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

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...' ”

Issac Asimov (1920-1992)

“The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.”

Thomas H. Huxley (1825-1895)

“You can never have too many shoes.”

Andy Warhol (1928-1987)

Abstract

The ovary provides a niche environment where female germ cells (or oocytes) are generated, stored within follicles and later matured in preparation for use during reproductive life. Following an extensive period of quiescence, which in human, may be up to forty years; the follicle surrounding the oocyte begins to grow, promoting maturation of the oocyte, and culminating 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. Cyclic follicle activation and growth is also associated with concurrent remodelling of the blood vasculature within the ovary, specifically the vessels surrounding the growing follicle. These blood vascular remodelling changes are well explored and have been demonstrated to be necessary for follicle growth, hormone synthesis, ovulation and for the development and function of the corpus luteum.

Physiologically, the lymphatic vasculature is known to closely interact with the blood vasculature and plays a number of important physiological roles including the return of extra-vascular fluid to the blood circulation, and in turn, maintenance of systemic fluid homeostasis. Additionally the lymphatic network is required for the trafficking of immune cells from the periphery to lymph nodes; during the initiation of an immune response and for the absorption of lipids and lipid soluble vitamins in the gastrointestinal tract. The lymphatic vasculature develops and functions concomitantly with the blood vasculature; however unlike the blood vasculature, the aetiology of lymphatic vasculature within the ovary is unknown. It is unclear whether lymphatic remodelling events occur in association with folliculogenesis and are necessary for fertility, as is seen with the blood vasculature.

To elucidate the mechanisms involved in the establishment and remodelling of the lymphatic vasculature within the ovary, I undertook expansive characterisation of its development and hormonal regulation. I exploited both hormonal manipulation and a known model of disrupted ovarian lymphatic development, the *Adamts1* null mouse line, to examine the mechanisms controlling ovarian lymphangiogenesis. Quantitative morphometric analysis of vessel size and number in postnatal mouse ovary revealed that the ovarian lymphatic vasculature develops postnatally between day 8.5 and 12.5, and in synchrony with induction of ovarian *CYP19a1* (Aromatase); the time when secondary follicles become FSH-responsive and estrogenic. The establishment of the lymphatic vasculature was also

associated with the induction of pro-lymphangiogenic growth factors *Vegfc* and *Vegfd*, and their receptor, *Vegfr3*.

Formation of ovarian lymphatics required the matrix-remodelling protease Adamts1, since ovaries from *Adamts1*$^{-/-}$ mice failed to undergo normal lymphatic vascular development. 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.

The concurrent establishment of the lymphatic vasculature with the first postnatal induction of ovarian Aromatase, and the hormone-regulated lymphangiogenesis suggests that a hormonal communication, likely estrogen, may synchronise lymphangiogenesis with folliculogenesis. Like FSH, exogenous estradiol was able to promote the expansion of a new lymphatic vascular network by increasing vessel number and size in *Adamts1*$^{-/-}$ ovaries. Additionally, FSH-analog eCG was able to enhance ovarian lymphangiogenesis during the window of lymphatic establishment (postnatal development of *Adamts1* null), whilst inhibition of the production of estradiol using the Aromatase inhibitor Letrozole, during this same window failed to have any effect.

This study is the first to investigate the relationship between ovarian lymphatic development and remodelling and folliculogenesis. The present studies reveal that the ovary undergoes lymphatic vascular remodelling, described elsewhere as adult or secondary lymphangiogenesis and that this process involves hormonal contributions from FSH and estradiol, as well as the extracellular matrix protease, Adamts1. 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. These results also significantly advanced the understanding of the tightly regulated processes controlling fluid dynamics within the ovary.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Hannah Mary Brown

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Abbreviations

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Adamts1$^{-/-}$	*Adamts1* null
Adamts1$^{+/-}$	*Adamts1* heterozygote
Amh	Anti-mullerian hormone
ANOVA	analysis of variance
ART	assisted reproductive technology
bp	base pairs
Ccnd2	Cyclin D2
cDNA	complementary DNA
CL	corpus luteum
COC	cumulus oocyte complex
COX	cyclooxygenase
CT	threshold cycle
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1
Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1
d	day
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
E	Embryonic day
E. coli	*Escherichia coli*
E_2	estradiol
$E_2\beta$	17-β-estradiol
eCG	equine chorionic gonadotrophin
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid $[CH_2N(CH_2CO_2H)_2]_2$
ELISA	Enzyme-linked immunosorbent assay
ER(s)	Estrogen receptor(s)
ER-X	Estrogen receptor X
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EtOH	ethanol

Flt1	fms-related tyrosine kinase 1
Flt4	fms-related tyrosine kinase 4
FSH	Follicle Stimulating Hormone
GIT	gastrointestinal tract
GnRH	Gonadotrophin releasing hormone
GPER	G-protein coupled estrogen receptor (aka. GPR30)
GVBD	germinal vesicle break down
h	Hour
H_2O	Water
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
HDL	high density lipoprotein
HGF	hepatocyte growth factor
Hsd3β	3β-hydroxysteroid dehydrogenase-isomerase
hVegf	human vascular endothelial growth factor
HZ	Heterozygous
i.p.	Intraperitoneal
IU	international units
Kdr	kinase insert domain receptor
KO	knock out
LB	luria broth
LDL	low density lipoprotein
LEC(s)	lymphatic endothelial cell(s)
LH	Luteinising Hormone
Lrh1	Liver receptor homolog 1
Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1
mg	Milligram
mg	Microgram
$MgCl_2$	magnesium chloride
MII	metaphase II
min	Minute
mL	Millilitre
mM	Millimolar

μm	micron
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
N	number of experimental animals or replicates
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
NaH_2PO_4	sodium dihydrogen phosphate
NCBI	National Center for Biotechnology Information
Ng	nanogram
NO	nitric oxide
O/N	overnight
^oC	degrees Celsius
OHSS	ovarian hyperstimulation syndrome
P	probability
PBS	phosphate buffered saline
PBST (0.025%)	phosphate buffered saline + Tween-20 (0.025%)
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
Pdgf-BB	platelet derived growth factor-BB
Pmol	picomol
PND	postnatal day
Prox1	Prospero homeobox 1
RNA	Ribonucleic acid
RpL19	Ribosomal protein L19
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
s.c.	subcutaneous
sec	second
SEM	standard error of the mean
sFkt1	soluble fms-related tyrosine kinase 1
siRNA	short interfering RNA
SMC	smooth muscle cell
SOC	Super Optimal broth with Catabolite repression

Sry	Sex-determining region Y
Star	steroid acute regulatory protein
svVegf	Snake venom vascular endothelial growth factor
TBE	tris/borate/EDTA
TE	tris/EDTA
Tris	tris(hydroxymethyl)aminomethane $(HOCH_2)_3CNH_2$
Tris-HCl	tris buffered with hydrochloric acid
UV	Ultraviolet
V	Volts
v/v	volume/volume percentage solution
Vegf	Vascular endothelial growth factor
Vegfa	Vascular endothelial growth factor a
Vegfb	Vascular endothelial growth factor b
Vegfc	Vascular endothelial growth factor c
Vegfd	Vascular endothelial growth factor d
Vegfe	Vascular endothelial growth factor e
Vegfr1	Vascular endothelial growth factor receptor 1
Vegfr2	Vascular endothelial growth factor receptor 2
Vegfr3	Vascular endothelial growth factor receptor 3
vSMC(s)	vascular smooth muscle cell(s)
VWF	Von Willebrand Factor
w/v	weight/volume percentage solution
ZP1	Zona pellucida protein 1
ZP2	Zona pellucida protein 2
ZP3	Zona pellucida protein 3