ANDROGEN RESPONSIVE ELEMENTS IN TRPM8 GENE IN PROSTATE CANCER CELLS

A THESIS SUBMITTED IN TOTAL FULFILMENT OF THE

REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

Yabin Zhou (M Biotech, BSc)

Department of Medical Biochemistry

School of Medicine

Faculty of Health Sciences

The Flinders University of South Australia

June 2010

TABLE OF CONTENTS

Table of Contents 2

List of Figures 9

List of Tables 12

Summary 13

Declaration 17

Acknowledgments 18

List of Abbreviations 20

Chapter 1: Introduction and Literature review 23

1.1 Introduction 23

1.2 Prostate cancer 24

1.2.1 Incidence and mortality of prostate cancer 24

1.2.2 Risk factors for prostate cancer 24

1.2.3 Pathology of prostate cancer 26

1.2.3.1 Prostatic intraepithelial neoplasia 26

1.2.3.2 Prostate adenocarcinoma 28

1.2.4 Clinical stages of prostate cancer 28

1.2.5 Treatment of prostate cancer 29

1.3 Androgen regulation and prostate cancer 32

1.3.1 Androgen receptor signalling 33

1.3.1.1 Androgen receptor structure 33

1.3.1.2 Genomic signalling pathway 36

1.3.1.3 Non-genomic signalling pathway 37

1.3.2 The role of abnormal AR signalling in the development of hormone-refractory prostate cancer 41

1.3.2.1 *In situ* synthesis of androgen in prostate tumours 42

1.3.2.2 Modifications of the AR gene in prostate tumours 42

1.3.2.3 Androgen independent activation of the AR 44

1.3.2.4 The effects of aberrant expression of AR coregulators on AR transactivation activity 45

1.3.3 The Androgen-responsive element 48

1.4 Experimental approaches for searching for AREs in genes directly regulated by the AR 50

1.4.1 Reporter-gene assay 50

1.4.2 Chromatin immunoprecipitation (ChIP) assay 52

1.4.3 DNase I hypersensitive site mapping 54

1.5 The TRPM8 non-selective cation channel 56

1.5.1 Overview of the TRP cation channel superfamily and the members of the TRPM plasma membrane cation channel subfamily 56

1.5.2 The *TRPM8* gene 59

1.5.3 Expression of TRPM8 in normal tissues 59

1.5.4 General properties of TRPM8 60

1.5.5 Structure and mechanisms of activation of TRPM8 61

1.5.6 Normal physiological functions of TRPM8 63

1.6 TRPM8 in normal prostate epithelial cells 66

1.6.1 TRPM8 expression 66

1.6.2 Androgen regulation of *TRPM8* expression 69

1.6.3 Hypothesis regarding the regulation of *TRPM8* transcription by the androgen receptor 72

1.7 *TRPM8* expression in prostate cancer 75

1.7.1 Change in *TRPM8* expression during prostate cancer progression 75

1.7.2 The relationships between AR activity and *TRPM8* expression 76

1.7.3 Proposed roles of TRPM8 in prostate cancer progression 76

1.7.4 TRPM8 as a potential diagnostic marker or therapeutic target in prostate cancer 78

1.8 Aims 78

Chapter 2: Materials and Methods 81

2.1 Materials 81

2.1.1 Plasmid vectors ... 81

2.1.2 Reagents, chemicals and other materials ... 81

2.2 Mammalian cell culture ... 82

2.2.1 Maintenance of cells ... 83

2.2.2 Androgen deprivation of LNCaP cells for luciferase reporter gene assay ... 83

2.2.3 Androgen deprivation of LNCaP cells for ChIP assay ... 84

2.2.4 Androgen treatment of LNCaP cells for luciferase reporter gene assay ... 84

2.2.5 Androgen treatment of LNCaP cells for ChIP assay ... 84

2.3 Transient transfection of LNCaP Cells with plasmids ... 85

2.4 Competent *E.coli* transformation ... 86

2.5 DNA and RNA purification ... 86

2.5.1 Genomic DNA purification ... 86

2.5.2 Purification of DNA from PCR reactions or other enzymatic reaction mixtures ... 87

2.5.3 DNA purification from agarose gel ... 88

2.5.4 Plasmid DNA Mini-prep ... 88

2.5.5 Plasmid DNA Midi-prep or Maxi-prep ... 88

2.5.6 Total RNA extraction and purification ... 89

2.6 DNA and RNA quantification and purity assessment ... 89

2.7 Agarose gel electrophoresis ... 90

2.8 PCR ... 90

2.8.1 Primers and oligos ... 90

2.8.1.1 Oligos for reverse transcription ... 91
2.8.1.2 qPCR primers for Human *TRPM8* mRNA amplification ... 91
2.8.1.3 qPCR primers for Human *HPRT* mRNA amplification ... 91
2.8.1.4 qPCR primers for ChIP assay-qPCR ... 92
2.8.1.5 PCR primers for amplifying 6 kb *TRPM8* promoter ... 92
2.8.1.6 PCR primers for amplifying AR Binding Site 1 ... 92

2.8.2 Reverse transcription ... 93

2.8.3 Conventional PCR 93

2.8.3.1 PCR amplifying 6 kb *TRPM8* promoter 93
2.8.3.2 PCR amplifying Androgen Receptor Binding Site 1 94

2.8.4 qPCR using SYBR Green dye for *TRPM8* mRNA level analysis . 94

2.8.4.1 Determination of qPCR amplification efficiency of *TRPM8* gene and *HPRT* gene 95
2.8.4.2 qPCR analysis of *TRPM8* and *HPRT* mRNA quantity 96

2.8.5 qPCR analysis of DNA from ChIP Assay 101

2.8.5.1 Performing qPCR 101
2.8.5.2 Raw data processing 101
2.8.5.3 Further data processing with an Excel SuperArray ChIP-qPCR Data Analysis Template 102
2.8.5.4 Final data presentation 102

2.9 Enzymatic reactions on DNA or RNA 103

2.9.1 DNAse I treatment of total RNA 103

2.9.2 Restriction enzyme digestion 103

2.9.3 DNA ligation 104

2.9.4 DNA recirculization 105

2.9.5 Blunting of both ends of a DNA fragment by *pfu* 105

2.10 Construction of luciferase reporter vectors 105

2.10.1 Construction of pGL-R-TRPM8pr-luc, pGL-TRPM8pr-luc and its deletion constructs 105

2.10.2 Construction of pGEM-T EASY-ARBS1, pGL-ARBS1EN/SV40-luc, pGL-R- ARBS1EN/SV40-luc and pGL-R-ARBS1EN/TRPM8pr-luc vectors 107

2.11 Luciferase assay 108

2.11.1 Reagent preparation 108

2.11.2 Cell lysis 109

2.11.3 Luminescence measurement for firefly luciferase and Renilla luciferase activity 109

2.11.4 Data processing 109

2.12 ChIP assay 111

2.12.1 Cell treatment 111

2.12.2 Crosslinking 111

2.12.3 Cell lysis and sonication 112

2.12.4 Immunoprecipiation 113

2.12.5 Elution and reversal of crosslinking 114

2.12.6 DNA purification 115

2.13 Statistical analysis 116

Chapter 3: Search for androgen-responsive elements in the 6 kb promoter region of *TRPM8* 118

3.1 Introduction 118

3.2 Results 119

3.2.1 Analysis of putative AREs within the 6 kb promoter region of the human*TRPM8* gene 119

3.2.2 Androgen induced endogenous *TRPM8* gene expression 120

3.2.3 Determination of optimal conditions for androgen depletion using *TRPM8* promoter-containing luciferase reporter gene assay 127

3.2.4 Luciferase reporter gene assay using the 6 kb promoter region of the*TRPM8* gene 129

3.2.4.1 Overall strategy 129

3.2.4.2 Construction of the pGL-TRPM8pr-luc, pGL-TRPM8pr-luc based deletion constructs and the negative control plasmid, pGL-R-TRPM8pr-luc .. 130

3.2.4.3 Transfection of LNCaP cells, androgen treatment and luciferase analysis 133

3.3 Discussion 138

3.3.1 DHT concentration for +DHT treatment prior to luciferase analysis. 138

3.3.2 Androgen responsiveness of the 6.1 kb *TRPM8* promoter........... 139

3.3.3 Androgen-independent inhibition of luciferase expression in the deleted 6 kb *TRPM8* promoter region 141

3.3.4 Time-dependent fluctuations in endogenous *TRPM8* mRNA expression 141

3.3.5 Interference by phenol red 142

Chapter 4: Search for Androgen-responsive elements in the 5' regulatory region of the *TRPM8* gene by ChIP assay 145

4.1 Introduction 145

4.2 Results 150

4.2.1 Overall strategy 150

4.2.2 Primer design, synthesis and validation 150

4.2.2.1 Primer design 151
4.2.2.2 Primer synthesis and validation 151

4.2.3 Optimization of chromatin fragmentation for ChIP assay 152

4.2.3.1 Sonication optimization for Misonix Sonicator S-4000 152

4.2.4 Identification of Androgen Receptor Binding Site 1 at approximately -28 kb upstream of the *TRPM8* gene by ChIP –qPCR analysis 159

4.2.4.1 ChIP assay 159
4.2.4.2 qPCR analysis 160

4.2.5 Determination of the boundaries of the ARBS1 region 161

4.3 Discussion 166

4.3.1 Time for DHT stimulation for AR binding prior to ChIP assay 166

4.3.2 ARBS1 is an AR binding site 167

4.3.3 Sonication conditions 168

Chapter 5: Evaluation of the regulatory function of the Androgen Receptor Binding Site 1 using luciferase reporter vectors 171

5.1 Introduction 171

5.2 Results 175

5.2.1 Construction of pGEM-T Easy-ARBS1 plasmid 175

5.2.1.1 PCR amplification of AR binding site 1 (ARBS1) 175
5.2.1.2 Ligation of ARBS1 and linear vector pGEM-T Easy 175

5.2.2 Construction of luciferase reporter plasmids, SV40- ARBS1, SV40-R-ARBS1 and TRPM8-R-ARBS1 177

5.2.2.1 Releasing ARBS1, linearizing SV40-empty and TRPM8-empty by *NotI* digestion 177
5.2.2.2 Ligation of *NotI*-ARBS1-*NotI* fragment with the linearized SV40-empty or TRPM8-empty. 178

5.2.3 Effects of androgen treatment on luciferase induction 184

5.3 Discussion 187

5.3.1 The inhibitory effects of ARBS1 on luciferase gene expression . 187

5.3.2 Colony screening 187

Chapter 6: General discussions 191

6.1 The hypotheses for androgen receptor regulation of *TRPM8* transcription 191

6.2 Discussion of the lack of androgen responses of the four putative androgen responsive elements identified by cross species analysis 193

6.3 A putative silencer of the *TRPM8* gene within the AR Binding Site 1 197

6.4 AR regulation of *TRPM8* gene expression 200

6.5 Future directions 203

6.5.1 Testing TRPM8-ARBS1 by luciferase assay 203

6.5.2 Identification of the ARE sequence within AR Binding Site 1 203

6.5.3 Further testing on the four putative AREs identified by cross species analysis 204

6.5.4 Searching for AREs in the 5' UTR, introns and the 3' UTR downstream of the *TRPM8* gene 205

6.5.5 Identification of the silencer ARE interaction site on the *TRPM8* promoter 205

6.5.6 Examination of the roles of identified AREs in different stages of prostate cancer 206

6.5.7 Future directions 207

Appendix 1: qPCR primers for the ChIP assay 210

Appendix 2: Plasmid constructs used for transient transfection and luciferase reporter gene assays 214

Appendix 3: Sequencing Data for the AR Binding Site 1 insert in pGEM-T EASY-ARBS1 222

Appendix 4: Composition of solutions 224

Bibliography 230

LIST OF FIGURES

Figure 1. 1 **Schematic illustration of Prostatic intraepithelial neoplasia** 27

Figure 1. 2 **Schematic TNM stages of prostate cancer** 31

Figure 1. 3 **Schematic illustration of the structures of the AR gene, AR protein, the two zinc fingers and the sequence of the consensus ARE motifs** 35

Figure 1. 4 **Genomic AR signalling pathway** 39

Figure 1. 5 **Non-genomic AR signalling pathway** 40

Figure 1. 6 **Abnormal AR signalling in prostate cancer** 47

Figure 1. 7 **Approaches for searching for ARE** 55

Figure 1. 8 **Predicted structure of the TRPM8 channel and activation pathways** 65

Figure 1. 9 **Schematic illustration of the *TRPM8* gene** 68

Figure 1. 10 **Putative AREs identified in the *TRPM8* gene** 71

Figure 1. 11 **Hypothesized mechanism by which the AR regulates *TRPM8* transcription** 74

Figure 2. 1 **Demonstration of how to determine qPCR amplification efficiency of the *TRPM8* gene and the *HPRT* gene** 98

Figure 2. 2 **Demonstration of the calculation of normalized *TRPM8* mRNA quantities** 100

Figure 2. 3 **Chemistry of firefly luciferase and Renilla luciferase reactions** 110

Figure 3. 1 Informatics analysis for putative AREs 123

Figure 3. 2 Time course showing a decrease in *TRPM8* mRNA level in LNCaP cells following removal of androgen 125

Figure 3. 3 Time course showing the induction of *TRPM8* mRNA by 10 nM DHT in androgen deprived LNCaP cells 126

Figure 3. 4 Effect of different times for androgen depletion on the induction of *TRPM8* expression in LNCaP cells 128

Figure 3. 5 Schematic illustration of the plasmid maps of pGL-TRPM8pr- luc and pGL-R-TRPM8pr-luc 134

Figure 3. 6 Schematic illustration of the construction of pGL-TRPM8pr-luc- 135

Figure 3. 7 Agarose gel image of restriction enzyme mapping for pGL-TRPM8pr-luc and pGL-R- TRPM8pr-luc 136

Figure 3. 8 Luciferase analysis of constructs that contain various sizes of the 5' flanking region of the *TRPM8* gene 137

Figure 4. 1 Model of ARE layout in the PSA gene 147

Figure 4. 2 ChIP-seq data of the low affinity AR binding site from Buchanan et al 148

Figure 4. 3 Schematic illustration of the human *TRPM8* gene and the 5' regulatory region to be studied using a ChIP assay 149

Figure 4. 4 PCR- gel electrophoresis validation of the size of amplicons from -1 kb to -20 kb primer pairs 154

Figure 4. 5 PCR- gel electrophoresis validation of the size of amplicons from -21 kb to -30 kb primer pairs 155

Figure 4. 6 Schematic representation of the location of the 5' flanking region of the *TRPM8* gene and the locations of the 32 qPCR amplicons 156

Figure 4. 7 Optimization of sonication conditions using a Misonix Sonicator S-4000 with microtip 158

Figure 4. 8 Enrichment of each tile on the 31 kb 5' regulatory region of the *TRPM8* gene. 164

Figure 4. 9 Schematic representation of the location of the ARBS1 at approximately -28 kb of the *TRPM8* gene. 165

Figure 5. 1 Schematic diagram of the strategy for ARBS1 regulatory function analysis using a luciferase reporter gene assay. 174

Figure 5. 2 PCR amplification of ARBS1 176

Figure 5. 3 Schematic representation of the construction SV40-ARBS1 and SV40-R-ARBS1 180

Figure 5. 4 Schematic representation of the construction of TRPM8-ARBS1 and TRPM8--R-ARBS1 181

Figure 5. 5 *NotI* restriction enzyme digestion of plasmids used to create constructs 182

Figure 5. 6 Agarose gel Images of restriction enzyme mapping for construct selection 183

Figure 5. 7 Analysis of ARBS1 regulatory functions on heterologous or endogenous promoters using luciferase reporter gene assays 186

Figure 6. 1 Models for AR regulation of *TRPM8* gene transcription 196

Figure 6. 2 Schematic representation of locations of AR Binding Site 1 and nearby genes 199

LIST OF TABLES

Table 1.1 Subfamilies of TRP cation channels and their functions --58

Table 1.2 TRPM8 expression patterns in normal tissues --62

Table 1.3 List of TRPM8 agonists, antagonists and their indicative potency --64

SUMMARY

On the basis of previous knowledge of the androgen receptor regulation of other genes in prostate epithelial cells and of prostate specific antigen gene expression, it was hypothesized that regulation of *TRPM8* gene expression by the androgen receptor is through a genomic pathway by binding at least two AREs at the *TRPM8* promoter: one in the proximal promoter initiates *TRPM8* transcription; the other one at a distal promoter site enhances the *TRPM8* transcription activity. The aim of this thesis was to test this hypothesis.

Transient Receptor Potential Maelastatin 8 (TRPM8) non-selective cation channel is a member of the TRP family of cation channels. The expression of TRPM8 is regulated by the androgen receptor. It has been proposed that the functions of TRPM8 may be to act as a cold sensor and/ or to regulate regulation of ion and protein secretion in the prostate gland. TRPM8 may also be involved in the regulation of prostate epithelial cell proliferation or apoptosis. In the benign prostate and in the early stages of prostate cancer, TRPM8 expression increases whereas its expression in advanced stages of prostate cancer decreases. In hormone refractory prostate cancer TRPM8 expression is lost. These observations indicate a potential role of TRPM8 in the progression of prostate cancer, especially the early stages of the cancer. The distinct expression patterns of TRPM8 in different stages of prostate

cancer also make it a potential marker for diagnosis and for prediction programs for prostate cancer.

The bioinformatic search for ARE in the *TRPM8* promoter was performed in human, chimpanzee and baboon. In the 6 kb *TRPM8* promoter, four putative AREs have been identified and are conserved across the three species. The 6 kb TRPM8 promoter was subsequently cloned into a luciferase reporter vector (pGL3 Basic) to investigate its androgen responsiveness. However, insignificant androgen response was found.

The endogenous TRPM8 mRNA expression pattern during androgen depletion and re-addition was also investigated by RT-qPCR. This was to predict the potential response pattern of the 6 kb *TRPM8* promoter during androgen treatment in the luciferase experiments. The endogenous TRPM8 mRNA expression study also provided information to optimize the conditions for the luciferase experiments.

The search for AREs was then expanded to the region from +1 kb to -30 kb (relative to the transcription start site) of the 5' regulatory region and the 5' untranslated region of *TRPM8* using ChIP –qPCR analysis to directly detect the *in vivo* androgen receptor binding sites. 32 pairs of qPCR primers were designed and synthesized.

They were validated by PCR-gel electrophoresis followed by sequencing confirmation. The ChIP-qPCR has identified an *in vivo* AR binding site, designated as Androgen Receptor Binding Site 1 which contains a putative ARE, located at around -28 kb of the 5’ regulatory region of the *TRPM8* gene.

The following functional test of Androgen Receptor Binding Site 1 by luciferase reporter gene assay showed that with androgen stimulation, it specifically inhibited luciferase expression in a luciferase reporter construct driven by the 6 kb *TRPM8* promoter.

In conclusion, we have detected a potential ARE that serves as a silencer in the 30 kb 5’ regulatory region of the *TRPM8* gene. This indicated a more complicated mechanism of androgen receptor regulation of *TRPM8* gene transcription: the androgen receptor may bind to some other ARE(s) to initiate and /or enhance TRPM8 gene transcription; under certain condition, AR may repress the *TRPM8* gene transcription through the binding to the silencer within Androgen Receptor Binding Site 1. However, further experiments are needed to test this hypothesis.

In future experiments, it would be helpful for our understanding of AR regulation of *TRPM8* gene expression to 1) identify the ARE sequence within the Androgen

Receptor Binding Site 1; 2) further test the four putative AREs identified by cross species analysis; 3) search for AREs in the 5’ untranslated region, *introns* of the TRPM8 gene; 4) identify the region in the *TRPM8* promoter that the Androgen Receptor Binding Site 1 interacts with to exert its regulatory function.

DECLARATION

I certify that the work embodied in this thesis is the result of original research conducted while I was enrolled as a PhD student in the Department of Biochemistry at Flinders University, South Australia. This thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is cited.

Yabin Zhou

ACKNOWLEDGMENTS

I would like to express my sincere thanks to my principle supervisor Professor Greg J. Barritt for his supervision, advice, and encouragement during the whole candidature of my PhD study.

Special thanks to my co-supervisor Dr. Michael Michael for his supervision, great help and valuable advice, especially on techniques regarding qPCR and ChIP assays. I would also like to thank Dr. Lisa for her comments and help during the course of this study.

I would like to thank Dr. Grant Buchanan and Dr. Eleanor Need for kindly sharing their ChIP-seq data, ChIP assay protocol, and helpful advice on the ChIP assay. I would also like to thank Ms Anne Rogers for her advice and helps with luciferase assays; Dr. Donggui Hu and Dr. Robyn Meech for their advice and help with ChIP assays and EMSA; Dr. Tim Chataway for his advice and help on using the Typhoon Imaging system and Misonix Sonicator S-4000 and especially the opportunity he provided for me to give a short talk to the Newell Foundation; Ms Angela Binns and Dr. Tadija Petronijevic for various advice and help during the course of this study; Dr. Hakan Muyderman for the interesting chat on a career as a scientist and for being a good companion to share the office with; Prof Neil Sims for his valuable comments

on my annual review of progress reviews, Dr. Roland Gregory for his advice of PBL tutoring.

I am grateful to the members of the Ca^{2+} signalling laboratory for their friendship and support， as well as the members from the Dept. of Medical Biochemistry, particularly Ms. Rachel Hughes and Ms. Diana.

I am always grateful to my parents and my wife Jin Hua for their love, understanding, encouragement, and support at all times during my studies.

LIST OF ABBREVIATIONS

AR	Androgen receptor
ARBS1	AR Binding Site 1
ARE	Androgen response element
ChIP	Chromatin immunoprecipitation
CMR1	Cold- and menthol-sensitive receptor
CPS	Count per second
DBD	DNA binding domain
DCS FBS	Dextran / Charcoal stripped FBS
DHEA	Dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DRE	Digital rectal exam
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
GRs	Glucocorticoid receptors
HDAC	Histone deacetylases
HGPIN	High-grade PIN
HPRT	Hypoxanthine guanine phosphoribosyl tranferase
iPLA2	Independent phospholipase A2
LARII	Luciferase Assay Reagent II
LPC	Lysophosphatidylcholine
LPI	Lysophosphatidylinositol
NE	Normalized gene expression
PIN	Prostatic intraepithelial neoplasia

PIP2	Phosphatidylinositol 4,5-bisphosphate
PLB	Passive Lysis Buffer
PSA	Prostate specific antigen
qPCR	Quantitative real-time PCR
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcription
SD	Standard deviation
TF	Transcription factor
TNM	Tumour, node, metastases
TRP	Transient receptor potential
TRPM	Transient receptor potential melastatin
TRPM8	Transient receptor potential cation channel, subfamily M, member 8
TSS	Transcription start site