FUNCTIONAL CHARACTERISTICS AND MOLECULAR REGULATION OF LYMPHANGIOGENESIS DURING GECKO TAIL REGENERATION: EVIDENCE FOR THE ROLES OF VEGF-C, VEGF-D AND THE RECEPTOR VEGFR-3

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Amendments to Thesis

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Thesis title:

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Reviewer One

Reviewer one stated he/she was happy with the thesis as is and no changes were requested. However, suggestions were provided for future publications and future research and these have been gratefully received and noted. A reference was added as requested on Page 150, line 16 to provide clarification.

Reviewer Two

Some page changes have occurred as a result of amendments to the thesis. The paragraph and line changes requested by the reviewer are given first (P= paragraph, L= line) and then where applicable the new paragraph and line numbers are provided in brackets.

Abstract – Reworded section to strengthen the description of the justification for the study.

Chapter 1

P.1-2. (P.2.L.1, 3 and 7) References added as requested

P.7, L.11. Typo corrected

P.9, L.2. 'Aquatic' deleted as requested

P. 9-10. L.25.Deleted this statement, as difficult to clarify (for simplicity)

Fig. 1.3. 17 is in the figure but is obscured by dark shading of the pelvic area in this region. I have deleted the reference to 17 in the figure legend given that it is not readily obvious.

P. 10, l.6. Changed sentence to remove ambiguity

Fig. 1.7. Have deleted this figure due to poor quality and inability to get a better quality image

P.42, L.20. Changed to Gekkonidae

P.43, L.5. *Lacerta* Italicized as requested

Fig. 1.9. Changed to lower case r in Representation as requested

Chapter 4

P. 96 (and others). A global search was performed highlighting the word "insight" and sentences were recast removing this word where appropriate. In particular, as a result of making these changes the last paragraph of the introduction (P.97. L.5, 6 and 7) was changed.

P. 96. (P.97, L.11-14) Added in a sentence stating that RNA was the same as that extracted in Chapter 3 (and therefore animals were the same and tails collected in the same way)

P107, L.7-8. (P108. L.8) Wording changed to 'using these primers', as suggested

Figs. 4.5, 4.6 and 4.7. Abbreviation of 'g' for gecko added to figure caption as requested. Gecko data have been highlighted by the addition of an arrow at each row

P.149, 2nd last line and P. 15-, L.2. (P.150, last line; P.151, L.3) Figs. 5.10A and 5.10B changed to 4.10A and 4.10B as requested

P.151, L.13-17. (P.152, L.13, 18 and 25) References added as requested

P.152, L.15. (P.153, L18, 19) Deleted "has also been found to" and "is believed to" as requested. Likewise performed global search on these terms to remove them where appropriate

P.163, L.7. (P.164, L.7) Deleted second full stop

P.163, L.16. (P.164, L.16) Deleted 'highly' as requested

Chapter 5

P.167, 5.2.1. (P.168, L.3-6) Details of gecko tails used in this chapter added as requested

Fig.5.6. Changed caption as requested

P.203, L.8. (P.205, L.8) Changed 'vessles' to 'vessels' as requested

P.204. A discussion regarding the homology of the immunogenic sites of the antibodies in comparison to the gecko sequence is provided on P.206, L.3-16. The specific immunogenic region (sequences) for the VEGF-C and VEGF-D antibodies were unavailable due to patents by the developers. The sequence for the VEGFR-3 immunogen is accessible and is discussed on P.206, L.13-16.

Chapter 6

6.1. (P.209, L.17) Added that lymphangiogenesis could occur as a combination of both *de novo* or sprouting mechanisms

As earlier addressed have removed "have been shown to" statements where appropriate

P. 220, L.5. (P.222, L.5) Word wasn't missing but an extra word had not been deleted. Deleted to clarify the sentence as requested

Further suggestions for future work

I thank the reviewer for his/her interest in this study and for the valuable suggestions for future studies/publications.

Specifically, the *Anolis carolinensis* sequence will be included in the manuscript of the work described in Chapter 4 for publication.

Further work in this area would almost certainly be warranted on the suggested skink species to examine the process in an animal with fewer lipid stores. A paragraph with this suggestion has been included in the Future Directions section of the thesis (P.218, L.5-10)

ABSTRACT

The Australian marbled gecko, *Christinus marmoratus* has the ability to voluntarily shed its tail (autotomy) and subsequently regenerate the lost tail. The lymphatic vessels of the gecko tail are severed during autotomy and yet the regenerated tail is not lymphoedematous, indicating that the mechanisms for interstitial fluid drainage are maintained, presumably by the growth of new lymphatic vessels (lymphangiogenesis). In contrast, disruption to the lymphatic system in humans can readily result in lymphoedema due to inadequate lymphatic regenerative capacity. Hence, the regenerating gecko tail offers an excellent model to study the process of and fundamental molecular mechanisms behind lymphatic regeneration. Here, I examine lymphangiogenesis within regenerating gecko tails. I hypothesise that physiological function of lymphatic vasculature is recovered by tail regeneration. Further, I hypothesise that lymphatic regeneration is, in part, regulated by vascular endothelial growth factor C (VEGF-C) and VEGF-D via binding to their receptor, VEGFR-3, a key lymphangiogenic pathway in mammals.

Lymphatic uptake and transport, of different sized radiolabelled tracers, were examined using lymphoscintigraphy. Basic lymphatic function is apparent at 6 weeks of regeneration, however lymph clearance and velocity are not restored to near original levels until 12 weeks of regeneration. Differential clearance and lymph velocity between tracers are likely influenced by changes in the cellular matrix and lymphatic vessel permeability.

Molecular control of lymphangiogenesis within regenerating gecko tails was studied by identifying and characterising VEGF-C, VEGF-D and VEGFR-3 in gecko tail tissue extracts. This is the first study to demonstrate the presence of these genes within any reptile. Sequence alignments and molecular modelling highlight conservation of many lymphangiogenic functional residues within the gecko proteins at both a sequence and structural level.

Real time PCR established differential expression profiles of VEGF-C, VEGF-D and VEGFR-3 mRNA throughout tail regeneration, with up-regulation during the early, late and mid-phases of regeneration, respectively. These data are consistent with mammalian studies in wound healing and suggest differing roles during gecko tail regeneration and potentially the lymphangiogenic process following autotomy.

Sites of expression of VEGF-C and VEGF-D in regenerating gecko tails, demonstrated by immunohistochemistry, include keratinocytes and fibroblasts. Positive staining lining blood and lymphatic-like vessels is demonstrated for VEGF-D and VEGF-C, respectively indicating possible associations of the proteins with VEGFRs on endothelial cell surfaces and hence angiogenic and lymphangiogenic capabilities. Strong positive staining of VEGF-C and VEGFR-3 is also observed in adipose tissue in both regenerated and original tail tissue suggesting potential roles in adipogenesis and lymphangiogenesis during fat store expansion.

Positive immunostaining using the LYVE-1 lymphatic endothelium marker demonstrates that lymphangiogenesis does occur during tail regeneration. Technical limitations, possibly related to antibody cross-reactivity prevented detection of VEGFR-3 staining on lymphatic (or blood) endothelial cells in all regenerated and original tails. A suspected lack of mammalian-derived antibody reactivity to the reptilian proteins was also encountered with ELISA and western blotting analyses, with both yielding inconclusive results.

In conclusion, this study demonstrates that adequate lymphatic vasculature and function are restored during gecko tail regeneration. Furthermore, this study provides several lines of evidence, through sequence conservation and mRNA and tissue expression profiles, that VEGF-C, VEGF-D and VEGFR-3 play a role in lymphatic regeneration in a reptile.

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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Helen Blacker
August 2011

*Publications included within this thesis:

Blacker HA, Tsopelas C, Orgeig S, Daniels CB, Chatterton BE. How regenerating lymphatics function: Lessons from lizard tails. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology (2007) 290: 108-114. © 2006 Wiley-Liss, Inc.

Blacker HA and Orgeig S. Differential mRNA and tissue expression of lymphangiogenic growth factors (VEGF-C and –D) and their receptor (VEGFR-3) during tail regeneration in a gecko. Journal of Comparative Physiology B (2011) DOI 10.1007/s00360-011-0604-0. © Springer-Verlag 2011

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All experiments were performed in accordance with the National Health and Medical Research Council guidelines for the use of animals and with approval from both the Adelaide University Animal Ethics Committee (S-18-2003) and Institute of Medical and Veterinary Sciences (IMVS) Animal Ethics Committee (47/04 and 117/08). Adult Australian marbled geckos were collected and housed with permission from South Australia National Parks and Wildlife (permit numbers: E24650 and A24420-7/8) This research was supported by Australian Research Council (ARC) grants to SO and also by a grant from the Breast Cancer Research Association Inc. as trustee for the Breast Cancer Research Trust.

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LIST OF ABBREVIATIONS

μCT	Micro-computed tomography
^{99m}Tc-ATC	^{99m}Technetium-antimony trisulphide
^{99m}Tc-DTPA	99mTcdiethylenetriaminepentaacetic acid
^{99m}Tc-TFC	99mTc-tin fluoride colloid
ADSC	Adipose tissue derived stem cell
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
BCA	Bicinchoninic acid
BEC	Blood endothelial cell
CAM	Chorioallantoic membrane
cDNA	Complementary DNA
cVEGF-C(-D)(R-3)	Chicken VEGF-C, VEGF-D or VEGFR-3
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FGFs	Fibroblast growth factors
Flt4	Fms-like tyrosine kinase 4
fVEGF-C(-D)(R-3)	Frog VEGF-C, VEGF-D or VEGFR-3
GSP	Gene specific primer
gVEGF-C(-D)(R-3)	Gecko VEGF-C, VEGF-D or VEGFR-3
HIER	Heat induced epitope recovery
hVEGF-C(-D)(R-3)	Human VEGF-C, VEGF-D or VEGFR-3
Ig-like	Immunoglobulin-like
IHC	Immunohistochemistry
LB	Luria-Bertani
LEC	Lymphatic endothelial cell
LYVE-1	Lymphatic endothelial hyaluronan receptor-1
MMF	Morgan-Mercer-Flodin
mVEGF-C(-D)(R-3)	Mouse VEGF-C, VEGF-D or VEGFR-3
NJ	Neighbour-joining
NRP	Neuropilin
PBS	Phosphate buffered saline

PBS-T	Phosphate buffered saline- Tween20
PCR	Polymerase chain reaction
PDB	Protein data base
PlGF	Placenta growth factor
QC	Quality control
qPCR	Quantitative real time PCR
rAAV	Recombinant adeno-associated virus
RACE	Rapid amplification of cDNA ends
rhVEGF-C	Recombinant human VEGF-C
rhVEGF-D	Recombinant human VEGF-D
rhVEGFR-3	Recombinant human VEGFR-3
rVEGF-C(-D)(R-3)	Rat VEGF-C, VEGF-D or VEGFR-3
rVEGF-C/D	Reptilian VEGF-C/VEGF-D homologue
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC	Secondary lymphoid chemokine
SOC	Super optimal broth with carbolite repression
SS	Signal sequence
sVEGFR-2	Soluble VEGFR-2
TBS	Tris buffered saline
UTR	Untranslated region
VEGF(-A)	Vascular endothelial growth factor (-A)
VEGF-B	Vascular endothelial growth factor B
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
VEGF-E	Vascular endothelial growth factor E
VEGF-F	Vascular endothelial growth factor F
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
zVEGF-C(-D)(R-3)	Zebrafish VEGF-C, VEGF-D or VEGFR-3