LARGE-SCALE PROTEOMIC SCREEN AND SIGNAL TRANSDUCTION ANALYSIS USED TO STUDY EMBRYONIC GONAD AND GERM CELL DEVELOPMENT

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B. Sc (Hons)

A thesis submitted to The University of Newcastle in partial fulfilment of the requirements of the Doctorate of Philosophy.

Discipline of Biological Sciences

The University of Newcastle

Callaghan, NSW, Australia

Original thesis submitted December 2009

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This thesis contains work performed in collaboration with my co-supervisor Professor Peter Koopman. Some work embodied in this thesis was carried out by me in the Koopman Laboratory at The Institute for Molecular Biosciences, The University of Queensland, St. Lucia, QLD, Australia.

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Katherine Ewen

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Professor Peter Koopman

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Professor R. John Aitken

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STATEMENT OF CONTRIBUTION BY OTHERS TO THIS THESIS

The work presented in this thesis is predominantly my own.

Chapter 1 is a published literature review (1A) and an addendum (1B) with additional information relevant to this thesis. This chapter was written by me and edited by Peter Koopman.

Chapter 2 is a published manuscript. I was responsible for the work presented in all figures. This chapter was written by me, with assistance from Peter Koopman, and edited by John Aitken and Mark Baker.

Chapter 3 is a manuscript currently under review. I was responsible for the work presented in all figures. This chapter was written by me and edited by Peter Koopman and Mark Baker.

Chapter 4 is a manuscript provisionally accepted for publication. I was responsible for the work presented in all figures except Figure 1. Andrew Jackson performed the Affymetrix microarray screen used to generate data for this figure. This chapter was written by me and edited by Peter Koopman and Dagmar Wilhelm.

Chapter 5 was written by me and edited by Peter Koopman.

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Katherine Ewen

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Professor Peter Koopman

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Professor R. John Aitken

PUBLISHED WORKS INCORPORATED INTO THIS THESIS

Mouse germ cell development: From specification to sex determination
Ewen KA, Koopman PA
Mol. Cell. Endocrinol. 2010; 323(1):76-93
- *Incorporated as Chapter 1A*

Global survey of protein expression during gonadal sex determination in mice
Ewen KA, Baker MA, Wilhelm D, Aitken RJ, Koopman PA
Mol. Cell. Proteomics. 2009; 8(12):2624-41
- *Incorporated as Chapter 2*

Quantitative comparison of sex- and stage-specific protein expression levels during gonad differentiation in mice
Ewen KA, Baker MA, Wilhelm D, Aitken RJ, Koopman PA
Submitted to J. Proteome Res.
- *Incorporated as Chapter 3*

A male-specific role for p38 mitogen activated protein kinase in germ cell sex differentiation in mice
Ewen KA, Jackson A, Wilhelm D, Koopman PA
Provisionally accepted in Biol. Reprod.
- *Incorporated as Chapter 4*

ACKNOWLEDGEMENTS

To my supervisors in Brisbane, Prof. Peter Koopman and Dr. Dagmar Wilhelm, and in Newcastle, Prof. R. John Aitken and Dr. Mark Baker, an immeasurable thanks. You have provided me with the opportunity to fulfil an academic milestone I was not sure that I would ever achieve. Thank you for sharing your knowledge, and for your support and guidance throughout the years, which has aided me to produce this thesis.

To my fellow students (both past and present), in particular, to Steve Bradford, Markus Kerr, Rodrigo Morales, Liam Town, Rehan Villani, Natalie Butterfield and Grant Challen, thank you for your company, advice and for going through this stage of my life with me. A special thanks to Alex Combes and John Abramyan, for your precious friendship, understanding and encouragement even when times were unbelievably tough, you guys are my rocks! Thanks also for all the great adventures and fun times we shared that helped keep me both sane and crazy. A special thanks also to John Harvey, who left us too soon.

To the staff of the Koopman and Aitken Labs (both past and present), thank you all very much for your assistance and stimulating conversations, especially Jo Bowles, Andrew Jackson, Deon Knight, Mathias Francoise, Terje Svingen, Brett Hosking, Des Tutt, Cassy Spiller, Rebecca van Kampen, Mei Goh, Simone Tannock, Brett Nixon and Angela DiSanto for providing not only much needed help, but friendship and laughter as well.

To the staff of the IMB, many thanks for keeping the place running and for lots of smiles. To Charles Nelson and Jill Bradley, thanks for keeping me safe. To the animal house staff, thanks for all your help with all my mice. A special thanks to Amanda Carozzi for being calm and reassuring when I wasn't.

To Dr. Sean Grimmond and Dr. Ewa Rajpert-de Meyts, thank you both for employing me and helping to keep my life in science afloat. Thanks also to the staff and students of the Grimmond lab (IMB), especially to Nicole Cloonan and Darrin Taylor, and the Dept. of Growth and

Reproduction (Rigshospitalet, Denmark), especially to Anne Jørgensen, David Kristensen and Martin Jensen, for welcoming me so warmly into your labs.

And finally, to my family, friends, loved ones (you know who you are), and especially to Tony, thank you for supporting me in all my endeavours, for having faith in me when I needed it most, and for loving me even when I was writing this thesis.

May the force be with you all.

ABSTRACT

In the fields of sex determination, embryonic gonad development and germ cell differentiation, much effort has been placed in performing large-scale screens anchored in RNA and DNA technologies. Whilst these technologies have identified new candidate genes, they are unable to provide expression data for functionally active gene products. Recent improvements in the sensitivity of proteomic screening technologies have made analysis of the embryonic gonadal proteome experimentally feasible. Thus, major aims of my PhD were to generate a data set of gonadal proteins expressed at the time of sex determination, and to identify differentially expressed proteins that potentially regulate early events in gonadogenesis, germ cell development and sex differentiation.

To detect and identify gonadal proteins, I used two-dimensional nano-flow liquid chromatography and tandem mass spectrometry. I identified 1037 proteins which primarily serve in RNA post-transcriptional modification and trafficking, protein synthesis and folding, and post-translational modification. Over 300 proteins were not identified in a similar transcriptomic study. Over 60 proteins were identified with potential links to human disorders of sexual development (DSDs). I identified four proteins up-regulated in the ovary and three proteins up-regulated in the testis at a critical time point in sex determination. I also identified five proteins increasing, and three proteins decreasing, in expression during early testis development. Two of these temporally-regulated proteins are associated with human DSDs. The majority of these expression differences have not been detected at the transcript level. These data provide novel targets potentially controlling events in gonadogenesis, sex determination and germ cell differentiation.

Recently, the field of germ cell sex differentiation was revolutionised with the discovery that retinoic acid (RA) initiates meiosis in female embryonic germ cells, and that the RA-degrading enzyme CYP26B1 inhibits meiosis in male germ cells, which consequently cease mitotic division till after birth. How these somatic environmental factors regulate the transition from mitosis to meiosis or mitotic arrest remains unanswered. p38 MAP kinase (MAPK) signalling initiates mitotic arrest in other differentiating cell types. Thus, a specific aim of my PhD was to investigate the role of p38 MAPK in controlling male germ cell differentiation.

To address this aim experimentally, I analysed expression of p38 MAPK in embryonic gonads and found it to be activated in differentiating male germ cells. I then blocked p38 MAPK

signalling and found that male germ cells expressed pluripotency and meiosis-associated proteins in a similar pattern to their female counterparts, and that testes exhibited more meiotic germ cells. These data suggest that p38 MAPK signalling contributes to meiosis inhibition in testicular germ cells, and potentially directs them towards quiescence.

The studies outlined in this thesis have identified new candidates potentially regulating early events in gonadogenesis, sex determination and germ cell differentiation. Further analysis of these proteins and signalling pathways may provide valuable insights into these important events which are essential not only for sexual development and reproductive competence, but also for fertilisation, genetic recombination and ultimately species evolution.

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ABBREVIATIONS – REAGENTS

ACN	acetonitrile
APS	ammonium persulphate
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propansulfonate
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulphoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiolthreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EtBr	ethidium bromide
FCS	fetal calf serum
H&E	hematoxylin and eosin
HRP	horseradish peroxidase
IgG	immunoglobulin G
MAPK	mitogen-activated protein kinase
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% Tween 20
PBTX	phosphate buffered saline with 0.1% Triton X100
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
RA	retinoic acid
SDS	sodium dodecyl sulphate
Taq	*Thermus aquaticus*
TBS	tris buffered saline
Tris	tris [hydroxymethyl] amino methane
Triton X100	polyethylene octyl phenyl ether
Tween 20	polyoxyethylene sorbitan monolaurate

ABBREVIATIONS – UNITS, PREFIXES AND SUFFIXES

1D	one-dimensional
2D	two-dimensional
3D	three-dimensional
A	Amperes
bp	base pair/s
°C	degrees Celsius
Da	Daltons
g	grams
g	gravity
h	hour/s
k	kilo (10^3)
l	litres
M	metre/s
m	milli (10^{-3})
M	molar
min	minute/s
m/*z*	mass-to-charge ratio
n	nano (10^{-9})
n	number of independent biological replicates
p	probability
RT	room temperature (25°C)
s	second/s
ts	tail somite/s
µ	micro (10^{-6})
U	Weiss unit/s
V	volts
v/*v*	volume-to-volume ratio
w/*v*	weight-to-volume ratio

ABBREVIATIONS – MISCELLANEOUS

cDNA	complementary deoxyribonucleic acid
Chr	chromosome
CID	collision-induced dissociation
CO_2	carbon dioxide
dH_2O	distilled water
DNA	deoxyribonucleic acid
dpc	days *post coitum*
dpp	days *post partum*
DSD	disorder of sexual development
ESI	electrospray ionisation
EST	expressed sequence tag
FPR	false positive rate
GC	germ cell
HPLC	high-powered liquid chromatography
IEX	ion exchange
IF	immunofluorescence
IHC	immunohistochemistry
IPA	Ingenuity pathway analysis
IPI	international protein index
LC	liquid chromatography
LTQ	linear trap quadrupole
MALDI	matrix-assisted laser desorption/ionisation
MDLC	multidimensional liquid chromatography
MGI	Mouse Genome Informatics
MQ	Milli-Q filtered
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NB	Northern blot
NCBI	National Center for Biotechnology Information
OMIM	online Mendelian inheritance in man
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PGC	primordial germ cell
pI	isoelectric focusing point
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
RP	reversed phase
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
TOF	time of flight
UGR	urogenital ridge
UV	ultra violet
WB	Western blot
XX	female
XY	male