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Investigating the Role of Extracellular Matrix Proteins in Ovarian Folliculogenesis and Ovarian Cancer

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Abstract

The extracellular matrix (ECM) is a highly organized, dynamic structure that maintains tissue integrity and regulates biological processes involved in organ development and function. To explore the role of ECM proteins in ovarian physiology and pathology, my thesis characterizes ECM proteins aberrantly overexpressed in the Estrogen Receptor (ER)β-null (βERKO) mouse ovary and epithelial ovarian cancer (EOC). The ECM undergoes extensive physical changes, and influences numerous cell functions, throughout folliculogenesis. This study identifies a role for ERβ in ovarian development earlier than previously believed. Nidogen 2 and Collagen 11a1 are aberrantly overexpressed in βERKO ovaries as early as postnatal day 13, and this dysregulation continues into adulthood, as determined by qPCR and immunofluorescence. Collagen IV, Nidogen 1 and Laminin are also more highly expressed in the βERKO ovary than in the wildtype ovary, suggesting that the repression of several ECM proteins in the ovary is ERβ-dependent. The molecular mechanisms that initiate gene repression by ERβ are not well understood; therefore a potential mechanism by which ERβ may act as a transcriptional repressor in the ovary is investigated. I characterized a novel ERβ transcriptional corepressor – transcription factor 21 (TCF21). In transient transfection and reporter assays, TCF21 represses ERβ transactivation of synthetic and natural estrogen-responsive promoters in various cell lines. As in the βERKO ovary, when the mechanisms regulating ECM dynamics during normal organ function are disrupted, the ECM becomes disorganized. This disorganization is associated with various pathologies, including cancers. The ECM protein, Spondin 1 (SPON1), is overexpressed in ovarian cancers and has been identified as a promising ovarian cancer marker, particularly for high-grade serous carcinomas; yet, its cellular functions and related mechanisms in EOC progression remain unknown. This study shows that SPON1 is expressed and secreted by immortalized EOC cell lines and human primary ascites-derived EOC cells. Treatment with exogenous SPON1 reduces EOC cell adhesion, viability and proliferation but not migration. Experiments utilizing a non-adherent culture surface suggest SPON1 does not effect EOC spheroid formation but is involved in spheroid anchoring and cell dispersion. These findings support an important role for ECM proteins in ovarian development and progression of ovarian carcinomas.

Keywords

Extracellular matrix, folliculogenesis, ovary, granulosa cell, Collagen 11A1, Nidogen 2, Estrogen Receptor β, TCF21, transcriptional coregulator, Spondin 1, ovarian cancer, high-grade serous ovarian carcinoma, patient samples

Co-Authorship Statement

Chapters 1: Written by Alexandra Carrier and edited by Dr. Bonnie Deroo

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Chapter 3: Written by Alexandra Carrier and edited by Dr. Bonnie Deroo. Coimmunoprecipitation assays were carried out in collaboration with Dr. Macarena Pampillo.

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Chapter 5: Written by Alexandra Carrier and edited by Dr. Bonnie Deroo.

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List of Abbreviations

ADAMTS	a disintegrin and metallopreoteinase with thrombospondin motif
AF	activation function
AMH	anti-Müllerian hormone
AP	alkaline phosphatase
ApoER2	apolipoprotein E receptor 2/ LRP8
APP	amyloid precursor protein
AR	androgen receptor
AREG	amphiregulin
ARID1A	AT-rich interactive domain-containing protein 1A
bHLH	basic helix-loop-helix
BM	basement membrane
BMP4	bone morphogenetic protein 4
BMP7	bone morphogenetic protein 7
BRACA1/2	breast cancer 1 and 2
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BSA	bovine serum albumin
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CG	ciliary ganglion
ChIP	chromatin immunoprecipitation
Chk2	checkpoint kinase 2
cho	chondrodystrophic
CHO	chinese hamster ovary
CL	corpus luteum

CNS	central nervous system
COC	cell-oocyte complex
Co-IP	coimmunoprecipitation
COL4A1	Collagen 4A1 protein symbol
Col11a1	Collagen 11A1 gene symbol
COL11A1	Collagen 11A1 protein symbol
CS-FBS	charcoal-stripped fetal bovine serum
Ctbp2	C-terminal-binding protein 2
CTNNB1	β-Catenin gene symbol
CYP11A1	cytochrome P450, Family 11, Subfamily A, Polypeptide 1
DAB-1	disabled-1
DAPI	6-Diamidino-2-Phenylindole
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle's Medium
dpc	days post coitum
αERKO	ERα Knockout
βERKO	ERβ Knockout
E-cadherin	epithelial cadherin
E2	17β-estradiol
ECL Plus	enhanced chemiluminescence reagent
ECM	extracellular matrix
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response elements
EREG	epiregulin
EMT	epithelial-to-mesenchymal transition

EOC	epithelial ovarian cancer
Esr1	estrogen receptor alpha gene symbol
Esr2	estrogen receptor beta gene symbol
FAK	focal adhesion kinase
FBS	fetal bovine serum
FOXL2	forkhead box L2
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GC	granulosa cell
GDF9	growth Differentiation Factor 9
GL	granulosa lutein cell
GnRH	gonadotropin-releasing hormone
GPR3	G-protein coupled receptor 3
GPR12	G-protein coupled receptor 12
Has2	hyaluronan synthase 2
HAT	histone acetyltransferase
HDAC	histone deacetylase
HGSC	high-grade serous ovarian carcinoma
HLH	helix-loop-helix
HUVEC	human umbilical vein endothelial cell
IF	immunofluorescence
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LBD	ligand-binding domain
LDL	low density lipoprotein
Lf	lactoferrin
LH	luteinizing hormone

LHCGR	luteinizing hormone/choriogonadotropin receptor
LRP	lipoprotein receptor–related protein
LRP8	low-density lipoprotein receptor-related protein 8
MAPK	mitogen-activated protein kinase
MCK	muscle creatine kinase
MEM	minimal essential medium
mh	minor triple helix
MMP	matrix metalloproteinase
MOF	multiple oocyte follicle
N-cadherin	neural cadherin
NCOR1	nuclear hormone receptor-corepressor 1
NCOR2	nuclear hormone receptor-corepressor 2/ SMRT
NF-Y	nuclear factor Y
Nid1	Nidogen 1 gene symbol
NID1	Nidogen 1 protein symbol
Nid2	Nidogen 2 gene symbol
NID2	Nidogen 2 protein symbol
Npp	amino-propeptide
NR	nuclear receptor
NTD	amino terminal domain
OSE	ovarian surface epithelium
P-cadherin	placental cadherin
Pbx1	pre-B-cell leukemia transcription factor 1
PBS	phosphate-buffered baline
P450SCC	cholesterol side chain cleavage enzyme
P/S	penicillin/streptomycin

PGC	primordial germ cell
PGE2	prostaglandin E2
PI3K/AKT	phosphoinositide-3 kinase/protein kinase B
PKA	protein kinase A
PLL	poly-l-lysine
PND	postnatal day
POD-1	Podocyte-1
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene difluoride membrane
qPCR	quantitative reverse transcription polymerase chain reaction
RANKL	receptor activator of NF-κB ligand
RIPA	radioimmunoprecipitation assay
Rspo	R-spondin gene symbol
ROC	reciever operating characteristic
Rpl7	ribosomal protein L7 gene symbol
RT	room temperature
S1P	sphingosine-1-phosphate
Scx	scleraxis
SCC	Santa Cruz cocktail
SF1	steroidogenic factor 1 /Ad4BP
SHP	small heterodimer partner
siRNA	small interfering RNA
SMC	smooth muscle cell
SPARC	secreted protein acidic and rich in cysteine
Spon1	Spondin 1 gene symbol
SPON1	Spondin 1 protein symbol

SRY	sex determining region
STIC	serous intraepithelial tubal carcinomas
TBST	tris-buffered saline with tween
TC	theca cell
Tcf21	Transcription factor 21 gene symbol
TCF21	Transcription Factor 21 protein symbol
TGFβ	transforming growth factor beta
TGFBI	transforming growth factor-beta-induced protein/ βig-H3
TIMP	tissue inhibitors of metalloproteinases
TNFAIP6	tumour necrosis factor, alpha induced protein 6
TSP	thrombospondin
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VSGP	vascular smooth muscle cell growth promoting factor
WT	wildtype

Chapter 1

1 Introduction

1.1 Overview of Chapter 1

This thesis examines the role of extracellular matrix (ECM) proteins in ovarian follicle development and ovarian cancer progression.

This chapter begins with an overview of folliculogenesis (Section 1.2) describing: a) the formation and growth of follicles, b) the events necessary for a follicle to mature into a preovulatory follicle capable of releasing a fertilizable oocyte, and c) the role of ECM components in follicular development. The next section (Section 1.3) describes Estrogen Receptor β (ERβ), its structure, mechanisms of action and the phenotype of the ERβ knockout mouse. The following two sections focus on the 17β-estradiol-regulated ECM proteins Collagen11A1 (Section 1.4) and Nidogen 2 (Section 1.5); in Chapter 2, I show data exploring the expression and localization of these two proteins in the ovaries of wildtype and ERβ knockout mice. Section 1.6 focuses on Transcription Factor 21 (TCF21); data suggesting a novel role for TCF21 as a co-regulator of ERβ-transcriptional activity is shown in Chapter 3. Spondin1, another 17β-estradiol-regulated ECM protein that is also implicated in ovarian cancer, is the focus of Section 1.7. Ovarian cancer is described in Section 1.8, with a focus on its origins, classification and the role of ECM components in ovarian cancer progression. The relevance of Spondin 1 to ovarian cancer progression is supported by my data in Chapter 4. The final section of Chapter 1 (Section 1.9) provides the rationale for these studies.

1.2 Folliculogenesis

1.2.1 Introduction to folliculogenesis

Folliculogenesis is a highly regulated process in the ovary by which an immature primordial follicle develops into a mature preovulatory follicle capable of releasing a mature oocyte. The ovary is also an important site of steroid production, including

testosterone, 17β-estradiol and progesterone, which are essential for the development of secondary sex characteristics and the maintenance of pregnancy.

The ovarian follicle is the basic functional unit of the ovary (Figure 1-1A). It consists of the oocyte and two somatic cell types, granulosa cells (GCs) and theca cells (TCs). GCs, an actively differentiating cell type, surround the oocyte and are essential for its growth. As GCs proliferate and the follicle grows, the GC layer becomes surrounded by a basement membrane and TCs. A fluid-filled cavity known as the antrum then forms, resulting in the development of a mature, preovulatory follicle. This cascade of events culminates in the rupture of one or more mature preovulatory follicles, and the release of a fertilizable oocyte. Although the timing of specific stages in folliculogenesis may differ, the overall pattern appears to be fairly well conserved in mammals.

1.2.2 Formation of primordial follicles

During mammalian embryonic development, primordial germ cells (PGCs) differentiate from the epiblast [1]. PGCs migrate to the genital ridge, then differentiate into oogonia once they reach the gonads [2]. Upon their arrival in the gonads, the germ cells undergo several rounds of mitosis until meiosis is initiated, which occurs approximately 13.5 days post coitum (dpc) in mice [3] and after 13 weeks of gestation in humans [4]. Just prior to the initiation of meiosis the germ cells are arranged into clusters connected by intercellular bridges. These clusters are known as oocyte “nests” or “cysts”. These nests break down to form primordial follicles, which are single oocytes surrounded by a layer of epithelial pre-granulosa cells [1] (Figure 1-1B). The first primordial follicles are histologically detected as early as postnatal day (PND) 1 in mice [5] and 15 weeks of gestation in humans [6]. Although many mechanisms involved in the assembly of primordial follicles remain to be elucidated, studies have identified a variety of genes involved in this process, including transcription factors, meiosis-specific enzymes, growth factors and proteins of the zona pellucida (the glycoprotein layer that surrounds the plasma membrane of the oocyte) [7]. This initial pool of primordial follicles is the stock from which all growing follicles are derived. Once primordial follicles are formed they remain dormant until they are activated at puberty [8].

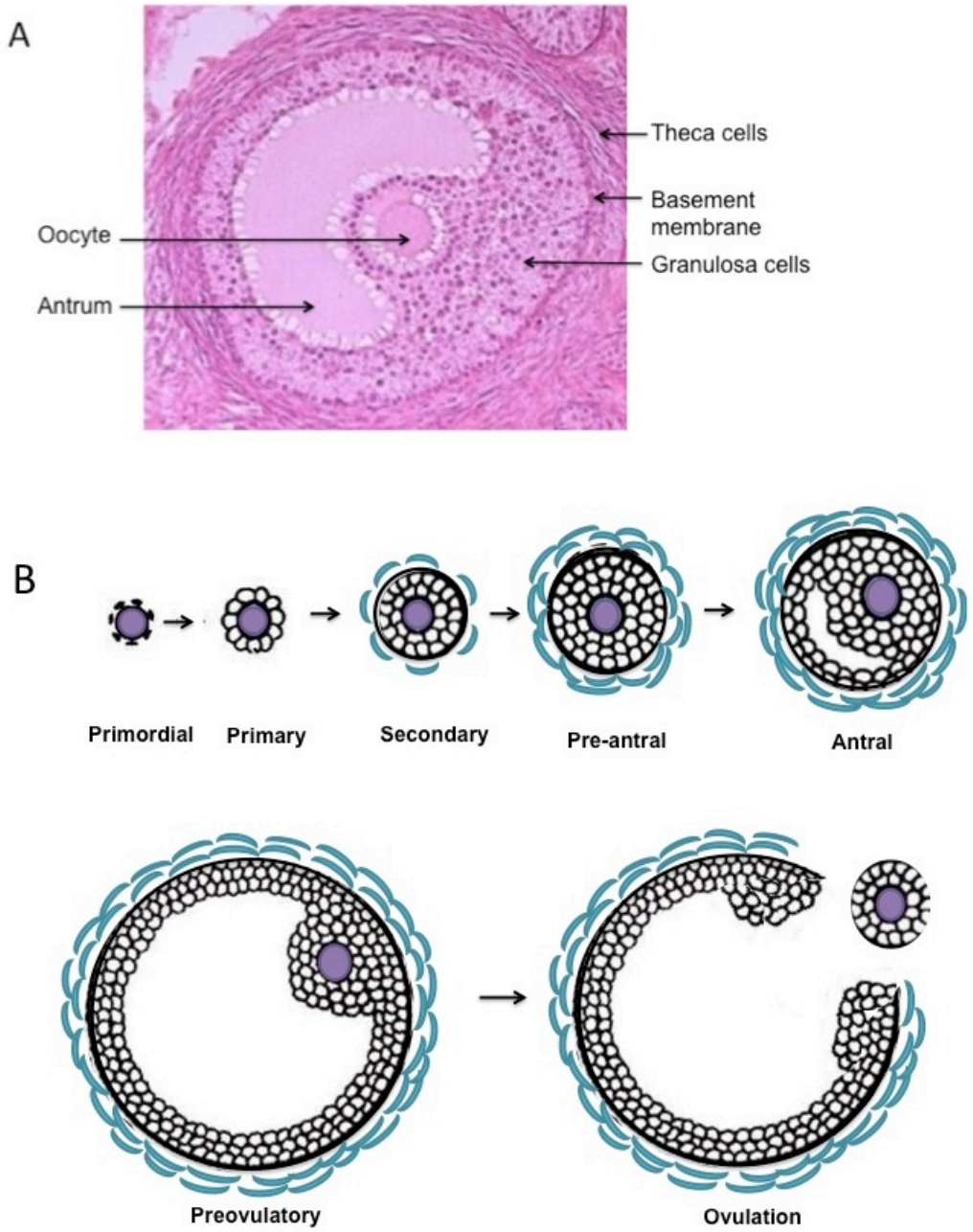

Figure 1-1: Folliculogenesis

A) A representative antral follicle. An antral follicle consists of an antrum as well as an oocyte, surrounded by granulosa cells, a basement membrane and thecal cells.

B) Stages of folliculogenesis. Growth of the follicle from the primordial to secondary stage is gonadotropin-independent. FSH is required for the formation of a large preovulatory follicle, which is capable of ovulation and forming a corpus luteum in response to an LH surge.

1.2.3 Primordial follicle to secondary follicle

Primordial follicles remain quiescent until they are selected for activation to become primary follicles. The factors controlling the activation of primordial follicles, i.e. why a particular follicle enters the growing pool while an adjacent one remains dormant, remain largely unknown, although it is well accepted that this is a multifaceted process involving the oocytes, somatic cells, ECM proteins and growth factors [9, 10].

According to studies using transgenic mouse models, inhibitory proteins and pathways exist to maintain primordial follicles in a dormant state. Anti-Mullerian hormone (AMH) controls the recruitment of primordial follicles [11]. An increased number of follicles are activated in AMH null mice [11], whereas overexpression of AMH suppresses primordial follicle recruitment and decreases the sensitivity of follicles to follicle stimulating hormone (FSH) [12]. Premature activation of primordial follicles is also observed following the loss of forkhead box O3 (Foxo3a) [13] or the oocyte-specific deletion of phosphatase and tensin homolog (*Pten*) [14], which leads to total depletion of the ovarian reserve and infertility in both animal models. It is ultimately the coordinated actions of inhibitory and activating signals within the ovary that initiate the recruitment of primordial follicles into the growing pool [2].

Once a primordial follicle enters the growing pool the squamous pre-granulosa cells differentiate into a single layer of cuboidal GCs surrounding the oocyte, forming a primary follicle. The transcription factor forkhead box L2 (FOXL2) is required for GC differentiation. Mice expressing an inactive form of FOXL2 do not complete the morphological transition from flattened pre-granulosa cells to cuboidal GCs, and the absence of functional GCs leads to an arrest at the primary stage and oocyte atresia [15]. The progression from primary to secondary follicles requires further GC proliferation (two or more layers), and the formation of both a basement membrane and a distinct layer of TCs. The oocyte-derived growth differentiation factor 9 (GDF9) mediates communication between the oocyte and GCs and is considered obligatory for this stage of growth, given that mice null for *Gdf9* are infertile, and lack secondary follicles or TCs around the basement membrane [16]. The factors involved in the recruitment of TCs are

not well understood; however it is believed that GC-derived signals are involved in the recruitment and differentiation of stromal cells into TCs [17]. Follicle growth up to the secondary stage is gonadotropin independent, as evidenced by transgenic mouse models and clinical studies in patients with FSH deficiency. Both *Fshb*$^{-/-}$ mice [18], which lack the follicle stimulating hormone (FSH) beta subunit and therefore cannot produce FSH, and women with inactivating mutations in *Fshb* [19] show follicular growth up to the secondary stage.

1.2.4 Secondary follicle to preovulatory follicle

Unlike the transition from the primordial to the secondary stage, the development of a large antral preovulatory follicle is dependent on the ability of GCs and TCs to respond to the gonadotropins, FSH and luteinizing hormone (LH). Mice with the inability to produce FSH (*Fshb*$^{-/-}$) or respond to FSH due to a loss of FSH receptor (*Fshr*$^{-/-}$) are infertile because follicles arrest at the secondary stage [18, 20]. Mice that lack the ability to respond to LH due to lack of LH receptor (*Lhcgr*$^{-/-}$) or produce it (*Lhb*$^{-/-}$) are also infertile, with folliculogenesis arrested at the early antral stage and a complete lack of preovulatory follicles [21, 22]. Therefore, FSH and LH are absolutely required for the careful coordination of mechanisms regulating the development of preovulatory follicles, steroid production and formation of a fluid-filled antrum.

Fluid collects in growing secondary follicles between the GC layers. Once these fluid-filled spaces coalesce to form the antrum, the follicle is termed antral [23]. The fluid within the antrum is similar in composition to serum but contains fewer proteins with a molecular weight above 100 kDa than serum [24]. Follicles at the antral stage have increased vascularization within the TC layer compared to follicles at the pre-antral stage, along with continued growth of the oocyte and proliferation of the somatic cells.

The primary role of TCs after the secondary stage of folliculogenesis is to produce androgens, specifically androstenedione and testosterone, to serve as precursors for 17β-estradiol production in GCs. The increase in androgens stimulates GC expression of FSHR. FSH then promotes GC proliferation and the expression of steroidogenic genes involved in the conversion of androgens to 17β-estradiol. The expression of Cyp19a1

(cytochrome P450, family 19, subfamily A, polypeptide 1) in GCs is the rate-limiting step in estrogen biosynthesis. Cyp19a1, commonly referred to as aromatase, converts TC-derived androgens to 17β-estradiol in GCs [25].

Early in FSH-dependent follicle growth the GCs produce low levels of 17β-estradiol, which suppresses GnRH (gonadotropin-releasing hormone) secretion by the hypothalamus, thereby reducing FSH secretion by the pituitary. As the follicle grows and the number of GCs increases, the production of 17β-estradiol is also increased, which raises GnRH levels and favours the production of LH [26]. In addition to 17β-estradiol GCs produce members the TGFβ family, activin and inhibin, named for their effect on FSH production. Activin, expressed by small and early antral follicles, stimulates FSH biosynthesis and release. Conversely, inhibin is expressed by late antral/pre-ovulatory follicles and has an inhibitory effect on follicle growth [27].

As GCs continue to proliferate in the early antral stage, they differentiate into two specialized GC subtypes, mural and cumulus. Layers of mural GCs are located along the basement membrane and are responsible for most of the follicle's steroidogenic activity, producing increasing amounts of 17β-estradiol as the follicle grows. Cumulus cells surround and are closely associated with the oocyte. They have lower steroidogenic activity than mural cells but promote oocyte growth by providing nutrients via gap junctions [28]. A defining feature of cumulus cells is their ability to undergo expansion later in folliculogenesis, a requirement for ovulation and therefore essential for fertility [29].

1.2.4.1 Selection of a dominant preovulatory follicle

Of the several thousand follicles in mice present at birth [30] or several million present in humans [31], only about 0.01% eventually develop into an ovulating dominant follicle while the rest undergo atresia, an apoptotic process [32]. The number of follicles selected to be dominant varies between species, from a select few in rodents to only a single follicle in humans. As noted earlier, the cyclical release of FSH at the antral stage initiates the growth of a cohort of follicles. Growth and follicle survival is FSH-dependent at this stage. As the follicle grows and the number of GCs increases, so do the

numbers of LHCGR expressed on mural cells. Gradually the mature follicle becomes more responsive to LH and less responsive to FSH. Furthermore, the expression of LHCGR leads to increased aromatase expression as well as abundant 17β-estradiol and inhibin production. The follicle that first acquires these characteristics is selected to become the dominant follicle because it is able to release 17β-estradiol more quickly and cause a switch in the gonadotropin output by the anterior pituitary, namely suppressing FSH levels and promoting LH production [30]. As follicle growth becomes LH-dependent and FSH levels fall, the remaining subordinate follicles in the growing pool that lack the LHCGR, and consequently the necessary LH responsiveness, undergo atresia. It has also been observed that dominant follicles are larger and faster growing than non-dominant follicles [31].

1.2.5 The “LH surge” and ovulation

As the dominant follicle continues to grow, it secretes high levels of 17β-estradiol and inhibin. Once a threshold concentration of estradiol is reached an acute surge of LH is released from the anterior pituitary [32]. The LH surge acts on the preovulatory follicle to initiate oocyte maturation, cumulus cell expansion, ovulation and the terminal differentiation of GCs and TCs to form the corpus luteum (CL). LH rapidly acts on the mural GCs to change gene expression and activate several pathways required for these events to occur [33]. The LH surge activates multiple pathways including protein kinase A (PKA), phosphoinositide-3 kinase/protein kinase B (PI3K/AKT), and RAS signaling cascades, all of which are critical for ovulation [34]. The epidermal growth factor (EGF)-like factors amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) are secreted from the mural GCs following the LH surge and activate EGF receptors (EGFR) in cumulus GCs [35]. The loss of these EGFR ligands in mice impairs ovulation, demonstrating that these signaling pathways are essential for ovulation to occur following the LH surge [36].

1.2.5.1 Oocyte maturation

During primordial germ cell formation, oocytes arrest at Prophase I of meiosis. The LH surge triggers oocyte reentry into the cell cycle; however, the exact mechanisms

involved are not well understood. It has been demonstrated that oocytes removed from follicles and placed in culture spontaneously resume meiosis [37], suggesting that follicle-derived factors are involved in maintaining the oocyte in meiotic arrest. It is generally accepted that high levels of the cyclic nucleotides, cAMP and cGMP, are involved in the maintenance of meiotic arrest because meiotic resumption coincides with either a reduction in cAMP [38] or cGMP [39] levels. It is expected that the oocyte produces cAMP itself [40], whereas cGMP is likely derived from somatic cells [41]. It has also been shown that GPR3 (G-protein coupled receptor 3) and GPR12 are necessary for the regulation of oocyte meiosis in mice [42] and rats [43], respectively. Oocytes from the *Gpr3*$^{-/-}$ knockout mouse resume meiosis independent of LH, while treatment of mouse oocytes with the GPR3/12 ligands SPC and S1P delayed meiotic resumption.

1.2.5.2 Cumulus expansion

Following the LH surge, cumulus GCs produce a hyaluronan (HA)-rich ECM that surrounds the oocyte, in preparation for ovulation [44]. This process is called cumulus expansion as a result of the increase in cumulus cell-oocyte complex (COC) volume. The HA-rich matrix contains various ECM proteins including laminin, collagen IV, fibronectin, and proteoglycans [45, 46]. The production of these proteins is dependent on the expression of both oocyte- and cumulus cell-derived factors. Many of the genes critical for COC expansion have been identified using knockout mouse models, including *Has2* (hyaluronan synthase 2), *Ptgs2* (prostaglandin endoperoxide synthase 2), *Tnfaip6*, and *Ptx3* (pentraxin 3). Prostaglandin signaling is necessary for the production of ECM proteins by cumulus cells and COC expansion, as mice lacking *Ptgs2* [47] or *Ptger2* (prostaglandin E2 receptor) [48] expression have impaired COC expansion and are infertile or subfertile, respectively. Tumour necrosis factor, alpha induced protein 6 (*Tnfaip6*)-null mice are also infertile [49]. Further studies have shown that isolated COCs that demonstrate poor expansion are unfertilizable because sperm are unable to degrade the HA-rich matrix, while oocytes isolated without cumulus GCs are unfertilizable due to reduced viability [29].

1.2.5.3 Follicle rupture

Ovulation involves the release of the COC from the follicle and ultimately from the ovary, as an oocyte surrounded by a single layer of expanded cumulus GCs. Before successful ovulation can occur the apical surface of the follicle must associate with the periphery of the ovary, and by proteolytic degradation weaken the follicle wall for follicle rupture. Following the LH surge, the follicle expresses proteases, including MMPs (matrix metalloproteinases) [50] and ADAMTSs (a disintegrin and metallopreoteinase with thrombospondin motifs) [51], involved in ECM degradation. Studies have indicated that members of both families are upregulated during follicle rupture in various species [52-55]; however, only the subfertile *Adamts1*$^{-/-}$ mice have exhibited a reproductive phenotype [56]. Several transgenic mouse models that lack genes encoding LH-induced proteases do not exhibit reproductive phenotypes, suggesting certain proteases may have a redundant role and may be compensated for by other proteins [57-60], thereby adding another level of complexity. The specific mechanisms responsible for selected ECM degradation remain to be elucidated although it has been proposed that localized expression of TIMPs (tissue inhibitors of metalloproteinases) may be involved [61].

1.2.5.4 Luteinization and formation of the corpus luteum

After the release of the oocyte, the remaining somatic cells differentiate into granulosa lutein (GL) and theca lutein (TC) cells in a process called luteinization. The new structure formed from the luteal cells is termed the corpus luteum (CL), and produces high levels of progesterone to prepare the endometrium for implantation and pregnancy. The follicular phase is now concluded and the luteal phase begins, characterized by the formation of the CL and consequent steroid production.

The CL produces high levels of 17β-estradiol and inhibin, which act on the pituitary to suppress FSH secretion [62]. The model of estrogen production by the follicle appears to be conserved by the CL because reminiscent of the GC and TC, the GL and TL cells have different roles in the CL. It is suggested that the GL and TL are the primary sites of estradiol synthesis and androgen production, respectively [63-65]. Furthermore, CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1) and HSD3B

(hydroxyl-delta-5-steroid dehydrogenase, 3 beta and steroid delta-isomerase) have been immunolocalized in GL and TL, demonstrating that both luteal cell types produce progesterone [65].

During the luteal phase, the CL also undergoes extensive neovascularization, which is required for the acquisition of large amounts of cholesterol as a substrate for progesterone. In the follicular phase, only the vasculature of the theca is well developed and does not penetrate the basement membrane. After ovulation, endothelial cells form new capillaries that invade the follicle. This process is primarily driven by vascular endothelial growth factor (VEGF) [66]. It has also been speculated that the anti-angiogenic factors, thrombospondin-1 (TSP-1) and TSP-2, are required for normal CL vascularization [67, 68].

If fertilization occurs the CL continues to produce progesterone until the placenta is able to produce its own. Throughout pregnancy, the CL continues to produce endocrine factors that suppress FSH secretion [62]. If fertilization does not occur the luteal cells become apoptotic and the CL regresses to become a corpus albicans. Consequently, the levels of 17β-estradiol and inhibin decrease and their suppressive effect on FSH is removed. The surge of FSH recruits a new cohort of preantral follicles, which initiates the follicular phase and another ovarian cycle begins.

1.2.6 ECM in follicular development

The ECM is composed of a diverse network of macromolecules with distinct properties; certain proteins are strictly structural components while others serve as signaling molecules. An important feature of the ECM is that it can be actively and specifically remodeled to serve its function in a certain tissue, at a particular physiological state. As described above, a developing follicle undergoes extensive morphological and biochemical changes during folliculogenesis. In addition to the HA-rich matrix produced during COC expansion (1.2.5.2), the composition of the ECM is dynamically remodeled throughout follicle growth, ovulation and atresia. The ovarian ECM has been characterized in several mammals, including bovine [69-73], human [74-76], mice [77, 78] and ovine [79, 80]. There are two main types of ECM observed in the

follicle: the basal lamina and the basal lamina-like focimatrix (abbreviated from **foc**al **i**ntraepithelial **matrix**) [69]. Granulosa cells are thought to produce many of the ECM components within the follicle [78, 81].

The basal lamina, often referred to as the basement membrane (BM), is a specialized sheet of ECM that underlies the epithelial cells and separates them from the interstitial matrix. The formation of a BM is required for normal tissue development and function, as BMs are able to regulate cell differentiation and proliferation as well as maintain the selective passage of cells and molecules [82]. The follicular BM is primarily composed of collagen type IV and laminin networks, which are connected and stabilized by nidogens [83]. The follicular BM possesses molecular exclusion capabilities and is necessary for the maintenance of the blood-follicle barrier, although its exact mechanism of accomplishing this remains largely unknown. Studies have suggested that not only is size a determinant (the mass cut-off of the follicular BM is between 100 and 500 kDa), so also is the charge of the molecule, because negatively charged molecules are excluded from movement across the barrier [84].

The focimatrix contains typical basal lamina components; however, it is neither basal nor laminate. Unlike basal laminas that envelop cells or groups of cells, the focimatrix develops as aggregates between the granulosa cells. Due to its morphology, the focimatrix is unable to perform the typical functions of the basal lamina, such as filtering material or creating microenvironments around cells. Instead, the focimatrix is predicted to be involved in initiating or assisting in the depolarization of granulosa cells, which is a prerequisite for luteinization [69]. Focimatrix aggregates are more abundant in apical than in basal GCs [73], and the amount of focimatrix increases as the follicle grows [69]. The expression of focimatrix genes has also been correlated with expression of steroidogenesis genes [72, 73].

Other proteins in follicles not associated with the basal lamina include proteoglycans (typically heparan sulphate proteoglycans such as perlecan, and small leucine-rich repeat proteoglycans such as decorin [71]), the matricellular glycoprotein

SPARC (secreted protein acidic and rich in cysteine) [85] as well as proteins involved in cumulus expansion and ovulation, including MMPs and TIMPs [86].

The presence and composition of the ECM influences numerous cell functions throughout follicle growth and atresia, including morphology, steroidogenesis, survival and proliferation [87, 88]. GCs *in vivo* have a rounded, epithelial morphology; however, when seeded onto uncoated tissue culture plastic they become flattened, resembling fibroblasts, and undergo rapid cell death. Interestingly, modifying the culture environment *in vitro* can greatly impact the features of isolated GCs. When GCs are plated on different matrices of ECM proteins, such as Matrigel (a gelatinous BM mixture rich in ECM proteins and growth factors), or individual BM components, they maintain their spherical morphology, are protected against apoptosis and proliferate more rapidly [79, 89-91].

The ECM also affects the production of steroids by somatic cells. When human GCs are cultured on serum-coated tissue culture plastic they produce significantly lower levels of estradiol, following treatment with the substrate androstenedione, than GCs cultured in the presence of collagen type I (50% higher) [92]. Furthermore, specific ECM proteins have been shown to influence GC production of progesterone in several species [87].

The presence of ECM components also impacts isolated follicles cultured *in vitro* [88]. For example, human ovarian tissue slices cultured on Matrigel possess a greater number of viable follicles over time than tissues cultured in the absence of ECM proteins [93], and mouse ovaries have higher follicle densities and growth initiation following culture on collagen type IV or laminin than on poly-L-lysine [10].

1.3 Estrogen Receptor Beta

1.3.1 Estrogen receptor discovery and structure

In 1958, Elwood Jensen discovered the existence of a receptor protein that mediated the actions of estrogen, and the corresponding gene was cloned in 1985 [94]. A novel subtype of the estrogen receptor (ER) was later cloned from the rat prostate and

ovary in 1996 [95]. This necessitated the renaming of the previously discovered ER to ERα, while the newly discovered ER was accordingly designated ERβ. The human ERα (*ESR1*) and ERβ (*ESR2*) genes are located on different chromosomes, 6q25.1 and 14q23.2, respectively. The human (and mouse) ERα and ERβ proteins have molecular weights of approximately 66 kDa and 54 kDa, respectively. As members of the nuclear receptor superfamily, ERs are found primarily in the nucleus, but also in the cytoplasm and mitochondria.

The ERs consist of five domains with distinguishable functions, designated domains A-F: an N-terminal domain (NTD) which includes the (A/B) domains, the DNA-binding (C) domain (also known as the DBD), a hinge (D) domain, a ligand-binding domain (LBD) (E), and a C-terminal (F) domain of unknown function that is unique to the ERs within the steroid receptor family (Figure 1-2). ERα and ERβ share the highest amino acid identity in the DBD (96%), while the NTD is the least conserved (30%) [96]. Interestingly, the LBDs are only ~ 50% identical, yet crystallographic studies indicate that the ligand binding pockets of both ERs have a similar structure [97]. ERα and ERβ show similar binding affinities towards most physiological and synthetic ligands, with some minor differences [98-100]. The ERs also possess two activation function (AF) domains that interact with distinct groups of coactivators: a ligand-independent AF1 in the NTD and a ligand-dependent AF2 in the DBD [96].

Several naturally-occurring splice variants have been described for both ER subtypes, with at least 3 ERα and 4 ERβ human isoforms known; however it is unclear whether all the variants are expressed as functional proteins and biologically active. The 530 amino acid (aa)-long human ERβ isoform is considered to be the wild type ERβ (rat and mouse, 549 aa), while the ERβ splice variants are referred to as $ERβ_{cx}$/2, ERβ3, ERβ4 and ERβ5 [94, 101]. All ERβ splice variants possess an altered C-terminus, and are unable to bind estrogens, coactivators, or other investigated ligands [94]. The full length and alternatively spliced forms of ERβ have been described in the normal ovary and ovarian tumours [102-104]. It is generally considered that ERβ2 is endogenously expressed only in rodents [102]; however, Fujimura *et al.* have detected expression of ERβ2 in human prostatic cancer, which makes it the only ERβ isoform identified at the

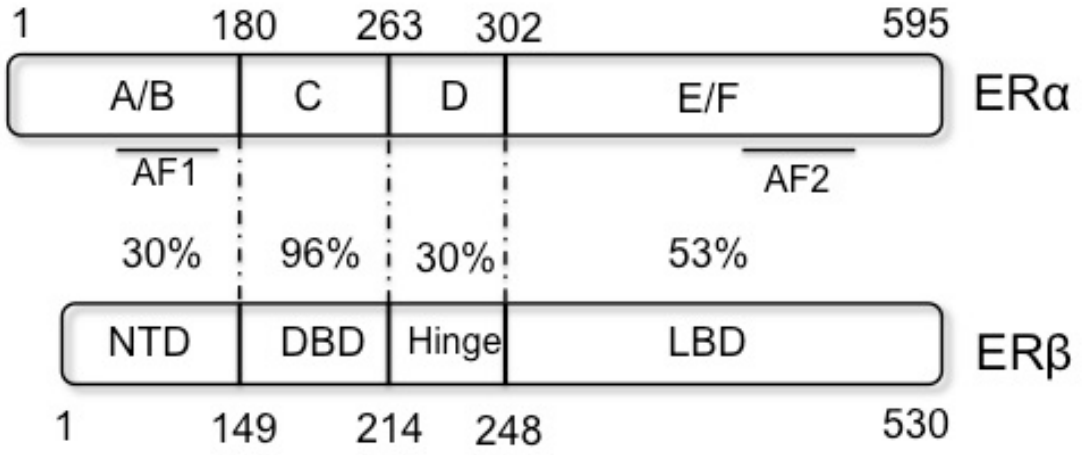

Figure 1-2: Comparison of the amino acid sequences of human ERα and ERβ. Schematic of human ERα and ERβ receptors. The amino acid sequence is numbered and steroid receptor domains are indicated. The protein sequence identity is shown between the ERα and ERβ diagrams as %. NTD: N-terminal domain, DBD: DNA-binding domain, LBD: Ligand-binding domain. AF: Activation function

protein level in human tissues [105]. The functional significance of these isoforms continues to be elucidated.

1.3.2 Classic mechanisms of ER action

The classic mechanism of ER action is similar to that of other receptors in the nuclear receptor superfamily. In the absence of ligand, the ERs are present in an inactive state within the nuclei, sequestered in a multiprotein inhibitory complex. Ligands are able to freely diffuse across the plasma and nuclear membranes, and bind the receptor. Ligand binding induces a conformational change within the ER and a release of chaperone proteins [106], which allows the receptors to dimerize and bind with high affinity to specific sequences, termed estrogen response elements (ERE) [100, 106, 107]. The consensus ERE is a 13 nucleotide inverted palindrome with a 3 base pair spacer of variable bases. Mutant variants/ imperfect EREs typically have a reduced ability to bind the ER and decreased transcriptional potency [106]. EREs are found throughout the genome, with only about 4% of ER binding sites mapped to 1-kb promoter-proximal regions [108]. ChIP-seq analysis has shown that the majority of ERβ binding sites (76%) are located within 50 kb of a gene. Approximately 25% of these sites are within a gene, 13% in proximal promoter regions of genes, 2% at the 3'-end of a gene, 36% elsewhere in a more distal region (5-50 kb), and the remaining 24% are over 50 kb from a gene [109]. Furthermore, ER binding sites are generally found overlapping Forkhead box (FOX) binding motifs [108]. Several studies have shown that FOXA1, for example, is a key determinant of ER function, and knockdown of FOXA1 decreases ER binding, cofactor recruitment and estrogen-stimulated transcription [110-113].

Once the ligand-ER complex is tethered to the ERE, coregulatory proteins are recruited and the receptors interact with the general transcription machinery either directly or indirectly via cofactor proteins [100, 114]. The cell and promoter involved will determine whether the ER will exert a positive or negative effect on the expression of the downstream target gene [114]. To activate transcription of a target, the structure of chromatin is disrupted through histone acetylation and other modifications, followed by initiation of transcription by RNA polymerase II. The molecular processes involved in

gene repression are not as well understood and continue to be elucidated; however, it is known that corepressor proteins recruit histone deacetylases (HDACs), which remove acetyl groups and restrict access of transcription factors to the promoter [115].

When acting through the classic ERE-driven mechanism, ERα homodimers and ERα/ERβ heterodimers tend to be stronger activators of transcription compared to ERβ homodimers. ERβ demonstrates lower binding affinity for ERE half-sites than ERα [116]. Both ERs contain a functional AF2 domain; however, the AF1 domain is only constitutively active in ERα whereas it contains a repressor function in ERβ. Once the AF1 domain is removed from ERβ, estradiol-ERβ-mediated transcriptional activity is increased [117]. Furthermore, it has been suggested that the effects of estrogen in a given tissue are a function of the relative levels of ERα and ERβ. Studies have shown that ERβ generally represses ERα-mediated gene transcription by competing with ERα for access to the DNA target [117-119]. For example, estradiol treatment of U2OS osteosarcoma cells engineered to express either ERα or ERβ demonstrated that each ER regulates a distinct and overlapping set of genes [120, 121]. Estradiol-treated U2OS cells expressing both ERs had a unique transcriptional profile compared to U2OS cells expressing either ERα or ERβ alone [122]. A study using ChIP-chip showed that the ratio between ERα and ERβ levels affects the ERβ-binding regions in MCF7 breast cancer cells, with ERα displacing ERβ when both ERs were present [123]. Lastly, T47D breast cancer cells that endogenously express ERα were engineered to also express ERβ. The expression of ERβ antagonized ERα-mediated transcription of the ERα target genes pS2 and PR [124]. Interestingly, although a role for ERβ2 has yet to be revealed, it has been shown to dimerize with wildtype ERs and dose dependently suppress ERα and ERβ1-mediated transcriptional activation *in vitro*, suggesting it to be a negative regulator of estrogen action [102, 104].

As mentioned previously, the cell and promoter involved will determine the nature of ER action on specific genes. For example, ERβ is a less potent transcriptional activator of both a consensus ERE and imperfect EREs than ERα in Chinese Hamster Ovary (CHO) cells [116]. Conversely, ERβ is a more potent transcriptional activator of a consensus ERE in response to estradiol in an osteoblast cell line, as compared to ERα

[125]. The different responses to the ERs by gene promoters in different cell types are most commonly attributed to the coregulatory proteins, which vary within cell lines and tissues.

1.3.3 Recruitment of transcriptional coregulators by ERβ

Coregulator proteins bind directly with nuclear receptors and general transcriptional machinery to regulate gene transcription. Two major groups of coregulators exist: coactivators that enhance transcription and corepressors that repress transcription [126]. Nuclear receptors interact with coregulatory proteins via two putative motifs: the NR box (LXXLL) and the CorNR box (L/IXXI/VI), which are present in coactivators and corepressors, respectively [127, 128].

Coregulators have enzymatic activities that produce posttranslational modifications of chromosomal proteins involved in gene regulation. For example, coactivator histone acetyltransferases (HATs) lead to the acetylation of histones, whereas HDACs are recruited by corepressors and modify chromatin in such a way that transcription is inhibited. It is well understood that multiple HDACs exist, each contributing their own combination of enzymatic requirements to the repression complex [114, 129]. Another level of complexity is added when other transcription factors are able to modify the stability of the transcription complex and/or suppress the enzymatic activities of the coregulatory proteins [130]. Coregulators can also function as bridging factors or adaptor proteins, by linking protein-binding partners together and facilitating the formation of larger signaling complexes [131].

In general, much less is known about the molecular processes involved in gene repression than gene activation. The best-characterized nuclear receptor corepressors are NCOR1 (nuclear hormone receptor-corepressor) and NCOR2/SMRT (silencing mediator of retinoid and thyroid hormone receptors). Both are conserved transcriptional repressors known to interact with several transcription factors, including ER [120]. They do not appear to have intrinsic repressive activity; rather they function as part of a larger repressor complex by recruiting other proteins involved in mediating the molecular actions required for repression, such as HDACs. HDACs act as corepressors in their own

right and may bridge between transcription factors to silence gene activity [129]. Nuclear receptors usually bind corepressors in the absence of ligand or in the presence of antagonists. The recruitment of NCOR1 and NCOR2 appears to be essential for the antagonist activity of ERα [132]; however, little is known regarding their ability to inhibit ERβ-mediated transcription. Interestingly, Webb *et al*. have shown by GST binding and mammalian two-hybrid assays that ERβ, through its AF-2 domain, binds to NCOR1 and NCOR2 in the presence of agonists, but not antagonists, via an LXXLL-like motif in the NCOR C-terminus [133].

Many coregulators that either enhance or inhibit ERα transactivation have been identified; however, ERβ coregulators have generally been understudied, and only a handful of coregulators have been shown to bind and affect transactivation by both ERs [96]. Furthermore, few studies have shown that ERβ recruits coregulators to endogenous genes. A U2OS cell line stably expressing a tetracycline-regulated ERβ was used to show that ERβ-specific agonists recruit NCOA2 to the endogenous TNFα promoter, thereby repressing TNFα gene expression [134]. Unliganded ERβ is also recruited to a known E2-induced gene in ERβ-expressing U2OS cells [135]. However, because U2OS cells do not endogenously express ERβ, it is unclear whether the effects observed in this artificial model would also be observed in primary bone cells.

Only a few coregulators that specifically regulate ERβ but not ERα transcriptional activity have recently been identified. A yeast two-hybrid assay using the ERβ A/B domain as "bait" found NM23-H2 to be an ERβ-associated protein, and its overexpression to increase ERβ-mediated transcription [136]. Whether NM23-H2 interacts with ERα or affects ERα-mediated transcription was not investigated. A comparable yeast-two hybrid assay identified HSP27/HSPB2 as an ERβ-binding protein, which subtly affects ERβ-mediated transcription [137]. Coimmunoprecipitation assays demonstrated that HSP27 specifically interacts with ERβ and not ERα. Although the role of coregulators in transcriptional regulation by ERs continues to be explored, there remains limited information in the literature regarding corepressors specifically involved in ERβ-mediated transcription or transactivation, and much remains to be revealed.

1.3.4 Alternative “non-classic” mechanisms of ERβ action

Over the years it has become apparent that ER can act through alternative signaling mechanisms that diverge from the classic model. These include the regulation of genes that lack an ERE (1.3.4.1), ER action in the absence of ligand (1.4.3.2), and membrane signaling often referred to as “nongenomic” action (1.4.3.3). These mechanisms are described briefly below.

1.3.4.1 Cross-talk with other transcription factors (ERE independent)

Ligand-bound ERs can also interact with other transcription factor complexes to stimulate the transcription of genes that are not regulated by ER binding to an ERE. This mechanism of ERE-independent ER activation involves “tethering” of the ER to other transcription factors, such as AP-1, SP-1 (GC-rich SP-1 motifs) or NFκB, that are directly bound to DNA by their respective response elements [106, 115]. ChIP-chip studies strongly suggest that this is a common mechanism of transcriptional regulation for both ERs [108, 123]. As with the classic model of ER action, ERα and ERβ differentially regulate target genes when acting via this mechanism [96]. Interestingly, ERβ interacts with SP-1 within the ERα promoter region and represses ERα expression [119].

1.3.4.2 Ligand-independent signaling

In addition to being activated by ligands, ERs can also be activated via ligand-independent pathways. Growth factors and intracellular messengers are able to activate ER-mediated gene transcription in the absence of ligand. Ligand-independent activation of ERs relies on the activation of kinases, which may phosphorylate the ER and/or its associated coregulators [138].

1.3.4.3 Membrane-associated ER signaling (nongenomic ER action)

The models of ER action described so far influence changes within a cell by acting in the nucleus and modulating the expression of target genes. This is a relatively lengthy process unlikely to offer measurable effects for hours following cell exposure to steroid. Yet rapid effects of estradiol treatment have been observed, within seconds or minutes of ligand treatment [139], which cannot be explained by the nuclear mechanism.

These rapid effects include activation of kinases, increases in ion fluxes across membranes and activation of nitric oxide synthase in endothelial cells [96]. The rapid responses of ER action are often attributed to a nongenomic mechanism, in which ligands bind to an ER localized to the membrane or cytoplasm. Although membrane ERs are becoming more accepted by the scientific community, they continue to be controversial, and it remains uncertain whether the classic ERs are involved or whether a distinct membrane-associated ER exists [96, 140, 141].

1.3.5 Localization of ERβ

The ERs exhibit species-, tissue-, and cell-specific patterns of expression and localization. ERβ is detected in the ovary, cardiovascular system, central nervous system, male reproductive organs, prostate, lung, colon, kidney and bone, whereas ERα is expressed in testis, ovary, mammary gland, uterus, breast, white adipose tissue, bone, heart and liver [94, 142]. Based on its broad expression ERβ regulates numerous physiological processes, such as bone density, the cardiovascular system, inflammation, and reproductive organ development and function [142].

ERβ is present in the ovaries of numerous species, including rodents, sheep, hamster, baboon, cow, pig and human [98], and total ERβ mRNA is more abundantly expressed than ERα mRNA in the rat ovary [143]. ERβ is predominantly expressed in the granulosa cells (GCs) of the ovary, while ERα is expressed in the ovarian theca cells [98]. An examination of immature and adult rat ovaries by *in situ* hybridization showed that ERβ is expressed in the GCs of small, growing and preovulatory follicles, with weak expression in a subset of corpora lutea (CL) [144]. Low levels of ERβ mRNA are detected as early as postnatal day (PND) 1 in the ovaries of mice [145] and PND 4 in the ovaries of rat [143], with levels increasing over time as the follicle grows. ERβ protein is detected in the primary follicles of mice on PND 4 [146]. It remains unclear whether ERβ is responsive to 17β-estradiol in these early follicles and influences the primordial to primary follicle transition; one study suggests it may be indirectly involved in the primordial to primary transition in rats by acting through various growth factors [146], whereas another shows 17β-estradiol has no effect in the ovaries of rats at this age or stage of follicle development [147]. Although it remains unclear at what precise stage

ERβ begins to influence folliculogenesis, it is well accepted that it has a critical role in the postnatal ovary, as it is required for optimal preovulatory follicle development, antrum formation, and ovulation [148, 149].

1.3.6 ERβ knockout mice

Both ERs are expressed within the ovary and the absence of either receptor affects ovarian function. ERβ knockout mice (βERKO) are subfertile [150-152] or infertile [153], ovulate less frequently and have smaller litters than wildtype females. The βERKO mouse has no distinct differences in the size or morphology of the ovary, and contains follicles at all stages of development (primordial to antral). However, it has been noted that there are fewer large antral follicles and CL, as well as a higher frequency of atretic follicles in the βERKO ovary than in the wildtype ovary [150, 152]. The GCs of βERKO preovulatory follicles have an impaired response to FSH-induced differentiation, as evidenced by decreased aromatase activity and estradiol synthesis, as well as a diminished LH surge and reduced expression of the LH receptor [149, 154, 155]. The attenuated response to FSH also affects the ovarian response to gonadotropins, consequently reducing the rate of follicle rupture, cumulus-oocyte expansion as well as the expression of prostaglandin synthase (Ptgs) and the progesterone receptor, which are essential for follicle rupture [149, 154]. Therefore due to an impaired response to the LH surge, ovaries of superovulated βERKO mice have numerous unruptured, preovulatory follicles [150, 154]. Furthermore ERβ is required for the production of cyclic adenosine monophosphate (cAMP) in GCs of preovulatory follicles, and insufficient cAMP levels may explain the reduced levels of estradiol synthesis in βERKO females [156].

The ERα knockout (αERKO) females are infertile [157]. The adult ovaries contain hemorrhagic, cystic follicles and no indication of ovulation. Folliculogenesis in αERKO ovaries progresses normally until the antral stage, but arrests before reaching the preovulatory stage [158]. FSH receptor and LH receptor mRNA expression is increased in αERKO ovaries compared to wildtype ovaries, while ovarian androgen receptor and progesterone receptor mRNA as well as ERβ protein levels are similar to wildtype levels [158]. Based on the ovarian phenotypes of the αERKO and βERKO mice it is evident that the receptors have different roles in folliculogenesis. It has been hypothesized that the

proliferative action of estrogen is carried out by ERα, whereas the differentiating effects of estrogen are mediated predominantly by ERβ [159].

Interestingly, the ovarian phenotype of the αβERKO mouse is different from that of either αERKO or βERKO mice [152]. The αβERKO mice are infertile, and while superovulation of immature females leads to the growth of antral follicles, no preovulatory follicles or CL are observed. The antral follicles in αβERKO ovaries are much smaller than those of wildtype or single mutant females. Furthermore, αβERKO ovaries possess fewer GCs than wildtype ovaries, and consequently the ovarian wall is abnormally thin due to the decreased number of mural GCs. The ovaries also have tubule-like structures that resemble seminiferous tubules of the testis, which are likely transdifferentiated from GCs [152, 160].

The identification of ERβ and its detection in numerous tissues expanded our understanding of estrogen signaling, physiology and pathophysiology [159]. The ERs have nearly a ubiquitous tissue distribution therefore it is not surprising that estrogens are involved in a variety of mechanisms in physiology and pathology. It is clear that ERβ plays a crucial role in the ovary, and changes in its expression may have clinical consequences, such as in infertility or cancer [98, 159]. ERβ levels decrease during the progression of many cancer types and it has been described as a potential tumour suppressor in epithelial ovarian cancer [161, 162].

We have shown that the mRNA levels of several ECM genes are disrupted in the ovaries of βERKO mice compared to their wildtype controls (Section 2.2.6, Table S2-1) [163]. Two of these genes identified in a microarray study by Dr. Deroo are *Col11a1* and *Nid2*, which I further investigated in this thesis (Chapter 2).

1.4 Collagen 11A1

1.4.1 Overview

Collagen is one of the most important components of the ECM. The collagen superfamily consists of 28 different types encoded by more than 40 genes [164]. All collagens are trimeric molecules consisting of three polypeptide chains that form a triple

helix structure. There are three subgroups within this diverse superfamily: fibrillar collagens, non-fibril collagens and fibril-associated collagens. Fibrillar collagens, capable of forming highly ordered fibrils in the ECM, are the most abundant subgroup. Fibril-associated collagens are targeted to the surface of collagen fibrils and help regulate fibrillar collagens. Non-fibril forming collagens are found in the basement membrane and do not form or associate with fibrils [165]. Collagen XI is a fibrillar collagen found in both cartilage and non-cartilaginous tissues [166, 167]. The mRNA levels of *Col11a1*, which encodes the collagen α1(XI) chain (COL11A1), is 1.7-fold higher in GCs of eCG-treated ERβ-null mice, than in eCG-treated ERβ heterozygous mice [156]. COL11A1 will be the focus of this section because the expression of COL11A1/*Col11a1* in the ovary is examined in Chapter 2 of this thesis.

1.4.2 Protein structure

Collagen XI is a heterotrimeric protein consisting of three alpha chains: α1(XI), α2(XI) and α3(XI). The α1(XI) and α2(XI) chains are unique gene products, whereas the α3(XI) chain is an overglycosylated form of the collagen α1(II) chain [168]. The alpha chains are initially synthesized as procollagens, with their amino and carboxyl termini subject to proteolysis to produce mature trimers [169].

The α1(XI) chain is encoded by the *Col11a1* gene, which consists of 67 exons [170]. COL11A1 has a globular amino terminal domain (NTD) that consists of the amino-propeptide (Npp), the variable region (Vr) and a minor triple helix (mh) (Figure 1-3A) [166, 171]. The structure of the α1(XI) chain is modulated by alternative splicing of the exons that make up the Vr domain (exons 6-8), which is both tissue-dependent and developmentally regulated [170, 172-174]. Due to the alternative splicing of the mRNA within this region, considerable structural diversity is generated, and at least eight different protein variants can be produced [170, 175, 176]. The Npp domain (exons 1-5) is common to all isoforms of the collagen α1(XI) chain irrespective of how the mRNA is spliced [169].

Proteolytic processing of procollagen 11A1 gradually removes the Npp [177]; however, the Vr region is maintained in the mature collagen XI and often localized to the

fibril surface [167, 178]. Preliminary data have suggested that the rate of proteolytic removal of the COL11A1 Npp is dictated by the splice forms in the Vr [169]. The presence of the Vr on the surface of the fibril indicates that the NTD will be exposed on the surface for an extended period of time after biosynthesis [167, 171]. This surface location of the NTD enables immunohistochemical analysis without requiring the disruption of fibril structure to access the major triple helix. Following secretion and proteolytic cleavage, the mature collagen molecules self-assemble into fibrils [179].

1.4.3 Binding partners and regulation

Collagen XI is best characterized for its copolymerization with collagen II and collagen IX to form an extensive network of thin fibrils in cartilage [168, 171, 180]. Collagen XI is located in the interior of the fibril; however, its NTD is located on the surface of collagen fibrils (Figure 1-3A) and therefore can interact with other proteins [168, 181]. The NTD of COL11A1 associates with several ECM components including collagens type II, IX, XI, XII and XIV; the proteoglycans perlecan, fibromodulin, epiphycan, and biglycan; the thrombospondins 1 and 5 (cartilage oligomeric matrix protein); chondroadherin and the matrilins 1 and 3 [182].

Surprisingly little is known about *Col11a1* regulation. Two research groups have shown that Nuclear factor Y (NF-Y) regulates the proximal promoter activity of *Col11a1* in cartilage and non-cartilage cells [183, 184]. Lymphocyte enhancer-binding factor 1 (Lef1), a transcription factor involved in the Wnt signaling pathway as well as mesenchymal/epithelial interactions during the development of several tissues [185], indirectly activates *Col11a1* transcription and negatively regulates osteoblast maturation [186]. The expression of *Col11a1* is also affected by TGF-β signaling [187]. Recently, Wu and colleagues showed that TGF-β1 treatment increases binding of NF-Y to the *Col11a1* promoter, which increases *Col11a1* expression [188].

1.4.4 Tissue distribution

Cartilage fibrils exist in distinct populations of thick and thin fibrils, and collagen XI is a minor fibrillar collagen restricted to the thin fibril group [178]. Collagen XI is most abundantly expressed in cartilage, but also found in several non-cartilaginous

tissues, including brain, skeletal muscle, placenta, lung, heart valve, skin, and vitreous fluid [166, 167, 173, 189]. *Col11a1* is detected in human fetal tissues [189], in the fetal mouse as early as embryonic day 11 [190], and in chick embryos on day 17 [168]. The α1(XI) transcripts are primarily found in cartilaginous tissues such as the chondrocranium and the developing limbs. They also accumulate in fetal non-cartilaginous sites, including odontoblasts, trabecular bones, the tongue, the atrioventricular valve of the heart, and the intestine [190]. Within the cartilage fibril the α1(Xl) chain forms a heterotrimer with α2(XI) and α1(II) [174]. In contrast, in non-cartilaginous tissues collagen α1(Xl) associates with chains of collagen V [174, 191].

1.4.5 Collagen XI function

Collagen XI is best characterized for its copolymerization with collagen II and collagen IX to form an extensive network of thin fibrils in cartilage [168, 171, 180]. The fibrils are composed of approximately 80% type II, 10% type IX and 10% type XI [192]. Although it makes up a relatively small amount of the total collagen in these fibrils, COL11A1 plays a critical role in cartilage assembly, organization and development [167].

It has been proposed that collagen XI regulates collagen II fibrillogenesis by steric hindrance, specifically through the COL11A1 NTD [193]. The major triple helical domains of collagen XI are sequestered within the collagen fibril, whereas the molecular dimensions of the NTD prevent it from being accommodated within the interior region; therefore, the Npp and Vr are localized on the fibril surface [167, 178]. Retention of the NTD at the surface of collagen II fibrils is postulated to restrict lateral growth by sterically hindering further addition of collagen II onto the fibril [167, 193], though the precise mechanism remains unclear.

The critical role of collagen XI in fibrillogensis is evidenced by the phenotype of chondrodystrophic (cho) mice, which present with a spontaneous mutation in the *Col11a1* gene that results in premature termination of the procollagen 11A1 (due to the introduction of a premature translation-termination codon), and therefore lack of a functional *Col11a1* [180]. The absence of Col11a1 disrupts the columnar arrangement

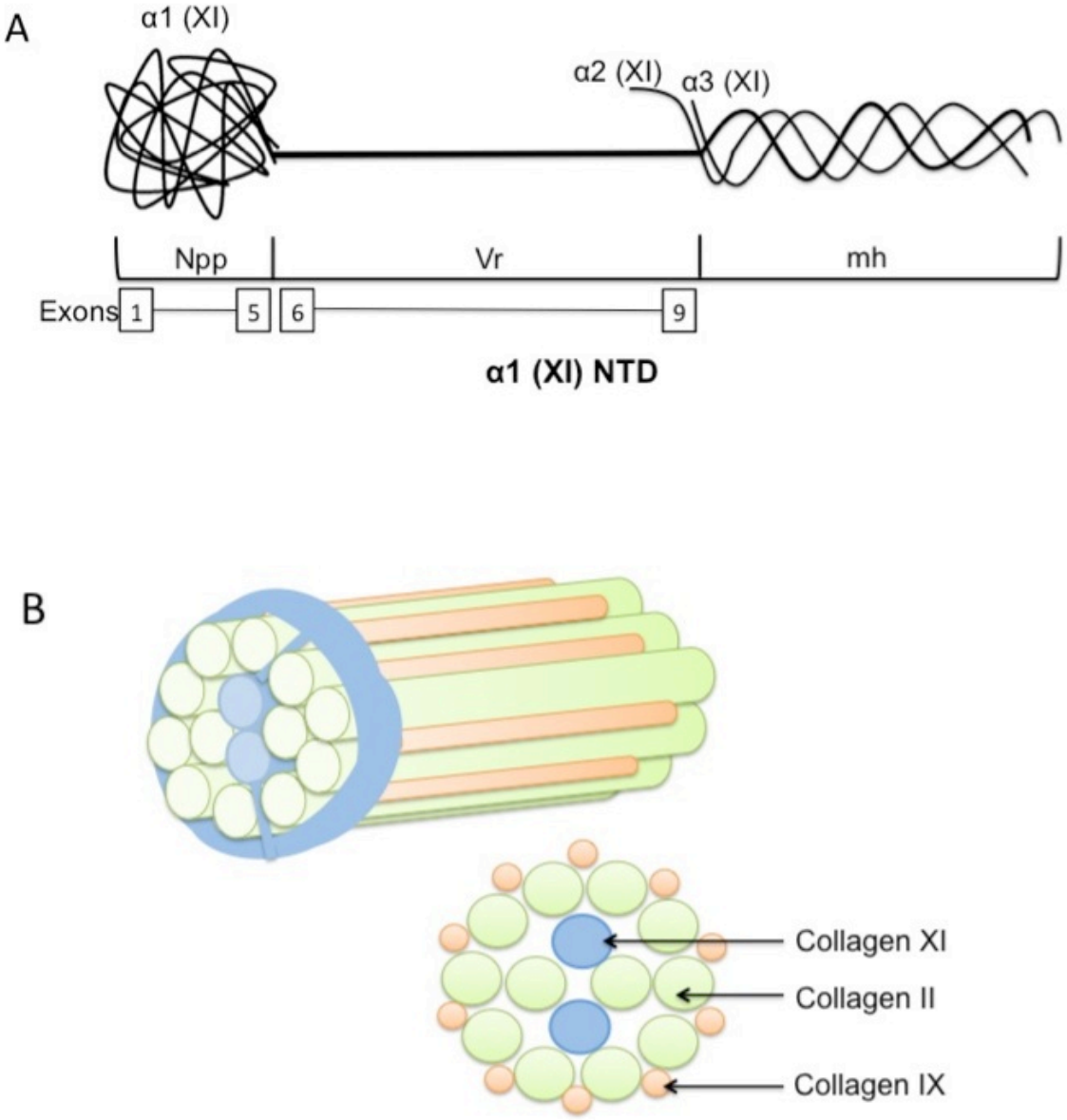

Figure 1-3: Collagen 11A1

A) Structure of the collagen 11a1 amino terminal domain (NTD). The NTD of the collagen XI chain contains an amino propeptide (Npp), a variable region (Vr) modulated by alternative splicing, and a minor collagen triple helix (mh). The Npp is coded by exons 1 through 5; the variable region is coded by exons 6 through 9.

B) Schematic of a cartilage collagen thin fibril. Collagen II (green), IX (orange) and XI (blue) copolymerize to form thin collagen fibrils. Collagen XI is located in the core of the fibril, and its NTD extends from the interior onto the fibril surface.

and maturation of growth plate chondrocytes, leads to thicker fibrils, and reduces the cohesive strength of the fibril matrices. The homozygous cho mutation is perinatal lethal, and a disproportionate frequency of dwarfism, short snouts and cleft palate are observed. Mice heterozygous for the cho mutation have no obvious phenotype. [180]

Mutations in the *Col11a1* gene are also associated with numerous human diseases, including Stickler and Marshall syndromes, which are characterized by altered facial appearance, eye abnormalities, joint alterations, and hearing loss [194, 195]. *Col11a1* mutations are also related to osteoarthritis [196], lumbar disc herniation [197], limbus vertebra [198], Achilles tendinopathy [199], and fibrochondrogenesis, a lethal form of dwarfism [200]. Furthermore, *Col11a1*/ COL11A1 is highly expressed in human invasive carcinomas of the ovary [188, 201], breast [202-204], stomach [205], pancreas [206], colon [207, 208], lung [209], head and neck [210], esophagus [211] and oral cavity/pharynx [212]. Changes in COL11a1 expression have been associated with carcinoma aggressiveness, progression and metastasis. Therefore, COL11A1 is not only an important stabilizing factor of cartilage fibrils, these studies suggest it plays a structural and organizational role in various tissues.

1.5 Nidogen 2

1.5.1 Overview

The vertebrate nidogen family consists of two members, nidogen 1 and nidogen 2, which are distinct gene products of *Nid1* and *Nid2* respectively. They are both ubiquitous BM components that have similar structure and affinity for other ECM proteins [213-215]. The predominant nidogen, nidogen 1, also known as entactin 1, is a 150 kDa protein originally isolated from the ECM of differentiating mouse embryonal carcinoma cells [216]. The second member of the nidogen family, nidogen 2, was initially cloned and isolated from human osteoblasts and named osteonidogen [83]. Once it was realized that this 200 kDa glycoprotein had a much broader expression pattern than osteoblasts alone, it was renamed to nidogen 2 or entactin 2 [83, 217]. Amino acid sequence similarity between nidogens 1 and 2 is species-dependent. For example, whereas mouse nidogens 1 and 2 are 27% similar, human nidogens 1 and 2 are 46% similar [218].

Nid2 expression is 3.9-fold higher in GCs of eCG-treated ERβ-null mice than in, eCG-treated ERβ heterozygous mice [156], and the expression of Nidogen 2/*Nid2* in the ovary is examined in Chapter 2 of this thesis. The nidogens have very similar protein structure, binding partners and expression patterns; therefore both proteins will be described in this section.

1.5.2 Protein structure

The nidogens share a similar domain structure, with three globular domains (G1-G3), separated by a link region between G1 and G2, and a rod region between G2 and G3 (Figure 1-4). A structural comparison between mouse nidogen 1 and nidogen 2 domains revealed 24%, 31.2% and 38.4% homology (common ancestry and similar structure) between G1, G2 and G3 domains, respectively. The rod domains showed 33.7% homology and the link region was least conserved with only 8% homology [217].

The N-terminal G1 globular domain is made up of a NIDO domain that resembles globular domains in the two ECM proteins α-tectorin and the transmembrane glycoprotein MUC4 [216]. G1 is often cleaved because its neighbouring link region is highly susceptible to proteolysis [219]. The G2 globular domain, which is important for perlecan and collagen IV interactions, contains EGF repeats as well as a G2F domain that is also found in fibulin-6 [216, 220]. The rod domain has four additional EGF-like repeats. There are consensus sequences for calcium binding within the second and fourth EGF-like repeats, followed by two thyroglobulin-like motifs (TY) in nidogen 2 and one TY motif in nidogen 1. Both mouse isoforms have an RGD motif within the rod domains, which acts as a site for potential integrin interaction. The RGD motif is present in the first EGF-like repeat in mouse nidogen 1, and the last EGF-like repeat in mouse nidogen 2, but absent in human nidogen 2. The G3 domain contains a low density lipoprotein (LDL) receptor-homology region, formed from six LY modules. Nidogen 1 has an additional EGF-like motif at the G3 C-terminal [216].

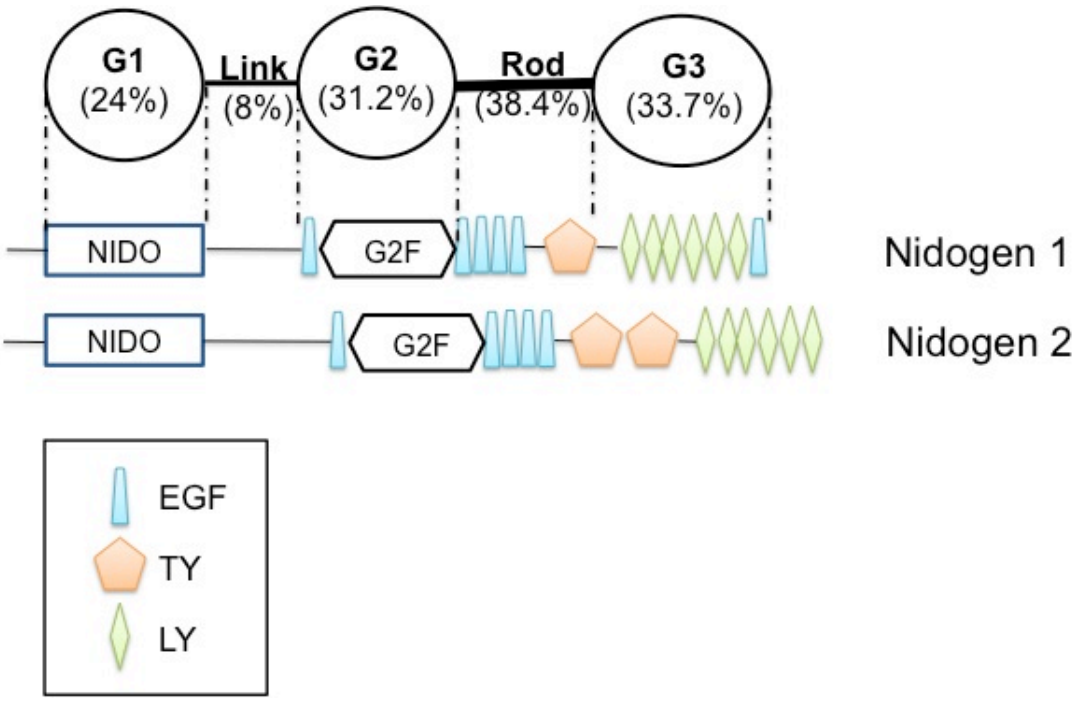

Figure 1-4: Comparison of Nidogen 1 and Nidogen 2 domains

Nidogens have three globular domains (G1-G3), a link region between G1 and G2, and a rod region between G2 and G3. The protein sequence homology between mouse Nidogen 1 and 2 is shown as %. NIDO: Nidogen like domain, EGF: Epidermal growth factor like module, G2F: G2 nidogen and fibulin module, TY: thyroglobulin-like motifs, LY: low density lipoprotein receptor module.

1.5.3 Binding partners

Both nidogen isoforms have been shown to interact with many binding partners, particularly the other BM components collagen IV, laminin, perlecan and fibulin [83, 213, 214]. These interactions suggest that nidogens are essential for BM organization [216]. Nidogen 1 and 2 show similar binding affinity towards collagen IV and perlecan through the central G2 domain [83, 220]. In contrast, *in vitro* binding assays for human nidogen 2 demonstrate that its affinity for laminin-1 is 100- to 1000-fold lower than for nidogen 1 [83]. Furthermore, mouse nidogen 2 binds laminin-111 with approximately 20-fold lower affinity than mouse nidogen 1 [214]. This differential affinity to laminin is unexpected since the interacting amino acids of the G3 domain are completely conserved in mammalian nidogens; therefore it has been speculated that the differences may be due to unconserved residues further from the interacting surfaces [83, 216]. Unlike nidogen 1, nidogen 2 is unable to bind fibulin -1 and -2 [83]. Interestingly nidogen 2, but not nidogen 1, binds strongly to endostatin, tropoelastin and the carboxyl-terminal domain of collagen XVIII [216].

The pericellular location of nidogens in the BM has prompted a search for specific cell receptors; however, limited information is available regarding nidogen 2-cell interactions. Salmivirta and colleagues showed that several human and rodent cell lines bind to recombinant nidogen 2 more efficiently than recombinant nidogen 1, and that nidogen 2-mediated binding primarily occurs through $\alpha_3\beta_1$ integrin (also a receptor for nidogen 1) with a minor role for $\alpha_6\beta_1$ integrin [214].

1.5.4 Tissue distribution

Both nidogen isoforms are found in all BMs of adult tissues, though they show variation in expression levels, and in particular nidogen 2 shows more restricted expression patterns throughout development and some tissue specificity in mature basement membranes than nidogen 1 [83, 214, 217, 221-223]. Nidogen 1 predominates in the adult mouse, as there is only a 3-18% molar equivalent of nidogen 2 protein [214]; however, the levels of the two nidogen proteins are more comparable in human tissues [83].

Nidogens are involved in mouse [221] and human [224] organogenesis. During human embryonic development both nidogen isoforms are ubiquitously expressed in BM zones underneath developing epithelia of most major organ systems as early as gestational week 6.5 [224]. Only nidogen 1, but not nidogen 2, is detected in the developing intestine and pancreas. Of note, gonadogenesis was not investigated in this screen [224].

Nidogen 2 is expressed in many adult mouse tissues, as demonstrated by Northern blots [83, 217] and immunofluorescence analyses [83]. Nidogen 2 is particularly enriched in blood vessels [83]. It is also strongly expressed in heart, lung, kidney, skeletal muscle, testis, placenta, and liver, with lower levels in the brain and spleen [83, 217]. Unlike nidogen 1, nidogen 2 is also expressed in non-BM matrices such as cartilage and the elastic tissues surrounding larger vessels [83, 214]. Double immunofluoresence microscopy demonstrated nidogen -1 and -2 colocalization in kidney, skin, and testis [83]. In the peripheral nervous system both nidogens are found in the BM of myelinated axons while nidogen 2 alone is found in the ECM surrounding unmyelinated axons [216]. Conversely, nidogen 2 is not expressed in certain BMs of the developing gastrointestinal tract, specifically the glands in the pancreas and in the small intestine [224].

1.5.5 Nidogen functions

Numerous *in vivo* and *in vitro* studies have examined the biological significance of nidogens. Both isoforms are primarily known for their role in maintaining the assembly and structural integrity of the BM, serving as the critical link between the collagen IV and laminin network during embryonic development and in adult tissues [214, 216, 218, 221, 225, 226]. *Nid1*/*Nid2* double-knockout mice die perinatally with abnormalities in heart, lung and limb development, directly related to BM defects [225, 227]. However, certain tissues, such as kidney [225] and skin [228], form in the absence of nidogens with ultrastructurally normal BMs, demonstrating that the requirements for nidogens are tissue specific. Interestingly single knockout mice have shown that absence of nidogen 1 or nidogen 2 alone does not affect BM formation or overall organ development [222, 223]. Mice lacking either nidogen 1 or 2 are generally, healthy, fertile, and have a normal life span. Nidogen 2 knockout mice do not have a known phenotype,

and no obvious change in the expression pattern of nidogen 1 or other BM components is observed [223]. In nidogen 1-null mice, nidogen 2 expression is redistributed; its level is higher in skeletal and cardiac tissues than in wildtype mice, suggesting that nidogen 2 can generally compensate for the loss of nidogen 1 in BM assembly [222]. Nidogen 1-null mice show specific neurological phenotypes [229, 230] and wound-healing defects [231] not observed in nidogen 2-null animals, indicating only partial redundancy and isoform specific function of the two proteins.

Based on the subtle phenotype of the nidogen 1 knockout mouse and lack of phenotype of the nidogen 2 knockout mouse it is not surprising that the majority of work in the literature has focused on the functional role and signaling events related to nidogen 1. For example, nidogen 1 has been shown to rescue mammary epithelial cells from apoptosis [232], and regulate laminin-dependent gene expression and differentiation in the mammary gland [233]. It accelerates epidermal wound healing [231], may regulate conformational changes to laminin [234] and regulates the formation and/or maintenance of neuromuscular junctions in *C. elegans* [235]. Some of these effects are likely mediated by signalling through integrin $\alpha_3\beta_1$ or $\alpha_v\beta_3$, to which nidogen 1 has been shown to bind [236, 237].

Few roles for nidogen 2 have been established. It has been shown that recombinant human nidogen 2 promotes cell adhesion and spreading of multiple cell lines more strongly than nidogen 1 [83]. After corneal injury stromal keratocytes upregulate nidogen 2 protein, which contributes to the regeneration of epithelial BM [238]. Several studies have implied that nidogen 2 may play a role in reducing tumour metastasis. For instance, the loss of nidogen 2, but not nidogen 1, significantly promotes lung metastasis of melanoma cells [239]. The loss of nidogen 2 expression may also have a pathogenic role in colon and stomach tumourigenesis [240]. It has been proposed that the absence of nidogen 2 likely causes subtle changes in the BM, weakening its strength and accelerating the passage of tumour cells across the BM, ultimately leading to a higher rate of metastasis and larger tumours [239, 240]. Conversely, elevated serum nidogen 2 levels are detected in ovarian cancer patients [241], however, the role of nidogen 2 in ovarian cancer is not known.

1.6 Transcription Factor 21

1.6.1 Discovery

Transcription Factor 21 (TCF21), encoded by *Tcf21*, was initially cloned in 1998 by four independent laboratories and shown to be expressed in the embryonic mesenchymal cells of developing organs, including the heart, lung, kidney, spleen and gonads [242-245]. At this time TCF21 was known by three different names based on the tissues in which each group originally identified it: “entactin” for its expression in the epicardium [245], “podocyte-1” (POD-1) for its presence in the podocytes of the developing kidney [244] and the term “capsulin” was derived from its expression pattern in the mesenchyme that “encapsulates” the developing organs [242, 243]. POD-1/Capsulin/Entactin has become most commonly referred to as TCF21 because of its predicted molecular weight of 21 kDa. TCF21 is member of the basic helix-loop-helix (bHLH) transcription factor family (Section 1.6.2) and as such is critical for the cellular differentiation and tissue development for a number of organ systems (Section 1.6.3).

1.6.2 Protein structure

Basic helix-loop-helix (bHLH) transcription factors are named for their functional domains. The helix-loop-helix (HLH) domain, composed of two amphipathic alpha helices separated by a loop region of variable length, is necessary for the formation of homo- and heterodimers between these proteins [242, 246, 247]. Immediately upstream of the HLH domain is a basic region, which mediates sequence-specific DNA binding. Once bHLH proteins dimerize the basic regions form a bipartite DNA-binding domain that recognizes a consensus CANNTG sequence known as the E-box. bHLH proteins must dimerize to exert their effects. Class I proteins are ubiquitously expressed and capable of binding to DNA on their own as homodimers, whereas Class II bHLH proteins have a more tissue-specific pattern of expression and require heterodimerization with a Class I factor to bind at the conserved E-box sequence of the target gene [246].

TCF21 contains an evolutionarily conserved 50 amino acid bHLH domain (Figure 1-5) and belongs to the Class II bHLH proteins [242-245]. It consists of 179 amino acids, and human and mouse TCF21 sequences are 95% identical – 92% within the amino-

terminal domain, 100% within the bHLH domain and 96% within the carboxyl-terminal domain [244]. A phylogenetic analysis and classification of the bHLH gene family has recently developed a unified nomenclature resulting in Tcf21 being renamed as bHLHa23 [247]. This analysis also found that the *Tcf21* gene is highly conserved between species. Interestingly, it was recently shown that TCF21 is phosphorylated at three sites within the N-terminal region on residues that are evolutionarily conserved across species, suggesting these post-translational modifications may play a functional role [248].

1.6.3 TCF21 functions during embryogenesis

Members of the bHLH family play important roles in cell fate specification, differentiation, and morphogenesis of several tissues during development [249-253]. Cell type-specific bHLH transcription factors, a group to which TCF21 belongs, are key regulators of organ development. TCF21 is a nuclear protein [254] expressed in mesenchymal cells at sites of epithelial-mesenchymal interactions in the developing urogenital, gastrointestinal, respiratory, and cardiovascular systems [242-245]. Furthermore, it promotes mesenchymal-to-epithelial (MET) transition of cells during organogenesis [252].

Tcf21 is first detectable in the mouse on embryonic day 8 (E8) in small clusters of cells in the branchial region [245], and has been detected in the gonads at E9.5 by *in situ* hybridization [253]. Throughout the mouse lifespan, it is expressed at the highest levels during embryogenesis, and its expression rapidly decreases in most postnatal tissues, with the exception of kidney, lung, heart and ovary [242, 245, 253, 255].

Phenotypic analysis of TCF21-knockout mice (*Tcf21*$^{-/-}$) demonstrates that it plays an important role in the formation of the spleen [256], kidney and lung [252], facial skeletal muscle development [257], and is critical for sexual differentiation [258]. Mice lacking TCF21 die in the perinatal period, and show male-to-female sex reversal [258]. Although TCF21 has a mesenchymal cell-specific pattern of expression during development, its loss results in major phenotypic defects in the adjacent epithelia.

Figure 1-5: TCF21

Schematic representation of human TCF21. The amino acid sequence is numbered. bHLH: basic helix-loop-helix domain

1.6.3.1 Heart development

TCF21 is primarily studied for its role in heart development and function, and is often used for the identification of proepicardial and epicardial cells [242, 259-262]. During development, TCF21 is highly expressed in epicardial progenitor cells and recent studies have demonstrated that TCF21 determines whether a precursor cell is fated to become a differentiated coronary smooth muscle cell (SMC) or a cell of the cardiac fibroblast lineages [259, 260]. Specifically, TCF21 is downregulated in cells that will become SMC while its expression is required for cardiac fibroblast development, as fibroblasts fail to develop in mice lacking TCF21. Interestingly, TCF21-null epicardial cells are unable to undergo epithelial-to-mesenchymal transition (EMT) suggesting TCF21 is also required for epicardial EMT [259].

1.6.3.2 Kidney development

Following its original discovery in podocytes, TCF21 continues to be studied in the kidney because it is one of only six transcription factors that are well characterized regulators of podocyte specification [263]. Unlike the heart, in the kidney TCF21 is not required for cell fate decisions of the mesenchyme or podocyte lineages [252]. However, it is clearly essential for kidney development as loss of TCF21 results in severely hypoplastic kidneys, decreased tubulogenesis and glomerulogenesis, as well as a failure of cells to undergo MET [252, 255, 264, 265].

1.6.3.3 Gonadal development

Several laboratories have shown that TCF21 is essential for normal development of the ovaries and testes [253, 258, 266, 267]. TCF21 is one of the earliest genes expressed in the mesodermal cells that later become the gonads [253]. It is the first Class II bHLH transcription factor with a sex and stage-dependent pattern of expression during gonadogenesis, with much higher expression in embryonic testes than the ovaries [253, 258]. Initially at E11.5 the levels of *Tcf21* expression are the same between the gonads of male and female mice; however at E13.5 the expression becomes stronger in the male gonad compared to the female [253]. Interestingly, a time course of *Tcf21* expression in the gonads from E13.5 to postnatal stages demonstrates that the testis and ovary have

opposite patterns of expression. *Tcf21* expression remains higher in the testis during embryonic development and slowly decreases over time at postnatal stages. Conversely, expression in the ovary remains relatively low during gonadogenesis but increases markedly after birth and becomes higher than in the testis by PND 21 [253]. Throughout testis development TCF21 is detected in the nucleus of somatic cells adjacent to germ cells (E13.5), Sertoli cells (E13) and Leydig cells (E16), but is absent in germ cells [266].

The gonads of TCF21-null mice are markedly hypoplastic, slightly shortened in length and have an irregular surface [258]. Although XX and XY *Tcf21*$^{-/-}$ mice are born in the expected 50:50 ratio the external genitalia are feminized in XY *Tcf21*$^{-/-}$ pups. The testes of XY mice begin to resemble ovaries at E12.5 and the urogenital tracts of XX and XY mice are indistinguishable for the latter part of gonadal development. Aspects of normal ovarian development are initiated by somatic and germ cells in XX *Tcf21*$^{-/-}$ mice, but are disrupted by E18.5 because no meiotic cells are observed at this stage. Although the ovaries of *Tcf21*$^{-/-}$ mice are similar in size to wildtype ovaries, their shape and location relative to the kidney are more variable. The ovaries of TCF21-null mice also lack a distinct mesenchymal zone [258]. Interestingly, when TCF21 is over-expressed in an embryonic E13 ovary culture system it promotes *in vitro* sex reversal and induces the expression of anti-Müllerian hormone (AMH), which is normally expressed by Sertoli cells in the embryonic testis but not expressed by the embryonic ovary [258]. These data suggest that TCF21 is involved in male sex determination and testis differentiation.

Both TCF21-null ovaries and testis express higher levels of steroidogenic factor 1 (SF1/ Ad4BP) and the cholesterol side chain cleavage enzyme (P450SCC) than wildtype mice [253, 258]. SF1 is an orphan nuclear receptor that regulates multiple genes involved in gonadal development and sexual differentiation, as well as steroidogenesis in adult mice [258]. It is proposed to be an essential regulator of P450SCC, an early marker for Leydig cell differentiation and a mitochondrial enzyme responsible for the initial reaction in the steroidogenic pathway [268]. Although P450SCC is required for the synthesis of steroid hormones in the postnatal ovary, it is not normally expressed during ovarian development; however, its expression is higher in fetal TCF21-null ovaries than in wildtype ovaries [258]. SF1 is localized to many of the same cell types as TCF21 within

the developing gonads, and has an interesting pattern of expression comparable to that of TCF21, namely that SF1 levels are similar in male and female gonads at E11.5, higher in the male gonad than the female gonads at E13.5, then higher in the ovary than the testis after birth [269].

Interestingly, initial reports of how TCF21 regulates SF1 activity indicated that TCF21 represses SF1 expression in an indirect manner because TCF21 is unable to bind to the E-box located within the SF1 promoter [258]. Since TCF21 was unable to bind directly to the SF1 promoter it was suggested that instead, TCF21 interacts with USF1, a known activator of SF1 [270], thereby preventing USF1 promoter binding and activation of SF1 expression [258]. Of note, the group also failed to show by co-immunoprecipitation that TCF21 directly interacts with USF1. More recently, however, TCF21 was successfully shown to bind to the SF1 E-box sequence and inhibit its expression [271]. The discrepancy in results is likely due to the different approaches used by each laboratory to analyze protein-DNA interactions; Franco *et al.* used chromatin immunoprecipitation (ChIP) assays, an *in vivo* method, whereas the earlier study by *Cui et al.* utilized electrophoretic mobility shift assay (EMSA), an *in vitro* method of analyzing protein-DNA interactions.

Lastly, TCF21 is a direct downstream target of the male sex-determining factor SRY, further supporting the essential role of TCF21 in sex determination [266]. SRY binds to the TCF21 promoter and initiates a cascade of transcriptional events related to Sertoli cell differentiation and testis development. Mutation of the SRY response elements within the TCF21 promoter disrupts the actions of SRY in fetal rat testis development.

Collectively, these data demonstrate that TCF21 is critically involved in gonadal development and part of a transcriptional network that coordinates cell fate decisions in gonadal progenitor cells.

1.6.3.4 Spleen, lung and skeletal muscle development

TCF21 regulates differentiation and cell fate decisions during the development of spleen, lung and skeletal muscles; however, its role in these tissues has not been

investigated as extensively as the organ systems discussed above. Briefly, in the absence of TCF21, splenic precursors undergo apoptotic cell death and TCF21-null mice fail to form a spleen, demonstrating that TCF21 controls a critical early step in spleen development [256]. The lungs of TCF21-null mice are severely hypoplastic and lack alveoli [252]. Furthermore, the expression of TCF21 in the mesenchyme is required for the appropriate expression of bone morphogenetic protein-4 (BMP-4) in the adjacent epithelium, otherwise the airway epithelium does not differentiate [252]. Lastly, TCF21 is closely related to the bHLH transcription factor MyoR, which acts as a transcriptional repressor and blocks myoblast differentiation [254]. TCF21-null mice do not have a musculature phenotype; however, specific facial muscles are absent in mice lacking both TCF21 and MyoR, demonstrating TCF21 is a negative regulator of myoblast differentiation [254, 257].

1.6.4 TCF21 functions in postnatal tissues

In addition to its essential role in embryonic development, TCF21 has recently been identified as a tumour suppressor, and deregulated in several types of cancer. TCF21 was first shown to function as a tumour suppressor in head and neck squamous cell carcinomas and non-small cell lung cancer in 2006 [272]. Since then, the loss or reduced expression of TCF21 has been found in many types of human cancers, including kidney [273, 274], lung [275-277], melanoma [278], urological [279], colon [280], gastric [281] and breast [282]. Decreased expression of TCF21 is often due to promoter hypermethylation and has been correlated with larger tumour size and decreased survival in patients [282]. It has also been suggested that histone modifications may be involved in TCF21 silencing [278], which requires further investigation.

Multiple studies have shown that restoration of TCF21 in cancer cells results in a reduction of tumour properties *in vitro*. For example, overexpression of TCF21 inhibits cell proliferation, induces apoptosis and suppresses migration and invasion in colorectal cancer [280], decreases cancer cell growth and colony formation in lung cancer [272], reduces motility of melanoma cells [278] and inhibits cell proliferation and epithelial-to-mesenchymal transition (EMT) in breast cancer [282]. As mentioned earlier, an important function of TCF21 in normal tissues is to promote mesenchymal-to-epithelial transition

(MET) during organogenesis [252]. The reversal of this process, EMT, is vital for tumour dissemination and progression [283]. Therefore, it is significant that the overexpression of TCF21 in cancer cells results in reduced expression of mesenchymal markers (SNAI1 and VIM) and increased expression of an epithelial factor (CDH1), as it suggests that TCF21 induces differentiation, likely through MET, *in vitro* [272, 282]. These studies suggest that TCF21 may act as a potential therapeutic target in certain cancers and its hypermethylation suggests is could be a useful methylation marker in various tumour types.

In addition to its role as a tumour suppressor, TCF21 is a coronary artery disease (CAD)-associated transcription factor. Briefly, it is suggested that following vascular injury, which is believed to be critical for vascular disease, TCF21 regulates the differentiation state of SMC precursor cells in the adult by a similar mechanism to the regulation of the developing epicardium [261]; i.e. TCF21 determines whether epicardial progenitor cells will give rise to SMCs or cardiac fibroblasts [260]. The SMC precursors in which TCF21 is expressed migrate into vascular lesions, and contribute to the stabilization of these lesions and prevent heart attacks [261]. It has also recently been found that a SNP of *Tcf21* is a susceptible locus for hypertension, a major risk factor for CAD [284].

Therefore, although TCF21 was identified for its role in embryonic development, it is apparent that it continues to play critical, yet largely undetermined, roles in postnatal tissues.

1.6.5 TCF21 as a transcriptional regulator

The molecular mechanisms by which Tcf21 acts to regulate transcription remain poorly understood in most systems. As mentioned previously, bHLH proteins must dimerize to exert their effects, and heterodimerization between Class I and Class II bHLH proteins is preferred. TCF21 heterodimerizes with four bHLH proteins, namely TCF3 (E12/E47) [243, 278], TCF4 (E2-2) [278], TCF12 (HEB) [242, 243, 266, 278] and ITF-2 [285]. Of note, TCF21 is unable to homodimerize [285]. TCF21 has also been shown to associate with HDAC-1 and AR (androgen receptor) in Sertoli cells [286], as well as

HDAC-2, Ctbp2 (C-terminal-binding protein 2) and Pbx1 (pre-B-cell leukemia transcription factor 1) in proepicardial cells [248].

Transcriptional assays and electrophoretic mobility shift assays in a number of *in vitro* systems have demonstrated that TCF21 has both activating and repressing transcriptional activity. It exhibits repressive activities in HepG2 cells from its C-terminal and N-terminal domains; constructs that include either of these domains retain repressive activity, while the bHLH domain alone had no activity. Conversely, transactivation activities in HT1080 and HeLa cells were only observed when TCF21 constructs retained the C-terminal domain. It is suggested that this dual function of the C-terminal domain may be cell-type dependent, and result from the presence of specific coactivators and corepressors [285]. Importantly, there are a limited number of direct transcriptional targets of TCF21 that have been reported and confirmed by functional assays. These include *MCK* (muscle creatine kinase) [243, 254], *Kiss1* (Kisspeptin-1) [278], *AR* [286], *p21* (cyclin-dependent kinase inhibitor) [254], *Sf-1* [253, 258, 287], *SHP* (Small Heterodimer Partner) [288] and *Scx* (Scleraxis) [267]. Bhandari et al recently identified 121 direct downstream binding targets of TCF21 using a modified ChIP-ChIP comparative hybridization analysis [267]. All targets contained an E-Box sequence, and 10 of the 121 targets were bHLH genes. To date, only one newly identified TCF21 target – *Scx* (*bHLHa41*), previously localized to Sertoli cells and shown to promote Sertoli cell differentiation in rats [267] – has been investigated in subsequent functional assays. The authors show that TCF21-induced SCX is required for the latter part of Sertoli cell differentiation associated with pubertal development.

TCF21 is the first bHLH protein suggested to act as a general repressor of nuclear receptors [286]. It was first identified as a repressor of the orphan nuclear receptor SF1 [253], and suppresses the promoter activity and transactivation of the AR, a nuclear receptor important in male sexual differentiation and testicular function [286]. Hong and colleagues propose that TCF21 acts as a repressor of several nuclear receptors, including Estrogen Receptor β, glucocorticoid receptor and retinoic acid receptor alpha [286]; however, this has not been tested by subsequent studies and warrants further investigation.

1.7 Spondin 1

1.7.1 Discovery

Spondin 1 (SPON1), encoded by *Spon1*, was originally identified in 1992 as a secreted ECM glycoprotein highly expressed it the floorplate of the developing rat [289]. It was originally named F-Spondin for its location in the floorplate of the neural tube (F) and its thrombospondin repeats (spondin), and is also known as vascular smooth muscle cell growth promoting factor (VSGP) [290]. Spondin 1 is a member of the neuronal subgroup of the diverse thrombospondin type 1 (TSR) repeats superfamily. The tissue expression pattern of *Spon1* is highly conserved in vertebrates [291].

1.7.2 Protein structure

Spondin 1 is a molecule of 807 amino acids with a predicted molecular mass of 87 kDa [289, 291]. The protein is subdivided into three domains (Figure 1-6). The first half of the N-terminal region (amino acids 1-200) is homologous with a domain in Reelin, and is aptly named the “reelin domain”, while the similar domain in Reelin is referred to as the “F-Spondin domain” [292]. The second half of the N-terminal region (amino acids 201-440) is named the “spondin domain”, and shares similarity with a domain in Spondin 2 (also known as Mindin), another member of the neuronal class of TSR proteins [291, 293, 294]. The C-terminal region (amino acids 441-807) contains six thrombospondin type 1 repeats (TSR).

Analysis of the crystal structure of the reelin domain revealed potential heparin-binding sites and weak dimerization between two reelin domains, which may be a location for glycosaminoglycan (GAG) binding [295]. The exact function of the GAG-binding site remains unclear; however, it may be used to for anchoring Spondin 1 to the ECM in such a way that the spondin domain and C-terminus are available for interactions with other proteins or cells [295]. The spondin domain is not well studied; however, it has been to shown to promote outgrowth of sensory neurons and may be involved in axonal regeneration following nerve injury [296]. TSRs are found in several protein families, and are involved in various functions including cell adhesion, GAG binding, inhibition of angiogenesis and activation of TGFβ [297, 298]. Most of the post-

translational modifications in Spondin 1 are found within the TSR repeats, as eight of ten tryptophan residues are C-mannosylated, while several serine and threonine residues are potential sites of O-fucosylation [299]. There are three N-glycosylation sites within the spondin and C-terminal domains [289]. Each TSR in Spondin 1 contains a WxxW sequence, which is known to bind TGFβ. The sixth TSR also has a KRFK motif, required for the activation of latent TGFβ [297].

Spondin 1 contains three proteolytic cleavage sites. The first was identified between the spondin and TSR domains [296]. Western blot analysis of conditioned medium from HEK293 (human embryonic kidney) and primary rat Schwann cells revealed SPON1 was cleaved into two half proteins - a 60 kDa amino-half with the reelin and spondin domains, and a 40 kDa half that contains the TSRs [296, 300]. Spondin 1 also has two plasmin-mediated cleavage sites, one between TSR-4 and TSR-5, and the second between TSR-5 and TSR-6. The fifth and sixth TSRs are bound to the ECM, whereas TSRs 1- 4 are not. By cleaving the first four non-adhesive TSRs from the fifth and sixth adhesive TSRs, plasmin generates a secreted protein [301].

1.7.3 Proteins with similar structure

In addition to the unique domains Spondin 1 shares with Spondin 2 and Reelin, it also shares the TSR domain with members of the diverse TSR superfamily, which contains numerous ECM and transmembrane proteins [302]. A few proteins that share domains with Spondin 1 are briefly described below.

Thrombospondin (TSP) -1 and -2: TSP-1 and TSP-2 are matricellular ECM proteins. As matricellular proteins they exist within the ECM but do not have a role in structural integrity [85]. Instead, TSP-1 and TSP-2 are involved in supporting cell attachment, cell motility and inhibiting angiogenesis [297]. Of note, TSP-1 and TSP-2 are both expressed in the early CLs of the rat ovary, with TSP-1 also expressed in the GCs of antral follicles [68].

Spondin 2: Spondin 1 and 2 are evolutionarily conserved, matrix-attached adhesion proteins involved in neural development [291]. They are the only proteins to contain the

spondin domain. Furthermore, Spondin 2 is the only protein to share two domains with Spondin 1 – the spondin domain and a TSR. Spondin 2 is involved in the regulation of axonal development and promotes the outgrowth and adhesion of embryonic hippocampal neurons [293]. It is also involved in the immune response and cardiac functions, and has been shown to bind integrins and block Akt signaling [303].

R-Spondin: The four R-Spondins (R-spondin 1-4) are relatively recent additions to the TSR family, with R-Spondin 1 being identified in 2004 and R-Spondins 2-4 discovered over the next four years [304]. The gene encoding R-spondin 1 (*Rspo1*) was first identified in the boundary region between the roof plate and neuroepithelium in the developing mouse, and thus was named R(oof plate specific)-spondin [305]. The four R-Spondins are small secreted proteins, with a single TSR near the C-terminus, and are all agonists of the canonical Wnt/β-catenin signaling pathway during embryogenesis [304]. R-Spondin 1 and -2 are both involved in ovarian development. Studies have demonstrated that mice lacking R-Spondin 1 display a partial sex reversal phenotype [306], and R-Spondin 2 is detected from the primary to antral stage in the oocyte and promotes follicle growth [307]. Currently, R-Spondin 3 and 4 have no known role in the ovary.

Reelin: The reelin domain is only shared by Reelin, Spondin 1 and ferric-chelate reductase 1 (FRRS1). Reelin is a large secreted glycoprotein involved in neural development. Its name is derived from the Reelin-deficient mouse that has a “reeling” gait (impaired motor coordination, tremors and ataxia) [292]. In humans, loss of Reelin expression has been linked to several neurological disorders [308]. Reelin expression has recently been detected in the TCs of dominant bovine ovarian follicles [309], and shown to promote proliferation of chicken GCs in culture [310].

1.7.4 Spondin 1 functions and mechanisms of action in the central nervous system

Following its discovery by Klar and colleagues in the floorplate of the embryonic rat [289], Spondin 1 has primarily been studied for its role as a neurological ECM

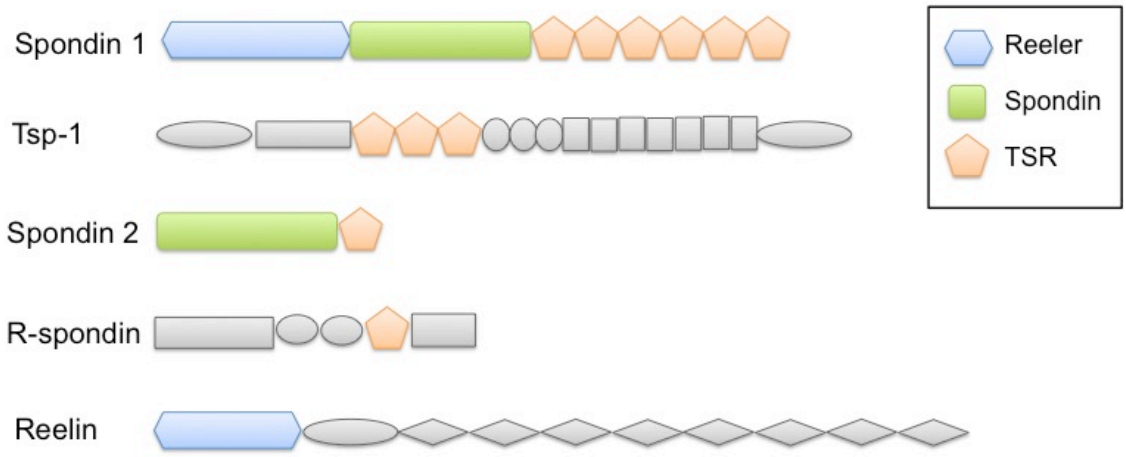

Figure 1-6: Domain diagram of Spondin 1 and similar proteins

Schematic representations of Spondin 1 and proteins with similar domains, thrombospondin 1 (Tsp-1), Spondin-2, R-spondin, Reelin. Reeler domain (blue), Spondin domain (green), TSR domain (orange) and other domains are shown in grey.

glycoprotein. In that first publication, Klar et al. demonstrated that Spondin 1 regulates cell adhesion and neurite outgrowth. Dorsal root ganglia cells plated on recombinant Spondin 1 grew to a greater length and were more adherent than those plated on the control bovine serum albumin (BSA) [289]. Since then, Spondin 1 has also been shown to promote adhesion and outgrowth of embryonic hippocampal neurons [293], and its expression is upregulated during axonal outgrowth following nerve injury [296]. Spondin 1-mediated cell adhesion is inhibited following treatment with heparin [289] and chondroitin sulfate [293], suggesting that binding by Spondin 1 is affected by proteoglycans. Further studies have demonstrated that Spondin 1 has a dual role in the accurate patterning of axons at the floorplate, because it acts as a chemoattractant that promotes the outgrowth of commissural axons [300] and as a contact-repellent molecule to inhibit outgrowth of motor neurons [311]. Spondin 1 also acts as an inhibitory signal for segmentation in somites in chicken embryos, patterning the migration of neural crest cells [312].

Neural outgrowth is mediated by the immobilization of the TSR 1-4 fragment of the secreted Spondin 1 protein by lipoprotein receptor–related protein (LRP) receptors expressed in the floorplate, including ApoER2 (apolipoprotein E receptor 2), also known as LRP8 (low-density lipoprotein receptor- related protein 8) [313]. Spondin 1 also interacts with the ApoER2 ligand, APP (β-amyloid precursor protein) via its reelin domain [314]. Spondin 1 affects APP processing, increases its expression at the cell surface, and appears to form an extracellular bridge between ApoER2 and APP [315]. Considering APP is a key protein in the pathogenesis of Alzheimer's disease, Spondin 1 is an intriguing target for Alzheimer's therapeutics [315].

In addition to affecting nerve growth and adhesion, Spondin 1 promotes nerve cell differentiation and viability. Treatment with Spondin 1 promotes the differentiation of a rat adult hippocampal precursor cell line, AHP, from neural precursor cells into cells with the biochemical and morphological features of nerve cells [316]. Furthermore, Spondin 1 increases the viability of chicken ciliary ganglion (CG) cells in culture by activating TGF-β signaling via its KRFK motif in TSR-6 [317]. Interestingly, Spondin 1 deletion mutants that contain the TSRs but lack the reelin and spondin domains were unable to

increase CG cell survival, suggesting that the N-terminal domains are required for complete TGF-β signaling and survival of CG cells [317]. This increased cell survival was also due to Spondin 1 binding to APP and inducing rapid phosphorylation of disabled-1 (DAB-1) [317]. Reelin has previously been shown to increase the phosphorylation of DAB-1, through APP receptor binding [308], further supporting the idea that the reelin domain is involved in promoting neuron cell survival.

1.7.5 Spondin 1 function and mechanism of action in non-neural tissues

The function of Spondin 1 is not restricted to the nervous system. Northern blot analysis revealed that *Spon1* is expressed in numerous tissues, including the ovary, kidney, bone and small intestine [290], where several functions for SPON1 have been identified.

Spondin 1 promotes cell-ECM adhesion in both neural and non-neural tissues of *C. elegans* [318]. It is localized to BMs and integrin-containing regions within body muscles of *C. elegans* embryos. Spondin 1 is responsible for maintaining strong attachments between muscles and epidermis, and muscles in *Spon1* mutants gradually detach from the epidermis [318].

Spondin 1 that was isolated and purified from bovine follicular fluid promoted growth of primary rat aortic vascular smooth muscle cells [290]. Alternatively Spondin 1 inhibits vascular endothelial growth factor (VEGF)-stimulated migration and tube formation of HUVECs (human umbilical vein endothelial cells) as well as angiogenesis in the rat cornea [319]. In this model system, Spondin 1 inhibits migration by blocking integrin $\alpha_v\beta_3$-mediated adhesion of HUVECs to vitronectin as well as VEGF-induced activation of Akt [319]

Spondin 1 has also been shown to increase cell survival of mouse Neuro-2a neuroblastoma cells [320]. Loss of viability following knockdown of Spondin 1 was attributed to the lower levels of IL-6, and correlated with decreases of NF-κB and p38 mitogen-activated protein kinase (MAPK). Treatment with Spondin 1 or IL-6 rescued the cells from death [320]. Furthermore, Spondin 1 expression is higher in osteosarcoma

specimens compared to benign osteochondroma samples. It promotes the migration and invasion of metastatic and non-metastatic osteosarcoma cell lines through Fak and Src dependent pathways [321].

Spondin 1 is upregulated in osteoarthritis articular chondrocytes where it is likely involved in the activation of latent TGF-β1. TGF-β1 activation leads to increased expression of prostaglandin E2 (PGE2) and matrix metalloproteinase 13 (MMP-13), which reduces proteoglycan synthesis and increases collagen degradation [322]. Spondin 1 is also highly expressed in embryonic cartilage, where it enhances chondrocyte terminal differentiation and mineralization by activating TGF-β1, which increases MMP-13 and alkaline phosphatase (AP) expression [323]. It promotes the differentiation of a human cementoblast-like cell line (HCEM) via bone morphogenic protein 7 (BMP7) [324]. Alternatively, Spondin1 inhibits the receptor activator of NF-κB ligand (RANKL)-mediated differentiation of osteoclastic precursors via LRP8 [325].

Surprisingly, despite the broad expression of Spondin 1 and its role in neural and non-neural tissues the SPON1-knockout mouse (*Spon1*$^{-/-}$) is viable and has a grossly normal phenotype [326]. The *Spon1*$^{-/-}$ mice undergo normal skeletal development without major skeletal abnormalities, which was unexpected considering the authors' prior detection of elevated Spondin 1 levels in embryonic cartilage and chondrocytes of osteoarthritic cartilage [322, 323]. At 6 months of age, the *Spon1*$^{-/-}$ mice have increased bone mass, likely as a result of increased bone synthesis. They also exhibit reduced levels of TGF-β1 in serum and cultured chondrocytes, and increased levels of phosphorylated SMAD1/5, BMP-regulatory SMADs. Palmer *et al.* speculate that the loss of *Spon1* decreases the activation of TGF-β, resulting in increased BMP signaling and bone deposition in adult mice [326]. Our laboratory has recently determined that *Spon1*$^{-/-}$ mice also have an ovarian phenotype. The *Spon1*$^{-/-}$ female mice are mildly subfertile, have smaller litters, decreased ovulation capacity, and smaller ovarian weights than wildtype mice (unpublished data).

1.7.6 Spondin 1 and the ovary

There is limited yet compelling evidence that Spondin 1 plays a role in the ovary. As mentioned above, Spondin 1 mRNA is abundantly expressed in the adult human ovary, as determined by Northern blot. Spondin 1 isolated and purified from the bovine follicular fluid has growth promoting properties towards vascular smooth muscle cells [290]. When ovariectomized mice are treated with 17β-estradiol, Spon1 mRNA expression increases in the uterus and mammary gland [327, 328]. Additionally, *Spon1* expression is 2.5-fold in GCs of eCG-treated ERβ-null mice than in eCG-treated ERβ heterozygous mice [156]. Our laboratory has recently demonstrated that *Spon1*$^{-/-}$ female mice are subfertile (unpublished data). These studies collectively suggest a role for Spondin 1 in ovarian folliculogenesis.

Furthermore, Spondin 1 mRNA is highly expressed in ovarian carcinomas compared to normal ovarian tissues [329]. An extensive screen of 500 ovarian carcinomas suggests Spondin 1 is a negative prognostic indicator and a promising biomarker for ovarian cancer, particularly for high-grade serous epithelial ovarian cancer [330].

1.8 Ovarian Cancer

1.8.1 Ovarian cancer classification

Ovarian cancers are divided into three categories – epithelial, stromal and germ cell – of which epithelial is the most common and lethal type, comprising over 85% of ovarian cancer cases [331, 332]. Epithelial Ovarian Cancer (EOC) is not a single disease, but consists of four main subtypes (endometrioid, mucinous, clear cell and serous) that are histopathologically, genetically and biologically distinct diseases [333, 334]. The four subtypes can be further classified as benign, borderline or malignant, and low- or high-grade [335]. EOC is the focus of my research and will be the focus of this section.

Serous ovarian cancer represents approximately 80% of EOCs [336]. As the most aggressive subtype, high-grade serous ovarian carcinoma (HGSC), accounts for 90% of serous cancers, two-thirds of all EOC deaths, and it is the most studied subtype [337].

HGSC often responds well to adjuvant platinum/taxane chemotherapy; however, recurrence occurs in the majority of cases [338, 339]. Clear cell and endometrioid carcinomas are less common, each accounting for approximately 10% of ovarian carcinomas [336]. Endometrioid carcinomas predominantly present at low stage and low grade therefore mortality associated with this subtype is relatively low. Clear cell carcinomas usually present with low-stage disease; however, all are considered high-grade and do not respond well to conventional treatments, which results in poor outcome for most patients [340, 341]. Only 3-4% of ovarian tumours are of the mucinous type, and most are confined to the ovary at presentation. As with clear cell carcinomas, current chemotherapeutics are ineffective for treatment of recurring mucinous carcinomas [336].

Over a decade ago, a new model for classification was proposed that separated EOCs into two broad categories – type I and type II tumours – by taking into account clinical and genetic findings in addition to tumour histopathology [342]. Type I tumours include all of the histotypes (serous, endometrioid, mucinous and clear cell) that are low grade and slow growing. These tumours have mutations rarely found in Type II tumours, such as *BRAF* (v-Raf murine sarcoma viral oncogene homolog B), *KRAS* (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), *CTNNB1* (β-catenin), *ARID1A* (AT-rich interactive domain-containing protein 1A), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) and *PTEN* (phosphatase and tensin homolog) mutations [342, 343]. Type II tumours include HGSCs, carcinosarcomas and undifferentiated carcinomas [342]. These tumours are highly aggressive, present at an advanced, metastatic stage and are associated with poor patient outcome. Type II tumours contain mutations in BRACA1/2 (breast cancer 1 and 2) and almost ubiquitously express TP53 mutations (97%) [344, 345]. Unlike earlier characterizations, this model does not consider low and high-grade tumours a spectrum of disease, but rather distinct diseases with different origins, epidemiology and mutations [334, 342].

1.8.2 Origins of ovarian cancer

The term ‘ovarian cancer’ can be misleading. Although the unifying clinical feature for all ovarian cancer is dissemination to the ovary and related pelvic organs, it has been proposed that a considerable number of tumours do not originate from ovarian

tissue [334]. The site of origin for HGSC remains strongly debated. Over the years it has been suggested that ovarian cancers can develop from any of the following origins: the ovarian surface epithelium (OSE), cells lining subsurface inclusion cysts, the abdominal peritoneum or the fallopian tube epithelium [332, 346].

Early theories hypothesized that all epithelial ovarian cancers arose from the OSE [335]. This traditional view is based on the idea that repeated ovulation during a woman's reproductive life increases the susceptibility of the OSE to transformation because of the frequent damage and repair to the OSE, as a result of follicle rupture to release oocytes [346]. Ovulation also leads to the formation of epithelial cell-lined inclusion cysts. It is hypothesized that the cells surrounding these intraovarian cysts differentiate into a Müllerian-like epithelium in response to the hormone-rich environment in the ovary, become dysplastic and lead to ovarian tumours [347]. These theories are consistent with epidemiological evidence showing that decreased ovulation, whether a result of multiple pregnancies, prolonged lactation or use of oral contraceptives, decreases the risk of developing ovarian cancer [346]. These models do not, however, address the fact that the different ovarian subtypes are histologically diverse and share few molecular similarities [348].

Recent studies indicate that the ovarian cancer subtypes arise from various sites of origin; certain subtypes that were traditionally believed to be primary ovarian tumours actually originate from non-ovarian tissues and involve the ovary secondarily [349]. Many studies have provided strong evidence that endometrioid and clear cell ovarian cancers are derived from endometriosis [350-355], whereas mucinous ovarian cancers are metastases to the ovary from gastrointestinal and appendiceal tumours [334]. The site of origin for serous cancers remains unknown. It continues to be strongly debated whether HGSC is derived from the surface of the ovary and/ or the distal fallopian tube [332, 335, 356-358]. The latter became a site of interest once pathologists examined fallopian tubes from patients who underwent prophylactic salpingo-oopherectomies and found that tubal lesions were present in women with germline *BRCA1/2* mutations [357]. Therefore a new model for the origin of HGSCs was proposed whereby cancer cells are shed from the fimbria of the fallopian tube, implant on the surface of the ovary and produce ovarian

carcinomas [359, 360]. These possible precursor lesions, designated serous intraepithelial tubal carcinomas (STICs), morphologically and molecularly resemble HGSCs [349]. Further studies supporting this mechanism have shown that 48% of women with HGSCs have STICs, and 92% of STICs from patients with HGSC have identical mutations in TP53 [335, 357]. Importantly, STICs have been found in patients in the absence of ovarian carcinomas, which negates the suggestion they formed as a result of metastases from HGSCs [335].

All of the current theories have something to offer regarding ovarian cancer development, yet none of them successfully merges all aspects of ovarian carcinogenesis [349]. While the data suggesting that HGSC originate from non-ovarian sites is compelling, serous ovarian cancers involve the ovaries and the peritoneum considerably more than the fallopian tubes. The relative importance of these two sites continues to be debated; nevertheless, all novel findings have critical implications for screening, prevention and treatment of this heterogeneous disease [334, 349].

1.8.3 Ovarian cancer prognosis and treatment

Ovarian carcinoma is the most lethal gynecological malignancy, and the fourth most common cause of female cancer death in the world [343]. The prognosis is more favourable for patients diagnosed at an early stage, before the tumour has spread beyond the ovary (stage I); surgical resection provides a 90% cure rate. Unfortunately, most patients present with advanced disease (stage III/IV) once the tumour has metastasized to the peritoneal cavity. The five-year survival rate for ovarian cancer patients is a discouraging 45% [361], and patients with HGSC have a further diminished survival rate between 35% and 40% [362]. Although most patients initially respond well to cytoreduction and platinum- and taxane-based chemotherapeutics, approximately 70% will develop chemoresistance and suffer recurrences [332]. The high mortality rates are also strongly influenced by the amount of residual tumour following cytoreductive surgery [362]. The unusual mechanism of EOC dissemination (Section 1.8.4) is a major contributor to the challenges associated with current treatment and the design of improved therapeutics.

1.8.4 Ovarian cancer metastasis

Compared to many epithelial cancers the initial dissemination of EOC is unique because it rarely involves the vasculature, although the vasculature is often associated with advanced stages of the disease [363]. Instead the characteristic early step of EOC metastasis is proteinase-mediated shedding of cancer cells from the primary tumour into the peritoneal cavity, where malignant cells are disseminated by peritoneal fluid or ascites (an increased volume of fluid in the abdomen) [364]. Shed EOC cells exist as single cells or multicellular spheroids within the abdominal cavity, where they attach to and invade the mesothelial lining to establish secondary lesions [365, 366].

1.8.4.1 Peritoneal ascites fluid

The peritoneal ascites fluid is a unique and complex environment in which EOC cells are forced to survive in suspension [363]. The typical composition of cellular components in ascites fluid from ovarian cancer patients (often as much as 4.5 litres accumulates) consists of 37% lymphocytes, 29% mesothelial cells, 32% macrophages, and <0.1% adenocarcinoma cells [364, 367]. Malignant ascites also contains an assembly of non-cellular factors that promote EOC cell survival and metastatic implantation, including growth factors, cytokines, chemokines and ECM fragments [368]. Over one-third of ovarian cancer patients present with ascites at diagnosis, and approximately 10% of patients with recurrent EOC are affected by malignant ascites [369]. Patients routinely undergo paracentesis to have the fluid removed, which provides a convenient source of tumour cells for EOC studies.

1.8.4.2 Multicellular spheroids

Appropriate cell-matrix interactions are an important aspect of cellular and tissue homeostasis [370]. Programmed cell death occurs when adhesion between epithelial cells and ECM components is disrupted, thereby preventing detached cells from reattaching to a new matrix and undergoing dysplastic cell growth [371]. The apoptosis induced by the loss of interaction between cells and the ECM is called anoikis [372]. The ability to resist anoikis is a critical mechanism in tumour metastasis [373].

Many tumour cells are unable to survive as single cells under anchorage-independent growth conditions; thus they form aggregates to avoid anoikis [365, 374, 375]. EOC cells that are shed from the primary tumour, and forced into suspension within the abdominal cavity, are believed to aggregate as multicellular spheroids in order to maintain cell-cell contact as part of their natural survival response [363]. Key cell-cell adhesion molecules such as cadherins and integrins facilitate cell compaction and create a tumour microenvironment that supports mechanisms of cell survival [363, 376, 377]. The formation of ovarian cancer spheroids is greatly disrupted, for example, when cells are treated with blocking antibodies against α5- or β1-integrin subunits [378]. Furthermore, the formation of multicellular aggregates is likely an important intermediate mechanism that facilitates metastasis. It has been suggested that spheroids should be considered in the dissemination of EOC and that the adhesion of spheroids initiates the conversion of floating cells within the ascites to an anchored metastatic lesion [364]. Of note, when spheroids implant on the mesothelium (a cell monolayer that covers all organs in the peritoneal cavity) they do not directly adhere to mesothelial cells, but rather to the ECM underneath [364-366]. The Brugge laboratory has demonstrated that ovarian cancer spheroids use myosin-generated force to displace the mesothelium, thereby gaining access to the ECM to promote invasion [379]. Burleson and colleagues have also shown that ovarian cancer spheroids adhere to, migrate on and invade into live human mesothelial cell monolayers *in vitro* [365, 380, 381].

Both single cells and spheroids can theoretically seed metastases [363]; however, multicellular spheroids *in vitro* more closely mimic the characteristics of solid tumours in the clinical microenvironment [334]. Spheroids grown in the presence of cellular or extracellular components normally found in the tumour microenvironment exhibit many histologic features similar to those of cells *in vivo* [364]. Ovarian cancer spheroids can be isolated from the peritoneal ascites (30-200 mm) or produced by culturing cells on non-adherent plates [382]. Of note, spheroids derived from ascites of patients with advanced disease vary in number and size, suggesting high variability between patients [332]. Non-adherent cell culture systems are an advantageous tool for simulating aspects of the peritoneal microenvironment and cells cultured in these conditions can provide further insights into ovarian cancer biology. The three-dimensional system also provides a

unique opportunity to examine the response of multicellular spheroids to many cancer therapies [383-385]. Various studies have shown that spheroids have increased resistance to common cytotoxic drugs and ionizing radiation compared to monolayer cultures of the same cells [386-388]. The majority of cytotoxic drugs target rapidly dividing cells; however, spheroids only have proliferating cells in the outer cell layers, whereas the cells in the center of the spheroid enter a quiescent state [389, 390]. Therefore it is not surprising that spheroids are generally more resistant to these therapeutic agents and may present a substantial impediment to effective treatment of late stage EOC.

1.8.5 ECM and cancer progression

The cellular components within the tumour microenvironment (also referred to as the tumour "niche") have been explored extensively for their role in the initiation of cancer development and its progression [391]. However, many studies have recently emphasized the important role that noncellular components of the tumour niche play as well, particularly the ECM [392-395]. The ECM is a complex, highly organized three-dimensional structure composed of several core components, including collagens, laminin, nidogen, fibronectin, and proteoglycans [396]. These components are produced and secreted by cells, and the composition and organization (including isoform expression and post-translational modifications) are specific to the particular requirements of a tissue [397, 398]. The ECM also serves as a reservoir for cytokines, growth factors and ECM-remodeling enzymes that work with ECM proteins to signal to cells [399]. Although the ECM was initially viewed as simply a support system involved in maintaining tissue morphology, it is now also recognized as a dynamic and flexible structure that either directly or indirectly influences many essential aspects of cellular biology [391, 399, 400]. Several mechanisms exist to closely regulate the production, degradation and remodelling of the ECM during normal development and organ function [401]. However, these control mechanisms can be disrupted, and when these pathways fail the composition and assembly of the matrix becomes disorganized, which disrupts ECM dynamics [402, 403]. Aberrant ECM dynamics are associated with various pathologies, including skeletal diseases, fibrosis and cancers [404, 405].

The role of tumour-associated ECM has emerged as an essential contributor to cancer progression [403, 406, 407]. The composition of the extracellular niche is a significant predictor of patient outcome, and classical pathology has demonstrated that excessive deposits of ECM are a common characteristic of tumours with poor prognosis [408]. Breast cancer tumours for instance can be divided into subcategories based only on their ECM composition, which are predictive of clinical outcome [409]. Recently, Rafii and colleagues analyzed data from a TCGA study where 316 serous EOC primary tumors underwent exome sequencing [410], and discovered that 89% of tumours have at least one mutation in cell adhesion related genes, including the ECM-receptor interaction pathway [411]. Gene expression screens have also shown that many genes encoding ECM components and ECM receptors are dysregulated during tumour metastasis [412-414]. Interestingly, using human melanoma xenografts in mice, Naba and colleagues have shown that the tumour matrix is produced by both tumor cells and stromal cells, and differs with metastatic potential [408].

Metastasis is a complex process that not only requires a microenvironment to support cancer cell growth at the primary site, but also a metastatic niche that will allow disseminated cancer cells to invade and migrate through new tissues to form secondary lesions [391, 415]. The ECM is an essential component of both niches. Ovarian cancer cells remodel the normal ECM by releasing proteases to degrade the pre-existing molecules, and by depositing new ECM with an altered composition and assembly [416]. It has been proposed, for example, that the loss of collagen IV and laminin in the ECM of primary tumours facilitates the shedding of ovarian cancer cells into the peritoneal cavity, and its restored expression later in tumour development promotes metastasis [417]. Deregulation of ECM dynamics facilitates cancer cell survival, migration and invasion, disrupts tissue polarity, and dysregulates angiogenesis [394, 418-420]. Abnormal ECM composition and dynamics have also been associated with resistance of tumour cells to conventional therapeutics [391, 421, 422]. The interactions between cancer cells and ECM are carefully balanced to support these pro-tumourigenic processes [407, 423]. Migration, for example, requires an intermediate level of cell-ECM adhesion because high adhesiveness prevents cells from fracturing the cell-matrix linkage, whereas low adhesiveness prevents cells from being able to generate enough traction to move

efficiently [424, 425]. During invasion, the proteolytic process of degrading and remodeling the ECM must be tightly controlled as well, so that the ECM becomes degraded enough for cell passage, but not so degraded that cells lose traction [423].

Ovarian cancer cell adhesion to the ECM initiates signaling pathways through specialized transmembrane ECM receptors and cell-cell adhesion molecules, such as integrins and cadherins [402, 403]. Integrins are the most prominent and likely the best characterized ECM receptors involved in adhesion interactions [426], and are essential in ovarian cancer metastasis [423]. They are a diverse family of glycoproteins that form heterodimers between covalently linked α- and β-subunits, with each pairing being specific for a set of ligands [402]. The β_1-integrin can heterodimerize with numerous α-subunits and promote the adhesion of disseminated cells to the mesothelium where integrin $\alpha_2\beta_1$-collagen type IV, $\alpha_6\beta_1$-laminin and $\alpha_5\beta_1$-fibronectin interactions occur [283, 332, 378]. Simultaneously, cancer-associated proteases degrade the existing ECM and allow invasion of the mesothelium [365, 366]. The adhesion of cancer cells to the mesothelial-lined peritoneal surfaces also triggers intracellular signaling pathways that can regulate cell growth, differentiation, migration and invasion [391, 403]. For example, an increase in collagen deposits or ECM stiffness increases integrin signaling, which promotes cell proliferation and survival [391]. Therefore, as a key component of the tumour niche, the ECM affects tumour initiation, progression and metastatic potential.

1.9 Scope of thesis

The ECM is a highly organized, dynamic three-dimensional structure with many physiological and pathological roles. It maintains tissue integrity, regulates various cellular and biological processes such as cell adhesion, migration, cellular differentiation, and proliferation, and acts as a reservoir of growth factors and cytokines. The functional diversity that allows the ECM to play an active role in developmental processes also makes it an interesting target whose deregulation can make it a rate-limiting step in cancer progression. Using several model systems we examined select ECM proteins that we predicted to have a possible role in normal ovarian development or ovarian cancer progression.

1.9.1 Hypothesis and Objectives

The work presented in this thesis was executed to test the *hypothesis* that ECM proteins play a functional role in ovarian folliculogenesis and ovarian cancer progression.

To test this hypothesis I pursued the following objectives:

1) To characterize the expression of ECM components in the immature and adult Estrogen Receptor β-null mouse ovary.
2) To determine whether TCF21 represses estrogen receptor-mediated transcription.
3) To characterize the ECM protein Spondin1 and its function in immortalized epithelial ovarian cancer (EOC) cell lines and primary ascites-derived ovarian cancer cells in order to discover a possible role for Spondin 1 in cellular processes essential for ovarian cancer progression.

1.9.2 Rationale and Studies

Microarray and cell-clumping studies performed by Dr. Deroo indicate that ECM composition and cell adhesion are disrupted in ERβ-null granulosa cells ([156] and unpublished data), which may contribute to the attenuated folliculogenesis observed in ERβ-null ovaries. Therefore, in Chapter 2, I characterize the expression and localization of two 17β-estradiol-regulated ECM proteins, Collagen 11A1 (*Col11a1*) and Nidogen 2 (*Nid2*), in the ovaries of ERβ-null and wildtype mice, which had been identified as differentially expressed in ERβ-null GCs in Dr. Deroo's microarray study. I also examine several other ECM proteins not identified as dysregulated by the original microarray, but previously identified in the ovary. I demonstrated that the expression of several ECM components is disrupted in the ovary of the immature and adult ERβ-null mouse. I identify several genes of the ECM whose protein levels are significantly higher in ERβ-null follicles than in wildtype follicles, suggesting that ERβ represses their expression.

The molecular mechanisms that initiate gene repression by ERβ are not well understood, and there remains limited information in the literature regarding corepressors specifically involved in ERβ-mediated transcription. Therefore, in Chapter 3 I investigate a potential mechanism by which ERβ may be acting as a transcriptional repressor in GCs. A yeast two-hybrid screen of a mouse granulosa cell cDNA library performed by Dr.

Deroo revealed a physical interaction between ERβ and the basic helix-loop-helix protein, Transcription factor 21 (TCF21) (unpublished data). Biochemical and genetic analyses have previously demonstrated that TCF21 can act as a transcriptional repressor [253, 254, 258, 285, 286]. Furthermore, it is well established that TCF21 is essential for normal gonadogenesis and sexual differentiation. Although TCF21 transcripts have previously been identified in ovaries of mice, the detailed mechanism of its role in postnatal ovaries is unknown. We demonstrate that TCF21 regulates estradiol-dependent transcriptional activity in an ER isoform-specific manner; TCF21 represses ERβ, but not ERα transactivation. Despite our best efforts we were unable to show that TCF21 forms a complex with ERβ *in vivo*. Therefore, we returned our focus to another 17β-estradiol-regulated ECM protein, Spondin 1, and its potential role in ovarian cancer progression.

As a key component of the tumour microenvironment, the ECM is essential at various stages of tumourigenesis. Spondin 1, a secreted glycoprotein, is abundantly expressed in the normal ovary and our laboratory has (unpublished) evidence identifying a role in folliculogenesis. Spondin 1 is highly overexpressed in ovarian cancer and has recently been identified as a promising ovarian cancer marker, particularly for high-grade serous epithelial ovarian cancer. Therefore, in Chapter 4 I characterize Spondin1 and its function in immortalized EOC cell lines and primary ascites-derived ovarian cancer cells. Spondin1 has previously been shown to have functional and mechanistic roles in various tissues, effecting cell adhesion, migration, and survival. Since many of the same cellular processes and behaviours that are necessary for normal development are also essential for cancer progression, I examined the effect of Spondin 1 on these processes in EOC cells. I demonstrate that Spondin 1 significantly reduces EOC cell adhesion, viability and proliferation; however, it does not effect cell migration.

The studies in this thesis have uncovered novel functions of ECM proteins in normal ovarian development and ovarian cancer progression, further demonstrating the diverse roles of ECM in infertility and tumourigenicity.

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Chapter 2

2 Expression of Extracellular Matrix Components is Disrupted in the Immature and Adult Estrogen Receptor β-null Mouse Ovary

This chapter is based on a peer-reviewed journal article:
Zalewski, A., E.L. Cecchini, and B.J. Deroo, *Expression of extracellular matrix components is disrupted in the immature and adult estrogen receptor beta-null mouse ovary.* PLoS One, 2012. **7**(1): p. e29937.

2.1 Introduction

It is well established that estrogens play a critical role in the ovary during folliculogenesis. 17β-estradiol (E2) synergizes with follicle stimulating hormone (FSH) to induce granulosa cell differentiation and the formation of a healthy preovulatory follicle capable of ovulation in response to luteinizing hormone (LH) [1]. E2 acts directly on granulosa cells [2, 3] via its receptor, ERβ [4, 5], which is the predominant ER form expressed in granulosa cells of both humans and mice.

E2 and ERβ are essential for folliculogenesis in mice. Adult ERβ-null females are sub-fertile or infertile [6-8], possess ovaries with reduced numbers of growing follicles and corpora lutea and, due to infrequent ovulation, have litters one-third the size of wildtype (WT) females or are completely sterile [6-8]. There is almost a complete lack of antral follicles in the prepubertal ERβ-null ovary [7]. Furthermore, ERβ-null granulosa cells isolated from post-natal day (PND) 23 mice have an attenuated response to FSH, resulting in reduced cAMP accumulation [5], and poorly differentiated granulosa cells [4]. This lack of differentiation results in attenuated follicular production of cAMP in response to LH [9], and reduced ovulation. Therefore, an important role for E2 and ERβ in the response to FSH in the ovaries of adult mice has been firmly established; however, a role for ERβ in the postnatal/immature ovary has not been explored. Lack of ERβ in the immature ovary might contribute to the impaired FSH response observed in ERβ-null granulosa cells.

Several lines of evidence indicate that both E2 and ERβ are not only present in the ovaries of immature rodents, but that E2 acting through ERβ regulates folliculogenesis at this time. E2 has been detected in neonatal circulation in the rat [10]. In addition, androstenedione (which can be converted to E2) is detectable at PND 7 in the mouse, and increases by PND 15 [11]. ERβ protein is present [12-14] and functional [13] in primary follicles in PND 4 mouse ovaries, consistent with earlier data indicating that ERβ mRNA is detectable in the mouse ovary as early as PND1 [14] or PND 4 [13, 14], and increases dramatically by PND 12 in the mouse [14] and rat [15]. Thus, both E2 and ERβ protein are simultaneously present in mice as early as PND 4, and increase around PND 12-15, when the ovary contains primordial and primary follicles, as well as secondary follicles with 2-3 layers of granulosa cells [16].

Evidence also suggests that E2, acting through ERβ, may regulate development of primordial and primary follicles. First, adult female *Cyp19a1*-null mice (which lack the enzyme Cyp19a1, also known as aromatase, which converts testosterone to 17β-estradiol in granulosa cells) have reduced numbers of primordial and primary follicles compared with WT mice [17], suggesting that production of E2 is required for optimal primordial and primary follicle development. Second, adult female ERβ-null mice have elevated numbers of primordial follicles, but reduced numbers of primary follicles [18]. Third, treatment of PND 20 mice with the ERβ-selective agonist 8β-VE_2 significantly increases the number of primary follicles, while the ERα-selective agonist, 16α-LE_2 did not [19]. These data suggest that E2 acting through ERβ may regulate the formation of primordial and/or primary follicles in young mice.

Based on these data, we hypothesized that disrupted gene expression would be observed in the ovaries of immature ERβ-null mice. The ERβ-null ovarian phenotype has been described almost exclusively in adult or gonadotropin-treated PND 23-29 mice; however, few studies have examined ERβ-null immature ovaries. Therefore, we examined the expression of a subset of genes (originally identified by microarray analysis [5] of granulosa cells isolated from PND 23-29 ERβ-null mice) in ERβ-null ovaries as early as PND 13. Specifically, we focussed our analysis on proteins of the extracellular

matrix because functional analysis of the microarray data revealed the novel observation that many ECM genes were dysregulated in ERβ-null granulosa cells, suggesting a novel phenotype in ERβ-null ovaries not previously reported.

It is well established that dramatic changes in the ECM occur throughout folliculogenesis to allow for the dramatic growth of the follicle from the primary to preovulatory stage [20-27]; the ECM regulates follicular cell morphology, aggregation, communication, differentiation, steroidogenesis, survival, and proliferation [27]. Two main follicular ECMs are the basal lamina and the "focimatrix," a basal lamina-like matrix located between granulosa cells, and granulosa cells are thought to produce many of these ECM components [22, 23]. In this study, we chose to further characterize the expression and ovarian localization of two ECM proteins whose expression was higher in ERβ-null granulosa cells than in WT cells, suggesting that ERβ may repress their expression: Collagen 11a1 (*Col11a1*) and Nidogen 2 (*Nid2*). We characterize Col11a1 and Nid2 localization and mRNA levels in the ovaries of immature mice at PND 13 and PND 23-29, as well as in adult mice. We also investigate several other ECM proteins (Col4a1, Nid1, and Laminin) which were not identified as differentially regulated in the original microarray, but whose ovarian expression has been previously characterized in the mouse [20, 24]. Surprisingly, many of these ECM proteins are elevated as well in the ERβ-null ovary, suggesting a general disruption of ECM composition, and a potential role for this disruption in the reduced fertility observed in ERβ-null mice.

Therefore, the overall aim of our study was to demonstrate that gene expression is dysregulated in the immature ERβ-null ovary, and in particular, that extracellular matrix (ECM) gene expression is dysregulated. We now report for the first time that the expression of several ECM genes is dysregulated in the ERβ-null ovary as early as PND 13, and that this dysregulation is maintained within the adult ERβ-null ovary, resulting in altered expression of ECM components compared to WT mice. Taken together, our data identify two novel findings: a) that ERβ regulates gene expression in the mouse ovary much earlier than previously thought, and b) that ERβ plays a role in the regulation of ECM composition in the immature and adult mouse ovary.

2.2 Materials and Methods

2.2.1 Mice

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). The generation of ERβ-null mice has been described previously [8]. Mice were obtained from Taconic Farms Inc., NY. Immature ERβ-null ($ER\beta^{-/-}$) female mice were generated via breeding homozygous ($ER\beta^{-/-}$) males with heterozygous ($ER\beta^{+/-}$) females. Wildtype (WT) C57BL/6 females were generated via breeding WT males and females. WT females were used as controls in all experiments. All females were weaned at PND 21 and genotyped as previously described [8]. All studies were conducted with untreated animals (ie. no gonadotropin or any other treatment).

2.2.2 Isolation of granulosa cells

Ovaries were removed from PND 23-29 mice and immediately transferred to a 100-mm cell culture dish containing 15 ml ice-cold M199 medium supplemented with 1 mg/ml BSA, 2.5 μg/ml Amphotericin B, and 50 μg/ml gentamicin (all reagents from Invitrogen, Carlsbad, CA). Ovaries were pooled according to genotype, and the granulosa cells from each were then expressed by manual puncture with 25-gauge needles followed by pressure applied with a sterile spatula. Follicular debris was removed manually and the granulosa cell suspension filtered through a 150-μm Nitex nylon membrane (Sefar America Inc., Depew, NY) mounted in Swinnex filters (Millipore, Billerica, MA). The granulosa cells were then pelleted by centrifugation at 250 x g for 5 min at 4°C, followed by two washes in DMEM/F-12 medium containing 1% Penicillin/Streptomycin solution (Invitrogen, Catalog # 15070-063). The final cell pellet was frozen at 80°C.

2.2.3 RNA isolation and quantitative qRT-PCR

Frozen pellets of granulosa cells (PND 23-29 mice) or frozen whole ovaries (PND 13 mice) were solubilized in Trizol (Invitrogen, Carlsbad, CA) and RNA was isolated according to the manufacturer's protocol. RNA was further treated with DNaseI, then

reverse-transcribed using Superscript II (Invitrogen). cDNA levels were detected using quantitative PCR with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) and Power Sybr Master Mix (Invitrogen). Primers were designed using the Applied Biosystems Primer Express Software version 2.0 (Table 2-1). Fold changes in gene expression were determined by quantitation of cDNA from target (ERβ-null) samples relative to a calibrator sample (WT). The gene for ribosomal protein L7 (*Rpl7*) was used as the endogenous control for normalization of initial RNA levels. Expression ratios were calculated according to the mathematical model described by Pfaffl [28], where ratio = $(\text{Etarget})^{\Delta\text{Ct(target)}}/(\text{Econtrol})^{\Delta\text{Ct(control)}}$ and E=efficiency of the primer set, calculated from the slope of a standard curve of log (ng of cDNA) vs. Ct value for a sample that contains the target according to the formula $E=10^{-(1/\text{slope})}$ and ΔCt=Ct(vehicle)-Ct(treated sample).

Table 2-1: Primer sequences used for quantitative RT-PCR.

Gene	Accession #	Forward Primer	Reverse Primer
Col11a1	NM_007729.2	5'- AGTTGGTCTGCAGTGGCAATTTCG -3'	**5'- AGATCCCAGATCCACCGTTTCGTT -3'**
Col4a1	NM_009931.2	5'- CTCCAGGTCCCTACGATGTC -3'	**5'- TCCAAAGGGTCCTGTCTCTC -3'**
Lama1	NT_039658.1	5'- TCCGTGGATGGCGTCAA -3'	**5'- TGTAGCGGGTCAAACACTCTGT -3'**
Nid1	NM_010917.2	5'- CACAGGCAATGGCAGACAGT -3'	**5'- CCCTTCACCTTGCCATTGA -3'**
Nid2	NM_008695.2	5'- GTCTGTTTGGCTGGCTCTTTGCTT -3'	**5'- TCCACGTCATGGACAAAGGTAGCA -3'**
Rpl7	**NM_011291**	**5'- AGCTGGCCTTTGTCATCAGAA -3'**	**5'- GACGAAGGAGCTGCAGAACCT -3'**

2.2.4 Immunofluorescence

Ovaries were dissected from PND13, PND 23-29, or two-month old adult WT and ERβ-null female mice and embedded in Cryomatrix (Fisher, Ottawa, ON). Using a cryostat, tissues were cut into 6 μm sections, mounted onto slides (Fisher) and stored at -20°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, blocked for 30 minutes with blocking solution (5% BSA in 0.1% Triton X-100), then rinsed three times with blocking solution. The tissue was then incubated for one hour with primary antibodies specific to each target, including rabbit polyclonal anti-nidogen 2 raised against a mouse epitope (1:50, Santa Cruz Inc. sc-33143), rat monoclonal anti-nidogen 1 raised against a mouse epitope (1:400, Abcam, Cambridge, MA, ab44944), rabbit polyclonal anti-collagen 11a1 raised against a human epitope (1:200, Abcam ab64883), rabbit polyclonal anti-collagen 4a1 raised against a mouse epitope (1:500, Abcam ab19808), rabbit polyclonal anti-laminin raised against a mouse epitope (1:200, Abcam ab11575), and rabbit polyclonal anti-calnexin raised against a dog epitope (1:50, Enzo Life Sciences ADI-SPA-860). Sections were then rinsed three times in blocking solution and incubated in secondary antibody (FITC-conjugated goat anti-rabbit secondary antibody, 1:250 Sigma F9887). The tissue was then washed twice in PBS followed by a 5 minute incubation in 4′,6-diamidino-2-phenylindole (1:1000, Sigma), and slides were mounted with Vectashield (Vector Laboratories, Burlington, ON). Slides were stored at 4°C and visualized the following day with an Olympus Provis AX70 upright microscope. Images were captured using Image-Pro 6.2 Software.

2.2.5 Statistical Analysis

Differences in average mRNA levels of *Nid2*, *Nid1*, *Col11a1*, and *Col4a1* between ERβ-null and WT granulosa cells as determined by qPCR were compared using an unpaired two-tailed Student's t-test. To estimate and quantify the amount of Nid2, Nid1, Col4a1, and laminin present in the focimatrix, the number of immunoreactive speckles per follicle in each follicle within the section was counted manually by an

experimenter blinded to genotype. Atretric follicles were not included in the count. Speckles were counted in 21-78 follicles per genotype for each protein of interest from a minimum of three mice per genotype per protein. Larger aggregates of speckles were estimated based on a pre-determined minimum speckle size. The number of speckles/follicle was compared between ERβ-null and WT using two statistical tests. First, averages were compared using an unpaired, two-tailed Student's t-test. Second, differences were investigated using the more stringent criteria of Receiver Operating Characteristic (ROC) analysis, an analysis that tests for differences over the entirety of both distributions.

2.2.6 Gene Ontology Analysis

The Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID 6.7) Functional Annotation tool [29, 30] was used to determine Gene Ontology Cellular Components [31] presented in Supplementary Table 2-1 from a previously published dataset by Deroo et al [5]. All analyses were conducted with Maximum EASE Score/P value set to 0.05.

2.3 Results

Our previous microarray studies (Gene Expression Omnibus accession number GSE11585) [5] comparing the gene expression profiles of granulosa cells isolated from gonadotropin-treated immature (PND 23-29) ERβ-het ($ER\beta^{+/-}$) and ERβ-null ($ER\beta^{-/-}$) mice indicated that the expression of numerous extracellular (ECM) proteins was dysregulated in ERβ-null granulosa cells compared to ERβ-het cells (Supplementary Table 2-1). From this set of ECM proteins (Supplementary Table 2-1), we chose to further characterize the expression and ovarian localization of two proteins whose expression was higher in ERβ-null granulosa cells than in ERβ-het cells: Collagen 11a1 (*Col11a1*) and Nidogen 2 (*Nid2*). We focussed on these two proteins because they met the following four criteria: 1) follow-up studies confirming the microarray data indicated that both genes were dysregulated in granulosa cells isolated from *untreated* ERβ-null PND 23-29 mice, suggesting an earlier role for ERβ in ovarian development than previously thought, 2) the higher levels of expression in ERβ-null granulosa cells

compared to ERβ-het cells suggested a novel inhibitory role for ERβ in the regulation of their expression (rather than an activational role), 3) there is previously-reported evidence for regulation of *Col11a1* (Gene Expression Omnibus dataset GDS884) and *Nid2* expression by 17β-estradiol [32, 33], and 4) the fold difference between ERβ-het and ERβ-null granulosa cells was greater than two, our predetermined cut-off value for further analysis. In addition, to our knowledge, expression of Collagen 11a1 had not been previously reported in the ovary, suggesting that its aberrantly high expression in ERβ-null granulosa cells may contribute to the disrupted folliculogenesis observed in ERβ-null mice. Note that untreated mice were used for all studies, ie. mice were not primed with gonadotropins or estradiol.

Therefore, we wanted to investigate Col11a1 and Nid2 expression and localization at PND 13 and PND 23-29 to determine when dysregulated gene expression could first be detected in the ERβ-null ovary. We also investigated these genes in adult ovaries to determine if the dysregulation observed in immature mice was maintained in the adult ovary.

2.3.1 Collagen 11A1

At PND 13, *Col11a1* mRNA levels were approximately two-fold higher in ERβ-null whole ovaries than in WT ovaries, as determined by quantitative RT-PCR (qPCR) (Figure 2-1A). Similarly, *Col11a1* mRNA levels were 2.5-fold higher in granulosa cells isolated from PND 23-29 ERβ-null mice than in WT granulosa cells isolated from age-matched mice (Figure 2-1A). We then wanted to determine, using immunofluorescence: a) if these increases in *Col11a1* mRNA levels correlated with increases in protein expression, and b) the localization of Col11a1 within the immature and adult ovaries of WT and ERβ-null mice. At PND 13, when the mouse ovary contains many preantral follicles with 2-3 rows of granulosa cells surrounded by a basal lamina, in addition to primary and primordial follicles [16], ERβ-null ovaries expressed higher levels of Col11a1 than WT mice of the same age (Figure 2-1B), and Col11a1 appeared to be localized to the cytoplasm and extracellular region of granulosa cells. AT PND 23-29 (Figure 2-1C) Col11a1 was almost undetectable in WT PND 23-29 ovaries. However,

Col11a1 was dramatically elevated in the follicles of ERβ-null mice (Figure 2-1C). Col11a1 protein was localized primarily to the cytoplasm of granulosa cells (Figure 2-1C, panel f). Similar localization in the follicle was observed for calnexin, which localizes to the endoplasmic reticulum and is frequently used as a cytoplasmic marker (Figure S2-1). Col11a1 expression was primarily observed in preantral follicles (both small and large), which predominate in the immature ERβ-null ovary. Only very weak Col11a1 staining was observed in the thecal layer or ovarian interstitium. In adult mice, as observed in the immature mice, Col11a1 expression was again higher in the granulosa cell cytoplasm in ERβ-null ovaries than in WT ovaries (Figure 2-1D).

2.3.2 Nidogen 2

At PND 13, *Nid2* mRNA levels were approximately 1.5-fold higher in ERβ-null whole ovaries than in WT ovaries, as determined by qPCR (Figure 2-2A). Similarly, *Nid2* mRNA levels were approximately 2.3-fold higher in granulosa cells isolated from PND 23-29 ERβ-null mice than in WT granulosa cells isolated from age-matched mice (Figure 2-2A). With respect to localization of Nid2 within the ovary as determined by immunofluorescence, while Col11a1 localized almost exclusively to the cytoplasm of granulosa cells (Figure 2-1C), Nid2 was localized to the follicular basal lamina, thecal matrix, sub-endothelial basal lamina of stromal blood vessels, and in a punctate pattern as "speckles" or "plaques" between granulosa cells (known as focimatrix) (Figure 2-2C) of PND 23-29 WT mice, as previously reported [24]. The focimatrix (**foc**al **i**ntra-epithelial **matrix**; a term coined by Irving-Rodgers et al. [34]), is a specialized ECM composed of basal-lamina like material that exists as plaques or aggregated deposits between granulosa cells, but does not surround the cells as a true basal lamina. Focimatrix is found in the ovaries of many species. In the mouse, primary focimatrix components include collagen, type IV α1 and α2, laminin α1, β1 and γ1, nidogens 1 and 2, perlecan, and collagen type XVIII [24]. Granulosa cells express mRNA encoding many focimatrix proteins [23, 35], and granulosa cells are thought to be the source of focimatrix protein production [25]. In our study, Nid2 localization was similar in both WT and ERβ-null ovaries (Figure 2-2B and 2-2C) at PND 13 and PND 23-29. However, as predicted by the

mRNA levels (Figure 2-2A), Nid2 expression was higher in the follicles of ERβ-null mice (Figures 2-2B and 2-2C) than in WT mice at both ages. However, this increase was only observed in the focimatrix of ERβ-null ovaries; Nid2 levels in the follicular basal lamina, thecal matrix, and sub-endothelial basal lamina of stromal blood vessels were similar in both genotypes. These differences in focimatrix Nid2 expression between WT and ERβ-null follicles were quantified in PND 23-29 ovaries by counting the number of focimatrix speckles per follicle, and the difference tested for statistical significance (Figure 2-2E). A statistically significant difference in the number of focimatrix speckles per follicle was observed between WT and ERβ-null follicles (Figure 2-2E) on average, as determined by Student's t-test (Figure 2-2E, left panel). In addition, a statistically significant difference was also detected using the more stringent criteria of Receiver Operating Characteristic (ROC) analysis (Figure 2-2E, right panel), an analysis that tests for differences over the entirety of both distributions. At PND 13, ERβ-null ovaries again expressed higher levels of Nid2 protein than WT mice of the same age (Figure 2-2B). Interestingly, Nid2 expression appeared higher throughout the ovary of ERβ-null mice at this stage: in the focimatrix, in the follicular basal lamina and in thecal matrix. (Focimatrix speckles were not counted due to difficulty of accurate counts resulting from the irregularity of follicle shapes and sizes at this stage). Expression of Nid2 was strikingly and significantly higher (Figure 2-2D and 2-2F) in adult ERβ-null focimatrix than in WT focimatrix, while expression of Nid2 in other follicular compartments was similar in both genotypes, as observed in younger mice (Figures 2-2B and 2-2C).

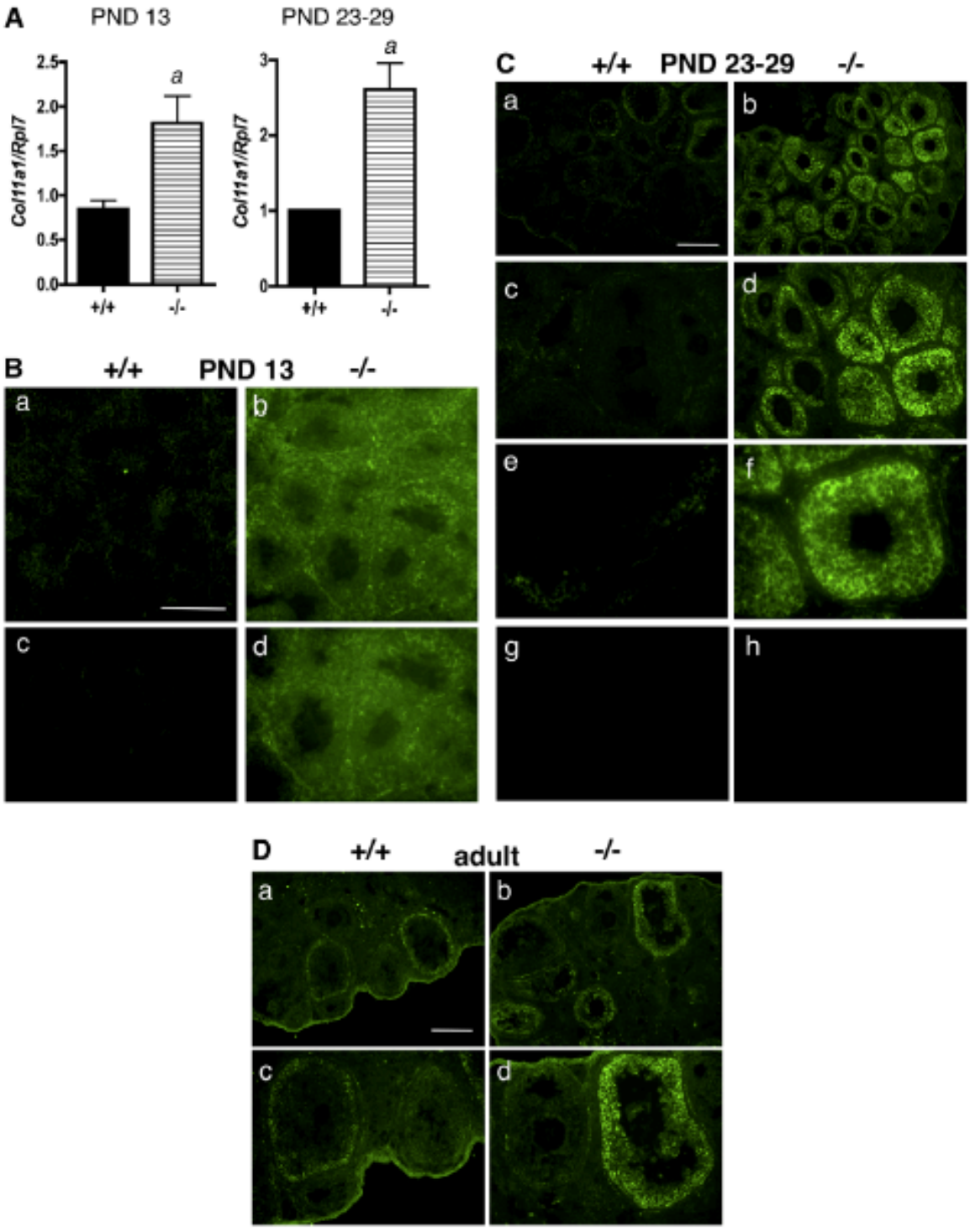
A
PND 13
PND 23-29
Col11a1/Rpl7
2.5
2.0
1.5
1.0
0.5
0.0
a
+/+
-/-
3
2
1
0
a
+/+
-/-
B
+/+
PND 13
-/-
a
b
c
d
C
+/+
PND 23-29
-/-
a
b
c
d
e
f
g
h
D
+/+
adult
-/-
a
b
c
d

Figure 2-1: Collagen 11a1 mRNA and protein levels are higher in granulosa cells and ovaries of ERβ-null mice than in wildtype mice.

(A) Granulosa cells were isolated and pooled from ovaries of untreated PND 13 or PND 23-29 wildtype (+/+) or ERβ-null (-/-) mice, and the levels of *Col11a1* mRNA were determined by quantitative RT-PCR compared to an *Rpl7* control (± SEM of three independent experiments). Wildtype and ERβ-null average mRNA levels were compared using an unpaired two-tailed Student's t-test. a: $p < 0.05$. B-D. Immunofluorescence with an anti- Col11a1 antibody was used to detect Col11a1 localization and expression in ovaries isolated from wildtype (+/+) and ERβ-null (-/-) mice at (B) PND 13 (a-d), (C) PND 23-29 (a-f; negative controls with secondary antibody only are shown in g and h), and (D) PND 60 (adult). Various magnifications are shown. (B) Scale bar = 100 μM for a-b, and 50 μM for c-d. (C) Scale bar = 200 μM for a-b and g-h, 100 μM for c-d, and 50 μM for e-f; (D) Scale bar = 200 μM for a-b, 100 μM for c-d.

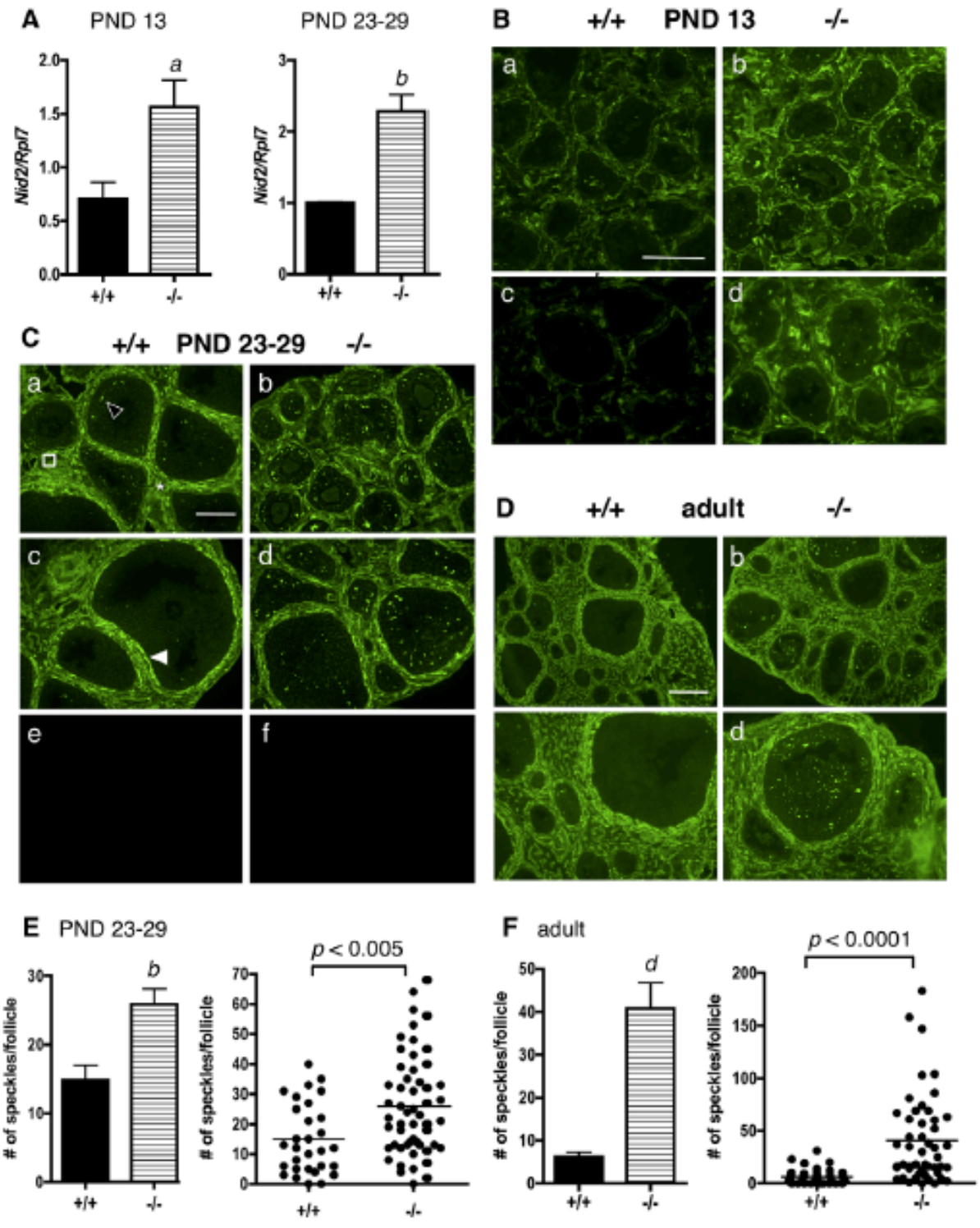
A
PND 13
PND 23-29
Nid2/Rpl7
a
b
+/+
-/-
B
+/+
PND 13
-/-
C
+/+
PND 23-29
-/-
D
+/+
adult
-/-
E
PND 23-29
# of speckles/follicle
p < 0.005
F
adult
p < 0.0001

Figure 2-2: Nidogen 2 mRNA and protein levels are higher in granulosa cells and ovaries of ERβ-null mice than in wildtype mice.

(A) Granulosa cells were isolated and pooled from ovaries of untreated PND13 or PND 23-29 wildtype (+/+) or ERβ-null (-/-) mice, and the levels of *Nid2* mRNA were determined by quantitative RT-PCR compared to an *Rpl7* control (± SEM of three independent experiments). Wildtype and ERβ-null average mRNA levels were compared using an unpaired two-tailed Student's t-test. a: $p < 0.05$; b: $p < 0.01$. B-D. Immunofluorescence with an anti-Nid2 antibody was used to detect NID2 localization and expression in ovaries isolated from wildtype (+/+) and ERβ-null mice (-/-) at (B) PND 13 (a-d), (C) PND 23-29 (a-d; negative controls with secondary antibody only are shown in e and f), and (D) PND 60 (adult). Various magnifications are shown at each age. (B) Scale bar = 100 μM for a-b, and 50 μM for c-d. (C) Two different sections from each genotype are shown (same magnification for both sections). Scale bar = 100 μM for a-f; (D) Scale bar = 200 μM for a-b, 100 μM for c-d. Nid2 is localized to the follicular basal lamina (white filled arrowhead), focimatrix (open arrowhead), thecal matrix (asterix), and endothelial basal lamina of stromal blood vessels (square). (E, F) Focimatrix speckles in the PND 23-29 and adult sections were counted per follicle, and the difference between genotypes analyzed by a two-tailed, un-paired Student's t-test (± SEM, left panel) and by Receiver Operating Characteristic analysis (right panel). Each dot in the scatter plot (right panel) represents one follicle. b: $p < 0.01$; d: $p < 0.0001$.

To show that this difference in expression between ERβ-null and WT granulosa cells was specific to *Nid2* and *Col11a1*, but not to all ECM genes, we also investigated the expression of Nidogen 1 *(Nid1),* Collagen, type IV (*Col4a1)*, and Laminin (*Lama1*). We chose the *Nid1*, *Col4a1,* and *Lama1* genes because their expression and localization has been previously characterized in the mouse ovary [20, 24, 36], and because neither gene had been detected as differentially expressed between WT and ERβ-null granulosa cells by our previously-conducted microarray (Supplemental Table 1). Nidogen 1 is structurally similar to Nidogen 2 and shares overlapping expression patterns during development and in many adult tissues [37, 38], and both Collagen, type IV and Laminin are ubiquitous ECM proteins found in many tissues, including the ovary.

2.3.3 Nidogen 1

Nid1 mRNA levels were similar in both ERβ-null and WT granulosa cells at PND 23-29 (Figure 2-3A). Similar to Nid2, Nid1 localized to the follicular basal lamina, thecal matrix, focimatrix, and basal lamina of stromal blood vessels (Figure 2-3B) of PND 23-29 WT mice as previously reported [24]. No differences in Nid1 expression levels were observed between genotypes in the follicular basal lamina, thecal matrix, or basal lamina of stromal blood vessels. Unexpectedly, Nid1 expression in the focimatrix was slightly higher in ERβ-null follicles than in WT follicles (Figure 2-3B), and this increase was statistically significant (Figure 2-3C). No significant differences were observed in Nid1 expression (Figures 2-3D and E) between adult wildtype and ERβ-null mice in the focimatrix, although the overall signal in the basal lamina and stroma appeared higher in ERβ-null ovaries than in WT ovaries.

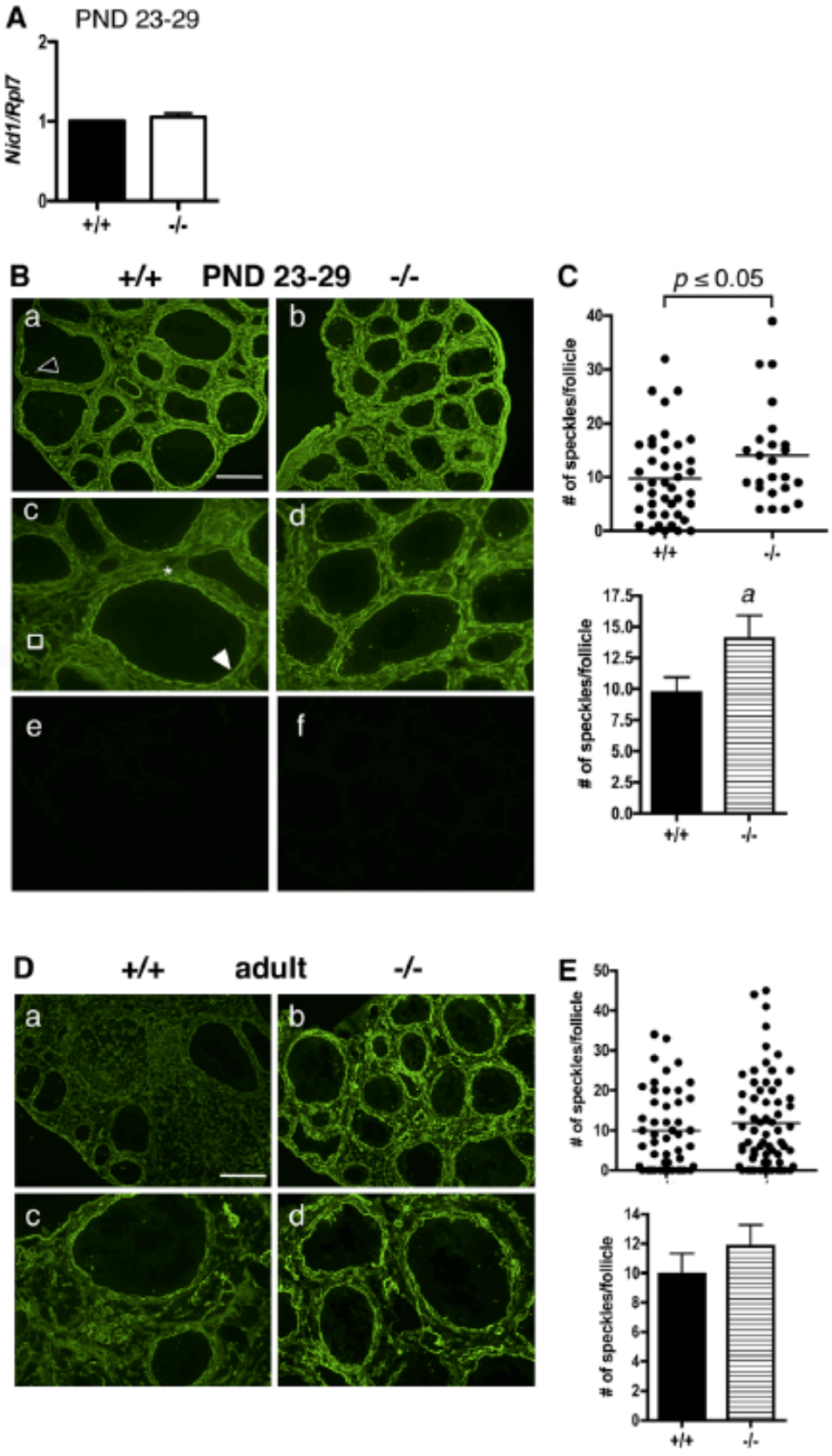
A
PND 23-29
Nid1/Rpl7
2
1
0
+/+
-/-
B
+/+
PND 23-29
-/-
a
b
c
d
e
f
C
p ≤ 0.05
# of speckles/follicle
40
30
20
10
0
+/+
-/-
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
a
D
+/+
adult
-/-
E
50
14
12
8
6
4

Figure 2-3: Nidogen 1 expression and localization in immature and adult ERβ-null and wildtype mouse ovaries.

(A) Granulosa cells were isolated and pooled from ovaries of untreated PND 23-29 wildtype (+/+) or ERβ-null (-/-) mice, and the levels of *Nid1* mRNA were determined by quantitative RT-PCR compared to an *Rpl7* control (± SEM of three independent experiments). (B) Immunofluorescence with anti-Nid1 antibodies was used to detect Nid1 localization and expression in ovaries isolated from wildtype (+/+) and ERβ-null (-/-) mice at PND 23-29 (a-d; negative controls with secondary antibody only are shown in e and f). Nid1 was localized to the follicular basal lamina (white filled arrowhead), focimatrix (open arrowhead), thecal matrix (asterix), and endothelial basal lamina of stromal blood vessels (square). Nid1 focimatrix expression is slightly higher in ERβ-null ovaries than in wildtype ovaries at PND 23-29. Scale bar = 200 μM for a-b and e-f, 100 μM for c-d. (C) A significant increase in Nid1 expression within the focimatrix of ERβ-null ovaries compared to wildtype ovaries was observed at PND 23-29, as determined by the number of focimatrix "speckles" counted per follicle. Differences in the number of speckles/follicle between genotypes were analyzed by Receiver Operating Characteristic analysis (top panel) and a two-tailed, un-paired Student's t-test (± SEM, bottom panel). Each dot in the scatter plot (top panel) represents one follicle. a: $p < 0.05$. (D) Nid1 expression in adult ERβ-null and wildtype mouse ovaries. Immunofluorescence with anti-Nid1 antibodies was used to detect Nid1 localization and expression in ovaries isolated from adult wildtype (+/+) and ERβ-null (-/-) mice. Two magnifications are shown. Scale bar = 200 μM for a-b, 100 μM for c-d. (E) Expression of Nid1 in the adult focimatrix was quantified by counting the number of focimatrix speckles/follicle, and these values were compared between genotypes by Receiver Operating Characteristic analysis (E, top panel) and a two-tailed, un-paired Student's t-test (± SEM, E bottom panel) in each case. Each dot in the scatter plot (E, top panel) represents one follicle. No statistically significant difference in NID1 focimatrix was observed between genotypes in the adult ovary.

2.3.4 Collagen 4a1

Col4a1 mRNA levels were similar in both ERβ-null and WT granulosa cells at PND 23-29 (Figure 2-4A). Similarly, Col4a1 protein levels were the same in WT and ERβ-null mice (Figure 2-4B). Interestingly, the localization of Col4a1 and Col11a1 was not the same within the WT or ERβ-null ovary. While Col11a1 localized almost exclusively to the cytoplasm of granulosa cells (Figure 2-1C), Col4a1 staining was observed in the follicular basal lamina, the focimatrix, the thecal matrix, and in the stromal sub-endothelial basal lamina of blood vessels (Figure 2-4B), as previously reported for WT mice [20, 24, 36]. Similar Col4a1 localization and staining intensity was observed in WT and ERβ-null PND 23-29 ovaries (Figure 2-4A). Focimatrix Col4a1 expression was quantified by counting the number of focimatrix speckles per follicle (Figure 2-4C). As predicted by the mRNA levels (Figure 2-4A), no statistically significant differences in the number of focimatrix speckles per follicle were observed between WT and ERβ-null follicles (Figure 2-4C). Expression of Col4a1 (Figure 2-4D) was strikingly and significantly higher (Figure 2-4E) in adult ERβ-null focimatrix than in WT focimatrix, while expression of Col4a1 in other follicular compartments was similar in both genotypes, as observed in younger mice (Figure 2-4D).

2.3.5 Laminin

Lama1 mRNA levels were similar in both ERβ-null and WT granulosa cells at PND 23-29 (Figure 2-5A). As previously reported [24], laminin was localized to the follicular basal lamina, the basal lamina of stromal blood vessels, the thecal matrix, focimatrix, and corpora lutea in both immature and adult mice (Figures 2-5B and 2-5D). At PND 23-29, ERβ-null follicles consistently possessed significantly higher numbers of focimatrix speckles per follicle than WT follicles (Figures 2-5B and 2-5C). Interestingly, laminin expression in the focimatrix of adult ERβ-null ovaries (Figure 2-5D) was again significantly higher than in WT focimatrix (Figure 2-5E).

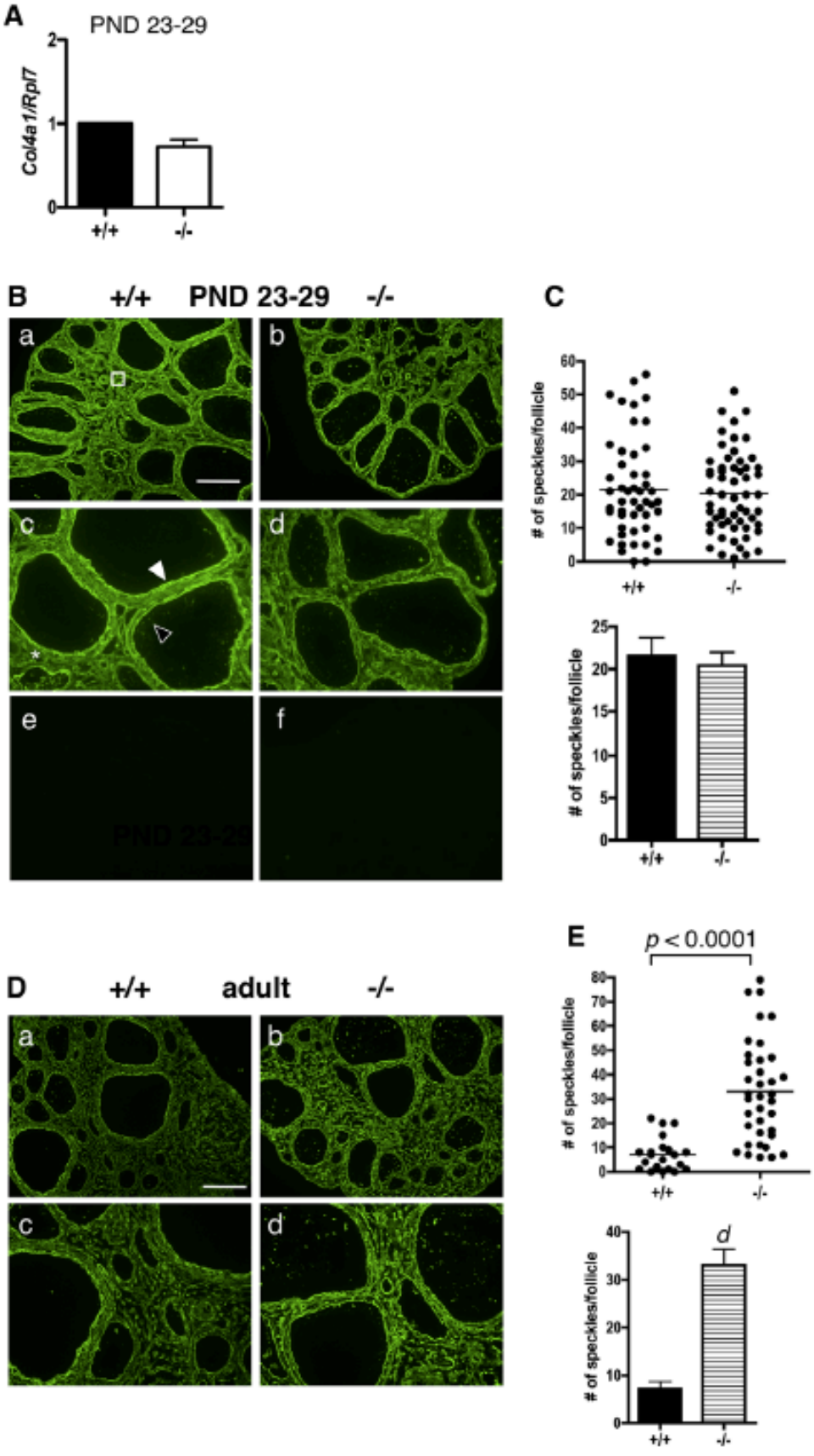
A
PND 23-29
Col4a1/Rpl7
2
1
0
+/+
-/-
B
+/+
PND 23-29
-/-
a
b
c
d
e
f
PND 23-29
C
# of speckles/follicle
60
50
40
30
20
10
0
+/+
-/-
# of speckles/follicle
25
20
15
10
5
0
+/+
-/-
D
+/+
adult
-/-
a
b
c
d
E
p < 0.0001
# of speckles/follicle
80
70
60
50
40
30
20
10
0
+/+
-/-
# of speckles/follicle
d
40
30
20
10
0
+/+
-/-

Figure 2-4: Collagen 4a1 expression and localization in immature and adult ERβ-null and wildtype mouse ovaries.

(A) Granulosa cells were isolated and pooled from ovaries of untreated PND 23-29 wildtype (+/+) or ERβ-null (-/-) mice, and the levels of *Col4* mRNA were determined by quantitative RT-PCR compared to an *Rpl7* control (± SEM of three independent experiments). (B) Immunofluorescence with an anti-Col4a1 antibody was used to detect Col4a1 localization and expression in ovaries isolated from wildtype (+/+) and ERβ-null (-/-) mice at PND 23-29 (a-d; negative controls with secondary antibody only are shown in e and f). Col4a1 was localized to the follicular basal lamina (white filled arrowhead), focimatrix (open arrowhead), thecal matrix (asterix), and endothelial basal lamina of stromal blood vessels (square). Scale bar = 200 μM for a-b and e-f, 100 μM for c-d. (C) No significant differences in Col4a1 expression within the focimatrix were observed, as determined by the number of focimatrix "speckles" counted per follicle analyzed by Receiver Operating Characteristic analysis (top panel) and a two-tailed, un-paired Student's t-test (± SEM, bottom panel). Each dot in the scatter plot (top panel) represents one follicle. (D) Col4a1 expression in adult ERβ-null and wildtype mouse ovaries. Immunofluorescence with anti-Col4a1 antibodies was used to detect Col4a1 localization and expression in ovaries isolated from adult wildtype (+/+) and ERβ-null (-/-) mice. Two magnifications are shown. Scale bar = 200 μM for a-b, 100 μM for c-d. (E) Expression of Col4a1 in the focimatrix was quantified by counting the number of focimatrix speckles/follicle, and these values were compared between genotypes by Receiver Operating Characteristic analysis (top panel) and a two-tailed, un-paired Student's t-test (± SEM, bottom panel). Each dot in the scatter plot (bottom panel) represents one follicle. d: $p < 0.0001$

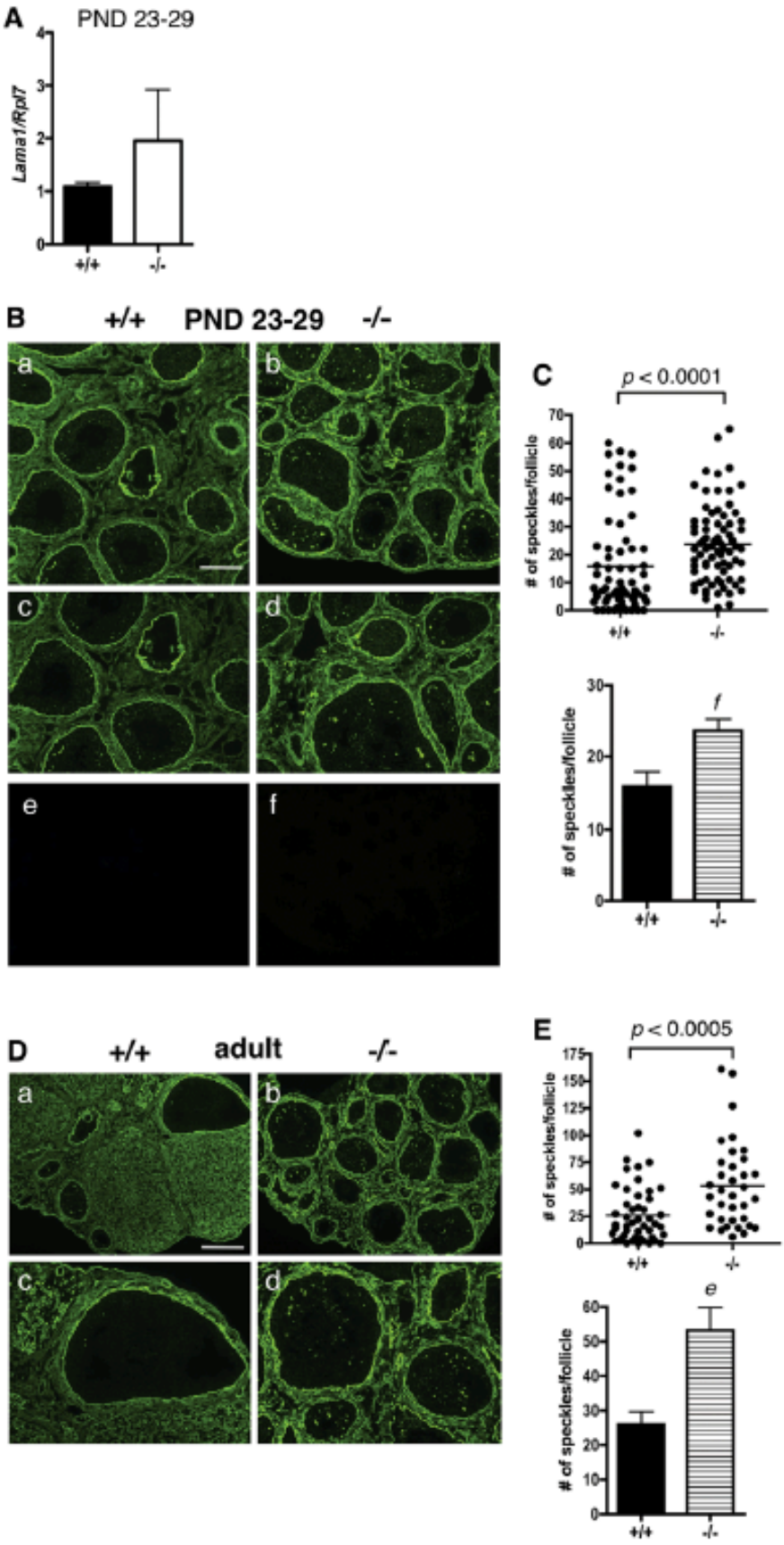
A
PND 23-29
Lama1/Rpl7
+/+
-/-
B
+/+
PND 23-29
-/-
a
b
c
d
e
f
C
p < 0.0001
# of speckles/follicle
+/+
-/-
f
D
+/+
adult
-/-
E
p < 0.0005
e

Figure 2-5: Laminin expression and localization in immature and adult ERβ-null and wildtype mouse ovaries.

(A) Granulosa cells were isolated and pooled from ovaries of untreated PND 23-29 wildtype (+/+) or ERβ-null (-/-) mice, and the levels of *Lama1* mRNA were determined by quantitative RT-PCR compared to an *Rpl7* control (± SEM of three independent experiments). (B) Immunofluorescence with an anti-laminin antibody was used to detect laminin localization and expression in ovaries isolated from wildtype (+/+) and ERβ-null (-/-) mice at PND 23-29 (a-d; negative controls with secondary antibody only are shown in e and f) wildtype (+/+) and ERβ-null (-/-) mice. Two magnifications are shown. Scale bar = 200 μM for a-b, 100 μM for c-d. (C) Focimatrix levels of laminin were quantified by counting the number of focimatrix speckles/follicle, and these values compared between genotypes by Receiver Operating Characteristic analysis (top panel) and a two-tailed, un-paired Student's t-test (± SEM, bottom panel). Each dot in the scatter plot (top panel) represents one follicle. f: $p < 0.005$. (D) Laminin expression in adult ERβ-null and wildtype mouse ovaries. Immunofluorescence with anti-laminin antibodies was used to detect laminin localization and expression in ovaries isolated from adult wildtype (+/+) and ERβ-null (-/-) mice. Two magnifications are shown. Scale bar = 200 μM for a-b, 100 μM for c-d. (E) Expression of laminin in the focimatrix was quantified by counting the number of focimatrix speckles/follicle, and these values were compared between genotypes by Receiver Operating Characteristic analysis (top panel) and a two-tailed, un-paired Student's t-test (± SEM, bottom panel). Each dot in the scatter plot (bottom panel) represents one follicle. e: $p < 0.0005$.

2.4 Discussion

In this study, we show that disrupted gene expression is observed in the ovaries of immature ERβ-null mice as early as PND 13, resulting in abnormal expression of ECM components in the ERβ-null ovary. We found that the mRNA levels of the ECM genes, *Col11a1* and *Nid2* were higher in granulosa cells isolated from ERβ-null PND 23-29 mice, or in whole ovaries isolated from PND 13 mice, than in age-matched WT controls. These elevated mRNA levels correlated with higher Col11a1 in the cytoplasm of granulosa cells and higher Nid2 expression in the focimatrix of the immature ERβ-null ovary, at both PND 23-29 and PND 13. Interestingly, the elevated expression of Col11a1 and Nid2 in ERβ-null follicles continued into adulthood. Finally, levels of the ubiquitous ECM proteins, collagen IV and laminin, were also higher in the adult ERβ-null ovary than in the WT ovary.

2.4.1 An early role for ERβ in ovarian development

Our results showing that gene expression is dysregulated in ovaries of ERβ-null mice at PND 13 are consistent with studies suggesting that both the levels of ovarian ERβ and its ligand, E2, increase during a similar time-frame in post-natal ovarian development, and that E2 may act through ERβ at this time to regulate gene expression, and possibly follicle development. The presence of circulating E2 or its precursors has been established in neonatal rats [10] and mice [39], and androstenedione is detectable at PND 7 and increases dramatically at PND 15 [11]. ERβ protein is present and functional in the ovaries of PND 4-5 mice, but not in younger mice [13, 14], and ovarian ERβ protein levels increase with age [14], with the most abundant expression in granulosa cells. ERβ mRNA is detectable at PND 1 [14] or PND 4 [13] in the mouse ovary, with a dramatic increase occurring between PND 1 and PND 12 [14]. Evidence supporting a role for both E2 and ERβ in regulating primary and primordial follicle development in the mouse ovary has been suggested using various model systems [17, 19, 40], and our results showing disrupted gene expression in ERβ-null mice at PND 13 support a role in ovarian development in the immature mouse. Interestingly, during the period of human gestation when primordial follicles are formed, the fetal ovary expresses both the

steroidogenic enzymes necessary for E2 production, and ERβ protein, suggesting that estrogen signaling may also regulate human primordial follicle formation [41]. While it may be possible that ERβ plays a role during prenatal ovarian development in the mouse, this is unlikely because ERβ mRNA is undetectable in the mouse ovary 26 days post-coitum [14] and only becomes detectable between PND 1 to PND 4 [13, 14]. Interestingly, although detectable at PND 8, we do not observe differences in gene expression by qPCR or protein levels by immunofluorescence in *Col11a1* or *Nid2* between ERβ-null and WT ovaries (data not shown) as we do at PND 13. One possible explanation for this lack of differential *Col11a1*/*Nid2* gene expression at PND 8 may be that ovarian ERβ levels are not high enough at PND 8 to detectably alter *Col11a1*/*Nid2* gene expression in WT mice, since there is a dramatic increase in ERβ mRNA between PND 1 and PND 12 in the mouse [14]. Thus it may not be until PND 13 that the lack of ERβ would result in significant differences in *Col11a1*/*Nid2* gene expression. On the other hand, there may be transcriptional coregulators required for ERβ-mediated transcription that are not present at PND 8 but are expressed at PND 13. Further experiments in WT and ERβ-null ovaries isolated from mice between PND 8 and PND 13 will be required to determine at which point during ovarian development ERβ activity is required for *Col11a1*/*Nid2* gene expression.

We have previously shown that ERβ-null granulosa cells isolated from PND 23-29 mice demonstrate an attenuated response to FSH, resulting in impaired *Lhcgr* and *Cyp19a1* expression, despite similar expression of FSH receptors [4, 5]. At least part of this attenuated response is due to reduced cAMP levels in response to FSH stimulation compared to WT granulosa cells [5]. Another important finding resulting from this previous study was that granulosa cells freshly-isolated from PND 23-29 ERβ-null ovaries produced significantly less cAMP than WT cells, even prior to stimulation by FSH. This reduced cAMP correlated with the elevated expression of phosphodiesterase 1c (PDE1C) in ERβ-null granulosa cells compared to WT cells (both isolated from untreated PND 23-29 mice) [5]. These results suggested that *prior* to PND 23, differences in granulosa cell gene expression between ERβ-null and WT mice are observed. Our current study supports and expands this observation, and provides strong evidence that

the impaired ERβ-null granulosa cell response to FSH at PND 23-29 is also due to the dysregulation of perhaps numerous ERβ-dependent genes prior to PND 23 that are required to prepare a granulosa cell to fully respond to FSH at the onset of puberty.

Thus, we propose that ERβ, acting either through E2 or in a ligand-independent manner, regulates granulosa cell gene expression in follicles at various stages of growth: in the primordial, primary, or preantral follicle, and in response to FSH during the formation of a preovulatory follicle, as has previously been shown. While it is well established that E2 acting through ERβ is required to augment the granulosa cell response to FSH for the formation of a preovulatory follicle [4, 5, 42-46], fewer studies exist establishing a role for E2 in folliculogenesis, prior to the gonadotropin surge at puberty. Several reports indicate that E2 enhances or is required for the production of primary follicles [17, 19], although others suggest that E2 inhibits primordial follicle assembly [12, 40]. It has been reported that the number of primordial and primary follicles are similar in immature (PND 23) ERβ-null and WT mice, suggesting that ERβ is not required for the formation of primordial or primary follicles [18]. In contrast, adult female ERβ-null mice have elevated numbers of primordial follicles, and reduced numbers of primary follicles [18], suggesting that ERβ may participate in primordial follicle recruitment and/or maintenance. Further experiments will be required to determine the function of ERβ in the PND 13 ovary, and whether ERβ's loss at earlier stages truly impacts primary follicle formation and/or granulosa cell function.

2.4.2 Disrupted Expression of ECM components in ERβ-null ovaries

This work is also novel in that we have characterized a significant elevation in multiple ECM proteins in immature and adult ERβ-null ovaries: a phenotype that has not previously been reported at either age. Col11a1 is expressed at very low levels in the WT immature ovary (Figure 2-1), but is robustly expressed in the cytoplasm of granulosa cells in the ERβ-null ovary at these ages. The localization of Col11a1 in the ovary of any species has, to our knowledge, not previously been reported, and in the mouse, *Col11a1* mRNA levels are highest in bone and cartilage [47]. In rat cartilage, Col11a1 is localized

in the ECM between chondrocytes [48]; however, in human colon tissue, Col11a1 is localized to the cytoplasm (specifically, the Golgi apparatus) of goblet cells [49]. We also observed Col11a1 in the cytoplasm of granulosa cells, and the function of Col11a1 in granulosa cell cytoplasm certainly merits further study, as does the possibility that granulosa cells may secrete Col11a1 and contribute to granulosa cell-cell adhesion or migration. We also observe Nid2 overexpression in the focimatrix in ERβ-null ovaries as early as PND 13 (Figure 2-2B), and Nid2 remains elevated in the adult (Figure 2-2D). Similarly, laminin expression (Figure 2-5) was higher in the focimatrix of both PND 23-29 and adult ERβ-null ovaries compared to their WT counterparts, while Col4a1 was elevated in ERβ-null adult but not PND 23-29 ovaries (Figure 2-4). Our results are consistent with two previous reports in which global collagen levels were higher in adult ERβ-null ovaries than in WT ovaries, in either: a) both stromal and thecal layers [50], or b) in the stroma only [6]. Our work supports and expands these observations, indicating that not just collagen, but a number of other ECM proteins are aberrantly highly expressed in the adult ERβ-null follicle, and in addition, these elevated levels are observed in immature mice. The fact that Nid2, laminin and Col4a1 expression was higher specifically in the focimatrix of ERβ-null ovaries, and not, for example, in the stroma, suggests that it is likely ERβ within granulosa cells regulating the expression of these genes (or other upstream genes required for their expression), since granulosa cells are the primary location of ERβ within the ovary, resulting in their secretion from the cell and localization to the extracellular region of granulosa cells. Further studies using *in situ* hybridization are needed determine which cells within the ovary produce these common ECM components.

2.4.3 Regulation of *Nid2* and *Col11a1* by Estradiol and ERβ

There is evidence that E2 regulates ECM composition in the ovary and other tissues. For example, E2 regulates collagen turnover and ECM maintenance in the uterus and vagina of ovariectomized rats [51], and neonatal estrogen treatment disrupts the ECM composition of the rat prostate [52]. Abnormal ECM composition and structure is also observed in lungs of ERβ-null mice [53]. Within the context of our study, several hypotheses can be put forward to explain how the lack of ERβ results in increased

expression of *Nid2* and *Col11a1* in the ERβ-null immature ovary. First, ERβ may directly repress the transcription of these genes either by binding EREs located proximal to or distant from the transcriptional start site, or by binding to other transcription factors, which themselves are bound to DNA (tethering). There is evidence that *Col11a1* and *Nid2* expression is regulated by E2 in other model systems. *Col11a1* mRNA is increased by E2 treatment of osteosarcoma cells expressing ERβ, but not ERα, indicating that not only is *Col11a1* regulated by E2 but that ERβ is selectively required for its regulation (Gene Expression Omnibus dataset GDS884) [33], although in this case E2 increases rather than decreases *Col11a1* expression, as would be predicted by the elevated *Col11a1* levels we observe in the absence of ERβ. Treatment of ovariectomized adult mice with E2 decreases uterine *Nid2* mRNA levels within six hours of treatment, consistent with a role for ERβ in repressing *Nid2* gene expression in the ovary [32]. It is also possible that ERβ indirectly decreases the transcription of *Nid2* and *Col11a1* by regulating the expression of other protein(s), such as transcription factors or transcriptional coregulators, or signaling molecules known to regulate folliculogenesis. In fact, in a whole ovary culture model in which PND 4 rat ovaries (which contain almost exclusively primordial follicles) were treated with Kit ligand [54], *Col11a1* expression was reduced, suggesting that Kitl signaling may be disrupted in ERβ-null neonatal ovaries. Lack of ERβ may also stabilize *Nid2* and *Col11a1* mRNA through regulation of a protein involved in RNA stability. Finally, it is possible that, ERβ may upregulate expression of a proteinase that degrades ECM proteins, resulting in the accumulation of Nid2 and Col11a1, and perhaps laminin and Col4a1 as well, in the absence of ERβ. Further experiments are required to determine which of these potential mechanisms is responsible for the elevated expression of *Col11a1* and *Nid2*, and the other ECM proteins we observed elevated in ERβ-null ovaries.

2.4.4 Potential impact of altered expression of ECM components on ERβ-null ovaries

What impact the elevated levels of ECM protein in the cytoplasm (Col11a1) or in focimatrix (Nid2, Col4a1, laminin) of granulosa cells might have on folliculogenesis or function of the ERβ-null ovary is not clear. It is well established that dramatic changes in

the ECM occur throughout folliculogenesis [24, 25, 27], and that the ECM carries out many functions within the ovary. Within the ovary and follicle, the ECM provides structural support, organizes and connects cells, and serves as a reservoir for signaling molecules that regulate follicle growth. The ECM also regulates establishment of the basement membrane, oocyte maturation, follicle atresia, steroidogenesis, and cell lineage [21, 26]. Further studies testing these specific functional endpoints in ERβ-null ovaries will help determine the potential impact of these overexpressed ECM proteins on ERβ-null ovary and granulosa cell function. The role of the focimatrix in granulosa cell and follicular function is less clear than that of the ECM, and very little is known regarding focimatrix function, although recent studies are beginning to address this question. Irving-Rodgers et al. have demonstrated that cholesterol side-chain cleavage cytochrome P450 (*Cyp11a1*) mRNA levels are highly and positively correlated with the expression of a number of focimatrix proteins in bovine ovaries, suggesting that the focimatrix participates in the selection of a dominant follicle [23, 35]. The same authors have also suggested that focimatrix may trigger the transition of an epithelial granulosa cell to a mesenchymal luteal cell by reducing the polarizing "cue" provided by the follicular basal lamina [34]. Thus, it is possible that the increased Nid2, Col4a1, and laminin expression we observe in the focimatrix of ERβ-null ovaries may impact the steroidogenic capacity of ERβ-null granulosa cells, and indeed, reduced E2 levels have been observed in cultured ERβ-null follicles [18]. Altered focimatrix composition may also affect ERβ-null granulosa cell luteinization, and this effect would be consistent with the dramatically reduced luteinization of ERβ-null granulosa cells in response to LH [4, 5, 41]. Also, given that focimatrix Nid2 levels are lower in bovine partially dominant follicles than in fully dominant follicles or subordinate follicles, it is also possible that increased Nid2 in focimatrix of ERβ-null ovaries may interfere with or alter follicle selection. Further experiments will be required to test these hypotheses.

A surprising finding was that Nid1 mRNA is not elevated in immature ERβ-null granulosa cells, but that its protein expression is significantly higher in the focimatrix of immature ERβ-null follicles than WT follicles. Given this elevated Nid1 expression observed in immature ERβ-null follicles, it was also surprising that Nid1 focimatrix

levels are similar in both genotypes in the adult mouse. One possible explanation for these findings is that ERβ may regulate export or secretion of focimatrix proteins such as Nid1, and that attenuation of this activity might occur with age, resulting in similar Nid1 protein levels in the adult ovaries of both genotypes. The ERβ-dependent regulation of focimatrix protein secretion may also explain the elevated focimatrix levels of Col4a1 and laminin observed in adult ERβ-null ovaries (and for laminin, also in immature ovaries), although Col4a1 and Lama1 mRNA levels were similar in both genotypes in both immature (Figures 2-4 and 2-5) and adult (data not shown) ovaries. A final possibility to explain Col4a1 accumulation in the adult but not the immature focimatrix is that Col4a1 protein may begin to accumulate in the ERβ-null immature focimatrix, but differences between WT and ERβ-null may not be detectable until sufficient Col4a1 has accumulated in the adult to detect these differences. In total, these results suggest that not all focimatrix genes are regulated via the same transcriptional mechanisms, and that ERβ may differentially regulate focimatrix protein export, as differential mechanisms of export have previously been observed for individual ECM proteins [55-57]. Although co-regulated expression of *Nid1*, *Nid2*, and *Col4a1* mRNA has been previously observed in bovine follicles [23], species differences may also account for the lack of coordinated regulation we observe in the ERβ-null ovary.

2.4.5 Conclusions

In summary, we have shown for the first time that disrupted gene expression is observed in the ovaries of immature ERβ-null mice as early as PND 13, resulting in elevated expression of ECM proteins in the extracellular regions within the focimatrix or surrounding granulosa cells within the ERβ-null ovary. This increased expression is also observed in the adult ERβ-null ovary. These findings suggest that ERβ regulates gene expression in the ovary prior to puberty, and we speculate that dysregulation of ERβ-mediated gene expression in early postnatal life may disrupt folliculogenesis and/or contribute to the impaired response of immature ERβ-null granulosa cells to FSH [4, 5].

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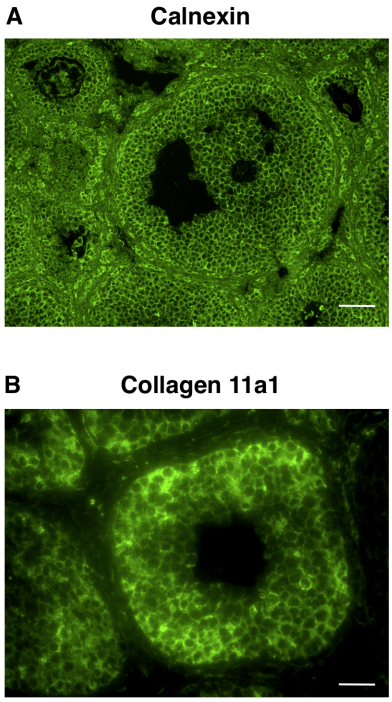

(image from Figure 1C)

Figure S2-1: Calnexin and Col11A1 localize to the cytoplasm of granulosa cells in ovaries of immature PND 23-29 mice

Immunofluorescence with anti-calnexin (A) and anti-Col11A1 (B) antibodies were used to confirm the cytoplasmic localization of (A) calnexin in PND 23-29 wildtype mice, and (B) Col11A1 in PND 23-29 ERβ-null (-/-) mice (identical image to that in Figure 2-1C, section f). (A): Scale bar = 100 μM; (B) Scale bar = 50 μM.

Table S2-1: Dysregulated extracellular matrix genes in ERβ-null granulosa cells compared to ERβ-het cells.

Genbank ID	Gene Symbol	Gene Name	Fold	*P*-value
NM_008695	Nid2	**nidogen 2**	3.9	6.5E-42
NM_178929	Kazald1	Kazal-type serine peptidase inhibitor domain 1	3.7	1.2E-40
NM_007729	Col11a1	**collagen, type XI, alpha 1**	2.3	1.4E-16
NM_175506	Adamts19	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 19	2.3	1.6E-07
NM_016762	Matn2	matrilin 2	1.7	9.3E-10
NM_008606	Mmp11	matrix metallopeptidase 11	1.6	0.00002
NM_011775	Zp2	zona pellucida glycoprotein 2	1.6	9.5E-10
NM_009369	Tgfbi	transforming growth factor, beta induced	1.6	0.00083
AK078108	Ptprz1	protein tyrosine phosphatase, receptor type Z, polypeptide 1	1.5	0.00001
NM_008482	Lamb1	laminin B1 subunit 1	1.4	4.2E-11
NM_009368	Tgfb3	transforming growth factor, beta 3	1.4	3.1E-06
NM_026439	Ccdc80	coiled-coil domain containing 80	1.4	0.00004
NM_009929	Col18a1	collagen, type XVIII, alpha 1	1.4	9.5E-10
NM_007833	Dcn	decorin	1.4	0.00002
NM_175148	N/A	RIKEN cDNA 2300002M23 gene	1.3	0.00002
NM_016696	Gpc1	glypican 1	-1.2	0.00007
AK003211	N/A	RIKEN cDNA 1110001D15 gene	-1.3	0.00093
NM_011261	Reln	reelin	-1.4	0.00041
NM_012050	Omd	osteomodulin	-1.5	5.6E-09
NM_010681	Lama4	laminin, alpha 4	-1.9	0.00007
NM_028266	Col14a1	collagen, type XVI, alpha 1	-1.9	0.00001
NM_145584	Spon1	spondin 1, (f-spondin) extracellular matrix protein	-2.5	6.1E-19
NM_019919	Ltgp1	latent transforming growth factor beta binding protein 1	-2.7	6.8E-20
NM_016685	Comp	cartilage oligomeric matrix protein	-15.7	7.2E-33

Chapter 3

3 The Basic Helix-Loop-Helix Transcription Factor TCF21 Represses Estrogen Receptor β-Mediated Transcription

3.1 Introduction

Estrogens are a class of steroid hormones that regulate cell differentiation, proliferation and function in many tissues. The biological effects of estrogens are facilitated by estrogen receptors (ERs), which are transcription factors that bind both natural and synthetic estrogens to regulate transcription of target genes [1]. The ER belongs to the nuclear receptor family of ligand-inducible transcription factors. Its classic mechanism of action is similar to that of other receptors in this superfamily. In the absence of ligand, the ERs are present in an inactive state within the nuclei. Ligand binding induces a conformational change within the ER, which allows the receptors to dimerize and bind with high affinity to specific sequences, called estrogen response elements (ERE), located within the regulatory regions of target genes and many other regions within the genome [2-4]. The receptors then interact with the general transcription machinery either directly or indirectly via cofactor proteins [3, 5]. The cell and promoter involved will determine whether the ER will exert a positive or negative effect on the expression of the downstream target gene [5].

Two isoforms of ER have been identified, ERα and ERβ. Although both are widely distributed throughout the body, they have distinct levels and expression patterns in different tissues and cell types [6, 7]. ERβ is expressed in fewer tissues than ERα, and is most highly expressed in the ovary [6]. Within the ovary ERβ is restricted to the granulosa cells, which nourish the oocyte and are essential for its growth [8]. Mice lacking ERβ ovulate less frequently and have smaller litters than wildtype mice due to an impaired response to follicle stimulating hormone, resulting in poor granulosa cell differentiation[9].

We have previously shown that the levels of expression of ECM components is disrupted in the ovary of the immature and adult ERβ-null mouse [10]. We have

identified a number of genes of the ECM whose protein levels are significantly higher in ERβ-null follicles than in wildtype follicles. This results in abnormally high expression of ECM components in the ERβ-null ovary, suggesting that these ECM genes are repressed, either directly or indirectly by ERβ. The molecular processes that initiate gene repression by ERβ in vivo are not well understood, and there remains limited information in the literature regarding corepressors specifically involved in ERβ-mediated transcription or transactivation *in vivo* [11-14]. Herein we look at the mechanism by which ERβ may be acting as a transcriptional repressor in granulosa cells.

Transcription factor 21 (TCF21) is a basic helix-loop-helix (bHLH) protein identified for its role in embryonic development [15-17]. Class II bHLH proteins, to which TCF21 belongs, have a tissue-specific pattern of expression. While Class I proteins are widely expressed and capable of binding to DNA as homodimers, a Class II bHLH factor typically requires heterodimerization with a Class I factor to bind at the target gene. TCF21 often binds to the E Box, a consensus CANNTG sequence, as a heterodimer with the ubiquitously expressed E12 protein [15, 16]. A phenotypic analysis of homozygous TCF21 mouse mutants demonstrates that TCF21 plays an important role in the formation of the spleen [18], kidney and lung [19], and is critical for sexual differentiation [20]. Mice lacking TCF21 die in the perinatal period and show male-to-female sex reversal [18-20]. TCF21 is one of the earliest genes expressed in the mesodermal cells that later develop into the gonads [21]. TCF21 transcripts have been identified in fetal and postnatal ovaries of mice, with the levels of TCF21 expression increasing from 13.5 dpc to PND28 [21]. Although it has become clear that TCF21 is essential for normal gonadogenesis, the detailed mechanism of its role in postnatal ovaries remains unclear.

Biochemical and genetic analyses have demonstrated that TCF21 can act as a transcriptional repressor [20-24]. It is the first bHLH protein suggested to act as a general repressor of nuclear receptors [24]. A detailed analysis of its effect on the Androgen Receptor (AR), another member of the nuclear receptor family, has suggested that TCF21 controls AR transcription and function [24]. A yeast two-hybrid screen of a mouse

granulosa cell cDNA library performed by Dr. Deroo revealed a physical interaction between ERβ (amino acids 1-254) and TCF21 (amino acids 23-149) (unpublished data).

To test the *hypothesis* that TCF21 represses ERβ-mediated transcription we utilized transient transfection of immortalized cell lines in combination with reporter assays. We also aimed to identify which regions of ERβ and TCF21 interact for this repression to occur. In the present study we demonstrate that TCF21 represses ERβ-mediated transcription, which is, to the best of our knowledge the first evidence of a bHLH transcription factor repressing the function of ERβ.

3.2 Materials and Methods

3.2.1 Cell Culture

HuH7 human hepatoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Wisent) supplemented with 5% (v/v) fetal bovine serum (FBS; Wisent) and 1% (v/v) penicillin/streptomycin (P/S; Wisent). The HuH7 cells were a gift from Dr. J. Matthews (University of Toronto). MCF7 human breast cancer cells (a gift from Dr. J. Torchia, University of Western Ontario) and COS-7 African green monkey kidney cells (a gift from Dr. J. Mymryk, University of Western Ontario) were both cultured in DMEM supplemented with 10% FBS and 1% P/S. HEC1 cells derived from a human endometrial adenocarcinoma were grown in McCoy's 5a medium supplemented with 10% FBS and 1% P/S. The HEC1 cells were a gift from Dr. B. Katzenellenbogen (University of Illinois) The HEK293 human embryonic kidney cell line (a gift from Dr. A. Babwah, Univerity of Western Ontario) was cultured in Minimal Essential Medium (MEM; Wisent) with 10% FBS and 1% P/S. The KGN cells derived from a human granulosa cell tumour were cultured in Dulbecco modified Eagle medium/F-12 (DMEM/F12; Wisent) supplemented with 10% FBS and 1% P/S. The KGN cells were obtained from the RIKEN BioResource Center. The GFSHR-17 rat granulosa cell line (obtained from Dr. A. Amsterdam, Weizmann Institute of Science) was grown in DMEM/F12 with 5% FBS +1% P/S. All cell lines were maintained at 37°C in 5% CO_2 and subcultured every 2–3 days or when cells reached 80% confluency.

3.2.2 Transient transfection and reporter assays

HuH7 cells were seeded 24 h before transfection in 12-well plates at a density of $1.5x10^5$ cells/well in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS (CS-FBS) and 1% P/S. Complete culture medium was freshly added 1 h before transfection. HuH7 cells were transfected with "GenJet Reagent for HuH7 Cells" (SignaGen) according to the manufacturer's protocol; 0.8 μg plasmid DNA was added to each well. Cells were transfected with 200 ng of receptor (pcDNA-hERβ), 500 ng of reporter (3x-ERE-luc, pS2-Luc, C3-luc or Lf-Luc), 5 ng of the pRL-SV40 renilla luciferase normalization vector, and either 200 ng bHLH expression plasmid (pCMV-SPORT-TCF21 (hTCF21), pcDNA-Mist1 (Mist1) or E12-pCLBabe (E12)) or the empty vector pcDNA3.1 to maintain the total amount of DNA constant per well. The pS2 and Lf-luc were generous gifts from Dr. T. Teng and Dr. K. Korach, respectively (NIH/NIEHS). The C3-ERE-luc reporter, TCF21 and E12 expression plasmids were purchased from Addgene.org. The Mist1 expression plasmid was a kind gift from Dr. C. L. Pin (University of Western Ontario). Transfections were performed in triplicate. Twenty-four hours after transfection cells were treated with 10 nM (10^{-9} M) of 17β-estradiol (estradiol) for 24 h. This dose was selected based on published studies in which HepG2 liver hepatocellular carcinoma cells were treated with estradiol (ranging from 10^{-11} to 10^{-4} M) and ERβ-specific induction of several promoters was investigated [2, 3]. Cells were then rinsed twice with PBS and 1X Passive Lysis Buffer (Promega) was added directly to the culture plates. The plates were gently rocked for 15 min at room temperature (RT) to ensure complete coverage of the cell monolayer. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's standard protocol, and each value was normalized to its Renilla luciferase control. Fluorescence was measured using a Synergy H4 Microplate Reader (Biotek).

HEC1, HEK293 and KGN cells were seeded 24 h before transfection in 24-well plates at a density of 8×10^4 cells/well in phenol red-free culture medium (McCoy's 5a, MEM and DMEM/F12 respectively) supplemented with 10% CS-FBS. Transfections were performed using FuGENE HD (Promega); 0.7 μg DNA was added to each well. A

reagent:complex ratio (µl FuGENE HD:µg DNA) of 7:2 was used to transfect HEC1 and HEK293 cells, and 5:2 to transfect KGNs. Cells were transfected with 90 ng hERβ, 500 ng reporter plasmid, 10 ng pRL-SV40 and 100 ng hTCF21 or pcDNA plasmid. Treatments with estradiol and luciferase assays were performed as described above for HuH7 cells.

MCF7 and COS7 cells were seeded 24 h before transfection in 6-well plates at a density of $2.5x10^5$ in phenol red-free DMEM with 10% CS-FBS. Both cell lines were transfected using FuGENE 6 (Promega) according to the manufacturer's protocol; a complex of reagent and plasmid was prepared at a ratio of 3:1 (µl FuGENE 6:µg DNA) with 1 µg DNA added per well. The amount of receptor plasmid, reporter plasmid and TCF21/pcDNA plasmid transfected was consistent with the HuH7 protocol described above. The lacZ expression plasmid pCMVβ (200 ng) was used as an internal transfection control in these reporter assays. Twenty-four hours after transfection cells were treated with 10 nM of estradiol for 24 h. Luciferase activity was assayed using the Luciferase Assay System (Promega) and normalized to β-galactosidase activity determined by X-gal staining.

All experiments were repeated a minimum of three times, and reproducible results were obtained in independent experiments.

3.2.3 Cell extracts and Western blotting

Protein lysates from adult mouse tissues were generated by homogenizing tissues in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) with a protease inhibitor cocktail (1:100; Sigma) until the resulting lysate was completely homogeneous. Lysates were incubated on ice for 20 min then clarified by centrifugation (15 000 × g for 20 min at 4°C). Total cellular protein from adherent cells was isolated using Pierce IP Lysis Buffer (Thermo Scientific) with 1X Halt Protease Inhibitor Cocktail (Thermo Scientific), clarified by centrifugation (13 000 × g for 10 min at 4°C) and quantified by DC Protein Assay (Bio-Rad).

Protein extract was boiled in Laemmli Buffer for 5 min and separated by SDS-PAGE (40 μg/lane) using a 12% gel. The separated proteins were then transferred to a polyvinylidene difluoride membrane (PVDF; Roche) at 100V for 1 h at 4°C, and blocked for 1 h at RT with 5% skim milk in Tris-buffered saline with Tween-20 (TBST). Following washes with TBST, the membrane was incubated with anti-TCF21 antibody (1:200 in 5% skim milk/TBST; Santa Cruz Inc. sc-15007) overnight at 4°C, then with a peroxidase-conjugated anti-goat (1:10 000 in 5% skim milk/TBST; Santa Cruz) for 1 h at RT. Immunoreactive bands were visualized using enhanced chemiluminescence reagent (ECL Plus; Amersham Biosciences) and Hyperfilm (Amersham).

3.2.4 Coimmunoprecipitation

Optimization of coimmunoprecipitation assays was performed with HuH7, HEC1, HEK293 and MCF7 cell lines. Cells were seeded 24 hours before transfection in 100 mm dishes and transfected with 10 μg of DNA (hERβ, FLAG-ERβ, hTCF21, hERβ + hTCF21, FLAG-ERβ + TCF21, hAR, hAR + hTCF21 or pcDNA3.1). The appropriate transfection reagent for each cell line was used as described above. Transfected cells were treated 24 and 48 hours later with 10 nM ligand (ERβ transfected cells with estradiol, AR transfected cells with testosterone) for 24 and 48 hours.

We tested four lysis buffers to isolate cellular proteins: Radioimmunoprecipitation assay (RIPA) buffer A (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a protease inhibitor cocktail (1:100; P8340; Sigma), RIPA buffer B (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 2.5 mM EGTA, 1% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM Na_3VO_4) supplemented with a protease inhibitor cocktail (1:100; Sigma), Pierce IP Lysis Buffer (Thermo Scientific) with 1X Halt Protease Inhibitor Cocktail (Thermo Scientific) and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Cells harvested with RIPA A or RIPA B Buffer were incubated for 20 (or 40 min) at 4°C with gentle rocking, then scraped and transferred to a 1.5-mL microcentrifuge tube. Lysates were then clarified by centrifugation (RIPA A: 15 000 × g for 20 min at 4°C; RIPA B: 23 000 × g for 20 min at 4°C). Pierce IP Lysis Buffer and NE-PER reagents were used

according to the manufacturer's protocol to isolate total cellular protein. Total protein was quantified using the DC Protein Assay (Bio-Rad).

Whole cell lysate (500 μg) was incubated with 50 μl of 50% protein G Sepharose Fast Flow (Sigma) slurry and one of the following antibodies [TCF21 (Abcam; ab32981); TCF21 (Santa Cruz; sc-15007); ERβ (Abcam; ab16813); ERβ (Abcam; ab92306); ERβ (Santa Cruz; sc-8974); AR (Santa Cruz; sc-815); FLAG (Sigma; F7425)] overnight, rotating at 4°C. The following day, the beads were washed four to six times with lysis buffer at 4°C, boiled in Laemmli Buffer for 5 min and separated by SDS-PAGE (50 μg/lane) using a 12% gel. The separated proteins were then transferred to a PVDF membrane (Roche) and probed for coimmunprecipitated proteins. Because we were attempting to optimize this protocol, we ran samples in duplicate whenever possible which allowed us to also probe for the immunoprecipitated protein with its own antibody. When this was not possible the single membrane was stripped and reprobed for the immunoprecipitated protein. The protein bands were detected with an enhanced chemiluminescence reagent (ECL Plus; Amersham Biosciences) and Hyperfilm (Amersham).

3.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data were expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey's post-hoc test with significances set at *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ as indicated.

3.3 Results

It has been reported that TCF21 is critical for sexual differentiation[20] and its expression increases in the murine whole ovary from the embryonic to postnatal stages[21]. Because ERβ is predominantly expressed in granulosa cells of the ovary we sought to determine whether granulosa cells also express TCF21. I have confirmed by Western blot that TCF21 is expressed in both primary mouse granulosa cells and in several granulosa cell lines, as well as various cell lines of other tissue origin, and mouse

tissues (Fig 3.1). HEK293 cells were transfected with hTCF21 expression plasmid to serve as a positive control in Western blot analysis.

3.3.1 TCF21 is a novel transcriptional repressor of ERβ-mediated transcription

The malignant liver cell lines HuH7[25] and HepG2[2, 3] are commonly used in the literature to study ER transcriptional activity because they do not have functional endogenous ER and therefore require exogenous ER to activate ERE-mediated transcription. Therefore, we elected to use HuH7 cells for each of our assays. Taking into account that different cell types will have different endogenous cofactors, we also used other cell lines to determine whether the effect of TCF21 on ERβ transcriptional activity is cell-line or cell type-specific.

To examine the possibility that TCF21 may modulate ERβ transcriptional activity we performed transient transfections and luciferase assays. We utilized both ER negative (HuH7 [25], HEC1 [26], COS7 [27], HEK293 [27]) and ER positive (MCF7 [high endogenous ERα, lower levels of ERβ] [28], KGN [ERβ positive, ERα negative] [29]) cell lines for these assays. Cells were co-transfected with an ERβ expression plasmid, a synthetic estrogen-responsive 3x-ERE (three copies of the vitellogenin estrogen response element) firefly luciferase reporter vector, a renilla luciferase or LacZ normalization vector and either hTCF21 or an empty vector. Because ERβ is a ligand-inducible transcription factor the cells were then treated with 10 nM estradiol (E2) for 24 hours. Luciferase assays were performed to determine the effect of TCF21 on ERβ-mediated transcriptional activity.

HuH7 cells were initially transfected with an increasing hTCF21:ERβ ratio (1:1, 2:1, and 3:1) to optimize transfection efficiency. Surprisingly, increasing the amount of TCF21 had no significant effect on ERβ-mediated transcription of the 3x-ERE luciferase reporter, which contains three copies of the canonical estrogen response element (Fig 3.2). Therefore, the 1:1 ratio was used for subsequent experiments.

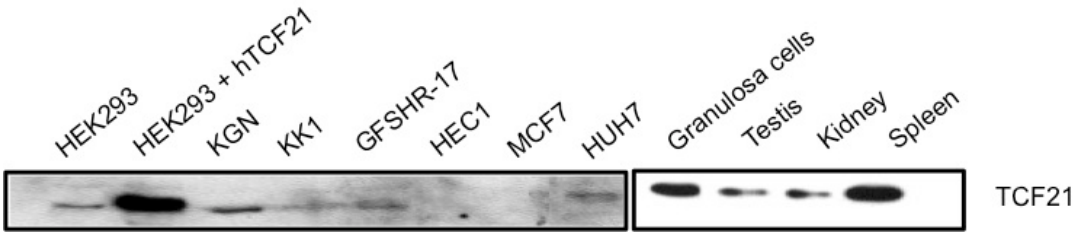

Figure 3-1: TCF21 expression in cell lines and mouse tissues.

Whole cell extracts from adult mouse tissues (granulosa cells, testis, kidney and spleen) as well as immortalized cell lines (HEK293, HEK293 transfected with a hTCF21 expression vector, KGN, KK1, GFSHR17, HEC1, MCF7 and HuH7) were analyzed by Western blot to detect TCF21 protein expression.

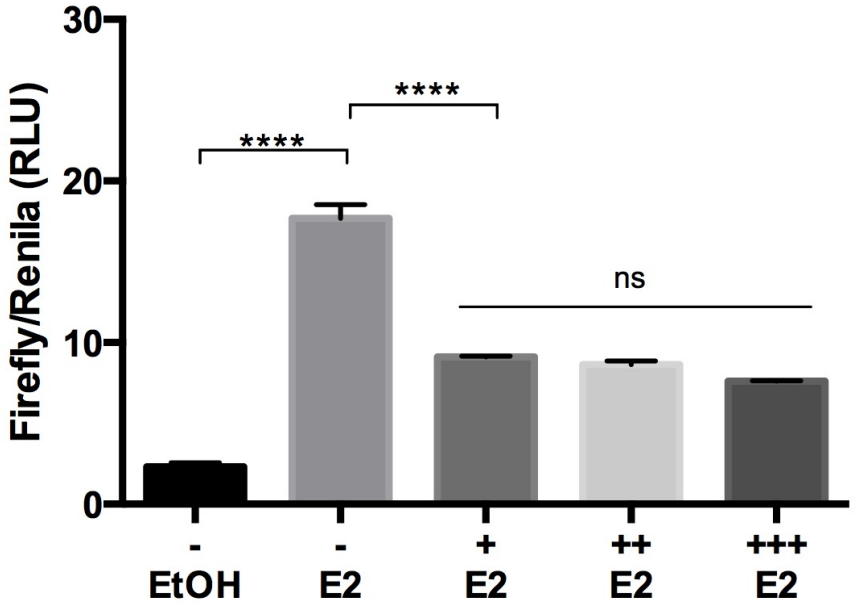

Figure 3-2: Effect of TCF21 on ERβ-mediated transcription.

HuH7 cells were transiently transfected with a 3x-ERE-luc reporter (200 ng), pRL-SV40 (5 ng) and increasing amounts of TCF21 expression plasmid (+, 200 ng; ++, 400 ng; +++, 600 ng). They were then treated with 10 nM estradiol (E2) (24 hours). Dual luciferase assays were performed. Data are expressed as means ±SEM of three independent experiments performed in triplicate. ****, $p < 0.0001$; ns = not significant (one-way ANOVA and Tukey's *post-hoc* test). RLU, relative light unit.

We have demonstrated that TCF21 represses ERβ transactivation of the 3x-ERE-luc reporter in six mammalian cell lines (Fig 3.3). The level to which E2 activates and TCF21 inhibits ERβ-mediated transcription is cell-line dependent. The lowest level of E2-induced activation was observed in the KGN cell line (Fig 3.3D), which is not surprising because ERE-luc is poorly activated by E2 when either ER form is transfected in KGN cells[29]. The relative level of repression by TCF21 is also low (33%), yet significant. In all remaining cell lines we studied TCF21 represses ERβ-mediated activation of the 3x-ERE-luc by at least 50%. TCF21 had no significant affect on ERα transactivation (Fig S3.1).

3.3.2 TCF21 inhibits ERβ transactivation of naturally occurring estrogen response elements

Estradiol does not activate transcription from all estrogen-responsive promoters in an equivalent manner and studies have shown that the transcriptional activity of ER is significantly affected by the nature of the target promoter [2]. Most naturally occurring EREs are imperfect, non-canonical EREs; therefore we used three well-known naturally-occurring estrogen-responsive promoters to determine if TCF21 would repress their estrogenic activity. Specifically, we studied the lactoferrin (Lf), pS2 and complement 3 (C3) promoters. We tested these promoters in two ER negative cell lines – HuH7 and HEC1; we elected to use these cell lines because the former is TCF21 positive, while the latter does not express endogenous TCF21 (Fig 3.1). We have demonstrated that TCF21 represses ERβ-mediated transcriptional activity of all three natural estrogen-responsive promoters; the extent to which TCF21 inhibits ERβ transactivation depended on the nature of the response element as well as being cell-type dependent (Fig 3.4). TCF21 has a significantly greater repressive effect on pS2 activation compared to the C3-ERE in HuH7 cells. Lactofferin is neither activated upon E2 treatment, nor repressed when TCF21 expression plasmid is co-transfected in HuH7 cells (Fig 3.4A). In HEC1 cells, the relative level of repression by TCF21 is C3>pS2>Lf. Surprisingly, pS2 was not activated by E2 treatment yet we still observed significant repression of the promoter by TCF21 (Fig 3.4B).

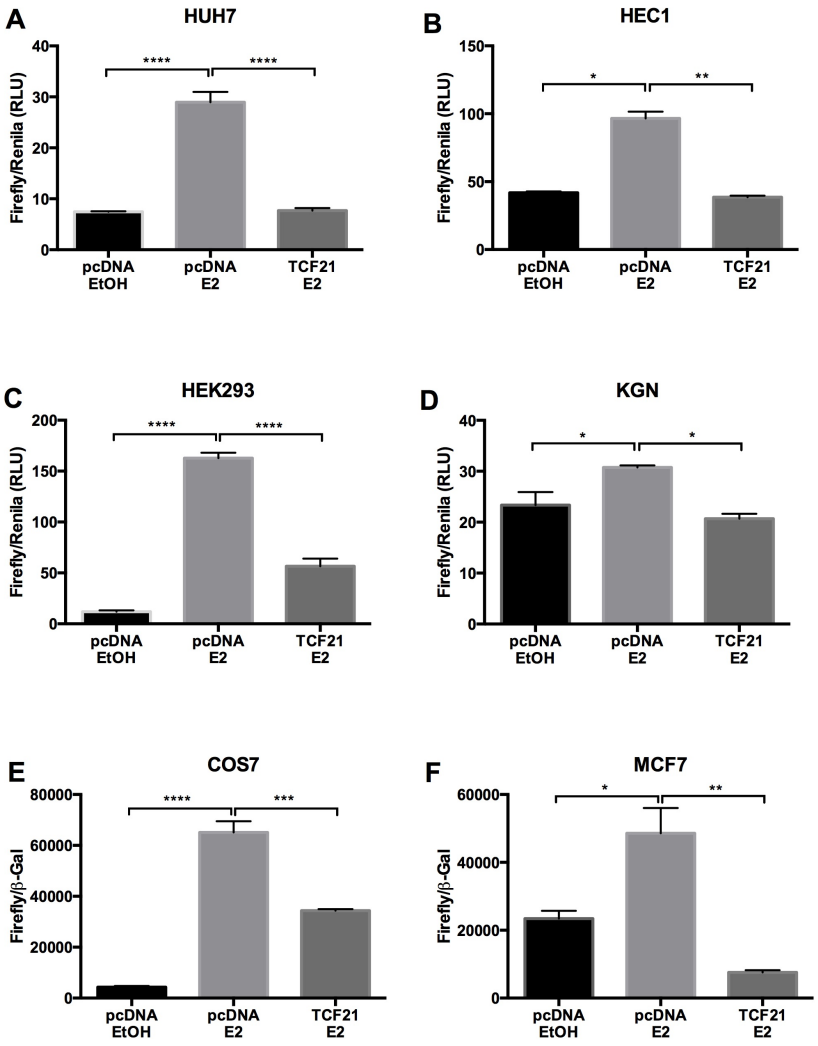

Figure 3-3: TCF21 represses ERβ-mediated transcription of a 3x-ERE reporter.

Various cell lines were transiently co-transfected with an ERβ expression plasmid, a 3x-ERE firefly luciferase reporter vector, a renilla luciferase (A-D) or lacZ (E-F) normalization vector, and either the TCF21 expression plasmid or empty vector (pcDNA). The cells were then treated with 10 nM estradiol (E2) for 24 hours. Dual luciferase assays were performed to determine ERβ-mediated transcriptional activity. All values represent mean ±SEM of three separate experiments. *p<0.01; **p<0.01; ***p<0.001; ****p<0.0001 as determined by one-way ANOVA and Tukey's *post-hoc* test. RLU, relative light unit.

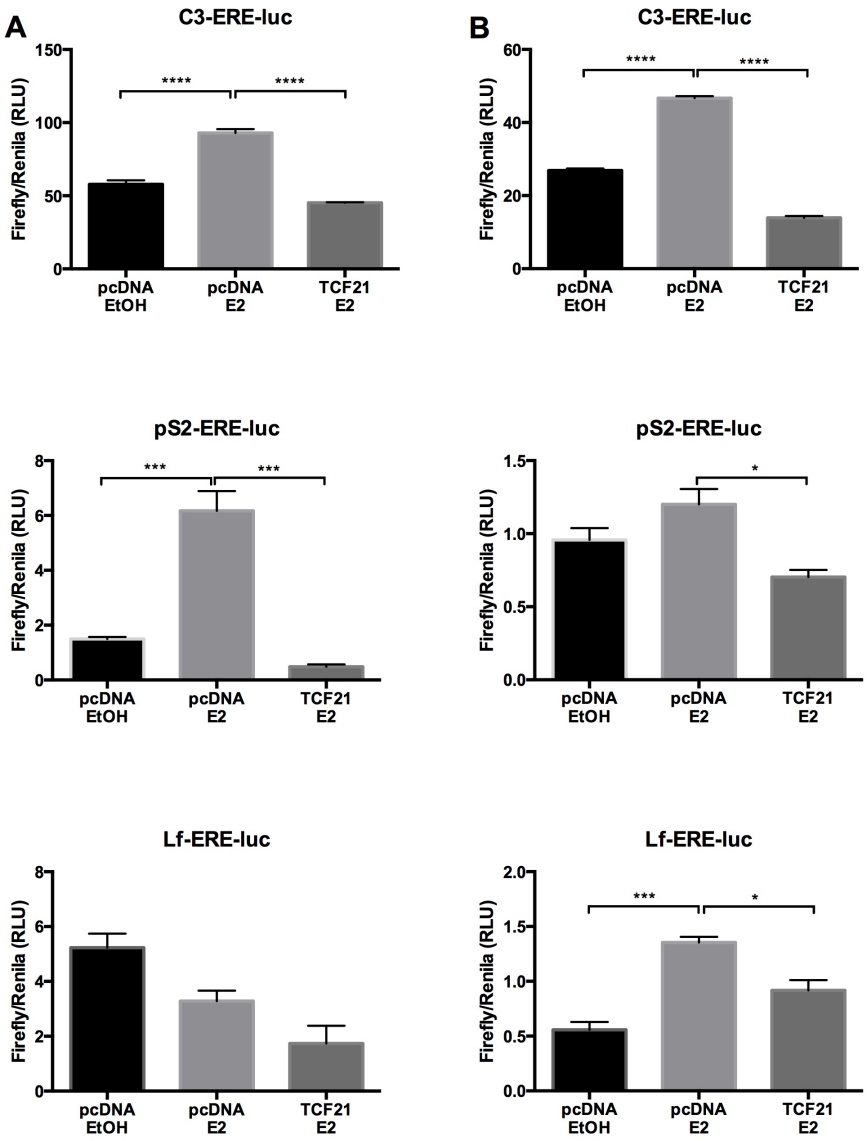

Figure 3-4: TCF21 represses ERβ transactivation of natural estrogen-responsive promoters.

HuH7 (A) and HEC1 (B) cell lines were transfected with ERβ expression plasmid, a native estrogen-responsive promoter (C3, pS2 or Lf), a renilla luciferase normalization vector, and either TCF21 or empty vector (pcDNA). The cells were then treated with 10 nM estradiol (E2) for 24 hours. Dual luciferase assays were performed to determine ERβ-mediated transcriptional activity. All values represent mean ±SEM of three separate experiments. *p<0.01; ***p<0.001; ****p<0.0001 as determined by one-way ANOVA and Tukey's *post-hoc* test. RLU, relative light unit.

3.3.3 ERβ transactivation is not inhibited by co-transfection with Mist1, another Class II bHLH protein

To confirm that the repression of ERβ transactivation by TCF21 is specific, rather than a broad response to co-transfection of ERβ with a Class II bHLH protein, we tested the effect of another Class II bHLH transcription factor on ERβ-mediated transcription. Mist1 is a bHLH transcription factor expressed in pancreatic acinar cells and other serous exocrine cells. It represses myogenic differentiation by targeting the MyoD gene[30]. We showed that Mist1 had no effect on ERβ-mediated transcription of the 3x-ERE reporter (Fig 3.5), demonstrating that the repression we have observed by TCF21 is specific and cannot be achieved by all Class II bHLH transcription factors.

3.3.4 Repression of ERβ transactivation by TCF21 does not require E12

E12 is a Class I bHLH transcription factor that often heterodimerizes with Class II bHLH transcription factors. TCF21 has previously been shown to transactivate promoters alone or in combination with E12[15, 16, 24, 31]; therefore we tested whether E12 was required for optimal TCF21-mediated repression of ERβ activity. We found that ERβ-mediated transactivation does not require TCF21 heterodimerization with E12 in this model (Fig 3.6). Unlike TCF21, E12 alone enhanced the effect of E_2 treatment on 3x-ERE-luc promoter activity. Interestingly, co-expression of E12 with TCF21 appeared to antagonize the ability of TCF21 to repress ERβ-mediated estrogenic activity.

3.3.5 ERβ and TCF21 interaction

After successfully demonstrating that TCF21 represses ERβ-mediated transcription *in vitro*, we next determined whether ERβ and TCF21 interact to form a complex *in vivo.* Both proteins are localized in the nucleus [23, 32] and Dr. Deroo's previous yeast two-hybrid screen suggested that the repressive effect of TCF21 on ERβ involved their physical association. Our objective was to assess their direct physical interaction using both GST-pull-down analyses and co-immunoprecipitation (Co-IP) experiments in TCF21 and ERβ-containing granulosa cell lines, with the ultimate goal of testing their interaction in primary granulosa cells.

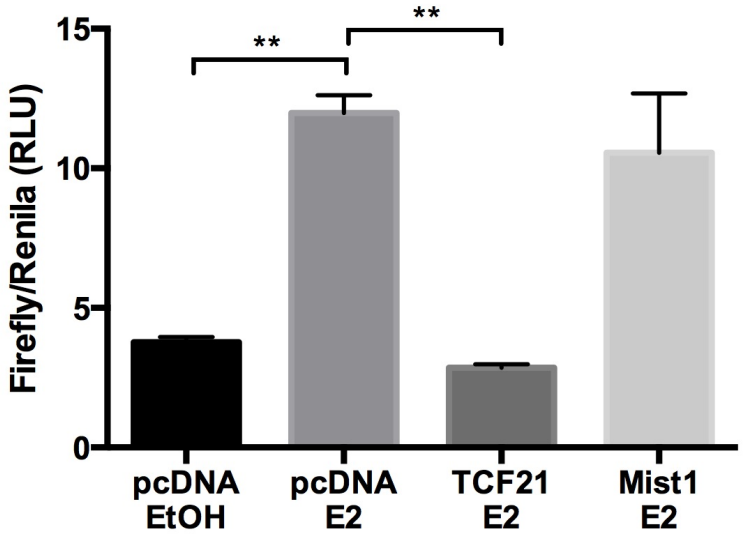

Figure 3-5: Mist1 does not repress ERβ-mediated transcription of a 3x-ERE reporter.

HuH7 cells were transfected with an ERβ expression plasmid, 3x-ERE firefly luciferase reporter vector, a renilla luciferase normalization vector, and either an empty vector (pcDNA), TCF21 or Mist1. The cells were then treated with 10 nM estradiol (E2) for 24 hours. Dual luciferase assays were performed to determine ERβ-mediated transcriptional activity. All values represent mean ±SEM of three separate experiments. $^{**}p<0.011$ as determined by one-way ANOVA and Tukey's *post-hoc* test. RLU, relative light unit.

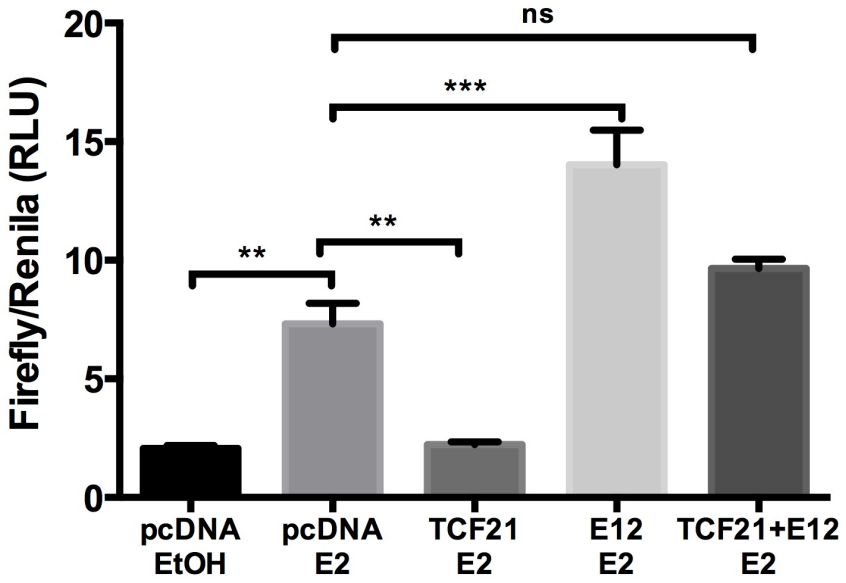

Figure 3-6: E12 is not required for TCF21 to repress transactivation of ERβ.

HuH7 cells were transfected with ERβ expression plasmid, 3x-ERE firefly luciferase reporter vector, a renilla luciferase normalization vector, and either an empty vector (pcDNA), TCF21 and/or E12. The cells were then treated with 10 nM estradiol (E2) for 24 hours. Dual luciferase assays were performed to determine ERβ-mediated transcriptional activity. All values represent mean ±SEM of three separate experiments. $^{**}p<0.01$; $^{***}p<0.001$; ns, not significant, as determined by one-way ANOVA and Tukey's *post-hoc* test. RLU, relative light unit.

Initial experiments focused on GST pull-down assays. However, we were unable to successfully purify ERβ or its deletion mutants (data not shown). We next turned our attention to Co-IP experiments. Our first objective was to optimize our protocol using the previously described protocol that demonstrated the interaction between TCF21 and the Androgen Receptor (AR) (Hong et al); however, we were unable to reproduce these data primarily due to non-specific binding by the AR antibody (data not shown). Therefore we proceeded to determine whether our proteins of interest, ERβ and TCF21, interact. Despite our best efforts we were unable to produce evidence of TCF21-ERβ interactions (data not shown). For over a year we tested a large variety of conditions including: four cell lines, four lysis buffers, duration of cell lysis, duration of transfection and ligand treatment, as well as numerous antibodies from different commercial sources. We were unable to show interaction, regardless of which protein we used for the IP. The most significant obstacle was the lack of a trusted ERβ antibody; all antibodies tested produced inconsistent results, significant background and non-specific bands in Western blot analyses [33]. We also attempted co-transfecting and detecting FLAG-ERβ without success. Therefore, although we were able to successfully demonstrate TCF21 repression of ERβ activity at the transcriptional level, we were unable to show they form a complex *in vivo*.

3.4 Discussion

Nuclear receptors (NR) and basic helix-loop-helix (bHLH) proteins are two superfamilies of transcription factors. The NRs regulate vital processes such as reproduction, development and metabolism, while the bHLH factors are involved in regulation of the cell cycle as well as many developmental processes. The interactions between members of the NR and bHLH families allow for accurate expression of downstream target genes.

Estrogens regulate many important physiological processes, including tissue-specific gene regulation in the reproductive tract. The biological responses to estrogens are mediated by estrogen binding to one of two specific estrogen receptors, ERα or ERβ. Both ERs are expressed in the body, yet there are considerable differences in their tissue

distribution [34]. Within the ovary ERβ is restricted to the granulosa cells, whereas ERα is expressed in the thecal cells [8]. Granulosa cells are responsible for estradiol synthesis, nourish the oocyte throughout folliculogenesis and are essential for its growth. Therefore, the function of ERβ in granulosa cells during follicle development has to be tightly regulated for proper ovarian function. As a member of the NR superfamily, ERβ regulates gene expression by binding to an estrogen response element and/ or by forming protein-protein complexes with other transcription factors.

3.4.1 TCF21 is a repressor of ERβ-mediated transcription

In this study, we investigated the role of the bHLH transcription factor TCF21 in regulating transcription of estradiol target genes. Our promoter assays in numerous mammalian cell lines demonstrated that TCF21 has an ER isoform-specific affect in regulating estradiol-dependent transcriptional activity. We observed significant repression of ERβ transactivation of the synthetic 3x-ERE promoter as well as three ER target genes that contain imperfect EREs (C3, pS2 and Lf), however, we saw no impact on ERα-mediated transcription. To our knowledge, this is the first bHLH transcription factor identified to act as a specific co-repressor of ERβ.

TCF21 has previously been reported to exert inhibitory effects on gene expression[20-24] and shown to associate with repressor complex proteins [35]. The transactivation property of TCF21 was first analyzed using the Gal4 fusion system, which found that TCF21 exhibits repressive activities in HepG2 cells from its C-terminal and N-terminal domains; constructs that include either of these domains retained repression activity, while the bHLH domain alone had no activity [22]. Conversely, transactivation activities in HT1080 and HeLa cells were only observed when constructs retained the C-terminal domain. It is suggested that this dual function of the C-terminal domain may be cell-type dependent, likely a result of specific coactivators and corepressors [22]. Interestingly, we found that the repression by TCF21 on ERβ-mediated transcriptional activity is not cell-type dependent.

3.4.2 TCF21 does not dimerize with E12 to repress ERβ-mediated transcription

TCF21 has previously been shown to interact with other bHLH transcription factors – E12 (TCF3), HEB (TCF12) and E2-2 (TCF4) [36]; E12 is the most likely Class I bHLH to heterodimerize with TCF21. We found that E12 does not improve the ability of TCF21 to repress ERβ transactivation; rather E12 acts as a coactivator and interferes with TCF21 repression. In this system E12 may be inhibiting the ability of TCF21 to bind an E box or preventing its interaction with ERβ. We cannot exclude the possibility that TCF21 heterodimerizes with another Class I bHLH to repress the activation of estradiol target genes.

3.4.3 Future studies

Many studies have focused on the molecular mechanisms that regulate the transcription of the ERα gene [37-39]; however, the mechanisms that regulate the ERβ gene remain largely unclear. Fujimoto et al. identified good homology between the human, mouse and rat ERβ promoters (80% identity between positions −1 and −550 between the rat and mouse, and 69% identity between −30 and −110, as well as −300 and −400 between the rat and the corresponding section of the human promoter) [40], and an evolutionarily conserved E box in the 5' promoter region of ERβ exists which has previously been shown to bind the bHLH-zip factor USF [40, 41]. The presence of an E-Box does not imply that it will be active for all bHLH proteins. However, considering TCF21 has previously been shown to act as a transcriptional repressor, including a repressor of the nuclear receptor AR, it may also function as a negative transcriptional regulator of the ERβ gene by binding to the E-Box within the ERβ promoter. In future studies it may be of interest to perform chromatin immunoprecipitation (ChIP) assays and determine whether TCF21 is recruited to the E-Box found in the ERβ promoter. If TCF21 and ERβ form a complex *in vivo*, further ChIP and GST-pulldown assays could be used to investigate the potential involvement of histone deacetylases (HDACs), well understood downstream recruits of corepressors that are often found within a repressor complex [11].

3.4.4 Limitations of study

The yeast two-hybrid screen suggests that the functional interaction we have observed between TCF21 and ERβ involves their physical association. Our objective was to identify what regions of ERβ and TCF21 interact using GST-pulldown assays; however, our attempts to purify GST-tagged ERβ and ERβ deletion mutants did not yield a detectable product. We also attempted to utilize Co-IP analysis to determine whether these proteins form a complex *in vivo*. In spite of our best efforts, testing numerous conditions and variables, we were unable to optimize the Co-IP protocol and confirm an ERβ/TCF21 complex. The most significant obstacle was the lack of a reliable ERβ antibody, an appreciated issue in the ERβ field [33, 42]. We also tried transfecting a FLAG-tagged ERβ into HuH7 and HEK293 cells to circumvent the need for an ERβ antibody. Unfortunately, we were unable to produce reliable results with a FLAG antibody as well. Despite our best efforts, we were disappointed with inconsistent results.

3.4.5 Potential mechanisms by which TCF21 inhibits ERβ transactivation

Since we were unable to obtain results from our Co-IP experiments due to the lack of a reliable ERβ antibody, we were also unable to conclusively determine whether it is by a passive or active mechanism that TCF21 inhibits ERβ transactivation. Therefore, we are proposing two potential mechanisms by which TCF21 may be acting as a corepressor. The first is a classic mechanism of transcriptional repression whereby a repression complex is formed and recruited to a target gene promoter. The second is based on a mechanism recently proposed for repression of ERα transcriptional activity [43]. 1) In the absence of TCF21, ERβ is recruited to the ER-target gene promoter upon ligand binding and target gene transcription is initiated. When TCF21 is expressed, it is recruited together with ERβ once ligand is introduced. Upon ligand binding, a TCF21/ERβ complex is formed and ERβ activity is inhibited. 2) Alternatively, TCF21 and ERβ could both be present on the ER-target gene promoter in the absence of ligand. Once ligand is introduced and ERβ is activated, TCF21 gradually is released from the promoter as more ERβ is recruited, and target gene transcription begins. Consequently, when we over-express TCF21 in promoter assays, more TCF21 remains bound to the ER-

target gene promoter, out-competing ERβ, and repression of ERβ transcriptional activity is observed.

3.4.6 Conclusions

We have demonstrated that TCF21 regulates estradiol-dependent transcriptional activity in an ER isoform-specific manner; TCF21 represses ERβ, but not ERα transactivation. Further studies with cell type-specific knockout of TCF21 in granulosa cells would allow for analysis of its role and its mechanism in the development and function of the granulosa cells and the ovary. Once a trusted antibody for ERβ is identified, it would be greatly beneficial to not only determine the interaction between TCF21 and ERβ in cell lines, but also in primary granulosa cells.

3.5 Bibliography

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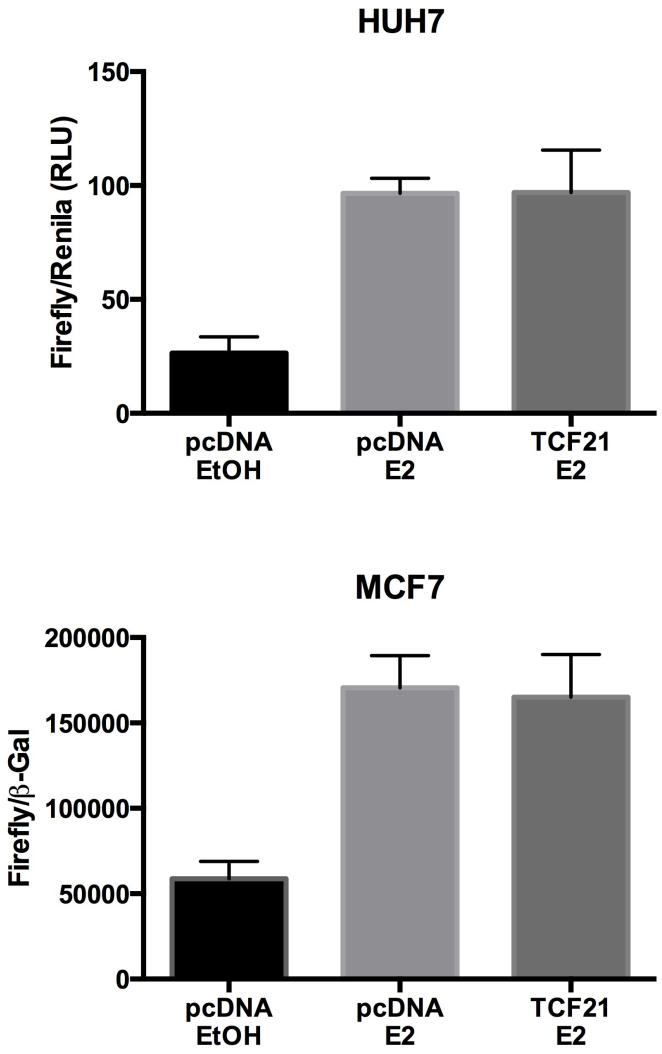


Figure S3-1: TCF21 does not repress ERα transactivation.

HuH7 and MCF7 cell lines were transfected with ERβ expression plasmid, 3x-ERE firefly luciferase reporter vector, a renilla luciferase normalization vector, and either TCF21 or empty vector (pcDNA). The cells were then treated with 10 nM estradiol (E2) for 24 hours. Dual luciferase assays were performed to determine ERβ-mediated transcriptional activity. All values represent mean ±SEM of three separate experiments. No significance was found by one-way ANOVA and Tukey's *post-hoc* test. RLU, relative light unit.

Chapter 4

4 Characterization of the extracellular matrix protein Spondin 1 and its function in immortalized ovarian cancer cell lines and primary ascites-derived cancer cells

4.1 Introduction

Ovarian carcinoma is the most lethal gynaecological malignancy, and the fourth most common cause of female cancer death, in the world [1]. Despite modern management the prognosis remains poor, with a five-year survival rate of 45% after initial diagnosis [2]. The prognosis is more favourable for patients with stage I/II tumours; however, most patients present with advanced disease (III/IV) [1].

Ovarian cancer is a heterogeneous disease [3]. Epithelial ovarian tumours are classified as serous, clear cell, endometrioid, and mucinous subtypes. Each subtype is correlated with different genetic risk factors and molecular events during oncogenesis [4], and each has a characteristic mRNA profile [5, 6]. Serous ovarian cancer represents approximately 70% of epithelial ovarian cancers (EOC) [7]. The most aggressive subtype, high-grade serous ovarian carcinoma, accounts for 90% of serous cancers, two-thirds of all EOC deaths, and it is the most studied subtype [8].

Metastasis of EOC is unique because unlike most solid tumours, ovarian cancer rarely disseminates through the vasculature; rather malignant cells spread from the primary tumour into the peritoneal cavity [9]. The peritoneal cavity accumulates ascites fluid containing malignant single EOC cells or EOC aggregates called spheroids [10]. The successful adhesion of these cells to the surfaces of abdominal organs is a key step controlling ovarian cancer metastasis and the formation of secondary tumours [9].

Many of the same cellular processes and behaviours that are necessary for normal tissue development are also essential for cancer progression. An example of such a process is remodelling of the extracellular matrix (ECM), which is closely regulated during normal development. When these remodelling pathways fail, cells can grow

uncontrollably, such that changes to the ECM composition can occur and tumours develop [11]. Another important aspect of cancer progression is the tumour microenvironment, which is composed of ECM proteins, fibroblasts and endothelial cells [12]. The tumour microenvironment can have a direct effect on cell proliferation, migration and differentiation through secreted proteins, cell–cell interactions and ECM remodelling [12].

Ovarian cancer cells are able to adjust the type and amount of ECM proteins they produce and secrete into their microenvironment, which can result in both stimulatory and inhibitory affects on tumour development [13]. The ECM is in direct contact with the tumour cells, thereby providing factors involved in growth, survival, motility, and angiogenesis, which affect the progression of the tumour [11, 14]. Furthermore, cell adhesion to the ECM through integrins and other cell surface receptors initiates signalling pathways, that are involved in regulating migration and differentiation [14]. ECM components can also contribute to tumour dormancy and enhance chemotherapy resistance [11, 15]. Therefore, as a key component of the tumour niche, the ECM is essential at various stages of tumourigenesis, and it may serve as a rate-limiting step in cancer progression and provide a potential target for therapeutics.

Spondin 1 (SPON1), also referred to as F-Spondin and vascular smooth muscle cell growth-promoting factor (VSGP), is a secreted ECM protein that was originally identified in the mouse embryonic floorplate [16]. SPON1 is a member of the diverse thrombospondin type 1 repeat superfamily, and possesses six C-terminal thrombospondin repeats (TSRs), an N-terminal reeler domain, and a spondin domain [16, 17]. The reeler domain is homologous with a domain in Reelin, a protein involved in neuronal migration [16, 18]. The spondin domain is homologous with regions in Mindin, a secreted protein and member of the thrombosondin type 1 family that binds to the ECM [16, 18, 19].

SPON1 has been primarily studied for its role in the central nervous system (CNS), where it regulates migration of neurons during embryonic development and cell adhesion in many cell types of the CNS [17, 19-22]. Apart from neuronal tissues, SPON1 has been shown to affect differentiation, proliferation and migratory functions in other

tissues. SPON1 localizes to integrin-containing tissues and basement membranes in *C. elegans*, coupling with integrins to maintain ECM adhesion [23]. SPON1 promotes growth of vascular smooth muscle cells [24] and increases cell survival of murine neuroblastoma [25]. It promotes the differentiation of chondrocytes [26], a human cementoblast-like cell line (HCEM) [27], and inhibits differentiation of osteoclastic precursors [28]. It has also been shown to promote the migration and invasion of osteosarcoma cells *in vitro* [29], and to inhibit the migration of HUVECs [30] and HCEMs [27].

SPON1 isolated from bovine follicular fluid has growth promoting properties [24], and our laboratory has recently demonstrated that SPON1 regulates steroidogenesis and increases proliferation of ovarian granulosa cell lines *in vitro* (unpublished data). Interestingly, Spondin 1 mRNA expression is higher in ovarian tumours than in normal ovarian tissues [31]. Furthermore, a recent study involving an extensive screen of ovarian carcinomas from 500 patients has suggested SPON1 is a promising biomarker for ovarian cancer, particularly high grade serous EOC, and a potential target for cancer immunotherapy [32]. However, the functional role of SPON1 in ovarian cancer cells has yet to be examined.

We hypothesized that SPON1 regulates properties essential for the progression and metastasis of epithelial ovarian cancer cells. Herein, we used EOC cell lines and primary ascites-derived ovarian cancer cells to examine the effect of exogenous recombinant human SPON1 on the adhesion, migration, viability and proliferation of ovarian cancer cells.

4.2 Materials and Methods

4.2.1 Culture of cell lines and ascites-derived cells

All experiments described below were performed in three established ovarian cancer cell lines: OVCAR3, OVCAR8 and HEY. OVCAR3 and OVCAR8 cells were originally established from the ascites fluid of patients with progressive adenocarcinoma that had prior chemotherapy treatment[30]. The HEY cell line is derived from a human ovarian cancer xenograft originally grown from a peritoneal deposit of a patient

diagnosed with moderately differentiated cystadenocarcinoma of the ovary[31]. Our preliminary studies also included the OVCAR5 and OVCA429 EOC cell lines. OVCA429 cells were cultured in Alpha Modified Eagle Medium (Wisent) supplemented with 5% (v/v) fetal bovine serum (FBS; Wisent), 1% (v/v) penicillin/streptomycin (P/S; Wisent) and 1x non-essential amino acids (Invitrogen). The remaining four cell lines were cultured in RPMI-1640 supplemented with 5% FBS and 1% P/S. All cell lines were a generous gift from Dr. T. Shepherd and Dr. G. DiMattia (University of Western Ontario).

Work with patient materials was approved by The University of Western Ontario Health Sciences Research Ethics Board and coordinated with Dr. Trevor Shepherd of the Translational Ovarian Cancer Research Group. Dr. Shepherd and Dr. Gabriel DiMattia generously provided all primary ascites cell cultures, collected from patients with advanced stage (III or IV) high-grade serous epithelial ovarian cancer. Patient cultures were generated from ascites fluid collected from patients at the time of paracentesis or debulking surgery. Patient EOC samples were cultured in Dulbecco Modified Eagle Medium/F12 (Wisent) supplemented with 10% FBS and 1% P/S. Fresh media was replaced every other day. All experiments with primary EOC cells were performed between passages 3 and 5. All cells were maintained at 37°C in 5% CO_2 and subcultured when cells reached 80% confluency.

4.2.2 Cell extracts and secreted protein concentration

Total cellular protein from adherent cells was isolated using Pierce IP Lysis Buffer (Thermo Scientific) with 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Lysates were clarified by centrifugation (13 000 × g for 10 min at 4°C) and quantified by DC Protein Assay (Bio-Rad).

Protein isolation from media: Conditioned media from cell lines and patient samples was concentrated using ultrafiltration columns (30K Amicon Ultra-0.5 mL Centrifugal Filters; Millipore). Prior to concentration of medium, cells were cultured as follows. Cells were plated in T25 flasks in complete medium and grown to 80% confluency. The cells were then rinsed twice with serum- and antibiotic-free medium to ensure that excess albumin from high serum was eliminated. Otherwise, the high albumin

levels during media concentration result in the formation of a gel and clogging of the Amicon columns. Fresh media with lower (0.1% FBS) or no serum was then added. Conditioned medium from cell lines was collected 24 and 48 h after the media change (1 ml at each time point). Conditioned medium from primary EOC samples was collected after 48 h only. Samples were centrifuged at 10 000 x g for 5 min at room temperature (RT) to remove cell debris prior to concentration. Amicon columns were used according to the manufacturer's protocol. Briefly, media was centrifuged twice at 14 000 x g for 10 min to concentrate the conditioned media (500 µl loaded onto the same column twice), followed by a final spin of 1000 x g for 10 min to recover the concentrated sample into a fresh microcentrifuge tube.

4.2.3 Western blotting

Protein extracts and concentrated media samples were boiled in Laemmli Buffer for 5 min and separated by SDS-PAGE (40 µg/lane and 20 µl/lane, respectively) using a 8% gel. The separated proteins were then transferred to a polyvinylidene difluoride membrane (PVDF; Roche) at 100V for 2 h at 4°C, and blocked for 1 h at RT with 5% skim milk in Tris-buffered saline with Tween-20 (TBST). Following washes with TBST the membrane was incubated with anti-SPON1 antibody (1 µg/ml in 5% skim milk/TBST; Abcam ab40797) overnight at 4°C, then with a peroxidase-conjugated anti-rabbit (1:10 000 in 5% skim milk/TBST; Santa Cruz) for 1 h at RT. Immunoreactive bands were visualized using enhanced chemiluminescence reagent (ECL Plus; Amersham Biosciences) and Hyperfilm (Amersham). The membrane was re-probed with anti-actin antibody (1:5000; Sigma A2668) overnight at 4°C, followed by incubation with peroxidase-conjugated anti-rabbit, and visualized as described for SPON1.

Additional antibodies used: anti-WNK-1 (1:1000; Abcam ab53151), anti-phospho-WNK-1 (1:500; Cell Signalling Technology 4946S) and anti-p53 (1:1000; Cell Signalling Technology 9282).

4.2.4 Cell viability assay

Cell viability was assessed using the MTS CellTiter 96®AQ_{ueous} One Solution assay (Promega), which detects the activity of NAD(P)H-dependent cellular

oxidoreductase enzymes, and serves as a measure of the relative number of viable cells. Each of the five ovarian cancer cell lines was seeded at 1.0 x 10^3 cells/well in 1% FBS-containing medium onto 96-well plates. Two hours (h) later the cells were treated with 5 μg/ml human recombinant SPON1 (R&D Systems 3135-SP) or 250 μg/ml BSA (BioShop) vehicle control. At a series of time points (24, 48 and 72 h after treatment), 20 μl of MTS reagent was added to each well, and the cells were incubated at 37°C for 2 h. Following incubation, the plate was shaken and the absorbance was measured at 490 nm using a microplate reader (Synergy H4 Microplate Reader; Biotek).

Cell viability was similarly assessed in patient samples using the MTS assay, with minor changes: 2.5 x 10^3 cells/well were seeded in 10% FBS-containing medium, and the MTS assays were performed on Days 2, 4 and 6 after recombinant SPON1 treatment.

4.2.5 Cell proliferation assay

Cell proliferation assays were performed in parallel with the Cell Viability Assays. The five established cell lines were seeded in triplicate (6.0 x 10^3 cells/well) in 1% FBS-containing medium in 24-well dishes and treated 2 h after seeding with human recombinant SPON1 (5 μg/ml) or a BSA (250 μg/ml) control. Cell counts were performed using the Coulter counter (Beckman Coulter) 24, 48 and 72 h after treatment; each cell suspension was counted in triplicate. Cell growth assays for patient samples were performed in parallel with the MTS assays; however, 6.0 x 10^3 cells were seeded in 96-well dishes in 10% FBS-containing medium (rather than 1% FBS for the cell lines).

4.2.6 Cell detachment assay

OVCAR3, OVCAR5 and OVCAR8 (3 x 10^4/well), HEY and OVCA429 (2 x 10^4/well) cells were seeded into 96-well plates and treated with carrier-free recombinant SPON1 (R&D Systems 3135-SP/CF) (1 μg/ml, 5 μg/ml or 10 μg/ml) or vehicle. After 48 h cells were detached first using weak trypsin (0.06% trypsin/EDTA) and counted using the Coulter counter [33]. Because we found that each cell line adhered with different strengths to the tissue culture surface, we optimized the length of time that each cell line was incubated in 0.06% trypsin so that 10-50% of cells detach yet no more than half of the total cells on the plate. The remaining cells were completely detached

from the culture dishes using full trypsin (0.25% Trypsin/EDTA) and counted using a Coulter counter. Cell adhesion was scored as the percentage of cells detached with 0.06% trypsin. Each experiment was conducted using three technical replicates; each experiment was carried out three times.

The cell detachment assay was performed in patient EOC samples as described above, except that 1 x 10^4 cells were seeded per well in 10% FBS-containing medium. All primary EOC cells were incubated with 0.06% trypsin/EDTA for 1 min.

4.2.7 Cell adhesion assay

96-well plates were incubated with increasing amounts (250 ng, 500ng or 1μg) of the following protein solutions for 12 h at 4°C: carrier-free recombinant SPON1, laminin or poly-L-lysine (positive control) or PBS (no coating control), to coat the wells with the specified proteins. After 12 h of incubation, the protein solution was aspirated and the plate air-dried at RT in the tissue-culture hood overnight. The following day, cells that had been serum starved for 12 h were seeded (2 x 10^4 cells/well) in serum-free media on the immobilized protein substrate and incubated at 37°C. After one hour, non-adherent cells were removed using gentle washes with a multi-channel pipettor, and full media replaced to allow adhered cells to recover from the serum starvation. After 4 h of recovery, 20 μl of the MTS CellTiter 96® AQ_{ueous} One Solution assay substrate (Promega) was added and the absorbance determined 2 h later (490 nm) using a microplate reader (Synergy H4 Microplate Reader; Biotek), which provided a relative measure of the number of adherent cells on the different protein substrates.

4.2.8 Spheroid formation and reattachment assays

Spheroid formation assays: Ultra Low-Attachment (ULA) plates (Corning) are commercially-purchased tissue culture plates that are coated with a hydrophilic, neutrally charged hydrogel to prevent cell attachment. Single-cell suspensions of HEY, OVCAR8 and OVCAR3 cells (5 x 10^4 cells/ml) were seeded onto ULA plates, and spheroids formed over time [34]. To determine whether SPON1 affects the formation of spheroids, cells were treated with recombinant SPON1 (10 μg/ml) or vehicle control (in triplicate) at the time of seeding. Spheroid formation was visualized after 24, 48 and 72 h and images

captured at the centre of each well. After 3 days in culture the spheroids were collected and re-plated onto 6-well tissue culture dishes with fresh growth medium, and allowed to reattach. Once re-attached the cells were fixed and stained using the HEMA-3 stain kit (Fisher). Phase contrast images of re-attached spheroids were captured using an Olympus IX70 inverted microscope and ImagePro software.

Spheroid reattachment assays: To determine whether SPON1 affects spheroid reattachment, spheroids were first formed on ULA plates for 3 days as described above, with the notable exception that cells were *not* treated with recombinant SPON1 at the time of seeding. HEY, OVCAR8 and one primary EOC sample (EOC 272) were used. Individual spheroids were collected, transferred onto 48-well dishes and treated with 10 μg/ml recombinant SPON1 (or vehicle control) at the time of spheroid re-plating. Phase contrast images of reattaching spheroids were taken prior to dispersion (3 h after re-plating) as well as 24 h (HEY and primary sample EOC 272) and 48 h (OVCAR8) following re-plating. Reattached spheroids were fixed and stained using the HEMA-3 stain kit (Fisher). Phase contrast images were captured using an Olympus IX70 inverted microscope and ImagePro software. Spheroid dispersion was quantified using the area measurement tool in *ImageJ* (NIH). Dispersion area at 24 h/48 h was calculated as a percentage of the original spheroid size at 3 h.

4.2.9 Cell migration assay

We utilized Transwell filters (8 μm pore size) (Corning) placed in 24-well plates to determine whether SPON1 affects cell motility. Cells were seeded in the upper chamber of a transwell dish in 0.5% FBS-containing medium, ± 5 μg/ml of SPON1. The bottom chamber was filled with 0.5% FBS-containing medium, 10% FBS-containing medium (positive control) or 5μg/ml SPON1 in 0.5% FBS-containing medium. After 24 h, cells that had not migrated through the membrane were gently removed from the membrane using a cotton swab. The remaining cells were fixed with 4% paraformaldehyde, and the nuceli of migrated cells stained using 4',6-diamidino-2-phenylindole (DAPI). Six random fields of view per membrane were imaged with an

Olympus IX70 inverted microscope and ImagePro software, and the nuclei counted. Each experiment was performed in triplicate.

4.2.10 Chemotaxis assay

We used the ibidi μ-Slide Chemotaxis^{2D} system to determine whether SPON1 may act as a chemoattractant or a chemorepellant. HEY cells were seeded according to the manufacturer's protocol. Briefly, cells were diluted to a concentration of 3 x 10^6/ml. 6 μl of cell suspension was applied to one filling port of the μ-Slide, after which 6 μl of air was aspirated from the opposite filling port. This procedure flushed the cell suspension through the observation channel. The slides were maintained at 37°C in 5% CO_2 in a moist chamber (a 10 cm dish lined with damp Kimwipes [Kimtech]) until the cells attached. To conduct the chemotaxis experiment each chamber was first filled with 80 μl medium supplemented with 0.5% FBS (without test reagent) or 10% FBS (positive control). One chamber previously filled with 0.5%-containing medium was then filled with 18 μl of 50 ng/μl SPON1 solution. The cells were tracked over time by capturing images at a series of time points using an Olympus IX70 inverted microscope and ImagePro software.

4.2.11 siRNA transfections

All siRNA transfections were performed in 6-well tissue culture dishes. The day before transfection, cells were plated at a density of 1 x 10^5 cells per well (OVCAR8 and HEY) or 2 x 10^5 cells per well (OVCAR3) in antibiotic-free media. The following day, Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) was used to transfect cells according to the manufacturer's protocol. A set of three predesigned SPON1 Stealth Select RNAi™ siRNA oligos was tested (siRNA 1: HSS115946, siRNA 2: HSS173622, siRNA 3: HSS115945) (Invitrogen). Briefly, for each well we diluted 4 μl of RNAiMAX in 196 μl Opti-MEM (Thermo Fisher Scientific) and 2 μl siRNA in 198 μl Opti-MEM. The diluted siRNA was added to diluted RNAiMAX and incubated for 5 min at RT. The siRNA-lipid complexes were then added directly to each well. Media was replaced 24 h after transection with fresh growth media. The cells were harvested 48 h and 72 h post transfection, and lysates prepared for Western blot analysis

as described above using Pierce IP Lysis Buffer (Thermo Scientific) with 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Densitometric quantification was carried out using *ImageJ* software. SPON1 expression was calculated relative to a β-actin control.

4.2.12 Functional blocking with antibody

To identify potential function blocking antibodies against SPON1, we utilized the Detachment Assay and Viability Assay protocols described above. For these assays, we used four antibodies that we had previously found to detect SPON1 by Western blot analysis (C-16, N-19, S17 from Santa Cruz and ab40797 from Abcam) were tested.

Detachment assay: Briefly, HEY cells were seeded as for the Detachment Assays described (Methods – 4.2.6), and diluted antibodies (1μg/ml), vehicle control or SPON1 (5μg/ml) were added at the time of seeding. Each of the three Santa Cruz antibodies detects a different fragment of SPON1 therefore they were combined together and the treatment referred to as the Santa Cruz cocktail (SCC). After 48 h cells were detached with weak trypsin (0.06%), then full trypsin (0.25%) and counted as described above.

Viability assay: Functional blocking of SPON1 was also attempted with the Viability Assay protocol. HEY cells were plated as before (Methods – 4.2.4), and diluted antibodies (1 μg/ml and 10 μg /ml), vehicle (250 μg/ml BSA) or SPON1 (5 μg/ml) were added 2 h after seeding. MTS assays were performed as described above: 24, 48 and 72 h following treatments.

The effects of SPON1-antibodies on detachment and viability were determined by using wells with no added antibodies as reference.

4.2.13 Phospho-kinase array

To screen for potential downstream targets of SPON1 in EOC cell lines we used the Human Phospho-Kinase antibody array (R&D Systems), which simultaneously detects the relative levels of phosphorylation of 43 kinases or their targets. OVCAR3 and HEY cells were cultured as described above (see Culture of cell lines section) in 10 cm dishes. Upon reaching 80% confluency the cells were serum starved for 12 h, then treated

with 5 μg/ml SPON1 or 250 μg/ml BSA (vehicle) for 15 min. This time point was selected based on published studies [25, 35] in which cultured cells were treated with recombinant SPON1 and SPON1-induced kinase activation was observed 10-30 minutes after treatment. Cell lysates (100 μg OVCAR3 and 350 μg HEY protein lysate) were applied to the phospho-kinase array and the blots developed according the manufacturer's instructions. Spot intensities were quantified using ImageJ software (National Institutes of Health).

4.2.14 Statistical analysis

Graphs were generated and statistical analysis was performed using GraphPad Prism software. Data were expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test or Analysis of Variance (ANOVA) and Tukey's Multiple Comparison Test with significances set at *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ as indicated.

4.3 Results

SPON1 is abundantly expressed in the ovary relative to other tissues in both human and mouse (Figure 4-1). Furthermore SPON1 is highly overexpressed in ovarian cancer and has been identified as a promising ovarian cancer biomarker [31, 32]. Therefore, herein, we investigated the functional role of SPON1 in ovarian cancer cells.

Ovarian cancer is a heterogeneous disease and many ovarian cancer cell lines are used as *in vitro* models in cancer research. Some of the most commonly used cell line models for high grade serous ovarian carcinoma (HGSC), particularly SKOV3 and A2780, are poor models for the disease as they do not accurately represent the molecular profiles of HGSC tumour samples[36]. The five epithelial ovarian cancer (EOC) cell lines we used in this study – OVCAR3, OVCAR5, OVCAR8, HEY and OVCA429 – more closely resemble tumour profiles [36]. We also utilized eight primary human EOC samples (EOC181, EOC183, EOC193, EOC196, EOC 200, EOC208, EOC25 and EOC272) generously provided by Dr. Shepherd and Dr. DiMattia. Carcinoma cell lines remain a fundamental tool in pre-clinical research; however the ascites-derived primary EOC cells provide greater clinical relevance. The ascites-derived EOC cells can only be

cultured for approximately three weeks and are relatively slow growing [37]. Therefore, due to the limited source material, we selected a few key functional assays to determine the effect of SPON1 on the primary EOC cells.

4.3.1 EOC cell lines and primary human EOC cells express and secrete SPON1

Spon1 mRNA expression is elevated in ovarian tumour tissues compared to the healthy ovary[31]. We first investigated whether our cell lines of interest and primary human EOC cells endogenously express SPON1 protein. Two granulosa cell lines, the human granulosa cell-like cell line, KGN[38] and the KK-1 cell line, an immortalized mouse ovarian granulosa cell line [39] were used as positive controls with the EOC samples because our laboratory has previously demonstrated that both cell lines express SPON1 protein as determined by Western blot. I found that, as expected, both KGN and KK-1 cell lines expressed SPON1 (Figure 4-2A-i) as determined by Western blot. Similarly, SPON1 was robustly expressed in all EOC cell lines (Figure 4-2A-i) and primary EOC samples (Figure 4-2B-i).

SPON1 is a secreted ECM glycoprotein[16]; therefore we next investigated whether the cell lines and primary EOC samples secreted SPON1 into the culture medium. SPON1 was recovered from the conditioned medium of cell cultures using centrifugal concentrators. The concentration of albumin in cell culture medium is typically high because of the presence of FBS; concentration of supernatant with a high albumin concentration will result in the formation of a gel and clogging of the concentration columns. To avoid this technical difficulty, cells were incubated in serum-free medium and reduced serum (0.1% FBS).

SPON1 was detected in conditioned medium from all five EOC cell lines after 24h. The level of secreted SPON1 increased over time, as higher SPON1 expression was observed after 48 h (Figure 4-2A-ii). Consistent with the cell line data, we found that primary EOC cells also secreted SPON1 into the medium (Figure 4-2B-ii). Due to the slower proliferation of primary cells, we only incubated them in reduced-serum and collected medium after 48 h.

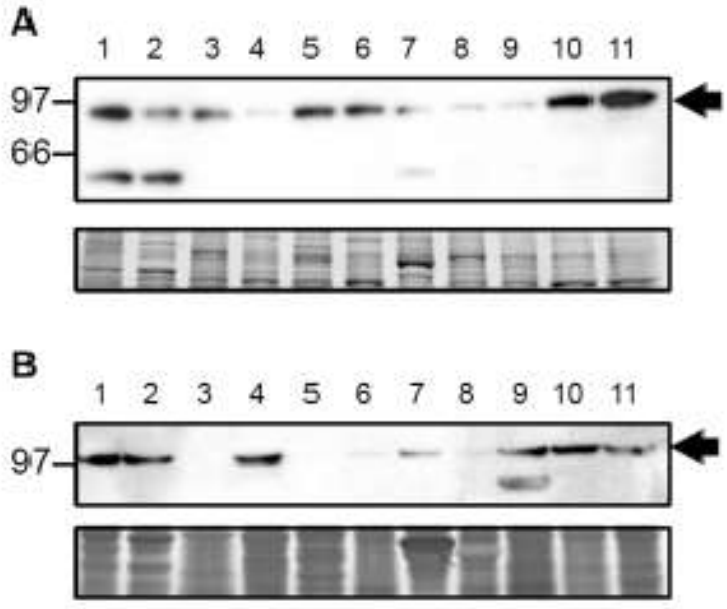
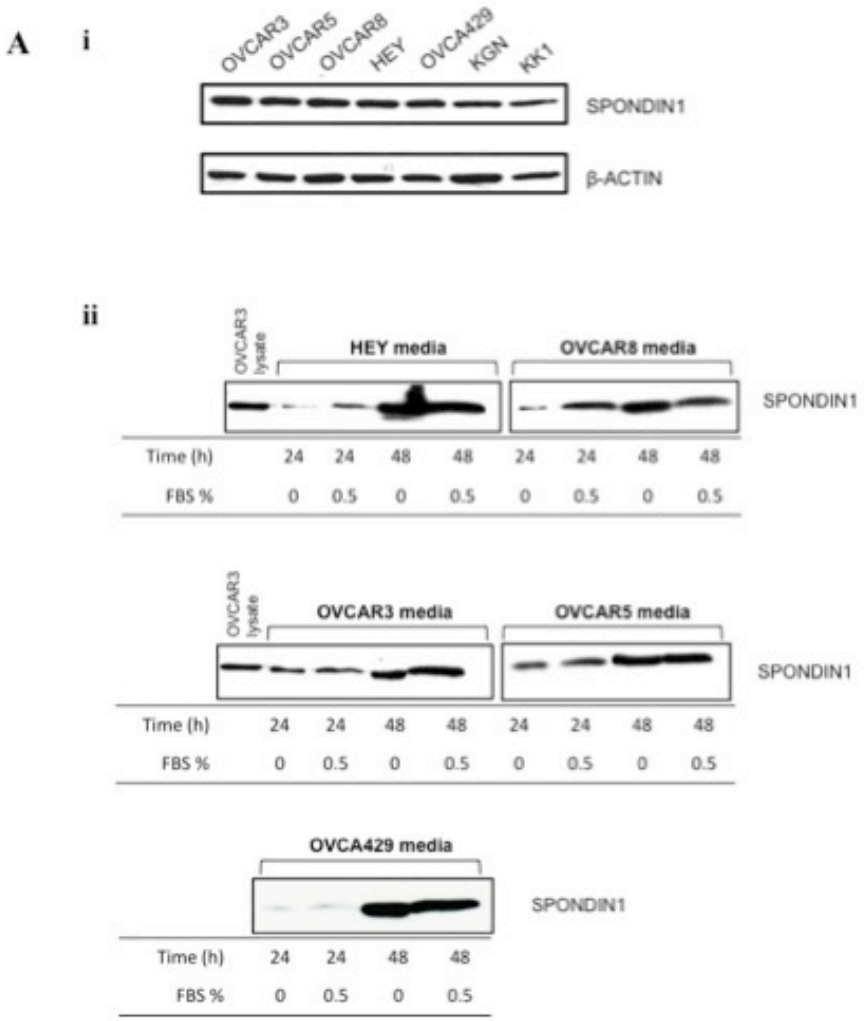


Figure 4-1: SPON1 expression in human and mouse tissues.

A) A human tissue INSTA-Blot™ was incubated with an anti-Spondin 1 antibody to detect SPON1. 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. 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).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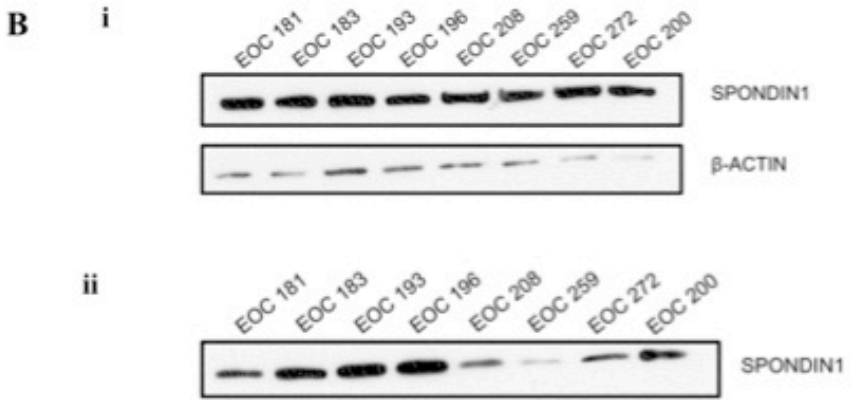
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Figure 4-2: SPON1 is expressed and secreted by EOC cell lines and primary human EOC cells.

A) (i) Western blot analysis of SPON1 expression in five EOC cell lines: OVCAR3, OVCAR5, OVCAR8, HEY and OVCA429. KGN, human granulosa cell tumour cell line, and KK1 mouse granulosa cell line were used as positive controls. β-actin was used as a loading control.

(ii) Western blot analysis of SPON1 in conditioned media of EOC cell lines (HEY, OVCAR8, OVCAR3, OVCAR5, OVCA429). Conditioned media was collected after cells were in culture for 24 h and 48 h, in media supplemented with 0% FBS or 0.5% FBS. OVCAR3 cell lysate was a positive control.

B) (i) Western blot analysis of SPON1 in primary EOC cells (β-actin was used as a loading control) and (ii) conditioned media of primary EOC cells.

4.3.2 SPON1 reduces adhesion of EOC cell lines and primary human EOC cells to tissue culture surfaces

Changes in cell-cell and cell-matrix adhesion play important roles in tumour development and metastasis, contributing to angiogenesis, cell migration and proliferation [40]. Given that Spondin 1 has previously been shown to affect adhesion properties *in vivo*, by promoting muscle-epidermal adhesion in *C. elegans* [23], we postulated that it also alters the adhesion properties of EOC cells *in vitro*. We tested this in monolayer adherent cell cultures by utilizing two adhesion assays – the *Detachment Assay* (Methods 4.2.6) and *Adhesion Assay* (Methods 4.2.7). We used the Detachment Assay to first measure the effect that carrier-free recombinant SPON1 treatment has on cell adhesion to cell culture plastic. BSA is a non-adhesive protein that has the ability to 'trigger' or 'activate' the attachment of cells to low levels of adhesion protein [41]; therefore, to avoid interference with BSA we used carrier-free recombinant SPON1 (BSA-free) for all adhesion studies. The Adhesion Assay was then used to examine whether coating of tissue culture plastic with carrier-free recombinant SPON1 affects cell adhesion.

Ovarian and/or circulating concentrations of SPON1 in humans or mice are currently unknown. Therefore, initially during Detachment Assay optimization, cell lines were treated with 1 μg/ml and 5 μg/ml of recombinant human SPON1. Treatment with 1μg/ml SPON1 did not have a significant effect on cell adhesion (Figure S4-1); cell detachment was comparable between untreated and 1μg/ml SPON1-treated cells when incubated with weak trypsin. Cells treated with 5 μg/ml SPON1 released more readily from the cell culture surface than untreated cells when incubated with weak trypsin. Therefore 5 μg/ml and 10 μg/ml SPON1 were used for subsequent Detachment Assays. These doses are comparable to those used in other studies in which cell lines were treated with recombinant SPON1, which ranged from 0.5 μg/ml [26, 28] to 10 μg/ml [25]. The cell detachment assays showed that SPON1-treated HEY, OVCAR8 and OVCAR3 cells had increased sensitivity to trypsinization compared to vehicle-treated cells (Figure 4-3). Indeed both SPON1 treatments initiate significantly higher cell detachment with weak trypsin incubation compared to vehicle-treated cells. A dose-response relationship exists

because significantly greater cell detachment occurred when these three cell lines were treated with 10 μg/ml SPON1 compared to 5 μg/ml SPON1. Interestingly, neither treatment with 5 μg/ml nor 10 μg/ml SPON1 changed the adhesion of OVCAR5 or OVCA429 cells (Figure S4-2).

To determine if the effect of SPON1 on cell adhesion in cell lines would also occur in a more clinically-relevant model, we conducted the Detachment Assay with primary EOC cells. All eight patient samples assayed demonstrated a significant increase in cell detachment with weak trypsin incubation following treatment with 10 μg/ml SPON1 compared to vehicle treated cells. The lower SPON1 dose significantly decreased the adhesion of one of the patient samples (EOC183, Figure 4-4B).

To further examine the effect of SPON1 on adhesion we used another model system and conducted Adhesion Assays with HEY, OVCAR8 and OVCAR3 cells; we excluded OVCAR5 and OCVA429 because they did not respond to SPON1 in the Detachment Assays. Plates were coated with serial dilutions of carrier-free recombinant human SPON1, Laminin, Poly-L-lysine (PLL) or PBS (no coating control). Based on our earlier results demonstrating that treatment of EOC cells with recombinant SPON1 decreases their adhesion, we wanted to compare the adhesion of EOC cells to another protein substrate previously shown to decrease EOC cell adhesion *in vitro*. Therefore we used laminin as a negative control to coat the plates because SKOV3 ovarian cancer cells do not adhere well when plated on a laminin-coated surface [42]. PLL was used as a positive control because it is a well-known promoter of cell adhesion [42, 43]. An MTS assay, a colorimetric method for determining the relative number of viable cells, was used as a read-out of the relative number of adherent cells on the different protein substrates, and therefore as a measure of adhesion. As in the Detachment Assay, we noticed a dramatic reduction in cells adhering to the SPON1-coated surface compared to uncoated wells or those coated with PLL (Figure 4-5). Interestingly, cells were also less likely to adhere to SPON1-coated wells compared to laminin-coated wells.

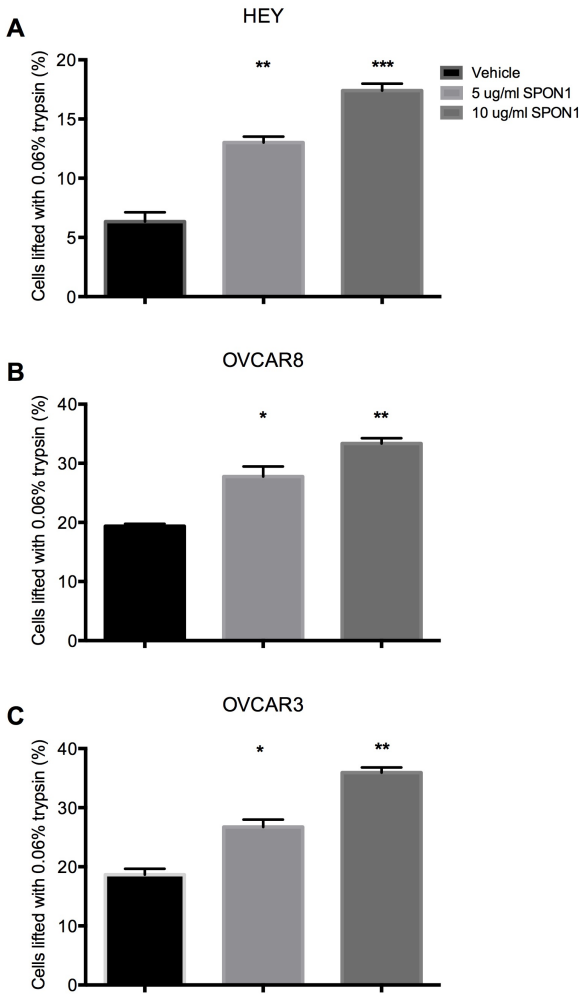
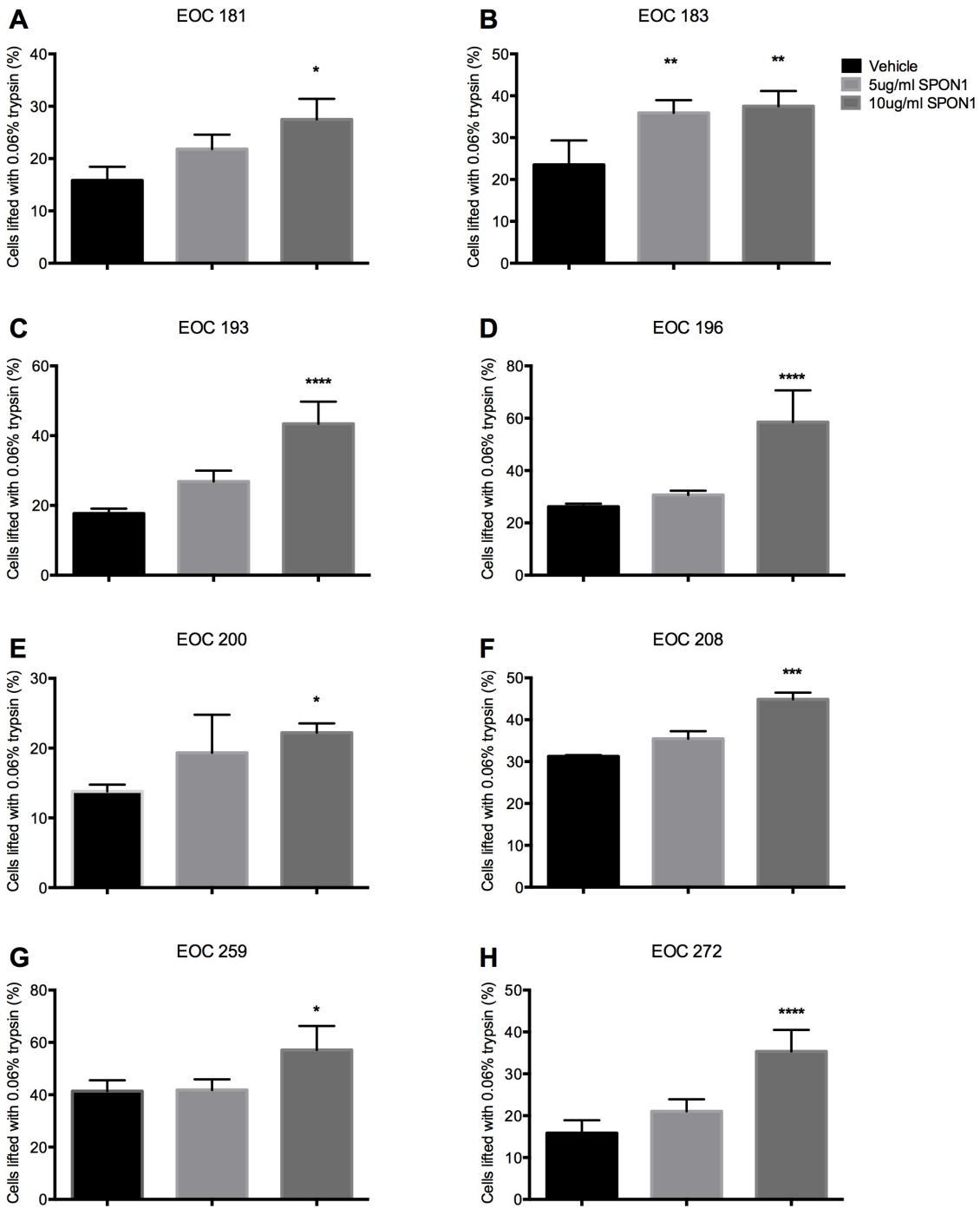


Figure 4-3: SPON1 reduces adhesion of ovarian cancer cell lines in a dose-dependent manner.

HEY (A), OVCAR8 (B) and OVCAR3 (C) cells were treated with vehicle, 5 µg/ml carrier-free recombinant human SPON1 or 10 µg/ml carrier-free recombinant human SPON1 at the time of seeding. After 48 h the cells were detached with 0.06% trypsin. Any adherent cells remaining were then detached with 0.25% trypsin and cells from each pool were counted. The proportion of cells detached by 0.06% trypsin is shown. SPON1 reduced adhesion in a dose-dependent manner because higher percentages of cells detach following weak trypsinization when treated with a higher SPON1 dose. The data represent the mean ± SD of triplicate measurements from at least two independent experiments. One-way ANOVA with Tukey's test was performed (*p<0.05; **p<0.01; ***p<0.001).


A
EOC 181
Cells lifted with 0.06% trypsin (%)
0
10
20
30
40
*
B
EOC 183
Cells lifted with 0.06% trypsin (%)
0
10
20
30
40
50
**
**
Vehicle
5ug/ml SPON1
10ug/ml SPON1
C
EOC 193
Cells lifted with 0.06% trypsin (%)
0
20
40
60
****
D
EOC 196
Cells lifted with 0.06% trypsin (%)
0
20
40
60
80
****
E
EOC 200
Cells lifted with 0.06% trypsin (%)
0
10
20
30
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F
EOC 208
Cells lifted with 0.06% trypsin (%)
0
10
20
30
40
50
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G
EOC 259
Cells lifted with 0.06% trypsin (%)
0
20
40
60
80
*
H
EOC 272
Cells lifted with 0.06% trypsin (%)
0
10
20
30
40
50
****

Figure 4-4: SPON1 reduces the adhesion of primary epithelial ovarian cancer cells. Eight primary EOC samples were treated with vehicle, 5 μg/ml carrier-free recombinant human SPON1 or 10 μg/ml carrier-free recombinant human SPON1 at the time of seeding. After 48 h the cells were detached with 0.06% trypsin. Any adherent cells remaining were then detached with 0.25% trypsin. Cells from each pool were then counted. The proportion of cells detached by 0.06% trypsin is shown. SPON1 decreased cell detachment in seven out of the eight EOC patient samples tested. All values represent the mean ± SD of triplicate measurements from at least two independent experiments. One-way ANOVA with Tukey's test was performed (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

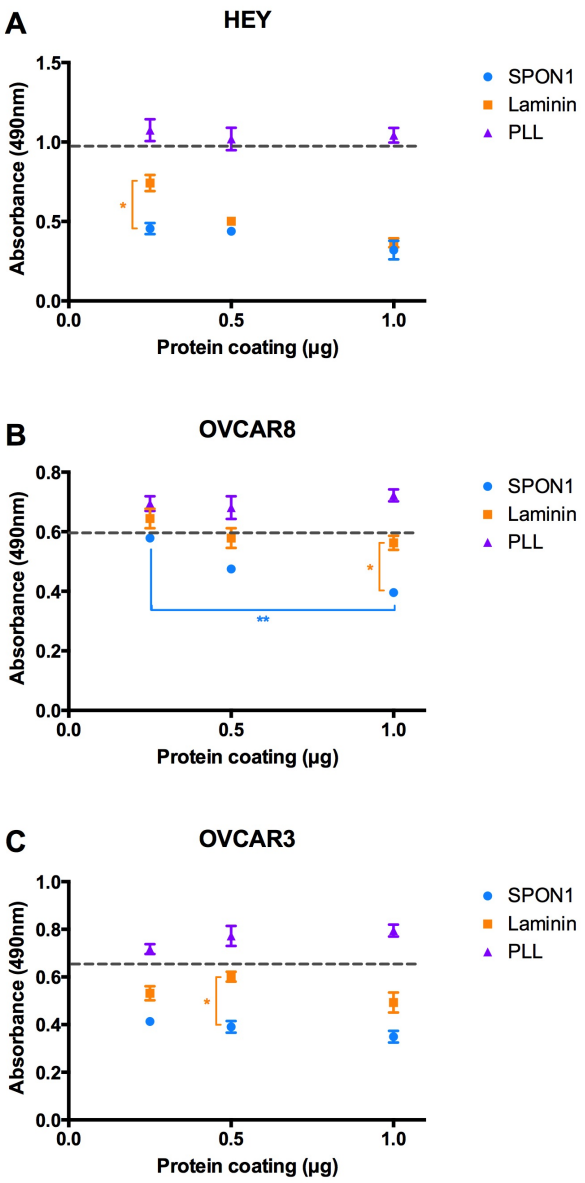


Figure 4-5: SPON1 inhibits adhesion of EOC cell lines to cell culture surfaces. Tissue culture surfaces were pre-coated with several dilutions of carrier-free recombinant human SPON1, Laminin, Poly-L-lysine (PLL) or left uncoated (horizontal dashed line). The MTS assay provides a relative measure of the number of cells attached to each protein substrate following 1 h incubation and 4 h recovery. HEY (A), OVCAR8 (B) and OVCAR3 (C) cells attach best to PLL and least to SPON1. Data represents mean ± SD of triplicate wells. Each assay is representative of three independent experiments. Two-way ANOVA with Tukey's test was performed (*p<0.05; ** p<0.001).

4.3.3 SPON1 affects reattachment of EOC spheroids but not spheroid formation

Within peritoneal ascites, shed EOC cells can be present as single cells and/or multi-cellular spheroids, and both populations of cells are capable of seeding secondary metastases [10]. EOC dissemination is significantly impacted by the formation of multicellular spheroids and their ability for reattachment and growth at secondary sites [10]. Given our results in monolayer cultures of EOC cells thus far, we postulated that treatment of EOC spheroids with SPON1 would decrease spheroid formation, reattachment and dispersion.

To determine whether SPON1 affected spheroid formation, at the time of seeding onto Ultra Low-Attachment (ULA) plates, we treated HEY, OVCAR8 and OVCAR3 cells with 10 μg/ml recombinant SPON1. Spheroid formation was visualized, as well as spheroid size and number, after 24, 48 and 72 h. No differences in spheroid size or number following SPON1 treatment were observed at these time points, and the spheroids did not appear more loosely aggregated, which suggested SPON1 does not affect cell-cell adhesion (Figure 4-6).

Since SPON1 treatment consistently reduced cell attachment in monolayer EOC cell cultures, we next sought to determine whether SPON1 treatment would affect the ability of EOC spheroids to reattach to standard tissue culture plastic. To test this, we again utilized ULA plates to form EOC spheroids; however we did not treat cells at the time of seeding; rather, cells were treated with 10 μg/ml recombinant SPON1 once the spheroids were re-plated onto standard tissue culture plastic. HEY, OVCAR8 and EOC 272 spheroids were formed on ULA dishes. OVCAR3 cells were excluded from these experiments because they form smaller, more delicate spheroids than the other cell lines, and are too difficult to transfer for a reliable experimental model. Spheroids were plated for reattachment by directly transferring them from ULA dishes onto standard tissue culture plastic with fresh culture medium.

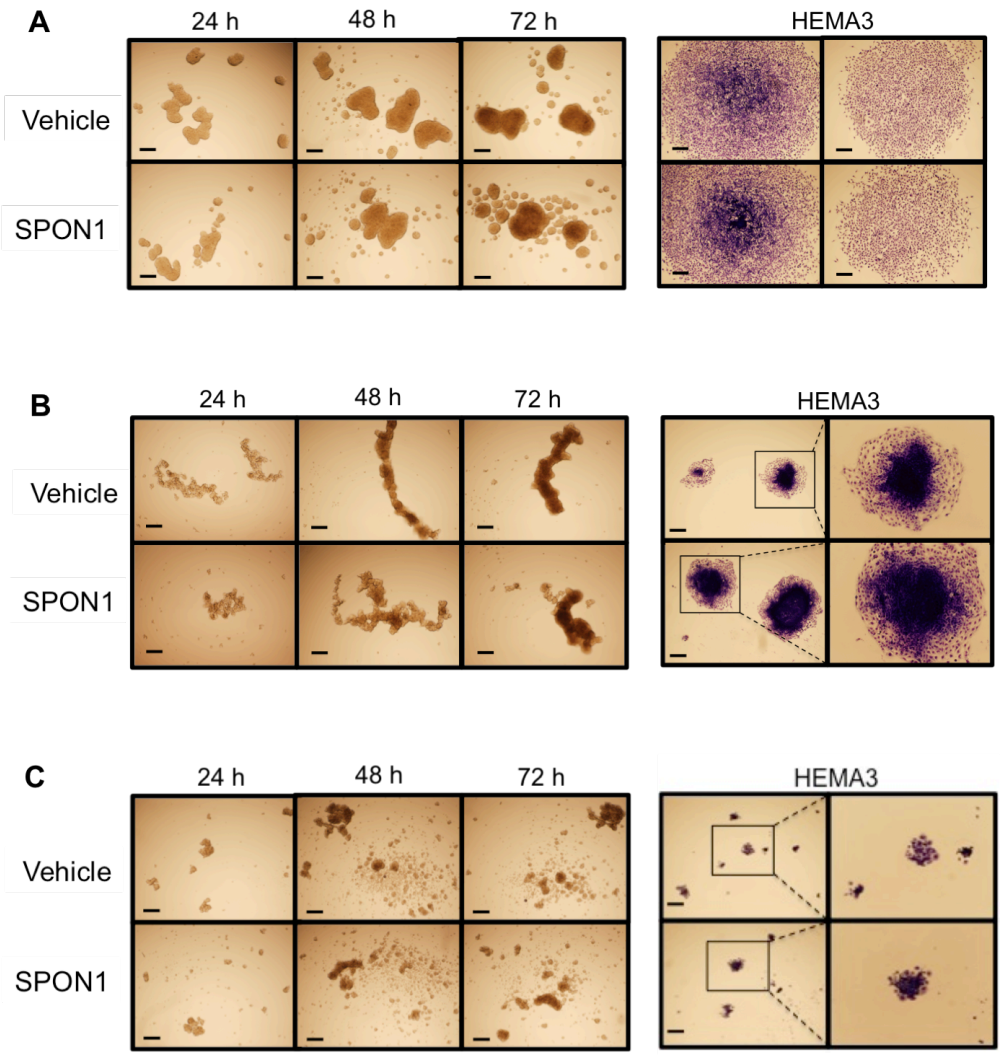


Figure 4-6: SPON1 treatment does not effect the formation of spheroids by EOC cell lines.

HEY (A), OVCAR8 (B) and OVCAR3 (C) cells were cultured with vehicle or 10 µg/ml recombinant SPON1 on ULA plates for three days (each treatment performed in triplicate/ cell line). Phase contrast images were captured daily at the centre of each well and representative images are shown. SPON1 does not affect the ability of EOC cell lines to form multicellular spheroids, as the size and number of spheroids formed were comparable to vehicle-treated controls. After 72 h on ULA dishes in the presence of SPON1, spheroids were re-plated in fresh medium onto standard tissue culture plastic and allowed to re-attach. Once re-attached the cells were fixed and stained using the HEMA-3 stain kit (Fisher). Scale bar = 50 µm.

Interestingly, we observed increased cell dispersion areas for HEY and EOC 272 SPON1-treated spheroids within the first 24 h of re-plating compared to controls; cell dispersion was increased by 59% and 51% for HEY and EOC 272 cells, respectively (Figure 4-7A and C). OVCAR8 cells form irregular spheroids and reattach more slowly than HEY or EOC 272 cells; therefore, dispersion areas of OVCAR8 cells were measured after 48 h, rather than 24 h. Consistent with the other cell lines, we found that SPON1-treated OVCAR8 spheroids also produced a greater dispersion area than control spheroids (68% increase) (Figure 4-7B). These results suggested that SPON1 reduced cell-cell adhesion upon spheroid reattachment to standard tissue culture plastic, as more cells were released from the SPON1-treated multicellular aggregates than controls. Taken together, these results suggest a functional role for SPON1 in reducing adhesion of adherent monolayer and spheroid EOC cells.

4.3.4 SPON1 does not affect EOC cell migration

Cell migration is an essential factor in tumour progression and metastasis. Although cells can move randomly, dissemination, invasion and migration are most efficient when cells are involved in directed migration in response to a chemical stimulus (chemotaxis) [44]. Adhesion of cancer cells to the ECM is a prerequisite for cells to develop the traction necessary for movement [45, 46]. Therefore, we sought to determine whether the decreased adhesion following SPON1 treatment impacts EOC cell migration. Furthermore, we wanted to investigate whether the increased dispersion area generated by SPON1-treated spheroids might be due in part to increased cell motility. Spondin1 has previously been shown to promote and inhibit the cell migration of other cell types. Spondin1 increases migration of osteosarcoma cells [29], and inhibits vascular endothelial growth factor (VEGF)-stimulated migration and tube formation of human umbilical vein endothelial cells (HUVECs) [30]. It has also been proposed that Spondin1 may act as a chemoattractant and/or chemorepellant, guiding commissural axons at the floor plate [18].

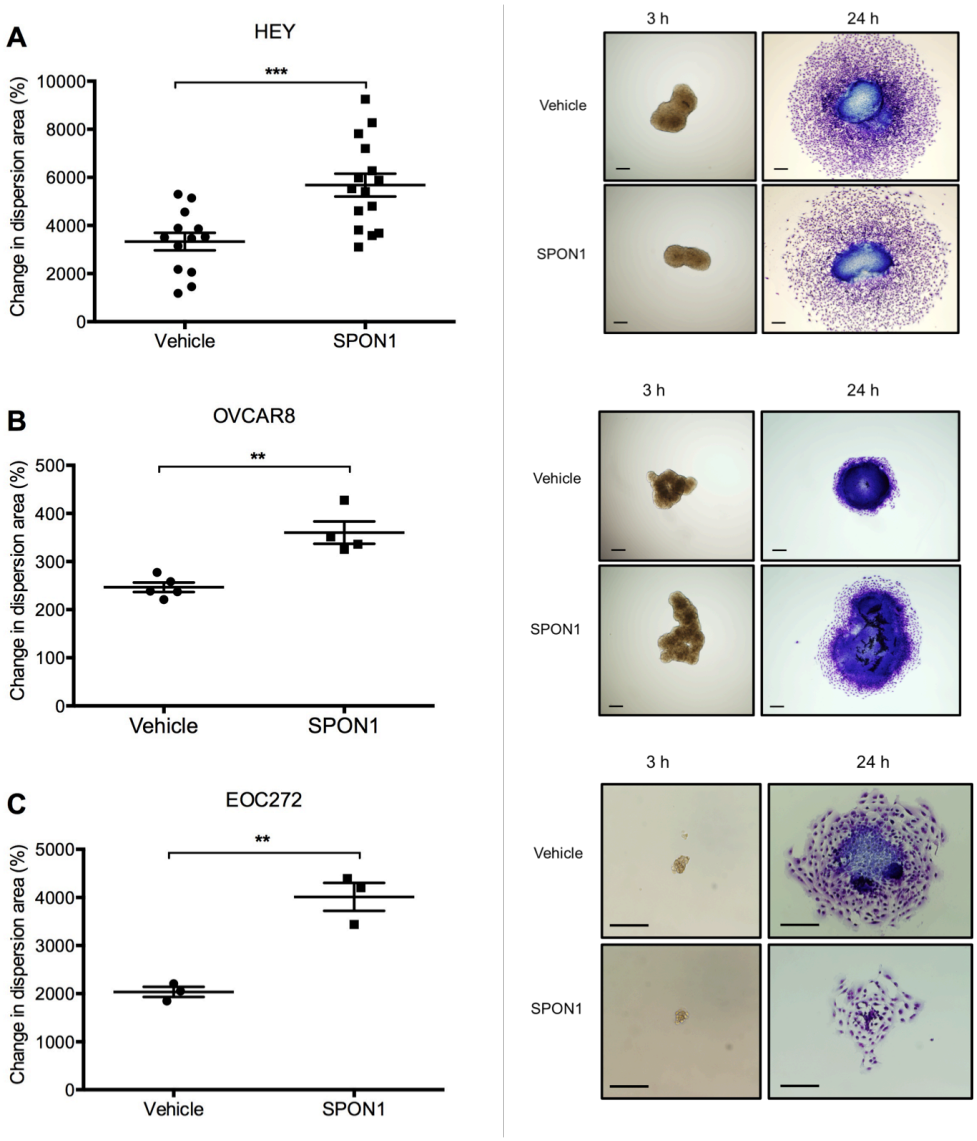


Figure 4-7: SPON1 enhances the dispersion area generated by reattached EOC spheroids.

HEY (A), OVCAR 8 (B) and EOC272 (C) spheroids were treated with 10 μg/ml SPON1 or vehicle at the time of re-plating onto standard tissue culture plastic. SPON1 increases the dispersion area generated by reattached EOC spheroids as quantified using *ImageJ* software. Dispersion area was calculated 24 h (A and C) or 48 h (B) after spheroids were re-plated, and the dispersion area normalized to the size of the original spheroid (3 h after re-plating). Representative images are shown of EOC cells at each time point. Each data point represents one spheroid. $^{**}p<0.01$; $^{***}p<0.001$ as determined by Student's *t*-test. Scale bar = 50 μm.

To test this hypothesis, we initially used Transwell filters (Corning) to evaluate the effect of SPON1 on EOC cell migration. HEY, OVCAR8 and OVCAR3 cells were seeded in the upper chamber of a transwell insert in low serum (0.5% FBS), ± recombinant SPON1. The cells were allowed to migrate for 24 h towards recombinant SPON1, low serum (0.5% FBS) or a high serum positive control (10% FBS) in the bottom chamber of the transwell insert. We found that the presence of SPON1 did not affect the migration of EOC cells across the transwell membrane (Figure S4-3).

We also utilized the ibidi μ-Slide Chemotaxis^{2D} system to determine whether SPON1 affects cell motility. The slides are designed for analysis of migrating adherent cells on a 2D surface. The small chambers of the ibidi system allowed us to use higher concentrations of recombinant SPON1 (10 μg/ml) than we used with the transwell model. HEY cells were used for the optimization of the ibidi system protocol because they are the fastest growing of the EOC cell lines we studied and their epithelial-like, slightly elongated morphology makes them the easiest to track over time (compared to the cobblestone-like morphology of the OVCAR3 and OVCAR8). The preliminary experiments demonstrated that SPON1 had no effect on chemotaxis; rather we observed HEY cells migrating randomly within the ibidi observation chamber (Figure S4-4). Based on these results and the transwell migration data we did not pursue SPON1's potential impact on migration further, with other EOC cell lines or with primary EOC cells.

4.3.5 SPON1 reduces cell viability and proliferation of EOC cells

To determine whether SPON1 affects EOC cell viability we treated EOC cell lines with recombinant human SPON1 or bovine serum albumin (BSA) vehicle as a negative control for 24, 48 and 72 h. We assessed viability using an MTS assay, which measures the conversion of a tetrazolium compound into formazan by a mitochondrial dehydrogenase enzyme in live cells. The amount of formazan is measured spectrophotometrically and provides a measure for the relative number of viable cells. We found that SPON1 reduced the viability of HEY cells modestly by 8% after 24 h, with a significant decrease in viability of 21% and 22% after 48 and 72 h, respectively (Figure 4-8A-i). Surprisingly, SPON1 did not significantly effect the viability of the other ovarian cancer cell lines (Figure 4-8B-i, 4-8C-i and Figure S4-5), even when a higher

SPON1 concentration (10 μg/ml) was used (Figure S4-6), or when SPON1 was replenished daily (Figure S4-7). Interestingly, we also did not observe a dose-response relationship between SPON1 and cell viability because the decrease in viability following treatment with 10 μg/ml SPON1 was not significantly different than the reduction observed with 5 μg/ml SPON1 (Figure S4-6). Therefore the lower SPON1 dose was used for subsequent experiments.

The effect of SPON1 on primary EOC cell viability was also assessed. Due to the slower proliferation of these cells the treatment times were extended to 2, 4 and 6 days rather than 24, 48 and 72 h, as used for the cell lines. Although the results are variable, SPON1 significantly decreased cell viability of each primary EOC sample (Figure 4-9). Of note, EOC 196 cell viability was significantly decreased after 2, 4 and 6 days of SPON1 treatment, compared to BSA-treated cells, by 21%, 11% and 10%, respectively (Figure 4-9D). We found that half of the primary EOC samples demonstrated a significant reduction in cell viability after 4 days of SPON1 treatment and this continued into day 6 (Figure 4-9A, E, F, H). On day 6, EOC 193 cell viability decreased dramatically with SPON1 treatment (Figure 4-9C), and in parallel, a subtle, yet significant decrease in EOC 183 viability (Figure 4-9B). The effect of SPON1 treatment on EOC 259 cells followed a different trend from the other samples; viability decreased early on day 2, however, there was no difference in viability at later time points (Figure 4-9G).

To determine whether the changes in cell viability might be due at least in part to decreased cell proliferation, we determined cell number under corresponding conditions to the viability assay treatments and time points. We found that the number of HEY cells decreased significantly compared to controls following SPON1 treatment at 48 and 72 h by 11% and 20%, respectively (Figure 4-8A-ii). SPON1 treatment had no effect on the proliferation of OVCAR8 (Figure 4-8B-ii) and OVCAR3 (Figure 4-8C-ii) cells.

We also found that SPON1 treatment decreased proliferation of primary human EOC cells (Figure 4-10). Generally in the EOC samples, a decrease in viability coincided with decreased cell numbers. Of the eight EOC samples assessed, EOC 193 was the only

sample that did not show a decrease in cell number following SPON1 treatment at any time point (Figure 4-10C), whereas SPON1 treatment decreased EOC 193 cell viability on Day 6 (Figure 4-9C).

4.3.6 Challenges with silencing SPON1 expression

Given that we observed significant effects of exogenous SPON1 treatment (gain-of-function assays) on adhesion, viability and proliferation of EOC cells, we wanted to complement these functional assays after blocking endogenous Spondin1 expression using "knockdown assays" to serve as loss-of-function experiments. We hypothesized that inhibiting endogenous SPON1 expression would increase EOC cell adhesion, viability and proliferation.

Knockdown of SPON1 was attempted using small interfering RNA (siRNA) contructs. A variety of conditions and protocols was tested; however, a successful knockdown was not achieved. The protocol that was the most successful was described earlier (4.3.11 – siRNA transfections). Densitometric quantification was carried out using *ImageJ* software, measuring SPON1 expression relative to β-actin control. The "no treatment" controls (Lane 1 and Lane 5) were considered as 100% SPON1 expression and all values were normalized to them. The greatest success we had with this approach was a 45% and 40% reduction of SPON1 protein in HEY and (Figure S4-8A) and OVCAR8 (Figure S4-8B) cells, respectively, that lasted only 24 h before SPON1 levels began to increase or were nearly restored to pre-knockdown levels. Knockdown of SPON1 in OVCAR3 cells (Figure S4-8C) was not achieved under any conditions tested.

Function-blocking antibodies can be an attractive experimental alternative to siRNA for clarifying the function of proteins [47]; therefore we next tried blocking SPON1 function in HEY cells using antibodies against SPON1. Four antibodies we previously found to detect SPON1 by Western blot analysis (C-16, N-19, S17 from Santa Cruz and ab40797 from Abcam) were tested within the Detachment Assay and Viability Assay protocols.

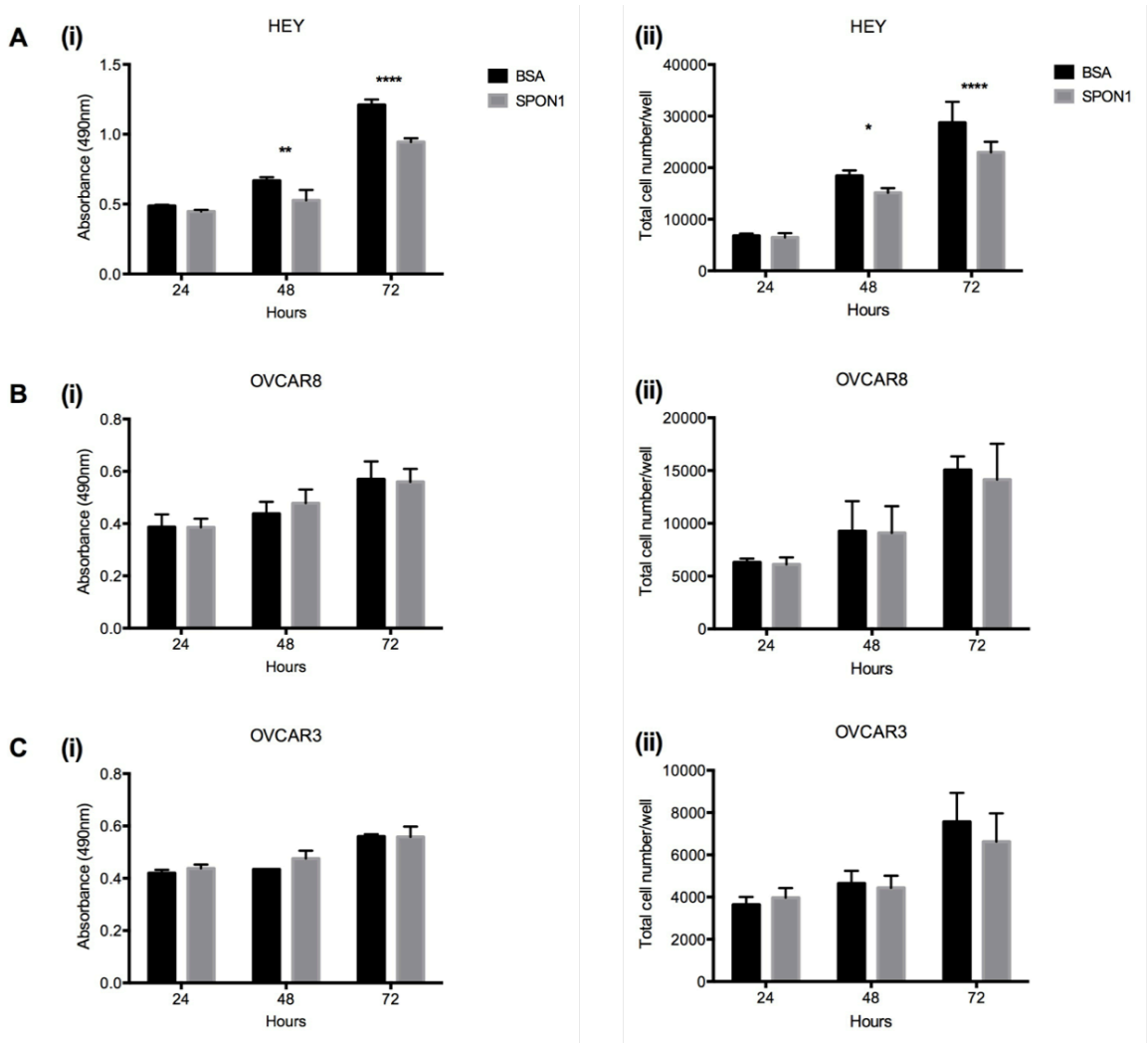
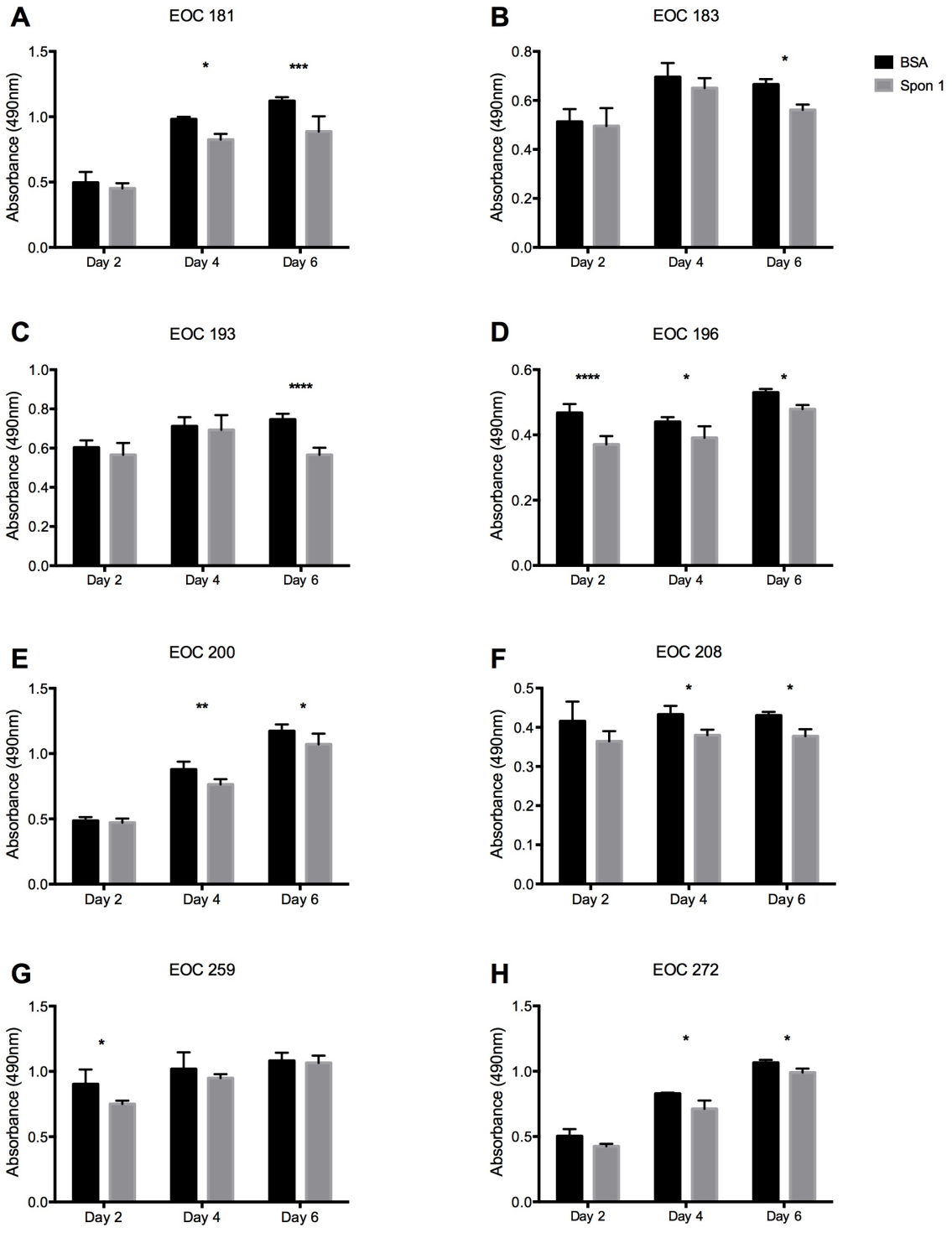


Figure 4-8: SPON1 has a cell line-dependent effect on the viability and proliferation of EOC cells.

Cell viability (i) was assessed in EOC cell lines by MTS assay in the presence or absence of SPON1. Cells were cultured in BSA vehicle (black bars) or human recombinant SPON1 (5 µg/mL) (grey bars). Cell proliferation (ii) was determined by measuring cell counts under corresponding conditions to the viability assay treatments and time points. Each bar represents the mean ± SD of triplicate measurements. Each assay is representative of a minimum of three independent experiments. SPON1 significantly decreased the viability and proliferation of HEY (A) EOC cells, but has no significant affect on the viability and proliferation of OVCAR8 (B) or OVCAR3 (C) cells. Two-way ANOVA with Tukey's test was performed (*p<0.05; **p<0.01; ****p<0.0001).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EOC 181
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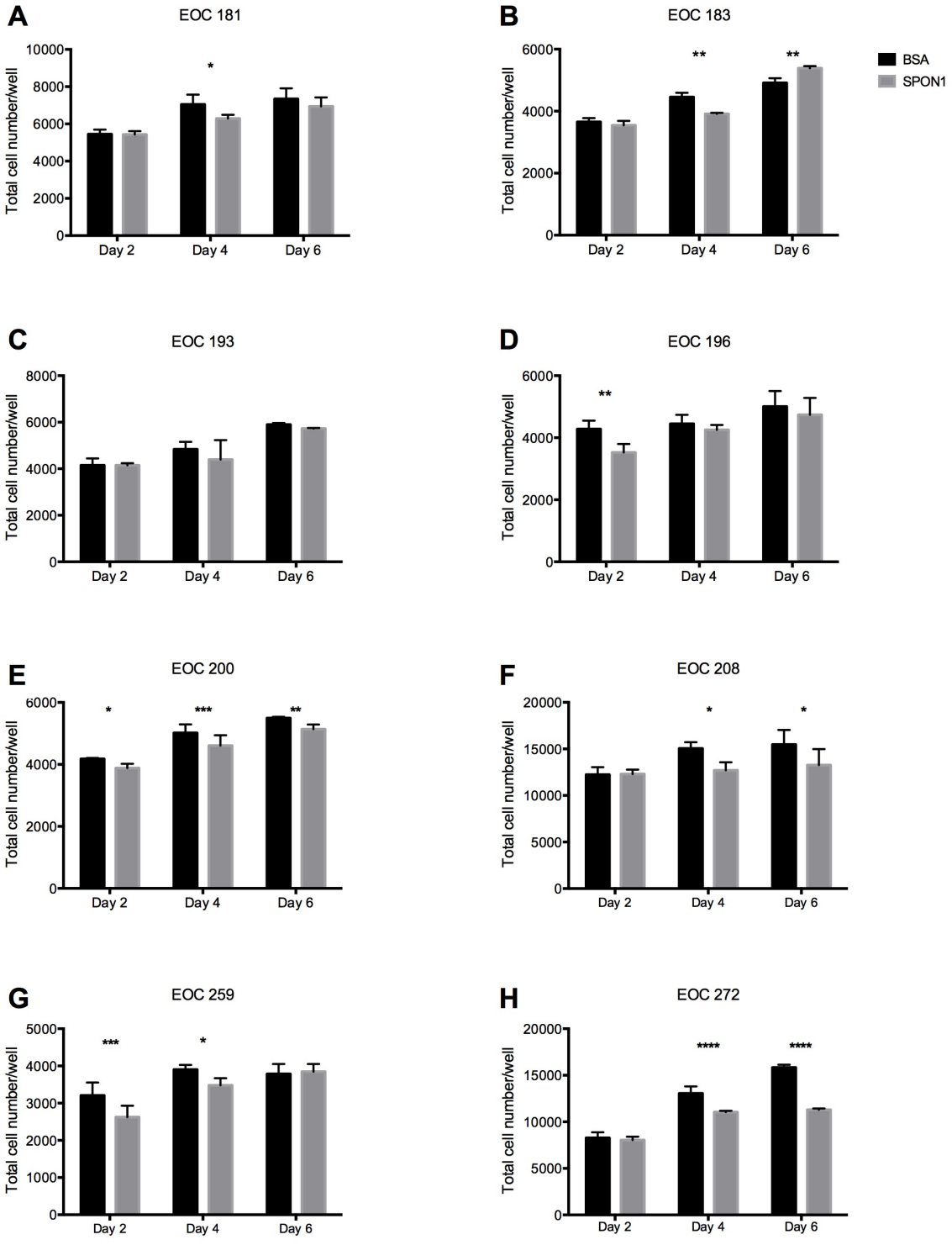
EOC 183
C
EOC 193
D
EOC 196
E
EOC 200
F
EOC 208
G
EOC 259
H
EOC 272
BSA
Spon 1
Absorbance (490nm)
Day 2
Day 4
Day 6

Figure 4-9: SPON1 decreases viability of primary human EOC cells.
Cell viability was assessed in ascites-derived primary EOC cells by MTS assay in the presence or absence of SPON1. Cells were cultured in BSA vehicle (black bars) or human recombinant SPON1 (5 μg/mL) (grey bars). Each bar represents the mean ± SD of six measurements. Each assay is representative of a minimum of three independent experiments. SPON1 significantly decreased the viability of each primary EOC sample. Two-way ANOVA with Tukey's test was performed (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).


A
EOC 181
Total cell number/well
10000
8000
6000
4000
2000
0
*
Day 2
Day 4
Day 6
B
EOC 183
Total cell number/well
6000
4000
2000
0
**
**
Day 2
Day 4
Day 6
BSA
SPON1
C
EOC 193
Total cell number/well
8000
6000
4000
2000
0
Day 2
Day 4
Day 6
D
EOC 196
Total cell number/well
6000
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EOC 200
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EOC 272
Total cell number/well
20000
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5000
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Day 2
Day 4
Day 6

Figure 4-10: SPON1 decreases proliferation of primary human EOC cells

Cell proliferation of ascites-derived primary EOC cells was determined by measuring cell counts under corresponding conditions to the viability assay treatments [BSA (black bars), SPON1 (grey bars)] and time points. Each bar represents the mean ± SD of six measurements. Each assay is representative of a minimum of three independent experiments. SPON1 significantly decreased the proliferation of each primary EOC sample. Two-way ANOVA with Tukey's test was performed (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Using the Viability Assay as a read out we attempted to block SPON1 function by adding each antibody independently to the EOC cells 2 h after seeding. Two concentrations of antibodies were used, 1 μg/ml and 10 μg /ml, which are comparable to doses previously used to block integrin function [47]. We postulated that one of the antibodies would successfully block endogenous SPON1 function, and EOC cell viability would increase. Surprisingly, this did not occur with any of the antibodies, at either concentration (Figure S4-9A). Of note, treatment with the higher dose of C-16, N-19 and S17 antibodies killed most of the cells after 48 h and 72 h.

We then tried blocking SPON1 function using the Detachment Assay as a read out. Since each of the Santa Cruz antibodies detects a different amino acid sequence of SPON1 we used a combination of the three (referred to as Santa Cruz Cocktail, SCC), which spanned the entire length of the protein. Each anti-SPON1 antibody was used at a concentration of 1 μg/ml. The morphology of HEY cells was affected by the Santa Cruz Cocktail in the Detachment Assay. Specifically, cells were rounded, and detached from the tissue culture plastic in multicellular clusters following weak trypsinization, which may have produced inaccurate cell counts despite sample triteration. The 1 μg/ml ab40797 treatment slightly reduced the percentage of cells that detached following weak trypsin incubation compared to vehicle-treated cells. However, neither antibody resulted in a significant effect on cell attachment. Therefore, we did not pursue this method of SPON1 function blocking in other assays. Although there are multiple SPON1 specific antibodies commercially available it appears that none of them block SPON1 function under the conditions we tested (Figure S4-9B).

4.3.7 Screening for downstream targets of SPON1-induced signaling in EOC cells

To screen for potential intracellular targets of SPON1 in EOC cells that could be responsible for decreased adhesion, 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 (nonphosphorylated) total proteins, and/or their targets. HEY and OVCAR3 cells were serum starved for 12 h to reduce background kinase activity, then treated with vehicle or

SPON1 for 15 minutes, after which cell lysates were prepared. The lysates were then applied to the Human Phospho-Kinase array, and the blots processed and spot intensities quantified according to the manufacturer's directions. Overall there were very subtle differences between the pixel densities of vehicle and SPON1-treated cells (Figure S4-10). The phosphorylation of WNK-1 (T60) decreased by 40% in HEY cells and by 35% in OVCAR3 cells after SPON1 treatment compared to control. SPON1 treatment of OVCAR3 cells also decreased the phosphorylation of p53 at three sites (S392, S46 and S15); however, this was not reproduced in HEY cells.

To validate the SPON1-mediated decreases in WNK-1 and p53 phosphorylation that we observed in the array, we conducted Western blot analysis with lysates from HEY and OVCAR3 cells serum starved for 12 h and treated with vehicle or SPON1 for 15 minutes. Despite multiple attempts, we were unable to confirm these results by Western blot because of high background and non-specific binding of the phospho-WNK-1 antibody, and inconsistent results with the total p53 antibody (data not shown).

4.4 Discussion

Epithelial ovarian cancer, a leading cause of death in women around the world, is a disease of dysregulated and aberrant protein expression and activation [1]. The extracellular matrix (ECM) is a complex, dynamic and essential component of the microenvironment of cells, particularly for providing mechanical support. The role of ECM in tumourigenesis has been intensively studied because ECM composition and organization undergo major changes in cancer that can affect properties of both tumor and stromal cells [48]. Although many details of the relationship between the ECM and cancer cells remain unknown, growing evidence suggests that the interactions of cells with ECM components can have either positive or inhibitory effects on cancer cell behaviour depending on the context [12, 13]. An extensive screen of 500 ovarian carcinomas suggests the ECM glycoprotein SPON1 is a promising biomarker for ovarian cancer, particularly high grade serous EOC [32]. Its use in combination with other markers could improve both specificity and sensitivity of monitoring and diagnosing the disease, yet its role in ovarian cancer had not previously been investigated. In this study we utilized established EOC cell lines to determine the affect of SPON1 on various

ovarian cancer cell phenotypes, and repeated select experiments in primary human ascites-derived EOC cells.

4.4.1 SPON1 is involved in changes of the adhesive, proliferative and viability phenotype of human ovarian cancer cells.

Firstly, we have shown that recombinant SPON1 treatment decreases adhesion of EOC cell lines and primary ovarian cancer cells. Dissemination of cells from the primary tumour to other organs is often the cause of patient morbidity and mortality [49]. Shedding of cells into the peritoneal cavity and subsequent adhesion to the serosal and organ surfaces is the initial step in further metastasis [50]. This progression of ovarian cancer relies on different forms of cell adhesion to maintain the signals necessary for sustaining and advancing tumour development [13]. We have demonstrated that either treating cells with exogenous SPON1 (Figure 4-3, 4-4) or plating cells on SPON1-precoated tissue culture plastic (Figure 4-5) reduces cell adhesion, as compared to control cells.

Interestingly, there is a dose-dependent relationship between SPON1 and the extent to which adhesion is affected in most EOC cell lines and primary EOCs. Patient EOC 259 cells were the only patient sample not significantly affected by recombinant SPON1 treatment. However, EOC 259 primary cells were prone to forming cellular aggregates upon trypsinization, despite sample triteration, which likely impacted cell-surface availability and cell counts and consequently led to SPON1-treated cells failing to reach significance.

Malignant cells survive in the peritoneum as single cells or multicellular spheroids until they are able to reattach to a hospitable substratum [10]. Adhesion of spheroids initiates a transition from a floating cell population to a metastatic lesion anchored in the peritoneal cavity. Given that malignant cells are capable of altering cell-substratum as well as cell-cell interactions, and the clinical relevance of multicellular spheroids has been documented [10, 51, 52], we also investigated the effect of SPON1 on spheroid formation and reattachment. Surprisingly, we did not observe a difference in spheroid size or density following SPON1 treatment of EOC cell lines in suspension

(Figure 4-6). If cell-cell adhesion was reduced during spheroid formation we would expect to see smaller, less dense-appearing spheroids [53]. Interestingly, treatment with exogenous SPON1 at the time of spheroid transfer from ULA plates did affect spheroid reattachment (Figure 4-7). The cell dispersion area was significantly higher in HEY, OVCAR8 and primary EOC 272 cells, which may suggest that cells detach from the spheroid more readily when treated with SPON1. The treated cells were more loosely dispersed than controls, which suggested that the increased cell dispersion area was unlikely due to an increase in cell proliferation. In fact, in this study we showed that SPON1-treated EOC cells had significantly decreased proliferation (Figure 4-8, 4-10) and viability (Figure 4-8, 4-9) than vehicle-treated controls. The development of secondary metastases is dependent on both successful cell proliferation and motility of reattached EOC cells from the peritoneum [10]. Unexpectedly, despite affecting migration in other tissues [18, 29, 30], the increase in cell dispersion was also unlikely due to an increase in cell motility because SPON1 did not affect migration of EOC cell lines (Figure S4-2 and S4-3).

Although we failed to observe an effect of SPON1 on the migration of EOC cell lines using trans-well dishes and the ibidi chemotaxis system, the effect of SPON1 on spheroid reattachment merits further attention, as larger studies may reveal a role for SPON1 in EOC cell motility. In this study, we were only able to test one primary human sample and with few replicates on ULA dishes due to limited sample availability. In the future, it would be beneficial to further examine the effect of SPON1 on primary EOC spheroid formation and reattachment with a greater number of samples and replicates.

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

A small number of studies in various model systems have examined the mechanisms and signaling pathways by which SPON1 regulates cellular functions, yet no common mechanism has been identified. Some studies have found that SPON1 acts either via integrin-mediated adhesion. In *C.* elegans, SPON1 localizes to integrin-containing structures on body muscles and to other basement membranes, and may be required for integrin-mediated muscle-epidermal adhesion [23]. SPON1 inhibits the

spreading of human umbilical vein endothelial cells (HUVECs) on vitronectin by specifically blocking integrin $\alpha_v\beta_3$ [30].

Integrins are known to associate with kinases to initiate cascades of signaling events [54]; therefore it is not surprising that SPON1 has also been shown to regulate kinase activity. SPON1 inhibits the VEGF-stimulated activation of Protein Kinase B (Akt) and phosphorylation of focal adhesion kinase (FAK) in HUVECs plated on vitronectin-coated dishes [30]. Conversely, SPON1 increases the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in murine neuroblastoma cells [25], the phosphorylation of the intracellular adaptor protein, disabled-1 (DAB-1) and Akt in chick ciliary ganglion [35], as well as phosphorylation of FAK and SRC in osteosarcoma cells, which promotes cell migration and invasiveness [29]. SPON1 also interacts with LRP8 (low-density lipoprotein receptor-related protein 8) which leads to inhibition of the TRAF6 and c-Fos signaling pathways in clastic cells [55], and acts as an adaptor protein and bridge between APP (amyloid precursor protein) and several apoE receptors (apoER2, LRP2 and LRP4) [56, 57]. Considering the diverse mechanisms and signaling pathways by which SPON1 exerts its effects, it is clear that much remains to be discovered regarding its actions in various tissues and model systems, including ovarian cancer progression.

4.4.3 Searching for mechanisms by which SPON1 may reduce adhesion and growth of epithelial ovarian cancer cells

Certain cell-ECM interactions in the tumour microenvironment induce cell adhesion, proliferation, migration and invasion by activating well-studied signaling pathways that include kinases such as MAPK and Akt [58]. To identify potential signaling pathways induced or repressed by SPON1 in ovarian cancer cells, quiescent HEY and OVCAR3 cell lines were treated with recombinant SPON1 and downstream signaling targets evaluated using the human phospho-kinase array. We quantified the spot intensities of targets that have previously been shown to influence tumourigenesis and appeared even slightly dysregulated by SPON1 treatment in either cell line. These studies identified p53, Chk2, ERK, GSK3, and WNK1 as potential SPON1 targets.

The p53 tumour suppressor protein has well-established functions in monitoring various stress signals and controlling cell cycle arrest and apoptosis. Many phosphorylation sites span the p53 protein, with the majority of these sites rapidly phosphorylated in response to cellular stress [59]. The phospho-kinase array targets three of these sites. One of the more widely studied N-terminal phosphorylation sites is S15. Its phosphorylation reduces p53 affinity for its negative regulator Hdm2 and encourages the recruitment of transcriptional co-activators. Phosphorylation of S46 is essential for p53-mediated induction of pro-apoptotic genes but is not necessary for the activation of cell cycle arrest targets. C-terminal S392 is phosphorylated in response to ultra-violet light. S392 phosphorylation stabilizes the p53 tetramer and activates specific DNA binding [59, 60]. SPON1 decreased p53 phosphorylation at all three sites in OVCAR3 cells (by 20-35%), which was not reproduced in HEY cells. We were unable to confirm this by Western blotting because of inconsistent detection of total p53 levels (data not shown).

Elements upstream or downstream of p53 can also be dysregulated in cancer, and Checkpoint kinase 2 (Chk2) is such a target. Chk2, a key regulator within the complex network of DNA damage checkpoints, is phosphorylated and recruited to DNA strand breaks to recruit several members involved in mediating cell cycle arrest, thereby delaying cell cycle arrest and allowing for DNA repair [61]. Chk2 is a direct regulator of p53 and mediates p53-mediated cell cycle arrest and apoptosis after DNA damage. For example, following activation by ionizing radiation Chk2 stabilizes p53 [62]. We observed only a subtle increase in phosphorylation of Chk2 following SPON1 treatment.

Disruptions of the ERK and PI3K/Akt/mTOR pathways are common in many types of cancer, including ovarian carcinomas. Specifically, genetic alterations are common in a high percentage of high-grade serous ovarian cancers, and therefore ideal targets for inhibitors in clinical development [50]. A 15 min treatment with recombinant SPON1 induced a slight increase in ERK1/2 phosphorylation and Akt S473 phosphorylation in HEY and OVCAR3 cells. SPON1 treatment did not affect Akt T308 phosphorylation.

SPON1 slightly increased the phosphorylation of GSK3 in both EOC cell lines, with more pronounced phosphorylation in HEY cells. GSK-3 has various roles in cancer, which even after years of study remain complex and controversial. GSK-3 is overexpressed in various tumour types including ovarian, in which it is believed to exert pro-proliferative effects [63].

In both EOC cell lines the most obvious change in phosphorylation levels following SPON1 treatment was a decrease of WNK1 T60 phosphorylation (39% reduction in HEY cells and 33% in OVCAR3 cells). The serine/threonine protein kinase WNK1 [with no lysine (K)] is ubiquitously expressed in tissues; however its biological functions and regulation are not well understood. The only known activators of WNK are changes in ionic strength, which is consistent with its role in regulating ion transport [64]. The down regulation of WNK1 in a mouse neural progenitor cell line greatly reduced cell growth and migration [65]. Despite our best efforts we were unable to confirm the phospho-kinase array results by Western blotting due to high background and non-specific antibody binding (data not shown).

Lastly, we analyzed the phosphorylation of Proline Rich AKT1 Substrate of 40 kDa (PRAS40) because our laboratory recently identified it to be a target of SPON1 in the KGN granulosa cell tumour cell line using the same phospho-kinase array. PRAS40 is a member of the mTORC1 complex, and when PRAS40 is not phosphorylated it is bound to mTORC1 and inhibits its activity. Phosphorylation of PRAS40 activates the mTORC1 complex and promotes ovarian cancer cell proliferation [66]. SPON1 does not significantly affect PRAS40 phosphorylation in either OVCAR3 or HEY cell lines (Figure S4-10).

The phospho-kinase array did not clarify which pathways SPON1 is using to affect biological events in ovarian carcinomas; consequently we will have to utilize other techniques when pursuing this question in the future, such as global gene expression analysis. We could also use a targeted approach and examine whether SPON1 interacts with integrins in this system. Not only are integrins one of the few known interacting partners of SPON1 (as discussed above), but they play an essential role in tumour

progression by providing a dynamic interface for the ECM to "integrate" with the cell interior [58, 67]. High levels of integrin expression increase tumourigenesis and impact cell shape, proliferation, and migration [68]. Integrin $\alpha_v\beta_3$ would be an interesting target to pursue considering it is expressed in most ovarian cancer cells [54] and SPON1 specifically blocks integrin $\alpha_v\beta_3$ in HUVECs [30]. Furthermore, it has been reported that interaction of $\alpha_v\beta_3$ with its ligand vitronectin promotes adhesion, proliferation, and motility of ovarian cancer cells [54]; blocking $\alpha_v\beta_3$ integrin function inhibits vitronectin-induced migration of ovarian cancer cells [69]. Although the precise mechanism of tumor progression promoted by $\alpha_v\beta_3$ remains inconclusive, various studies support a role for $\alpha_v\beta_3$ in ECM-induced phenotypic changes of ovarian cancer cells [54]. Therefore, it is reasonable to postulate that SPON1 may be acting through integrin $\alpha_v\beta_3$ to exert its effects on EOC cells, specifically, binding of SPON1 to $\alpha_v\beta_3$ integrin may decrease integrin $\alpha_v\beta_3$/vitronectin-mediated ovarian cancer cell adhesion and growth.

4.4.4 Examples of molecules known to inhibit biological properties of EOC cells

Many ECM components enhance adhesion, proliferation, migration and invasion of ovarian cancer cells. For example, collagen, laminin and fibronectin have previously been shown to enhance all of these biological properties in both HEY and OVCAR3 cell lines [58]. There are relatively far fewer examples in the literature of proteins that inhibit these functions in ovarian carcinomas, which make our observations of SPON1's function in EOC cells all the more intriguing. Some examples from the literature include TSP-1, SPARC, ADAM15 and S1P.

Thrompbospondin-1 (TSP-1), like SPON1, is also a member of the thrombospondin superfamily, and is an adhesive glycoprotein and a potent inhibitor of tumour growth, migration, invasion and angiogenesis [70]. The therapeutic use of TSP-1 has been a topic of research for many years because studies have shown that TSP-1 and its mimetic molecules can inhibit the growth of tumours of melanoma, pancreatic, lung and ovarian origin [71-73].

Secreted protein acidic and rich in cysteine (SPARC) is a secreted glycoprotein that interacts with various ECM macromolecules. SPARC is involved in the regulation of cell adhesion, proliferation, and migration, as well as in processes requiring ECM turnover such as tumor progression. The mechanism through which SPARC modulates cancer progression is complex and depends on tumor cell type and the surrounding microenvironment. SPARC has anti-proliferative and pro-apoptotic functions in ovarian cancer, and has also been shown to abolish ovarian carcinoma cell adhesion by inhibiting integrin-mediated cell adhesion to extracellular matrix proteins. [74]

ADAM15 is known to inhibit various biological functions in ovarian cancer. The ADAM proteins, a family of transmembrane and secreted glycoproteins, have diverse functions that include cell adhesion, cell fate determination, migration and intracellular signalling [75]. Double immunostaining has shown that ADAM15 and $\alpha_v\beta_3$ have a similar distribution pattern on the surface of ovarian cancer cells [75]. When ADAM15 is overexpressed in ovarian cancer cells it binds to integrin $\alpha_v\beta_3$ thereby decreasing integrin $\alpha_v\beta_3$/vitronectin-mediated ovarian cancer cell growth, adhesion and motility [75].

Sphingosine-1-phosphate (S1P) is a bioactive lipid molecule that inhibits the growth and survival of ovarian cancer cells. S1P has an inhibitory effect on cell attachment and cell adhesion; S1P inhibits cell attachment to the surface of uncoated culture dishes as well as dishes pre-coated with laminin, collagens I and IV and fibronectin [76]. Interestingly, Hong *et al.* suggest that the inhibitory effect of S1P on cell growth is preceded by its inhibitory effect on cell attachment or cell adhesion.

4.4.5 Does SPON1 have an oncogenic or tumour suppressive role in ovarian cancer progression?

Further *in vitro* and *in vivo* studies are required to determine whether SPON1 has an oncogenic or tumour suppressive role in ovarian cancer tumour progression. Survival analyses have suggested that SPON1 is a negative prognostic indicator [32]; therefore the SPON1-induced decrease in cell adhesion we observe may increase cell detachment at the primary tumour and thereby promote dissemination and tumour progression. However, we have also shown that SPON1 decreases EOC cell viability and

proliferation, suggesting it may be suppressing tumour growth. Therefore the reduced cell adhesion may inhibit cell attachment at secondary sites in the peritoneal cavity and reduce tumour progression.

The SPON1-induced decrease in adhesion, viability and proliferation I have observed *in vitro* can be explored further *in vivo* using the SPON1$^{-/-}$ mouse, which is viable and develops normally to adulthood [26]. Following implantation of ovarian cancer cells in the SPON1$^{-/-}$ mouse, tumour burden can be assessed by comparing tumour weight, ascites volume, and number of metastases to tissues within the peritoneal cavity.

4.4.6 Future Directions

Beyond the future experiments we have already proposed, further investigations into the acellular fraction of ascites and the potential role of SPON1 in tumour angiogenesis will help create a more comprehensive picture of SPON1's role in ovarian cancer.

Over one-third of ovarian cancer patients present with large amounts of ascites at the time of diagnosis [77]. Ascites accumulates when fluid production in the peritoneal cavity exceeds fluid reabsorption [78]. Ascites is composed of a cellular fraction containing ovarian cancer cells, lymphocytes, and mesothelial cells and an acellular fraction consisting of angiogenic and growth factors, bioactive lipids, cytokines and ECM components [77]. All of these factors have been shown to promote cell growth, survival and/or invasion. Clinical observations have revealed that the presence of ascites correlates with more extensive tumour spread [78]. *In vitro* studies have shown that the acellular fraction of ascites can affect proliferation, apoptosis, migration and invasion of EOC cell lines; both positive and inhibitory regulators of tumour progression can be present in the acellular fraction [77, 79]. In our study, we detected endogenous SPON1 in lysates generated from five EOC cell lines and eight primary ascites-derived samples, as well as conditioned medium from all EOC cells (Figure 4-2). Future studies could focus on examining the acellular fraction of ascites to confirm that EOC cells are secreting SPON1 *in vivo*.

It would also be beneficial to determine whether SPON1 regulates angiogenesis during ovarian cancer progression. Angiogenesis is the development of new blood vessels from the preexisting vasculature. This process is a key factor in the progression of cancer and has been shown to strongly correlate with risk of invasion and metastasis. A balance of pro- and anti-angiogenic factors controls angiogenesis. During tumour progression and metastasis there is a disruption in this balance that favours angiogenesis [80]. The unique method of ovarian cancer metastasis, by shedding of EOC single cells or small clusters from the primary tumour into the peritoneal cavity where they establish secondary lesions on abdominal organs, is one of the reasons ovarian cancer is difficult to treat. Unlike many epithelial cancers the initial dissemination of EOC rarely involves the vasculature; however, the vasculature is often involved in advanced stages of ovarian carcinomas [10]. Though SPON1 may either have a pro- or anti-angiogenic effect, there is evidence in the literature to support an inhibitory role for angiogenesis in ovarian cancer [30]. Future investigations could utilize established *in vitro* angiogenesis models, such as growing HUVEC cells in supernatant from EOC cells and establishing whether the presence of SPON1 impacts the formation of complex tube network, a hallmark of angiogenesis [81].

4.4.7 Limitations of study

We were unable to use a loss-of-function approach to establish a requirement of SPON1 for the phenotypic changes to EOC cells we have demonstrated by a gain-of-function approach. This was not due to omission or lack of effort. Firstly, we attempted to reduce SPON1 levels using an siRNA approach. Another member of our laboratory also had previously attempted to optimize the knockdown of SPON1 in granulosa cell lines using a number of different protocols, culturing conditions, primer sets and transfection reagents, without success. Nevertheless, due to the variability between cell types and cell lines, we attempted to optimize the conditions for knockdown of SPON1 in OVCAR3, OVCAR8 and HEY cell lines. The most success we had with this approach was a 45% and 40% reduction of SPON1 protein in HEY and OVCAR8 cells, respectively, that lasted only 24 h before SPON1 levels began to increase, and eventually were nearly restored to pre-knockdown levels.

We also attempted to block SPON1 function using anti-SPON1 antibodies, without success. The four antibodies we tested successfully detect SPON1 using Western blot analysis; therefore it is possible that either these antibodies are only able to bind to the denatured SPON1 protein following SDS-PAGE or are simply unable to act as functional blocking antibodies. Interestingly, the lower dose of the Abcam antibody (1 μg/ml) showed promise because we observed a slight decrease in cell adhesion with weak trypsinization compared to control cells; however the reduction in adhesion failed to reach statistical significance. Higher doses of this antibody caused the cells to take on a rounded morphology and detach in clusters following weak trypsinization. Therefore, we were unable to use a loss-of-function approach to further support the results and conclusions of our study.

4.4.8 Conclusions

This is the first study to determine cellular functions of SPON1 in ovarian carcinomas. We have shown for the first time that SPON1 decreases the adhesion, viability and proliferation of ovarian cancer cells. We have also demonstrated that SPON1 is endogenously expressed in, and secreted by, established ovarian cancer cell lines and primary human epithelial ovarian cancer cells. Further investigations are necessary to determine whether SPON1 plays an oncogenic, tumour suppressive or dichotomous role in ovarian cancer development and metastasis, and whether it may serve as a potential treatment target for the progression of ovarian cancer.

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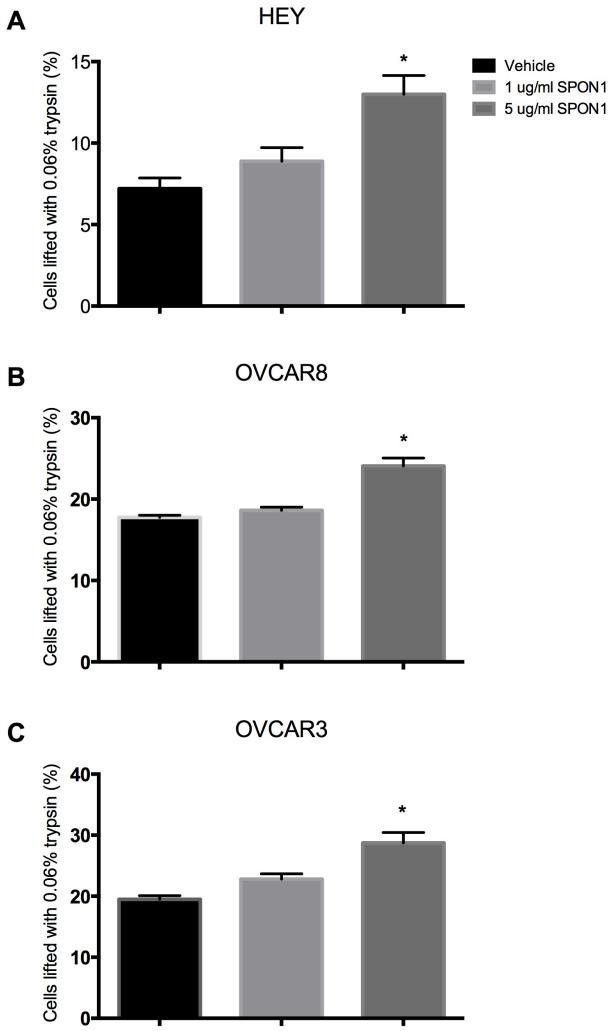

Figure S4-11: SPON1 affects EOC cell adhesion in a dose-dependent manner. HEY (A), OVCAR8 (B) and OVCAR3 (C) cells were treated with vehicle, 1 μg/ml recombinant human SPON1 or 5 μg/ml recombinant human SPON1 at the time of seeding. After 48 h the cells were detached first with 0.06% trypsin. Any adherent cells remaining were then detached with 0.25% trypsin. Cells from each pool were then counted. The proportion of cells detached by 0.06% trypsin is shown. Cells remained attached when incubated with 1 μg/ml SPON1 after weak trypsinization compared to controls. Treatment with 5 μg/ml SPON1 significantly increased the proportion of total cells that detached with weak trypsinization compared to vehicle. The data represent the mean ± SD of triplicate measurements. *$p<0.05$ as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

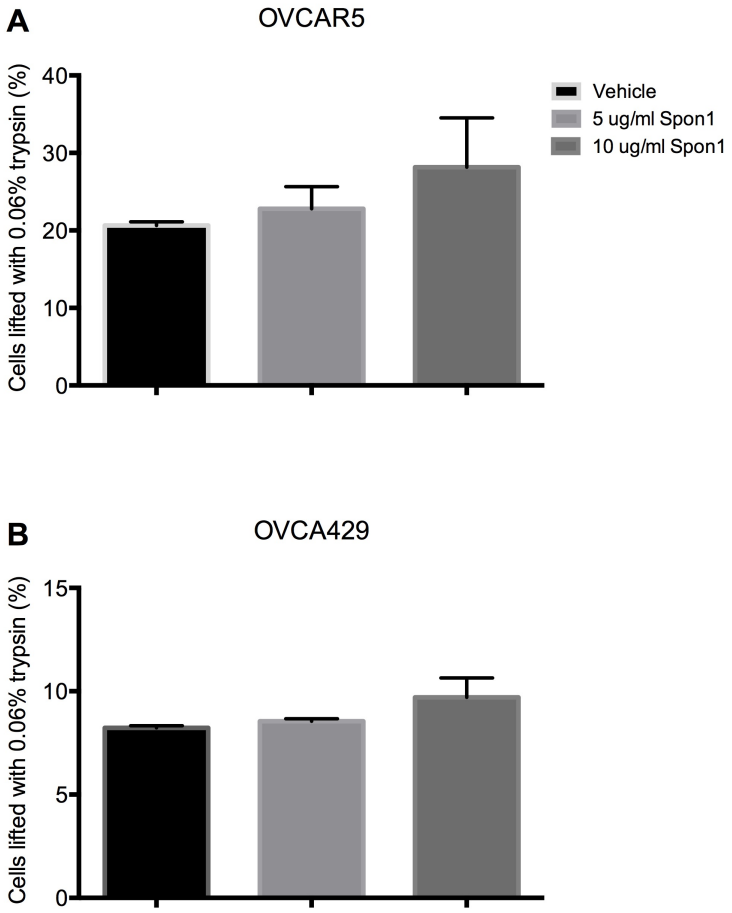

Figure S4-12: SPON1 does not affect the adhesion of OVCAR5 and OVCA429 cell lines.

OVCAR5 (A) and OVCA429 (B) cells were treated with vehicle, 5 μg/ml carrier-free recombinant human SPON1 or 10 μg/ml carrier-free recombinant human SPON1 at the time of seeding. After 48 h the cells were detached first with 0.06% trypsin (OVCAR5 incubated for 2 min, OVCA429 for 1 min in 0.06% trypsin). Any adherent cells remaining were then detached with 0.25% trypsin. Cells from each pool were then counted. The proportion of cells detached by 0.06% trypsin is shown. Treatment with SPON1 did not have an affect on the proportion of total cells that detach with weak trypsinization compared to vehicle. The data represent the mean ± SD of triplicate measurements from two independent experiments. Differences in cell detachment between vehicle and SPON1 treatments were analyzed by one-way ANOVA with Tukey's test.

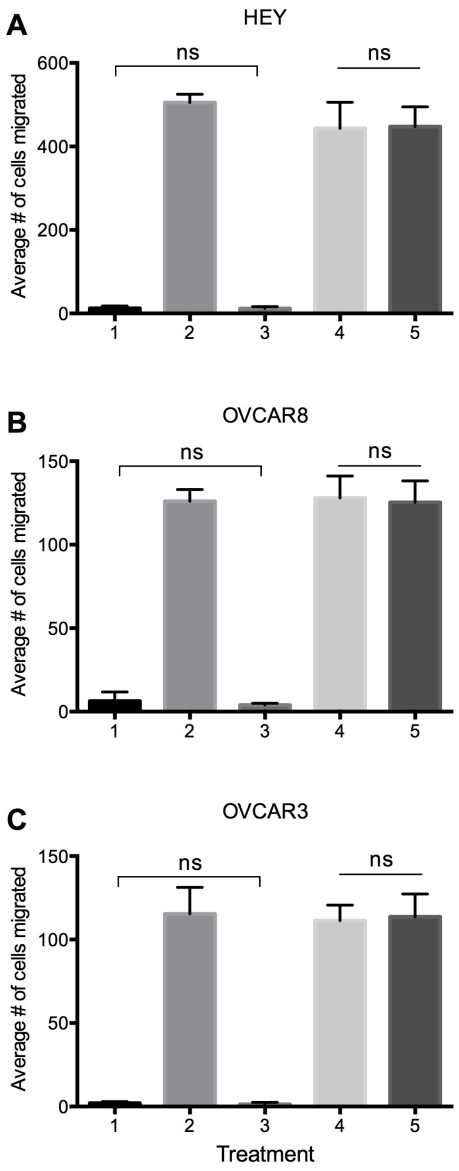

Figure S4-13: Ovarian cancer cell migration is not affected by SPON1.

EOC cell lines were treated at the time of seeding in the upper chamber of transwell inserts with 5 µg/ml recombinant human SPON1 or left untreated. Cells were allowed to migrate across the transwell membrane towards 5 µg/ml recombinant human SPON1 in 0.5% FBS or 10% FBS. EOC cell migration was not effected by the presence of SPON1. Effect of treatment determined by one-way ANOVA. Treatments (Top of insert/ Bottom of insert): (1) 0.5% FBS/ 0.5% FBS; (2) 0.5% FBS/ 10% FBS; (3) 0.5% FBS/ 0.5% FBS+5 µg/ml SPON1; (4) 0.5% FBS+5 µg/ml SPON1/ 10% FBS; (5) 0.5% FBS+5 µg/ml SPON1/ 10% FBS+5 µg/ml SPON1.

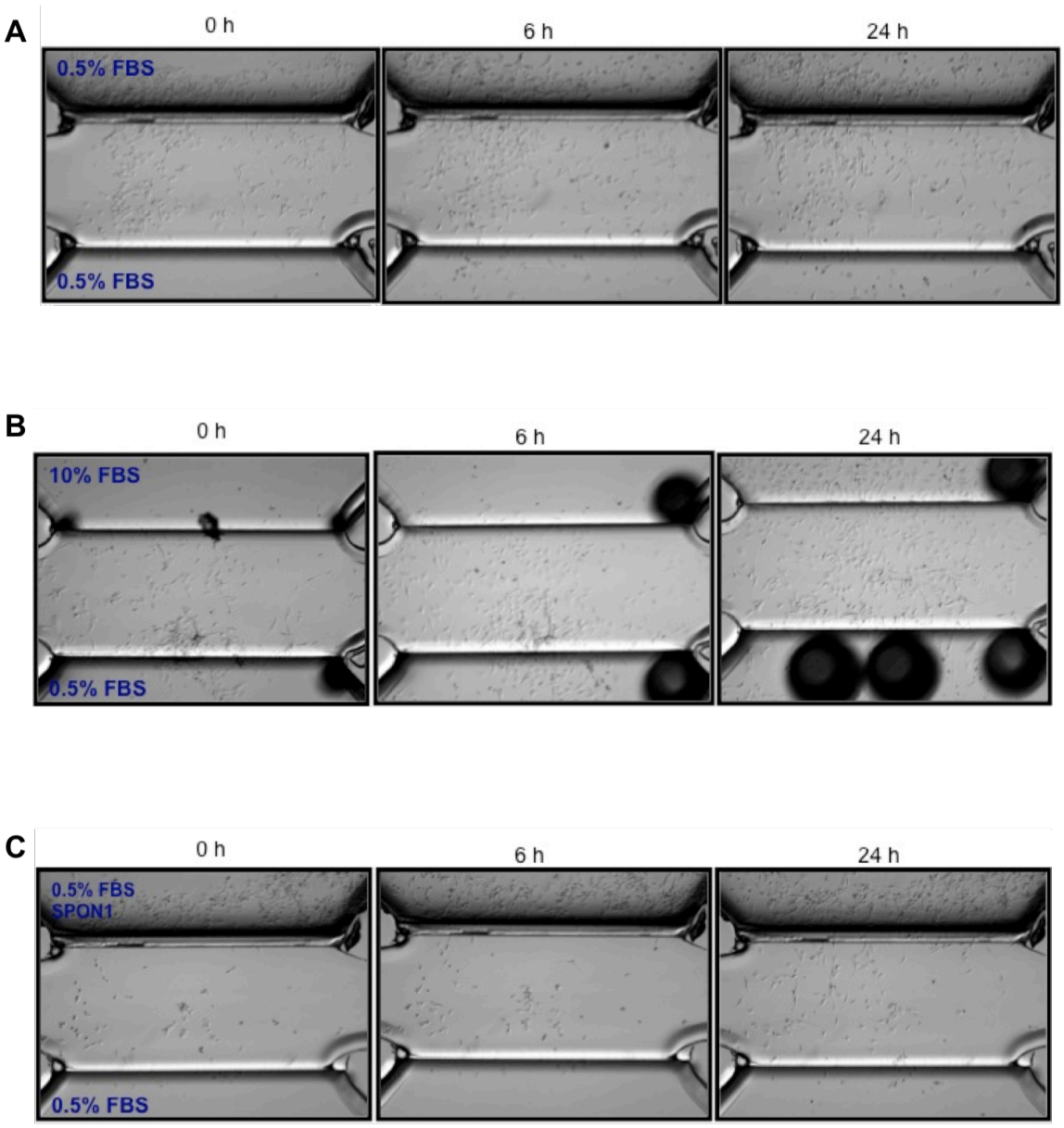

Figure S4-14: SPON1 does not affect the chemotaxis of HEY cells.

The ibidi µ-Slide Chemotaxis^{2D} system was used to determine whether SPON1 acts as a chemoattractant or chemorepellant. HEY cells were seeded in the observation chamber of the slide and the bottom chamber was filled with 0.5% FBS. The top chamber was filled with (A) 0.5% FBS, (B) 10% FBS or (C) 0.5% FBS+10 µg/ml recombinant human SPON1. Cells were allowed to migrate over 24 h. Images were captured at 0 h, 6 h and 24 h. The experiment was repeated three times and representative images are shown. HEY cells migrated randomly indicating SPON1 does not affect their chemotaxis.

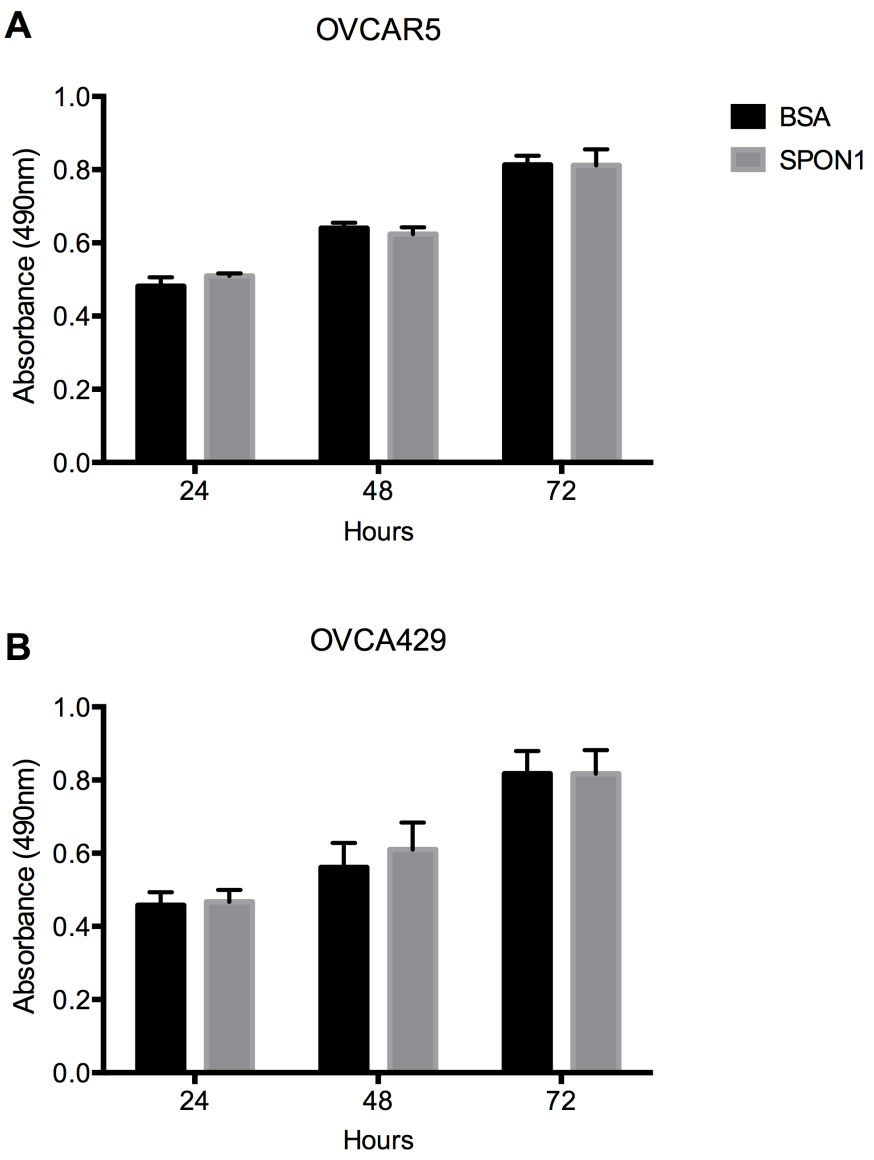

Figure S4-15: SPON1 does not affect the viability of OVCAR5 and OVCA429 ovarian cancer cell lines.

Cell viability was assessed in OVCAR5 (A) and OVCA429 (B) cells by MTS assay in the presence or absence of SPON1. Cells were cultured in BSA vehicle (black bars) or 5 μg/mL human recombinant SPON1 (grey bars). Each bar represents the mean ± SD of triplicate measurements. Each assay is representative of three independent experiments. SPON1 does not significantly affect the viability of these EOC cell lines. Two-way ANOVA with Tukey's test was performed.

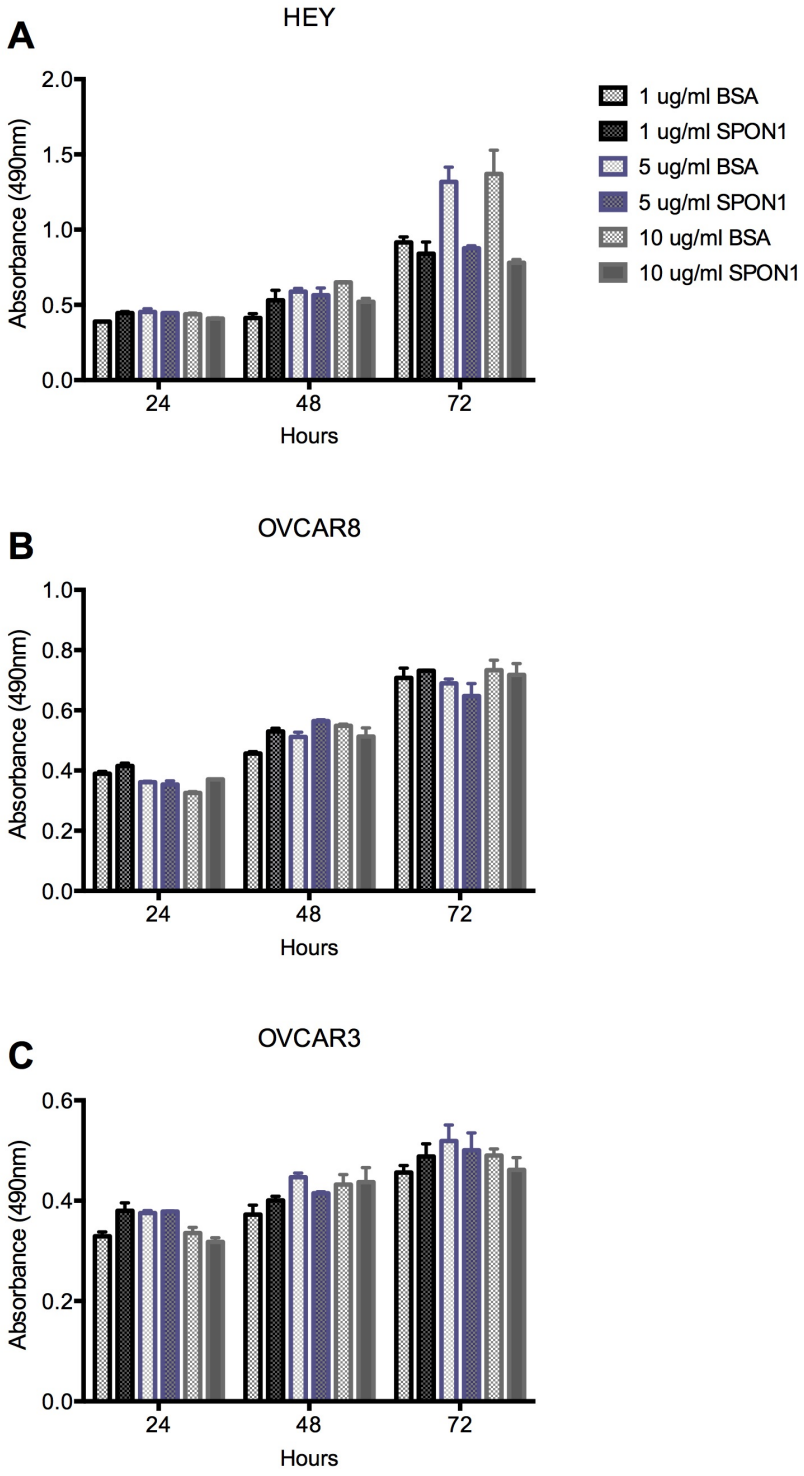
A
HEY
Absorbance (490nm)
2.0
1.5
1.0
0.5
0.0
24
48
72
Hours
1 ug/ml BSA
1 ug/ml SPON1
5 ug/ml BSA
5 ug/ml SPON1
10 ug/ml BSA
10 ug/ml SPON1
B
OVCAR8
Absorbance (490nm)
1.0
0.8
0.6
0.4
0.2
0.0
24
48
72
Hours
C
OVCAR3
Absorbance (490nm)
0.6
0.4
0.2
0.0
24
48
72
Hours

Figure S4-16: The effect of serial dilutions of recombinant human SPON1 on the viability of EOC cell lines.

Cell viability was assessed in HEY (A), OVCAR8 (B) and OVCAR3 (C) cells by MTS assay. Cells were cultured in BSA (vehicle) or serial dilutions of human recombinant SPON1 (1 μg/mL, 5 μg/mL or 10 μg/mL). Each bar represents the mean ± SD of triplicate measurements. The viability of EOC cells was not affected by 1 μg/mL SPON1 treatment. HEY cell viability was significantly decreased with 5 μg/mL and 10 μg/mL SPON1 treatment, but a dose response relationship does not exist because there was not a significant difference in HEY cell viability between 5 μg/mL and 10 μg/mL SPON1. The viability of OVCAR3 and OVCAR8 cells was not affected by SPON1 treatment.

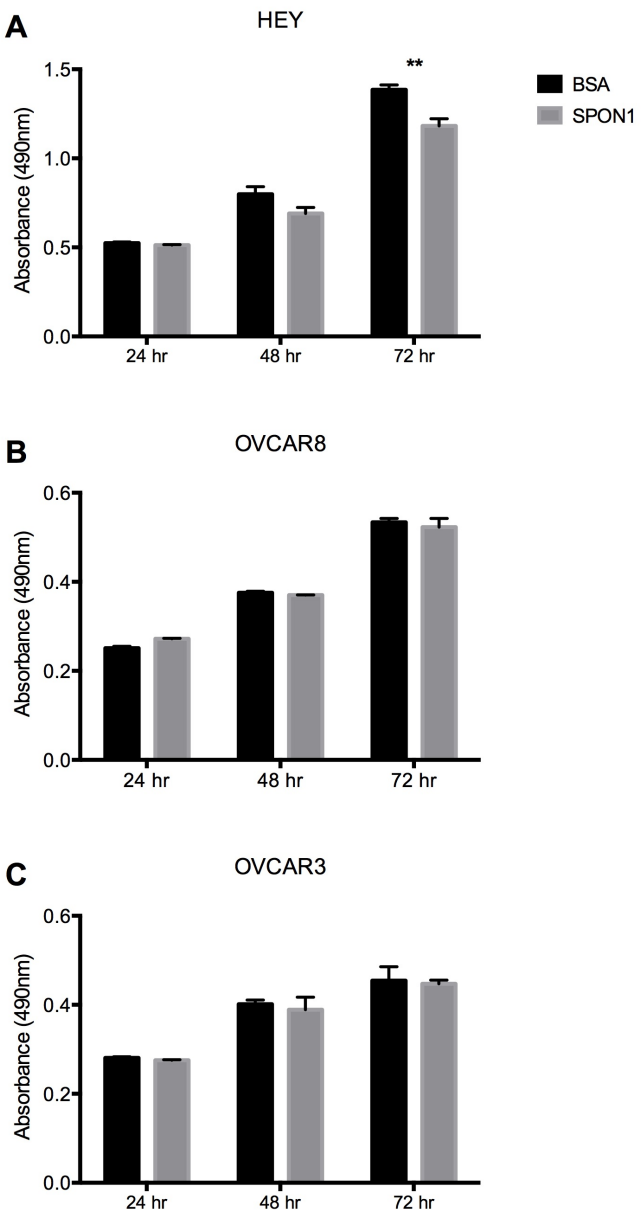

Figure S4-17: Replenishing recombinant human SPON1 treatment daily does not increase the effect of exogenous SPON1 on viability of EOC cell lines.

Cell viability was assessed in HEY (A), OVCAR8 (B) and OVCAR3 (C) cells by MTS assay. Cells were cultured with BSA (vehicle) or 5 µg/mL human recombinant SPON1, and treatments were replenished daily. The effect on viability of EOC cell lines was the same whether cells were treated with a single dose of recombinant SPON1 (Figure 4-7) or when recombinant SPON1 was replenished daily. Two-way ANOVA with Tukey's test was performed (**p<0.01).

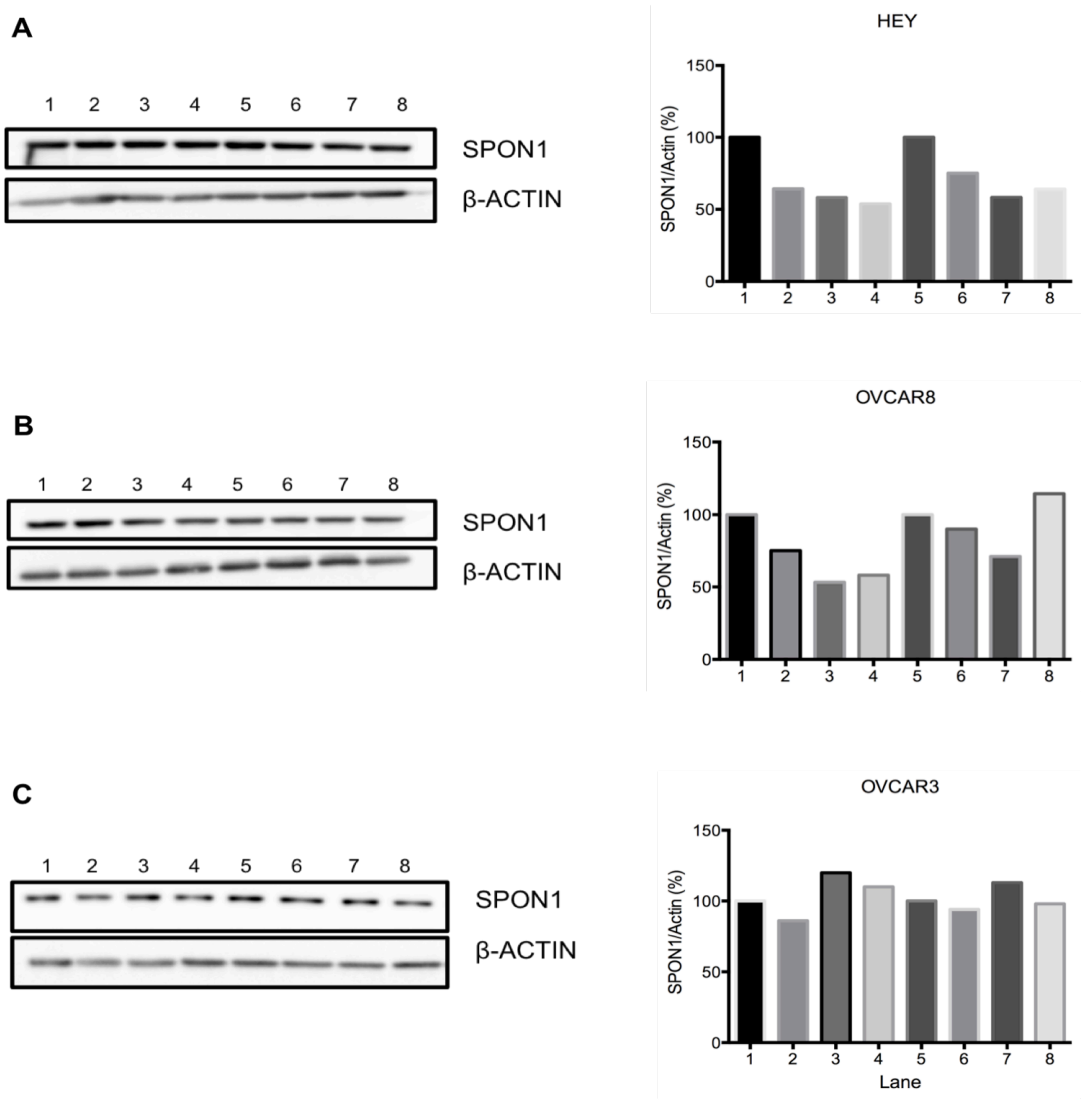

Figure S4-18: siRNA-mediated knockdown of SPON1 was not achieved.
Western blot performed for SPON1 as indicated on HEY (A), OVCAR8 (B) and OVCAR3 (C) cells 48 and 72 h after transfection. β-actin was used as a loading control. Lanes: (1) 48 h No treatment (NT); (2) 48h 10nM siRNA 1; (3) 48h 10nM siRNA 2; (4) 48h 10nM siRNA 3; (5) 72 h NT; (6) 72h 10nM siRNA 1; (7) 72h 10nM siRNA 2; (8) 72h 10nM siRNA 3. Densitometric quantification was carried out using *ImageJ* software, and calculating SPON1 expression relative to β-actin control. The NT controls (Lane 1 and Lane 5) were considered as 100% SPON1 expression and all values were normalized to these values (Lanes 2-4 were normalized to Lane 1, Lanes 6-8 were normalized to Lane 5).

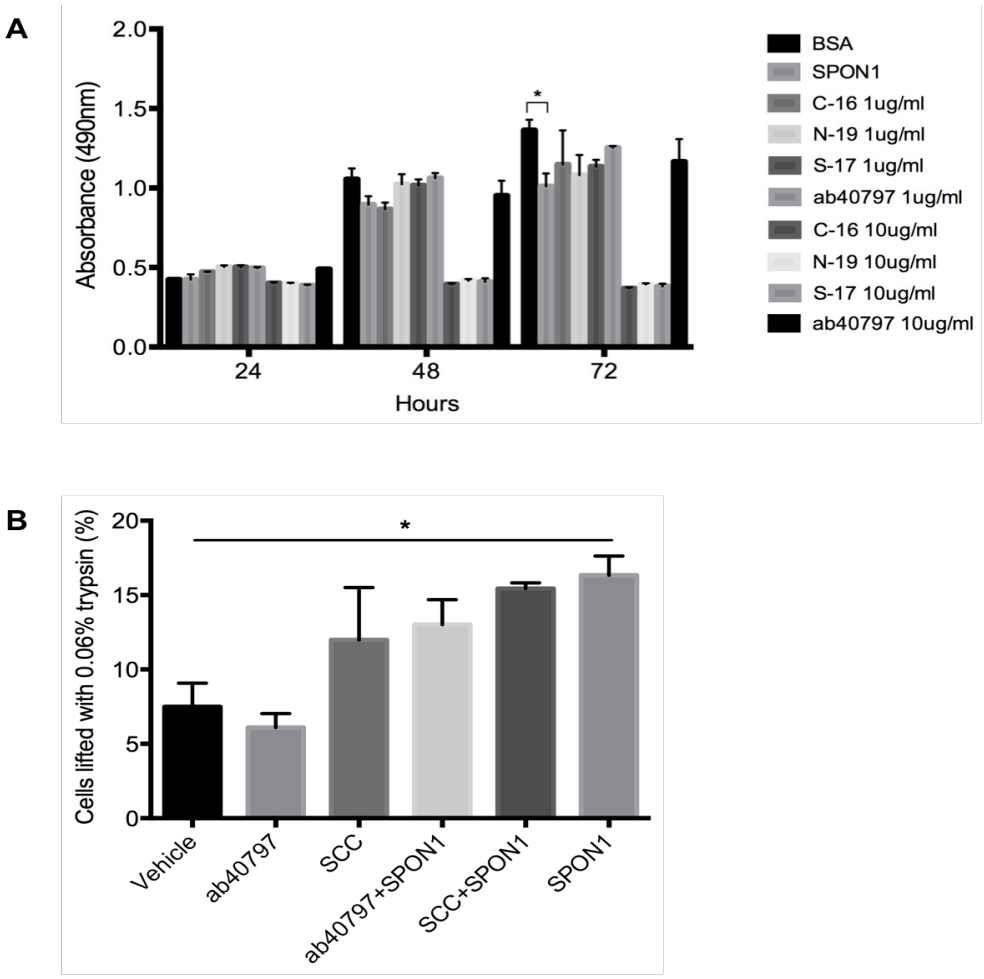

Figure S4-19: Anti-SPON1 antibodies did not block SPON1 function.

A) Cell viability was assessed using the MTS assay. Four antibodies generated against SPON1 (1 μg/mL and 10 μg/mL) were co-incubated with HEY cells in an attempt to block endogenous SPON1 function. Co-incubation with the antibodies did not increase cell viability, which indicated SPON1 function was not blocked. Two-way ANOVA with Tukey's Test was performed (*p<0.05).

B) HEY cells were co-incubated with anti-SPON1 antibodies – ab40797 and 3 Santa Cruz antibodies combined (SCC) ± 5 μg/ml recombinant human SPON1. After 48 h the cells were detached first with 0.06% trypsin and any adherent cells remaining were then detached with 0.25% trypsin. Cells from each pool were then counted. The proportion of cells detached by 0.06% trypsin is shown. Co-culture with anti-SPON1 antibodies did not block SPON1 function. The data represent the mean ± SD of triplicate measurements. One-way ANOVA with Tukey's Test was performed (*p<0.05)

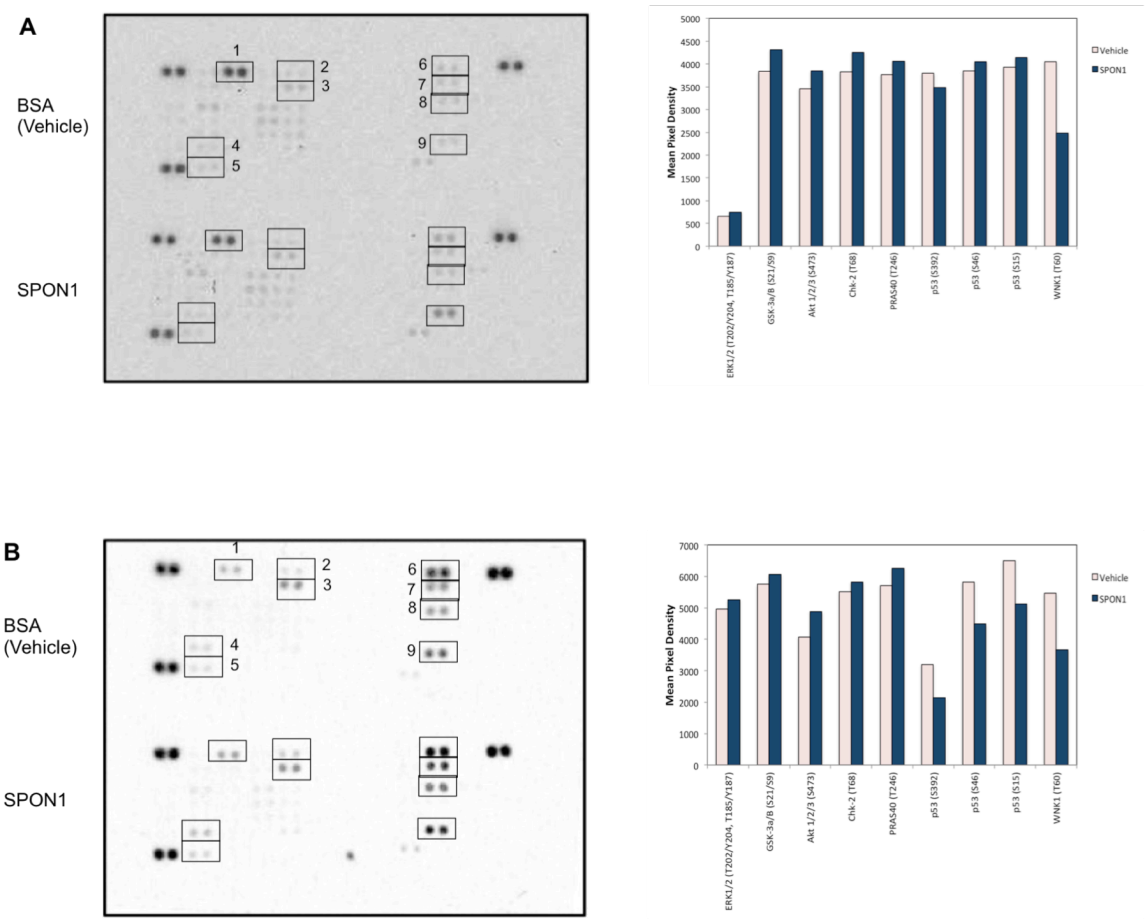

Figure S4-20: Phospho-kinase array in control and SPON1-treated EOC cells. HEY (A) and OVCAR3 (B) cells were incubated for 15 min with 250 µg/ml BSA (vehicle control) or 5 µg/ml SPON1. Total protein lysates were incubated with membranes containing capture antibodies (spotted in duplicate) against kinase phosphorylation sites (R&D Systems). The membranes were then incubated with biotinylated detection antibodies, streptavidin-HRP, and proteins were detected using chemiluminescence. Densitometric quantifications were done using *ImageJ* software, data are presented in the graphs as a mean pixel density (n = 2 spots). Spots: 1) ERK1/2 (T202/Y204, T185/ Y187); 2) GSK-3α/β (S21/S9); 3) Akt 1/2/3 (S472); 4) Chk-2 (T68); 5) PRAS40 (T246); 6) p53 (S392); 7) p53 (S46); 8) p53 (S15); 9) WNK1 (T60).

Chapter 5

5 Discussion

5.1 Summary of findings

The ECM plays an active and complex role in regulating cell growth, survival, motility, polarity and differentiation. It also provides the structural foundation required for tissue function and regulates the availability of growth factors and cytokines. The studies within this thesis examined the expression of several ECM components within the ovary of the ERβ-knockout mouse (βERKO) and the role of the ECM protein, Spondin 1, in the progression of epithelial ovarian cancer (EOC).

Microarray analysis performed by Dr. Deroo indicates that ECM expression is disrupted in βERKO GCs [1] and may contribute to the attenuated folliculogenesis observed in βERKO ovaries. To further investigate these observations (Chapter 2), I used qPCR and immunofluorescence (IF) assays to characterize the ovarian expression and localization of two ECM proteins that had been identified as differentially expressed in ERβ-null GCs, namely Collagen 11A1 (*Col11a1*) and Nidogen 2 (*Nid2*) [1]. I found that expression of both Collagen11a1 and Nid2 is significantly higher in βERKO ovaries than in wildtype ovaries as early as PND 13, and this heightened expression continues through PND 23–29 into adulthood. Similarly, I examined the expression and localization of the ECM proteins Collagen IV (*Col4a1*), Nidogen 1 (*Nid1)* and Laminin (*Lama1*), which had not been identified by the original microarray, but are well-known mouse ovarian ECM proteins. Collagen IV, Nidogen 2 and Laminin were also more highly expressed in the βERKO ovary than in wildtype. This data suggests that ERβ represses the expression of several ECM proteins in the mouse ovary. In addition, given that dysregulation was observed as early as PND 13, my data also indicates that granulosa cell (GC) gene expression is regulated by ERβ prior to puberty—an unexpected and novel finding.

In Chapter 3, I investigated a potential mechanism by which ERβ may be acting as a transcriptional repressor in GCs. I used transient transfection assays to show that the bHLH transcription factor, TCF21, regulates estradiol-dependent transcriptional activity

in an ER isoform-specific manner, and represses ERβ, but not ERα-driven transactivation. TCF21 represses ERβ-mediated transcription of a 3x-ERE reporter in several cell lines, as well as three naturally occurring estrogen responsive promoters (pS2, C3 and Lf). This repression does not require TCF21 heterodimerization with E12. Lastly, the bHLH transcription factor Mist1 does not repress ERβ-mediated transcription of the 3x-ERE reporter, suggesting that not all bHLH proteins repress ERβ-mediated transcription. Despite our best efforts we were unable to show that TCF21 forms a complex with ERβ *in vivo*. Therefore, we turned our focus to another ERβ-dependent ECM protein identified in Dr. Deroo's microarray, SPON1, and its potential role in ovarian cancer progression.

SPON1 is highly overexpressed in ovarian cancer and has recently been identified as a promising ovarian cancer marker, particularly for high-grade serous EOC. Therefore, in Chapter 4, I examined whether SPON1 affects key ovarian cancer cell functions in immortalized EOC cell lines and in human primary ascites-derived ovarian cancer cells. I confirmed by Western blot that SPON1 is expressed and secreted by both immortalized ovarian cancer cell lines and primary ascites-derived ovarian cancer cells. Although I was unable to silence SPON1 expression in EOC cell lines using siRNA or functional blocking with antibodies, I performed several functional assays to assess whether treatment of EOC cells with recombinant SPON1 affects specific cellular processes. My data demonstrated that SPON1 significantly reduced EOC cell adhesion, viability and proliferation; however, it did not affect cell migration. Finally, using a non-adherent culture system I examined whether SPON1 affects EOC spheroid formation and subsequent reattachment to adherent tissue culture plastic. Treatment of EOC cells with recombinant SPON1 prior to spheroid formation did not impact the formation or reattachment of spheroids. However, treatment of spheroids with recombinant SPON1 following transfer to adherent tissue culture plastic did increase the cell dispersion area of spheroids that had re-attached. These data suggest that SPON1 regulates a subset of functions of ovarian cancer cells.

In summary, my thesis work has shown that ECM proteins are aberrantly overexpressed in the βERKO mouse ovary, as well as high-grade serous EOC. The

expression of the ECM proteins investigated herein is ERβ-dependent. This body of work contributes to our understanding of the role ECM proteins have in ovarian development and ovarian cancer progression.

5.2 Potential mechanisms by which the ECM regulates ovarian follicle development/ growth and EOC progression

The histological analysis of mammalian organs demonstrates the incredible complexity of cellular organization required to build and maintain normal tissues [2]. The disruption of this structural organization usually leads to disease and neoplastic transformation. Several biological functions are mediated by the interaction of ECM proteins with binding partners, which include other ECM components, growth factors, signal receptors and adhesion molecules [3, 4]. Normal tissue organization requires two key components: (1) organized cell-cell and cell-ECM adhesion, and (2) the establishment and maintenance of cell polarity. The dysregulation of these two functions is a hallmark of cancer. In this section of the Discussion, I will briefly introduce the key features of organized adhesion and polarity, and describe how these features relate to ovarian follicle growth and EOC progression. Finally, I will discuss how the ECM proteins I investigated may be affecting these two critical components of tissue organization. Specifically, I believe that the ECM proteins I investigated in Chapter 2 help maintain ovarian structural integrity, whereas Spondin 1 (Chapter 4) is a matricellular protein that lacks a structural role but is involved in cell-matrix interactions.

5.2.1 Adhesion

Within tissues, cells physically interact with the ECM (cell-ECM adhesion) as well as neighbouring cells (cell-cell adhesion). The correct adhesion of a cell to ECM components determines whether the cell is in the correct location and consequently regulates cell survival. Cells that lose cell-cell or cell-ECM adhesion undergo apoptosis to restrict inappropriate cell growth [5]. Various adhesion molecules mediate cell-cell and cell-ECM interactions, and functional units of cell adhesion can be grouped into three

general classes: adhesion receptors, ECM proteins, and cytoplasmic membrane proteins [6].

Cell adhesion receptors are typically transmembrane glycoproteins, and include members of the cadherin, integrin, immunoglobulin, selectin and proteoglycan superfamilies [6]. These receptors bind to other adhesion receptors on neighbouring cells or to proteins of the ECM. ECM proteins include members of the collagen, fibronectin, nidogen, laminin, and proteoglycan families.

Cadherins are one of the most important and ubiquitous cell adhesion receptors involved in cell-cell adhesion and recognition [7]. They are associated with adherens junctions, which link adjacent cells, and exhibit functional adhesion activity by forming a complex with catenins and the actin cytoskeleton [6]. Classic members of the cadherin family are named for the tissue in which they were originally discovered, and include E (epithelial), N (neural), P (placental), and VE (vascular endothelial) cadherin [8].

Focal adhesions are large, dynamic, integrin-containing complexes that connect cells to the ECM. Integrins are the major adhesion receptors within focal adhesions, and facilitate crosstalk between the ECM and the cell. The stimulation of integrins triggers intracellular signals and activates signalling proteins such as focal adhesion kinase (FAK) [9]. Cells adhere to the ECM via specific integrin-matrix ligand interactions.

Several studies have demonstrated that cell-cell and cell-ECM adhesions are interdependent, and that modulation of extracellular factors that alter cell-ECM interactions can directly impact cell-cell interactions [10-12]. The interdependence of cell-cell and cell-ECM forces is likely disrupted by the overexpression of ECM components, which has implications in development and disease.

5.2.1.1 Adhesion in folliculogenesis and its disruption in the βERKO ovary

There are several changes to cell-cell and cell-matrix adhesions that occur during the functional and morphological changes of folliculogenesis; consequently both cadherins and integrins are involved in the maintenance and remodeling of the ovary [13, 14]. E-cadherin and N-cadherin are expressed throughout prepubertal development in the

oocyte and GCs, respectively. Adherens junctions expressing N-cadherin exist between GCs and between GCs and the oocyte [15]. Blocking N-cadherin reduces follicle formation, whereas blocking E-cadherin accelerates follicle formation, suggesting the latter is likely involved in maintaining oocytes in nests (clusters of germ cells that break down to form primordial follicles) [16]. Multiple laboratories have reported the presence of integrins in the ovary [14, 17]; however, unlike the cadherins, the role of integrins in ovarian function is less clear. Integrin $\alpha_6\beta_1$, for example, is likely necessary for maintaining a healthy, nonluteal GC phenotype [14]. Burns *et al.* found that several integrin subunits were expressed at various stages in folliculogenesis; however, their role in follicle growth has not been pursued [14]. The integrin subunit β3 has been detected in GCs, TCs and interstitial cells and it is postulated that FSH controls its expression [17].

My data suggest that cell-cell and/or cell-ECM interactions may be disrupted in the βERKO ovary. The effect of overexpressed ECM components on GC adhesion and adhesion complexes can be examined using several techniques. One mechanism, by which the overexpressed ECM proteins may be affecting cell adhesion, is by dysregulating the expression of adherens junction and/or focal adhesion components [18-21]. A potential preliminary experiment to determine whether cell-cell or cell-ECM adhesion complexes are disrupted in the βERKO mouse would be to characterize the expression (qPCR and Western blot) and localization (IF) of cadherins and integrins known to be expressed throughout folliculogenesis. Secondly, adhesion can be assessed with the Detachment and Adhesion Assays that I used in Chapter 4; GC cell lines can be treated with recombinant protein and cell adhesion measured using these established techniques. Alternatively, we could create transgenic mice that overexpresses Col11a1 or Nid2 using GC-specific promoters (CYP19A1-Cre for FSH-responsive stage, PRE-Cre for the luteal stage), and examine the expression of adherens junction and focal adhesion components.

The potential impact that ECM proteins within the focimatrix have on GC adhesion is of particular interest, because unlike the follicular basal lamina, the function of the focimatrix remains largely unknown. Although its punctate morphology suggests it is does not perform typical basal lamina functions, such as filtering material or creating

microenvironments for enclosed cells [22], it has been postulated by Irving-Rogers et al. that GCs interact with the focimatrix via integrins [23]. I propose that the overexpressed ECM proteins in the βERKO focimatrix compete for integrin receptors [24-26]. Therefore, the "integrin binding competition" disrupts accurate integrin-matrix adhesions, thereby disrupting integrin-signaling. Of note, Nidogen 2, which was significantly overexpressed in the βERKO mouse focimatrix, has previously been reported to interact with integrin $\alpha_6\beta_1$ *in vitro* [27], which is expressed in the mouse ovary [14]

There is compelling evidence in the literature that in addition to biochemical signaling and hormonal cues, mechanical signaling from the physical environment regulates the development and function of the ovary [28, 29]. This regulation can occur in a number of ways. Firstly, the physical environment regulates follicle growth in culture when hormonal stimulation is constant [29, 30]. For example, the substrate on which a follicle is cultured (collagen, laminin or a poly-L-lysine control) affects theca cell (TC) development and antrum formation [28]. Secondly, a rigid, dense environment within the ovary maintains primordial follicles in a dormant state. As a follicle migrates towards the medulla of the ovary it moves into a less dense matrix, which permits follicle growth; therefore, the ovarian physical environment may initiate the growth of an immature, dormant follicle [31]. *In vitro*, decreasing matrix stiffness and solids concentration of alginate hydrogel enhances follicle growth and function, whereas a stiff environment hinders follicle development [30]. If the overexpressed ECM proteins in the βERKO ovary maintain a higher-than-normal ECM density compared to wildtype, the βERKO follicles would be more rigid, thus restricting follicle growth. The movement of follicles from a dense to a less dense environment may also be hindered by this increased matrix density, and consequently contribute to the premature arrest of folliculogenesis observed in the βERKO ovary.

In summary, I predict that the higher expression of ECM components disrupts the maintenance of the adherens junction and focal adhesion complexes as well as matrix stiffness within the follicle. Aberrant expression of adhesion complex proteins may impact mechanical signals that are relayed to the cells by cell adhesion receptors. Consequently, these dysregulated mechanically induced signaling cascades may impact

cell form and function, ultimately leading to the arrest of follicle growth and subfertility observed in βERKO mice.

5.2.1.2 Adhesion and EOC tumour progression

Reversible changes in cell-cell and cell-matrix adhesion occur to facilitate ovarian tumour progression. The initial dissemination of cells from the primary tumour necessitates a disruption of cadherin-mediated cell-cell adhesions as well as integrin-matrix contacts. Subsequently, tumour cells in suspension utilize cell-cell adhesions to form multicellular spheroids. The development of metastases involves remodeling of the cadherin-based adhesions as the spheroid disaggregates on the mesothelium of the peritoneal cavity, while the integrin-matrix adhesions anchor the cells to the mesothelium [32-34]. Therefore, it would be of interest to examine the role of SPON1 in mediating the adhesion between tumour cells, as wells as its contribution to tumour-stroma interactions. Furthermore, cell survival and proliferation are adhesion-dependent phenomenon in anchorage-dependent tumor cells, therefore the decreased viability and proliferation of EOC cells I have observed following treatment with recombinant SPON1 may be a result of decreased cell adhesion.

The ovarian surface epithelium (OSE) is unlike the majority of epithelia because it generally lacks E-cadherin expression; rather its cell-cell integrity is maintained by N-cadherin [35]. E-cadherin expression becomes more abundant during ovarian carcinogenesis; however, its expression is reduced at advanced stages and in ascites-derived tumour cells. This is referred to as the “cadherin switching”, whereby N-cadherin and P-cadherin compensate for the loss of E-cadherin in advanced tumours and EOC spheroids [36]. The switch in cadherin expression is indicative of an EMT (discussed in Section 5.2.2).

The ability to resist anoikis is a critical mechanism in tumour metastasis [37], and EOC cells in suspension within the abdominal cavity form multicellular spheroids to maintain cell-cell contact as part of their natural survival response [34]. Both integrins and cadherins facilitate this cell compaction [38]. EOC spheroids acquire E-cadherin-mediated adhesion as a means to suppress anoikis [35], and downregulation of E-

cadherin has been shown to decrease EOC cell viability [39]. The formation of ovarian cancer spheroids is also mediated by integrins, and spheroid formation is disrupted when cells are treated with antibodies that block α5- or β1-integrin subunits [40]. In Chapter 4, I found that treatment of spheroids with recombinant SPON1 after the spheroids were transferred from non-adherent to adherent tissue culture dishes significantly increased the cell dispersion area of the attaching spheroid. SPON1 is likely affecting cell adhesion mechanisms in this model system. In the future, it would be interesting to investigate whether SPON1 affects the cadherin switch by measuring the relative levels of E-cadherin, N-cadherin and P-cadherin, following spheroid formation, as well as the expression of integrins involved in the disaggregation and re-attachment of spheroids. The potential effect of exogeneous SPON1 on the mRNA and protein expression levels of these adhesion components should also be examined.

As when formed into multicellular spheroids, individual ovarian cancer cells express several integrins that allow them to bind ECM proteins, and many integrins have been shown to affect the adhesion of EOC cells [41-44]. To examine whether integrins and their associated signaling proteins are involved in the SPON1-mediated decrease in EOC cell adhesion that I observed in both ovarian cancer cell lines and EOC patient samples, select molecules can be investigated in future studies. FAK and $\alpha_v\beta_3$ integrin would be ideal preliminary targets because both are expressed in ovarian cancers (both promote tumour progression) [43, 45-47], and SPON1 has been shown to interact with or signal through them in other model systems [25, 48]. Phosphorylation of FAK promotes cancer cell growth; therefore, if SPON1 inhibits FAK phosphorylation (as observed in HUVECs [25]) it may be a mechanism by which SPON1 reduces EOC cell adhesion and proliferation, as I observed in Chapter 4. SPON1-induced changes in FAK phosphorylation can be examined by immunoblot. Additionally, FAK can be silenced using siRNAs, and functional assays can be performed to determine whether SPON1 is acting through FAK to effect cell adhesion and proliferation.

Alternatively, SPON1 may be acting through integrin $\alpha_v\beta_3$ to exert its effects on EOC cell adhesion, potentially by disrupting the interaction of this integrin with vitronectin. Vitronectin is a ligand of integrin $\alpha_v\beta_3$, and this interaction promotes

adhesion, proliferation, and motility of ovarian cancer cells [43]. Blocking $\alpha_v\beta_3$ integrin function inhibits vitronectin-induced migration of ovarian cancer cells [47]. SPON1 has previously been shown to block the integrin $\alpha_v\beta_3$/vitronectin interaction in HUVECs [25]. Therefore, binding of SPON1 to integrin $\alpha_v\beta_3$ (over vitronectin) may decrease integrin $\alpha_v\beta_3$/vitronectin-mediated ovarian cancer cell adhesion and growth, thereby disrupting the intracellular signals regulating cell survival and progression. The expression of integrin $\alpha_v\beta_3$ can be blocked using neutralizing antibodies against its subunits, an approach that has been successfully utilized with several integrins in ovarian cancer [41]. Considering we were unable to successfully silence SPON1 expression with an siRNA approach, identifying and silencing its downstream targets will be a valuable alternative method to continue exploring the role of SPON1 in the metastatic progression of EOC.

There are many examples in the literature where a change in cell adhesion corresponds to a change in cell motility and invasion [41, 43, 47-51]. Therefore, it was surprising that the SPON1-induced decrease in adhesion did not correspond to an effect on EOC cell migration in trans-well assays (Chapter 4, Fig. S3). This may be a limitation of the technique I used. An alternative functional assay, for example the scratch-wound assay, may demonstrate that SPON1 affects cell motility (as suggested by the increased cell dispersion area following treatment of spheroids with SPON1).

5.2.2 Cell polarity

Cells have a defined organization, with an asymmetric distribution of proteins and physical features of the cell, including the cell surface, cytoskeleton and organelles. An internal axis of polarity is created during morphogenesis and this creates apical-basal polarity of the cell. Polarity is involved in the biological processes of cells and tissues that necessitate an asymmetrical symmetry. These processes include growth, survival, migration and epithelial-to-mesenchymal transitions (EMT) [52].

The synchronized actions of three protein complexes direct the establishment and maintenance of apical-basal polarity: the Crumbs (Crumbs–Patj–Pals), Scribble (Scribble–Lgl–Dlg) and Par (Par3–Par6–aPKC) complexes [2]. Atypical protein kinase (aPKC) is the catalytic component of the Par complex [53]. There are two homologues of

aPCK, aPCKι and aPKCζ, both of which have been implicated in human cancers; aPCKι expression is upregulated in ovarian cancer and correlated with poor prognosis [54]. Polarity is achieved by the interaction of these three complexes with the structural components of the cytoskeleton and adherens junctions between cells. Several extracellular cues are required for the epithelial cells to exhibit all aspects of polarity, and attachment to the ECM prompts the formation of the apical-basal axis [2].

Adhesion and polarity are closely interrelated; cell polarity mechanisms rely on the formation and maintenance of adherens junction complexes, and the activities of the polarity complexes are required for the maintenance of adherens junction complexes [2]. EMT, which occurs in normal physiological processes but has also been linked to cancer progression, requires the disruption of cell-cell and cell-matrix adhesions, as well as the loss of apical-basal polarity [52]. EMT is a process by which a polarized epithelial cell, which normally interacts with the basement membrane (BM), undergoes several biochemical changes that allow it to take on a mesenchymal phenotype, which involves greater motility, invasiveness, resistance to apoptosis and an increased production of ECM components [55]. The establishment and maintenance of both somatic and germ cell polarity are essential features in ovarian development and folliculogenesis [56].

5.2.2.1 Cell polarity and folliculogenesis

It has previously been established that aberrant polarization of GCs can affect all stages of folliculogenesis, from the recruitment of primordial follicles to the atresia of preovulatory follicles [57]. It remains a point of contention whether GCs lose their polarity prior to ovulation or once they luteinize. The original theory was that GCs are polarized, whereas luteal cells are not [58]. Mora and colleagues, however, have recently suggested that GCs undergo a partial and contained EMT, which is completed at ovulation [15]. A transition such as this is unusual in adult tissues, because EMT usually occurs during development or tumour progression. In support of the concept of partial EMT, Irving-Rodgers and colleagues have proposed that prior to the expression of the focimatrix the follicular basal lamina dictates the polarity of GCs, which enables directional secretion, uptake of molecules, and other polarized functions [59]. Once the focimatrix is expressed basal lamina components are interspersed between the GCs,

which may reduce the polarization cue from the follicular basal lamina. The change in polarity may initiate the partial EMT, which is only completed after ovulation when GCs luteinize and lose their epithelial nature.

Considering that the ECM can define positional information and differentiation cues in tissues [52], I hypothesize that the elevated expression of ECM proteins in the βERKO ovary disrupts cell polarity cues within the follicle, ultimately compromising folliculogenesis.

The increased expression of Col11a1 around individual βERKO GCs may influence the expression of adherens junction proteins, and therefore compromise follicular cell polarity. The pattern of expression of Col11a1, specifically, encircling individual GCs, very subtly resembles that of the adherens junction components N-cadherin and β-catenin in wildtype ovaries; unlike Col11a1, N-cadherin and β-catenin do not encircle the GCs completely, they are are localized to one side of GCs [57]. Interestingly, the expression of N-cadherin and β-catenin is disrupted in another mouse model with compromised ovarian folliculogenesis and fertility ($Wnt4^{mCh/mCh}$; transgenic mouse with disrupted Wnt4 activity due to the insertion of the *mCherry* fluorescent protein), and the disrupted expression pattern of both N-cadherin and β-catenin is strikingly similar to that of Col11a1in the βERKO mouse ovary (completely encircling individual GCs) [57]. Previously published results have shown that collagens can affect the expression of cadherins and catenins [18, 20, 21]; therefore I postulate that the overexpression of Col11a1 at sites of adherens junctions disrupts the expression of cadherins and catenins in the mouse ovary, which dysregulates polarity. The Col11a1-induced disruption of cadherin and catenin expression in the βERKO ovary can be determined by measuring gene expression in isolated GCs using qPCR. Furthermore, primary GC and GC cell lines can be plated on uncoated and Col11a1-coated dishes, and the expression of adherens junction components determined by qPCR and Western blot [19]. The assembly of adherens junctions can also be determined by IF [20]. If adherens junction complexes are dysregulated, polarity markers should then be assessed using similar techniques.

My studies have also shown that the expression of Nidogen 2 is elevated in the βERKO focimatrix. At present, the role of the focimatrix in follicle development remains unknown; however, the suggestion that it impacts polarity is worth investigating. Examining the role of the focimatrix in maintaining cell polarity would be difficult. Specifically, replicating *in vitro* the punctate localization of the focimatrix around the GCs, and thereby the polarity cues from all sides of the GCs would be challenging.

I have carried out preliminary IF experiments to investigate cell polarity in βERKO and WT mouse ovaries by examining the relative expression aPKCζ in ovarian sections (Appendix, Figure 6-1). Previous studies have shown that aPKCζ is expressed in preovulatory GCs of the rat [60] and suggested aPKCζ may be involved in the regulation of ovulation [61]. Interestingly, I found that the localization pattern of aPKCζ differs between prepubertal (PND 23) βERKO and WT ovaries. In WT ovaries aPKCζ is easily detected in GCs closest to the BM; however, its expression is low or undetectable in GCs closer to the oocyte. In contrast, in βERKO ovaries I consistently observed expression of aPKCζ in *both* GCs closer to the oocyte and BM. Future studies are required to confirm and expand on these preliminary results. Initially, these preliminary results require confirmation. Subsequently, the expression of aPKCζ in the βERKO mouse ovary can be characterized at earlier (for example, PND 13 when we see dysregulation of ECM proteins) and later stages to determine when this dysregulation is occurring. If this disrupted aPKCζ expression persists to the antral stage, I postulate, based on the localization at the pre-antral stage, that βERKO cumulus *and* mural GCs will express aPKCζ, whereas only mural GCs will express aPKCζ in wildtype follicles. This disrupted polarity may impair cumulus cell differentiation, thereby disrupting cumulus cell-oocyte complex (COC) expansion, which is known to be inhibited in βERKO ovaries [62]. There are several techniques that could be utilized to pursue these hypotheses, including the development of a GC-specific aPKCζ, knockout mouse, as well as utilizing qPCR to compare the levels of polarity markers (e.g. aPKCζ and Par6) between isolated COC and mural GCs from βERKO and wildtype mice.

5.2.2.2 Cell polarity and ovarian cancer

The loss of cell polarity and consequent tissue disorganization is a hallmark of cancer and increased malignancy [52]. Several studies have demonstrated that changes in the activation or expression of core cell polarity proteins are implicated in the development of human cancers (reviewed in [2]). Importantly, the loss of apical-basal cell polarity (along with the loss of cell-cell adhesion) is necessary for EMT, which is a key step in cancer cell migration and invasion. The loss of polarity also permits growth factors and receptors, which are normally compartmentalized by tight junctions in polarized cells, to induce aberrant autocrine cell activation [53].

Oncogenic signaling has been shown to directly disrupt cell polarity mechanisms [54]. It is well-accepted in the literature that TGFβ is a major inducer of EMT, and TGFβ-induced EMT often coincides with a loss of E-cadherin expression [63]. TGF-β receptors bind directly to Par6, leading to the recruitment of aPKC and interference with apical-basal polarity by changing the binding partners, composition and localization of Par6-aPKC [64, 65]. The TGF-β-induced inactivation of the Par complex induces the cells to undergo EMT.

SPON1 signaling mechanisms in ovarian cancer cells (and most other cells) remains to be elucidated. However, SPON1 has previously been shown to activate latent TGFβ in embryonic and osteoarthritis articular chondrocytes [66, 67]. Therefore, SPON1 may have a role in TGFβ signaling in ovarian cancer and be indirectly involved in the regulation of PKCι. It would also be of interest to examine whether treatment of EOC cells with recombinant SPON1 or silencing SPON1 expression impacts EMT. Future studies could utilize qPCR to determine whether SPON1 affects the cadherin switch (E-cadherin, N-cadherin) and examine the expression of several transcription factors involved in EMT (e.g. Snail, Slug, Twist and ZEB) during the formation of spheroids and subsequent re-attachment.

5.3 An early role for ERβ-regulated ECM proteins in the ovary

While it is well established that estradiol acting through ERβ is required to augment the GC response to FSH for the formation of a preovulatory follicle, fewer studies exist establishing a role for estradiol in folliculogenesis, prior to the gonadotropin surge at puberty, and the role of ERβ prior to puberty remains largely unexplored. My data demonstrate that ERβ regulates gene expression in the mouse ovary much earlier than previously thought. Further studies are required to determine at what stage the expression of ECM components is disrupted and when this disruption impacts follicle growth.

I have performed preliminary IF experiments using βERKO ovaries that suggest Col11a1 may be overexpressed as early as PND 5 or PND 8 (Appendix, Figure 6-2), while the expression of Nid2 is comparable in βERKO and WT ovaries at these earlier ages. Oktay *et al.* isolated ovaries from mice on PND 5 and showed that follicle growth *in vitro* is not only affected by the presence of the ECM but also by the specific ECM component on which the ovary is cultured [68]. Furthermore, the production of estradiol is required for the optimal growth of follicles from the primordial to primary stage, and specifically ERβ, but not ERα, is involved in this transition [69, 70]. Therefore, ERβ-regulated ECM composition may impact the growth of follicles from the primordial stage.

Furthermore, ERβ-regulated ECM proteins could be involved during some of the earliest stages of postnatal ovarian development, specifically by influencing oocyte nest breakdown. In mice, the majority of nest breakdown occurs between PND 2 and PND 4; however, small nests can be found in mice as old as PND 8 [71]. Nest breakdown and primordial follicle formation are inhibited by estradiol, progesterone, and the phytoestrogen genistein [72], and inhibition of nest breakdown can lead to the development of multiple oocyte follicles (MOFs) in WT mice. βERKO mice, treated neonatally with genistein, do not develop MOFs [73], suggesting that ERβ may be involved in the regulation of nest breakdown. Future studies utilizing qPCR and IF will

be required to assess whether ECM components are disrupted in βERKO ovaries at these early stages.

5.4 TCF21-ERβ interactions: Novel findings and future studies

Using a model of transient transfection of ERE-driven luciferase reporters, and co-transfected expression plasmids of human ERβ and TCF21, I have shown that TCF21 represses ERβ-dependent activation of both synthetic and natural estrogen-responsive promoters in several cell lines (Chapter 3). Based on these data, I hypothesized that TCF21 and ERβ interact to form a complex *in vivo*. My objective was to utilize GST-pull-down analyses and co-immunoprecipitation (Co-IP) experiments to demonstrate and further understand their interaction.

Despite our best efforts, we were unable to produce evidence of TCF21-ERβ interactions using Co-IP. We tested a large variety of conditions including four different lysis buffers, varying duration of cell lysis (20 min or 40 min), four cell lines, several antibodies (three anti-ERβ and two anti-TCF21 antibodies), as well as transfection of FLAG-ERβ and an anti-FLAG antibody. However, we were unable to show interaction, regardless of which protein we used for the IP. Furthermore, all antibodies caused technical difficulties due to inconsistent results, high background signal and non-specific binding. One future approach to improving this assay might involve using a different tag for ERβ, and using a tagged TCF21 construct. An alternative approach would be to create our own ERβ antibody because the lack of a reliable, commercially available ERβ antibody that does not cross-react with ERα is a well-known obstacle in the field [74, 75]. However, whether we could successfully create a specific ERβ antibody when so many others have failed is unknown.

I was also unable to successfully purify GST-ERβ or GST-tagged ERβ deletion mutants using an established protocol [76], and future troubleshooting is required. The protein purification protocol will require optimization of several conditions including, but not limited to, buffers, temperature at which bacterial cultures are grown as well as incubation time, to achieve successful purification of functional proteins.

Additional studies for consideration to investigate ERβ-TCF21 interaction include 1) chromatin immunoprecipitation (ChIP) assays to determine whether TCF21 binds to the ERβ promoter (using a tagged TCF21), and 2) qPCR and Western blot analysis to assess whether TCF21 affects endogenous levels of ERβ mRNA and protein in granulosa cell lines and primary granulosa cells. Binding of TCF21 to the ERβ promoter could theoretically repress transcription of ERβ by either inhibiting the binding of RNA polymerase to the promoter or inhibiting its release from the promoter [77].

5.5 ERβ- and 17β-estradiol-regulated ECM components: An effect on ovarian cancer progression?

Epidemiological evidence indicates that the induction and progression of ovarian cancer is related to estrogen exposure [78], and high estradiol levels are often observed in EOC patients because both OSE and EOC cells secrete estradiol [79, 80]. Several studies, using various model systems, have shown that estradiol treatment contributes to the initiation and promotion of ovarian cancer growth. For example, treatment of ovariectomized mice with estradiol increases tumour growth by over 400% compared to controls [81]. Estradiol treatment has also been shown to promote growth, migration and invasion of several ovarian cancer cell lines [78, 80]. Whether estradiol increases tumour burden *in vivo* and decreases survival times is unclear because of variable results in the literature [78, 82].

The expression of the ER in ovarian cancer is variable. Of the four EOC subtypes, endometrioid ovarian cancer exhibits the highest occurrence of ER expression. Fujimura *et al.* found that although all subtypes of clinically resected ovarian adenocarcinomas express ERβ, ERβ expression is most often observed in endometrioid tumours (75% of cases), as compared to serous (41%), clear cell (39%) and mucinous (30%). ERα is expressed by all (100%) endometrioid tumours, 97% of serous tumours, 70% of mucinous and is absent (0%) in clear cell samples. As in normal ovarian development, the role of the two ERs differs in EOC development – high ERα levels are associated with a worse prognosis, whereas high ERβ levels are associated with longer survival [80]. ERβ expression is weak in ovarian tumour tissues compared to normal ovarian tissues,

likely decreasing over time with tumour progression, suggesting that ERβ has tumour-suppressive functions and a protective role [83, 84].

5.5.1 Spondin 1 in ovarian caner

The expression of Spon1 is disrupted in the βERKO ovary, suggesting it is regulated by ERβ [1]. There is also evidence that Spon1 is regulated by 17β-estradiol; when ovariectomized mice are treated with 17β-estradiol, Spon1 mRNA expression increases in the uterus and mammary gland [85, 86]. Since endometrioid EOC is estradiol responsive and has the highest occurrence of ER expression, future studies could utilize this subtype, in addition to serous EOC, to assess whether estradiol and/or ERβ affect the expression and activity of SPON1 in EOC cells. EOC cells can be treated with estradiol and Spon1 expression measured by qPCR.

Repeating the functional assays used in Chapter 4 with endometrioid EOC cells would be intriguing because survival analyses demonstrate that SPON1 is a higher risk factor in endometrioid ovarian cancer, as compared to high-grade serous or all four subtypes combined [87]. Although this thesis focused on high-grade serous cancer, the most common and aggressive EOC subtype, it is possible that the other subtypes of ovarian cancer would be affected differently by SPON1.

5.5.2 Collagen11A1 and Nidogen 2 in ovarian caner

Interestingly, both Col11a1 and Nid2, which are more highly expressed in the βERKO ovary than in WT ovaries (Chapter 2), are elevated in serous histotypes of ovarian cancer [88, 89]. There is no evidence in the literature that the disrupted expression of these ECM components in EOC is related to ERβ or estradiol. However, it is intriguing that their expression is elevated in two models where ERβ expression is lost (βERKO ovary) or weakened (EOC). Furthermore, there is an inverse relationship between the expressions of COL11A1 and NID2, and ERβ in ovarian tumours – expressions of these ECM proteins is higher in more aggressive, late-stage serous ovarian tumours than in earlier stages, whereas ERβ expression decreases with tumour progression. Considering the ERβ-mediated repression of Col11a1 and Nid2 in the normal mouse ovary, it would be of interest to determine whether their expression is also

ERβ-dependent in EOC cells. Following the reintroduction of ERβ into EOC cells using an adenoviral vector, the mRNA and protein expression of COL11A1 and NID2 could be examined by qPCR and IF, respectively.

5.6 Does SPON1 have a dichotomous or context-specific role in EOC progression?

My data suggest that SPON1 could be either tumour promoting or tumour suppressive. I have shown that treatment with SPON1 decreases the viability and proliferation of EOC cell lines as well as primary ascites-derived tumour cells, suggesting that expression of SPON1 inhibits tumour growth. On the other hand, I have also shown that treatment with SPON1 decreases EOC cell adhesion; however, it is unclear whether this feature is oncogenic by promoting metastasis, or tumour suppressive, by impeding the anchoring of cells within the peritoneal cavity to form secondary metastases. One would expect that the overexpression of SPON1 in ovarian cancer tissues is oncogenic. since it seems unlikely that EOC cells would make and secrete a protein that only hinders their growth and metastasis. A possible explanation is that SPON1 has a dichotomous or context-specific role in ovarian cancer development.

The concept of proteins having a dichotomous role in cancer development has been described previously in several human cancers, including ovarian [90-95]. The activity of a protein may vary based on the tumour environment, signaling pathways driving tumour formation, available cellular binding partners (e.g. ECM, integrins), and the status of malignancy. One of the most studied examples of this dichotomy is TCF-β, which induces apoptosis and cell cycle arrest in normal or less transformed cells, but enables metastasis in advanced tumours [96]. Transforming growth factor-beta-induced protein (TGFBI/βig-H3) has been described as a “double-edged sword” in ovarian cancer because its loss promotes tumourigenesis and a more chemoresistant phenotype; however, in the peritoneal cavity the peritoneal cells express βig-H3 to facilitate metastasis [93]. Furthermore, βig-H3 induces migration and invasion of OVCAR5 and SKOV3 ovarian cancer cell lines, but does not affect the OVCAR3 cell line that is known to be less metastatic [93].

Therefore, the role of SPON1 in ovarian cancer progression *in vivo* may depend on the tumour microenvironment and tumour stage, and may not be defined simply as either tumour suppressive or tumour promoting.

5.7 Would SPON1 make an appropriate therapeutic target for EOC?

The high expression levels of SPON1 in ovarian carcinomas make it an appropriate ovarian cancer biomarker. Its prospect as a therapeutic target remains to be determined, especially considering it is unclear whether SPON1 has a tumour suppressive or promoting role in ovarian cancer progression.

If SPON1 is shown to be tumour promoting, it may not be an ideal therapeutic target because despite the diverse functions attributed to SPON1 in the literature, the $SPON1^{-/-}$ mouse has a grossly normal phenotype, which suggests SPON1 is functionally redundant. Therefore, silencing SPON1 expression or activity may result in the increased expression of another protein(s), likely another member of the thrombospondin superfamily that has similar domains (Section 1.7.3 - Proteins with similar domains), which will compensate for the loss of SPON1 and assume its functions.

Alternatively, if SPON1 is tumour suppressive in certain contexts or stages of malignancy, it may be beneficial to promote its expression once a mechanism for its regulation is uncovered. For instance, if SPON1 expression is regulated by ERβ in EOC cells (Section 5.5.1), using an ERβ-specific agonist may serve as an effective therapeutic strategy. ERβ has previously been identified as a tumour suppressor in ovarian cancer *in vitro*. The overexpression of ERβ in SKOV3 cells reduces proliferation, inhibits motility and increases apoptosis [97]. SKOV3 cell growth is also inhibited following treatment with an ERβ agonist (DPN) [98]. Furthermore, the proliferation of the EOC cell line, BG-1, is decreased following introduction of ERβ, and the expression of ERβ strongly inhibits the expression and activity of ERα [83].

5.8 Summary

Either directly or indirectly, the ECM regulates almost all fundamental aspects of cell biology. The goal of this thesis was to contribute to our knowledge of its role in ovarian development and ovarian cancer progression. The data presented herein characterizes aberrantly overexpressed ECM proteins in a model of subfertility (Chapter 2) as well as high-grade serous EOC (Chapter 4), and discusses the potential impact of this dysregulation. This body of work provides rationale for future investigations into the mechanisms by which these ECM components are regulated. The expression of the ECM proteins investigated is ERβ-dependent, and Chapter 3 describes a novel corepressor of ERβ-mediated transactivation. Understanding the unique roles of ECM components in these model systems may improve current therapeutic options for infertility and ovarian cancer.

5.9 Bibliography

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Appendix: Additional Figures

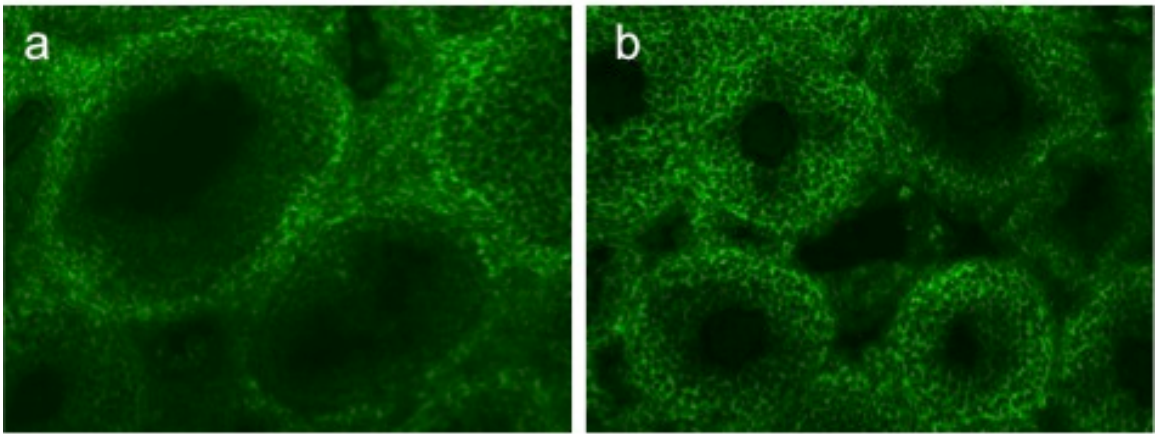

Figure 6- 1: PKCζ protein expression in prepubertal (PND 23) ERβ-null and wildtype mouse ovaries.

Immunofluorescence with an anti-PKCζ antibody was used to detect PKCζ localization and expression in ovaries isolated from (a) wildtype and (b) ERβ-null mice. 200x magnification.

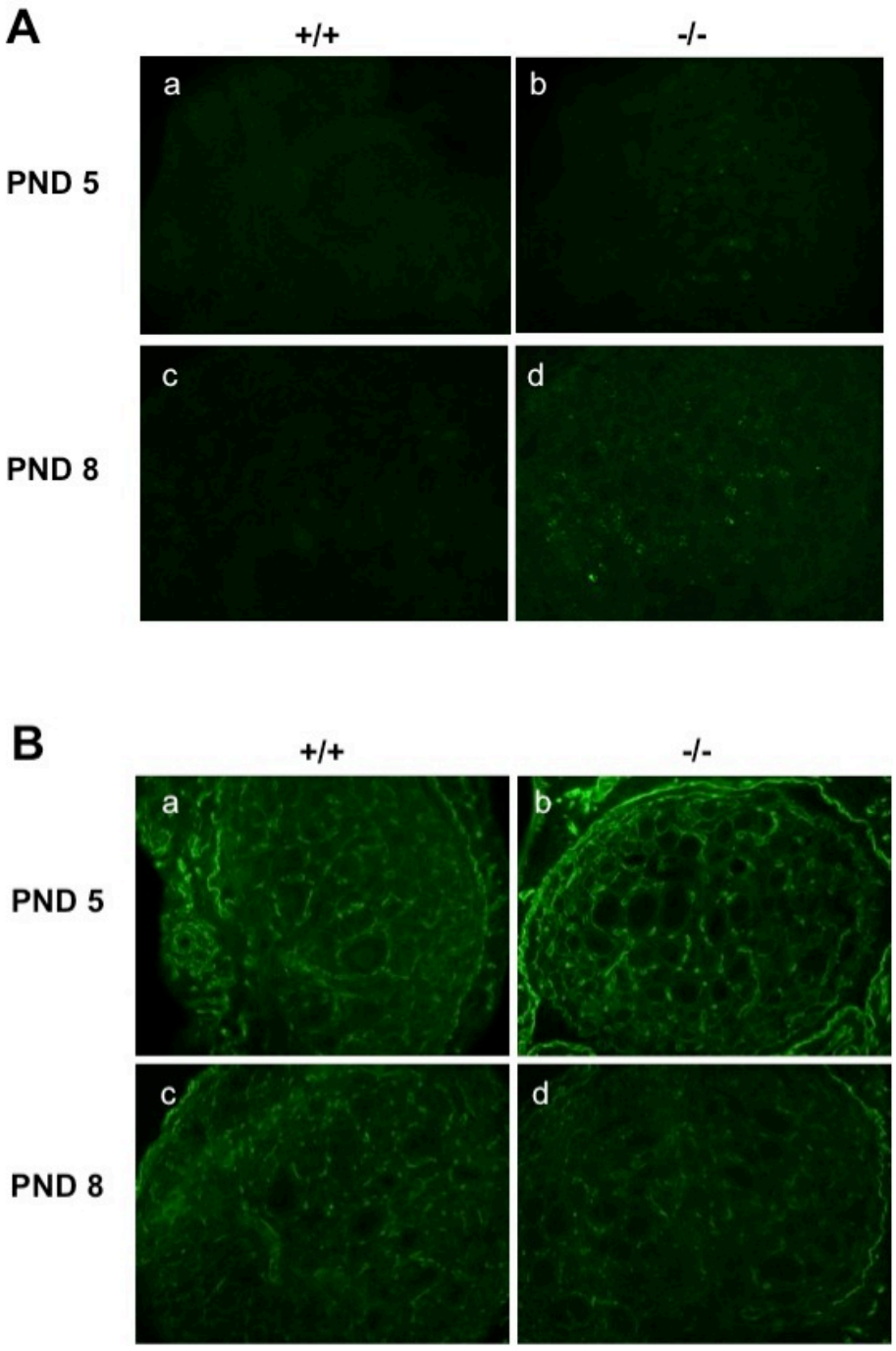

Figure 6- 2: Col11a1 and Nid-2 protein expression in ERβ-null and wildtype mouse ovaries on PND 5 and PND 8.

Immunofluorescence with an anti-Col11a1 (A) or anti-Nid2 (B) antibody was used to detect Col11a1 and Nid2 localization and expression in ovaries isolated from wildtype (+/+) and ERβ-null (-/-) mice at PND 5 (a, b) and (c, d) PND 8. 200x magnification.

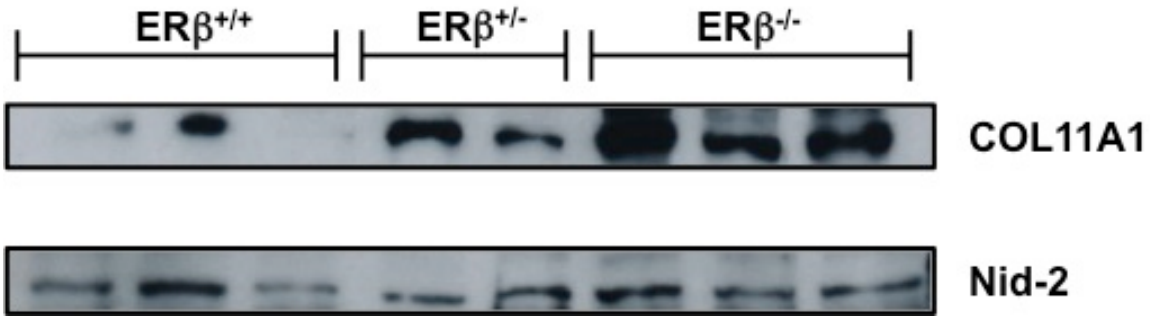

Figure 6- 3: Collagen 11A1 and Nidogen 2 expression in wildtype, ERβ$^{+/-}$ and ERβ$^{-/-}$ mouse ovaries.

Whole cell extracts from adult wildtype (+/+), ERβ-het (+/-) and ERβ-null (-/-) mouse ovaries were analyzed by Western blot to detect Col11a1 and Nid-2 protein expression.

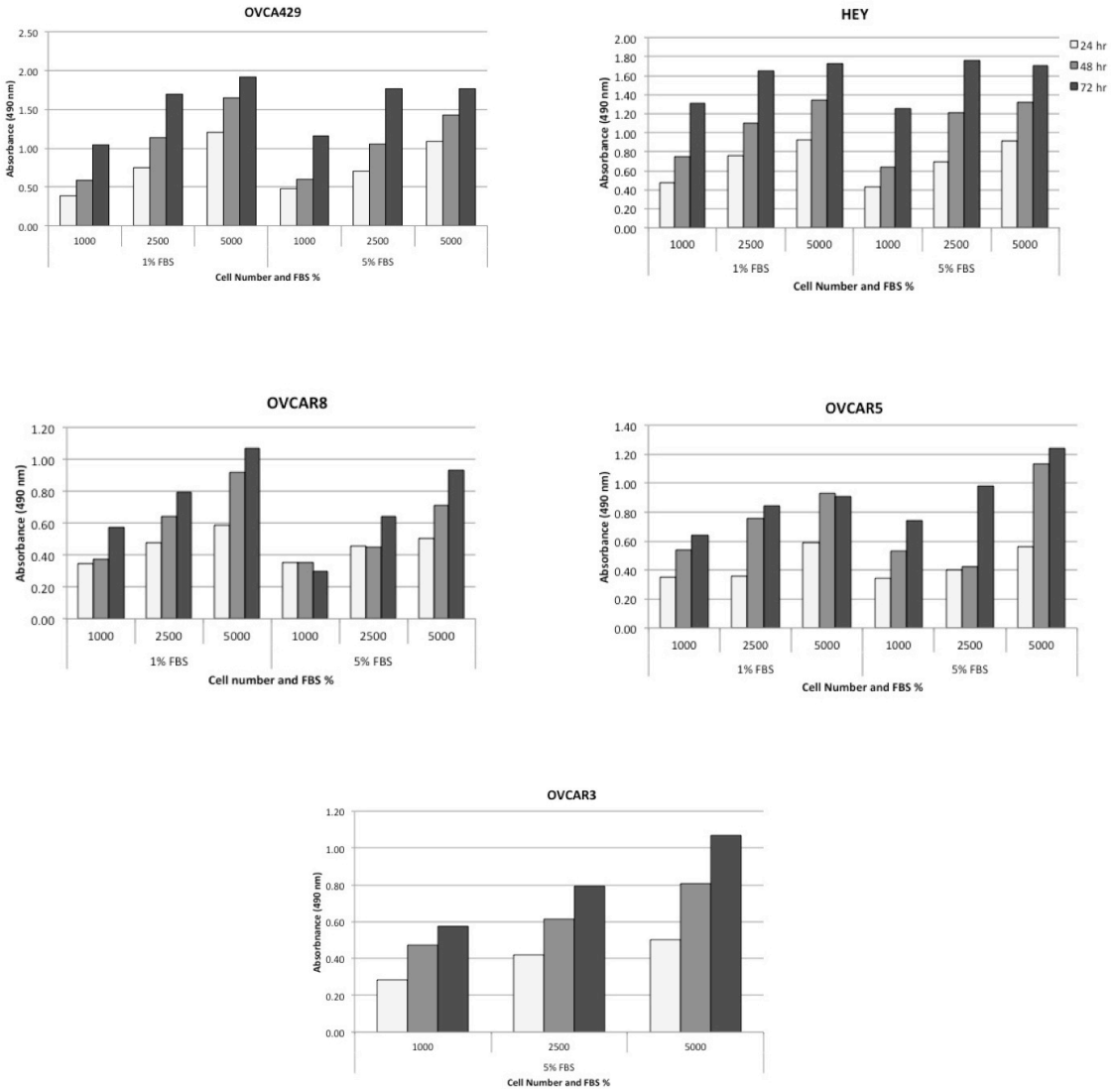

Figure 6- 4: Optimization of cell number and FBS% for viability assays.

The cell number and % FBS were optimized for MTS viability assays for the five established cell lines. 1000, 2500 and 5000 cells/ well were tested. Two FBS concentrations were tested: 1% FBS and 5% FBS. OVCAR3 cells were only tested in 5% FBS due to their slow rate of proliferation. Optimization of conditions was performed to ensure that the assay signal remains within the linear range throughout the assay (an absorbance of ~0.5 – 1.5 at 490 nm).

Curriculum Vitae

Alexandra Carrier

EDUCATION

2009-2016 Ph.D. Biochemistry
University of Western Ontario, London, Ontario

2005-2009 Honours B.Sc. Biochemistry and Molecular Biology
Trent University, Peterborough, Ontario

RESEARCH-RELATED EXPERIENCE

2009-2016 *Doctoral Candidate,* Biochemistry, University of Western Ontario
Thesis Supervisor: Dr. Bonnie Deroo
Thesis title: Investigating the role of extracellular matrix proteins in ovarian folliculogenesis and ovarian cancer progression

2008-2009 *Fourth year honours thesis project*, Aquatic Biology, Trent University
Thesis Supervisor: Dr. Paul Frost
Thesis title: Effects of gut flora on the assimilation of elements by *Daphnia magna*

2007-2009 *Undergraduate Research Assistant,* Aquatic Biology, Trent University
Hired to independently complete biological stoichiometry experiments involving the effect of food quality on the physiological processes of *Daphnia*

TEACHING EXPERIENCE

2014 Farah Ashgar, Biochemistry Undergraduate Summer Research Program

- Solely responsible for training student in appropriate laboratory techniques, managing her schedule, coordinating and overseeing daily experiments, and providing expertise crucial for project completion

2014 Benjamin Withers, summer student

- Responsible for training student in appropriate laboratory techniques, as well as coordinating and overseeing daily experiments

2011-2012 Susan Kuruvilla, Honours Student, Department of Biochemistry
- Solely responsible for training student in appropriate laboratory techniques, managing her schedule, coordinating and overseeing daily experiments, and providing expertise crucial for project completion

2009 Tutor for the biochemical concepts CHEM-BIOL 231H course at Trent University

2007-2009 Trained summer students and new employees in appropriate laboratory techniques

AWARDS

2013-2014 Ontario Graduate Scholarship
2012-2013 Queen Elizabeth II Scholarship
2011-2012 Ontario Graduate Scholarship
2009-2012 Schulich Graduate Scholarship, The University of Western Ontario
2009 President's Honour Roll. Otonabee College, Trent University
2006-2009 Dean's Honour Roll. Otonabee College, Trent University

PUBLICATIONS

1. **Zalewski, A.**, E.L. Parker and B.J. Deroo. Expression of Extracellular Matrix Components is Disrupted in the Immature and Adult ERβ-null Mouse Ovary. 2012, *PLoS One* 7, e29937.
2. **Zalewski, A.**, N.D. Wagner and P.C. Frost. Antibiotics affect the growth responses of *Daphnia magna* to poor food quality. 2011, *Aquat Ecol*. 45, 493-504.
3. Frost, P.C., D. Ebert, J.H. Larson, M.A. Marcus, N.D. Wagner and **A. Zalewski**. Transgenerational effects of poor elemental food quality on *Daphnia magna*. 2010, *Oecologia* 162, 865-72.

PRESENTATIONS

2011 **A. Zalewski** and B. Deroo. Characterization of Nidogen-2 and Collagen11a1 in ERβ-Null Ovarian Folliculogensis. Paul Harding Research Awards Day, London, ON, April 27, 2011.

2010 Deroo, BJ, Raulic, S, and **Zalewski, A**. Loss of ERβ activity results in dysregulation of follicular extracellular matrix and cell adhesion genes in response to follicle stimulating hormone. The Endocrine Society Annual Meeting, San Diego, CA, June 19-22, 2010.

2010 **A. Zalewski** and B. Deroo. Characterization of Nidogen-2 and Collagen11a1 in ERβ-Null Ovarian Folliculogensis. Paul Harding Research Awards Day, London, ON, May 19, 2010.

2008 Frost, P.C., M.A. Marcus, **A. Zalewski** and D. Ebert. Variable effects of phosphorus-poor food on *Daphnia magna*: Is there a mediating role of maternal elemental nutrition? American Society of Limnology and Oceanography Conference, St. John's, Newfoundland and Labrador, June 8-13.