Investigating Lymphatic Vascular Remodelling During Postnatal Mouse Mammary Gland Morphogenesis

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BLabMed (Hons)

Thesis submitted for the degree of

Doctor of Philosophy

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SA Pathology

July 2011

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ABSTRACT

The lymphatic vasculature, an essential component of the cardiovascular system, serves several functions critical to embryonic development and adult homeostasis. Lymphatic vessels return interstitial protein-rich fluid to the bloodstream, transport immune cells during immune surveillance and infection and absorb lipids from the digestive tract (Alitalo et al., 2005; Tammela and Alitalo, 2010). The aberrant growth and development of lymphatic vessels (lymphangiogenesis) is a common feature of human disorders including lymphoedema, inflammatory diseases and tumour metastasis (Alitalo et al., 2005; Tammela and Alitalo, 2010). Lymphatic vessels are of key importance to breast cancer patients. Lymphatic vessels are exploited as a key route of metastasis for tumour cells and the ability of a tumour to promote lymphangiogenesis has been linked with metastasis and poor patient prognosis (Gu et al., 2008; Nakamura et al., 2005; Nakamura et al., 2003; Ran et al., 2010; Skobe et al., 2001). Moreover, lymphatic vascular damage incurred during the surgical resection of lymph nodes commonly results in secondary lymphoedema, a debilitating complication for up to 40% of breast cancer patients (Armer et al., 2009). Despite the involvement of lymphatic vessels in breast cancer, the genes and molecular mechanisms that regulate lymphangiogenesis in the breast remain relatively uncharacterised.

The mammary epithelium and blood vasculature undergo dynamic remodelling events in response to hormonal signals and functional demands during postnatal mouse mammary gland morphogenesis (Djonov et al., 2001; Matsumoto et al., 1992; Richert et al., 2000; Watson and Khaled, 2008). The aims of this project were:

1. To investigate the spatial organisation of lymphatic vessels in the mouse mammary gland.
2. To investigate whether lymphatic vessels, like blood vessels and the mammary epithelial tree, are temporally remodelled during mouse mammary gland morphogenesis.
3. To define signals that regulate lymphangiogenesis during postnatal mouse mammary gland morphogenesis.

This study provides the first evidence demonstrating that the lymphatic vasculature is dynamically remodelled along with the mammary epithelial tree and blood vasculature during postnatal mouse mammary gland morphogenesis. In addition, this study reveals an intimate association of lymphatic vessels with epithelial ducts, a finding that has important implications for tumour metastasis, as well as the spatial organisation of lymphatic vessels in other branched epithelial tissues, including the lung, kidney, pancreas and prostate. Furthermore, we established that vascular endothelial growth factor

(Vegf) C (*Vegfc*) and *Vegfd* mRNA levels are significantly increased early during pregnancy and that proteolytically-processed, active VEGF-D is expressed selectively in pregnant, but not virgin mouse mammary glands, corresponding with the stage of peak lymphatic vessel density. In accordance with these data, we demonstrated that a tyrosine kinase inhibitor specific for VEGF receptor 3 (Kirkin et al., 2001; Kirkin et al., 2004), the principal receptor for mouse VEGF-C and VEGF-D, can block the proliferation of primary dermal lymphatic endothelial cells that is stimulated by mammary epithelial and stromal cell conditioned media *ex vivo*. These data suggest that VEGF-C and VEGF-D, two of the best characterised lymphangiogenic stimuli to date, are likely to play key roles in the stimulation of lymphangiogenesis in the pregnant mouse mammary gland. Elucidation of the molecular mechanisms controlling lymphangiogenesis in the mammary gland has the potential to reveal important targets for the future generation of pro- and anti-lymphangiogenic therapeutics, with the ultimate goal to repair surgically damaged lymphatic vessels and prevent breast cancer metastasis, respectively.

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 to Kelly Louise Betterman 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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Kelly Louise Betterman

ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank my supervisor, mentor and friend, Dr Natasha Harvey, for her endless guidance, encouragement, support, patience and most of all, positivity over the past four years. There have been many times when I felt beaten, but you made sure that I never lost sight of the big picture! It has been a great honour and privilege to undertake my PhD in your laboratory. Tash, you are an inspiration to any woman embarking on a career in research. I can only hope that one day I will be half as good a scientist as you!

Thank you to past and present members of the Lymphatic Development Laboratory for filling my days with gossip, laughter, food and coffee (the latter two obviously not consumed in the PC2 lab of course) and for developing my taste for wine! I am especially grateful to my two 'lab mums', Jan and Gen, for keeping me grounded, their friendship and their ever-willingness to provide assistance when needed. Jan, you are a walking scientific encyclopaedia! How can I ever repay you both?! Emma, although you are now on the other side of the world, rest assured that your pipettes are in very safe hands!

Thank you also to my co-supervisor, Dr Lisa Butler, for her valuable technical advice and constructive feedback in lab meetings, to the staff and students of the Division of Haematology and Centre for Cancer Biology for help when required and for providing a fun environment to work in, and to the National Breast Cancer Foundation for funding me with a prestigious Doctoral Research Scholarship, without which this study would not have been possible.

Last, but by all means not least, I could not have completed this PhD without the endless support of my friends and family, to whom I am most grateful!

To my APP girlies, Darshika and Harshani, thank you for listening to my constant whinging and whining, but most of all thank you for your friendship over the past few years. I hope we can remain friends for life. This *Chief* will miss you dearly! To Diana, Chantelle, Rebecca, Tori, Felix and of course the 'Big GM' Robert, for all the laughter and lab-related shenanigans making those long nights at the lab all-the-more bearable!

To Laura and Chun Chun, *Saltcake* and *Lil' soldier*, my fellow PhD buddies who have battled alongside me from the beginning. Thank you for the laughter, your understanding, countless coffee breaks and for

the constant reminders that I am not alone...we can all have Permanent Head Damage together! Now, it's time for the *Commander* to make sure her battalion isn't left behind in combat. Girls, you can do it!!! I am eagerly awaiting sharing a drink…or two…or three…or more…with you both at the end of this 'war'. Pick up those weapons and fight…or should I say write?!

To Nas, my running buddy, how could I have made it through 'thesisanity' without all those gym sessions, 'Mt Everest' challenges, pancakes and late night motivational messages…'toughen up princess'…actually I probably would have made it through a whole lot less sore!

Most importantly, to my family, Mum, Dad and Nanna, thank you from the bottom of my heart for believing in me!

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ABBREVIATIONS

αSMA	alpha smooth muscle actin
β-gal	beta-galactosidase
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
ADAM10	a disintegrin and metalloproteinase 10
AGE	agarose gel electrophoresis
Angpt	angiopoietin
ANOVA	analysis of variance
AnxA1	annexin A1
AP	alkaline phosphatase
ApoER2	apolipoprotein E receptor 2
APS	ammonium persulphate
BABB	benzyl alcohol:benzyl benzoate
BC	Before Christ
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bcl2	B-cell lymphoma 2
BDNF	brain-derived neurotrophic factor
BEC	blood endothelial cells
Bfk	B-cell lymphoma 2 family kin
BH	Bcl2 homology
bp	base pair
BSA	bovine serum albumin
BVI	blood vascular invasion
ccbe1	collagen and calcium binding EGF domains 1
CCL21	chemokine (C-C motif) ligand 21
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLEC-2	C-type lectin-like receptor 2

cm	centimetre
CM	conditioned media
cm^2	square centimetre
CO_2	carbon dioxide gas
COUP-TFII	chicken ovalbumin upstream promoter transcription factor 2
CXCL15	chemokine (CXC motif) ligand 15
DAB	3,3'-diaminobenzidine
Dab1	disabled homolog 1
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethlypyrocarbonate
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle's Medium
DMEM-10%FBS	Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal bovine serum
DMEM-20%FBS	Dulbecco's Modified Eagle's Medium supplemented with 20% (v/v) foetal bovine serum
DMEM-5%FBS	Dulbecco's Modified Eagle's Medium supplemented with 5% (v/v) foetal bovine serum
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	1,4-dithiothreitol
E	embryonic day
EBM®-2	Endothelial Cell Basal Medium®-2
EC	endothelial cells
ECF	enhanced chemifluorescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELR^+	ELR motif; glutamic acid-leucine-arginine
EphB4	Eph receptor B4
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta

FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FGF	fibroblast growth factor
FGF-1	fibroblast growth factor 1
FGF-2	fibroblast growth factor 2
FGFR-1	fibroblast growth factor receptor 1
Fiaf	fasting-induced adipose factor
FITC	fluorescein isothiocyanate
Flt4	FMS-like tyrosine kinase 4
FOXC2	forkhead box C2
G	gauge
g	gram
GFP	green fluorescent protein
Gli	glioma-associated
Gli1	glioma-associated 1
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HGF	hepatocyte growth factor
HGFR	hepatocyte growth factor receptor
Hh	Hedgehog
HHF	Hank's balanced salt solution containing 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid and 5% (v/v) foetal bovine serum
IDC	invasive ductal carcinoma
IDL	invasive lobular carcinoma
IGF-1	insulin-like growth factor 1
IGF-2	insulin-like growth factor 2
IPTG	isopropyl-β-D-thiogalactosidase
JLS	jugular lymph sac
K14	cytokeratin 14
K18	cytokeratin 18
KCl	potassium chloride
kDa	kilodalton
KOH	potassium hydroxide

L	litre
LB	Luria Bertani
LEC	lymphatic endothelial cells
LVI	lymphatic vascular invasion
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
M	Molar
mA	milliamp
MACS	magnetic-activated cell sorting
MAZ51	3-(4-dimethylamino-naphthalen-1-ylmethylene)-1,3-dihydro-indol-2-one
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
MMTV	mouse mammary tumour virus
MQ-H_2O	Milli-Q water
mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
N	Normality
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium chloride
NFATc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
ng	nanogram
nm	nanometre
Nrp2	neuropilin 2
O.C.T	optimal cutting temperature
ºC	degree Celsius
pBS	pBluescript II SK (+)
PBS	phosphate-buffered saline

PBS-0.1%TW20	phosphate-buffered saline with 0.1% (v/v) Tween®20
PBS-0.1%TX100	phosphate-buffered saline with 0.1% (v/v) Triton®X-100
PBS-0.3%TW20	phosphate-buffered saline with 0.3% (v/v) Tween®20
PBS-0.3%TX100	phosphate-buffered saline with 0.3% (v/v) Triton®X-100
PBS-0.3%TX100-1%BSA	phosphate-buffered saline with 0.3% (v/v) Triton®X-100 and 1% (w/v) bovine serum albumin
PBS-2%FBS	phosphate buffered saline with 2% (v/v) foetal bovine serum
PC	proprotein convertase
PCA	principal component analysis
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFRα	platelet-derived growth factor receptor alpha
PDGFRβ	platelet-derived growth factor receptor beta
PE	phycoerythrin
PFA	paraformaldehyde
pg	picogram
pmol	picomole
PR	progesterone receptor
Prox1	Prospero-related homeobox 1
Ptch1	Patched 1
PVDF	polyvinylidene fluoride
PyMT	polyomavirus middle T antigen
RIN	RNA Integrity Number
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Shh	sonic hedgehog
SLC	secondary lymphoid organ chemokine
SLP-76	SH2 domain containing leukocyte protein of 76kDa
Smo	smoothened

Sox18	SRY-box containing gene 18
SSC	saline-sodium citrate
ST-cDNA	sense transcript-complementary deoxyribonucleic acid
SU5402	3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl)methylene]-2-indolinone
SU5416	3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin-2-one
sVEGFR-2	soluble vascular endothelial growth factor receptor 2
Syk	spleen tyrosine kinase
T25	25cm^2 tissue culture flask
TBE	Tris-borate-ethylenediaminetetraacetic acid
TBS	Tris-buffered saline
TBS-0.1%TW20	Tris-buffered saline with 0.1% (v/v) Tween®20
TDLU	terminal ductal lobular unit
TE	Tris-ethylenediaminetetraacetic acid
TEB	terminal end bud
TEMED	N,N,N',N'-tetramethylethylenediamine
TFB	transformation buffer
TIMP	tissue inhibitor of metalloproteinase
Tnc	tenascin C
TrkB	tropomyosin-related kinase B
tRNA	transfer ribonucleic acid
TSP1	thrombospondin 1
TSP2	thrombospondin 2
U	unit
UV	ultraviolet
V	volt
v/v	volume per volume
VE-cadherin	vascular endothelial-cadherin
VEGF	vascular endothelial growth factor
VEGF-A	vascular endothelial growth factor A
VEGF-C	vascular endothelial growth factor C
VEGF-D	vascular endothelial growth factor D
VEGFR-1	vascular endothelial growth factor receptor 1
VEGFR-2	vascular endothelial growth factor receptor 2
VEGFR-3	vascular endothelial growth factor receptor 3

VHD	VEGF homology domain
VLDLR	very low density lipoprotein receptor
w/v	weight per volume
WECHE	weird chemokine
WT	wild-type
x g	relative centrifugal force
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside