

Genetic Control of Hypothalamo-Pituitary Axis Development and Function in Mice

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A thesis submitted for the degree of

Doctor of Philosophy

March 2011

School of Molecular and Biomedical Science

Discipline of Biochemistry

The University of Adelaide

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*Discipline of Biochemistry
School of Molecular and Biomedical Science
The University of Adelaide
Adelaide Australia 5000*

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For my parents,

Jan and Dorota Szarek

“Glands rarely become ill, but when they do,
they give their disease to the rest of the body”

Hippocrates, *Glands*, circa 500 B.C.

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A NOTE ON NOMENCLATURE

Relevant nomenclature guidelines were taken into account when referring to genes and gene products throughout this thesis. To unambiguously refer to mouse (*Mus musculus*) genes and gene products, and to distinguish these from mammalian nomenclature, the following conventions were adhered to. Mouse gene names are italicized and in lower case, whereas gene products are non-italicized and the first letter is capitalized. Human gene names are italicized and all capitalized, whereas proteins are non-italicized and all capitalized. In addition, reference may be given to *Drosophila* genes and gene products. To differentiate these from mouse and/or human genes and gene products *Drosophila* genes are italicized and the protein are non-italicized and in lower case. Additionally, when referring to both the gene and protein, the protein name is given.

Species	Gene (abbreviation)	Protein (abbreviation)
Mouse	<i>Sox3</i>	Sox3 or mSox3
Human	SOX3	SOX3 or hSOX3
<i>Drosophila</i>	<i>sox3</i>	sox3

With reference to the Sox3 knock-out, transgenic and reporter mice, these will be referred within this thesis as follows:

Mouse Line	Nomenclature within thesis
<i>Sox3</i> -null	<i>Sox3</i> -null
Sox3-transgenic (Sox3 ^{iRES-eGFP})	Extra-Sox3
Sox3-GFP reporter	Green-Sox3

With reference to the novel dwarf mouse line described herein, we have given this mouse line the name Tukkuburko. The name is the Kurna Aboriginal word referring to “small mouse”. The Kurna Indigenous people are the custodians of the greater Adelaide region and their cultural and heritage beliefs are still important to the living Kurna people today.

ABSTRACT

Congenital dysfunction of the hypothalamic-pituitary (HP) axis occurs in approximately one birth per 2,200 and is associated with a broad range of common disease states including impaired growth (short stature), infertility, hypogonadism poor responses to stress and slow metabolism (Pescovitz and Eugster, 2004). Although, a number of genes have been linked to diseases of the HP axis, the genetic cause in many patients remains unknown.

This thesis examines two aspects of HP axis development and function. The first aim was to identify *Sox3* targets by examining gene expression differences between three mouse lines: *Sox3*-null (mice lacking *Sox3*; loss of function), Extra-*Sox3* (mice over-expressing *Sox3*; gain of function) and wild-type, by genome wide profiling using the Illumina BeadChip microarray platform. The second aim was to characterize the downstream effects relative to HP development in a novel recessive dwarf mouse model with pituitary hypoplasia and growth hormone (GH) deficiency, generated by N-ethyl-N-nitrosourea (ENU) mutagenesis that produces a point mutation in the gene for the enzyme tryptophanyl-tRNA synthetase (WARS).

The first project (*project 1*) examined *Sox3*, the causative gene associated with X-linked hypopituitarism (XH), in wild-type and transgenic mice. SOX3 is a member of the SOX (SRY-related HMG box) gene family of transcription factors that is expressed in progenitor cells of the mouse embryonic central nervous system (CNS) including the developing and postnatal hypothalamus (Rizzoti et al., 2004). It is the only member of the SOXB1 subfamily positioned on the X chromosome (Collignon et al., 1996; Stevanovic, 2003). Appropriate dose- and time-dependent expression of *Sox3* in the developing hypothalamus is required for normal neuroendocrine function, particularly related to growth and growth hormone (GH). Changes associated with a loss-of-function and/or gain-of-function of *Sox3* may contribute to a better understanding of other important genes, currently not known, involved in XH and/or X-linked mental retardation. At this point, however, the mechanisms linking SOX3 to its direct targets and their interplay within other downstream signaling cascades regulating HP axis development remain unknown. In order to identify *Sox3*-dependent genes, in mice, I performed microarray analysis of RNA extracted from embryonic mouse heads at 10.5 days post coitum (dpc) and compared RNA from wild-type, loss-of-function (*Sox3*-null) and gain-of-function (Extra-*Sox3*) mice. Several emergent candidate genes were further tested by quantitative mRNA expression analysis (qPCR). One of these was Neurogenin-3 (Ngn3), which showed a 2.5-

fold decrease ($P < 0.001$) in expression by microarray in *Sox3*-null ($n=6$), compared with wild-type (WT; $n=6$) mice and 1.8-fold decrease ($P < 0.001$) by qPCR between *Sox3*-null ($n=6$) and WT ($n=6$) mice. To evaluate the relationship between *Ngn3* and *Sox3* at a cellular level immunohistochemistry was performed on 10.5 dpc and 12.5 dpc brains. In WT mice at 10.5 dpc and 12.5 dpc *Ngn3* and *Sox3* expression overlapped in a subset of cells across the ventral-midline of the developing hypothalamus. In addition and in contrast to WT mice, in *Sox3*-null mice, there were few *Ngn3* positive cells, localized to the arcuate hypothalamic nucleus. Neurogenin-3 (*Ngn3*) is a member of the Neurogenin gene family of proneural basic helix-loop-helix proteins. Although previous data show the importance of *Ngn3* during pancreatic development, there is no information on the mechanisms and actions of *Ngn3* or a relationship between *NGN3* action and *SOX3* during hypothalamic development. These results suggest *Ngn3* is a downstream target of *Sox3* that is contributing to appropriate development of the hypothalamic-pituitary axis.

The second study (*project 2*) aimed to characterize and further examine a novel recessive ENU mouse mutant, called *Tukku*¹, exhibiting HP axis dysfunction resulting in dwarfism, pituitary hypoplasia and GH deficiency. Adult *Tukku* mice are 30-40% smaller than their WT littermates. The primary focus was to characterize the dwarfism phenotype in relation to the somatotrophic axis and to identify the causative gene. The mutation was identified as a leucine to proline substitution in tryptophanyl-tRNA synthetase (*WARS*), a member of the aminoacyl-tRNA synthetase (*AARS*) enzyme family that link amino acids to their specific tRNAs. For proper function of this enzyme the specific recognition of substrates is critical for the fidelity of protein synthesis. The *Wars* mutation is contained within the N-terminal WHEP domain, from residue 16-69, and likely causes the disruption of the alpha helical structure. The N-terminal WHEP domain has only been found in eukaryote *Wars* enzyme. Importantly, *AARS* have been linked to regulating the noncanonical activity of angiogenesis (Otani et al., 2002; Wakasugi, 2010; Wakasugi and Schimmel, 1999; Wakasugi et al., 2002b). Along with pituitary hypoplasia, *Tukku* mice show a significant reduction in pituitary GH and serum levels of IGF-1, suggesting the defect leading to pituitary hypoplasia involves brain regions implicated in growth of the anterior pituitary. The reduction in pituitary GH levels may also involve delivery of GH-releasing hormone (GHRH) to GH-secreting cells since preliminary data also indicate that *WARS* is expressed within blood vessels of the pituitary and hypothalamus. To assess this, quantitative mRNA expression analysis (qPCR) of GHRH and somatostatin (*Sst*) was

¹ *Tukku*, meaning 'small' in Kurna Aboriginal language.

performed. qPCR revealed a decrease in both GHRH and Sst (fold change >2) indicating that the defect is likely to be within the hypothalamic hypophysial vasculature that extends and makes a connection with the pituitary. To evaluate the relationship between Wars and pituitary vasculature, immunohistochemistry was performed on pituitaries at 8-weeks postnatal. Pituitary sections were co-stained with antibodies against platelet endothelial cell adhesion molecule (PECAM) + Wars or vascular-endothelial cadherin (VE-Cadherin; an endothelial specific, transmembrane protein, which clusters at adheren junctions where it promotes homotypic cell-cell adhesion) + Wars. Wars immunostaining was expressed within the endothelial cells of the pituitary vasculature, both in the anterior and posterior pituitary. Both PECAM and Wars appeared co-expressed within the vascular wall. VE-Cadherin was expressed in vessels together with Wars.

Overall, the data gathered from these projects highlight important insights into the identification of *Ngn3* as a likely *Sox3* target gene (*project 1*) and have identified a novel dwarf mouse model with a genetic determinant of HP axis function (*project 2*). These results have application to the study of HP axis development, to the study of vascular development during embryology and postnatally, and to possible avenues of genetic screen testing and development of new treatments related to GH deficiencies.

STATEMENT OF CONTRIBUTION BY OTHERS TO THIS WORK

Ms Sandra Piltz contributed to routine technical assistance in the maintenance of mouse colonies as well as purification of genomic DNA and genotyping by PCR.

Mr Dale McAninch contributed to the validation of genes identified by microarray (Chapter 3. Identification of *Sox3* Target Genes, p.105). This work formed part of his honors thesis in 2008.

Dr Stuart Reed, Dr Chris Goodnow and the team from The Australian Phenomics Centre (Canberra, ACT, Australia) the dwarf mouse line generated and identified the mutated gene by sequencing used in Project 2 (Chapter 4. Novel Dwarf Mouse Generated by ENU Mutagenesis, p.151).

Ms Carlie Delaine, Ms Siti Hadzir and Dr Briony Forbes contributed to the analysis of pituitary growth hormone and serum IGF-1 levels (Figure 4-5, p.162).

Ms Nadia Gagliardi performed paraffin embedding of tissues, sectioning and histological staining of mouse ovaries and testis (Figure 4-17, p.188) and brains (using Cresyl violet stain; Figure 4-3, p.158).

Ms Chin Ng contributed to the analysis of the dwarf mouse line by generating murine Wars constructs for analysis of angiostatic activity in cell culture (Figure 4-14, p.181). Ms Chin Ng also performed western blotting of mouse brain, pituitary and kidney samples (Figure 4-12, p.178). This work formed part of her honors thesis in 2010 (Ng, 2010).

A/Prof Paul Thomas and Prof Jeffrey Schwartz provided critical reading and proofing of the thesis manuscript.

Eva Szarek was responsible for the remainder of the work.

DECLARATION OF ORIGINALITY

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 Eva Szarek 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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* Szarek, E., Cheah, P. S., Schwartz, J., Thomas, P., 2010. Molecular genetics of the developing neuroendocrine hypothalamus. *Mol Cell Endocrinol.* 323, 115-23

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ACKNOWLEDGEMENTS

There are many people that I would like to thank for making my Ph.D possible and for offering support along the way.

My sincerest gratitude goes to my advisors A/Prof Paul Thomas and Prof Jeffrey Schwartz for their guidance, encouragement and support. It has been an honor to work under your guidance and supervision. I am grateful to A/Prof Paul Thomas for his patience, persistence and wealth of knowledge in molecular biology; I have learned an enormous amount. I am grateful to Prof Jeffrey Schwartz for introducing me to and sharing with me his wealth of knowledge in the wonderful world that is the pituitary. Working with both of you has been an amazing experience, at the bench and over a few drinks. I would like to thank you both for giving me an amazing opportunity to pursue my Ph.D in the fascinating field of brain development and neuroendocrinology. Thank you for the discussion of ideas, for giving me the freedom to follow my intuition, for offering invaluable advice.

I would also like to thank all the members of the Thomas lab (past and present), for discussions, useful tips at the lab bench, and for being a fun group to work with.

To all my friends outside the lab, thank you for being so supportive and getting my mind off the Ph.D every now and then. I really appreciated it!

A very special thank you to my loving parents and family for believing in me and taking an interest in my project. Thank you for your encouragement, and ongoing support. A special thank you to my wonderful husband for putting up with my late nights and weekends in front of the computer and my less than seldom grumpy mood. I love you and... I can finally say... "koniec!"

ACRONYMS AND ABBREVIATIONS

3'UTR	3' untranslated region	FCS	fetal calf serum
ACTH	Adrenocorticotrophic hormone	FSC	forward scatter
ADH	antidiuretic hormone (same as AVP)	FSH	Follicle Stimulating Hormone
AGRF	Australian Genome Research Facility	G1	First Generation
AH	anterior hypothalamus	gDNA	genomic DNA
ARC	arcuate nucleus	GFP	green fluorescent protein
AVP	arginine vasopressin (same as ADH)	GFP+	GFP-positive
BAC	Bacterial Artificial Chromosome	GH	Growth hormone
BCIP	5-Bromo-4-Chloro-3-Indolyl phosphate	GHRH	Growth-hormone-releasing hormone
BM	basement membrane	GHRHR	growth hormone-releasing hormone receptor
BMP	bone morphogenic protein	h	hour
bp	base pair	H ₂ O	water
BSA	bovine serum albumin	HEPES	N-[2-hydroxyethyl]-piperazin-N'-[2-ethansulfonic acid]
C-terminal	carboxyterminal	HISS	heat-inactivated horse serum
cAMP	cyclic adenosine mono phosphate	HMG	high mobility group
cDNA	complimentary deoxyribonucleic acid	HP	hypothalamo-pituitary
CH	congenital hypopituitarism	IGF	insulin-like growth factor
ChIP	chromatin immunoprecipitation	IGHD	isolated growth hormone deficiency
CNS	Central nervous system	IP	immunoprecipitation
CoIP	co-immunoprecipitation	IPTG	isopropylthiogalactosid
DEPC	diethylpyrocarbonate	IRES	internal ribosome entry site
DIG	digoxigenin	kb	kilobase pair = 1000bp
DMEM	Dubelcco's Modified Eagle Medium	kDa	Kilo Dalton
DMN	dorsal-medial nucleus	KO	Knockout
DMSO	dimethylsulfoxide	LH	Luteinizing hormone
DNA	Deoxyribonucleic acid	M	Molar
dpc	days post coitum	m	mouse
E	Embryonic day	MAPK	mitogen-activated protein kinase
E. coli	Escherichia coli	ME	median eminence
ECM	extracellular matrix	min	minute
EDTA	ethylene diaminetetra acetic acid	ml	millilitre
EGF	epidermal growth factor	mM	millimolar
eGFP	enhanced green fluorescent protein	MQ-H ₂ O	milliQ H ₂ O
EGTA	ethyleneglycolbis-(2-aminoethyl)-tetraacetic acid	mRNA	messenger ribonucleic acid
ENU	N-ethyl-N-nitrosurea	mRNA	messenger RNA
FACS	fluorescence activated cell sorting		

NBT	4-nitroblue tetrazolium chloride	SSC	Salt Sodium Citrate
N-terminal	aminoterminal	Sst	somatostatin
ng	nanograms	TE	Tris-EDTA
NGN/Ngn	neurogenin	tg	transgenic
NGN3/Ngn3	neurogenin-3	TGF β	transforming growth factor-beta
nM	nanomolar	TRH	Thyrotropin-releasing hormone
ORF	Open reading frame	TRIS	Tris-(hydroxymethyl)-aminomethan
OT	oxytocin	TrpRS	tryptophan-tRNA synthetase (see also WARS)
P	postnatal day	TSH	Thyroid-stimulating hormone
PAGE	polyacrylamide-gel electrophoresis	U	units
PBS	Phosphate buffered saline	UTR	untranslated region
PCR	Polymerase Chain Reaction	VEGF	vascular endothelial growth factor
PDGF	platelet-derived growth factor	VMN	Ventro-medial nucleus;
PFA	paraformaldehyde	WARS	see also TrpRS
PI	propidium Iodide	WT	wild-type
PKA	protein kinase A	XH	X-linked hypopituitarism
PKC	protein kinase C	zf	zebrafish
POA	preoptic area;	μ g	microgram
POMC	Pro-opiomelanocortin	μ M	micromolar
PVN	paraventricular nucleus;		
qPCR	quantitative real-time polymerase chain reaction		
qRT-PCR	quantitative real-time polymerase chain reaction		
r	rat		
RE	restriction enzyme		
RIN	RNA integrity number		
RNA	ribonucleic acid		
rpm	revolutions per minute		
rRNA	ribosomal RNA		
RT	reverse transcription		
rt	room temperature		
RT-PCR	reverse transcriptase-polymerase chain reaction		
SCN	supra-chiasmatic nucleus;		
SDS	sodium dodecyl sulfate		
SHH	sonic hedgehog		
SOCM	Sox consensus motif		
SON	supra-optic nucleus;		
SOX	Sry-related HMG box containing		

PUBLICATIONS

First author publications arising from the work presented within this thesis. A copy of this publication can be found in the Publications section of this thesis.

Szarek, E., Cheah, P. S., Schwartz, J., Thomas, P., 2010. Molecular genetics of the developing neuroendocrine hypothalamus. *Mol Cell Endocrinol.* 323, 115-23.

CONFERENCE PRECEEDINGS

The results described in this thesis have been presented as seminar communications at the following conferences:

Szarek, E., Read, S., Forbes, B., Delaine, C., Schwartz, J., Thomas, P. A novel ENU mutation, WARS, causes dwarfism in mice. *Gold Coast Health and Medical Research Conference, Gold Coast, Queensland, Australia.* December 2nd-3rd 2010

Szarek, E., Read, S., Forbes, B., Delaine, C., Schwartz, J., Thomas, P. A Novel ENU mutation, WARS, causes dwarfism in mice. *Program in Developmental Endocrinology and Genetics (PDEGEN) Research Conference, National Institutes of Health, Bethesda, MD, USA.* July 9th 2010.

Szarek, E., Read, S., Forbes, B., Schwartz, J., Thomas, P. Identification of the sequence responsible for and further phenotypic characterization of a novel dwarf mouse produced by ENU-induced mutagenesis. *Gold Coast Health and Medical Research Conference, Gold Coast, Queensland, Australia.* December 3rd - 4th 2009.

Szarek, E., Lovell-Badge, R., Schwartz, J., Thomas, P.Q. Expression of NGN3 in the developing hypothalamus: dependence on and co-localization with SOX3 in the mouse model of altered pituitary function. *ENDO2009, Washington DC, USA.* June 10th - 13th 2009.