. These receptors activate DAB-1, which activates the AKT/PI3K pathway. AKT is responsible for phosphorylation of PRAS40, which removes inhibition of mTORC1 activity. Increased activity of mTORC1 would then increase the viability and possibly proliferation of GCs. This could also be achieved through the interaction of Spondin 1 and APP, although expression of APP in the ovary is currently unknown. It is also possible that increased TGFβ signalling activates the AKT/PI3K pathway and subsequently mTORC1 (Appendix E).

5.3.2 Steroidogenesis

Based on our data, interpretation of Spondin 1’s effects on steroidogenesis is much more complex than its role in viability. In Chapter 2, Spondin 1 decreased progesterone levels following dbcAMP treatment in the human granulosa tumour cell line, KGN, while in Chapter 3 Spondin 1 increased progesterone levels following dbcAMP or FSH and testosterone treatment in mouse primary GCs. The simplest explanation for this is that neither KGNs nor primary mouse GCs are a perfect model for GCs in vivo and differences between the two models are not unexpected. This is especially true for KGNs in terms of the steroidogenesis pathway. KGNs produce little 17β-estradiol but respond to dbcAMP to produce progesterone [21]. Mouse primary granulosa cells in culture must adapt to loss of interactions with oocytes and theca cells and, although in vivo they primarily produce 17β-estradiol and minimal progesterone, in vitro they produce higher levels of progesterone. It is possible that Spondin 1 inhibits
expression of progesterone production genes in cells it “recognizes” as GCs while promoting expression of progesterone production in cells it “recognizes” as luteinized. This could occur due to the vastly different expression patterns of genes in granulosa cells under the influence of FSH compared to LH.

The functional interaction of Spondin 1 with LRP8 and VLDLR, as well as its interaction of unknown purpose with LRP2 and LRP4 [22], is intriguing, as these receptors are involved in cholesterol transport across the cell membrane. In the cow ovary, where LRP8 and VLDLR are proposed to deliver cholesterol to follicular cells, these receptors were expressed at different times and in different locations depending on follicular stage and cell type [23]. If Spondin 1 signalled through these receptors it could affect steroidogenesis differently depending on the specific receptor present (Appendix E).

Finally, the variable action of Spondin 1 in steroidogenesis could simply be due to the difference in species of KGNs (human) and primary mouse GCs. Several processes in folliculogenesis are known to occur in a species-specific manner, for example, larger mammals, such as humans and cows, possess dominant follicles while rodents do not. The specifics of steroidogenic regulation by Spondin 1 will need to be more thoroughly studied in the future, but the identification of Spondin 1 as a factor in the process is extremely novel.

5.3.3 Angiogenesis

As discussed in 5.2, the upregulation of Spon1 mRNA after eCG/FSH treatment and downregulation following hCG/LH treatment suggests that Spondin 1 is involved in FSH-regulated processes such as granulosa cell proliferation and steroidogenesis, discussed in 5.3.1 and 5.3.2. However, expression of Spon1 is not lost completely following LH exposure, and the presence of Spondin 1 in the corpus luteum suggests that, following downregulation by LH, Spondin 1 still plays a role in the ovary. In Chapter 3, Spondin 1 co-localized with markers of vascularization, and PECAM1 in particular showed a similar pattern of localization to Spondin 1. We also co-localized Spondin 1 with PECAM1 in the kidney and found that Spondin 1 is localized to the vasculature,
particularly glomerular capillaries, in this tissue, as well (Appendix F). This suggests that Spondin 1 may play a role in vascularization in general. The proximity of Spondin 1 to vasculature in theca cells and to developing capillaries within the corpus luteum, as well as previous in vitro studies with endothelial and vSMCs [1], lead us to hypothesize that Spondin 1 is involved in ovarian angiogenesis.

A possible signalling mechanism for Spondin 1 regulation of angiogenesis is blockade of integrin $\alpha_v\beta_3$, which caused inhibition of HUVEC migration [24]. In the ovary, integrin $\beta_3$ is expressed in the theca cells and in the corpus luteum in a pattern similar to Spondin 1, but integrin $\alpha_v$ is only expressed in granulosa cells of multi-layer follicles [25]. This suggests that, although Spondin 1 can interact with integrin $\alpha_v\beta_3$, this is not the case within the ovary. However, if Spondin 1 interacts directly with integrin $\beta_3$, a different integrin $\alpha$ subunit coupled with $\beta_3$ may be involved in Spondin 1 regulation of angiogenesis within the ovary (Appendix E).

The ovarian mechanism of anti-angiogenesis of TSP-1 may provide insight into the role of Spondin 1 in angiogenesis. TSP-1 inhibits endothelial cell proliferation and induces apoptosis [26, 27], while Spondin 1 did not inhibit proliferation of endothelial cells, just migration and tube formation [24]. TSP-1 directly regulates levels of vascular endothelial growth factor (VEGF) in the ovary by binding and internalizing it via LRP1 [28], a receptor which has never been shown to interact with Spondin 1. TSP-1 knockout ovaries are significantly hypervascularized with increased levels of PECAM1 and VEGF. We saw no such phenotype in Spon1$^{-/-}$ ovaries and found no mRNA expression differences for either gene following hCG treatment (Appendix G). The expression and localization of Tsp-1 in the ovary also greatly differs from Spondin 1. In rat primary granulosa cells Thbs1 (the gene encoding TSP-1) expression was not affected by FSH treatment but was increased following LH treatment [29]. In rat ovaries TSP-1 is found in GCs of all follicles, and all cells of the CL early in its formation, but not in its later stages [29]. In contrast, Spondin 1 localized to theca cells and faintly in granulosa cells and was present in CLs at early and later stages. Therefore the role Spondin 1 is playing in angiogenesis within the ovary is not likely to be similar to that of TSP-1. I propose that Spondin 1 is involved in limiting the growth of blood vessels, particularly capillaries, by
promoting their maturation, which halts growth. Spondin 1 may even be involved in recruiting pericytes or vSMC to stabilize new blood vessels. If Spondin 1 signals to stop growth and promote maturation it could explain why, in vitro, Spondin 1 inhibits migration and tube formation of endothelial cells and promotes proliferation of vSMC. The presence of Spondin 1 at capillaries in the kidney, where blood vessels are maintained but do not grow and regress, also supports this hypothesis.

5.4 Influence of Spondin 1 on Female Fertility

In the Spon1−/− female, we report a diminished ovulatory capacity of 27% that strongly coincides with the 23% decrease in litter size and 35% decrease in ovarian weight. A decrease in ovulatory capacity could arise from defects at any stage of folliculogenesis. Loss of primordial germ cells such as in deleted in azoospermia-like, Dazl−/− [30] and Smad5−/− [31] female mice, or improper nest breakdown, seen in Ngf (nerve growth factor) knockouts [32], would result in complete loss of ovulation. Complete loss of ovulation would also occur following improper primordial follicle formation as seen in Foxl2LacZ mice where GCs fail to differentiate from squamous to cuboidal, resulting in a lack of primary and secondary follicles [33]. Inhibition of primordial follicle activation is required to avoid premature ovarian failure but in several knockout mouse models, activation is accelerated or overly inhibited. AMH treatment of ovaries results in activation of fewer primordial follicles than controls, while loss of AMH results in premature activation of all primordial follicles. The same over-activation is seen in Pten−/− females [34]. If follicles fail to respond to FSH and estrogen they will not grow to the antral and pre-ovulatory stages. These effects are seen in estrogen receptor knockout females and Fshb−/− and Fshr−/− mice. If pre-ovulatory follicles fail to fully respond to LH, ovulation will not occur. Lhcgr−/− mice lack pre-ovulatory follicles and have no CLs. Pgr−/− and Bmp15−/− females both respond to LH and form CLs but often oocytes are not expelled and remained trapped. It is unlikely that Spon1−/− females are not responding to FSH or LH as all follicle stages are present, from primordial to corpus luteum and we did not see any trapped oocytes. With our current knowledge of the Spon1−/− ovary the most likely explanation of subfertility is that the decrease in ovulated
oocytes results from inhibition of activation of a subset of primordial follicles due to overexpression of AMH.

A similar mechanism involving TGFβ and mTOR could also be hypothesized. mTOR signalling is important but not essential for primordial follicle activation and, as discussed in 5.3.1, TGFβ has been shown to activate mTOR signalling. In the Spon1<sup>−/−</sup> mouse levels of circulating active TGFβ are decreased and it is therefore possible that mTOR activity is also decreased. If mTOR inhibition is occurring in the Spon1<sup>−/−</sup> ovary it could result in subfertility, especially if both AMH overexpression and mTOR inhibition were acting to suppress activation.

5.5 Spondin 1 as a Matricellular Protein

Within the ECM, a subclass of proteins has been designated as “matricellular”. The definition of matricellular is continuously being updated as new research expands our knowledge of these proteins. In the most general terms, matricellular proteins are ECM proteins that are involved in cell-matrix signalling but are not required for structural integrity. Some thrombospondins are often classified as matricellular proteins, especially TSP-1 and -2, however mention of Spondin 1 as a member of this group is extremely rare, and in fact only one example could be found in the literature. Important characteristics of matricellular proteins, besides being non-structural ECM signalling molecules, include: a) expression in areas of development, growth, or high turnover, b) interactions with multiple signalling pathways via ECM structural components, cell surface receptors, and other ECM signalling proteins, such as growth factors, c) context-dependent, possibly opposing, functional roles, d) a subtle or normal phenotype when knocked out, and e) status as a biomarker for a specific disease (reviewed in [35-38]). We know that Spondin 1 is expressed in areas of development (nervous system), and growth and turnover (ovary and bone), that Spondin 1 interacts with multiple different proteins including cell surface receptors (LRP8, VLDLR, APP) and growth factors (TGFβ), and that Spondin 1 is a biomarker for ovarian cancer. In this work I have shown that Spondin 1 may act in a context-dependent manner to affect angiogenesis and possibly steroidogenesis in the ovary and that the phenotype of the Spon1<sup>−/−</sup> mouse is indeed subtle. Observed within the context of a matricellular protein, as I believe Spondin 1 should be
classified, the seemingly conflicting roles of Spondin 1 in progesterone production and corpus luteum angiogenesis, as well as the mild subfertility of the Spon1−/− female mouse, are to be expected.

5.6 Future Directions

5.6.1 Functional Redundancy of Spondin 1

In this work I have shown that Spondin 1 has functional roles in the ovary, including cell viability, proliferation, steroidogenesis, and possibly angiogenesis. In the literature, examples of Spondin 1 function include: nerve development and outgrowth, nerve cell viability and differentiation, neural and non-neural cell adhesion, proliferation of vascular smooth muscle cells, inhibition of endothelial cell tube formation, and chondrocyte and cementoblast differentiation. If Spondin 1 were indispensable within these roles then loss of function via knockout or mutation would be expected to result in infertility, severe impairment of nervous system development, vascularization, cartilage and bone development, and potential lethality. However, besides being mildly subfertile, exhibiting increased bone mass, and decreased TGFβ1 serum levels, the Spon1−/− null mouse has a grossly normal phenotype. None of the aforementioned functions seem to be impaired, suggesting that Spondin 1 is functionally redundant in most cases. This is not unexpected as there are several proteins, sharing domains with Spondin 1, which may be differentially regulated following loss of Spondin 1. To determine if this is the case expression studies of these proteins could be undertaken. Thrombospondin-1, -2, and Reelin have already been studied within the ovary of some species for both their expression and localization, while Spondin 2 has not. mRNA and protein expression of these candidates in untreated and gonadotropin treated Spon1+/+ and Spon1−/− ovaries would determine if regulation is altered following loss of Spondin 1, while immunofluorescence following the same treatments would determine if these proteins have altered their locations within the ovary to compensate for loss of Spondin 1. If any of these candidate proteins were found to have significantly altered expression in the Spon1−/− ovary, double knockouts of Spon1 and that protein could provide further insight into Spondin 1’s roles, both in the ovary and elsewhere.
5.6.2 Elucidation of Spondin 1 Signalling Mechanisms

The temporal expression and location of Spondin 1 receptors, LRP8, VLDLR, and APP, is currently unknown in the mouse ovary. Therefore the model that I propose of Spondin 1 signalling through these receptors remains a theory, but provides a testable hypothesis. Characterization of these receptors and their target, DAB-1, by expression and localization studies in gonadotropin treated mice could shed light on their involvement. Functional assays with blocking of these proteins by antibody or siRNA in cell lines or primary GCs could test the hypothesis. These could include viability assays and steroid assays using our current methods, as well as measuring phosphorylation of downstream targets, DAB-1, AKT, and PRAS40. Other proteins potentially involved in the proposed mechanisms could be studied to determine their roles in Spondin 1 functions, including AKT, mTOR, and TGFβ. The role of TGFβ in Spondin 1 function is particularly interesting as TGFβ signalling is involved in regulating many cellular functions and a decrease in circulating active TGFβ, as seen in the Spon1<sup>−/−</sup> mouse, could have many implications. The interaction of Spondin 1 with integrins could also be examined as blockade of integrin α<sub>v</sub>β<sub>3</sub> by Spondin 1 was implicated in inhibiting HUVEC migration. Spondin 1 may interact with this and other integrins to promote viability and affect steroidogenesis. A broader approach whereby global gene expression changes were measured either by microarray or RNA-seq would also be useful.

5.6.3 Spondin 1 as a Diagnostic Biomarker

Now that ovarian roles in viability and angiogenesis have been identified for Spondin 1, its status as an ovarian cancer biomarker should be studied more extensively. mTORC1 is currently a target for therapy in ovarian cancer due to its increased activity in the disease, so Spondin 1, as a possible mTORC1 regulator, could be a target as well. If Spondin 1 does indeed play a role in stabilizing blood vessels following angiogenesis, its role in disease progression may be two-fold. Studies should be undertaken to evaluate the suitability of Spondin 1 as a serum biomarker, as it could possibly be a better diagnostic and prognostic indicator than the currently used biomarker, CA125.
5.7 Conclusion

It is now clear that Spondin 1 is functional within the ovary. I have shown not only that Spondin 1 affects viability and steroidogenesis of ovarian granulosa cells in vitro, but that Spondin 1 is important for complete fertility, as evidenced by the subfertility of the Spond1$^{-/-}$ female mouse. Therefore further study of Spondin 1 in the ovary, particularly in humans, could provide insight into currently unexplained fertility. Spondin 1-mediated increased viability and proliferation, and increased stability of vasculature as proposed in this work could contribute to development and progression of ovarian cancer. Expression status of progesterone and estrogen receptors is considered a prognostic marker for several cancers, including ovarian [39, 40]. Often cancers overexpressing these receptors are reliant on continued production of their respective hormones. Therefore, Spondin 1-mediated changes in steroidogenesis could affect the survival and progression of ovarian cancer. The potential role of Spondin 1 in angiogenesis is intriguing considering its localization to capillaries in tissues with angiogenic growth (ovary and uterus) and tissues where vessels are maintained (kidney). This suggests that Spondin 1 may be involved in vascular maintenance in general, and may perform this function in other tissues as well.

This thesis presents evidence that Spondin 1 plays an important role in maintaining ovarian fertility and expands the current knowledge of Spondin 1 functions. Future investigations into the specific mechanisms of Spondin 1-mediated increases in viability and proliferation, Spondin 1 regulation of steroidogenesis, and Spondin 1’s role in angiogenesis will help to create a more complete picture of Spondin 1’s role in the ovary. Hopefully, these studies and future work will have implications for therapeutic intervention in infertility and ovarian cancer.
5.8 Bibliography


39. Ho S-M. Estrogen, progesterone and epithelial cancer. Reproductive Biology and Endocrinology 2003; 1:

Appendix A

Appendix A. Steroid hormones and the steroidogenesis pathway.

The steroidogenesis pathway in the ovary from cholesterol to 17β-estradiol. Steroid hormones are written in bold and not enclosed. Steroid enzymes are written beside the reactions they catalyze. STAR – steroid acute regulatory protein, transports cholesterol from the outer to the inner mitochondrial membrane. CYP11A1 – cholesterol side chain cleavage enzyme, cleaves cholesterol to form pregnenolone. HSD3B1 - 3β-hydroxysteroid dehydrogenase, dehydrogenates pregnenolone, 17α-pregnenolone or dehydroepiandrostenedione to form progesterone, 17α–progesterone or androstenedione, respectively. CYP17A1 - 17α-hydroxylase, adds a hydroxyl group to pregnenolone or progesterone and splits the side chain off of 17α-hydroxy–pregnenolone or –progesterone to form dehydroepiandrostenedione or androstenedione, respectively. HSD17Bx - 17β-hydroxysteroid dehydrogenases, convert androstenedione to testosterone
and estrone to 17β-estradiol by hydrogenation. CYP19A1 – aromatase, converts androstenedione to estrone and testosterone to 17β-estradiol by oxidation and elimination of a methyl group. Inset: Chemical structures of progesterone, testosterone, and 17β-estradiol.
Appendix B. Apoptosis array comparing BSA-treated and Spondin-1 treated KGNs.

Apoptosis array comparing protein lysates from BSA-treated (control) KGNs with Spondin 1-treated KGNs. Proteins present on the assay include but are not limited to: Bad, Bax, Bcl-2, Pro-caspase-3, cleaved caspase-3, Claspin, Hif-1 α, HSPs, Livin, Phospho-p53s, and Survivin. The assay was performed twice and no differences between treatments were found.
Appendix C

Red-green intensity plots of merged images in Chapter 3.
Plots of the intensity of red and green fluorescence in merged images of localization of SPON1 (green) with PECAM1, DES, or ACTA2 (red). Higher intensity of individual colours represents areas where there is little to no overlap between red and green, while lower intensity represents overlap. Fig. 3-3, A, B, and C represent SPON1 with PECAM1 in the ovary and shows high overlap. Fig. 3-4 represents SPON1 with A) DES and B) ACTA2 and shows overlap of SPON1 with DES but localization of DES to places where SPON1 is not, and not very much overlap between SPON1 and ACTA2. Fig. 3-5 shows high overlap between SPON1 and PECAM1 in the uterus. Fig. 3-6 A shows little overlap overall between SPON1 and DES in the uterus (a-c), with some overlap taking place in the myometrium (d-f). Fig. 3-6 B shows almost no overlap between SPON1 and ACTA2 in the uterus.
## Appendix D

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role in Folliculogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Akr1c18</em></td>
<td>Aldo-keto reductase family 1, member C18 or 20α-hydroxysteroid dehydrogenase, converts progesterone to 20α-dihydroprogesterone during breakdown of the corpus luteum</td>
</tr>
<tr>
<td><em>Amh</em></td>
<td>Anti-mullerian hormone, suppresses activation of primordial follicles and response of secondary follicles to FSH</td>
</tr>
<tr>
<td><em>Cyp11α1</em></td>
<td>Cholesterol side chain cleavage enzyme, converts cholesterol to pregnenolone</td>
</tr>
<tr>
<td><em>Cyp19α1</em></td>
<td>Aromatase, converts androstenedione to estrone and testosterone to 17β-estradiol</td>
</tr>
<tr>
<td><em>Lhcgr</em></td>
<td>Luteinizing hormone/chorionic gonadotropin receptor, involved in response to LH</td>
</tr>
<tr>
<td><em>Pgr</em></td>
<td>Progesterone receptor, involved in response to progesterone</td>
</tr>
<tr>
<td><em>Ptgfr</em></td>
<td>Prostaglandin F receptor, involved in corpus luteum formation and breakdown</td>
</tr>
<tr>
<td><em>Ptgs2</em></td>
<td>Prostaglandin-endoperoxide synthase 2, important for cumulus expansion and ovulation</td>
</tr>
<tr>
<td><em>Scarb1</em></td>
<td>Scavenger receptor class B member 1, receptor involved in uptake of cholesterol by ovarian cells</td>
</tr>
<tr>
<td><em>Sox9</em></td>
<td>SRY-box 9, development gene involved in female to male sex reversal, regulates transcription of <em>Amh</em></td>
</tr>
<tr>
<td><em>Star</em></td>
<td>Steroid acute regulatory protein, moves cholesterol across the mitochondrial membrane</td>
</tr>
</tbody>
</table>

### Appendix D. List of Genes measured for changes in mRNA expression between *Spon1*−/− ovaries and *Spon1*+/+ ovaries in Chapter 4.
Appendix E. Simplified model of proposed Spondin 1 pathways in the ovary.

Proposed mechanisms by which Spondin 1 might affect cell viability and proliferation, steroidogenesis, and angiogenesis. A) Signalling through either APP, LRP8, VLDLR or some combination of these receptors, SPON 1 promotes phosphorylation of DAB1. DAB1 activates PI3K signalling which phosphorylates AKT. AKT phosphorylation of PRAS40 removes PRAS40 from the mTORC1 complex thereby activating mTORC1 signalling which affects many cellular processes including cell growth and viability.
SPON1 may also signal to LRP8 and VLDLR to increase cholesterol transport into the cell for steroidogenesis. B) SPON1 binds the latent TGFβ/LTBP/LAP complex and removes LTBP and LAP which activates TGFβ. TGFβ can then signal through one of its receptors, here represented as TGFβR, and turn on TGFβ signalling, either through PI3K to mTORC1 (as shown here) or via another pathway, to promote cell viability and proliferation. C) SPON1 may signal through or block signalling of an integrin to effect the process of angiogenesis and potentially other cellular processes, such as viability and steroidogenesis.

SPON1 – Spondin 1; APP – amyloid precursor protein; LRP8 – low-density lipoprotein receptor related protein 8; VLDLR – very low density lipoprotein receptor; DAB1 – disabled 1; PI3K – phosphoinositide 3-kinase; AKT – protein kinase B; mTORC1 – mammalian target of rapamycin complex 1; PRAS40 – proline-rich AKT substrate of 40 kDa; TGFβ – transforming growth factor β; LTBP- latent TGFβ binding protein; LAP – latency-associated peptide; TGFβR – TGFβ receptor.
Appendix F. Co-localization of SPON1 and PECAM1 in the kidney.

Immunofluorescence by confocal microscopy showing co-localization of SPON1 (red) and PECAM1 (green) in the kidney. DAPI (blue) shows nuclei of cells. The small round structures are glomeruli, spherical networks of capillaries that filter blood. SPON1
localizes to the vasculature, as marked by the location of PECAM1, especially to the capillaries within the glomeruli. B is a magnified image of A, and the merged image of B is shown larger for clarity.
Appendix G

Appendix G. Expression of Vegfa and Pecam1 is not altered following gonadotropin treatment in Spon1+/+ and Spon1−/− ovaries.

mRNA expression of Vegfa and Pecam1 in Spon1+/+ and Spon1−/− ovaries following eCG treatment or eCG treatment followed by hCG treatment for 4, 16, or 24 hours. There was significant differences for treatment for both genes as determined by two-way ANOVA, Vegfa – F (3, 38) = 6.452, P < 0.01, Pecam1 – F (3, 38) = 7.925, P < 0.001. There was no significant difference for genotype overall or by individual treatment.
Curriculum Vitae

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Submitted Manuscripts

Prepared Manuscripts