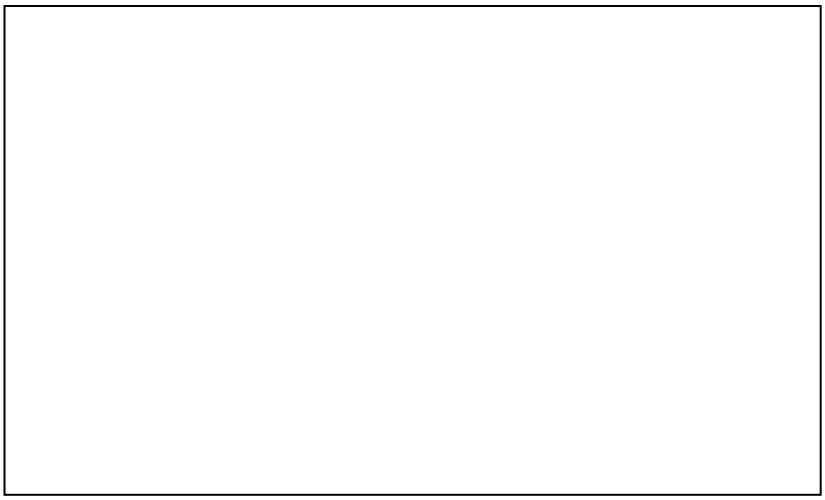
The Characterisation, Genomic Organisation, Expression and Function of the mEphA1 Receptor Tyrosine Kinase

A thesis submitted for the degree of Doctor of Philosophy at the University of Queensland in April 2007



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Dedication

For my parents John and Elsie and my family Helen, Philippa, Harriet and Julian

From: ***The Prophet*** by Khalil Gibran

On Children

And a woman who held a babe against her bosom said, "Speak to us of Children."
And he said:
Your children are not your children.
They are the sons and daughters of Life's longing for itself.
They come through you but not from you,
And though they are with you, yet they belong not to you.
You may give them your love but not your thoughts.
For they have their own thoughts.
You may house their bodies but not their souls,
For their souls dwell in the house of tomorrow,
Which you cannot visit, not even in your dreams.
You may strive to be like them, but seek not to make them like you.
For life goes not backward nor tarries with yesterday.
You are the bows from which your children as living arrows are sent forth.
The archer sees the mark upon the path of the infinite, and He bends you with His might that His arrows may go swift and far.
Let your bending in the archer's hand be for gladness;
For even as He loves the arrow that flies, so He loves also the bow that is stable.

Statement of Originality

In accordance with the Rules and Regulations governing the degree of Doctor of Philosophy at the University of Queensland, the following statements are made:

I assess my contribution to the work described in each chapter as follows:-

Chapter 1: 100%

Chapter 2: 100%

Chapter 3: 80% in collaboration with Dr Jason Lickliter, Dr Ke Chen, Dr Graham Webb, Dr Mirella Dottori and Dr Ian Tonks

Chapter 4: 80% in collaboration with Dr Cuong Do

Chapter 5: 80% in collaboration with Ms Jacinta Carter, Dr Shannon Duffy, Dr Nirmitha Herath, Ms Jenny McCarron and Dr Mark Spanevello

Chapter 6: 80% in collaboration with Ms Jacinta Carter, Dr Shannon Duffy, Dr Nirmitha Herath, Ms Jenny McCarron and Dr Mark Spanevello

Chapter 7: 100%

In conclusion, my overall contribution to this thesis is more than 80%.

This thesis is less than 100,000 words in length exclusive of tables, figures and references.

The work described in this thesis has been published in peer-reviewed journals as listed below.

Signed ……. Date / /2008

Dr Mark D Spanevello

Signed ……. Date / /2008

Professor Andrew W Boyd

Signed ……. Date / /2008

Dr Mark G Coulthard

Acknowledgments

I thank Professor Andrew Boyd for his guidance and considerable patience in allowing me to work towards my goal of becoming a clinician-scientist in his laboratory.

I am grateful to all the LFQ laboratory members past and present who have assisted me over the past 10 years. There are too many to list individually here.

I could not have generated the knockout mice without the assistance of Dr Graham Kay, Dr Iank Tonks, Ms Carol Paterson and Ms Anna Zournazi in the QIMR Transgenic Laboratory.

I thank Dr Gael Phillips for reviewing the histological sections.

I thank Dr Bob Munford for giving me my first chance in the laboratory, and Dr Alan Varley, Dr Richard Kitchens and Dr Mike Hamilton for proving to me that you can most definitely complete a PhD after the age of 40 (but before the age of 50).

I thank my parents for instilling in me sufficient self-belief, or is it stubbornness, to have completed this journey.

Lastly, I thank my wife and children for all their love and support.

Publications arising directly from this Thesis

Coulthard MG, Lickliter JD, Subanesan N, Chen K, Webb GC, Lowry AJ, Koblar S, Bottema CD, Boyd AW. (2001) Characterisation of the EphA1 receptor tyrosine kinase: expression in epithelial tissues. Growth Factors 18, 303–17.

Coulthard MG, Duffy S, Down M, Evans B, Power M, Smith F, Stylianou C, Kleikamp S, Oates A, Lackmann M, Burns, GF, Boyd AW. (2002) The role of the Eph-ephrin signalling system in the regulation of developmental patterning. Int J Dev Biol 46, 375–84.

Coulthard MG, Duffy SL, Herath NI, Spanevello MD, McCarron JK, Carter JC, Kay GF, Phillips, GE, Boyd AW. (2007) EphA1 reporter knockout mice demonstrate restricted EphA1 expression in epithelial tissues and developmental defects of the tail and female genital tract. *Submitted genesis (The Journal of Genetics and Development).*

Abstract

The Eph receptor tyrosine kinases and their ephrin ligands are cell surface molecules with a wide range of biological functions. Specifically, the Eph/ephrin receptor-ligand family influences cell behaviour during both embryogenesis and adult life, principally through modification of cytoskeletal organisation and cell adhesion. *EphA1* (previously referred to as *eph*) was isolated during a search for novel tyrosine kinases with oncogenic potential. The murine homologue of *hEphA1,* formerly *Esk* and now *mEphA1*, was cloned by reverse transcriptase PCR using degenerate oligonucleotide primers and RNA prepared from embryonic stem cells in culture. Northern blot analysis revealed expression in day 12 mouse embryo, and adult mouse thymus, liver, kidney, lung and placenta.

The work in this thesis investigates the expression and function of *EphA1* in mutant mouse and other animal models. This has been achieved by a number of different techniques including:- (1) the classical techniques of molecular biology; (2) a search for the zebrafish homologue of *mEphA1*; (3) generation and phenotypic analysis of the hPLAP EphA1 reporter knockout mouse and (4) generation of the EphA1 conditional knockout mouse.

The chromosomal localisation and Southern blotting of genomic digests confirmed that *Esk* (*mEphA1*) is the murine homologue of *eph* (*hEphA1*). The binding of soluble mEphA1 to a panel of ephrin ligands analysed by surface plasmon resonance (BIACore), and the binding of various ephrin-Fc molecules to cell surface expressed EphA1, confirmed that EphA1 is the cognate receptor for the ephrin-A1 ligand. The *mEphA1* genomic sequence was isolated, sequenced and the exon-intron boundaries mapped. Interestingly, Exon 3, which includes the ligand binding domain, is split into two smaller exons (Exon 3a and Exon 3b). This pattern was also found in *hEphA1*; however, it is a novel finding compared with the other Ephs, and the reason underlying this difference remains speculative. *In situ* hybridisation analysis confirmed epithelial expression of *mEphA1* in the basal layer of the epidermis, developing hair follicles, thymic epithelial cells and adult kidney.

At the commencement of the zebrafish (ZF) library screening project in 1997, it seemed likely that there was an ZF orthologue of EphA1. However, over 50 clones were

isolated by degenerate PCR of zebrafish cDNA and genomic libraries, and although some of the sequences had homology to known Ephs, none matched *EphA1*. The ZF genome has now been sequenced completely [http://wwwmap.tuebingen.mpg.de/ ; http://zfin.org/] and has confirmed that there is indeed no zebrafish orthologue of EphA1.

The hPLAP EphA1 reporter knockout mouse was generated with the technical assistance of Dr Graham Kay (Queensland Transgenic Laboratory). The homozygous null mice have a kinky tail in two separate embryonic stem cell lines with a high degree of penetrance. A proportion of female null mice display the imperforate vagina phenotype. The null mice are otherwise grossly normal, with equal sex ratios and normal growth, health and life expectancy. The microscopic examination of haematoxylin and eosin stained sections of all the major organs revealed no histological abnormalities. The expression of hPLAP, (hence mEphA1), analysed in frozen sections confirmed the previous work which defined the epithelial expression of mEphA1 to the basal epidermis and hair follicle. There was also previously undescribed hPLAP (mEphA1) expression in the uterus, vagina and small intestine.

The EphA1 conditional knockout mouse was also generated with the assistance of the Queensland Transgenic Facility. The homozygous null mice were grossly normal with equal sex ratio and normal health and life expectancy. The kinky tail phenotype was observed infrequently and has not yet been fully characterised in these mice. Similarly the imperforate vagina phenotype has not been observed in this strain of mice. This strain of genetically modified EphA1 knockout mice can be mated with various strains of *Cre*-deleter mice to achieve tissue specific silencing of *EphA1* and consequently allow more precise analysis of EphA1 function.

In summary, the studies described in this thesis have confirmed the importance of the Eph/ephrin receptor-ligands in both embryonic development and the maintenance of adult tissues, and have generated several new findings which add to our knowledge of the biology of EphA1. The generation of the hPLAP EphA1 reporter mice and EphA1 conditional knockout mice has provided us with very useful tools. These knockout mice will allow further analysis of the role of EphA1 in mouse models of human diseases, including skin and colon cancer, severe sepsis and post-traumatic injury.

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List of symbols

α	alpha
β	beta
γ	gamma
Δ	delta
μ	mu
λ	lambda
Φ	phi
©	Copyright
®	Registered
™	Trade-Mark
+/+	wild type
+/-	heterozygote
-/-	homozygous null or knockout

List of abbreviations

A_{260}	absorbance at light wavelength 260 nm
A-loop	activation loop (kinase domain)
Abl	Abelson (kinase)
ABP	AMPA receptor binding protein
AChE	acetylcholinesterase receptor
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ABP	AMPA receptor-binding protein
Arg	Abelson related gene
AP	anterior-posterior
ARC	Animal Resource Centre
ARC(S)	Animal Resource Centre (Swiss)
ARMS	ankyrin repeat-rich membrane spanning
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMP	bone morphogenetic protein
bp	base pairs
BRCA1	breast cancer gene 1
BSA	bovine serum albumin
CA	cornus ammonis (region of the hippocampus)
C.elegans	*Caenorhabiditis elegans*
CAP	Cbl-associated protein
CAS	Crk-associated protein
Cbl	Casitas B-lineage lymphoma (Cbl family of proteins)
Cdk	cyclin-dependent kinase
cDNA	complementary DNA
CFNS	craniofrontonasal syndrome
cfu	colony forming units
Ci	curies
CIP	calf intestinal alkaline phosphatase
CNS	central nervous system

Cre	Cre recombinase gene (cyclisation recombination protein)
CST	corticospinal tract
CXCR4	Chemokine receptor 4
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
ddH_2O	double distilled water (usually Milli-Q water)
DEPC	diethylpyrocarbonate
DG	dentate gyrus
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpc	days post coitus
DH	Dbl homology (domain)
D. melanogaster	*Drosophila melanogaster*
DTT	dithiotreitol
DV	dorsal-ventral
E11	embryonic day 11
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	ethylene-diamine-tetraacetic-acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
Eph	erythropoietin producing hepatocarcinoma
ERK	extracellular signal-regulated kinase
ES	embryonic stem (cell)
EtBr	ethidium bromide
F1	first generation of offspring
FAP-1	Fas associated peptide-1
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FBS	foetal bovine serum
Flp-e	Flp-e recombinase gene
FLIM	fluorescence lifetime imaging microscopy
FRET	fluorescence resonance energy transfer
G418	neomycin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCG	Genetics Computer Group
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GIT1	G protein-coupled receptor kinase-interacting protein
GluR	glutamate receptor
GPI	glycosylphosphatidylinositol
Grb	growth factor receptor bound protein
GRIP	glutamate receptor interacting protein
GST	glutathione-S-transferase
HA	haemagglutin

HeV	Hendra virus
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
HMBS	hydroxymethylbilane synthase
HP	high power
hPLAP	human placental alkaline phosphatase
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ
IRES	internal ribosomal entry site
Jak	Janus kinase
JH	Jak homology (domain)
JNK	c-Jun N terminal kinase
kB	kilobase
kDa	kilodalton
KAc	potassium acetate
Kidins220	kinase-D-interacting substrate of 220 kDa (= ARMS)
LB	Luria-Bertani
LD	leucine-rich motif/domain (LDXLLXXL)
LIF	leukaemia inhibitory factor
LIM	Lin 11, Isl 1, Mec3 (proteins required for developmental decisions)
LM	lateral-medial
LMC	lateral motor column
LMW-PTP	low-molecular weight phosphotyrosine phosphatase
loxP	locus of crossing over of bacteriophage P
LP	low power
LTP	long-term potentiation
MDCK	Madin-Darby canine kidney
mGlu1	metabrotopic glutamate receptor type 1
mJ	milliJoules
Milli-Q	Milli-Q ion exchange purified water
MMC	medial motor column
MMP	matrix metalloprotease
MMTV	mouse mammary tumour virus
MO	morpholino
MOPS	3-(*N*-Morpholino)-propane-sulphonic acid
MS	multiple sclerosis
NaOAc	sodium acetate
NBT	nitroblue tetrazolium
NCC	neural crest cell
NEB	New England Biolabs
NF-κB	nuclear factor kappa B
NIK	Nck interacting kinase
NiV	Nipah virus
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
NMR	nuclear magnetic resonance
N-WASP	neuronal Wiskott Aldrich Syndrome protein
OD^{600}	optical density at wavelength of 600 nm
OT	optic tectum

P5	post-natal day 5
PAC	plasmid artificial chromosome
PAK	p21-activated kinase
pBst	pBluescript (Stratagene)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDZ	Post-synaptic density protein-95, *Drosophila* disc large tumour suppressor (Dlg), Zona occludens-1
PDZ-RGS3	PDZ - regulator of heterotrimeric G-protein signalling
PEG	polyethylene glycol
pfu	plaque forming units
PH	pleckstrin homology (domain)
PHIP	ephrin interacting protein
PIX	p21-activated kinase interacting exchange factor
PTP-BL	phosphotyrosine phosphatase-basophil like
PI3K	phosphatidylinositol 3-kinase
Pick	protein kinase Cα interacting
PIPES	piperazine-1,2-bis[2-ethanesulphonic acid])
PLC-γ	phospholipase Cγ
poly(A)+	polyadenylated ribonucleic acid
PTB	phospho-tyrosine binding
QIMR	Queensland Institute of Medical Research, Herston QLD 4029
Q-PCR	quantitative real-time polymerase chain reaction
Ras-GAP	Ras GTPase-activating protein
RET	Ret proto-oncogene = “rearranged in transfection”
RGC	retinal ganglion cell
RhoGEF	Rho associated guanine nucleotide exchange factor
RNA	ribonucleic acid
ROCK	Rho-associated kinase
rpm	revolutions per minute
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SAM	sterile alpha motif
SC	superior colliculus
SCID	severe combined immunodeficiency
SDF-1	stromal-cell derived factor 1 (CXCR4 ligand)
SDS	sodium dodecyl sulphate
SFK	Src-family kinase
SH	Src homology (domain)
Shh	sonic hedgehog
SIRS	systemic inflammatory response syndrome
SLAP	Src-like adaptor protein
SOCS	suppressor of cytokine signalling
SPF	specific pathogen free
SSC	salt sodium citrate
SSPE	salt sodium phosphate EDTA
STAT	signal transducers and activators of transcription
SVZ	sub-ventricular zone
Taq	*Thermus aquaticus*

TBE	tris-borate-EDTA
TBS	tris-buffered saline
TCF	T-cell transcription factor
TE	tris-EDTA (10mM Tris, 1mM EDTA, pH 7.5)
Tris	trishydroxymethylaminomethane
Tris	2-amino-2-hydroxymethyl-1,3-propanediol.
TsAP	thermosensitive alkaline phosphatase
TN	temporal-nasal
TNF	tumour necrosis factor
TZ	termination zone
TX-100	Triton X-100
UTR	untranslated region
UV	ultra-violet (light)
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WEHI	Walter and Eliza Hall Institute, Melbourne, Victoria
WASP	Wiskott-Aldrich syndrome protein
X. laevus	*Xenopus laevus*
ZF	zebrafish