CHARACTERISATION OF THE DEVELOPMENT AND HORMONAL REGULATION OF THE OVARIAN LYMPHATIC VASCULATURE

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CHAPTER 5
GONADOTROPHIC REGULATION OF KEY ANGIOGENIC AND LYMPHANGIOGENIC MEDIATORS
5.1 INTRODUCTION

Angiogenesis in normal adult tissues is a rare event and the vascular endothelium represents a stable population of cells with low mitotic activity [200]. Adult angiogenesis is most often associated with pathologies including, wound repair and tumourigenesis. The female reproductive organs, including the ovary and uterus, however, undergo repeated cycles of growth and remodelling in response to gonadotrophin hormones, as part of the menstrual cycle. Tight control of these processes is mandated in the ovary, as oocytes must develop in an avascular environment within the follicle, while in stark contrast the corpus luteum which develops from those follicles is highly vascular [29, 188]. The high rate of vessel growth and proliferation within the ovary is matched only by that of the fastest growing and most highly aggressive tumours [201, 202] and is associated with a high metabolic requirement for very rapid steroidogenesis. For this reason, the necessity for continuous and rapid cyclic vascular remodelling is essential to provide for the rapidly changing energy demands.

During each menstrual cycle follicles grow rapidly under hormonal control of a large number of follicular/ovarian genes mediating cell proliferation and remodelling of the stroma and extracellular matrix (ECM) in and surrounding the follicle. While mouse models with mutations of the genes involved in angiogenesis and lymphangiogenesis have not been studied in terms of fertility due to embryonic lethality, inhibitors of angiogenic and lymphangiogenic signalling clearly indicate important roles for the vascular endothelial growth factor (Vegf) family of growth factors and receptors in folliculogenesis and ovulation [67-74]. Inhibition of Vegfa signalling throughout the follicular phase in marmosets results in a loss of thecal vascularisation leading to suppression of follicle development and atretic loss of antral follicles [71, 72]; while inhibition of Vegfa or Vegfr2 signalling during the late follicular phase causes inhibition of the endocrine changes necessary for follicle maturation and ovulation [62, 68, 69, 74]. Suppression of Vegfa signalling during and following follicle selection in the marmoset decreased endothelial cell proliferation and thecal vascularisation and resulted in anovulation and non-functional CLs [70]. Similarly, in rodents, inhibition of Vegfr2 prevents gonadotrophin-dependent follicle growth, ovulation and luteinisation [67, 73].
While the function of Vegfa has been thoroughly investigated in the ovary, other members of the Vegf family and their receptors are yet to be identified or characterised. The mammalian vascular endothelial growth factor family includes six members: Vegfa (or Vegf), Vegfb, Vegfc, Vegfd, Vegfe, and placental growth factor (Pgf). All members of the family have been implicated in vasculogenesis, angiogenesis and lymphangiogenesis [34, 35, 44, 203]. The Vegf family of ligands act predominantly through the actions of the three Vegf receptors, Vegfr1 and Vegfr2 which are responsible for the development and expansion of the blood vasculature and Vegfr3, involved in lymphangiogenesis. To date, Vegfa is the only family member to have been described in detail within the ovary, and known to be hormonally regulated. Koos et al described an approximate eight-fold induction of Vegfa in whole rat ovary four and ten hours following hCG administration using semi quantitative-PCR [84], and many others have described the in vitro effects of Vegfa production by granulosa cells in response to hormones in primates and rodents [29, 184, 185, 204, 205].

The known lymphangiogenic mechanism involves pro-lymphangiogenic growth factors, Vegfc and Vegfd, which act via Vegfr3 signalling pathways (reviewed in [102]) and has not been well described in the ovary to date. Vegfc has been reported to be present in human cultured granulosa-lutein cells [206], whilst both Vegfc and Vegfd have been reported to be present in human ovarian carcinoma [207]. Vegfr3 has not been described within the ovary. Consequently, I hypothesised that the ovary may produce known lymphangiogenic mediators under hormonal control which may act in an autocrine or paracrine manner to promote lymphangiogenesis. To investigate this hypothesis, I analysed the hormonal regulation of mRNA expression of genes known to be involved in the lymphangiogenic mechanism, as well as angiogenic genes, in isolated ovarian cell types.

5.2 MATERIALS AND METHODS

5.2.1 Animal treatment and tissue preparation

Inbred C57BL/6 mice were purchased from Laboratory Animal Services (University of Adelaide, Australia). All mice were maintained in 12 h/12 h light dark and given water and rodent chow ad libitum.
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All experiments were approved by the University of Adelaide Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice were injected i.p. at 1400 h on d 21 of age with 5IU eCG and 5IU hCG at 1000 h two days later to stimulate follicle growth and subsequent ovulation, followed by cervical dislocation and collection of ovaries at the required time points (Figure 5.1). Cumulus oocyte complexes, granulosa cells and the residual cell (thecal and stromal cells) compartments were isolated independently, by firstly removing cumulus oocyte complexes from follicles by puncturing with a 27½ gauge needle, and then subsequently removing the granulosa cells and snap freezing independent cell types in liquid nitrogen. For an initial survey of the temporal pattern of ovarian gene expression (Experiment 1), tissues from ovaries of eight mice (16 ovaries) were pooled after no treatment or after four hour increments from 20-44 h after eCG, or two hour increments (2-16 h) after hCG (Figure 5.1). For statistical analysis of the major expression changes (Experiment 2), three additional independent replicates were conducted with tissues collected from three mice (six ovaries) per replicate pooled at time points of main interest (untreated, 44 h eCG, 6 h hCG, 12 h hCG; Figure 5.1). Additional ovaries were also collected and fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemistry.

5.2.2 Immunohistochemistry

Immunohistochemical localisation of Lyve1 and Vegfa were undertaken as per Chapter 2.2.5. The Lyve1 antibody required Proteinase K antigen retrieval whilst the Vegfa antibody (Abcam; Cambridge, MA, USA) required citrate buffer and Proteinase K antigen retrieval. Images were captured at high resolution using NanoZoomer Digital Pathology technology (Hamamatsu Photonics K.K., Japan).

5.2.3 Real time RT-PCR

Real Time RT PCR was performed as per Chapter 2.2.7 with primers listed in Table 2.1. Genes measured were Lyve1, Prox1, Vegfa, Vegfc, Vegfd, Vegfr1, Vegfr2 and Vegfr3. For Experiment 1, granulosa cell and residual cell data was normalised to the untreated 0 h eCG time point and presented as fold change from untreated. For Experiment 2, data was presented as mean raw 2-ΔΔCT ± SEM to demonstrate fold differences and relative expression differences between the two ovarian tissue types.

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Figure 5.1 Schematic representation of sample collection schedule

Isolated ovarian samples were collected following eCG and hCG stimulation to analyse temporal ovarian gene expression. In Experiment 1, mice were collected untreated or received treatment with eCG and subsequent hCG and were collected at the following time points; untreated, 20-36 h eCG (four h increments), 0-16 h hCG (two hour increments). In Experiment 2, mice were collected untreated or received treatment with eCG and subsequent hCG and were collected at the following time points; untreated, 44 h eCG, 6 h hCG and 12 h hCG.
5.2.4 Statistical analysis

Following tests of normality and equal variances, real time RT-PCR data was analysed using One Way ANOVA with Tukey Post-Hoc and statistical significance considered if \( P \leq 0.05 \) (SPSS 11.5; SPSS Inc., Chicago, IL).

5.3 RESULTS

5.3.1 Vegfa is expressed and localised within granulosa cells and the stromal/residual ovarian compartment and is induced after hCG treatment

Following treatment with eCG and hCG, Vegfa was significantly induced in granulosa cells 3.2 and 5.3-fold respectively after 6 and 12 h hCG, and was also significantly higher after 12 h hCG in the residual ovary (Figure 5.2 A, B). The detailed time course of assessment of Vegfa expression in Experiment 1 indicated a rapid increase within 2 h of hCG administration peaking at 14-16 h, 16.8-fold in granulosa cells (Figure 5.2 A). The relative level of Vegfa expression between granulosa and residual tissues showed that Vegfa expression in granulosa cells was almost identical to the residual ovary prior to treatment (Figure 5.2 B). Vegfa protein localisation was not detectable in granulosa cells of 44 h eCG treated ovaries but was highly abundant in the theca/stromal compartment (Figure 5.2 C, D). Following 12 h treatment with hCG, supporting the immunolocalisation, mRNA had increased by more than five-fold, in the granulosa cells, but by only two-fold in the residual compartment, such that expression and protein abundance was now greater in the granulosa cells (Figure 5.2 E, F).

5.3.2 Vegfc and Vegfd are expressed within granulosa cells and are hormonally regulated

Vegfc was detectable within granulosa cells and the stromal/residual compartment of the ovary, with 5.4-fold lower expression in granulosa cells compared to the residual compartment prior to treatment. There was no significant difference in Vegfc expression between immature versus preovulatory eCG 44 h treated granulosa or residual tissues. Following the ovulatory hCG stimulus, Vegfc was dramatically
Figure 5.2 Regulation and localisation of Vegfa following treatment with eCG and hCG

Isolated granulosa cells (red) and residual ovary (blue) were collected following eCG and hCG stimulation to analyse temporal ovarian gene expression.

In Experiment 1 (A) (n = one replicate, eight pooled samples), Vegfa expression (normalised to Lrp19) is presented relative to the level of expression detected in unstimulated samples to characterise the temporal expression changes within granulosa or stromal ovarian compartments. Data represent fold change from untreated (normalised to 1). Dotted line indicates hCG stimulation.

Experiment 2 (B) (n = four independent replicates, samples pooled from at least three mice) represents Vegfa expression at key stages during follicular development and ovulation. Data represent mean 2^{-ΔΔCT} ± SEM. Different letters represent statistical significance of F≤0.05.

Vegfa protein localisation was identified by immunohistochemical localisation in ovarian tissue sections following 44 h eCG (C, D) and 12 h hCG (E, F) stimulation. Magnification 20X (C, E) and 60X (D, F). Gran = granulosa cells, Theca = theca cells.
induced (7.5-fold) within 4 h, specifically in granulosa cells (Figure 5.3 A) and was significantly increased in granulosa cells after 6 h (five-fold, Figure 5.3 B). The detailed temporal profile of Vegfd expression in Experiment 1 showed a striking induction after 20 h eCG in granulosa cells, however, replicated analyses in additional groups of mice sampled after 10 or 20 h eCG showed induction in some, but not others resulting in no significant changes after eCG or hCG in granulosa cells or residual ovary (Figure 5.3 D. 10 and 20 h time points; data not shown). Vegfd was detected in levels between six and 12-fold higher in the residual ovary than the granulosa cells at all time points (Figure 5.3 D).

5.3.3 Vascular endothelial growth factor receptors are expressed in granulosa cells and are hormonally regulated

Vegfr1 was expressed within granulosa cells but was more than 30-fold higher in the residual ovary prior to treatment (Figure 5.4 B). There was no significant difference between immature (unstimulated) or eCG 44 h treated ovaries, however, significantly higher expression was evident in hCG treated granulosa cells compared to those from immature (0 h eCG) follicles (Figure 5.4 B). The blood angiogenic receptor, Vegfr2 showed a sharp increase in expression within 2 h of hCG stimulation (Figure 5.4 C). After 6 h of hCG treatment, a significant 10.1-fold increase (compared with unstimulated 0 h eCG) in Vegfr2 was evident in granulosa cells which was transient and declined after 12 h (Figure 5.4 D). The major lymphangiogenic growth factor receptor, Vegfr3, unlike the other Vegf receptors, was strikingly induced in granulosa cells during the period of follicular growth (following treatment with eCG) with a peak 7.5-fold increase within 36-44 h after eCG treatment (Figure 5.4 E). The significant induction of Vegfr3 detected in granulosa cells 44 h following eCG (5.3 fold) was rapidly down-regulated after hCG, returning to basal (non-stimulated) levels within 6 h of hCG treatment (Figure 5.4 F).

5.3.4 Lyve1 is produced by granulosa cells in response to gonadotrophin administration

The lymphatic endothelial cell hyaluronan receptor Lyve1 is known to be expressed almost exclusively by lymphatic endothelial cells, as well as a sub-population of macrophages and some hepatic blood sinusoidal endothelial cells [174]. We found unexpectedly, that while Lyve1 mRNA was low in
Figure 5.3 Regulation of Vegfc and Vegfd following treatment with eCG and hCG

Isolated granulosa cells (red) and residual ovary (blue) were collected following eCG and hCG stimulation to analyse temporal ovarian gene expression.

In Experiment 1 (A, C) (n = one replicate, eight pooled samples), Vegfc and Vegfd expression (normalised to Lrp19) are presented relative to the level of expression detected in unstimulated samples to characterise the temporal expression changes within granulosa or stromal ovarian compartments. Data represent fold change from untreated (normalised to 1). Dotted line indicates hCG stimulation.

Experiment 2 (B, D) (n = four independent replicates, samples pooled from at least three mice) represents Vegfc and Vegfd expression at key stages during follicular development and ovulation. Data represent mean $2^{-\Delta\Delta CT}$ ± SEM. Different letters represent statistical significance of $P \leq 0.05$. NS represents no statistical significance.
Figure 5.4 Regulation of *Vegfr1*, *Vegfr2* and *Vegfr3* following treatment with eCG and hCG

Isolated granulosa cells (red) and residual ovary (blue) were collected following eCG and hCG stimulation to analyse temporal ovarian gene expression.

In Experiment 1 (A, C, E) (n = one replicate, eight pooled samples), *Vegfr1*, *Vegfr2* and *Vegfr3* expression (normalised to *Lrp19*) are presented relative to the level of expression detected in unstimulated samples to characterise the temporal expression changes within granulosa or stromal ovarian compartments. Data represent fold change from untreated (normalised to 1). Dotted line indicates hCG stimulation.

Experiment 2 (B, D, F) (n = four independent replicates, samples pooled from at least three mice) represents *Vegfr1*, *Vegfr2* and *Vegfr3* expression at key stages during follicular development and ovulation. Data represent mean $2^{-\Delta\Delta CT} \pm$ SEM. Different letters represent statistical significance of $P \leq 0.05$. NS represents no statistical significance.
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A. Vegfr1
Gene expression (Relative to unirradiated)

B. Vegfr1
Relative gene expression

C. Vegfr2
Gene expression (Relative to unirradiated)

D. Vegfr2
Relative gene expression

E. Vegfr3
Gene expression (Relative to unirradiated)

F. Vegfr3
Relative gene expression

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granulosa cells of growing follicles (eCG treatment) it was strikingly, and significantly up-regulated by ovulatory hCG treatment. Between 6-10 h following treatment with hCG, Lyve1 mRNA was induced 90-110-fold (Figure 5.5 A). This observation was reproducible and significant in repeated experiments (Figure 5.5 B). To confirm this novel expression of Lyve1 by a non-lymphatic cell type, the Lyve1 RT-PCR product was cloned, sequenced and subjected to BLAST analysis which confirmed absolute homology with published Lyve1 sequence (data not shown). Furthermore, Lyve1 protein was detectable by immunohistochemical localisation on endothelial cells of lymphatic vessels within the stromal compartment of the ovary (Figure 5.5 E, F) and in granulosa and cumulus cells following 12h hCG treatment (Figure 5.5 G, H). The homeobox transcription factor Prox1, like Lyve1, was significantly induced in granulosa cells following eCG and hCG treatment with a five-fold induction 6 h and 12 h after hCG (when compared to the 0 h eCG unstimulated time point) (Figure 5.5 C, D).

5.4 DISCUSSION

Cyclic tissue growth and remodelling within the ovary requires vascular remodelling in order to sustain the constantly regenerating follicles and corpora lutea through provision of nutrients and waste removal. The importance of a robust vascular supply around follicles is demonstrated by repeated association of blood flow with oocyte quality in clinical IVF [208, 209] and angiogenesis in CL formation is one of the most vigorous neovascularisation processes known [188]. In spite of the importance of vascular remodelling during these processes, details of the hormonal regulation of blood- and lymph-angiogenic growth factors have not been reported. Here I demonstrate the first detailed characterisation of hormonal control of the family of Vegf growth factors and their receptors. Furthermore, I have identified Prox1 and Lyve1 as novel granulosa cell products induced by ovulatory hormone stimulation.

While the distinct effects of the Vegf family of growth factors and receptors are well described during development and under pathophysiological circumstances, their expression and function within the ovary has received limited investigation. The originating member of this family Vegfa has been identified within the ovary in species including cow, pig, sheep, rat, mouse and human [66], and its critical importance in folliculogenesis, ovulation and luteinisation identified in mouse [67, 68] and primate model [62]. Vegfa is a pro-angiogenic molecule, promoting blood endothelial cell proliferation [35].
Figure 5.5 Regulation and localisation of novel granulosa cell products *Lyve1* and *Prox1* following treatment with eCG and hCG

Isolated granulosa cells (red) and residual ovary (blue) were collected following eCG and hCG stimulation to analyse temporal ovarian gene expression.

In Experiment 1 (A, C) (n = one replicate, eight pooled samples), *Lyve1* and *Prox1* expression (normalised to *Lrp19*) are presented relative to the level of expression detected in unstimulated samples to characterise the temporal expression changes within granulosa or stromal ovarian compartments. Data represent fold change from untreated (normalised to 1). Dotted line indicates hCG stimulation.

Experiment 2 (B, D) (n = four independent replicates, samples pooled from at least three mice) represents *Lyve1* and *Prox1* expression at key stages during follicular development and ovulation. Data represent mean 2^ΔΔCT ± SEM. Different letters represent statistical significance of *P*≤0.05. NS represents no statistical significance.

*Lyve1* protein localisation was identified by immunohistochemical localisation in ovarian tissue sections following 44 h eCG (E, F) and 12 h hCG (G, H) stimulation. Magnification 20X (C, E) and 60X (D, F). Gran = granulosa cells, Theca = theca cells, COC = cumulus oocyte complex, BV = blood vessel, LV = lymphatic vessel.
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A. **Lyve1**
   - Gene expression (Relative to untreated)

B. **Lyve1**
   - Relative gene expression

C. **Prox1**
   - Gene expression (Relative to untreated)

D. **Prox1**
   - Relative gene expression

E. Theca Gran COC
F. Gran LV BV
G. COC Gran Theca
H. Gran Theca
Inactivation of Vegfa using soluble Vegf-receptor trap approaches demonstrates that Vegfa is essential for blood vessel growth around growing follicles and in the developing CL and is essential to survival and growth of these tissues [68, 69, 71-76, 82]. My results indicate that Vegfa is a product of both granulosa cells and cells of the theca/stroma, and up-regulation in response to LH, in both ovarian compartments, and may contribute to neovascularisation of the CL. Additionally Vegfa is the most potent promoter of vascular permeability [210-212], and as such, has been proposed to promote accumulation of antral fluid during follicle development [84]. Vegfa acts predominantly through Vegfr2 following embryonic blood vessel development. Our identification of the hormonal regulation of Vegfr1 in granulosa cells during the ovulatory cascade suggests a novel signalling role for Vegfr1, potentially as an autocrine mediator of cell survival.

A growing body of evidence suggests that dysregulation of Vegfa is associated with both Polycystic Ovarian Syndrome (PCOS) and Ovarian Hyper Stimulation Syndrome (OHSS). For this reason, understanding the normal regulatory events associated with modulation of the Vegf family, and subsequently the vasculature, is necessary for further progress in this area. The pathology of PCOS, includes inappropriate accumulation of fluid-filled ovarian cysts, and abnormal hypervascularisation of the theca [88]. Recently PCOS has been associated with dysregulation of granulosa cell, serum and follicular fluid Vegfa [89-91]. Furthermore, excess generation of Vegfa in response to hyperstimulation during assisted reproductive technology (ART) is thought to promote/exacerbate OHSS [93, 94]. Follicular fluid Vegfa levels have also been associated with follicle maturation, oocyte quality and fertilisation competence in PCOS patients; however more research is certainly warranted in this area [92]. My demonstration of dynamic regulation of Vegfa and its receptors within the ovary in response to gonadotrophins provides further evidence that this system may be subject to dysregulation in altered hormonal environments such as superovulation. In the case of OHSS our data support the suggestion of others [95-97] that super-physiological doses of gonadotrophins administered during ART directly alter the abundance of Vegfa or its receptors. This may also result in a perturbed balance of other blood and lymphatic vessel mediators, affecting vessel development or permeability and normal fluid flow dynamics in the ovary and/or abdomen and resulting in symptoms of extravascular fluid retention, hypervolaemia and the pathological development of OHSS.
A small number of studies have reported Vegfc and Vegfd in ovarian cells, associated with ovarian carcinoma [207] and in cultured human granulosa-lutein cells collected from ART patients [206]. Vegfc is selectively involved in the promotion of lymphangiogenesis via binding Vegfr3 on lymphatic vascular endothelial cells [178], however it is also able to promote new blood vessel formation via activation of Vegfr2 [213]. I identified unique granulosa cell expression and regulation of both Vegfc and Vegfr2 following treatment with hCG suggesting that a paracrine (or autocrine) signalling loop of Vegfc-Vegfr2 signalling may exist in peri-ovulatory granulosa cells. In other tissues, Vegfc has been described as a stimulator of motility [214, 215]. During formation of corpus luteum, endothelial cells migrate from the thecal layer into the granulosa cells to provide the functional vasculature for the CL. The expression of Vegfc by granulosa cells following hCG treatment may be involved in the promotion of chemoattraction and motility of Vegfr2 expressing blood endothelial cells or Vegfr3 expressing lymphatic endothelial cells.

I identified a strong and consistent up-regulation of Vegfr3 in granulosa cells during eCG stimulated folliculogenesis. Granulosa cells at this stage of differentiation also expressed both Vegfc and Vegfd, thus an autocrine signalling loop may also be proposed in the granulosa layers of growing follicles, or paracrine signalling through Vegfc/d production in the theca/stromal compartment acting on granulosa cells. The rapid down-regulation of Vegfr3 in granulosa cells after hCG indicates that any role for this signalling is only important during follicle growth and not the ovulation/luteinisation response to hCG.

The hyaluronan (HA) receptor Lyve1 is a close homolog of CD44, the principal hyaluronan binding receptor, known to promote cell-cell and cell-matrix interactions in immune and metastatic cells [216]. CD44 is also important in fertility through an essential role in the formation of the HA-rich expanded cumulus matrix that assembles in the periovulatory period [217-220]. Surprisingly I found dynamic regulation of Lyve1 in the granulosa cells also in response to ovulatory LH (hCG) signalling. Lyve1 has been widely characterised and used diagnostically as a marker to identify lymphatic vessels [174]. The function of Lyve1 is yet to be elucidated. Lyve1 knockout mice show neither lymphatic nor fertility defects providing little insight [173], however it has been suggested to promote immune cell trafficking via binding of immune cells and HA internalisation [221]. The temporal pattern of CD44 expression is very similar to Lyve1 but is highly specific to the cumulus cells and not mural granulosa cells [217-220].
While the role for another HA receptor in mural and cumulus cells is not clear, I speculate that via its HA-binding capacity, *Lyve1* within granulosa cells may be involved in HA-matrix polymerisation at cumulus and mural granulosa cell surfaces. This may be necessary for growth factor sequestration, angiogenesis or cell migration required for the formation of the mature, functional CL. *Lyve1* may also be involved in the attraction of immune cells, particularly macrophages during tissue remodelling following ovulation [162]. What is clear, however, is that the granulose cell production of *Lyve1* does not appear to be as a result of infiltrating/migrating LECs. During the infiltration process, endothelial cells migrate into the ovulatory follicle/developing CL at localised foci, and it is clear that these foci are void of *Lyve1*-positive vessels or LECs during this period (Figure 4.14 B).

In Chapter 4, I proposed that in response to gonadotrophins, the ovary may produce key lymphangiogenic mediators in temporal association with FSH-mediated follicle growth. The data in this chapter clearly demonstrates that in response to gonadotrophins, virtually all members of the Vegf family were regulated within the ovary. Interestingly, during the period of FSH-mediated follicle growth (in response to eCG) *Vegfc* and *Vegfd* were produced by granulosa cells. While they do not appear to be regulated, the data obtained gives an indication of the gene expression on a per cell basis, when normalised to housekeeper *Rpl19*. In response to FSH, and the subsequent production of estradiol, granulosa cells proliferate extensively, which means that although there is no measurable difference in output on a per cell basis, the follicle may be producing significantly more growth factors as a unit as seen in whole ovary expression of *Vegfc* and *Vegfd* in response to eCG (Figure 4.10). Similar to the way in which granulosa derived Vegfa acts in a paracrine manner to stimulate follicular blood vasculature [29, 184, 185], the data presented indicate a similar mechanism involved in ovarian lymphangiogenesis. This is the first detailed description of the regulation of vascular endothelial growth factor family and their receptor expression in ovarian compartments during folliculogenesis and ovulation/luteinisation and along with its implication for the ovarian lymphangiogenic mechanism, provides new pathways for exploration of granulosa cell proliferation and survival.
CHAPTER 6
ESTROGENIC MODULATION OF THE OVARIAN LYMPHATIC VASCULATURE
6.1 INTRODUCTION

In previous chapters, I demonstrated that FSH (eCG) is able to promote ovarian lymphangiogenesis in association with follicle growth, and that pro-lymphangiogenic growth factors and their receptors are regulated by gonadotrophins. Within the ovary, FSH acts via the FSH receptor on granulosa cells to promote a number of functions, one of which includes the induction of Cyp19a1 (Aromatase) the enzyme responsible for the production of estradiol. Given the FSH-mediated changes seen within the lymphatic vasculature, the temporal similarities between the induction of Aromatase and postnatal ovarian lymphatic establishment and the fact that estrogen is an important modulator of blood endothelial cells, I proposed that estradiol may directly mediate ovarian lymphangiogenesis.

Estradiol is the most potent of the endogenous estrogen-related steroids which include estrone and estriol that exert their action by binding Estrogen Receptors (ERs), of which there are four described. ERα and ERβ are members of the nuclear receptor family of ligand activated transcription factors [222], that have genomic actions, but have also recently been associated with non-genomic or membrane initiated effects [223]. GPER or GPR30 is a G-Protein coupled Estrogen Receptor and the least well defined receptor ER-X, a putative receptor found in the brain [224, 225].

The estrogen receptors have been described on a wide range of cell types, and as such, estrogens have wide and varied actions within the body in both reproductive and non-reproductive systems. Estradiol is responsible for growth of the breast and reproductive epithelia, maturation of long bones and development of the secondary sexual characteristics. Estrogen controls growth of the uterine lining during the first part of the menstrual cycle, causes changes in the breasts during adolescence and pregnancy, and regulates a number of other metabolic processes, including bone mineralisation and cholesterol levels. Both ERα and ERβ are localised to the vasculature in both the endothelial cells and smooth muscle cells in many species (reviewed in [226]) and are linked with both vascular health and function.
Additionally, blood vessels express Aromatase, and are therefore capable of producing estrogens [227]. Estrogenic effects on the blood vasculature include improved vascular health and promotion of re-endothelialisation and repair of vessel damage by improving circulating lipid profiles [228] and inhibition of LDL lipid peroxidation [229-232]. Estradiol has a vasodilatory function and is known to enhance blood flow in the reproductive tract [233, 234] as well as many other tissues [228, 235]. In murine models where estrogen signalling is disrupted (ERα−/−), mice display a reduction in endothelium-dependent vasodilation [236-238] and abnormal vascular function and hypertension (ERβ−/−) [239]. Estrogen promotes vasodilation via alteration or modulation of ion flux within smooth muscle cells or modulation of factors derived from the endothelium, including nitric oxide (NO) and cyclooxygenase (COX) metabolites. Within the reproductive organs, estradiol levels modulate blood vasculature and blood flow in animal models. In mares, there are characteristic changes in blood supply to the uterus and ovaries throughout the estrus cycle, with negative correlations between resistance to blood flow in the uterine and ovarian arteries and the plasma estrogen levels during estrus [240]. In sheep, prolonged estradiol administration effects uterine production of endothelial nitric oxide synthase (eNOS), mediating blood flow changes [241, 242]. The effects of estrogens on the lymphatic circulation in response to estrogen, however is unexplored. Unlike the blood vasculature, the majority of the lymphatic vasculature lacks a smooth muscle layer, and as such, effects of estrogen would most likely be different. Additionally, recent microarray analysis identified the presence of ERβ on the lymphatic vascular endothelium, and in higher abundance than on the blood vascular endothelium [243], so it is certainly reasonable to propose that the lymphatic vascular endothelium may be responsive to estrogen.

A range of evidence exists to suggest that female sex hormone dysregulation effects lymphatic function. Lymphedema, a disease characterised by regional swelling and fluid accumulation is more common in women, and commonly presents at puberty [244]. Previous clinical reasoning for the gender differences has included differences in subcutaneous pressure between the sexes and a possible association with estrogen [244, 245], which would also justify the exacerbation at puberty. Lymphedema is also associated with (exacerbated by) pregnancy, menses and menarche, all of which are associated with significant fluctuation in the serum hormone profile. Turner’s Syndrome, a chromosomal disorder, affecting females in which all or part of one of the X chromosomes is absent, presents with lymphedema in conjunction with severe gonadal dysfunction and reduced levels of female hormone production [246], with reports of exacerbation of the lymphedema following induced menarche [247]. Additionally, ovarian
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Hyper-stimulation syndrome (OHSS) is a severe complication resulting from superphysiological hormone treatment of women in preparation for assisted reproduction, presenting with abdominal bloating, ascites and life threatening hypovolaemia. While the pathogenesis of OHSS is unknown symptoms closely resemble lymphedema and may arise through dysregulated vascular permeability and/or lymphatic function.

To explore the hypothesis that estrogen directly modulates lymphangiogenesis of the ovary several approaches were used. I analysed the direct effect of estradiol treatment on lymphangiogenesis in the pre-pubertal model used previously to demonstrate a role for FSH (Chapter 4). The Aromatase inhibitor, Letrozole was used to block estrogen production in the secondary follicles after PND 8.5 and expected to block estradiol-mediated primary lymphangiogenesis. Furthermore, to examine the role of Adamts1 in estradiol-mediated ovarian lymphangiogenesis, I utilised the ovarian lymphatic deficient Adamts1 null mouse line and examined the effect of estradiol in the prepubertal model.

6.2 MATERIALS AND METHODS

6.2.1 Animal treatment and tissue collection

Adamts1+/− and Adamts1−/− mice were generated as per Materials and Methods 2.2.2. All mice were maintained in 12 h/12 h light dark and given water and rodent chow ad libitum. All experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Adamts1+/− and Adamts1−/− mice were collected unstimulated (immature untreated) at d 23 and at two months of age (estrous, metestrus, diestrus and proestrus) for serum estradiol assessment. Estradiol treatment: Estradiol was dissolved and used as previously described [248]. Briefly, 17-β-estradiol (Sigma) was dissolved to a concentration of 200µg/mL in 100% Ethanol. It was then further diluted in 95% Ethanol to a working concentration of 100µg/mL. Immediately prior to injection, 100µg/mL solution was further diluted 1:10 in sesame oil (Sigma). Adamts1+/− and Adamts1−/− nulls female mice were subcutaneously (dorsal skin) injected with 100ng (10mg/kg) 17-β-estradiol at 1400 h on 2 consecutive days (Figure 6.1 A). On day three, mice were sacrificed at 1000 h via cervical dislocation and ovaries were either snap frozen whole (independently) in liquid nitrogen for mRNA analysis or collected and
Figure 6.1  Schematic representation of sample collection schedule/experimental design

A). *Adamts1*^+/−* and *Adamts1*^−/−* ovaries were collected on d 23 following either no treatment or 44 h 17-β-estradiol (10mg/kg) treatment. Ovaries were collected into paraformaldehyde for immunohistological analysis of lymphatic vessels, or snap frozen in liquid nitrogen for subsequent mRNA analysis (n=4-5 mice/treatment/genotype). B). Postnatal day (PND) 8.5 mice were injected with Letrozole (2mg/kg), Letrozole (2mg/kg) plus B-estradiol (10mg/kg) or vehicle s.c. at 1400h and for three subsequent days. At PND 12.5 mice were sacrificed via decapitation, uteri weighed and ovaries snap frozen whole in liquid nitrogen for mRNA analysis or fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemistry. Ovaries from 6-7 mice were collected for each treatment.
Chapter 6: Estrogenic modulation of the ovarian lymphatic vasculature

A

No treatment or 10mg/kg β-estradiol 14:00 h
0 (d 21 of age)

Adams1−/− and Adams1−/− Follicle growth

Ovary collection at 10:00 h for protein detection and mRNA analysis

B

Group 1: No treatment (vehicle)

Group 2: Letrozole (2mg/kg)

Group 3: Letrozole (2mg/kg) + β-estradiol (10mg/kg)

PND 0.5 PND 8.5 PND 10.5 PND 12.5

Early postnatal ovarian development

Ovary collection at 10:00 h for protein detection and mRNA analysis
fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemistry. A total of 4-5 mice were collected for each treatment and genotype. Experiments were performed alongside eCG experiments, and as a result, compared to the same un-stimulated controls. Letrozole treatment: Inbred C57BL/6 mice were purchased from Laboratory Animal Services (University of Adelaide, Australia). Letrozole was obtained from Novartis. PND 8.5 mice were injected with Letrozole (2mg/kg) (as previously described [249]), Letrozole (2mg/kg) plus B-estradiol (10mg/kg) or vehicle s.c. at 1400h and for three subsequent days (Figure 6.1 B). At PND 12.5 mice were sacrificed via decapitation, uteri weighed and ovaries snap frozen whole (independently) in liquid nitrogen for mRNA analysis or collected and fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemistry. A total of 6-7 mice were collected for each treatment and analysed independently.

Letrozole is a specific, non-steroidal Aromatase inhibitor which acts via its direct binding to the heme group of the cytochrome P450 unit of the enzyme. I chose Letrozole for the following experiments due to its known efficacy in other systems, and its specificity for inhibiting the production of estrogen without effecting production of mineralo- or corticosteroids (Reviewed in [250]). I chose an Aromatase inhibitor in preference to a selective estrogen receptor modulator (SERM) because I wanted to be sure that all the actions of estradiol were being blocked in our system. SERMs are known to have altered tissue-specific efficacy dependent on the ratio of co-activator and co-repressor [251, 252] and given our interest in complete inhibition of the production of ovarian estradiol, an Aromatase inhibitor was deemed the most suitable for these experiments.

6.2.2 Estradiol assays

Adamts1+/− and Adamts1−/− mice were collected at d 23 (immature) (n=4-6 mice per genotype) or adult cycling (n= at least 3 mice per genotype) at estrus, metestrus, diestrus and proestrus. Vaginal cytology smears were used to confirm estrus cycle stage. Mice were anaesthetised with Avertin and whole blood was collected from each mouse via the sub-orbital venous plexus using heparinised micro-haematocrit tubes (Chase Scientific Glass Inc, Rockwood TN, USA). Mice were then sacrificed via cervical dislocation. Serum was collected following centrifugation at 4000 rpm for 10 minutes. Ultra-sensitive Estradiol RIA (Diagnostic Systems Laboratories, Texas USA) were performed as per manufacturer’s instructions. Estradiol levels were presented as ± SEM.
6.2.3 Immunohistochemistry

Immunohistochemical localisation of Lyve1 was performed as per Chapter 2.2.5 and 3.2.2. The Lyve1 antibody required Proteinase K antigen retrieval, whilst the ERα (Santa Cruz Biotechnology, CA, USA) antibody required Proteinase K and citrate buffer antigen retrieval.

6.2.4 Image capture and morphometric analysis

Image capture and morphometric ovarian and lymphatic vessel analysis was performed as per Sections 2.2.6 and 4.2.3.

6.2.5 Real Time RT-PCR

Real Time RT PCR was performed as per Chapter 2.2.7 with primers listed in Table 2.1. Genes measured were Lyve1, Vegfa, Vegfc, Vegfd, Vegfr3, Aromatase, Ccnd2 and Amh. Data was presented as mean raw 2^ΔΔCT ± SEM to demonstrate fold and relative expression differences.

6.2.6 Statistical analysis

Data was analysed using One Way ANOVA with Tukey Post-Hoc and statistical significance considered if $P \leq 0.05$ (SPSS 11.5; SPSS Inc., Chicago, IL). Alternatively, when comparing the difference between only two groups, Student’s t test was used where statistical significance considered if $P \leq 0.05$ (SPSS 11.5; SPSS Inc., Chicago, IL).

6.3 RESULTS

6.3.1 *Adams1* null mutation does not affect serum estradiol

Serum estradiol was measured at d 23 (immature) and in adult cycling mice (two months of age). There was no difference between the level of serum estradiol in immature *Adams1*+/- and *Adams1* +/- mice (17.9±1.8 pg/mL vs. 16.0±0.8 pg/mL) (Figure 6.2 A). In adult cycling mice, there was no difference in
Figure 6.2 Serum estradiol does not differ between *Adams1* genotypes

Serum estradiol was measured by ELISA in *Adams1*+/- (red) and *Adams1*-/ (blue) female mice at d 23 (immature untreated) or in cycling adult mice (d 60) at proestrus, metestrus, estrus and diestrus. (n= at least three mice/treatment/genotype). NS represents no statistical significance (non-significant).
serum estradiol between *Adamts1*<sup>+/−</sup> and *Adamts1*<sup>−/−</sup> mice at any stage of the estrus cycle (Figure 6.2 B), indicating that while ovarian follicle morphogenesis is disrupted in the *Adamts1*<sup>−/−</sup>, this was not sufficient to alter estradiol production.

### 6.3.2 Estradiol altered ovarian characteristics in *Adamts1*<sup>+/−</sup> and *Adamts1*<sup>−/−</sup> mice

Following estradiol administration in *Adamts1*<sup>+/−</sup> mice, total ovarian area was unchanged, however in *Adamts1* null mice, ovarian area significantly increased such that it was no longer different to that of their *Adamts1*<sup>+/−</sup> counterparts (Figure 6.3 A). While not significant, estradiol treatment increased the percentage of ovarian follicle area and decreased the percentage of stromal area in *Adamts1*<sup>−/−</sup> mice suggesting that increased ovarian area is in part due to increased follicle size. Prior to and following treatment with estradiol, *Adamts1*<sup>+/−</sup> mice had a higher proportion of ovarian area made up by growing follicles when compared to their *Adamts1* null counterparts, and in turn, less stromal volume (Figure 6.3 B, C) as expected due to the disrupted folliculogenesis previously reported in *Adamts1*<sup>−/−</sup> ovaries [142].

Uterine weight, a bioassay of circulating active estradiol, was significantly increased following estradiol administration in both *Adamts1* genotypes, and was not significantly different between genotypes (Figure 6.4) further indicating that circulating estradiol was equivalent and that estradiol administration was effective in each genotype.

### 6.3.3 Estradiol promotes ovarian lymphatic vessel growth and expansion

Estradiol treatment in *Adamts1*<sup>+/−</sup> mice had no effect on total lymphatic vessel number per ovary (Figure 6.5 A) or mean lymphatic vessel size (Figure 6.6). When vessels were classified according to vessel size, *Adamts1*<sup>+/−</sup> ovaries had significantly less small vessels following estradiol administration. The number of vessels in the medium, large and extra large categories was not significantly changed (Figure 6.5 B). Alternatively, in *Adamts1*<sup>−/−</sup> ovaries that were almost devoid of lymphatic vessels at d 21, showed significantly increased ovarian vessel number by 6.3-fold after estradiol treatment (Figure 6.5 A). Furthermore, when classified according to vessel size, estradiol significantly increased the number of small and medium sized vessels (Figure 6.5 C), as well as significantly increasing overall mean vessel size (Figure 6.6). The increase in mean vessel size was comparable to that seen following eCG
Figure 6.3 Effect of estradiol on ovarian and follicle area

*Adamts1*+/− (red bars) and *Adamts1*−/− (blue bars) ovaries were collected on d 23 following either no treatment (untreated; filled bars) or 44 h estradiol treatment (checkered bars). Total ovarian area (μm²) (A), percent of ovarian area occupied by growing follicles (B) and percent of ovarian area occupied by stroma (non-follicle area) (C) were calculated based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 4-5 animals per genotype and treatment. Different letters represent statistical significance (P≤0.05).
Figure 6.4 Estradiol increases uterine weight equally in $Adamts1^{+/+}$ and $Adamts1^{-/-}$ mice

Uterine weight was measured in $Adamts1^{+/+}$ and $Adamts1^{-/-}$ following either no treatment (untreated; filled bars) or 44 h estradiol treatment (checkered bars) at d 23. Data represents mean ± SEM, n = 4-5 animals per treatment. Different letters represent statistical significance ($P≤0.05$).
Figure 6.5 Estradiol modulates ovarian lymphatic vessel number and size

Ovarian lymphatic vessels were counted and area measured in Adamts1+/− (red bars) and Adamts1−/− (blue bars) ovary sections following either no treatment (untreated; filled bars) or 44 h estradiol treatment (checkered bars). Total ovarian lymphatic vessel number was analysed (A) and vessels were classified by size (B, C). Morphometric analysis was based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 4-5 animals per genotype and treatment. Different letters (or *) represent statistical significance (P≤0.05).
Figure 6.6 Estradiol increases ovarian lymphatic vessel size in *Adams*1−/− ovaries

Mean ovarian lymphatic vessel size was calculated in *Adams*1+/− (red bars) and *Adams*1−/− (blue bars) ovaries following either no treatment (untreated; filled bars) or 44 h estradiol treatment (checkered bars) or 44 h eCG treatment (for comparison from Figure 4.7; striped dull bars). Morphometric analysis was based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 4-5 animals per genotype and treatment. Different letters represent statistical significance (P≤0.05).
administration (Figure 6.6; dull blue bars) and is consistent with formation of new vessel networks in the ovary in response to estradiol. Unlike the increases seen following eCG stimulation, estradiol treatment had no effect on total lymphatic area when expressed as a percentage of ovarian or stromal area in Adamts1+/− mice. Contrastingly in Adamts1−/− mice, estradiol treatment significantly increased the percentage of both total ovarian area and stromal area occupied by lymphatic vessels (Figure 6.7 A-B) indicating that increased total lymphatic number and area is not simply the result of increased ovarian size in these mice.

6.3.4 Estradiol mediates spatial location-specific changes to the ovarian lymphatic vasculature

Following estradiol treatment, ovarian lymphatic vessels were classified by their spatial location (Figure 4.2). Vessels were present in all three regions independent of treatment or genotype, although in very minimal numbers in Adamts1−/− ovaries without exogenous hormone treatment (Figure 6.8 B, filled bars). In Adamts1+/− ovaries, a decrease in the number of vessels within the follicle associated region of the ovary, but no effect on the number of vessels in either the cortical or medullary regions was seen after estradiol treatment (Figure 6.8 A). In Adamts1−/− ovaries, estradiol caused a 22-fold increase in the number of vessels in the medullary region (medulla and stalk vessel) and a nine-fold, non-significant, increase in the number of vessels in the cortical region (Figure 6.8 B). Estradiol treatment had no effect on the size of vessels in any location in either genotype (Figure 6.8 C, D).

6.3.5 Estradiol treatment induces Lyve1 and Vegfc in the Adamts1−/− ovary

Whole ovary mRNA expression of Lyve1, Vegfc, Vegfd and Vegfr3 were analysed using real time RT-PCR. Lyve1 expression increased two-fold in the Adamts1−/− ovaries in response to estradiol (Figure 6.9 A), consistent with the morphological changes seen in the ovarian vasculature. Estradiol treatment also significantly increased Vegfc expression in the Adamts1 null ovary, but was unchanged in the Adamts1+/− mice (Figure 6.9 B). Vegfd and Vegfr3 expression were not significantly different following estradiol treatment in either genotype (Figure 6.9 C-D). Interestingly, estradiol administration stimulated large three and four-fold increases in Lyve1 expression in the uterus of Adamts1+/− and Adamts1−/− mice respectively, however, this was only significant in the Adamts1 null (Figure 6.9 E).
Figure 6.7 *Adams1*, but not estradiol effects total ovarian lymphatic area

Lymphatic area as a fraction of total ovarian area (A) and stromal area (B) was calculated in *Adams1^+/−* (red bars) and *Adams1^−/−* (blue bars) ovaries following either no treatment (untreated; filled bars) or 44 h estradiol treatment (checkered bars). Morphometric analysis was based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 4-5 animals per genotype and treatment. Different letters represent statistical significance (*P*≤0.05).
Figure 6.8 Estradiol causes location specific changes in lymphatic vessel size and number

Ovarian lymphatic vessels were characterised by number, size and spatial distribution in $Adamts1^{+/−}$ (red bars) and $Adamts1^{-/-}$ (blue bars) ovaries following either no treatment (untreated; filled bars) or 44 h estradiol treatment (checkered bars). Lymphatic vessel number and distribution were analysed (A, B) as well as size and distribution (C, D). Morphometric analysis was based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 4-5 animals per genotype and treatment. * represents statistical significance ($P<0.05$).
Figure 6.9  Estrogenic regulation of ovarian lymphangiogenic genes

Real time RT-PCR was used to analyse *Lyve1* (A), *Vegfc* (B), *Vegfd* (C) and *Vegfr3* (D) expression in *Adamts1*+/− (red bars) and *Adamts1*−/− (blue bars) ovaries following either no treatment (untreated; filled bars) or 44 h estradiol treatment (checkered bars). *Lyve1* expression was also analysed in whole uterus (E). Data represents mean (∆∆*CT*) ± SEM, *n* = 4-5 animals per genotype and treatment. Different letters represent statistical significance (*P*≤0.05).
6.3.6 Estrogen receptor α localises to the nucleus of ovarian lymphatic vessels

Immunohistochemical localisation of lymphatic vessel marker Lyve1 and Estrogen receptor α (ERα) in serial ovarian sections revealed that the lymphatic vasculature express ERα. ERα was localised to the nucleus of cells associated with the Lyve1 positive vessels (Figure 6.10 B, arrows).

6.3.7 Aromatase inhibitor Letrozole alters ovarian composition

In order to further explore the contribution of estrogen to ovarian lymphangiogenesis, the Aromatase antagonist, Letrozole was administered to inhibit the rise in estrogen occurring at PND10.5 and the corresponding effect on ovarian lymphatic vascularisation was examined (Figure 6.1 B). Letrozole administration from PND 8.5 to 12.5 had no effect on ovarian size, or mean follicle size (Figure 6.11 A-B). Similarly, Letrozole plus estradiol replacement had no effect on total ovarian area, but did however lead to an unexpected increased average follicle cross sectional area when compared to the vehicle treated control (Figure 6.11 A-B). When examining the proportion of growing follicles within the ovary, Letrozole administration alone increased the growing follicle area when expressed as a percentage of total area, while the estradiol replacement group was not significantly different. The inverse was seen in stromal volume, with the vehicle treated control having a higher proportion of stroma than the Letrozole treated group (Figure 6.11 C-D).

6.3.8 Aromatase inhibition decreases ovarian lymphatic vessel size and alters ovarian gene expression

Ovarian lymphatic vessel number was not different between control, Letrozole and Letrozole + estradiol treated ovaries (Figure 6.12 A). Letrozole treatment significantly decreased ovarian lymphatic vessel size (Figure 6.12 B) suggesting an effect of estradiol on developing primary ovarian lymphatics, however lymphatic vessel size was not significantly restored by treatment with Letrozole plus estradiol (Figure 6.12 B). As a percentage of ovarian area or stromal area, the lymphatic vessel area in Letrozole treated ovaries was significantly lower than the Letrozole plus estradiol treated group, however in this
Figure 6.10  Estrogen receptor alpha (ERα) is found on lymphatic vascular endothelium

Lymphatic vessels (Lyve1-positive; A) and Estrogen receptor alpha (ERα; B) were localised in serial ovarian sections. ERα positive cells (arrows, B) were found in vessels which were Lyve1 positive (A). Images were captured at 60x.
Figure 6.11 Letrozole administration alters follicle and stromal area

Mice were treated with vehicle (untreated; green bar), Letrozole (yellow bar) or Letrozole + estradiol (yellow and black checkered bar) from postnatal day (PND) 8.5 to PND 12.5. A) Total ovarian cross sectional area (μm²) B) mean cross sectional area of follicles, C) percent of ovarian area occupied by growing follicles D) percent of ovarian area occupied by stroma. Morphometric analysis was based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 6-7 animals per treatment. Different letters represent statistical significance (P≤0.05).
Figure 6.12 Letrozole treatment has no effect on ovarian lymphatic vessel number, but decreases vessel size

Lymphatic vessel number (A) and cross-sectional size (B) were analysed following either vehicle treatment (untreated; green bar) or Letrozole treatment (yellow bar) or Letrozole plus estradiol replacement (yellow and black checkered bar). Morphometric analysis was based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 6-7 animals per treatment. Different letters represent statistical significance (P≤0.05).
case neither treatment group were different to vehicle (Figure 6.13 A-B). When vessels were classified by location, there were no differences in the size or number of vessels between any treatment groups (data not shown).

Assessment of ovarian Lyve1 expression as another indicator of lymphatic volume fraction showed unexpected significantly lower ovarian Lyve1 expression in the Letrozole plus estrogen replacement group compared to control, but not to Letrozole treatment alone (Figure 6.14 A). Vegfc, Vegfd and Vegfr3 were not affected by any treatment (Figure 6.14 B-D). Vegfa was not different from control after Letrozole treatment, but was significantly higher in the Letrozole plus estrogen group when compared to Letrozole alone (Figure 6.14 E). The expression of Anti-Mullerian hormone (Amh), a granulosa cell specific gene product measured to determine whether the granulosa cell number was significantly altered in estradiol replacement group (Figure 6.14 G). To answer whether estrogen responsive gene expression were inhibited in Letrozole treated ovaries, Ccnd2 expression analysis was surprisingly unchanged from control following either treatment (Figure 6.14 H). Strikingly Aromatase expression in ovaries was significantly five-fold higher in Letrozole treated than Letrozole plus estradiol treated groups (Figure 6.14 F). Further investigation of Letrozole efficacy came from uterine weight which was unchanged in Letrozole treated compared to control PND12.5 mice, but significantly increased after Letrozole plus estrogen replacement (Figure 6.15).

### 6.4 DISCUSSION

In Chapter 4, I was able to demonstrate that treatment with exogenous FSH-analogue, eCG stimulates ovarian lymphatic vessel growth, while in Chapter 3 I showed a temporal association between the induction of ovarian Aromatase and ovarian lymphatic vascular establishment. Given these observations, I hypothesised that estradiol, produced by granulosa cells in response to FSH, is at least in part responsible for the hormone mediated ovarian lymphatic vascular changes described. In these experiments, I exploited both the postnatal lymphatic establishment model as well as the lymphatic vessel deficient Adamts1-/- model to examine the specific lymphangiogenic effects of estradiol.
Figure 6.13 Estradiol restores Letrozole-mediated effects on ovarian lymphatic vascular area

Lymphatic vessel area was calculated and expressed as a percentage of both total ovarian area (A) and total stromal area (B) following either vehicle treatment (untreated; green bar) or Letrozole treatment (yellow bar) or Letrozole plus estradiol replacement (yellow and black checkered bar). Morphometric analysis was based on analysis of serial ovarian sections at 35μm increments through all ovaries. Data represents mean ± SEM, n = 6-7 animals per treatment. Different letters represent statistical significance (P≤0.05).
Figure 6.14  Ovarian mRNA expression is altered following Letrozole ± estradiol administration

Real-time RT-PCR was used to analyse ovarian mRNA expression of *Lyve1* (A), *Vgfc* (B), *Vegfd* (C), *Vegfr3* (D), *Vegfa* (E), *Aromatase* (F), *Amh* (G) and *Cend2* (H) following either vehicle (untreated; green bar), Letrozole (yellow bar) or Letrozole + estradiol treatment (yellow and black checkered bar). Data represents mean \( (2^{\Delta \Delta CT}) \pm \text{SEM} \), n = 6-7 animals per genotype and treatment. Different letters represent statistical significance \( (P \leq 0.05) \).
Figure 6.15 Letrozole has no effect on uterine weight, while estradiol increases uterine weight

Uterine weight was measured in following either vehicle treatment (untreated; green bar) or Letrozole treatment (yellow bar) or Letrozole plus estradiol replacement (yellow and black checkered bar) at PND 12.5. Data represents mean ± SEM, n = 5 animals per treatment. Different letters represent statistical significance (P≤0.05).
Firstly, when considering estradiol as an endocrine stimulator of ovarian lymphatic vessels, it was necessary to confirm whether serum estradiol levels are altered in mice lacking Adamts1, potentially explaining the lymphatic deficiency phenotype. In immature (d 23) mice, and adult cycling mice, there was no difference between the genotypes when comparing serum estradiol levels. This outcome, the observed equivalent uterine weight in each Adamts1 genotype and a parallel study showing no difference in mammary gland development [Russell and Ingman; personal communication] indicates that circulating estradiol is not deficient in Adamts1–/– mice. It would be expected that during the normal estrus cycle, serum estradiol would be significantly increased in the pro-estrus period, coinciding with the rapid growth of follicles within the ovary. It is unclear why we did not see this within our experiment, but it is likely due to the low number of animals within these groups in both genotypes (Adamts1+/– n=3; Adamts1–/– n=3) and the short duration of the pro-estrus period in mice compared with other species (which have extended follicular phases). It can be concluded that altered serum estradiol is not responsible for the ovarian lymphangiogenesis phenotype of the Adamts1–/– mice and hence that Adamts1 has a role in lymphangiogenesis distinct from the hormone mediated effects. This however does not invalidate the possibility that estrogen promotes ovarian lymphangiogenesis.

In many ways, treatment with estradiol mimicked the effects previously described in response to FSH supporting the hypothesis that FSH-mediated estradiol production stimulates ovarian lymphangiogenesis. The limited effectiveness of estradiol on the total number or area of lymphatic vessels in normal Adamts1+/– mice is consistent with a maximal estrogen effect and a fixed threshold number of lymphatic vessels already being established in these mice. This may indicate that in addition to hormone mediated induction of lymphangiogenic factors some undefined inhibitory factors maintain a fixed vessel density. Contrastingly, in mice lacking lymphatic vessels (Adamts1–/–), estradiol treatment increased the number of ovarian lymphatic vessels; an outcome virtually identical to that seen in response to FSH. Estradiol administration significantly increased the number of small and medium vessels within the Adamts1 null ovary and also significantly increased the average lymphatic vessel size by 50%, again, virtually identical to the changes observed in response to FSH. The close similarities in the FSH and estradiol-mediated lymphatic vascular changes in the ovary, especially in the Adamts1–/– mice strongly suggest that the FSH-regulated ovarian lymphangiogenesis is mediated by estradiol. However eCG showed effects in Adamts1+/– mice causing a two-fold increase in vessel size while
estradiol had no similar effect suggesting that FSH has actions independent of estradiol on ovarian lymphangiogenesis.

As discussed earlier, estrogen receptors (ERα and ERβ) have been localised to the blood vasculature in both the endothelial cells and smooth muscle cells in many species (Reviewed in [226]) and have been linked with both vascular health (and the pathophysiology of atherosclerosis) and function. More recently, ERβ has been localised to the lymphatic endothelium [243]. Here I was able to localise ERα to the lymphatic endothelium. Estrogens promote cell survival and proliferation in a wide range of tissues, whilst anti-estrogens inhibit proliferation by promoting cell cycle arrest in the G0/G1 phase [253-255] and promote apoptosis via caspase activity/activation [256-259]. Estrogens promote cell proliferation under a number of normal and pathological circumstances including folliculogenesis, bone turnover and a large number of cancers [260-262] via ERα. Additionally, estradiol had been demonstrated to be capable of promoting vascular endothelial cell proliferation via a non-genomic estrogen pathway involving production of Cyclin D1 (CcnD1) and the activation of Erk1/Erk2 signalling [263, 264]. Estrogens acting via ERα in lymphatic endothelial cells may mediate any one of a wide range of changes in the lymphatic vascular endothelium; however this certainly requires further exploration and may be best examined in in vitro culture systems.

Interestingly, estradiol treatment failed to mediate many of the gene expression changes seen following eCG treatment. eCG increased whole ovary expression of Lyve1, Vegfc, Vegfd and Vegfr3 in one or both Adamts1 genotypes (Chapter 4), whilst estradiol regulated expression of Vegfc only in the Adamts1 nulls (Chapter 6). This change in Vegfc expression, however was virtually identical to the response following eCG administration. Vegfc promotes lymphangiogenesis and is known to promote cell migration [214, 215] and, whilst speculative, it is plausible that the stromal or granulosa cells may produce Vegfc as a chemotactic stimulus to enhance vascular expansion within the ovary. This was especially evident in the case of the Adamts1−/− ovary, which was virtually devoid of lymphatic vasculature unless exogenous hormone was administrated. This may be similar to the process of primary or embryonic lymphangiogenesis where Vegfc is secreted by the tissue adjacent to the newly differentiated lymphatic precursor cells of the venous circulation, promoting migration and proliferation, and subsequent formation of the primary lymph sacs, from which the lymphatic vessels sprout (reviewed
in [102]). That eCG could induce VegfC and increased vessel size supports a role in vessel maturation. The induction by eCG but lack of estradiol-mediated regulation of Vegfd and Vegfr3 suggests that the action of FSH is mediated both through estrogenic and non-estrogenic lymphangiogenic mechanisms. It would appear that FSH is able to mediate pathways, independently of estradiol, which regulate changes in Vegfd and Vegfr3 expression, as well as promote lymphangiogenesis in the Adamts1+/− ovary; both of which were absent following stimulation with estradiol alone.

Inhibition of estradiol production using the Aromatase inhibitor, Letrozole did not produce the expected outcome. I hypothesised that the inhibition of estradiol production during the window when Aromatase was induced and lymphatics established within the ovary, would negatively impact on lymphatic establishment. However Letrozole treatment did not affect ovarian vessel number during lymphatic establishment. Letrozole did significantly decrease average lymphatic vessel size (compared to control), however, but not when compared to the estradiol replacement group, failing to support that the lack of estradiol had caused the decreased vessel size. Evidence that Letrozole effectively decreased ovarian Aromatase action was limited. Uterine weight, which we used as a bioassay for circulating estradiol levels, was expected to have been lower than controls, and this was not the case, but the increased uterine weight after estrogen replacement indicates that this was clearly an effective treatment. The five-fold increased Aromatase expression in ovaries of Letrozole treated mice and significant reversal of this effect after estradiol replacement suggests that an increase in Aromatase synthesis may have compensated for the loss of estrogen at this dose of the antagonist. On balance this experiment does not compellingly disprove, or invalidate the effect of exogenous estrogen on lymphangiogenic events observed in d 21 mice. Furthermore, there is little question, that in the Adamts1 nulls, estrogen alone promoted the development of the ovarian lymphatic vasculature. What remains unclear is what contribution and to what extent estradiol mediates FSH action on ovarian lymphangiogenesis. This could be determined by a future investigation of the effect of Letrozole on eCG stimulated lymphangiogenesis. Measuring serum estradiol during this experiment would allow definitive confirmation of the successful inhibition of Aromatase.

My hypothesis that FSH mediates ovarian lymphangiogenesis via the induction of downstream factors capable of acting in a paracrine manner in the ovary is supported in this chapter by the observed effect
of estradiol on the ovarian lymphatic vascular number and size. Estrogen is produced by granulosa cells in response to FSH signalling, may act on ERα which was localised to the lymphatic vascular endothelium. These results, in combination with the temporal association of the induction of Aromatase with ovarian lymphatic vascular establishment suggest that ovarian lymphangiogenesis is hormonally regulated in response to both FSH and estradiol, which promote expression of known lymphangiogenic factors. These novel findings provide significant new knowledge surrounding the control of ovarian fluid homeostasis, and may provide a new model, and concepts in ongoing research in lymphatic disease and disorders.
CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS
The ovary provides a niche environment where the female germ cells (or oocytes) are generated, stored and matured for use during a female's reproductive years. Following an extensive period of quiescence, the follicle and ovary undergo extensive remodelling events whereby the follicle grows the oocyte matures and which culminates in the expulsion of a fully mature oocyte in preparation for fertilisation. These events occur cyclically as part of the menstrual (or estrus) cycle and involve extensive remodelling of both the follicle and its surrounding extracellular tissue. Integral to these cyclic events is the concurrent remodelling of the blood vasculature within the ovary. These changes are well explored and have been demonstrated to be necessary for follicle growth, hormone synthesis, ovulation, as well as for development and function of the corpus luteum.

In other systems, the lymphatic vasculature is known to closely interact with the blood vasculature and plays a number of important physiological roles. The lymphatic vasculature is responsible for return of extra-vascular fluid to the blood circulation, and in turn, maintenance of systemic fluid homeostasis, trafficking of immune cells from the periphery to lymph nodes in the initiation of an immune response and the gastrointestinal absorption of lipids and lipid soluble vitamins. Unlike the blood vasculature, the development aetiology and remodelling lymphatic vasculature had been poorly described within the ovary until now.

### 7.1 OVARIAN LYMPHATIC VASCULAR ESTABLISHMENT

To elucidate the mechanisms involved in lymphatic establishment I examined in detail the lymphatic vasculature, as well as the genes responsible for the mediation of lymphangiogenesis during postnatal ovarian development. Remarkably the lymphatic vasculature was not detectable within the ovary until PND 8.5 and fully populated the ovary and was associated with growing follicles by PND 12.5. The establishment of the ovarian lymphatic vasculature was associated with increased expression of Lyve1, as well as known lymphangiogenic growth factors Vegfc, Vegfd and the lymphangiogenic receptor Vegfr3 (Chapter 3). The timing of the lymphatic vascular expansion was especially interesting for two reasons. Firstly, the postnatal development of the lymphatic vasculature lags dramatically behind blood vascular establishment. Secondly, the concurrent establishment of lymphatics with the induction of ovarian Aromatase, estrogen synthesis and the development of secondary follicles during first wave of
foliculogenesis suggests an important interaction between the lymphatic system and these rapidly developing follicles.

7.1.1 The ovarian lymphatic vasculature is established postnataally

In other organs, including the gut, heart and lungs, the lymphatic vasculature is described as early as E15 [109, 110], however within the ovary, the lymphatic vasculature was not present until postnatal day PND 8.5. Appropriate trafficking of fluid and immune cells through most tissues requires that blood and lymphatic vasculature work in close functional association, however blood vascularisation of the ovary develops during early gonadogenesis in the embryo [57, 59-61]. It is entirely comprehensible that as soon as the gonad is established, it requires a blood vasculature providing oxygen and other factors for cell survival. It is unclear why a unique, organ-specific, disparity in the development of the vascular systems, and why the ovary lags behind other systems with its establishment of a lymphatic network. Unlike most organs, the ovary undergoes extensive remodelling postnataally, during which the primordial follicles are assembled. Possibly it is important for this process to be completed prior to the establishment of the lymphatic system. What is apparent is that its functions must not be required within the ovary until after PND 8.5. Given its well described roles in fluid homeostasis in association with the blood vasculature, it would appear that the mouse ovary does have a functioning fluid homeostatic mechanism present until during postnatal development, or that the blood vasculature alone, can execute all necessary actions. Physiologically, it is plausible that major fluid homeostatic mechanisms may not be required until the first wave of follicles reach antral growth, where both rapid changes in blood vessels and fluid accumulation occur; and my results suggest that by this time, the lymphatic vasculature would be present.

7.1.2 The ovarian lymphatic vasculature is established concurrently with estrogenic follicle growth

The concurrent establishment of the lymphatic vasculature with the first postnatal induction of ovarian Aromatase, suggests that hormonal communication may synchronise lymphangiogenesis with foliculogenesis. Estrogen receptors ERα (this study) and ERβ [243] are present on lymphatic endothelial cells, and considering the array of known actions of estrogens on blood vascular endothelial
cells, it is plausible to consider that estradiol acts directly on the lymphatic endothelium to promote ovarian lymphatic vascular establishment in the developing ovary and remodeling in the cycling ovary. An alternative possibility is that the production of estrogen induces other factors, either within ovarian follicles or within the stroma which could act in either a paracrine or autocrine fashion to promote lymphangiogenesis. Additionally, FSH-analog eCG was able to enhance ovarian lymphangiogenesis during the window of lymphatic establishment (postnatal development or Adamts1 null), the evidence suggesting that this acts both via estrogen as well as through FSH specific actions, perhaps the induction of Adamts1 [143].

### 7.2 OVARIAN LYMPHATIC VASCULAR REMODELLING

Research to date has primarily focused on the primary embryonic establishment of the lymphatic vasculature from the venous system, and has failed to uncover, or describe any normal physiological models of lymphatic vascular remodelling. This is probably not remarkable, given the close similarities between the lymphatic and blood vasculatures, and taking into consideration both systems are thought to predominantly remodel under the pathological stress of wound healing, inflammation and tumour progression. The cyclic nature of the reproductive system, however, provides the only known model of normal adult blood vascular remodelling (or angiogenesis). The present studies reveal that the ovary undergoes lymphatic vascular remodelling, described elsewhere as adult or secondary lymphangiogenesis [119, 120, 265], and that this process involves hormonal contributions from FSH and estradiol, as well as the extracellular matrix protease, Adamts1 (Figure 7.1).

#### 7.2.1 The role of FSH and estradiol

FSH promoted remodelling of the existing lymphatic vascular maturation by increasing lymphatic vessel size in normal (Adamts1+/−) ovaries, and promoted the expansion of a new lymphatic vascular network by increasing vessel number and size in Adamts1−/− ovaries. These vessel changes were also associated with the induction of pro-lymphangiogenic factors, Vegfc and Vegfd, as well as their receptor, Vegfr3 providing a mechanistic explanation for the hormonal mediated lymphangiogenesis. Interestingly, in the Adamts1−/− model, where normal postnatal ovarian lymphatic vascular establishment occurs, only vessel size increased in response to FSH. This suggest that once the vasculature is
Figure 7.1  Schematic representation of the factors regulating ovarian lymphangiogenesis

Ovarian lymphangiogenesis is regulated by FSH and estradiol, and involves protease Adamts1. FSH is likely to mediate ovarian lymphangiogenesis by inducing the expression of pro-lymphangiogenic growth factors Vegfc and Vegfd, which promote lymphatic vascular remodelling via Vegfr3 (found on lymphatic endothelium). FSH also induces the granulosa cell production of estrogen, which can act directly on the lymphatic endothelium through its receptors ERα and ERβ. Exogenous estrogen is likely to regulate lymphangiogenesis via its induction of Vegfc, and/or via estrogen receptors on the lymphatic endothelium. Adamts1, regulated by FSH, is essential for normal lymphatic development, and is likely to contribute to ovarian lymphangiogenesis via direct interactions with lymphangiogenic growth factors (Vegfc/d), the extracellular matrix of the ovary, or the proliferative nature of the lymphatic endothelial cells.
established within the ovary, a finite and functionally sufficient number of ovarian lymphatic vessels exist, and that functional adaptations are made in response to hormone. In both Adiams1 genotypes, the lymphatic vascular changes described would support an increased functional capacity of the ovarian lymphatic vasculature coordinated with FSH-mediated follicle growth. The granulosa layers and oocyte of ovarian follicles are avascular but have high metabolic activity and during FSH-mediated follicle growth changing ovarian fluid dynamics result in the formation of a fluid filled antrum. Antrum formation is a unique physiological event in itself, and intriguingly, resembles the pathophysiological formation of edema. The follicle is thus dependent on dynamic fluid transport and homeostasis. While it remains unclear what contribution the lymphatic vasculature makes to antrum formation, its remodelling during this period suggests it is of importance, as does the association of failed lymphangiogenesis, failed folliculogenesis and infertility in Adiams1−/− mice.

Exogenous estradiol mimicked many FSH-mediated lymphangiogenic effects. A significant induction of Vegfc in eCG or estradiol treated Adiams1−/− ovaries corresponding with strikingly increased vessel number and size suggesting that this mechanism of lymphangiogenic induction is common to both hormones. The presence of ERα on lymphatic vessels suggest the possibility that estradiol acts directly on the lymphatic vascular endothelium to mediate vessel changes. Estrogen alone is not sufficient to promote antrum formation, which may in part explain the lack of lymphangiogenesis in response to estradiol in normal ovaries. The results suggest that while estradiol alone is capable of promoting ovarian lymphangiogenesis, FSH mediates changes distinct from those of estradiol.

In the future, a number of other techniques may be used to further describe the proliferation, remodeling and functional significance of the ovarian lymphatic vasculature. In order to examine the proliferation of the vascular endothelium in response to hormonal stimulation, the introduction of bromodeoxyuridine (BrdU), a synthetic thymidine analogue used to detect proliferating cells could be used to identify the presence of proliferating LECs. Also, immunolocalisation of Ki67, a protein present during cell replication (proliferation) may also be useful in localizing the proliferating LECs. The use of other estrogen mediators may also be useful in further examining the contribution of estradiol to the ovarian lymphatic remodeling events within the ovary, particular in combination with eCG/FSH. It is also possible that with the development and early clinical applications of tyrosine kinase inhibitors for Vegfr3
(and therefore Vegfc and Vegfd signalling), that these compounds could also be used in our system to evaluate and elucidate the functional significance of the ovarian lymphatic vasculature [266]. Given the granulosa cell production of these growth factors, it is also likely that the use of these compounds may also reveal novel roles for these growth factors during folliculogenesis, also.

7.2.2 The role of Adamts1

I previously established that Adamts1 null mice have an ovarian lymphatic vessel defect, whereby at PND 10 and d 21, lymphatic vessels were not detectable within the ovary [142]. Here I clearly demonstrate that ovarian lymphatic vascular establishment was stimulated, although not fully restored in the Adamts1 nulls following FSH-analog eCG or estradiol administration, suggesting that Adamts1 is essentially and non-redundantly involved in ovarian lymphangiogenesis. Adamts1 is known to promote both cell migration and proliferation [153, 159, 160], and it is possible that one or both of these mechanisms are involved in the process of ovarian lymphangiogenesis. It is likely that both the establishment and remodelling of the ovarian lymphatic vasculature involves the degradation of extracellular matrix to allow cell motility, intrinsic motile capacity of the lymphatic endothelial cells and cell proliferation. Additionally, Adamts1 has been described to be expressed by blood vessels [155] and to have both pro- and anti-angiogenic properties [156-158], and most recently, to promote blood endothelial cell and fibroblast migration during wound healing [159] and under hypoxic stress [160]. These ideas support the all hypothesis that Adamts1 is directly involved in the promotion of ovarian lymphangiogenesis. Further exploration, involving the Adamts1 null model and cell culture techniques to inhibit or over-express the actions of Adamts1 in lymphatic vascular endothelial cells may elucidate the specific roles of Adamts1 in this process. Another possibility warranting future investigation is that Adamst1 directly cleaves Vegfc and/or Vegfd, which require N- and C-terminal truncation by proteases for activity [267, 268].

7.3 CLINICAL RELEVANCE

The results in this thesis have provided significant insight into the mechanisms controlling fluid homeostasis and immune cell trafficking within the ovary through FSH and estradiol-mediated ovarian lymphangiogenesis and a non-redundant role for extracellular matrix protease, Adamts1. These
research findings are highly relevant and translatable to both the reproductive and lymphatic disease areas. Polycystic ovarian syndrome (PCOS) and ovarian hyperstimulation syndrome (OHSS) are two common pathologies directly associated with reproductive function; both of which involve perturbations to or dysfunctional ovarian fluid homeostasis. While research in these areas to date has focused on potential dysfunction of the blood vasculature, the lymphatic vasculature (systemically responsible for maintenance of blood volume and avascular fluid homeostasis) has been overlooked as an area of interest. One reason for this is the lack of understanding to date of the aetiology and relationship with the endocrine system of the ovarian lymphatics. The present study has resolved this shortcoming at least for the mouse ovary. What role the lymphatic vasculature will play in either or both of these pathologies must yet be investigated in appropriate model systems and in human patients. Continuing research in this area, focussing on ovarian lymphatic vascular function will likely provide new insight. It is also likely that this work will impact the lymphatic disease research area. Current therapies for lymphatic disease are focused on physical therapy, compression bandages and pharmaceutical manipulation of osmolarity, none of which are curative. This work provides the first evidence of a malleable lymphatic system and a model for regulation of normal adult lymphangiogenesis, and may one day be used to explore ways in which to regenerate damaged vessels to cure lymphatic diseases and disorders.