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

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CHAPTER 1
INTRODUCTION
Reproductive success requires an extensive and highly orchestrated series of events whereby oocytes, or eggs are generated before birth and stored within the ovary until required; a process which can be more than forty years in human and relies on intrinsic factors as well as extrinsic factors such as age, diet, lifestyle and environment. Successful fertility relies on the blood vasculature in order to meet the metabolic demands of such a highly active tissue, as well providing necessary central endocrine support. The necessity and function of the ovarian blood vasculature are well understood, however the presence, regulation and function of its most closely related physiological system, the lymphatic system, is yet to be described within the ovary. In other tissues, the lymphatic network is important for maintaining fluid homeostasis, immune cell trafficking, hormone and lipid transport, and similar functions are expected to be important in the ovary. Elucidating this currently unexplored system within the ovary will improve our understanding of normal ovarian function, and may provide insight into reproductive pathologies.

1.1 OVARIAN DEVELOPMENT

1.1.1 Female gonadogenesis and early ovarian development

The growth and maturation of a developmentally competent oocyte requires firstly the embryonic establishment of a finite number of oocytes. The first distinguishable development of the ovary occurs during the first trimester of human gestation, or around embryonic day (E) 12.0-13.5 in the mouse when the primordial germ cells form loose aggregate structures called ovigerous cords [1-4]. Primordial germ cells cluster in the centre of these cords and form a syncitium or cyst [5] which is surrounded by somatic cells. These somatic cells eventually separate the germ cells into individual oocytes, with each oocyte surrounded by a single, flattened layer of squamous epithelial cells known as granulosa cells, forming the primordial follicle [6]. It is at this very early stage of development (before birth) when germ cells cease dividing by mitosis and enter into their first stage of meiotic division; their progress halted during the first meiotic prophase, at the diplotene stage, with their chromosomes enveloped within the germinal vesicle. The primordial follicle and its oocyte may remain quiescent for an extended period of time until activation and follicle growth begins. The oocyte, however, will remain in meiotic arrest even after follicle activation until ovulation is initiated and the oocyte receives signals to resume meiosis.
1.2 FOLLICULOGENESIS

1.2.1 Pre-antral follicle growth and development

Folliculogenesis initiates when the flattened layer of squamous pre-granulosa cells are activated and undergo transition to cuboidal granulosa cells; known as a primary follicle (Figure 1.1) [7]. It is during this time when the oocyte develops a zona pellucida, a glycoprotein layer around the oocyte which has been shown to be involved in sperm binding and fertilisation, and critical to reproductive success [8-10]. The oocyte grows dramatically in size and the follicle expands to allow this growth, however at this stage, there is only one layer of granulosa cells. From the primordial stage of folliculogenesis, the follicle is surrounded by a basement membrane made up of extracellular matrix (ECM) proteins which excludes vasculature and acts as an acellular filter.

The transition into a secondary (pre-antral) follicle requires heightened proliferation of granulosa cells into a multilayer, and the development of a newly formed layer of cells on the outside of the follicle called the thecal cells. These thecal cells are made up of steroidogenic cells which will later provide the follicle with steroid precursors (predominantly androgens) necessary for its growth and maturation, as well as blood vascular endothelial cells which provide the follicle with vascular support. Extensive growth of the follicle occurs during this stage and there is rapid expansion of the basement membrane.

1.2.2 Antral follicle growth

The development of small, fluid-filled antral spaces, which eventually form an antral cavity, marks the next stage of follicle growth. This phase is one of high cellular proliferation and is under the control of gonadotrophin hormones follicle stimulating hormone (FSH) and luteinising hormone (LH), secreted by the anterior pituitary gland. During antral follicle growth, LH on the LH receptor, found on thecal cells, to stimulate the production of androgens, which are transported to the granulosa cells. FSH is necessary for the production of estrogens by the granulosa cells. The FSH receptor, located on granulosa cells is a G-protein coupled receptor, which when FSH binds, activates more than 100 target genes [11, 12]. During folliculogenesis, an essential function of FSH is the induction of the Cyp19a1 (or Aromatase) gene [13, 14]. The product of this gene, Aromatase is the enzyme responsible for the
Figure 1.1  Simplified schematic diagram of follicle activation, growth, maturation, ovulation and corpus luteum development

Primordial follicles are activated and grow in the absence of gonadotrophic support. Under the control of FSH, follicles develop a fluid-filled antrum, and under the control of LH, a dynamically regulated series of events culminates in the expansion and expulsion of the cumulus oocyte complex from the ovary, in a process known as ovulation. If fertilisation occurs, the corpus luteum (CL) matures and produces the hormones required for maintenance of pregnancy, or alternatively, failing fertilisation, the CL regresses in preparation for the next cyclic wave of folliculogenesis.
conversion of the thecal derived androgens to estrogens within granulosa cells. Estrogen stimulates the proliferation of granulosa cells [15-17], resulting in growth and expansion of the follicle; and facilitates the actions of both FSH and LH [18]. Estrogen receptors (ERs) are nuclear receptors found on granulosa cells which act as ligand-activated transcription factors [18]. The proliferative nature of estrogen increases the number of estrogen producing granulosa cells, which accounts for the continual rise in estradiol during folliculogenesis. In addition to the autocrine ovarian actions of estrogens, estrogen acts in a negative feedback fashion to inhibit the hypothalamus and anterior pituitary [19, 20]. The low but rising level of estradiol acts directly on the hypothalamus to inhibit the secretion of gonadotrophin releasing hormone (GnRH), thus suppressing the GnRH-mediated release of FSH from the anterior pituitary, whilst also acting at the anterior pituitary itself to decrease the sensitivity of the FSH-producing cells to GnRH; all culminating in a decrease in circulating FSH. Additionally, inhibin which is also produced by the granulosa cells of the growing follicle also acts to inhibit the secretion of FSH at the pituitary gland. In contrast to the fall in plasma FSH, LH continues to rise, despite the inhibition of GnRH by estradiol; requiring the addition of progesterone for complete inhibition of LH production [19, 20]. This rise in LH is associated with the continual production of estradiol by the growing follicle, until peak estradiol levels are reached. Conversely, these high estradiol levels now act in a positive feedback fashion at the hypothalamus and anterior pituitary, increasing both FSH and LH secretion, and increasing the sensitivity of the LH producing cells to GnRH, culminating in the LH surge, which promotes ovulation (see Section 1.2.3).

During secondary follicular development, the majority of growing follicles undergo atresia. Atresia is the process by which follicles are lost from the growing pool and involves programmed cell death of the oocyte and follicular cells. More than 99% of activated follicles are lost from the reproductive pool via this process, which is tightly regulated by central gonadotrophic support [7, 21].

1.2.3 Peri-ovulatory follicle growth

The final stage of follicle growth involves a surge of LH from the pituitary which is required to induce terminal changes in the oocyte and follicle which promote ovulation and also to modify the endocrinology of the follicle to a progesterone producing corpus luteum. LH acts on the granulosa cells, which have concurrently induced the expression of LH receptor, to promote a cascade of gene expression changes and events. These include the rapid expansion of the cumulus cells, loss of gap
junctional communication and the production of an extracellular matrix around them and the oocyte (Reviewed in [22, 23]); and all culminating in the expulsion of the oocyte from the ovary at ovulation. During this peri-ovulatory period meiosis is resumed within the oocyte [20]. Following the release of the oocyte, the follicle undergoes transformation into the corpus luteum (CL), a transient endocrine gland that produces the steroid hormone, progesterone, which is required for the establishment and maintenance of early pregnancy. This process involves the differentiation of the granulosa cells into progesterone producing luteal cells (or granulosa-lutein cells) and the migration of the blood vascular endothelial cells into the corpus luteum to provide a rich vascular network responsible for hormone trafficking.

This orchestrated series of follicular events occur cyclically. Follicles are continually recruited and grow as part of the estrus (or menstrual) cycle, requiring a continually changing endocrine environment, whilst promoting the remodelling events necessary to accommodate the rapid expansion required during folliculogenesis and luteal development. Ovarian folliculogenesis requires the development and expansion of an intricate blood vascular network to support both folliculogenesis and luteal function (see Section 1.4). While the role and development of the blood vascular network has been clearly documented, it is currently unclear whether the lymphatic vasculature is also associated with, and necessary for follicle growth and fertility.

1.3 THE BLOOD VASCULATURE

The blood vasculature is part of the circulatory system, functions to transport blood throughout the body and is made up of vessels of varying sizes. Capillaries are vessels formed by endothelial cells and supported by surrounding pericytes, whilst arteries and veins are vessels consisting of a number layers including endothelial cells, pericytes, basement membrane and smooth muscle. The blood vascular circulation is necessary for the transport of oxygen, nutrients and hormones systemically and to remove waste products generated as a result of energy utilisation and metabolism.

1.3.1 Blood vascular development and remodelling

There are three major processes by which blood vessels are formed and remodelled; vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis is the de novo formation or establishment of the
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blood vasculature during embryogenesis and involves the migration, differentiation and proliferation of circulating and tissue-resident endothelial stem cells (angioblasts) and is virtually complete at birth [24-26]. Angiogenesis refers to the process by which established vascular trees are then pruned and extended; the physiological process involving the budding and expansion of new blood vessels (capillaries) from pre-existing vessels [27]. Angiogenesis is a normal physiological process in growth and development, however is most commonly associated pathologically with wound healing and cancer growth and metastasis [28]. The unique cyclical remodelling capacity of the ovary is one of the only known non-pathophysiological events which require angiogenesis throughout adulthood [29].

1.3.2 Control of blood vessel growth by Vascular Endothelial Growth Factors

Vasculogenesis and angiogenesis are predominantly controlled by the vascular endothelial growth factor (Vegf) family of growth factors which includes seven members; Vegfa, Vegfb, Vegfc, Vegfd, Vegfe, svVegf (found in snake venom) and placental growth factor (Pgf), many of which have isoforms and splice variants. Three Vegf receptors exist; Vegfr1, Vegfr2 and Vegfr3, which are found predominantly on vascular endothelial cells. All members of the family have been implicated in some way with the development and remodelling of the blood and lymphatic vasculatures.

Vegfa, commonly known as Vegf, is produced by a wide range of cell types including endothelial cells, macrophages and activated T-cells and is a potent regulator of vascular development. Vegfa exists in a number of cell surface and secreted isoforms, with varying affinities for heparin [30-35]. Modifications to the Vegfa gene in mice have elucidated a number of roles for the angiogenic growth factor, and in fact, even the loss of a single Vegfa allele (Vegf<sup>−/−</sup>) results in embryonic lethality due to severe vascular defects [36, 37] including myocardial angiogenesis and ischemic cardiomyopathy [38] as well as a number of capillary and temporal vascular branching [38] and growth plate defects [39]. Similarly, ablation of the receptors by which Vegfa signals, Vegfr1 and Vegfr2 (Figure 1.2) also results in embryonic lethality and have defects in blood vessel formation [40-42]. It is clear that Vegfa plays extensive roles in a wide range of normal physiological processes and has also been demonstrated to be highly necessary for normal ovarian function (Section 1.4.2). Vegfc and Vegfd, the lymphangiogenic growth factors are closely related and share C and N terminal extensions, which are not found in the
Figure 1.2  Simplified schematic diagram illustrating the receptor-binding specificity of Vegf family members

The activation of downstream signal transduction molecules leads to changes in gene expression which influence several different endothelial cell functions such as migration, vascular permeability, survival and proliferation. Dotted lines indicate binding prior to N and C-terminal truncation. Image modified from [43].
other Vegf family members [44]; and which alter receptor affinity [45]. Vegfc signals predominantly through Vegfr3 (Figure 1.2) which is found almost solely on lymphatic endothelium and has been shown to promote endothelial cell mitogenesis in vitro and in vivo [46-48], whilst Vegfd has been shown to stimulate mitogenesis and migration of cultured endothelial cells [44]. Gene ablation and overexpression studies have demonstrated roles for Vegfc in lymphatic development, with Vegfc null mice lacking a lymphatic vascular network [49]; whilst ablation of the Vegfr3 gene results in defective blood vessel development in the early embryo, implicating the receptor in both angiogenesis and well as lymphangiogenesis [50]. Over-expression of Vegfd has been shown to promote formation of lymphatic vessels within tumours and promotion of metastasis of tumour cells [51]; whilst in contrast, ablation of Vegfd results in only subtle lymphatic phenotype involving a decrease in the abundance of lymphatic vessels in the lungs [52, 53]. Vegfb, Vegfe and svVegf are less well described and will not be discussed further.

1.4 ANGIOGENESIS AND THE OVARY

The ovary undergoes cyclic remodelling throughout each menstrual/estrus cycle. This process requires significant vascular remodelling to supply each new cohort of growing follicles. A rich, and highly fenestrated vascular network supplies the ovary, and growing oocyte with hormones, the nutrients required for metabolism as well as factors that are incorporated into the cumulus oocyte complex prior to ovulation and later to transport the CL derived hormone, progesterone, to the uterus to support endometrial development in preparation for (and during) pregnancy [54-56].

Ovarian blood vascular development first occurs during gonadogenesis. At E 11.5, the indifferent gonad contains a primitive vascular system, in which small branches from the mesonephric vessels extend into the undifferentiated gonads of both sexes [57]. Sex-specific development of the vasculature occurs after the expression of the sex-determining gene, Sry in the XY gonad. Vascularisation of the presumptive testes involves the migration of endothelial cells from the mesonephros into the tissue [58-60]. These cells form a meshwork of smaller branched vessels in the coelomic domain of the XY gonad that resolves into a distinctive coelomic vessel by E 13.5 [57], making it morphologically distinguishable from the ovary. Between E 12.5 and 13.5, vascular branches extending from the coelomic vessel can be
identified along the outside of testis cords and can be identified as the primordial arterial system of the testis by their Notch1, Notch4 and ephrinb2 expression [59]. In contrast, the XX gonad lacks a coelomic vessel, and XX vascular development does not involve migration of cells from the mesonephros. Vascular development of the XX gonad appears to be the result of proliferation and extension of the branches of the primordial gonadal vasculature [57, 59-61]. At E 13.5, dense networks of vessels are found in close proximity to the strings of germ cells known as ovigerous cords or germ line cysts [61]. The blood vascular changes associated with primordial follicle assembly and early post-natal ovarian development however remain unexplored.

1.4.1 Follicular angiogenesis

Follicular angiogenesis occurs concurrently with folliculogenesis and continues throughout follicle growth. Follicles prior to the pre-antral stage (primordial and primary follicles) have no vascular supply of their own and rely on the passive diffusion of oxygen and nutrients from stromal vessels [29, 62]. At the time antrum formation begins, a wreath-like structure of vessels forms around the follicle, consisting of two concentric networks of vessels within the thecal layers, and is closely linked with growth and development of the follicle [54]. The blood vasculature sequesters an increasing supply of gonadotrophins, growth factors, oxygen, lipids and steroid precursors required for folliculogenesis and oocyte maturation, as well as removing waste products. The vessels increase in size and number, but never penetrate the basement membrane, thus keeping the granulosa layers and oocyte completely avascular until just prior to ovulation (Reviewed in [29]). In mono-ovular species, where a single, dominant follicle is selected for peri-ovulatory growth and ovulation, selection may be dependent on the development of a rich vascular network with increased vascular permeability [63]. Research also suggests that follicles which fail to acquire satisfactory vascular support may undergo atresia, and that vascular loss is also associated with the earliest stages of follicular atresia [64].

Vegfa has been identified within the ovary of several species including cow, pig, sheep, rat, mouse and human (Reviewed in [66]), and its critical importance in folliculogenesis, ovulation and luteinisation identified in mouse [67, 68] and primate models [62]. Gene ablation models have not been particularly useful in elucidating angiogenesis and lymphangiogenesis in the ovary due to embryonic lethality arising from vascular defects in early development, however inhibitors of angiogenic signalling have
Figure 1.3 Simplified schematic diagram of the structure association of the blood vasculature with ovarian folliculogenesis

Simplified schematic illustrating the stages of follicular maturation, ovulation and corpora lutea (CL) formation and maturation. Blood vessels (red) appear within follicles at the late secondary stage, prior to antrum formation. They remain in the thecal layer because of the presence of a basement membrane. At ovulation, the basement membrane breaks down and, in association with intense angiogenesis, the blood vessels invade the resulting CL. Image modified from [65].

NOTE: This figure is included on page 11 of the print copy of the thesis held in the University of Adelaide Library.
have revealed important roles for the Vegf family of growth factors and receptors in normal ovarian function [67-74]. Approaches including ligand and receptor immuno-neutralisation, tyrosine kinase inhibitors of Vegf receptor signalling activity, soluble receptor treatment (s-Flt1) and receptor based antagonists (Vegf-traps) have been exploited within reproductive biology to closely examine and elucidate the role of Vegfa [67, 68, 71-76]. In vivo inhibition of Vegfa signalling at the beginning of the 10-day follicular phase (in marmosets) results in the production of normal healthy pre-antral follicles, but an uncharacteristic absence of large antral follicles. Thecal proliferation is decreased in late secondary and tertiary follicles, with morphometric analysis revealing loss of both endothelial and non-endothelial cell proliferation, as well as granulosa cell proliferation in antral follicles. The decrease in follicle growth and maturation suggests that disruption to the vascular density or permeability, both of which are controlled by Vegfa signalling, may prevent the follicle from reaching its growth potential during the follicular phase [71, 72].

1.4.2 Peri-ovulatory and luteal angiogenesis

Just prior to ovulation, the basement membrane separating the avascular granulosa layers of the follicle and the vascular thecal and stromal layers breaks down. The capillaries become fenestrated and rapidly sprout and invade the granulosa cell layer, proliferating extensively [77-79]. Blood vascular remodelling within the ovary is often compared to that of the most rapidly growing tumors and the CL has one of the highest rates of blood flow of any organ [80]. Inhibition of Vegfa or Vegfr2 during the late follicular phase and the peri-ovulatory period in primates has drastic effects on ovarian function. During the late follicular phase, inhibition of Vegfa signalling using a Vegfr2 antibody results in disruption of the endocrine and autocrine changes necessary for follicular maturation and ovulation, with suppression of the follicular phase rise in estradiol and inhibin B, and abnormal pituitary secretion of both LH and FSH [81]. During peri-ovulatory follicle growth, suppression of Vegfa signalling leads to decreased endothelial cell proliferation and thecal vascularisation, and in turn, anovulation and non-functional CLs [70]. Similarly, in rodents, inhibition of Vegfr2 prevents gonadotrophin-dependent follicle growth, ovulation and luteinisation [67, 73].

Modulation of angiogenesis during luteal phase by treatment with Vegfa signalling inhibitors either at, or shortly following ovulation significantly reduced the number of proliferative and endothelial cells within
the CL, and significantly decreased progesterone secretion [76, 82] whilst a more prolonged treatment, well into the luteal phase resulted in complete ablation of microvascular branching [71, 83]. Inhibition of LH signalling using GnRH antagionists mimicked this effect, also supressing early luteal angiogenesis, and implicating the LH surge in normal luteal angiogenesis, consistent with described induction of Vegfa by LH [84, 85]. In vivo inhibition of Vegfa signalling throughout the luteal phase (days 3-10; marmoset) also caused marked decreases in luteal angiogenesis and a fall in plasma progesterone [76, 86]. All of these modulations suggest that Vegfa plays essential, non-redundant roles in the cyclic vascular remodelling required to promote successful reproduction. The lymphangiogenic growth factors, Vegfc and Vegfd and their expression, regulation and functional significance within the ovary has not been explored to date.

1.4.3 The Vegf family in ovarian pathophysiology

In addition to the in vivo characterisation of the function of Vegfa during normal ovarian physiology using animal species, a growing body of evidence suggests that dysregulation of Vegfa is associated with both polycystic ovarian syndrome (PCOS) and ovarian hyper stimulation syndrome (OHSS). PCOS is an endocrine disorder associated with androgen excess and ovulatory dysfunction, and effecting 6-7% of women of reproductive age worldwide [87]. The ovarian pathophysiology 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]. Follicular fluid Vegfa levels have also been associated with follicle maturation, oocyte quality and fertilisation competence in PCOS patients [92], however more research in this area is required to better understand these relationships. Furthermore, excess generation of Vegfa in response to hyperstimulation during assisted reproductive technology (ART) is thought to promote/exacerbate OHSS [93, 94]. Ovarian hyperstimulation syndrome is an exaggerated response to ovulation induction therapy, in particular, exogenous gonadotrophins, occurs in approximately 1% of cases, and results in symptoms of extravascular fluid retention and hypervolaemia and the pathological development of OHSS [95-97].
1.5 THE LYMPHATIC VASCULATURE

The lymphatic vascular system has been recognised anatomically for centuries [98, 99] but only recently have gene ablation models and other molecular strategies led to the discovery of lymphatic specific growth factors, receptors and markers, and the elucidation of the molecular mediators underlying lymphatic development. The lymphatic vascular system plays three major physiological roles. Firstly, it is responsible for mediating tissue-fluid homeostasis by providing a transport network for extra-vascular fluid and proteins, and therefore contributing to fluid reabsorption and tissue perfusion [100]. Secondly, the lymphatic system is involved in absorption of lipids and fat soluble vitamins A, D, E and K within the gastrointestinal tract. Thirdly, it is the system responsible for the trafficking of immune cells, including antigen-presenting cells to lymph nodes from the periphery. More recently, pathophysiological roles including cancer metastasis to lymph nodes and other organs has been associated with the lymphatic vasculature, and has prompted a rejuvenated interest in research into the lymphatic vasculature.

The lymphatic system is part of both the circulatory system and the immune system, and its functions stretch as widely as the gastrointestinal system, and has only been reported to be absent from the brain and retina and other known avascular structures including epidermis, hair, nails, cartilage and the cornea [101]. The lymphatic vascular system is prevalent in the organs in direct contact with the external environment, including the skin, lungs and gastrointestinal tract (GIT) and as such, mediates the first recognition of foreign antigens by the immune system. The lymphatic vasculature is comprised of blind-ended capillaries, without a structural basement membrane, consisting of an overlapping layer of lymphatic vascular endothelial cells, lacking either pericyte or smooth muscle coverage (Figure 1.4). The endothelium is anchored to the underlying extracellular matrix (ECM) by filamentous structures, allowing for large inter-endothelial openings and permitting the transport of large protein moieties. Increased interstitial pressure transfers tension to the filaments and therefore the lymphatic endothelium, resulting in a widening of the vessel lumen and fenestrations, facilitating transport of fluid, macromolecules and cells. The remainder of the lymphatic vascular system is comprised of a one way, open-ended network of larger collecting vessels through which lymph fluid is returned to the venous circulation via the inferior vena cava [103]. These larger collecting vessels are supported by a basement membrane and smooth muscle which helps moderately with lymph propulsion. Without a
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Figure 1.4 Simplified schematic diagram of the structure and function of the lymphatic vessels

The endothelial cells of lymphatic capillaries (green) lack tight junctions. Instead, the neighbouring endothelial cells partly overlap, forming valve-like openings, which allow easy access for fluid, macromolecules, and immune cells into the vessel lumen. Lymphatic capillaries lack or have an incomplete basement membrane (yellow). Elastic fibres known as anchoring filaments connect lymphatic capillary endothelial cells to the surrounding stroma and maintain vessel patency during increased interstitial pressure. The lymph drains from the lymphatic capillaries to pre-collecting and collecting lymphatic vessels, which are finally emptied into veins in the jugular region. The pre-collecting and collecting lymphatic vessels have a basement membrane, are surrounded by vascular smooth muscle cells (vSMCs; red) with intrinsic contractile activity to promote lymph flow, and, like veins, contain valves that prevent backflow of the lymph. The valve regions are devoid of vSMCs. Lymph is filtered through a series of lymph nodes. In contrast, the endothelial cells of blood vessels form tight junctions, have a distinct basement membrane, and are surrounded by pericytes/vSMCs, which form one or multiple layers increasing in thickness with vessel size. Image modified from [102].
direct pump (as found in the circulatory system) the pressure gradient of the lymphatic vascular system relies on one-way valves found within the vessels as well as changes in intrathoracic pressure generated by respiration and musculoskeletal contraction within the limbs in order to stimulate lymphatic flow [104].

1.5.1 Lymphatic vascular development (primary lymphangiogenesis)

Recently, murine gene ablation in mice has allowed insight into the differentiation and subsequent development of the lymphatic vascular system. Prior to lymphatic commitment (embryonic day (E) 8.5), mouse embryonic cardinal vein endothelial cells (and presumptive lymphatic endothelia) all expressing Vascular endothelial growth factor receptor-3 (Vegfr3, Flt4) and Lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1). Prospero-related homeobox 1 (Prox1) is induced (under unknown control) on a pole of the cardinal vein [105-107], which marks the initiation of lymphatic endothelial cell commitment (Figure 1.5). Between E 10.5-11.5 presumptive lymphatic vascular endothelial cells (LECs) bud off the cardinal vein, acquire an altered gene expression profile and adopt the LEC phenotype. Maturation into differentiated lymphatic vessels begins at E 11.5-12.5, with the formation of rudimentary lymph sacs [105]. At E 12.5 Prox1 positive, specified LECs sprout from the sac and spread throughout the embryo, whilst the Prox1 negative venous system switches off Vegfr3 and Lyve1. LECs then migrate peripherally, under the control of Vegfc, and undergo higher levels of differentiation, including the accumulation of a number of LEC-specific markers, to achieve lymphatic specification [98]. At E 14.5, terminal differentiation is virtually complete, although the lymphatic vasculature continues to organise through the first few days of life [108] whereby the two vascular networks become separated with connections maintained in only a few specific locations, serving the function of fluid return. The established primary vessel network is extensively remodelled during late embryonic and early postnatal development. This involves the transformation of a uniformly-sized primary capillary plexus into a hierarchical vascular tree, composed of lymphatic capillaries and collecting vessels. This process involves the formation of new capillaries via sprouting from pre-existing vasculature as well as specification of collecting vessels, which involves recruitment of smooth muscle cells (SMC) and formation of luminal valves to ensure unidirectional pumping of lymph fluid. By E 15, the lymphatic vasculature is present within distal organs, including the heart, diaphragm, lungs, gut and skin.
Figure 1.5 Schematic diagram of mouse lymphatic vascular development

Lymphatic vascular development occurs during fetal development in mice. Upon stimulation by, as yet, unidentified signal(s), a subset of venous endothelial cells becomes committed to the lymphatic endothelial fate (E 9.0-9.5). These differentiating lymphatic endothelial cells (LECs) express Lyve1, Prox1, and Vegfr3. Stimulated by Vegfc, which is secreted by the adjacent tissue, they migrate and proliferate to form primary lymph sacs, from which the lymphatic vessels start sprouting (E 10 onwards). The primary lymphatic vascular plexus becomes separated from the blood vessels. It undergoes remodelling and maturation to create the lymphatic vasculature consisting of a lymphatic capillary network (which lacks pericytes) and collecting lymphatic vessels, which contain valves and are associated with vascular smooth muscle cells. Molecules involved at these later stages of the lymphatic development include Syk, Slp76, Ang2, EphrinB2, Foxc2 and Podoplanin. Image taken from [100].
Expansion continues throughout embryonic development and early postnatal life [109, 110], however the mechanisms controlling the generation and expansion of the lymphatic vasculature within organs during early development remain elusive, and virtually unexplored.

1.5.2 Adult Lymphangiogenesis (or Secondary lymphangiogenesis)

Like the blood vasculature, lymphatic vascular development is primarily completed during fetal development and its remodelling is not known to occur under normal physiological circumstances in adults. As a result, very few models of secondary (or postnatal) lymphatic growth and development currently exist. A number of animal post-surgical models have been described [111-114], however the mechanism by which lymphangiogenesis is achieved and regulated is poorly understood and likely to involve multiple factors such as inflammation, physical changes to interstitial fluid flow, matrix metalloproteinase (MMP) activity (extracellular matrix remodelling) and Vegfc/Vegfr3 signalling [115-119]. Like blood vascular remodelling, the process of secondary lymphangiogenesis has important implications for wound healing [44, 120] and is clearly important for the field of metastatic tumour/cancer biology. Vegfc, Vegfd, hepatocyte growth factor (Hgf) and platelet derived growth factor (Pdgf)-BB have been implicated in the promotion of tumour lymphangiogenesis and lymph node/systemic metastasis (Reviewed in [102]). During secondary lymphangiogenesis, new lymphatic vessels have been reported to sprout from existing vessels [121], although circulating endothelial progenitor cells have been reported in other systems [122, 123].

1.6 THE LYMPHATIC VASCULATURE IN REPRODUCTIVE TISSUES

The lymphatic vasculature plays essential roles maintaining fluid homeostasis, immune cell trafficking and lipid absorption and transport; roles likely to be necessary within the ovary. The lymphatic system within the ovary is yet to be described in detail; however it has been described in the uterus and testes. There are currently no reports in any species describing the ovarian lymphatic vasculature surrounding follicles and the vasculature has been described to a minimal extent in the CL. One report suggests that lymphatic vessels are found in the peripheral zone of the CL in later stages of development in rats [124] whilst another reports the presence of lymphatic capillaries observed among the theca lutein, but
not granulosa lutein cells three days following hCG stimulation in the rabbit [125]. Both of these observations were made prior to the identification of a lymphatic specific marker and fail to describe a more extensive network of vessels within the ovary. Other studies in pig and sheep have examined lymph flow from the ovary and the constituents of this lymph, without actually describing the vascular network within the ovary itself. Lymph flow from the ovine ovary is highest when active corpora lutea are present, and higher (per unit weight) than any other organ measured, whilst lymph flow at estrus was minimal [126]. Hormones including progesterone, estradiol and inhibin have been measured in ovarian lymph, and have been proposed to be transferred back to the ovarian arteries via retrograde transfer to promote feedback regulation within the ovary [126-129]. This suggests that there is a rich network of lymphatic vessels within the ovary, but fails to describe the location of the vasculature in and detail. What is clear is that a detailed description of the lymphatic vasculature is required within the ovary, in particular, the follicular associated lymphatic vasculature to better understand its function and significance, given the importance of fluid homeostasis and blood vascular contributions to ovarian function.

Within the uterus, lymphatic vessels have been identified within the endometrium and myometrium. In the myometrium, lymphatic vessels were found within the extracellular matrix between smooth muscle bundles [130]. There is some contention with regard to the vessels within the endometrium, with some groups reporting small and sparsely distributed lymphatic vessels within the functionalis and larger vessels associated with the spiral arterioles in the basalis region [130, 131]; whilst others failed to locate vessels within the functionalis [132] or entire endometrium [133, 134]. Possible reasons for these conflicting findings may include cycle differences, the sparse nature of the vessels and lack of effective lymphatic specific markers.

As in the uterus, there is some contention regarding the lymphatic vasculature within the testes, however, what is uniformly agreed is that uninterrupted and efficient lymphatic flow from the testicular lymphatics is necessary for function [135]. Lymphatic vessels have been described in the interstitium of the testis in human [136] bull, ram, [137] rabbit [138], guinea pig and chinchilla [139], in an extensive system of sinusoidal lymphatics in close association with Leidig cells and the tunica propria of the
seminiferous tubules, and outside the parenchyma (in dog and human) [140, 141]. Given the presence of the lymphatic vasculature within other reproductive tissues, and considering that the blood vasculature is uniquely remodelled within female reproductive tissues, it is likely that the ovary also has a functional lymphatic vascular network and entirely possible that it is remodelled in association with cyclic ovarian remodelling.

1.7 **ADAMTS1 IS A MATRIX METALLOPROTEINASE NECESSARY FOR OVARIAN LYMPHANGIOGENESIS**

One recent and novel observation identified the lymphatic vasculature within the ovary and demonstrated that normal ovarian lymphatic development is dependent on the protease Adamts1 (Figure 1.6) [142]. Adamts1 (a disintegrin and metalloproteinase with thrombospondin motifs 1) is an extracellular protease, expressed within a wide range of tissues. Within the ovary, Adamts1 is produced by granulosa cells and regulated in part by FSH [143], and then rapidly induced following the LH surge, in a progesterone-receptor dependent manner [143-145]. Within the ovary, Adamts1 cleaves the proteoglycan, Versican during the expansion of the cumulus oocyte complex [145] and mice null for *Adamts1* display severe fertility defects which have been well characterised [142, 146-148]. In other systems, Adamts1 is implicated in a large number of physiological and pathophysiological roles.

Adamts1 is involved in bone remodelling [149], ischemic heart disease and the remodelling of atherosclerotic plaques [150, 151] and is highly up-regulated during myocardial infarction [152]. Adamts1 promotes cell migration and is highly associated with the metastatic capacity of cancer cells [153]. It protease activities have been reported to disrupt cell adhesion and promote cell migration [154]. Most recently, Adamts1 has been described to have a distinct association with the blood and lymphatic vasculature. It has been described to be expressed by blood vessels [155] and to have both pro- and anti-angiogenic properties [156-158]. Interestingly, Adamts1 has also been reported to promote endothelial cell and fibroblast migration during wound healing [159] and under hypoxic stress [160].
Figure 1.6 *Adams1* null mice have disrupted ovarian lymphatic development

Lymphatic vessels staining positive for Lyve1 are absent from immature *Adams1*−/− ovaries. Immunofluorescent staining of Lyve1 (a, b red; c, d green) positive lymphatic endothelial cells detects fully formed vessel networks in PND 10 and d 21 day *Adams1*+/− ovaries (a, c, arrowheads). No Lyve1 positive vessels were detectable in *Adams1*−/− ovaries at either developmental stage (b, d), despite Lyve1 positive vessels being strongly detected in extra-ovarian fat of the same tissue sections (b, d, arrows). Cell nuclei were counterstained with DAPI (blue), images taken at 20x original magnification, (Scale bar = 50µm). *Image taken from [142].*
We demonstrated that mice null for the *Adamts1* gene lack normal lymphatic development in the prepubertal mouse ovary (Figure 1.6) [142]. The ovarian lymphatic vasculature was absent at postnatal day (PND) 10 and 21, by which time counterparts with an intact *Adamts1* gene had a complex lymphatic vascular network. This finding was ovary specific, since Lyve1-positive lymphatics present in the extra-ovarian tissue (fat, uterus, and oviduct) and skin [142]. Interestingly, along with the lymphatic defect, *Adamts1* null mice display ovaries with dysgenic follicle growth and abnormal follicle loss, aberrant cumulus matrix formation and ovulatory disruption, which together culminate in a severely sub-fertile phenotype. Whilst *Adamts1* is a protease with known function and substrates within the ovary, its association with the development of the ovarian lymphatic defect is currently unclear. It is possible that the lymphatic defect has a functional impact on ovarian function, and we speculate from its known function in other systems that it may be involved in maintaining ovarian fluid homeostasis during the cyclic remodeling events, immune cell transport, and/or steroid precursor and hormone transport to and from the ovary. The lack of knowledge surrounding the presence and function of lymphatic vessels within the reproductive tissues and the role for the *Adamts1* protease clearly warrants exploration.
1.8 SUMMARY, HYPOTHESIS AND AIMS

The ovary provides a niche environment where oocytes are generated, stored within follicles and later matured in preparation for use during reproductive life. Follicles are activated and grow, a process known as folliculogenesis, cyclically as part of the menstrual (or estrus) cycle. Folliculogenesis involves extensive remodelling of both the follicle and its surrounding extracellular tissue to allow for the rapid growth and expansion of the follicle.

Angiogenesis, or blood vascular remodelling, occurs concurrently with folliculogenesis, specifically involving the vessels surrounding the growing follicle and is well explored and documented. These vascular changes are vital to ovarian functions including follicle growth, hormone synthesis, ovulation and for the development and function of the corpus luteum.

Systemically, the blood vasculature is known to closely interact with the lymphatic vasculature. The lymphatic vasculature performs a number of important physiological roles including the return of extracellular fluid to the blood circulation to maintain fluid homeostasis, trafficking of immune cells and the absorption of lipids in the gastrointestinal tract. Unlike the blood vasculature, the lymphatic vasculature is yet to be described within the ovary. It has been described within other reproductive organs, including the testes and uterus, and is likely to be necessary for a number of ovarian functions including the control of fluid dynamics. The developmental aetiology is currently unexplored, and it is unknown whether lymphatic vascular remodelling events occur in association with folliculogenesis, as is seen with the blood vasculature.

The general hypothesis underpinning the studies described in this thesis is that a functional lymphatic vascular network exists within the ovary which is hormonally regulated in co-ordination with folliculogenesis through endocrine and paracrine factors.
Based on this hypothesis, the following aims have been developed:

1. To characterise primary lymphatic establishment within the ovary and its regulation.

2. To characterise secondary lymphangiogenic events within the ovary and its association with folliculogenesis and cyclic ovarian remodelling.

3. To identify the mechanism(s) responsible for the regulation of ovarian lymphangiogenesis.

4. To investigate the role of *Adamts1* in ovarian lymphangiogenesis.

Characterisation of this virtually unexplored physiological system within the ovary will begin to yield an understanding of the factors regulating the lymphatic vasculature and its function in normal ovarian physiology. It may also provide translational relevance to a number of disorders affecting the reproductive and lymphatic vascular systems.
2.1 MATERIALS
Equine chorionic gonadotrophin (eCG/Gestyl) was purchased from Professional Compounding Centre of Australia, (Sydney, NSW). Human chorionic gonadotrophin (hCG/Pregnyl) was purchased from Organon, Australia (Sydney, NSW). Secondary antibody (biotin-labelled goat-anti-rabbit) was purchased from Millipore (North Ryde, NSW, Australia).

2.2 METHODS

2.2.1 Animals
C57BL/6 mice were purchased from Laboratory Animal Services (University of Adelaide, Australia) and the Australian Research Council Animal Facility (Perth, Australia). All mice were maintained in 12 h/12 h light dark conditions and given water and rodent chow ad libitum. 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.

2.2.2 Adamts1 null mouse generation and genotyping
The Adamts1 null mutant mouse line on a 129SvJ/C57BL/6 genetic background used in this study was described previously [146]. Heterozygous female (Adamts1+/−) and homozygous null male (Adamts1−/−) breeding pairs were maintained. At days 18-23 of postnatal age, mice were sexed, weaned and tail tips and ear tag biopsies collected from anaesthetised mice. Genotyping of offspring was performed by PCR analysis of tail and/or ear DNA. Genomic DNA was extracted from tail tip and ear biopsies, by incubation at 55°C for two hours (180 rpm shaking) in tail digest buffer (10mM Tris-HCL pH 7.8, 75mM NaCl, 25mM EDTA, 1% w/v SDS) containing 0.16mg/ml Proteinase K. Phenol chloroform (250µL) was added to the digested tail solution and inverted three times, followed by centrifugation at 13000 rpm for 10 minutes. Following transfer of the upper aqueous phase to a clean tube, DNA was precipitated by addition of 500mL of ice cold 100% ethanol. Precipitated DNA was pelleted by centrifugation for 10
minutes and 13000 rpm. The precipitated DNA was resuspended in 100mL TE (10mM Tris-HCl pH 8.0, 0.1mM EDTA pH8.0 ), warmed at 37°C for 30 minutes to dissolve and stored at 4°C. The DNA was then used in two polymerase chain reactions using the primer pairs shown as follows. Reaction 1 contained the primer pair Wild type-F (AGTTACCTCAATGCAGCTCTCA) and Wild type-R (ATCCCGAGAGTGTCACACGTGT) which spanned exons 2 and 3 producing a product of 576 base pairs (bp) from the wild type gene when exon 2 was present, indicating the progeny carried one or more wild type alleles. Reaction 2 contained the primer pairs Knockout-F (TCCTCAAGCCCCACCCCTTGG) and Knockout-R (TCCTGCTGGGGTCACATACAG), which spanned intron 1 and exon 3 producing two possible products of 278bp and 1323bp. A product of 278bp was amplified when exon 2 was deleted, and an additional product of 1323bp was amplified in the presence of exon 2. Presence of the 278bp product in the absence of a product in reaction 1 represented an Adamts1 null genotype, while presence of the reaction I product and both products in reaction 2 represented an Adamts1 heterozygous genotype, with one normal and one mutant allele present. Genotyping PCRs were conducted in a 25μL volume containing 5μL 5X GoTaq Flexi® buffer (Promega Corporation, Annandale, NSW, Australia) 3mM MgCl2, 0.5mM each dNTP, 5ng of each primer, 1.25 units of GoTaq® polymerase and H2O to 25mL. Cycling conditions used were 94°C, 5 minutes (1 cycle); 94°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute (40 cycles), 72°C, 5 minutes (1 cycle). Amplified products were evaluated by agarose gel electrophoresis.

2.2.3 Gel electrophoresis

PCR products were separated by agarose gel electrophoresis in a gel containing 1% (w/v) agarose (Promega Corporation, Annandale, NSW, Australia)/0.5X TBE (44.5mM Tris, 44.5mM Boric acid, 1mM EDTA pH8.0) and 1μg/ml ethidium bromide. Electrophoresis was performed in 0.5X TBE buffer at 80V for approximately 30 minutes. To confirm size of resolved DNA fragments 100bp ladder (Promega Corporation, Annandale, NSW, Australia) was run concurrently. Gels were visualised and photographed using a UV transilluminator and gel documentation system (Kodak DC120).
2.2.4 Tissue preparation

Ovarian and uterine tissues were collected from mice directly into 4% paraformaldehyde fixative in phosphate buffered saline (PBS; 80mM Na₂HPO₄, 20mM NaH₂PO₄, 100mM NaCl) and fixed at 4°C for 24 h. Following fixation, tissues were washed three times in PBS over 24 h before being transferred to 75% ethanol in preparation for processing and paraffin embedding. Tissues were processed and embedded in the Leica TP1020 Tissue Processor (Leica Microsystems, NSW, Australia) by the following dehydration and embedding protocol; 1 h 75% EtOH, 1 h 85% EtOH, 1 h 90% EtOH, 1 h 96% EtOH, 2 x 1 h Absolute EtOH, 2 x 1 h 100% Xylene, 2 x 1.5 h paraffin wax under vacuum conditions. Tissues were then molded into wax blocks and stored for sectioning on a Leica Rotary Microtome (Leica Microsystems, NSW, Australia). All tissue sections were cut in serial 7μm sections and fixed to slides using a 45°C water bath. Tissue sections were then further fixed to slides and dehydrated O/N at 37°C prior to histological processing.

2.2.5 Immunohistochemistry

Seven micron tissue sections were dewaxed in xylene and rehydrated through graduated dilutions of ethanol and antigen retrieval was performed by incubating slides (20 minutes at 95°C) in citrate buffer solution (10 mM sodium citrate, pH 6.0) and/or incubation with 1 μg/mL Proteinase K (Sigma, Perth, Australia) for 10 min at RT, following washing with phosphate-buffered saline containing 0.025% Tween-20 (PBST, pH 7.4). Slides were then blocked with 10% serum (species of host of secondary antibody) in PBST for 1 h and then incubated with primary antibodies [1:500] O/N in a humid chamber, then washed well with PBST and incubated with biotinylated secondary antibodies (Biotinylated goat-anti-rabbit [1:500], biotinylated rabbit-anti-rat [1:500], Millipore Corporation; Billerica, MA) for 1 h. Sections were treated with Strepdavidin-conjugated horseradish peroxidase (HRP) (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and detection was performed using diaminobenzadine (Vector Laboratories) according to manufacturer's instructions. Sections were counterstained with haematoxylin prior to mounting. Negative controls were incubated with species and irrelevant isotype matched primary antibodies.
2.2.6 Morphometric ovary and lymphatic vessel analysis

For morphometric ovarian and lymphatic analysis, serial sections were cut through the entirety of each ovary for every experiment. Every 5th section, the equivalent of 35μm increment depths, through the entirety of the tissues were mounted independently and analysed (between 15 and 50 sections per ovary depending on size and treatment as previously described [142]). As a result, analysis was representative of the entire ovary, and any bias was minimised and equal between groups. Images of all ovary sections were captured at high resolution using NanoZoomer Digital Pathology technology (Hamamatsu Photonics K.K., Japan). Sections were identified and multipoint focusing was used. Following scanning, images were then captured using NDP View (Digital Slide Viewer; Hamamatsu Photonics K.K., Japan) to allow for transfer into image analysis software. Analysis™ software (Olympus, NSW Australia) was used for all ovarian and lymphatic vessel morphometric analysis. Images were calibrated followed by measurement of cross-sectional area, and classification of vessel location for every vessel in every section. Whole ovarian area and growing follicle area were also measured. For analysis, ovarian and growing follicle area was calculated by adding the total areas from all sections analysed for each ovary independently, whilst stromal area was calculated by subtracting the total follicle area from the total ovarian area. Similarly, total lymphatic vessel number was derived from the addition of all lymphatic vessels counted in all serial sections for each ovary, and likewise, mean vessel size and total lymphatic vessel area. All ovaries from each of the experiments were analysed independently.

2.2.7 Real Time RT-PCR

Total RNA was isolated using Trizol according to manufacturer’s instructions (Invitrogen Australia Pty. Ltd), with the following modifications; 10μg of Blue Glycogen (Ambion Inc., Austin TX, USA) was included in the precipitation step to allow for detection of the RNA pellet. Total RNA was then treated with 1IU DNAse (Ambion Inc.) at 37°C for 1 h as per manufacturer’s instructions. First strand complementary DNA (cDNA) was synthesised from 500-5000ng (experiment specific) of total RNA using random hexamer primers and Superscript III reverse transcriptase (Geneworks, Hindmarsh SA, Australia; Invitrogen, Australia Pty. Ltd.). Gene primers for Real Time RT-PCR (Table 2.1) were designed against published mRNA sequences from the NCBI Pubmed Database using Primer Express software (PE Applied Biosystems, Foster City, CA) and synthesised by Sigma Genosys (Sigma-Aldrich...
Real time RT-PCR was performed in triplicate for each sample on a Rotor-Gene™ 6000 (Corbett Life Science, Sydney Australia). In each reaction, cDNA from 10ng total RNA, 0.2μL forward and reverse primers and 10μL SYBR ® Green Master Mix (Applied Biosystems CA, USA), and water was added to a final volume of 20μL. All primers were used at an optimised concentration of 25μM with the exception of the Vegfd primers, which were optimal at a concentration of 50μM. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec followed by 60°C for 60 sec. Single product amplification was confirmed by analysis of disassociation curves and ethidium bromide stained agarose gel electrophoresis. Controls included the absence of cDNA template or the reverse transcriptase enzyme, in otherwise complete reactions; and each showed no evidence of product amplification or genomic DNA contamination. All gene expression was normalised to an RpL19 (L19) internal loading control that was amplified in parallel for each sample. Results were then expressed relative to the calibrator sample using the 2⁻(ΔΔCT) method [161], and then normalised to the level of expression detected in samples from untreated (0 h eCG) mice.

Real-time RT PCR was chosen as the method of choice for analysing gene expression in preference to analysing protein abundance to reduce the numbers of mice required for the experiments and to maximise the number of different factors that could be explored. Majority of the growth factors and cell markers analysed are regulated at the mRNA level which makes RNA analysis an appropriate method for detection.

2.2.8 Cloning of Lyve1 Real Time RT-PCR product

Lyve1 PCR product was cloned to confirm sequence specification of the novel and unexpected granulosa cell expression. Products were generated by standard PCR in a 50μL volume containing 10μL 5X GoTaq Flexi® buffer (Promega Corporation, Annandale, NSW, Australia) 3mM MgCl₂, 0.5mM each dNTP, 10ng of each gene specific primer, 1.25 units of GoTaq® polymerase and H₂O to 50mL. Cycling conditions used were 94°C, 5 minutes (1 cycle); 94°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute (40 cycles), 72°C, 7 minutes (1 cycle). Single products were confirmed and visualised by agarose gel electrophoresis. Products were cloned using the TOPO TA Cloning ® Kit with pCR®2.1-TOPO® and One Shot® chemically competent cells. Products were cloned into pCR® 2.1-TOPO® as per manufacturer’s instructions. Briefly, the cloning reaction contained 1μL PCR product, 1μL Salt Solution, 1μL TOPO® vector and water to 6μL. Reaction was gently mixed and incubated at room temperature.
### Table 2.1 Real Time RT PCR Primers

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<th>NM (Pubmed database)</th>
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for 30 minutes, and transferred to ice. Plasmids were then transformed into One Shot® TOP10 chemically competent cells. Briefly, 2μL of cloning reaction was added to one vial of One Shot® TOP10 chemically competent cells, with pUC19 control DNA. The tubes were further incubated on ice for 30 minutes then heat-shocked in a 42°C water bath for 45 seconds followed by incubation for two minutes on ice. The cells were resuspended in 250μL of room temperature SOC media (20% tryptone, 5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgSO4, 10mM MgCl2, 20mM glucose), transferred to 15mL sterile tubes and incubated at 37°C with aeration (230rpm) for one hour. The transformed E. coli were plated on LB-Agar plates (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 15g/L agar) containing ampicillin (100μg/mL, Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) and incubated at 37°C for 16 hours. Transformants were analysed by PCR. Briefly, 20 colonies per plate were picked and added to a 25μL reaction (5μL 5X GoTaq Flexi® buffer; Promega Corporation, Annandale, NSW, Australia) 3mM MgCl2, 0.5mM each dNTP, 5ng of M13 primer (M13 Forward 5’-GTAAAACGACGGCCAG-3’; M13 Reverse 5’-CAGGAAACAGCTATGAC-3’), 1.25 units of GoTaq® polymerase and H2O to 25μL. Cycling conditions used were 94°C, 5 minutes (1 cycle); 94°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute (40 cycles), 72°C, 5 minutes (1 cycle) and products of appropriate size were confirmed using gel electrophoresis. Plasmids were further amplified by Luria broth (LB) inoculation and culture. 2mL of inoculated LB (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) containing 100μg/ml ampicillin (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) was incubated at 37°C with aeration for eight hours (230rpm). From these cultures, plasmid constructs were purified using the Wizard® Plus SV Miniprep kit (Promega Corporation, Annandale, NSW, Australia). Luria broth (1.5mL) was removed, bacteria were pelleted by centrifugation at 13 000rpm for 1 minute and the supernatant removed. The pellet was resuspended in 250μL of Cell Resuspension Solution followed by the addition of 250μL of Cell Lysis Solution and mixed by inversion. Alkaline Protease Solution (10μL) was added, mixed by inversion, incubated at room temperature for five minutes, followed by the addition of 350μL of Neutralization Solution and mixed by inversion. The bacterial lysate was centrifuged at 13 000rpm for 10 minutes. The supernatant was then added to the Spin Column, centrifuged at 13 000rpm and the flow through removed from the collection tube. The column was washed with 750μL of Column Wash Solution, centrifuged again at 13,000rpm for 1 minute, followed by a second 250μL wash and centrifugation for 2minutes. The spin column was transferred to a sterile 1.5mL microfuge tube and the plasmid DNA eluted using 30μL of sterile H2O.
2.2.9 Sequencing

Sequencing of plasmid inserts was performed using BigDye Terminator 3.1 kit (Applied Biosystems, Scoresby, VIC, Australia). Sequencing reactions consisted of 190 ng of plasmid DNA, 2.4 pmol of designated primer, 0.4 μL Terminator Ready Reaction Mix 3.1, 2 μL of 5X Big Dye Sequencing Buffer and H₂O to 20 μL. Cycling conditions used for sequencing were as follows, 96°C, 1 minute (1 cycle); 96°C, 10 seconds; 50°C, 5 seconds; 60°C 4 minutes (25 cycles). Sequencing products were precipitated by addition of 80 μL of 75% isopropanol (v/v), brief vortexing and precipitation at room temperature for 15 minutes, followed by centrifugation at 13,000 rpm for 20 minutes and removal of the supernatant. The pellets were washed with 250 μL of 75% isopropanol (v/v) and re-centrifuged at 3,000 rpm for 5 minutes. The supernatant was aspirated and residual isopropanol evaporated by incubating the tubes, with the lids open, in a 90°C heat block for 1 minute. Samples were analysed at the Molecular Pathology Sequencing Facility (Institute of Medical and Veterinary Science, IMVS, Adelaide Australia) by an ABI 3730 Capillary sequencer (Applied Biosystems, Scoresby, VIC, Australia). Resultant chromatograms were aligned and compared with published sequences for homology using NCBI BLAST software.