



**INVESTIGATION OF THE MOLECULAR FUNCTION OF
THE NUCLEAR ONCOPROTEIN HOX11 IN HUMAN
T-CELL LEUKAEMIA**

THESIS

**PRESENTED FOR THE AWARD OF THE DEGREE
OF DOCTOR OF PHILOSOPHY (Ph.D)**

BY

MANSOUR HEIDARI

(B.Sc, M.Sc)

**DIVISION OF BIOMEDICAL SCIENCE
MURDOCH UNIVERSITY**

2003

DECLARATION

Apart from the assistance stated in the acknowledgments, and where due reference is made in the text, the work presented in this thesis was performed by myself. Segments of the work have been published under the author's name, as shown in the publications and scientific communications.

Mansour Heidari

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr Wayne Greene for supervising my Ph.D studies with insight and enthusiasm, and for his support that made the few difficult times bearable. These investigations would not have been possible without his consistent help, kind encouragement, and interest in the progress of the study. Special thanks are also due to Professor Ursula Kees, Children's Leukaemia and Cancer Research, ICHR for enhancing my academic experience and for efforts in revision, comment, and constructive criticism during my studies. Thanks also to Dr David Berryman, Manager/Research and Development Officer, Western Australian State Agricultural Biotechnology Centre, Murdoch University for guiding me through the use of the laboratory equipment.

I am indebted to several colleagues for their help and advice during my postgraduate work. Firstly, Dr Mohammad Bassami for critical advice provided during the expression and purification of HOX11. Deep appreciation is also expressed to Dr Peter Dallas for providing advice and for his suggestions regarding the chromatin immunoprecipitation technique. I also thank Dr Jacqueline Phillips for providing antibody conjugate and for her critical advice about confocal microscopy. Dr Geoffrey Dwyer was always helpful and provided me with many suggestions on an academic and technical level. A big thank-you also goes to Jette Ford for generously supplying the leukaemic T-cell lines used in this study, Dr John H. Blinco for his help during the immunocytochemical study and Dr John Murphy for his help with the fluorescent microscopy work. I also acknowledge the help and advice on the proteomic work provided by Dr Richard Lipscombe and colleagues of Proteomics International.

On a personal note, I would like to thank my Ph.D student colleagues Kim Rice and Darcelle Dixon for their help and cooperation during the course of this dissertation.

This work would not have been possible without financial assistance. Therefore, I deeply thank the Iranian Ministry of Health and Medical Education for supporting my study by providing a Ph.D. Scholarship.

I am deeply thankful to my wife for her understanding patience, for her inspiration and constant encouragement. Finally, a deeply heartfelt thank-you to my children, Masoud and Matin, for tolerating me during these studies.

PUBLICATIONS

1. Heidari M., Kim L. Rice, Ursula R. Kees, and Wayne K. Greene. (2002). Expression and purification of the human homeodomain oncoprotein HOX11. *Protein Expression & Purification* 25; 313-318.
2. Heidari M., J.K. Phillips, U.R. Kees, and W.K. Greene (2003). The Nuclear Oncoprotein HOX11 Associates with Pericentromeric Heterochromatin in Leukaemic T-cells (manuscript in preparation).
3. Heidari M., U.R. Kees and W.K. Greene (2003). Identification of proteins within HOX11-containing nuclear complexes by mass spectrometry (manuscript in preparation).

CONFERENCE ABSTRACTS

1. Heidari M., J.K. Phillips, U.R. Kees and W.K. Greene (2003). The Nuclear Oncoprotein HOX11 Associates with centromeric DNA in Leukaemic T-cells. 3rd Annual Medical Research Symposium, Perth, Western Australia.
2. Heidari M., Kees U.R. and Greene W.K. (2003). The Nuclear Oncoprotein HOX11 Associates with Pericentromeric Heterochromatin in Leukaemic T-cells. 15th Lorne Cancer Conference Lorne, Victoria, Australia.
3. Heidari M., J.K. Phillips, U.R. Kees and W.K. Greene (2003). The Nuclear Oncoprotein HOX11 Associates with Pericentromeric Heterochromatin in Leukaemic T-cells. Keystone Symposium, Chromatin: Organizing the Genome for Patterns of Gene Expression in Health and Disease, Big Sky, Montana, USA.
4. Heidari M., U.R. Kees and W.K. Greene (2001). Identification of direct chromosomal target sequences of HOX11, a nuclear oncoprotein misexpressed in human T-cell leukaemia. The Second Iranian Medical Sciences Postgraduate Students Conference, Teheran, Iran.

ABSTRACT

HOX11, the prototypical member of the HOX11 family (HOX11, HOX11L1 and HOX11L2) was originally discovered as a transcriptional regulator aberrantly expressed in tumours with an immature T-cell phenotype (T-ALL) as a result of specific chromosomal translocations involving T-cell receptor loci. Subsequently, it was revealed that HOX11 is required for normal spleen development since newborn *Hox11*^{-/-} mice exhibit asplenia. In both its normal and abnormal roles, HOX11 has been postulated to function by binding regulatory elements within specific target genes to control gene transcription. However, very few genomic targets of HOX11 have been identified and little is known about its mode of action. In this study, we sought to further understand the role of HOX11 in controlling differentiation and cell growth by 1) determining the identity of genomic sequences that are directly bound by HOX11 and 2) determining the identity of proteins which exist within HOX11-containing nuclear complexes.

To identify direct HOX11 target sequences, a whole genome PCR-based screening method was employed using immobilised recombinant HOX11 that had first been expressed as a biologically active GST fusion protein. Using this approach, restriction enzyme-cleaved human genomic DNA was selected for high-affinity HOX11 binding sites. Unexpectedly, almost all clones isolated contained sequences derived from satellite 2 DNA that, together with related satellite 3 DNA, is found on most chromosomes at transcriptionally inactive pericentromeric heterochromatin. The specific binding of HOX11 to satellite 2 DNA was verified by bandshift assays using both recombinant HOX11 protein and nuclear extract derived from the T-ALL cell line,

ALL-SIL. DNA-protein complexes containing HOX11 were identified by their ablation upon addition of HOX11 antibody.

To confirm that HOX11 associates with pericentromeric heterochromatin *in vivo*, HOX11 was characterised in terms of its nuclear localisation during interphase in unsynchronised leukaemic T-cells (ALL-SIL) harbouring a translocation involving the *HOX11* locus. Using indirect immunofluorescence and confocal microscopy, HOX11 antibody produced a punctate pattern of staining in the nucleus with discrete areas of dense staining superimposed on a diffuse distribution of HOX11 protein. By dual staining, the bright HOX11 foci correlated with centromeres since they overlapped with signals detected by an antibody specific for the centromeric protein CENP-B. Further evidence for a direct interaction of HOX11 with satellite 2 DNA was provided by chromatin immunoprecipitation assay. In the presence of HOX11 antibody, DNA fragments containing satellite 2 sequences were immunoprecipitated from sheared, cross-linked ALL-SIL chromatin but not from chromatin isolated from the HOX11-negative T-cell line PER-117. Finally, using a combination of immunoprecipitation with HOX11 antibody, gel electrophoresis and mass peptide fingerprinting, a set of nuclear proteins were identified as potential HOX11 interactors which are known to either localise to centromeric regions or act as regulators of gene expression. Together, these results implicate HOX11 in a functional interaction with centromeric heterochromatin, which may be a key feature of this oncoprotein in terms of both its T-cell transformation and transcriptional regulatory functions.

LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APS	ammonium persulphate
B-ALL	B-cell acute lymphoblastic leukaemia
BP	broad pass
bp, kb	(base pair) length of DNA, kilobase pairs
BSA	bovine serum albumin
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CO ₂	carbon dioxide
Da, kDa	dalton, kilodalton
DDW	double deionised H ₂ O
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetra-acetic acid
EMSA	electrophoretic mobility shift assay
FRET	fluorescence resonance energy transfer
gm;mg;µg;ng;pg	gram, milligram, microgram, nanogram, picogram
GST	glutathione S-transferase
HCl	hydrochloric acid
HD	homeodomain
hr, min	hour, minute
IF	immunofluorescence
Ig	immunoglobulin
IP	immunoprecipitation
IPTG	isopropyl thio-β-D-galactoside
LB	Luria-Bertani medium
M;mM;µM;nM	molar; millimolar; micromolar; nanomolar
M	marker of DNA
Mr	relative molecular mass
ml;µl	millilitre, microlitre
MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight
nm	nanometre
NS	non-specific
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
Poly dI-dC	polydeoxyinosinic-deoxycytidylic acid
rHOX11	recombinant HOX11
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	short pass
SN	supernatant
TAE	Tris-acetate-EDTA
T-ALL	T-cell acute lymphoblastic leukaemia
TCR	T-cell receptor
TE	Tris-EDTA
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetra methyl ethylene diamine
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
uCi	microcurie
UV	ultraviolet
v/v	volume per volume
w/w	weight per weight
w/v	weight per volume
WG-PCR	whole genome PCR
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
g	relative centrifugal field

LIST OF TABLES

Table 1.1 Functional classification of transforming genes at translocation junctions associated with T-ALL (p. 8).

Table 1.2 *HOX* genes affected by genetic alterations in human cancer (p. 21).

Table 1.3 Putative MYC target genes (p. 27).

Table 2.1 Cloning, PCR, sequencing and EMSA assay primers (p. 49).

Table 2.2 PCR thermocycling conditions for HOX11 and HOX11 Δ H3 amplification (p. 51).

Table 2.3 Composition of a typical PCR reaction for HOX11 and HOX11 cDNA PCR amplification (p. 53).

Table 2.4 PCR thermocycling conditions for ABI PrismTM Ready DyeDeoxyTM Terminator Cycle Sequencing Kit (p. 53).

Table 2.5 Summary of HOX11 protein yields from representative 1 L of *E. coli* (p. 70).

Table 3.1 Reaction mix and conditions used for human genomic DNA digestion using *Sau3A* I and *Tsp509* I restriction enzymes (p. 84).

Table 3.2 Oligonucleotides used in the whole genome PCR procedure (p. 86).

Table 3.3 Sequence identity and lengths of the HOX11-selected fragments obtained from from two adapted libraries, (*Sau3A* I and *Tsp509* I) by whole genome PCR (p. 97).

Table 4.1 Oligonucleotides used for ChIP-assay PCR and EMSA (p. 117).

Table 5.1 Summary of co-immunoprecipitated proteins identified by mass spectrometry in ALL-SIL cells (pp. 152).

Table 5.2 Number and location of identified tryptic peptides within TFIIB (p.154).

TABLE OF CONTENTS

Declaration.....	i
Acknowledgements	ii
Publications	iiI
Conference Abstracts	iii
Abstract	iv
List of Abbreviations.....	vi
List of Tables.....	viii

CHAPTER 1: Introduction

1.1 Childhood Leukaemia.....	1
1.1.1 Introduction.....	2
1.1.2 Acute lymphoblastic leukaemia (ALL)	3
1.1.3 The molecular genetics of childhood leukaemia.....	4
1.1.4 T-cell Acute lymphoblastic leukaemia.....	6
1.2 The Normal Role of HOX11.....	9
1.2.1 Developmental role of homeobox genes.....	9
1.2.2 The role of homeoproteins in gene regulation.....	11
1.2.3 The HOX11 protein.....	12
1.2.4 Developmental role of HOX11.....	13
1.2.5 HOX11 homologs.....	15
1.3 The Abnormal Role of HOX11.....	16
1.3.1 The oncogenic role of transcription factors in T-ALL.....	16
1.3.2 HOX genes and cancer.....	19
1.3.3 The role of HOX11 as an oncogene and involvement in T-ALL.....	21
1.4 Target Genes and Interacting Co-Factors	
of Oncogenic Transcription Factors	25
1.4.1 Target genes	25
1.4.2 Protein partners.....	29
1.4.3 HOX11 target genes and protein partners.....	32
1.5 Project Aims.....	36

CHAPTER 2: Expression and Purification of HOX11 as GST Fusion Protein

2.1 Introduction	39
2.2 Materials and Methods	42
2.2.1 Agarose gel electrophoresis.....	42
2.2.2 DNA Purification.....	43
2.2.2.1 Ethanol Precipitation.....	43
2.2.2.2 Gel Purification of DNA.....	44
2.2.2.3 BRESAspin PCR Purification Kit.....	44
2.2.3 Plasmid purification.....	44
2.2.3.1 GeneElute™ Plasmid miniprep kit (Sigma)	44
2.2.3.2 QIA Prep Spin Plasmid Kit.....	45
2.2.3.3 Qiagen Plasmid Midi Kit.....	45
2.2.4 Determination of DNA concentration.....	46
2.2.4.1 Fluorometric method	46
2.2.4.2 Agarose gel electrophoresis method	47
2.2.5 Preparation of competent <i>E. coli</i>	47
2.2.6 Bacterial transformation.....	47
2.2.7 PCR primer design.....	48
2.2.8 Polymerase chain reaction (PCR)	49
2.2.9 Cloning into pGEX Expression Vector.....	52
2.2.10 Screening for recombinant plasmids.....	52
2.2.10.1 Screening of recombinant plasmids by PCR.....	52
2.2.10.2 Screening of recombinant plasmids by restriction digestion.....	53
2.2.10.3 PCR sequencing.....	54
2.2.11 HOX11 Recombinant Protein Expression.....	55
2.2.11.1 Small Scale	55
2.2.11.2 Large Scale.....	55
2.2.12 Polyacrylamide gel electrophoresis.....	57
2.2.12.1 SDS-PAGE.....	57
2.2.12.2 Non-denaturing Gel for Electrophoretic Mobility Shift Assay.....	57

2.2.13 Western Blotting.....	58
2.2.14 DNA labeling for Electrophoretic Mobility Shift Assay (EMSA).....	58
2.3 Results.....	61
2.3.1 Cloning of HOX11 and HOX11 cDNA into pGEX Expression Vector.....	61
2.3.2 Expression and purification of GST-HOX11 fusion proteins.....	65
2.3.3 Verification of the biological activity of rHOX11	72
2.4. Discussion.....	75

CHAPTER 3: Identification of Genomic DNA Target Sequences of HOX11 by Whole Genome PCR

3.1 Introduction.....	79
3.2 Materials and Methods.....	83
3.2.1 Genomic DNA Isolation.....	83
3.2.2 Preparation of genomic DNA fragments	83
3.2.3 Whole genome PCR (WG-PCR)	84
3.2.4 Subcloning of PCR products from WG-PCR.....	85
3.2.5 DNA Sequencing.....	87
3.2.6 PCR product labelling for EMSA.....	87
3.3 Results.....	88
3.3.1 Whole genome PCR.....	88
3.4 Discussion.....	100

CHAPTER 4: Confirmation of the Physical Association HOX11 with Pericentromeric Satellite 2 DNA

4.1.Introduction.....	108
4.2. Materials and Methods.....	111
4.2.1 Cell lines and cell culture..	111
4.2.2 Trypan blue viable cell counting.....	111
4.2.3 Preparation of nuclear extracts.....	111
4.2.4 Determination of nuclear extract protein concentration.....	112
4.2.5 Western blotting.....	113

4.2.6. Chromatin Immunoprecipitation.....	113
4.2.7 Chromatin Immunoprecipitation (ChIP) Assay.....	114
4.2.8 PCR amplification of immunoprecipitated DNA.....	116
4.2.9 Electrophoretic Mobility Shift Assay (EMSA).....	117
4.2.10 Immunocytochemistry.....	117
4.2.10.1 Slide Mounting.....	118
4.2.10.2 Fluorescence microscopy.....	119
4.2.10.3 Confocal microscopy.....	119
4.3 Results.....	120
4.3.1 HOX11 interacts with a single repeat unit of satellite 2 DNA in leukaemic T-cells.....	120
4.3.2 Validation of Anti-HOX11 antibody.....	125
4.3.3 HOX11 interacts with Satellite 2 DNA <i>in vivo</i>	129
4.3.4 Nuclear Localisation of the HOX11 Oncoprotein.....	131
4.3.5 Localisation of HOX11 Protein to centromeric heterochromatin in Leukaemic T-cells.....	133
4.4 Discussion.....	135
4.4.1 Identification of HOX11 DNA binding sites <i>in vivo</i>	135
4.4.2 Biological significance and model(s) of HOX11 interaction with centromeric DNA.....	137

CHAPTER 5: Identification of Potential Protein Partners of HOX11

5.1 Introduction.....	144
5.2 Materials and Methods.....	157
5.2.1 Co-immunoprecipitation.....	157
5.2.2 Protein visualization and analysis.....	157
5.2.2.1 Silver staining.....	157
5.2.3 Peptide-mass fingerprinting by MALDI-TOF.....	157
5.3 Results.....	149
5.3.1 Screening for HOX11 Partner Proteins.....	149
5.1 Discussion.....	156

CHAPTER 6: General Discussion

6.1 General Discussion.....	164
-----------------------------	-----

6.1.1. Sequestration Model.....	170
6.1.2. Dynamic Gene Repositioning Model.....	171
6.1.3. Centromere /Kinetochore Regulation Model.....	173
REFERENCES.....	177