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

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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	*Sox3*	Sox3 or mSox3
Human	*SOX3*	SOX3 or hSOX3
Drosophila	*sox3*	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**
Sox3-null	*Sox3*-null
Sox3-transgenic ($Sox3^{iRES\text{-}eGFP}$)	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 Kaurna Aboriginal word referring to “small mouse”. The Kaurna Indigenous people are the custodians of the greater Adelaide region and their cultural and heritage beliefs are still important to the living Kaurna 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 somatotropic 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

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[1] *Tukku*, meaning 'small' in Kaurna 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 instituition 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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ACRONYMS AND ABBREVIATIONS

3'UTR 3' untranslated region
ACTH Adrenocorticotropic hormone
ADH antidiuretic hormone (same as AVP)
AGRF Australian Genome Research Facility
AH anterior hypothalamus
ARC arcuate nucleus
AVP arginine vasopressin (same as ADH)
BAC Bacterial Artificial Chromosome
BCIP 5-Bromo-4-Chloro-3-Indolyl phosphate
BM basement membrane
BMP bone morphogenic protein
bp base pair
BSA bovine serum albumin
C-terminal carboxyterminal
cAMP cyclic adenosine mono phosphate
cDNA complimentary deoxyribonucleic acid
CH congenital hypopituitarism
ChIP chromatin immunoprecipitation
CNS Central nervous system
CoIP co-immunoprecipitation
DEPC diethylpyrocarbonate
DIG digoxigenin
DMEM Dubelcco's Modified Eagle Medium
DMN dorsal-medial nucleus
DMSO dimethylsulfoxide
DNA Deoxyribonucleic acid
dpc days post coitum
E Embryonic day
E. coli Escherichia coli
ECM extracellular matrix
EDTA ethylene diaminetetra acetic acid
EGF epidermal growth factor
eGFP enhanced green fluorescent protein
EGTA ethylenglycolbis-(2-aminoethyl)-tetraacetic acid
ENU N-ethyl-N-nitrosurea
FACS fluorescence activated cell sorting
FCS fetal calf serum
FSC forward scatter
FSH Follicle Stimulating Hormone
G1 First Generation
gDNA genomic DNA
GFP green fluorescent protein
GFP^+ GFP-positive
GH Growth hormone
GHRH Growth-hormone-releasing hormone
GHRHR growth hormone-releasing hormone receptor
h hour
H_2O water
HEPES N-[2-hydroxyethyl]-piperazin-N'-[2-ethansulfonic acid]
HISS heat-inactivated horse serum
HMG high mobility group
HP hypothalamo-pituitary
IGF insulin-like growth factor
IGHD isolated growth hormone deficiency
IP immunoprecipitation
IPTG isopropylthiogalactosid
IRES internal ribosome entry site
kb kilobase pair = 1000bp
kDa Kilo Dalton
KO Knockout
LH Luteinizing hormone
M Molar
m mouse
MAPK mitogen-activated protein kinase
ME median eminence
min minute
ml millilitre
mM millimolar
MQ-H_2O milliQ H_2O
mRNA messenger ribonucleic acid
mRNA messenger RNA

NBT 4-nitroblue tetrazolium chloride

N-terminal aminoterminal

ng nanograms

NGN/Ngn neurogenin

NGN3/Ngn3 neurogenin-3

nM nanomolar

ORF Open reading frame

OT oxytocin

P postnatal day

PAGE polyacrylamide-gel electrophoresis

PBS Phosphate buffered saline

PCR Polymerase Chain Reaction

PDGF platelet-derived growth factor

PFA paraformaldehyde

PI propidium Iodide

PKA protein kinase A

PKC protein kinase C

POA preoptic area;

POMC Pro-opiomelanocortin

PVN paraventricular nucleus;

qPCR quantitative real-time polymerase chain reaction

qRT-PCR quantitative real-time polymerase chain reaction

r rat

RE restriction emzyme

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

SSC Salt Sodium Citrate

Sst somatostatin

TE Tris-EDTA

tg transgenic

TGFβ transforming growth factor-beta

TRH Thyrotropin-releasing hormone

TRIS Tris-(hydroxymethyl)-aminomethan

TrpRS tryptophan-tRNA synthetase (see also WARS)

TSH Thyroid-stimulating hormone

U units

UTR untranslated region

VEGF vascular endothelial growth factor

VMN Ventro-medial nucleus;

WARS see also TrpRS

WT wild-type

XH X-linked hypopituitarism

zf zebrafish

μg microgram

μM micromolar

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.